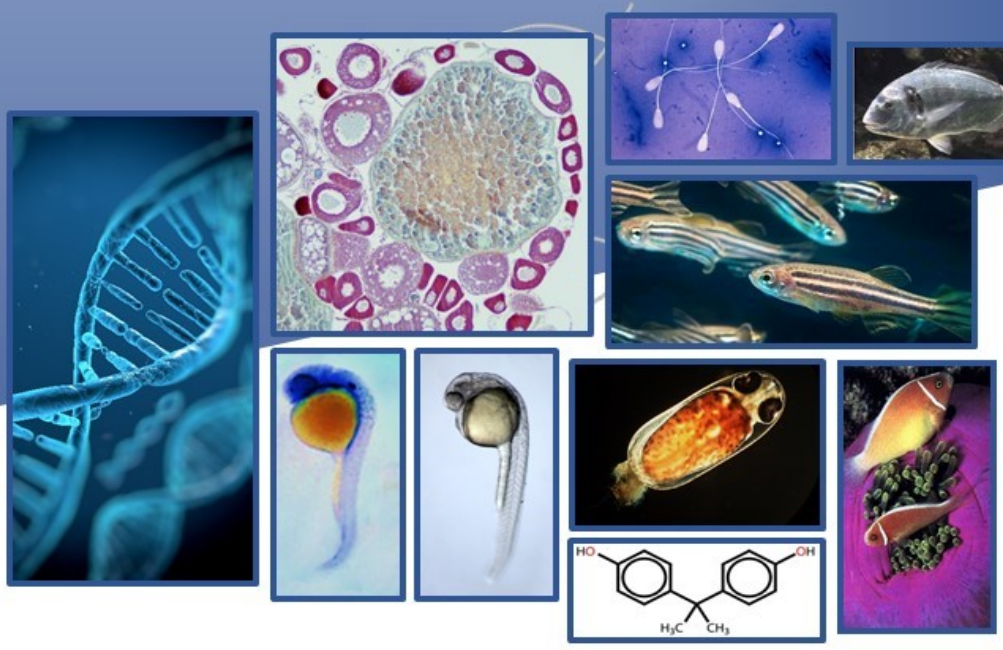
5th International Workshop on the Biology of Fish Gametes

Ancona Italy

5th International Workshop on the Biology of Fish Gametes ~ Ancona, 7-11 September 2015 ~

Sept. 7-11 2015



AQUAGAMETE

cost EUROPEAN COOPERATION IN SCIENCE AND TECHNOLOGY

UNIVERSITÀ POLITECNICA DELLE MARCHE

BioTecnologie BT

TECNIPLAST innovation through passion

5th International Workshop on the Biology of Fish Gametes
7-11 September 2015, Ancona (Italy)
Book of Abstracts
290 pages
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PREFACE

During the past six years, four international workshops on fish gametes demonstrated a rapid development of methodologies that encompass extensive opportunities for promising use in basic reproductive biology, genetic research, biotechnology and aquaculture practice. All of these can have far-reaching consequences on conservation of endangered species, assessment of anthropogenic and climatic impacts on aquatic species and application in aquaculture, as well as in fisheries management. In particular, it has been recognized that there are many highly diverting details in the practical application of these new methods used by most scientists and laboratories, which, although experimental species are kept constant, can cause highly variable, if not contradicting, results.

The workshop, arrived at its fifth edition and taking place in Ancona in the coming September, is organized within the framework of the AQUAGAMETE COST Action FA1205 coordinated by Dr. Juan F. Asturiano, funded by the European Cooperation in Science and Technology, with Università Politecnica delle Marche as local organizer.

It will represent a relevant occasion during which the latest research related to various aspects of the field of fish reproductive biology will be presented to an international audience. Special emphasis will be given to the dissemination of innovative protocols to be adopted in order to achieve a standardization of sound and common application in aquaculture research and commerce, as aimed by the Action.

In particular, the highlights of the event will be:

1. To focus on new and cutting edge research in order to gain insights about the latest science and enhance knowledge beyond the participants' scientific fields of interest.
2. A robust scientific program that merges selected sessions into cross-disciplinary themes.
3. An increase visibility of participants' science by appealing both luminaries in fish reproduction and up and coming talents, as well as decision makers. To this audience, a plenty of opportunities avail to seek out finding for new research and get quality feedback before publishing the results in high ranking journals.
4. Networking among participants from various backgrounds and different countries.
5. To give leads to participants' careers, especially to young researchers willing to start achieving their career goals and increasing their worth to potential employers.

The local organizing committee wishes all participant will enjoy this workhop's edition and would like to thank them for their contribution and interest.

Oliana Carnevali

Head of the organizing committees

COMMITTEES

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Stefania **Santangeli**

Andrea **Miccoli**

PROGRAMME AT A GLANCE

Programme at a glance
5th International Workshop on the Biology of Fish Gametes

Monday, September 7th

- 14:00 - 18:00 Congress registration
- 15:00 - 18:00 Aquagamete MC Meeting
- 18:00 - 18:30 Authorities' greetings
- 18:30 - 20:00 Welcome party

Tuesday, September 8th

- 08:00 - 09:00 Congress registration
- 09:00 - 10:50 Symposium 1: Spermatology: basic and strategic research leading to high quality sperm
- 10:50 - 11:10 Coffee break
- 11:10 - 12:50 Symposium 1: Spermatology: basic and strategic research leading to high quality sperm
- 12:50 - 14:00 Lunch
- 14:00 - 15:50 Symposium 2: Anthropogenic contaminants in the environment: effects on fish gametes
- 15:50 - 16:10 Coffee break
- 16:10 - 17:10 Side event
- 17:10 - 19:00 Poster session I

Wednesday, September 9th

- 09:00 - 10:50 Symposium 3: Oogenesis: the molecular basis behind oocyte growth, egg quality, fertilization and embryo development
- 10:50 - 11:10 Coffee break
- 11:10 - 12:50 Symposium 3: Oogenesis: the molecular basis behind oocyte growth, egg quality, fertilization and embryo development
- 12:50 - 14:00 Lunch
- 14:00 - 18:30 Social event at Frasassi caves

Thursday, September 10th

- 09:00 - 10:50 Symposium 4: Progress and perspectives on fish gametes cryopreservation
- 10:50 - 11:10 Coffee break
- 11:10 - 12:50 Symposium 4: Progress and perspectives on fish gametes cryopreservation
- 12:50 - 14:00 Lunch
- 14:00 - 15:50 Symposium 5: Fish germ cell: from basic sciences to applied biotechnologies
- 15:50 - 16:10 Coffee break
- 16:10 - 17:50 Symposium 5: Fish germ cell: from basic sciences to applied biotechnologies
- 17:50 - 19:00 Poster session II
- 20:00 - 23:00 Social dinner at Passetto Restaurant

Friday, September 11th

- 09:00 - 11:10 Symposium 6: Reproduction and development in ornamental fish and invertebrates
- 11:10 - 11:30 Coffee break
- 11:30 - 12:30 Symposium 7: Epigenetic programming: from gametes to embryo
- 12:30 - 12:45 Closing remarks and awards ceremony

SCIENTIFIC PROGRAMME

Monday, September 7th

14:00-18:00 Congress registration (Arcade of the Faculty of Economics)

15:00-18:00 Aquagamete MC meeting (Room T37)

18:00-18:30 Opening and Authorities' greetings (Room A1)

18:30-20:00 Welcome party (Arcade of the Faculty of Economics)

Tuesday, September 8th

08:00-09:00 Congress registration (Arcade of the Faculty of Economics)

***Symposium 1*: Spermatology: basic and strategic research leading to high quality sperm** - Room A1

Chairs: Viveiros A, Mylonas C.

09:00-09:30 #SOA1 - STATE OF THE ART - **Vanesa Robles*,** Paz Herráez, Catherine Labbé, Elsa Cabrita, Martin Psenicka, David G. Valcarce, Marta F Riesco.
MOLECULAR BASIS OF SPERM QUALITY.

09:30-09:50 #O1 - **Meiri-Ashkenazi I**, Solomonovich R, Rosenfeld H*.
LONG TERM EFFECTS OF MASCULINIZING TREATMENTS ON THE REPRODUCTIVE CHARACTERISTICS OF GREY MULLET (*Mugil cephalus*).

09:50-10:10 #O2 - **Vílchez MC**, Morini M, Peñaranda DS, Gallego V, Asturiano JF, Pérez L*.
THE ROLE OF ION SODIUM IN THE EUROPEAN EEL SPERM MOTILITY.

10:10-10:30 #O3 - **Dietrich MA**, Dietrich GJ, Mostek A, Ciereszko A*.
CHANGES IN CARP SPERM PROTEOME AFTER MOTILITY ACTIVATION.

10:30-10:50 #O4 - **Peñaranda DS,** Gallego V, Caccia R, Vílchez MC, Pérez L, Gómez A, Giménez I, Asturiano JF*.
FIRST FULL SPERMATOGENESIS AND SPERMIATION INDUCED WITH SPECIFIC RECOMBINANT GONADOTROPINS IN A TELEOST FISH, THE EUROPEAN EEL.

10:50-11:10 Coffee break

11:10-11:30 #O5 - **Tunçelli G***, Memiş D.
EFFECTS OF TWO DIFFERENT WATER RESOURCES ON SPERM QUALITY OF BROODSTOCK OF TROUT (*Salmo coruhensis*).

11:30-11:50 #O6 - **Dzyuba B***, Cosson J, Dzyuba V, Bondarenko O, Boryshpolets S, Fedorov P, Rodina M, Linhart O.
SPECIFIC SPERM MATURATION DETERMINED BY UROGENITAL SYSTEM ANATOMY IN STURGEON.

11:50-12:10 #O7 - **Fedorov P***, Dzyuba B, Fedorova G, Grabic R, Martínez-Pastor F, Cosson J, Dzyuba V, Rodina M.
MACROERGIC PHOSPHATES CONTENT AND CLUSTER ANALYSIS OF MOTILITY IN SPERMATOZOA OF WHITEFISH *Coregonus lavaretus maraena* IN RESPONSE TO ENVIRONMENT OSMOLALITY.

12:10-12:30 #O8 - Nynca J*, Dietrich GJ, Liszewska E, Judycka S, Karol H, Dobosz S, Krom J, **Ciereszko A**.

USEFULNESS OF PORTABLE FLOW CYTOMETER FOR SPERM CONCENTRATION AND VIABILITY MEASUREMENTS OF RAINBOW TROUT SPERMATOZOA.

12:30-12:50 #O9 **- Levavi-Sivan B***, Naama M, Jakob B, Matan G.
HYPOTHALAMIC REGULATION OF THE RELEASE OF FSH AND LH.

12:50-14:00 Lunch

Symposium 2 **- Anthropogenic contaminants in the environment: effects on fish gametes**

Chairs: Weltzien FA, Kestemont P.

14:00-14:30 #SOA2 **-** STATE OF THE ART **- Hamid R Habibi*.**
ANTHROPOGENIC CONTAMINANTS IN THE ENVIRONMENT: EFFECTS ON FISH GAMETES.

14:30-14:50 #O10 **-** Agbohessi P, Toko II, **Kestemont P***.
PESTICIDES USED IN COTTON PRODUCTION AFFECT EMBRYO-LARVAL STAGE, REPRODUCTIVE DEVELOPMENT, ENDOCRINE REGULATION AND OFFSPRING FITNESS IN AFRICAN CATFISH *Clarias gariepinus* (Burchell, 1822).

14:50-15:10 #O11 **-** Rojo-Bartolomé I, **Cancio I***.
DUPLICATION OF THE TRANSCRIPTION FACTOR IIIA (TFIIIA) GENE IN TELEOST GENOMES, AND OOCYTE-SPECIFIC TRANSCRIPTION OF TFIIIAb: APPLICATIONS IN THE ENVIRONMENTAL MONITORING OF XENOESTROGENICITY.

15:10-15:30 #O12 **- Valencia A**, Rojo-Bartolomé I, Bizarro C, Cancio I, Ortiz-Zarragoitia M*.
ALTERATION IN MOLECULAR MARKERS OF OOCYTE DEVELOPMENT AND INTERSEX CONDITION IN MULLETS IMPACTED BY WASTEWATER TREATMENT PLANT EFFLUENTS.

15:30-15:50 #O13 **- Maradonna F**, Santangeli S, Kocikowski M, Carnevali O*.
EFFECTS OF ENVIRONMENTAL PLASTICIZERS ON ZEBRAFISH MALE REPRODUCTION.

15:50-16:10 Coffee break

Side Event

Chairs: Asturiano JF, Vilella S.

16:10-16:30 #SE1 **- Borini A.**
USE OF ZEBRAFISH MODEL FOR BUILDING UP THE KNOWLEDGE TO IMPROVE HUMAN ASSISTED REPRODUCTIVE TECHNOLOGY.

16:30-16:50 #SE2 **- Rosenthal H.**
DO WE NEED TO MAKE SCIENCE QUALITY EVALUATION MORE SCIENTIFIC? THE PROS AND CONS OF VARIOUS COMMONLY USED ASSESSMENT INDICATORS.

16:50-17:10 #SE3 **- Weltzien FA.**
IMPRESS: IMPROVED PRODUCTION STRATEGIES FOR ENDANGERED FRESHWATER SPECIES.

17:10-19:00 POSTER SESSION I

Wednesday, September 9th - Room A1

Symposium 3 **- Oogenesis: the molecular basis behind oocyte growth, egg quality, fertilization and embryo development**

Chairs: Lubzens E, Cancio I.

09:00-09:30 **#SOA3 - STATE OF THE ART -** Xin Qi, Wenyi Zhou, **Chun Peng.**
PARACRINE REGULATION OF FOLLICLE DEVELOPMENT IN ZEBRAFISH.

09:30-09:50 **#O14 - Yilmaz O**, Com E, Lavigne R, Pineau C, Bobe J*.
PROTEOMICS OF EGG QUALITY IN ZEBRAFISH.

09:50-10:10 **#O15 - Schaerlinger B***, Staub C, Touze JL, Chardard D, Krauss D, Ledore Y, Alix M, Fontaine P.
SEX DETERMINATION AND EGG QUALITY EVALUATION IN THE EURASIAN PERCH, *Perca fluviatilis*, USING ULTRASONOGRAPHY.

10:10-10:30 **#O16 - Rojo-Bartolomé I**, Martínez-Miguel L, Lafont AG,Peñaranda DS, Vílchez MC, Asturiano JF, Pérez L, Cancio I*.
RIBOGENESIS MOLECULAR MARKERS OF OOCYTE DIFFERENTIATION IN EUROPEAN EEL *Anguilla anguilla*: TRANSCRIPTIONAL REGULATION DURING ARTIFICIALLY INDUCED OOGENESIS.

10:30-10:50 **#O17 - Mylonas C***, Salone S, Biglino T, de Mello PH, Fakriadis I, Sigelaki I, Duncan N.
OPTIMIZED SPAWNING INDUCTION PROTOCOL FOR MEAGRE (*Argyrosomus regius*) USING WEEKLY INJECTIONS OF GnRHa.

10:50-11:10 Coffee break

11:10-11:30 **#O18 - Siddique M***, Linhart O, Krejszeff S, Żarski D, Pitcher TE, Butts IAE.
GAMETE BIOCHEMISTRY AND GENETIC ARCHITECTURE ON THE RATE OF EYED EMBRYOS IN A EXTERNALLY FERTILIZING FRESHWATER FISH, IDE *Leuciscus idus*.

11:30-11:50 **#O19 - Żarski D***, Palińska-Żarska K, Łuczyńska J, Krejszeff S.
THE EFFECT OF HORMONAL TREATMENT ON EMBRYONIC SURVIVAL, PROXIMATE COMPOSITION AND FATTY ACID PROFILE OF EURASIAN PERCH, *Perca fluviatilis* L., EGGS.

11:50-12:10 **#O20 - Bouleau A**, Pasquier J, Desvignes T, Traverso JM, Nguyen TV, Chesnel F, Fauvelle C, Bobe J*.
CONTRIBUTION OF NUCLEOPLASMIN GENES TO EGG DEVELOPMENTAL COMPETENCE IN FISH.

12:10-12:30 **#O21 - Mandiki R***, Milla S, Nkogo Robles S, Kestemont P.
CORTICOSTEROIDS DEEPLY DEPRESS THE *in vitro* STEROIDOGENIC CAPACITY OF PERCH OVARY AT THE END OF THE REPRODUCTIVE CYCLE.

12:30-12:50 **#O22 - Du H**, Zhang S, Luo J, Li P, Shen L, Qiao X, Liu Z, Wei Q*.
SEX STEROID AND ULTRASOUND DIAGNOSIS OF MATURATION IN CAPTIVE CHINESE STURGEON *ACIPENSER SINENSIS.*

12:50-13:30 Group photograph and lunch bags collection

13:30-18:30 ***Social event at Frasassi Caves***

Thursday, September 10th - Room A1

Symposium 4 **- Fish germ cell: from basic sciences to applied biotechnologies**

Chairs: Cabrita E, Pšenička M.

09:00-09:30 **#SOA4 - STATE OF THE ART - Goro Yoshizaki***, Seungki Lee.
PRODUCTION OF VIABLE TROUT OFFSPRING DERIVED FROM FROZEN WHOLE FISH.

09:30-09:50 **#O23 - Curran E**, Gautier A, Saito T, Patinote A, Depincé A, Labbé C, Le Gac F, Lareyre JJ*.
MOLECULAR SIGNATURE AND STEMNESS PROPERTIES OF PAIRED SPERMATOGONIA IN ZEBRAFISH.

09:50-10:10 **#O24 - Pšenička M***, Saito T, Pocherniaieva K, Rodina M.
CRYOPRESERVATION OF TESTICULAR AND OVARIAN CELL SUSPENTION VS WHOLE TISSUE IN STURGEON.

10:10-10:30 **#O25 - Iegorova V***, Psenicka M, Saito T.
ABNORMAL CLEAVAGE PATTERNS PRODUCE HAPLOID/DIPLOID MOSAICISM IN STURGEON (*Acipenser ruthenus*).

10:30-10:50 **#O26 - Leininger S***, Wargelius A, Skaftnesmo KO, Kleppe L, Andersson E, Taranger GE, Schulz R, Edvardsen RB.
A CANDIDATE GENE APPROACH TO PRODUCE STERILE, GERM CELL-FREE ATLANTIC SALMON BY CRISPR-CAS9 MEDIATED GENOME EDITING.

10:50-11:10 Coffee break

11:10-11:30 **#O27 - Saito T***, Psenicka M.
STERILIZATION OF STERLET STURGEON (*Acipenser ruthenus*) EMBRYOS BY ULTRAVIOLET IRRADIATION.

11:30-11:50 **#O28 - Güralp H***, Pocherniaieva K, Blecha M, Policar T, Pšenička M, Saito T.
EMBRYOGENESIS AND PRIMORDIAL GERM CELLS DEVELOPMENT IN PIKEPERCH, *Sander lucioperca.*

11:50-12:10 **#O29 - Linhartová Z***, Saito T, Kašpar V, Rodina M, Prašková E, Hagihara S, Pšenička M.
STERILIZATION OF STERLET *Acipenser ruthenus* BY USING KNOCK DOWN AGENT, ANTISENSE MORPHOLINO OLIGONUCLEOTIDE, AGAINST DEAD END GENE.

12:10-12:30 **#O30 - Lubzens E*.**
STAGNATION IN FISH CRYOBIOLOGY RESEARCH? THINK OF DESICCATION.

12:30-12:50 **#O31 - Miccoli A**, Falcinelli S, Gioacchini G, Maradonna F, Skobo T, Dalla Valle L, Carnevali O*.
MATERNAL FACTORS MODULATING *Danio rerio* DEVELOPMENT ARE AFFECTED BY BENEFICIAL BACTERIA.

12:50-14:00 Lunch

Symposium 5 **- Progress and perspectives on fish gametes cryopreservation**

Chairs: Horváth A, **Ciereszko A**.

14:00-14:30 **#SOA5 - STATE OF THE ART - Juan F. Asturiano***, Elsa Cabrita, Ákos Horváth.
PROGRESS, CHALLENGES AND PERSPECTIVES ON FISH GAMETE CRYOPRESERVATION.

14:30-14:50 **#O32 - Horokhovatskyi Y***, Dzyuba B, Rodina M, Cosson J, Linhart O.
INVESTIGATION OF VARIABILITY OF COOLING PROFILES IN TWO FREEZING METHODS APPLIED TO STERLET *Acipenser ruthenus* SPERM CRYOPRESERVATION.

14:50-15:10 **#O33 - Marinovic Z***, Lujić J, Bernáth G, Urbányi B, Horváth A**.**
CRYOPRESERVATION OF TENCH *Tinca tinca* L. ISOLATED TESTICULAR CELLS.

15:10-15:30 **#O34 - Bernáth G***, Kása E, Szentes K, Staszny A, Várkonyi L, Kollár T, Hegyi A, Bokor Z, Urbányi B, Horváth A.
IMPROVEMENT OF COMMON CARP (*Cyprinus carpio*) SPERM CRYOPRESERVATION: THE APPLICABILITY OF A CONTROLLED-RATE FREEZER AS A STANDARD DEVICE.

15:30-15:50 **#O35 - Horváth A***, Bernáth G, Bokor Z, Kása E, Ősz A, Urbányi B, Cabrita E, Gavaia P, Snoj A, Bravničar N, Jesenšek D.
PROGRESS TOWARDS STANDARDIZATION OF CRYOPRESERVATION METHODS FOR SALMONID SPECIES OF THE ADRIATIC DRAINAGE BASIN.

15:50-16:10 Coffee break

16:10-16:30 **#O36 - Félix A**, Riesco MF, Martinez-Páramo S, Matias D, Joaquim S, Soares F, Suquet M, Cabrita E*.
PRELIMINARY STUDIES ON PORTUGUESE OYSTER (*Crassostrea angulata*) SPERM CRYOPRESERVATION.

16:30-16:50 **#O37 - Dietrich G***, Judycka S, Dobosz S, Ciereszko A.
EFFECT OF DILUTION OF FROZEN/THAWED SPERM OF SEX-REVERSED RAINBOW TROUT ON SPERM MOTILITY AND FERTILIZING ABILITY DURING POST-THAW STORAGE**.**

16:50-17:10 **#O38 - Kása E***, Bernáth G, Kollár T, Żarski D, Lujić J, Marinović Z, Bokor Z, Hegyi A, Urbányi B, Vílchez MC, Morini M, Peñaranda DS, Pérez L, Asturiano JF, Horváth A.
VITRIFICATION OF FISH SPERM.

17:10-17:30 **#O39 - Viveiros A***, Di Chiacchio IM, Almeida ILG, Taffarel TR.
STORAGE AND TRANSPORTATION OF *Prochilodus lineatus* (Characiformes) SPERM PRIOR TO CRYOPRESERVATION.

17:30-17:50 **#O40 - Bozkurt J***, Yavaş I, Bucak MN.
CRYOPRESERVATION OF NILE TILAPIA (*Oreochromis niloticus*) SPERMATOZOA: EFFECT OF EXTENDER SUPPLEMENTED WITH DIFFERENT CRYOPROTECTANTS ON POST-THAW SPERM MOTILITY, VIABILITY, DNA DAMAGE AND FERTILIZATION.

17:50-19:00 **POSTER SESSION II**

20:00-23:00 ***Social dinner at Passetto Restaurant***

Friday, September 11th - Room A1

Symposium 6 **- Reproduction and development in ornamental fish and invertebrates**

Chairs: Suquet M, Maradonna F.

09:00-09:30 **#SOA6 - STATE OF THE ART - Ike Olivotto***, Giulia Chemello,Chiara Carla Piccinetti, Oliana Carnevali.
MARINE ORNAMENTAL SPECIES CULTURE: THE PAST, THE PRESENT AND THE FUTURE.

09:30-09:50 **#O41 - Suquet M***, Gourtay C, Malo F, Le Goïc N, Quere C, Donval A, Le Grand J, Ratiskol D, Fauvel C.
THE QUALITY OF GREAT SCALLOP (*Pecten maximus*) SPERM AFTER THAWING.

09:50-10:10 **#O42 - Cosson J*.**
SPERM GUIDANCE STRATEGIES IN BROADCAST SPAWNERS.

10:10-10:30 **#O43 - Pocherniaieva K**, Sidova M, Psenicka M, Saito T, Kaspar V*.
qPCR TOMOGRAPHY OF THE *Acipenser ruthenus* OOCYTE.

10:30-10:50 **#O44 -** Erdogan M, **Arslan T*.**
EFFECTS OF VITAMIN E ON REPRODUCTIVE PERFORMANCE OF PINDANI (*Pseudotropheus socolofi* Johnson, 1974).

10:50-11:10 **#O45 - Prokopchuk G***, Dzyuba B, Rodina M, Cosson J.
FLAGELLAR BEHAVIOR OF FISH SPERM AT MOTILITY INITIATION.

11:10-11:30 Coffee break

Symposium 7 **- Epigenetic programming: from gametes to embryo**

Chairs: Labbé C, Linhart O.

11:30-11:50 **#O46 - Juanchich A**, Skatnesmo KO, Thermes V, Bobe J, Wargelius A*.
ANALYZING MICRO-RNA REPERTOIRES IN EGGS FROM 4 FISH SPECIES CAN REVEAL SECRETS OF BOTH LIFE HISTORY TRAITS AND EMBRYO VIABILITY/QUALITY.

11:50-12:10 **#O47 - Santangeli**,S, Cobellis G, Gioacchini G, Maradonna F, Carnevali O*.
ENVIRONMENTAL DOSES OF BPA INHIBIT ZEBRAFISH REPRODUCTION THROUGH A DEREGULATION OF EPIGENETIC PATTERN.

12:10-12:30 ***Closing remark and awards ceremony***
Rosenthal H.

POSTER SESSION (Arcade of the Faculty of Economics)

17:10-19:00 POSTER SESSION I (Tuesday, September 8th)

Anthropogenic contaminants in the environment: effects on fish gametes

#P01. Nathanailides C*, Perdikaris C, Chantzaropoulos A, Kokokiris L, Barbouti A.
EFFECT OF TRICLOSAN ON SPERM QUALITY PARAMETERS AND VITELLOGENIN LEVELS OF GOLDFISH.

#P02. Bonaldo M, Ramoino P, Traversi I, Viganò L, Mandich A*.
EFFECTS OF THE EXPOSURE TO POLLUTED RIVER SEDIMENTS ON THE DEVELOPMENT OF THE GONADS OF BARBEL: MORPHOLOGICAL ENDPOINTS.

#P03. von Krogh C, Andersen IG, Weltzien FA*, Schulz RW, Erik Ropstad E. EFFECTS OF BISPHENOL A OR TETRABROMOBISPHENOL A EXPOSURE ON SPERMATOGENESIS AND STEROIDOGENESIS IN A ZEBRAFISH (*Danio rerio*) EX VIVO TESTICULAR MODE

#P04.Forner Piquer I, Santangeli S, Zanardini M, Maradonna F, Carnevali O*. DINP AS ENDOCRINE DISRUPTOR: THE CASE OF ENDOCANNABINOID SIGNALING IN REPRODUCTIVE SYSTEM OF *Danio rerio.*

Progress and perspectives on fish gametes cryopreservation

#P05. Bozkurt Y*, Yavaş I.
EFFECT OF STRAW VOLUMES AND THAWING RATES ON MOTILITY, VIABILTY AND FERTILIZATION CAPACITY OF CRYOPRESERVED COMMON CARP (*Cyprinus carpio*) SPERM.

#P06. Bozkurt Y*, Yavaş I.
EFFECT OF DOCOSAHEXAENOIC ACID (DHA) ON MOTILITY, VIABILITY AND FERTILIZATION ABILITY OF CRYOPRESERVED BROWN TROUT (*Salmo trutta macrostigma*) SEMEN.

#P07. França TS*, Leal MC, Almeida ILG, Isaú ZA, Viveiros A. COMPOSITION AND OSMOLALITY OF THE ACTIVATING AGENT ON FRESH SPERM QUALITY OF *Prochilodus lineatus.*

#P08. Nogueira Vasconcelos AC*, Freitas Garcia RR, Varela AS Jr, Goulart K, Corcini CD, Cabrita E, Streit DPJr.
PRELIMINARY RESULTS OF CRYOPRESERVATION EFFECT ON THE SEMINAL PROTEIN PROFILE OF *Colossoma macropomum.*

#P09. Nogueira Vasconcelos AC*, Oliveira Felizardo V, Ferreira Souza de Carvalho A, Freitas Garcia RR, Solis Murgas LD, Streit DPJr.
EFFECT OF CRYOPROTECTANTS COMBINED IN SPERM CELLS OF *Prochilodus lineatus*.

#P10. Judycka S*, Szczepkowski M, Ciereszko A, Dietrich GJ.
OPTIMAL GLUCOSE CONCENTRATION IN EXTENDER IS CRUCIAL FOR SUCCESSFUL CRYOPRESERVATION OF STERLET (*Acipenser ruthenus* L.) SPERM.

#P11. Isau ZA*, Leal MC, Almeida ILG, França TS, Viveiros ATM.
EFFECTS OF COMPOSITION AND OSMOLALITY OF THE ACTIVATING AGENT ON POST-THAW SPERM QUALITY OF *Prochilodus lineatus*.

#P12. Streit PD Jr*, Marques L, de Godoy LC, Silva L, Maschio D, Zhang T, Bos-Mikich A.
VIABILITY OF ZEBRAFISH (*Danio rerio*) OVARIAN FOLLICLES AFTER VITRIFICATION.

#P13. Asturiano JF*, Riesco MF, Martins G, Vílchez MC, Pérez L, Gavaia PJ, Cabrita E.
CRYOPRESERVATION OF ZEBRAFISH SPERM, FIRST TRIALS AND RESULTS.

#P14. Lujić J*, Bernáth G, Marinović Z, Radojković N, Simić V, Ćirković M, Urbányi B, Horváth A.

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#P15. Kollár T*, Horváth A, Labesse C, Milon P, Csenki Z, Kovács R, Bernáth G, Béres T, Depincé A, Urbányi B, Labbé C.
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#P16. Sanches EA*, Caneppele D, de Camargo TW, da Cunha Pinto A, da Rocha Brande M, Romagosa E.
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#P17. Sarosiek B*, Dryl K, Palińska-Żarska K, Żarski D.
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#P20. Ninhaus-Silveira A*, Bashiyo-Silva C, da Silva Costa R, de Castro Ribeiro D, Senhorini JA, Veríssimo-Silveira R.
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#P21. Freitas Garcia RR*, Streit Jr DP, Salomão PE, Corcini CD, Cabrita E, Varela AS Jr.
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#P22. Król J*, Krejszeff S, Żarski D, Palińska-Żarska L, Kucharczyk D.
EFFECT OF TIME AFTER HORMONAL STIMULATION ON SPERMATOZOA MOTILITY AND SHORT-TERM STORAGE OF SEMEN GOLDFISH *Carassius auratus* (L.).

#P23. Król J, Żarski D, Krejszeff S, Palińska-Żarska K.
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#P24. Król J*, Krejszeff S, Żarski D, Palińska-Żarska K, Stańczak K, Stabińska A, Łączyńska B, Hliwa P.
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#P25. Bondarenko O*, Yoshida M, Yoshida M, Ono C, Dzyuba B, Cosson J.
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#P27. Dziewulska K*, Domagała J, Pilecka-Rapacz M.
COMPARATIVE CHARACTERISTICS OF SPERMATOZOA MOTILITY TRAITS IN SOME SPECIES OF CYPRINIDS.

#P28. Dadras H*, Dzyuba B, Cosson J, Linhart O.
COMPARATIVE ANALYSIS OF SPERM MOTILITY OF THREE SPECIES, *Cyprinus carpio*, *Oncorhynchus mykiss* AND *Acipenser ruthenus* AT VARIOUS TEMPERATURES.

#P29. Dzyuba V*, Dzyuba B, Cosson J, Rodina M.
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#P30. Gazo I*, Chenevert J, Dietrich MA, Cosson J.
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#P31. Valcarce DG, Chereguini O, Herráez MP, Rodríguez C, Robles V*.
OPTIMAL SPERM SUBPOPULATION SELECTION IN *Solea senegalensis.*

#P32. Shaliutina-Kolesova A*, Cosson J, Lebeda I, Dzuyba B, Shaliutina O, Gazo I, Linhart O.
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#P33. Cabrita E*, Oliveira C, Riesco MF, Livramento M, Mañanós EL, Conceição LEC, Serradeiro R.
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#P34. Vílchez MC*, Riesco MF,. Asturiano JF, Cabrita E, Pérez L.
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#P35. Vílchez MC*, Morini M, Peñaranda DS, Asturiano JF, Pérez L.
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#P36. Vílchez MC*, Pla D, Gallego V, Sanz L, Pérez L, Asturiano JF, Calvete JJ, Peñaranda DS.
IDENTIFICATION OF MAJOR PROTEINS FROM THE SEMINAL PLASMA OF HORMONE-INDUCED SEXUAL MATURED EUROPEAN EELS (*Anguilla anguilla*). CORRELATION WITH SPERM QUALITY.

#P37. Pérez L*, Riesco MF, Vílchez MC, Asturiano JF, Cabrita E.
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#P38. Zilli L, Schiavone R, Vilella S*.
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#P39. Burow S, Hollander L, Shpilman M, Nourizadeh-Lillabadi R, Weltzien FA*, Levavi-Sivan B.
DEVELOPMENT OF SPECIFIC ELISAS FOR MEDAKA (*Oryzias latipes*) GONADOTROPINS LH (LUTEINIZING HORMONE) AND FSH (FOLLICLE-STIMULATING HORMONE) USING RECOMBINANT PROTEINS.

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Epigenetic programming from gametes to embryo

#P40. Shih YA, Gwo JC, Juang BT*.
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#P41 de Mello F*, Garcia JS, de Godoy LC, Depince A, Labbé C, Streit DP Jr.
CRYOPROTECTANTS ALTER DNA METHYLATION PATTERN OF SPERMATOZOA AND DECREASE REPRODUCTIVE SUCCESS IN *Colossoma macropomum*.

Fish germ cell from basic sciences to applied biotechnologies

#P42. Nynca J*, Arnold GJ, Fröhlich T, Ciereszko A.
COMPARATIVE ANALYSIS OF BLOOD AND SEMINAL PLASMA PROTEOME OF RAINBOW TROUT.

#P43. Golpour A*, Pšenička M.
OPTIMIZATION OF STORAGE OF STURGEON TESTICULAR CELLS IN -80°C.

#P44. Leal MC*, de Godoy LC, Affonso EG, Costa GMJ, Lacerda SMSN, França LR.
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#P45. Poursaeid S*, Kalbassi MR, Hassani SN, Yoshizaki G, Baharvand H. SHORT-TERM *in vitro* EXPANSION OF SPERMATOGONIAL STEM CELLS IN CASPIAN BROWN TROUT (*Salmo trutta caspius*).

#P46. Damasceno DZ, Rodrigues ML, dos Santos Sanchez MS, Weiler KA, Frei GR, Feiden A, Boscolo WR, Signor A, Bittencourt F*.
CAN THE REPLACEMENT OF CORN BY SORGHUM SUPPLEMENTED WITH PHYTASE AFFECT THE GONADOSOMATIC AND HEPATOSOMATIC INDEXES OF SILVER CATFISH MALES KEPT IN CAGES?

#P47. Damasceno DZ, Sanches EA, Maccari GR, da Costa MRL, Damasceno DZ, Bilha Moro E, Alves Fernandes DR, Reidel A, Buglione Neto CC, Boscolo WR, Bittencourt F*, Romagosa E.
CAN ARGININE AFFECT THE REPRODUCTIVE RATES OF *Rhamdia quelen* FEMALES KEPT IN CAGES?

#P48. Kleppe L*, Edvardsen RB, Furmanek T, Andersson E, Wargelius A.
SCREENING FOR STERILITY CANDIDATE GENES IN ATLANTIC SALMON (*Salmo salar*).

#P49. Veríssimo-Silveira R*, Zonatto AB, de Siqueira-Silva DH, Ninhaus-Silveira A.
GONADAL MORPHOGENESIS IN *Brycon orbignyanus* (Characiformes, Characidae).

#P50. Kumari J*, Flaten GE, Škalko-Basnet N, Tveiten H.
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Oogenesis: the molecular basis behind oocyte growth, egg quality, fertilization and embryo development

#P51. Palomino J*, Rodríguez J, Hernández E, Martínez V.
CATHEPSINS EXPRESSION IN FLOATING AND NONFLOATING EARLY EMBRYOS IN THE YELLOW-TAIL KINGFISH *Seriola lalandi*.

#P52. Blecha M*, Svacina P, Kristan J, Lebeda I, Flajshans M, Policar T.
THE FIRST RESULTS OF HEAT SHOCK TRIPLOIDISATION IN PIKEPERCH (*Sander lucioperca*).

#P53. Domagała J, Kirczuk L*, Dziewulska K, Pilecka-Rapacz M.
SEXUAL CYCLE OF GONAD IN THE RUDD (*Scardinius erythrophthalmus*) IN RIVER SECTIONS INFLUENCED DIFFERENTLY BY THE HEATED EFFLUENTS FROM A POWER PLANT.

#P54. Linhart O*, Shelton WL, Tučková V, Rodina M, Siddique MAM. EFFECTS OF TEMPERATURE ON SHORT-TERM STORAGE OF STERLET (*Acipenser ruthenus*) OVA.

#P55. de Godoy LC*, Pereira Santos FB.
DEVELOPMENT OF STAINING TECHNIQUES TO ASSESS VIABILITY OF TAMBAQUI (*Colossoma macropomum*) OOCYTES.

#P56. Falahatkar B*, Ghiasi B, Arslan M.
EGGS AND LARVAL QUALITY OF STERLET STURGEON *Acipenser ruthenus* INJECTED WITH THIAMINE.

#P57. Fatira E, Psenicka M, Arai K, Saito T*.
NUCLEAR TRANSFER IN STERLET EGGS: A FIRST ATTEMPT.

#P58. de Cerio OD, Rojo-Bartolomé I, Valencia A, Ortiz-Zarragoitia M, Cancio I*.
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#P59. Pereira MM, Ribeiro MJP, Evangelista MM, Gisbert E, Romagosa E*.

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#P60. Ribeiro MJP, Evangelista MM, Pereira MM, Sanches EA, Tachibana L,. Gonçalves GS, Romagosa E*.
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#P61. Fujimoto T*, Nagasaka T, Aoki T, Arai K.
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#P62. Endoh M*, Fujimoto T, Arai K.
IMPROVED FERTILIZATION RATE BY TEMPORARY PRESERVATION IN ZEBRAFISH**.**

#P63. Dobroslavić T*, Mozara R, Bartulović V, Glamuzina B.
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#P64. Maud A*, Chardard D, Omid Feizbakhsh, Ledore Y, Schaerlinger B, Fontaine P.
HISTOLOGICAL STUDY OF EMBRYOGENESIS AND CLASSIFICATION OF DEVELOPMENTAL IMPAIRMENTS IN THE EURASIAN PERCH, *Perca fluviatilis*.

#P65. Du H*, Wu J, Wang C, Yang H, Wei Q.
ULTRASOUND ASSISTED DETERMINATION OF THE FINAL MATURATION OF HORMONE INDUCED SICHUAN TAIMEN *Hucho bleekeri* KIMURA, 1934.

#P66. Candelma M*, Colella S, Santojanni A, Radaelli G, Dalla Valle L, Carnevali O.
MOLECULAR CHARACTERIZATION AND RELATIVE mRNA EXPRESSION PROFILES OF GONADOTROPIN RECEPTORS IN EUROPEAN HAKE (*Merluccius merluccius*) DURING PUBERTY.

#P67. Miccoli A*, Leonori I, De Felice A, Estonba A, Carnevali O.
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#P68. Cejko BI*, Judycka S, Kujawa R.
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#P69. Fabbrocini A*, D'Adamo R, Maurizio D, Gasparro MR, de Oliveira LFJ, Silvestri F, de Sousa ECPM.
STANDARDIZATION OF A CRYOPRESERVATION PROTOCOL FOR THE GREEN SEA URCHIN *Lytechinus variegatus* SPERMATOZOA: EFFECTS OF CRYOPROTECTANTS ON SPERM MOTILITY.

#P70. Bernáth G*, Ittzés I, Szabó Z, Horváth A, Krejszeff S, Koncsek A, Lujić J, Urbányi B, Bokor Z.
SHORT-AND LONG-TERM PRESERVATION OF SPERM IN DIFFERENT GOLDFISH TYPES.

#P71. Marinović Z*, Petri E, Čelić A, Lujić J, Urbányi B, Horváth A.
THREE-DIMENSIONAL STRUCTURAL MODELS OF VASA PROTEIN IN SELECTED FISH SPECIES BY HOMOLOGY MODELING.

#P72. Kokokiris L*, Mylonas C, Minos G, Rosenfeld H, Nathanailides C, Monokrousos N, Papadaki M, Simeonidis C.
HORMONAL INDUCTION OF SPAWNING AND EGG PRODUCTION IN THICK LIPPED GREY MULLET, *Chelon labrosus.*

#P73. Kokokiris L*, Mylonas C, Minos G, Monokrousos N, Nathanailides C, Papadaki M, Simeonidis C.
SPERM CHARACTERISTICS AND ANDROGENS IN THICK LIPPED GREY MULLET (*Chelon labrosus*) AFTER ADMINISTRATION OF GnRHa IN THE FORM OF MULTIPLE INJECTIONS OR IMPLANTS.

#P74. Sevinç BM, Özdemir RC, Atmaca S, Ekici A*.
BROODSTOCK AND SPERM QUALITY DIFFERENCES IN GENETICALLY MODIFIED ZEBRAFISH AND WILD-TYPE ZEBRAFISH (*Danio rerio* HAMILTON-BUCHANAN, 1822).

#P75. Arturo Vargas*, Nuno Simões, Mascaró Maite.
ANALYSIS OF THE SURVIVAL OF NEWBORN SEAHORSES: PRELIMINARY RESULTS.

#P76. Olivotto I*, Piccinetti CC, Chemello G, Maradonna F.
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ABSTRACTS OF STATE OF ART AND ORAL PRESENTATIONS

Spermatology: basic and strategic research leading to high quality sperm

SOA1

MOLECULAR BASIS OF SPERM QUALITY

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INTRODUCTION

It is generally accepted that the best indicator for sperm quality is fertilization ability. Other parameters have been correlated with fertilization such as motility and sperm viability. Plasma membrane composition, mitochondrial functionality, spermatozoa metabolic activity, spermatozoa morphology and analysis of chromatin integrity have been also used in order to provide a general picture of sperm quality. But, taking into account the inherent ability of germ cells to transmit their genetic and epigenetic information to the progeny, it is necessary to introduce a molecular component in the equation of sperm quality assessment.

RNAs IN THE CONTROL OF SPERM PRODUCTION AND SPERM QUALITY

The specialized spermatozoa are the result of a complex differentiation process tightly controlled at transcriptional and epigenetic levels but also controlled at post-transcriptional level. Matured spermatozoa are transcriptionally inactive cells, but they have a pool of miRNAs and mRNAs remnant from spermatogenesis that have been pointed as crucial factors not only for fertility but also for a proper embryo development.

Low sperm quality could be reflecting a problem in spermatogenesis and differences in remnant molecules from such process could be good indicators of failures at crucial differentiation steps. In this sense, the study of RNAs in the control of sperm production and sperm quality takes special interest. It is known that germ cells of diverse animal species have the Chromatoid Body (CB) that is crucial in the control of postmeiotic differentiation of these cells, having an important function in mRNA storage, metabolism and even in the mRNA transport between nucleus and cytoplasm. CB moves during spermatogenesis stages. The composition of CB includes RNA-binding proteins such as VASA (ATP dependent DEAD-box RNA helicase) (Yuan et al. 2014) PIWI proteins (which bind to piRNAs) and RNAs (small RNAs and mRNAs) (Wang et al. 2009). Other proteins such as the kinesin K1F17b also accumulates in CBs (Nagamori and Sassone-Corsi 2008). The alteration of these CB components could result in the arrest of spermatogenesis.

Interestingly, the non-coding RNAs could also play an important role in epigenetic inheritance. It is well known that epigenetic information can be transgenerational transmited via germ cells, and piRNAs have been proposed as potential mediators of epigenetic transgenerational inheritance (Yadav and Kotaja 2014).

NEW TOOLS AND TECHNIQUES AT THE SERVICE OF SPERM QUALITY EVALUATION AND SPERM SUBPOPULATIONS SELECTION

The importance of the RNAs stored in the spermatozoa has been highlighted by recent studies, which confirmed (using microarrays and/or quantitative PCR) that the presence of certain RNAs is crucial for fertilization and for successful early embryo development (Guerra et al 2013). Quantitative PCR has been also used for lesion quantification in specific genes or genome regions (Cartón-García et al 2012). This methodology provides the perfect complementary tool for global DNA integrity assays. All these techniques provide novel and rigorous methods to

define molecular aspects leading to high sperm quality. But in addition to this, we need methods that provide, from a practical point of view, an efficient tool for selecting high quality spermatozoa. The presence of different spermatozoa subpopulations within the same seminal sample is an important point to take into account, particularly in low quality samples. Magnetic Activated Cell sorting has been recently used for selecting non-apoptotic spermatozoa from *Solea senegalensis* F1 low quality seminal samples. This method is based in the fact that Annexin V binds to phosphatidylserine, which is externalized to the outer surface of the sperm membrane in early apoptosis. Those spermatozoa linked to Annexin V-MACS beads are retained in the column, and non-apoptotic spermatozoa are recovered.

Finally, it is important to mention that new approaches, such as surrogate production, could be also understood as potential techniques to discover new key players in spermatogenesis. The possibility of xenotransplanting germinal cells, could allow the study within the same gonad of the interaction of germ cells and somatic cells from different species. To know these molecular interactions will undoubtedly help in the understanding of the molecular basis of sperm quality.

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O1

LONG TERM EFFECTS OF MASCULINIZING TREATMENTS ON THE REPRODUCTIVE CHARACTERISTICS OF GREY MULLET (*MUGIL CEPHALUS*)

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INTRODUCTION

The grey mullet, *Mugil cephalus,* are fished and farmed world-wide. To avoid the continuous pressure on the wild populations, we developed at IOLR-NCM a breeding protocol for captive mullets (Aizen *et al.* 2005). Following a successful domestication of this species, improved economics of production is expected by culturing all-female mullet populations, largely due to their highly prized roe used for preparing a seafood delicacy called "botarga/Karasumi". To achieve this goal, the current study has adopted the indirect feminization strategy (Piferrer 2001), involving the masculinisation of genotypic females, and crosses of the produced neomales with normal females, to produce a female monosex mullet population. Previous studies have demonstrated effective sex reversals in mullet using both an androgen to masculinize (Chang *et al*. 1999) and an oestrogen to feminize (Chang *et al*. 1995) the fish. However, there was no information whether the masculinized fish turned to be functional phenotypic males at maturation. Therefore, our specific objectives were to follow the sex-reversed fish to sexual maturity and to evaluate their phenotypic sexual stability and fertility.

MATERIALS AND METHODS

Experimental Fish - Hatchery produced mullet fry at our facility (IOLR-NCM) were maintained in 1- 5 m^3 tanks, supplied with ambient (Gulf of Eilat, Red Sea) seawater at 40 ppt salinity and subjected to natural fluctuations of photoperiod, light and temperature. Fish were fed daily at the rate of 1.5% of their body weight using a 35% crude protein and 7.2% lipid diet, according to our (IOLR-NCM) feed formulation and feeding regime.

Induced masculinization - A masculinized phenotype was obtained by exposing for 4 months, mullet fry, at 3 age categories (3, 6 and 9 month old), to food supplemented with methyltestosterone (MT; 15 or 10 mg MT/Kg of food). In order to study the phenotypic sexual stability, the 6 month old group that obtained the primary treatment of 15 mg MT/Kg food was divided randomly into two groups: one received repeated MT exposure via a slow release vehicle (administered via ethylene-vinyl acetate copolymer [EVAc] implants; 5 mg MT/pellet) at 2, 3 and 4 years after the primary treatment. The second group was used as untreated controls, which obtained only the primary treatment (see above). Sex was determined by vitellogenin dot blot analysis and/or gonadal biopsies as in Aizen *et al.* (2005). Presence of milt in male mullets was checked by applying gentle abdominal pressure and a sample of milt was collected to evaluate sperm motility, morphology and spermatocrit.

RESULTS AND DISCUSSION

As in wild stocks, the sex ratio among control mullet groups did not differ significantly ($P > 0.05$) from the expected 1:1 male vs. female ratio. These results rule out the occurrence of skewed sex ratios due to culture conditions, and are consistent with those of previous studies carried out in Taiwan (Chang *et al.* 1995, 1999) and North Carolina (Bichy, 2004). The results of the masculinizing studies further define, in mullets, the period of 6 to 9 month of age, as a labile phase when the differentiating gonads are most susceptible to androgens. The most potent treatment [MT-6] gave rise to 100 % males upon the completion of sexual differentiation. Nevertheless, at sexual maturity (3 years after treatment) markedly lower male percentages (70%) were observed in this group, suggesting that the grey mullet can spontaneously sex reverse. Interestingly, higher male percentages (90%) were detected among retreated fish boosted with MT containing EVAc implants. The milt produced by the latter fish revealed

characteristics (i.e. sperm count, motility and morphology) resembling those found for untreated males.

CONCLUSIONS

The grey mullet appears to have high sexual plasticity also away from the sex-differentiation period, which is atypical to most other gonochoric fish that exhibit fixed sexuality once sex differentiation is being completed. Similar sexual plasticity was documented also in trout (Yoshizaki *et al.* 2010) which attests to a spontaneous sex reversal and stresses the need to preserve functional phenotypic sex by repeated treatments. Besides their aquaculture applicability, our results propose the grey mullet as an important model for further studying germ cells plasticity in fish.

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O2

THE ROLE OF ION SODIUM IN THE EUROPEAN EEL SPERM MOTILITY

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INTRODUCTION

In marine teleosts, the osmotic shock faced by the spermatozoa when they are released in the marine environment leads to a rapid flux of ions and water across the sperm membrane which activates the sperm motility (Morisawa, 2008), but the exact mechanism through which this happens is still unknown. In mammals sperm it has been demonstrated the presence of epithelial sodium channels (ENaC), and a decrease in intracellular sodium during sperm capacitation (Escoffier et al. 2012). Thus, Na^+ seems to have some role on mammal sperm motility. However, the role of Na^+ fluxes on fish sperm motility has been poorly studied, and intracellular Na^+ changes related to sperm motility have been determined only for a freshwater species, the common carp (Krasznai et al. 2003). In the present work, a method for the quantitative analysis of $[Na^+]_i$ was set up for eel sperm, and with this method it have been studied by first time, the variations in $[Na^+]_i$ during the motility activation in a marine species, the European eel (*Anguilla anguilla*).

MATERIALS AND METHODS

Male eels were matured with hCGrec weekly injections (1.5 IU/g b.w.). After 10 weeks of treatment sperm samples were extracted and maintained at 4 ºC until use. Sperm motility was determined after activation with sea water and only those samples having >50% of motile cells were selected for the following trials.

Trial 1: Quantification of intracellular sodium. The response calibration of $[Na^+]_i$ was performed measuring the fluorescence intensity of the CoroNa Green indicator by Flow Cytometry (Cytomics FC500). Sperm cells were suspended in solutions with precisely known free Na^+ concentrations, in presence of 20 µM of ionophore monensin, which equilibrate internal $[Na^+]$ with external concentrations. The dissociation constant for the indicator-ion complex (K_d) was determined using the following equation, where F denotes the fluorescence intensity and the subscripts *min* and *max* identify the data points corresponding to the minimum and maximum ion concentrations, respectively.

$$[Na^+] = K_d\ (F - Fmin)/(Fmax - F)$$

Once known K_d, the same equation was used to obtain $[Na^+]$ values corresponding to measured fluorescence intensities (F) of experimental samples.

Trial 2: Role of ion sodium in sperm motility. Sperm samples were washed in sodium-free extender, or in control extender (P1, Peñaranda et al. 2010, containing sodium), and sperm motility was activated in presence (with seawater, SW) or absence of sodium (in choline chloride, Na-free activator). The effect of the ionophore monensin was also studied in these conditions. Sperm motility and morphometric analysis were carried out using a CASA/ASMA software (ISAS, Proiser R+D, S.L., Spain), and the intracellular Na^+ fluorescence was evaluated by Flow Cytometry (Cytomics FC500).

RESULTS

Trial 1. The fluorescence emission response of CoroNa Green in sperm suspensions increased its intensity as a function of increasing $[Na^+]$. The K_d calculated from these data (58.25 mM) was used to calculate the values of intracellular sodium ($[Na^+]_i$) in quiescent state (96.72 mM) and post-activation with seawater (152.21 mM).

Trial 2. The elimination of extracellular Na^+ by washing reduced the sperm motility (Fig. 1A), in a non-reversible way. All the samples (washed with/without extracellular Na^+) showed a significant increase of $[Na^+]_i$ post-activation with SW

or Na-free activator (Fig. 1B). This increase of $[Na^+]_i$ after activation, coincided with a significant decrease of the area of the cells.

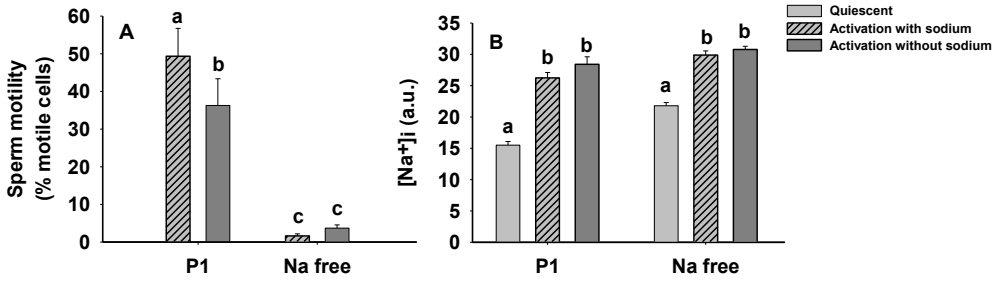

Fig. 1: A) Percentage of motile spermatozoa after washing in control extender (P1) or Na-free extender and activated with or without sodium. **B)** Emitted fluorescence by intracellular Na^+, on quiescent sperm after washing in control extender (P1) or Na-free extender and activated with or without sodium. Data are expressed as mean ± SEM (n= 9) and different letters indicate significant differences ($P<0.05$) between activation media.

DISCUSSION AND CONCLUSIONS

This work determines by first time the $[Na^+]_i$ concentration before and after sperm activation in the European eel. European eel sperm showed higher values of $[Na^+]_i$ in comparison with those reported by Krasznai et al. (2003) in carp sperm. Also, during post-activation, $[Na^+]_i$ increased significantly in contrast to what was reported in carp (Krasznai et al. 2003). As $[Na^+]_i$ increased post-activation even in absence of Na^+ in the extender and the activator, the only explanation for that increase is the reduction in cell volume, which has been probed by sperm size measurements before and after activation.

The present results confirm that: a) the presence of Na^+ in the extender medium is important for the preservation of the sperm capacity for further motility post-activation, b) the presence/absence of Na^+ in the activation medium does not affect sperm motility parameters, and c) although $[Na^+]_i$ increases after activation, this increase does not seem related with the sperm motility, as it happens even in samples with low motility, like those washed in Na-free extender.

ACKNOWLEDGEMENTS

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O3

CHANGES IN CARP SPERM PROTEOME AFTER MOTILITY ACTIVATION

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INTRODUCTION

In freshwater cyprinid teleosts spermatozoa are quiescent at osmolarity of seminal plasma and sperm motility is initiated by the hypoosmotic shock after discharge into the aqueous environment. In carp, extracellular Ca^{2+} is a prerequisite for the initiation of sperm motility. The flagellar axoneme is regulated by calcium/calmodulin-dependent protein phosphorylation (Krasznai et al. 2000). To the best of our knowledge only a few proteins involved in the initiation of fish sperm motility have been identified. Recently, proteomic approach were introduced to study activation of sperm motility in sea bream and allowed to identify three proteins that play a key role in the initiation of sperm motility (Zili et al., 2008). However, it is unknown at present which proteins are involved in carp sperm motility activation process. Therefore the present study was undertaken in order to examine the changes in carp sperm proteome after activation using 2D-DIGE approach.

MATERIALS AND METHODS

Semen from six carp (*Cyprinus carpio* L) males were diluted with immobilizing solution 1:4 (Rurangwa et al. 2001), then one part was activated with hatchery water at 1:19 ratio while the other was diluted at the same ratio with immobilizing solution. After 30 s, samples were centrifuged at 1:1500 x g for 30 min. and lysis buffer was added to the sperm pellet for protein extraction. After sonication (5x5s), sperm extract was centrifuged and obtained supernatant was used for future analysis. Immobilized spermatozoa (n=6) and activated spermatozoa (n=6) was labeled with fluorescent dyes (Cy3, Cy5, Cy2, GE Healthcare) and applied to two-dimensional electrophoresis (2-DE) on 18 cm gel strips (pH 3-10 NL). After isoelectrofocusing, strips were equilibrated with dithiothreitol and iodoactetamide, and then SDS-PAGE was performed. The gels were analyzed using DeCyder software (GE Healthcare), selected protein spots were excised, digested with trypsin and subjected to mass spectrometry MALDI-TOF/TOF (Bruker, Germany). The MS/MS data were searched with Mascot against the NCBIr Cyprinidae databases. CASA system was used to measure sperm motility parameters after activation. Moreover immobilized and activated spermatozoa after 2DE were stained with Pro-Q Diamond (Life Technology) for detection of phosphorylated proteins. This work is currently underway to identify phosphorylated proteins.

RESULTS

The quantitative comparison of proteome profile between immobilized and activated spermatozoa yielded 91 protein spots significantly changed in abundance during sperm motility activation (56 protein spots increased in abundance after motility activation, $p < 0.05$ with FDR correction). We successfully identified 77 protein spots represented 50 proteins (Tab. 1). The majority of identified proteins (21) were involved in metabolism and energy production. Within this group enzymes of major metabolic pathways were recognized, including glycolysis, tricarboxylic acid cycle, fatty acid oxidation and respiration. We also identified Ca^{2+} binding and structural proteins as well as proteins associated with oxidative stress and ubiquitin-proteasome mediated pathways. Sperm motility after activation were within 70-90%.

Metabolism and energy production		
Glycolysis and gluconeogenesis		
1	↑	putative glyceraldehyde-3-phosphate dehydrogenase
2	↓	triosephosphate isomerase B Tpi1b protein
3	↑	L-lactate dehydrogenase A chain
Fatty acid oxidation		
4	↓	3-ketoacyl-CoA thiolase, mitochondrial
5	↓	enoyl-CoA delta isomerase 1, mitochondrial Dci protein
Tricarboxylic acid cycle		
6	↑	isocitrate dehydrogenase 2 (NADP+), mitochondrial
7	↓	malate dehydrogenase, mitochondrial
8	↑	succinate-CoA ligase, ADP-forming, beta subunit
9	↑	aconitase 2, mitochondrial
10	↑	citrate synthase Cs protein
11	↓	glutamate dehydrogenase 1a
Amino acids metabolism		
12	↑	aspartate aminotransferase, mitochondrial Got2a protein
13	↑	hydroxyphenylpyruvate dioxygenase-like protein
Respiration		
14	↑	ATP synthase subunit beta, mitochondrial; Flags: Precursor
15	↑	ATP-citrate synthase
16	↑	NADP-dependent malic enzyme, mitochondrial
Other pathways		
17	↓↑	brain creatine kinase
18	↓	nucleoside diphosphate kinase-Z1
19	↑	adenosylhomocysteinase
20	↑	phosphopantothenate-cysteine ligase
21	↑	10-formyltetrahydrofolate dehydrogenase
Signal transduction		
22	↑	Ran (GTP binding nuclear protein)
Other function		
23	↑	glyoxalase domain containing 4
Protein folding and turnover		
24	↓	proteasome subunit beta type-6
25	↑	proteasome subunit, beta type, 5
26	↑	proteasome 26S subunit, non-ATPase, 13
27	↓	26S proteasome non-ATPase regulatory subunit 8
28	↓	ubiquitin fusion degradation 1-like
29	↓	ubiquitin-conjugating enzyme E2 variant 2
30	↓	heat shock 60 (Hspd1)
31	↓	valosin containing protein ; Transitional endoplasmic reticulum ATPase
32	↓	cullin-associated NEDD8-dissociated protein 1
33	↑	COP9 signalosome complex subunit 5
34	↑	T complex protein 1
Cytoskeleton, flagella and cell movement		
35	↑	tubulin alpha 6
36	↑	beta-actin 1
37	↓	muscle cofilin 2
38	↓	septin-8-A
39	↑	sperm associated antigen 6
Reproduction		
40	↑	pachytene checkpoint protein 2 homolog
Nucleotide, ion and lipid binding and transport		
41	↑↓	parvalbumin 6
42	↓	calreticulin
43	↓	EH-domain containing 1
44	↓	EF-hand domain (C-terminal) containing 2
45	↑	calmodulin
Oxidative stress		
46	↑	glutathione S-transferase mu
47	↓	protein disulfide-isomerase
48	↓	constitutive heat shock protein 70
49	↓	heat shock protein 9
50	↓	heat shock 10 protein 1 (chaperonin 10)

Tab.1. Proteins altered after carp sperm activation (arrows indicate up and down regulated proteins in activated spermatozoa).

DISCUSSION AND CONCLUSION

The dominance of metabolic enzymes within proteins changed after carp spermatozoa activation such as mitochondrial ATP synthase together with creatine kinase coincides with their involvement in generation and maintenance of essential ATP level required for ATP hydrolysis by dynein ATPase localized within the flagellar motile apparatus (Dzyuba and Cosson, 2014). Moreover the identification of different Ca^{2+} binding proteins support the importance of calcium binding proteins in the mechanism of calcium ion action in the sperm cell and indicated the complexity of the calcium ion metabolism. Our results also suggest that structure of proteins is also altered by activation. In conclusion, our results demonstrated the involvement of different proteins in carp spermatozoa motility which implies the complex network of signaling molecules such as Ca2+ binding proteins and metabolic enzymes involved in carp sperm motility activation and regulation. The future studies are necessary to define specific role of particular proteins in mechanism of sperm activation.

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O4

FIRST FULL SPERMATOGENESIS AND SPERMIATION INDUCED WITH SPECIFIC RECOMBINANT GONADOTROPINS IN A TELEOST FISH, THE EUROPEAN EEL

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INTRODUCTION

Recombinant FSH and LH have been synthesised for a few fish species, including eel, using different expression systems as baculovirus in silkworm larvae (Japanese eel; Kobayashi et al., 2010), or drosophila (Japanese eel; Kazeto et al., 2008). Eel recombinant gonadotropins induced spermatogenesis *in vitro* in Japanese eel, but had limited effect *in vivo* (Kazeto et al., 2008). Therefore, in the current work, the aim will be to check the functionality *in vivo* of new specific recombinant gonadotropins in European eel.

MATERIALS AND METHODS

European eel males (n=72; 100.1±1.9 g) from a local fish farm were distributed in four 150-L aquaria and progressively adapted to sea water and 20 ºC. Single-chain recombinant FSH and LH were obtained by transfection of a mammalian cell line (CHO) with further partial purification and concentration (Rara Avis Biotec S.L.; Valencia, Spain). Fish were submitted to three hormonal treatments administered weekly during a total of 12 weeks. Males received rFSH in three doses (2.8, 1.4 and 0.7 µg/fish; high, medium and low treatments) during 3 weeks. After that, an increasing (every 3 weeks) dose of rLH: 1, 2 or 6 µg/fish was combined with the different rFSH doses.

Three males/treatment were sacrificed every 3 weeks. External morphometric parameters (fin color and eye index, EI) were noted. Fish and testis were weighed to calculate the GSI. Blood samples were taken and plasma levels of 11-ketosterone (11KT) and testosterone (T) determined by commercial ELISA kits. A portion of testis was collected for histological determination and as it was possible, sperm quality was analysed: volume, density and motility (by CASA software, ISAS, Proiser R+D, S.L., Spain).

RESULTS

The administration of only rFSH was enough to initiate the spermatogenesis (W3), inducing darker fins, T levels, EI increase (Fig. 1) and testis development until the SPG2 stage in fish reared at high rFSH group (Table 1), but no differences were observed in the GSI. Higher 11KT values were observed in the high group respect to lower doses, which did not showed significant increases throughout the treatment.

Table 1. Distribution of stages of testis development reached by the different males through the samplings (W3-12) in the three experimental groups: (●) High (2.8 µg/fish); (●) Medium (1.4 µg/fish); and (○) Low (0.7 µg/fish). Stages: SPG1: Spermatogonia 1; SPG2: Spermatogonia 2; SPC1: Spermatocyte 1; SPC2: Spermatocyte 2; SD: Spermatid; SPZ1: Spermatozoa 1; SPZ2: Spermatozoa 2.

		SPG1	SPG2	SPC1	SPC2	SD	SPZ1	SPZ2
rFSH + 6µg rLH/fish	W12		○	○○	●● ○	●●● ●●● ○○○	●● ●	● ●
rFSH + 2µg rLH/fish	W9	○		● ○○	●	●●	●	
rFSH + 1µg rLH/fish	W6	● ○○○		●	●● ●●			
rFSH	W3	● ●●● ○○○	●●					

The rLH administration (W6) promoted further maturation to SPC2 stage in medium and high experimental groups, but no gonadal progression was observed in the low group. Only the high group showed darker fins respect to previous weeks. The highest T levels were achieved in all the experimental groups, and bigger 11KT values in the rFSH high group.

A second increase of rLH (W9) induced further maturation to SD-SPZ1 and SPC1 stages in the high and low groups, respectively. The medium group showed no further maturation (staying at SPC1-2 stages), although the fish registered higher EI and darker fins. Low group fishes showed a first significant increase of EI in comparison with untreated fish. Any group showed significant GSI increases. The highest dose of rLH (W12) was necessary to find fish in the most advanced stage (SPZ2), being also the dose which caused the highest values of EI and the darkest fin colour. The GSI increased gradually during the treatment but at this moment showed the first significant increase in all the experimental groups, being especially higher in the fish receiving the high rFSH treatment. A progressive 11KT (high group) and T (all treatments) decrease was observed from W9 to W12, but without differences respect to W6.

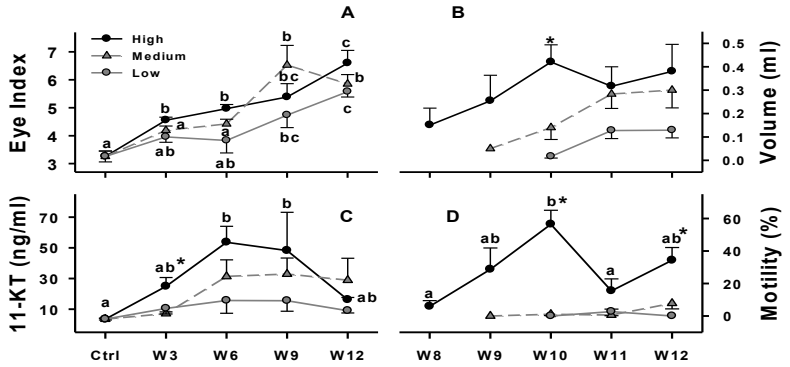

Figure 1. Sperm parameters throughout the FSH/LH treatments: A) Eye index; B) Sperm volume; C) 11KT plasma levels; D) Total motility. Different letters indicate differences during the treatment and asterisk indicates differences between treatments.

The sperm production in high, medium and low experimental groups started at 8th, 9th and 10th weeks, respectively (Fig. 1). Although the sperm quality was variable and not all the spermiating males produced samples with high sperm quality, the fish treated with the highest rFSH dose and progressive increase of rLH registered the best sperm quality samples, with motilities ≥50% motile cells, densities around $7*10^9$ cells/ml and volumes of approximately 0.4 ml.

DISCUSSION AND CONCLUSIONS

By first time in teleosts, specific recombinant gonadotropins have produced good quality sperm, demonstrating that the half-life of these recombinant gonadotropins is long enough to induce *in vivo* effects. The group treated with the highest rFSH and increasing doses of rLH provided the best sperm quality samples. However, ~20% of non-responders were registered and the sperm quality was variable. Therefore, further experiments combining these recombinant hormones are required to improve the hormonal treatments.

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O5

EFFECTS OF TWO DIFFERENT WATER RESOURCES ON SPERM QUALITY OF BROODSTOCK OF TROUT (*Salmo coruhensis*)

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INTRODUCTION

Turkey have some of endemic species of trout. The most of researches are on taxonomic. Studies on gamete quality under aquaculture conditions are barely.
Gamete quality in fish depends on broodstock husbandry conditions, management and nutrition, sperm storage, environmental factors such as water quality and presence of pollutants, other aspects such as stress and disease (Rurangwa et al., 2004).
Sperm quality and quantity of fish they produce varies according to the species. In salmonid species, direct factors that effects to sperm quantity are culture conditions, stripping time, age, feeding conditions and presence of male (Büyükhatipoğlu and Holtz, 1984; Rurangwa et al., 2004). It is reported that inside culture conditions, temperature of water can strongly effect quality of fish sperm, especially duration of motility (Williot et al., 2000; Rurangwa et al., 2004).
In this study, spermatological parameters of Çoruh trout (*Salmo coruhensis*) broodstock (age 3+) which are kept during the spawning season in two different water sources (well water and river water) were investigated.
The aim of present study was to determine the effect of different water resource on fresh sperm parameters such as motility (%), volume (ml), curviline are velocity (VCL) (µm/sn), motility duration (s), progressive motility (%) and seminal plazma osmolality (mOsm/kg).

MATERIALS AND METHODS

The study was carry out at Istanbul University, Fisheries Faculty, Sapanca Inland Fish Water Production, Research and Applied Station, during the spawning season of Çoruh trout. The brood fish were held in four circular fiber glass tanks ($2m^3$) by eight weeks.
Broodstock divided into two experimental groups. One group received from well water (Group A) while the other received from stream water from Kurtköy Stream (Group B).
We carried out this study with 3+ years old 20 male fish in two groups. Average fish weight was 2147±647g and average fish total lenght 56±2cm. We constituted this study with four groups and there was five fish each group.
During the experiment the temperature of the water from the tank (°C), oxygen (O_2) and the pH was measured daily.
Sperm was collected by abdominal massage from all individuals in early december. Motility (%), Progressive motility (%) and VCL (µm/sn) were determined with CEROS II (Hamilton-Thorne, Beverly, MA, USA) computer-assisted sperm analysis (CASA) system. Volumes of the sperm samples were measured with glass beaker. To determine cell density used a hemocytometer chamber (thoma).
We checked semen extender osmolality by a freezing-point osmometer. Duration of motility measured with a stopwatch, was used to measure the time period from initial activation to the end of mass motility.

RESULTS

The temperature of the river water varied from 8.1 to 12.8°C during the experiment and well water was constant at 12.4 - 12.7°C. Physicochemical parameter of two groups are shown (Table 1).

Table **1**. The mean physicochemical parameters of two groups (Group A and B) during the experiment.

Date	Group A (Well Water)			Group B (River Water)		
	Temperature	Oxygen	pH	Temperature	Oxygen	pH
22.10.14 02.12.14	12,52±0,13	8,98±0,63	7,55 ±0,02	11,22±1,26	7,66 ±0,09	12,52±0,13

In the end of the study, the first sperm obtained from the Group B on 02.12.2014. The motility value varied from %11.20 to %65.50 inside this group. Second sperm sample obtained on 16.01.2014 from the one individual of Group A. The motility value was %27.78.

DISCUSSION AND CONCLUSIONS

The normal gonadal development as can be expected under optimal (natural) conditions. There was differences between two groups about time of collecting and parameters of sperm. It was found that when the temperature of rearing conditions not changed during all spawning season, means the temperature stay constant (12.4 - 12.7°C), testes development of fish can prolonged. Sperm could get from fish of Group A, but not from all broodstocks. These sperm samples more poor than sperm samples which is taken from broodstock that reared with changing river water temperature (Group B).

In the end of the study, sperm quality and quantity of river water Group B has been found to be better than well water Group A.

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O6

SPECIFIC SPERM MATURATION DETERMINED BY UROGENITAL SYSTEM ANATOMY IN STURGEON

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INTRODUCTION

Recently, we discovered that acquisition of capability for sperm motility and fertilization (maturation) in sturgeon takes place outside the testes. In contrast to teleostean species, sturgeons possess an excretory system in which spermatozoa pass through kidney before release into environment (Hoar, 1969, Fig 1d). In current report we summarize results of studies performed in our laboratory focused on physiological processes potentially involved in sturgeon sperm maturation, namely changes in: 1) ionic environment; 2) sensitivity of spermatozoa to calcium ions (Ca^{2+}); 3) antioxidant enzymes and proteolytic activities; and 4) content in macroergic phosphates arising during this maturation process.

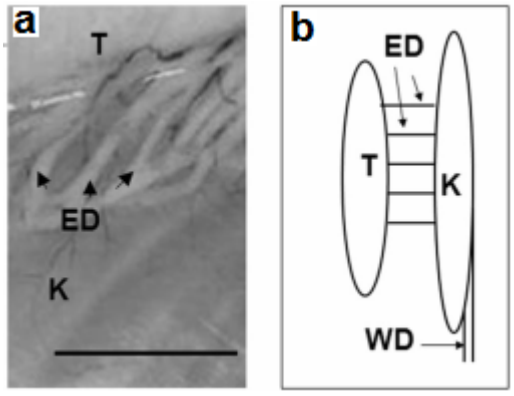

Fig. 1. Structure of sterlet male urogenital tract. T – testis, K – kidney, WD – Wolffian ducts, ED – efferent ducts. a – testis–kidney junction. b – schematic view of sturgeon urogenital system. Bar = 1cm (Dzyuba et al., 2014a).

MATERIALS AND METHODS

Spermiation in sterlet *Acipenser ruthenus* was stimulated by hypophysation. Sperm was collected from the Wolffian ducts and from testes. Wolffian duct sperm was collected by catheterization of urogenital sinus (Fig.1a). Testicular sperm was collected by incision of efferent ducts (Fig. 1b). Seminal fluid (SF) from Wolffian duct sperm was used for *in vitro* testicular sperm maturation as described by Dzyuba et al. (2014b). Chelator of Ca^{2+} (EGTA), Ca^{2+} channels blocker (verapamil), Ca^{2+} ionophore (A23187), or oxidative phosphorylation uncoupler (CCCP) were added in different experiments to affect *in vitro* sperm maturation process. Amidase and anti-proteolytic activities as well as activity of antioxidant enzymes (SOD, CAT), uric acid and thiobarbituric acid-reactive substance (TBARS) content were estimated in seminal fluids by spectrophotometric methods. Gelatinolytic and caseinolytic activities were investigated in seminal fluids and spermatozoon extracts and were detected electrophoreticaly. Sperm motility was analyzed applying activating medium (AM: 10 mM Tris-HCl, pH 8.0) and AM containing different Ca^{2+} concentrations or CCCP. Determination of ATP, ADP and creatine phosphate (CP) content was performed using liquid chromatography/high-resolution mass spectrometry in sperm samples before and after maturation and also before and after sperm dilution with activating medium.

RESULTS

Concentration of testicular sperm was significantly higher than that of Wolffian duct sperm. Osmolality and cations (Na^+, K^+ and Ca^{2+}) content were significantly lower in Wolffian duct sperm than in testicular sperm. Testicular spermatozoa

were not able to activate motility in AM. Testicular spermatozoa motility was only possible in AM, containing increased Ca^{2+} concentration. Incubation of testicular spermatozoa in seminal fluid from Wolffian duct sperm led to motility activation in AM (*in vitro* maturation). Addition of verapamil or EGTA to the seminal fluid during *in vitro* maturation suppressed the maturation process.
Testicular SF was characterized by the higher level of TBARS, uric acid and activity of SOD and CAT compared with SF from Wolffian duct sperm. No significant difference in proteolytic profiles of seminal fluids from Wolffian duct and testicular sperm was found. Gelatin and especially casein zymography revealed essential difference in spermatozoon extracts: WS extracts were characterized by the presence of a broad proteolytic band ranging from 48 to 41 kDa, while TS extracts did not possess such activity and expressed it only after *in vitro* maturation.
Testicular sperm *in vitro* maturation significantly decreased CP content, while ADP and ATP content were not affected. Addition of CCCP during maturation stopped the maturation process.

DISCUSSION AND CONCLUSIONS

Observed differences in osmolality and ionic composition of testicular and Wolffian duct seminal fluid and differential sensitivity of sperm to Ca^{2+} before and after maturation suggest significant differences in maturation processes among sturgeon and teleostean fishes (Schulz et al., 2010). We suggest that it is not the definite levels of proteolytic and anti-proteolytic activities, but rather the changes in their inter-relations and triggering by such a way some cascade of signaling events that could be crucial for the maturation process. Energy dependency of TS maturation in sturgeons was established by our results.
We conclude that sturgeon sperm maturation represents a complex, energy dependent process, associated with involvement of proteolytic activities of enzymes located in spermatozoa. Speculatively, activation of this activity is leading to changes in sensitivity of spermatozoa to Ca^{2+}, which is required for acquisition by sperm the ability for fertilization.

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O7

MACROERGIC PHOSPHATES CONTENT AND CLUSTER ANALYSIS OF MOTILITY IN SPERMATOZOA OF WHITEFISH *COREGONUS LAVARETUS MARAENA* IN RESPONSE TO ENVIRONMENT OSMOLALITY

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INTRODUCTION

In spermatozoa bioenergetics, a major role is devoted to creatine- and adenylate-phosphates metabolism: At activation of fish sperm motility, flagellar dynein ATPase causes a rapid intracellular ATP (Adenosine Tri-Phosphate) consumption, as described in several species (Ingermann, 2008). ATP can be regenerated from ADP (Adenosine Di-Phosphate) in axoneme by adenylate kinase activity. Creatine-Phosphate (CP) also supports ATP level during motility via creatine-phosphokinase (Cosson, 2012). After activation of fish spermatozoa, motility duration and intracellular levels of macroergic phosphates (ATP, ADP, creatine phosphate - CP) are greatly affected by environmental factors such as osmotic pressure and ionic composition (Cosson, 2004), the latters being the main factors controlling axonemal motility. The relationship between activities of membranous water- or ion-channels and bio-energetic of fish spermatozoa is poorly understood. Based on this, our aim was to investigate how activating medium osmolality influence subpopulation distributions of motile sperm, in relation with the contribution of creatine- and adenylate-phosphates changes observed at initiation and during the motility period.

MATERIALS AND METHODS

Study was performed using sperm samples from six males collected during natural spawning of whitefish *Coregonus lavaretus maraena.* Differences in energy consumption between motile and immotile spermatozoa and in motility parameters were tested by changing environment osmolality and ions composition: spermatozoa were diluted in non-activating (NAM) or activating (AM) media of osmolalities100, 200, 300 mOsm L^{-1} NaCl. Sperm motility in NAM was prevented by 5 mM KCl,

Determination of ATP, ADP, AMP, CP, and creatine was performed in perchloric extracts of whitefish sperm samples obtained at different post-activation time using a Q-Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Sperm motility video records were analyzed using CASA-automated plugin (http://www.ucs.mun.ca/~cfpurchase/CASA_automated-files.zip) for ImageJ software (National Institutes of Health, USA). Obtained data were cleaned up and processed into a single dataset. Total motility and the median values of the kinematic variables were calculated for each individual sample. Subpopulation and statistical analyses were carried out using the R statistical environment (R Core Team, Vienna, Austria) and the methodology based on hierarchical clustering in each sample (Gallego et al., 2015).

RESULTS

The levels of ATP and CP were significantly decreased after dilution of sperm by both AM and NAM solutions, but significantly greater in AM. However, no differences were observed between the consumption of the aforementioned compounds in both AM and NAM with different osmolalities (Fig 1a, b).

Four clusters with different values of motility parameters of spermatozoa were distinguished after subpopulation analysis. Cluster analysis revealed that the most abundant at initial stage of motility in each AM was spermatozoa cluster 1 (CL1), possessing spermatozoa with high VCL and linearity. Significant decrease in percentage of CL1 was observed at 40 s post-activation in 100 and 200 mOsm L^{-}

^{1} compared to 300 mOsm L^{-1} AM. The longest motility duration and significantly highest VCL starting from 20s post-activation was observed in 300 mOsm L^{-1} AM (Fig.1c)

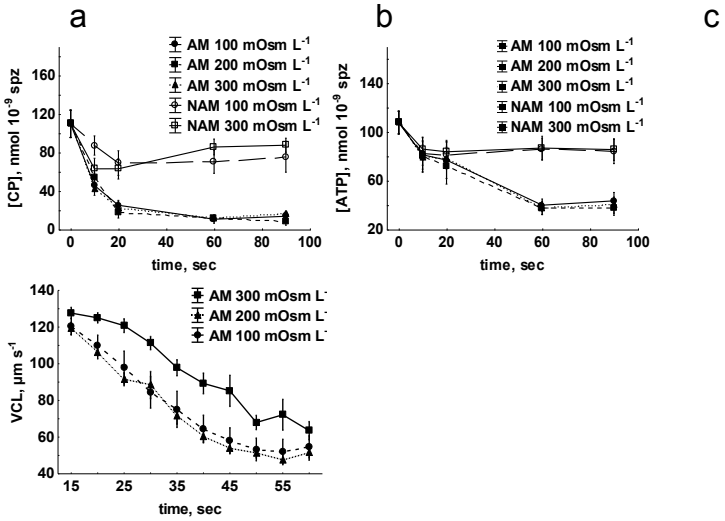

Fig. 1. Creatine phosphate (a), ATP (b), and VCL (c) after whitefish sperm dilution in AM and NAM. Results are mean ± SE.

DISCUSSION AND CONCLUSIONS

Presented data show that changes in sperm environment composition lead to various degrees of energy consumption, part of which would be associated with ionic pumps increased activity. Energetic consumption significantly increased in motility activating condition suggesting flagellar dynein-ATPases are the main energy consumers, but a basal level of energy consumption occurs in non-motile cells, probably needed by fish sperm to control ionic balance across its membrane after to osmotic stress. Following activation signal, sperm cluster analysis allows the detection of a gradual switch from straight-line to circular tracks populations hypothetically related to rise of internal Ca^{2+} ions concentration (Cosson et al., 1989).

We conclude that isotonic conditions favor the sub-population of spermatozoa presenting longer period of straight-line and fast motility without increase in energy consumption. This suggests that fish sperm energy management is more efficient after activation in isotonic conditions.

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O8

USEFULNESS OF PORTABLE FLOW CYTOMETER FOR SPERM CONCENTRATION AND VIABILITY MEASUREMENTS OF RAINBOW TROUT SPERMATOZOA

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INTRODUCTION

Sperm concentration is one of the major semen parameters that is evaluated as part of the standard fish semen analysis (Cuevas-Uribe and Tiersch, 2011). Flow cytometry can be potentially used for counting cells, but until now it has been restricted to laboratory conditions (Hossain et al., 2011). However, recently a portable flow cytometer Muse Cell Analyzer (Millipore, USA) has been developed, which for the first time enables flow cytometry analysis to be carried out in field conditions. The aim of this study was to test the usefulness of a portable flow cytometer in relation to the measurement of sperm concentration and viability in rainbow trout. This method was compared with the currently used methods, including the spectrophotometric method, computer-aided fluorescence microscopy and a haemocytometer.

MATERIALS AND METHODS

Sperm concentration of mature rainbow trout males (3 years of age, n=12) was measured independently using four methods: (1) by measuring the absorbance of sperm suspensions (Ciereszko and Dabrowski, 1993); (2) by computer-aided fluorescent microscopy NucleoCounter SP-100 (Chemometec, Denmark) as described by Nynca and Ciereszko (2009); (3) using a Muse Cell Analyzer (Millipore, Billerica, MA, USA) according to the instructions supplied by the manufacturer; (4) using Bürker haemocytometer (BT, Brand, Germany). Sperm viability was analysed by NucleoCounter SP-100 (Nynca and Ciereszko 2009) and Muse flow cytometer using the Muse Count and Viability kit. Statistical tests were performed at a significance level of 0.05 using GraphPad Prism software (GraphPad Software Inc. USA). Regression equations were also calculated between estimated parameters.

RESULTS

Significant differences between values of sperm concentration evaluated by the applied methods were noticed (Table 1). The mean concentration obtained using the spectrophotometer and NucleoCounter SP-100 was significantly lower (by 15–20 %) than that obtained by the flow cytometer and haemocytometer. The flow cytometer produced concentration outcomes similar to the outcomes obtained by the haemocytometer.

Table 1. The average sperm concentration and viability parameters of rainbow trout (n=12) evaluated using four methods.

Parameter	*Flow cytometer*	*Fluorescence microscope*	*Spectrophotometer*	*Haemocytometer*
Sperm concentration $\times 10^9$ spz ml^{-1}				
Mean ± SD	12.35± 3.88[a]	10.46± 3.60[b]	9.86 ± 3.69[b]	11.73 ± 4.78[a]
Range	5.92-18.82	5.20 –16.91	4.57 –16.48	5.55 –20.10
Viability (%)				
Mean ± SD	97.00± 0.99[a]	86.22 ± 1.16[b]		
Range	95.95–9.05	84.82 –88.97		

Values (mean ± SD) with different superscripts are significantly different ($P < 0.05$) among parameters estimated by different methods.

Significant regressions ($p<0.0001$) between sperm concentration obtained by using a flow cytometer, a spectrophotometer ($y=0.93x-1.64$; $r^2=0.95$), a fluorescence microscope ($y=0.90x-0.69$; $r^2=0.94$) and a haemocytometer ($y=1.16x-2.57$; $r^2=0.87$) were found (Fig. 1). The sperm viability determinations obtained using a flow cytometer were significantly higher (97.00 ± 0.99%) than the values obtained by using fluorescence microscopy (86.22 ± 1.16%). However, a significant regression was found between these two viability measurements (Fig. 2).

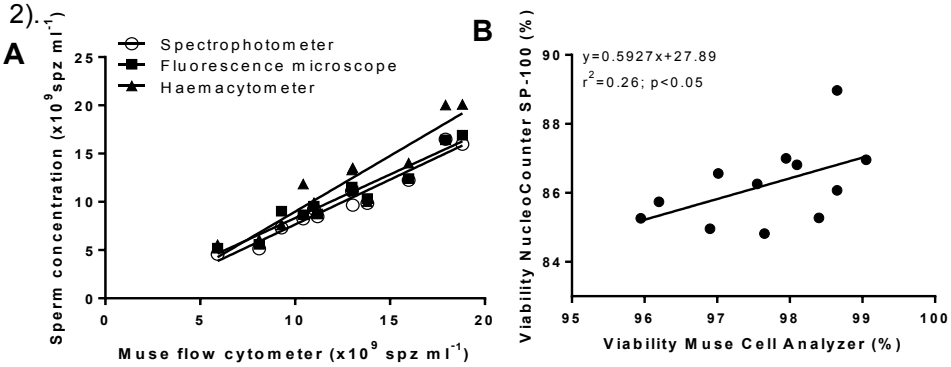

Fig. 1. (A) Regression between sperm concentration measured using flow cytometer (Muse), NucleoCounter SP-100 apparatus, haemocytometer (Bürker) and evaluated by spectrophotometer (Eppendorf). **(B)** Regression between sperm viability evaluated with the application of NucleoCounter SP-100 and Muse flow cytometer apparatus (n=12).

DISCUSSION AND CONCLUSIONS

To the best of our knowledge, this is the first study related to the application of a portable flow cytometer in sperm analysis. The Muse Cell Analyzer provides fast measurement of sperm concentration and viability. However, the application of this instrument depends greatly on the research needs. The portable spectrophotometer is still the method of choice for measuring a single parameter, such as sperm concentration. For sperm concentration and viability, the NucleoCounter SP-100 and Muse flow cytometer can both be used. The advantage of a compact flow cytometer is the incorporation of additional sperm analysis, such as apoptosis detection, changes in mitochondrial potential, counting of cells undergoing oxidative stress, DNA fragmentation and cell cycle distribution, in future fish reproductive studies.

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O9
HYPOTHALAMIC REGULATION OF THE RELEASE OF FSH AND LH

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INTRODUCTION
Both oogenesis and spermatogenesis are deeply controlled by the gonadotropins FSH and LH: FSH stimulates follicular development in the ovary and gametogenesis in the testes, while LH stimulates processes leading to final oocyte maturation and ovulation in females, and spermiation in males (Levavi-Sivan *et al.*, 2010). Gonadotropin release is tightly regulated by a complex network of central and peripheral factors, where the most important is GnRH. Recently, the neuropeptides kisspeptin (encoded by Kiss1), neurokinin B or F (NKB and NKF encoded by tachikinin 3 [Tac3]) and GnIH (encoded by lpxrf) have been placed as crucial for reproduction (Levavi-Sivan *et al.*, 2010; Biran *et al.*, 2012). However, the differential regulation of FSH and LH release is not fully understood yet. We aimed to differentiate between the organization and the control of FSH and LH. We targeted this aim in tow different aspects. The first was to identify novel modulators of gonadotropin secretion, and the second was to study the functional and anatomical aspects of gonadotrope regulation, in terms of the function and components of the hypothalamic–pituitary axis. In fish a neuroglandular mode of delivery was considered dominant, whereas in tetrapods hypothalamic signals are relayed via the hypophysial portal blood system.

MATERIALS AND METHODS
Using gonadotropin regulatory elements from tilapia, we generated transgenic zebrafish and tilapia lines where FSH cells were labeled with EGFP and LH with mCherry (Golan *et al.*, 2014; Golan and Levavi-Sivan, 2013). Cloning, in vivo experiments and in situ hybridization (ISH) were carried out as described in (Biran *et al.*, 2014a; Biran *et al.*, 2014b).

RESULTS
Two hours after ip injection of tilapia (ti) NKB, the plasma levels of both FSH and LH were increased, whereas tiNKF was more effective in increasing LH levels. However, tiNKB was more effective than tiNKF in increasing both FSH and LH from tilapia pituitary dispersed cells. Using ISH and fluorescent immunohistochemistry, we have shown that LH cells possess tac3, tac3ra, and tac3rb mRNAs, whereas FSH cells possess mainly tac3rb and tac3ra and tac3 to a much lesser extent (Biran *et al.*, 2014a). Administration of the tilapia LPXRFa-2 peptide to primary cell culture of tilapia pituitaries, or to reproductive female tilapia by ip injection, positively regulated both LH and FSH release *in vivo* and *in vitro*. Using double-labeled fluorescent ISH and immunofluorescence, βLH cells were found to co-express both tilapia lpxrf and lpxrf-r mRNA, whereas some of the βFSH cells coexpressed only lpxrf-r mRNA. No coexpression of tilapia lpxrf-r was identified in GH-positive cells (Biran *et al.*, 2014b).
We found significant differences in the accessibility of LH and FSH cells to the vasculature and to GnRH signals. Surprisingly, we also identified a much closer association of GnRH axons and permeable blood vessels, than with the gonadotropes, indicating vascular delivery of GnRH rather than direct, neuroglandular gonadotrope regulation (Golan *et al.*, under revision). Using the transgenic tilapia we found that FSH cells reacted to stimulation with GnRH by proliferating and showed increased transgene fluorescence, whereas estrogen exposure caused a decrease in cell number and transgene fluorescence (Golan *et al.*, 2014).

DISCUSSION AND CONCLUSIONS

Our findings have important implications regarding the differential regulation of LH and FSH and contradict the traditional view of the teleost pituitary that assumes direct innervation of endocrine cells by hypothalamic fibers. Instead, we provide evidence favoring a non-direct, neurovascular control of gonadotropes which is more reminiscent of tetrapods. Furthermore, we showed that the members of the NKB and GnIH systems may serve as paracrine/autocrine regulators of gonadotropin release in fish pituitary. These findings can be applied to develop new methods for the manipulations of the reproductive axis in fish.

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Anthropogenic contaminants in the environment: effects on fish gametes

SOA2

ANTHROPOGENIC CONTAMINANTS IN THE ENVIRONMENT: EFFECTS ON FISH GAMETES

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INTRODUCTION

The presence of anthropogenic chemicals related to agricultural activities, municipal, and industrial operations have been reported in various rivers around the world. These environmental contaminants have been linked to adverse health effects in humans and wildlife. Over the past decade our group and other investigators have identified over 200 contaminants in the water samples collected from three rivers in Southern Alberta, Canada (Jeffries et al., 2008, 20110; Sosiak and Hebben, 2005). The compounds detected include synthetic estrogens, industrial chemicals and pharmaceuticals downstream of municipal wastewater effluents as well as natural hormones, pesticides and herbicides in agricultural areas. In field studies, we observed significant increase in female to male adult ratio from 50 to 90%, indicating possible sex reversal and feminization of fish (Jeffries et al., 2008, 20110). The observed female bias correlated with high levels of vitellogenin expression in male fish, indicating the presence of compounds with estrogen-like activity. To investigate the effects of chemicals detected, we performed controlled laboratory experiments in which fish in aquaria were exposed to the same concentrations of a selected number of chemicals detected in the river system, individually and as mixtures. We investigated the adverse physiological and metabolic outcome in fish exposed to contaminants, using transcriptomics, metabolomics and physiological approach.

MATERIALS AND METHODS

Well-established methods were used to investigate hormone and hormone receptor levels, apoptosis and pituitary function as described previously (Andreu-Vieyra et al., 2005; Nelson and Habibi, 2010). Following exposure to chemicals, serum, liver, testis, ovary and hypothalamic samples were removed and immediately frozen at -80C for biochemical analyses. Multiple end points, including expression of various genes involved in reproduction and gonadal development were measured. Tissue samples were also used for metabolite extraction as described previously (Jordan et al., 2012), and 2D spectroscopy was performed to validate metabolite chemical shift assignments. Transcriptomics were carried out using well-characterized microarray system to identify pathways affected and identify novel biomarkers.

RESULTS

To investigate the health impact of chemicals detected, we performed controlled laboratory experiments in which fish were exposed to the same concentrations of a selected number of chemicals detected in the river system (Bisphenol-A, BPA; nonylphenol, NP; phthalate esters, DEHP; and the fungicide vinclozolin, VZ), individually and as mixtures. We used various biological end points, including transcriptomics and metabolomics in samples obtained from liver, gonads and brain of fish. In goldfish exposure to BPA, NP, DEHP and the fungicide vinclozolin (VZ) resulted in disruption of reproduction in male and female. Parameters affected included changes in circulating levels of gonadal steroids, including 11-ketotesteosterone (11-KT) in make fish and E2 level in both male and female. Contaminant exposure also altered circulating gonadotropin levels as well as transcripts of genes encoding gonadotropin and GnRH receptors, StAR, CYP17, and 3ß-HSD in the testis and ovary of goldfish. The results demonstrate that exposure to environmental contaminants induced significant changes in the hormones of brain–pituitary–gonadal axis, as well as significant changes in amino acid, lipid, energy, carbohydrate, nucleotide and cofactor/vitamin metabolism in the liver and testis of goldfish. The effects of mixture of contaminants were significantly different from all the individual treatments in the liver. However, in

testes, the effect of the mixture resembled that of BPA and DEHP, presumably due to differential distribution of steroid receptors. We also used microarray approach, and observed significant changes in a number of canonical pathways, including cell cycle & proliferation, inflammatory, innate immune response, stress response, and drug metabolism.

DISCUSSION AND CONCLUSIONS

Endocrine regulation of gonadal development involves interaction between hormones of brain-pituitary gonadal axis, as well as local factors involved in the control of gametogenesis in teleosts. Availability of sufficient energy is particularly important to support ovarian development in oviparous species such as fish. In the present study, exposure of fish to environmental contaminants, caused disruption of steroidogenesis, pituitary hormone concentrations, and energy metabolism. Therefore the adverse impact of contaminants with hormone-like activity are directed at multi levels causing disruption of brain-pituitary gonadal axis, leading to negative reproductive outcome. In case of ovarian development, positive folliculogenesis/ atresia ratio results in ovarian development, characterized by primary oocyte growth, formation of lipid bodies, cortical alveoli, and expression of polysialoglycoproteins, and vitellogenesis. In each reproductive cycle, a large number of oocytes develop simultaneously and undergo synchronous maturation and ovulation either as a whole or in several batches depending on the species. Among the factors affected by the contaminants were, gonadotropin and gonadotropin receptors as well and GnRH and GnRH receptors. Our studies in the past demonstrated importance of GnRH as a paracrine regulator of follicular and testicular development in fish. GnRH peptides produced in the gonads regulate steroidogenesis and apoptosis in the ovary and testis, and work in concert with hypophyseal and thyroid hormones as well as gonadal steroids to maintain gonadal synchronicity. The presence of gonadotropin hormones and E2 prevent apoptosis. In goldfish, follicles and testis at late stages of development are susceptible to GnRH-induced apoptosis (Andreu-Vieyra et al., 2005; Habibi and Andreu-Vieyra, 2007). BY contrast, GnRH exerts survival action on mid-vitellogenic follicles without influencing gonadotropin-stimulated functions. Under normal conditions, gonadotropins and steroids oppose GnRH-induced apoptosis. Thus high ratio of gonadotropin/GnRH, which is achieved during the narrow period of ovulation results in re-initiation of meiosis and ovulation. Environmental stressors disrupt brain-pituitary-gonadal function, leading to reduced gonadotropin hormone levels and high GnRH/gonadotropin ratio that favours apoptosis, and ultimately leading to follicular atresia, demise, and impaired gametogenesis.

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O10

PESTICIDES USED IN COTTON PRODUCTION AFFECT EMBRYO-LARVAL STAGE, REPRODUCTIVE DEVELOPMENT, ENDOCRINE REGULATION AND OFFSPRING FITNESS IN AFRICAN CATFISH *CLARIAS GARIEPINUS*

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INTRODUCTION

In several West-African countries, cotton is considered as the white gold of the agricultural production (AIC, 2009). Tihan 175 O-TEQ, containing flubendiamide (100g/L) and spirotetramat (75g/L) as active ingredients, and Thionex 350 EC (endosulfan as active substance) are among the main pesticides used in Benin to fight against cotton pests. Aquatic ecosystems are the final destination of pesticides used in agricultural production (Chao et al. 2009). Endosulfan is known to be very toxic to fish. However, the effects of flubendiamide and spirotetramat on tropical aquatic ecosystems are rather unknown. Two experiments were done. The first compared the acute effects of Tihan and its active compounds on the African catfish *Clarias gariepinus* with those of endosulfan (Thionex 350 EC). The second experiment aimed to compare the effects of a chronic exposure of endosulfan and Tihan on the sexual development, endocrine regulation, reproductive performances and larval fitness in African catfish.

MATERIALS AND METHODS

In the first study, 200 mg of artificially fertilized eggs (±100 eggs) were incubated in a trough placed in aquarium with graded doses of Tihan, as well as its active ingredients, and of Thionex. At 4-h intervals, the proportions of hatched larvae, dead eggs, and eggs/larvae with abnormalities (e.g., loss of swimming coordination, dead embryo within the egg, immotile larvae, and larvae with curved or short tails) were recorded. In a second experiment, fish were exposed from embryo stage to adult stage (13 months old) to chronic doses of Tihan and endosulfan and the impact of this exposure was assessed on endocrine regulation and offspring fitness.

RESULTS AND DISCUSSION

The results of the acute exposure indicated that all these pesticides decreased hatching rates. Similarly, Nguyen et al. (1999) showed that concentrations of NaPCP >1 ppm delayed embryo development of *C. gariepinus*. Tihan and spirotetramat disturbed larval swimming coordination while flubendiamide induced tail cleavage. Penetration of Tihan into the eggs possibly led to energy depletion to levels insufficient to support escape from the eggshell (Varo et al. 2006). This could explain the observation of dead embryos within the eggshell, particularly at 30 ppm Tihan. Thionex and flubendiamide might also have caused energy depletion, albeit to a lesser extent, thus explaining the observation of immotile newly hatched larvae at concentrations higher than 100 and 0.5 ppm, respectively. Beyger et al. (2012) observed immotile larvae of Florida flagfish *Jordanella floridae* after exposure to 10 ppb endosulfan for 96 h. Loss of swimming coordination in larvae was observed in the Tihan-treated groups, but this was much more marked in spirotetramat treatment, even at the lowest concentration of 2.5 ppm. This behavior was observed by Beyger et al. (2012) in Florida flagfish at concentrations of 10 ppb endosulfan. Curved or short-tailed larvae were particularly noted in flubendiamide-treated groups.

In the second experiment, plasma estradiol-17β (E2) and 11 ketotestosterone (11-KT) of 13 month-old fish decreased in both sexes of African catfish exposed to Tihan. Singh and Singh (2008a) also indicated a significant decline of 11-KT levels in Asian stinging catfish *Heteropneutes fossilis* exposed to sub-lethal

concentrations of cypermethrin for 45 days, but the plasma levels of E2 were not modified. In our study the simultaneous decline of E2 and 11-KT plasma levels, while testosterone (T) levels remained stable could be explained by a blocking effect of T conversion by Tihan. Indeed, T was present but did not play its precursor role of the two latter hormones. In contrast, in endosulfan exposed fish, plasma levels of E2 increased and T decreased while 11-KT levels did not change. This could be explained by the stimulation of 17β-aromatase, and not of 11β-hydroxylase, by endosulfan. Endosulfan doses altered gonad histology and induced high proportions (18–30% of males) of ovotestis in males and follicular atretic oocytes in females, indicating occurrence of feminization in fish (Pieterse et al., 2010). Tihan also altered gonad histology but only one case of ovotestis was observed at the highest dose. Presence of foam cells in lobular lumen, fibrosis, necrosis, and immature cells released in lobular lumen were found in male gonads while melano-macrophage centers (MMCs), necrosis, fibrosis and vacuolation were observed in female gonads. Chronic exposures to Tihan and Thionex decreased fertilization rate, hatching rate, ova and larval weight, as well as larval resistance to osmotic choc. They also delayed hatching and increased abnormalities in the F1 generation, all these indicators suggesting transgenerational effects of these compounds (Velasco-Santamarıa et al., 2011).

CONCLUSIONS

The changes in sex steroid profiles caused by exposure to endosulfan or Tihan clearly suggest a disruption of the hypothalamus–pituitary–gonadal axis by these agricultural pesticides. The increase of plasma E2 with other alterations caused by these pesticides, such as regressive changes observed in the histological analysis of gonads may gradually lead to the death of affected individuals and the disappearance of sensitive species in the watershed receiving the effluents from cotton fields.

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O11

DUPLICATION OF THE TRANSCRIPTION FACTOR IIIA (TFIIIA) GENE IN TELEOST GENOMES, AND OOCYTE-SPECIFIC TRANSCRIPTION OF TFIIIAb: APLICATIONS IN THE ENVIRONMENTAL MONITORING OF XENOESTROGENICITY

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INTRODUCTION

Oogenesis in fish is characterized by a massive growth of the oocytes that will be spawned each reproductive season. Maternal production and stockpiling of certain molecules in the oocytes, is essential to ensure proper development of the future embryo. In this way, rRNAs accumulated during oogenesis assist rapid ribosomal assembly for proper protein synthesis in the newly formed embryo. Thus, the massive 5S rRNA expression in oocytes serves as marker of their presence in gonads, and of the intersex condition in male fish exposed to xenoestrogens. This production of 5S rRNA by RNA polymerase III is regulated by transcription factor IIIA (TFIIIA), which in turn binds 5S rRNA for accumulation in the cytosol. As a consequence, high TFIIIA mRNA level is also sensitive indicator of intersex condition in fish as demonstrated in thicklip grey mullets (*Chelon labrosus*) captured in polluted Basque estuaries and harbours (Diaz de Cerio et al., 2012; Ortiz-Zarragoitia et al., 2014).

The molecular process that activates *tfiiia* transcription in the oocytes within the testis of intersex male fish is not known yet. What it is known is that anurans possess one single *tfiiia* gene that through alternative promoter usage produces two transcripts; the shortest one exclusively expressed in oocytes. The aim of this study was to analyze the *tfiiia* gene complement in teleost genomes and the regulation of its expression.

MATERIALS AND METHODS

The teleost fish genomes available in Ensembl (*D. rerio, G. aculeatus, O. latipes, T. rubripes, T. nigroviridis, G. morhua, O. niloticus, X. maculatus, A. mexicanus*), were searched for 5S rRNA and TFIIIA genes. The same was done with the genomes of other vertebrates that did not suffer the teleost specific third round of genome duplication; an early sarcopterygian, the coelacanth *L. chalumnae,* a non-teleost actinopterygian, the spotted gar *L. oculatus*; and Xenopus and human as outgroups. We then studied the gene transcription profile of TFIIIA in different organsand early embryo stages of zebrafish, *D. rerio* (and tilapia for comparative purposes).

Zebrafish newly fertilised embryos were also exposed to 100 ng/L estradiol (E_2) and methyltestosterone (MT), diluted in 0.01% ethanol (ETOH), for 61 days. 22 individuals from each treatment, and ETOH controls, were sampled at 26 and 61 dpf. 12 whole individuals were independently frozen at -80ºC for molecular analysis and the rest fixed in formalin and embedded in paraffin for histological analysis. RNA extraction was performed with TRIzol and transcription levels of *amh*, *dmrt1*, *cyp19a1*, tfiiiab, and *tfiiiaa* quantified by RT-qPCR analysis.

RESULTS

In analysed teleost genomes two TFIIIA paralog genes were identified, placed in different chromosomes and identified as *tfiiiaa* and tfiiiab. In contrast, one single gene was found in the genomes of *X. tropicalis*, humans, coelacanth and spotted gar. Synteny analysis revealed linkage of a conserved set of genes with *tfiiia* in all non teleost species, which is also maintained in the case of teleost *tfiiiab*. Only in the zebrafish genome, linkage of *tfiiiaa* with two genes neighbouring *tfiiia* in non-teleosts was identified, indicating apparition of the duplicated gene through genome duplication in teleosts.

PCR analysis revealed that *tfiiiaa* is ubiquitously expressed in zebrafish and tilapia organs, while *tfiiiab* is exclusively expressed in ovary.

In early developing zebrafish embryos *tfiiiab* transcription begins only at the onset of ovarian differentiation, while *tfiiiaa* transcription begins with the activation of the zygotic genome (~8 hpf, Figure 1). At 2 hpf maternally inherited transcripts of both genes can be observed-

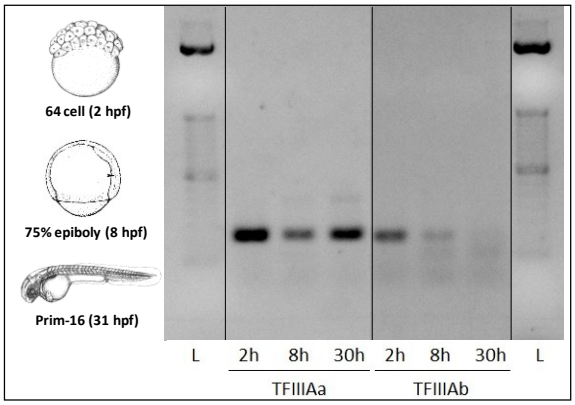

Figure 1. Transcription levels of *tfiiiaa* and *tfiiiab* in early developing zebrafish embryos, 2 to 30 hpf.

Under laboratory exposure to E2, all 60 dpf zebrafish population showed developed ovary, showing transcription of ovarian *tfiiiab* and *tfiiiaa*. On the other hand, MT masculinised zebrafish only showed transcription of *tfiiiaa*. The transcription levels of *tfiiiab* coincided with those of *cyp19a1*, while in masculinised zebrafish significant upregulation of both *amh* and *dmrt1*, in comparison to controls, was observed. *cyp19a1* and *tfiiiab* were downregulated in masculinised fish. *Tfiiiab* transcription was able to identify females and males in 60 dpf fish, with elevated transcript levels in females. At 26 dpf, where no differentiated gonad was histologically observed yet, no expression of any of the sex differentiation genes was detected.

DISCUSSION AND CONCLUSIONS

In anuran oocytes during the massive 5S rRNA production stage, *tfiiia* could constitute 10% of the total protein. The TFIIIA protein isoform in charge (38 kDA) is exclusively synthesized in oocytes. As suggested by synteny analysis two paralog *tfiiia* genes exist that arouse after the third round of genome duplication in teleosts. *Tfiiiab*, which has retained the same neighbouring genes as *tfiiia* in non-teleost vertebrates, displays in zebrafish a deduced molecular weight of 37 kDa, and is exclusively transcribed in ovaries. *Tfiiiaa* (deduced MW 42 kDa) has been maintained in fish genomes possibly as a gene specifically expressed outside oocytes.

Thus, *tfiiiab* transcription is a consequence of oocyte differentiation and not a direct result of estrogen exposure and constitutes a very potent marker of intersex condition and fish feminization. These observations reveal the importance of the ribosomal molecule stockpiling process during fish oocyte differentiation and maturation.

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O12

ALTERATION IN MOLECULAR MARKERS OF OOCYTE DEVELOPMENT AND INTERSEX CONDITION IN MULLETS IMPACTED BY WASTEWATER TREATMENT PLANT EFFLUENTS

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INTRODUCTION

Wastewater Treatment Plant (WWTP) discharges are one of the main sources of endocrine disrupting chemicals (EDCs) into the aquatic environment. Fish populations inhabiting areas downstream of WWTP effluents can show alterations in gamete development such as intersex condition and xenoestrogenic specific gene responses (up-regulation of vitellogenin and *cyp19a1b*) (WHO/UNEP, 2013). However, molecular mechanisms mediating the development of intersex condition and the formation of oocytes in testis tissue in fish exposed to WWTP effluents are not elucidated. Recently, differential regulation of molecular markers of oocyte development such as 5S rRNA and transcripts coding for proteins participating in 5S rRNA production, stockpiling and trafficking (*tfiiia, 42sp43* and *importins*) were described in intersex mullets from polluted sites in the Basque coast (Diaz de Cerio et al., 2012).
The aim of this study was to assess the impact of two WWTPs effluents with different contaminant load in mullet populations inhabiting downstream waters, examining the presence and severity of intersex condition. Molecular markers of xenoestrogenicity and oocyte differentiation and development (*vtg*, *cyp19a1a*, *cyp19a1b, foxl2*, *tfiiia*, 5S/18S rRNA ratio) were also studied in order to establish an association with intersex condition. Thicklip grey mullets (*Chelon labrosus*) were used as sentinel organisms since mugilids have been proposed as suitable sentinel species of reproductive endocrine disruption effects in estuarine and coastal waters (Ortiz-Zarragoitia et al., 2014).

MATERIALS AND METHODS

Adult thicklip grey mullets were captured downstream of the wastewater treatment plants (WWTPs) of Gernika and of Galindo in the Bilbao metropolitan area (South East Bay of Biscay) in June 2013 and February 2014. Samples of gonads, liver and brain were obtained. Histological and histopathological study of the gonad was carried out in order to identify alterations in gamete development such as intersex condition. Intersex severity index was calculated according to Jobling et al. (2006). Additionally, transcript levels of genes associated with xeneostrogenic response and gamete differentiation and development were studied by qPCR. Assessed genes included *cyp19a1a*, *tfiiia*, *dmrt1* and *foxl2* in gonads, vitellogenin in livers and *cyp19a1b* in brain. For this purpose dmrt1 and foxl2 expression sequence tags were previously cloned and sequenced using degenerate primers. The 5S/18SrRNA ratio, proposed as suitable index of oocyte development, was also calculated in total RNA 2100 Bionalayzer (Agilent) electropherograms.

RESULTS

Intersex individuals were detected in mullet populations from Gernika and Galindo at both seasons. Prevalence of intersex ranged from 36% in June to 90% in February in Gernika while in Galindo was 9% in both periods. Most intersex fish showed oocytes in previtellogenic stage dispersed through the testicular tissue. Only one individual collected in Gernika in June showed vitellogenic oocytes within the testis tissue. Intersex severity index ranged from 1 to 3, showing low to moderate incidence in studied mullet populations. Higher transcript levels of oocyte markers *tfiiia* and *cyp19a1a* were quantified in females than in males, intersex fish showing intermediate values. Further, 5S/18S rRNA index was high

in females and low in males, values obtained in intersex mullets being similar to those of females.
Accordingly, xenoestrogen specific responses, such as upregulation of *vtg* and *cyp19a1b*, were detected in male mullets from both Gernika and Galindo. No differences were observed between males and intersex fish for these two genes.

DISCUSSION AND CONCLUSIONS

Intersex individuals were detected in both populations but at higher prevalence in Gernika than in Galindo. The low to moderate severity of the intersex condition was similar to levels reported in other mullet populations from the Basque coast (Bizarro et al., 2014). Xenoestrogenic responses, such as *vtg* up-regulation, detected in mullets from Gernika and Galindo suggests exposure to xenoestrogenic EDCs. However, no association between these responses and intersex condition could be established.
Differences in the transcription levels of oocyte molecular markers were not detected between mullets of both populations. However, sex related differences were found. Female mullets showed higher transcription levels for *cyp19a1a* and *tfiiia* than male mullets. Intersex individuals showed intermediate values, more similar to females as a result of the presence of oocytes within their male gonad tissue. This was reflected by an increase in the 5S rRNA relative proportion, due to its strong expression in developing oocytes (Diaz de Cerio et al., 2012; Ortiz-Zarragoitia et al., 2014).
In conclusion, no direct association between xenoestrogenic responses and intersex condition was established in studied mullet populations. This suggests that intersex condition can be the result of earlier exposures to EDCs. 5S/18S rRNA index and *tfiiia* transcription levels enabled molecularly distinguishing females and males and showed potentiality in the analysis of intersex condition development in fish. Forthcoming results on *foxl2* and *dmrt1* transcript levels will allow a more comprehensive picture of the molecular processes occurring in the development of intersex condition in fish.

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O13

EFFECTS OF ENVIRONMENTAL PLASTICIZERS ON ZEBRAFISH MALE REPRODUCTION

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INTRODUCTION

In recent years, a variety of synthetic compounds with hormone-like activity developed, they are collectively named endocrine disruptors (EDCs) and have the ability to generate adverse health outcomes by impairing master physiological processes (Richter et al., 2007). Among them, bisphenol A, a chemical produced in large quantities for use primarily in the production of plastics and resins (Lang et al., 2008), has been identified by the scientific community as a high-priority research area (American Recovery and Reinvestment Act of 2009 – ARRA). One reason for such concern is related to its extensive employment in a wide variety of consumer products (Tsai, 2006) and its presence as environmental contaminant in rivers and drinking water (Kolpin et al., 2002). Opposite, one poorly characterized plastic additive, recently approved by Food and Drug Administration is the diethylene glycol dibenzoate (DGB). The aim of this study is to compare the hormonal impact of DGB respect to the well-studied BPA. The DGB hormone like effects and impact on spermatogenesis have been analyzed using *Danio rerio* as experimental model.

MATERIALS AND METHODS

Adult male zebrafish, *Danio rerio*, were exposed for 3 weeks to a range of environmental relevant concentration of BPA (5,10 and 20 µg/L) and DGB (1,10 and 100 µg/L) via water. RNA samples were extracted from testis and analysed by real time PCR. The expression of a set of genes involved in reproduction was analyzed (*fshr, lhcgr, amh, ar, cyp11a1 and cyp19a1*).

RESULTS

The effects of BPA exposure in the zebrafish testis evidenced the increase of *fshr* and *lhcgr* expression by the 10 µg/L dose while the expression of the *amh* was increased by the 20 µg/L dose. No variations have been found in the expression of *ar* and *cyp11a1.* Interestingly, the expression of cyp19a, which in male testes is directly involved in the acquirement of sperm motility, is increased even though not in a significant way in 10 µg/L dose. On the contrary, the exposure to all DGB doses induced a down regulation of all the genes analyzed *(fshr, lhcgr, amh, ar, cyp11a1 and cyp19a1)*.

DISCUSSION AND CONCLUSIONS

The results obtained confirm the well-known feminizing effect of BPA and among the doses tested the 10 µg/L appears to be the less detrimental. Interestingly, the reduction of gene transcription in the testis by all DGB doses let hypothesize its effects on steroidogenesis and the impairment of male reproduction. These results shed new light on the neglected issue of DGB biological activity in the testis and attempts to estimate the risk associated with its use in the replacement of phtalates. However, additional study are requested to better elucidate the hormone like activity of DGB.

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Oogenesis: the molecular basis behind oocyte growth, egg quality, fertilization and embryo development

SOA3

PARACRINE REGULATION OF FOLLICLE DEVELOPMENT IN ZEBRAFISH

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INTRODUCTION

Follicle development in zebrafish is divided into five stages, primary growth (stage I), cortical alverolus (stage II), vitellogenesis (stage III), oocyte maturation (stage IV) and mature egg (stage V) (Selman et al., 1993). While regulation of early stages of follicle development is poorly understood, it is well established that vitellogenesis is mainly controlled by follicle-stimulating hormone (FSH) and estrogen whereas oocyte maturation is primarily regulated by luteinizing hormone (LH), maturation-inducing hormone (MIH) and maturation promoting factor (MPF). In addition, peptide hormones and growth factors produced within the ovary, including members of the transforming growth factor β (TGF-β) superfamily, have also been shown to play important roles in regulating oocyte maturation.

MATERIALS AND METHODS

In vivo and in vitro studies were performed to determine the effects of TGF-β ligands on oocyte maturation. Real-time PCR and Western blot analyses were carried out to investigate genes regulated by the TGF-β ligands.

RESULTS

We have demonstrated that activin stimulates oocyte maturation in zebrafish (Wu et al., 2000) while TGF-β (Kohli et al., 2003) and BMP-15 exert inhibitory effects on LH-induced oocyte maturation (Clelland et al., 2007) by regulating the expression of membrane progestin receptors (mPRs) (Tan et al., 2009). More recently, we found that two Nodal transcripts, ndr1 and ndr2, are expressed in follicles and their mRNA levels declined during with the progression of follicle development. Treatment of intact follicles or denuded oocytes with Nodal significantly reduced basal and hormones-induced oocyte maturation.

DISCUSSION AND CONCLUSIONS

Our findings suggest that the TGF-β superfamily plays important regulatory roles in fine-tuning the response of follicles to LH and MIH.

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O14

PROTEOMICS OF EGG QUALITY IN ZEBRAFISH

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INTRODUCTION

Most cultured fishes have problems producing eggs with high embryo developmental potential, which constrains fish farming and global food security. Maternally-inherited RNAs and proteins regulate early development until the activation of the zygotic genome (Bouleau et al., 2014). Numerous mRNA transcripts may be differentially expressed in good quality (GQ) versus poor quality (PQ) eggs of several species of cultured fishes, but there has been little consistency between studies in the specific transcripts involved (Sullivan et al. 2015). Additionally, in zebrafish oocytes and other complex biological systems, the correlation between mRNA transcripts and their product protein abundances is poor (Knoll-Gellida et al. 2006; Maier et al., 2009). As the zebrafish egg proteome has not previously been related to developmental competence, the aim of this study was to deliver the first proteomic portrait of GQ versus PQ eggs and to identify potential egg quality markers in this important model species for vertebrate development.

MATERIALS AND METHODS

Aliquots of zebrafish eggs were sampled just after fertilization and stored at -80^{0}C for proteomic analyses. Sixty eggs per spawn were incubated to assess embryo survival after 24 h. Spawns with <30% and >90% survival were considered to be of PQ and GQ, respectively. Eggs from 4 spawns of each quality type were pooled (40 eggs/spawn) for protein extraction followed by SDS-PAGE, fractionation, in-gel digestion and LC-MS/MS (Chalmel et al. 2014). Spectra were searched against the Uniprot zebrafish proteome (zfPDB) to identify proteins with >1 returned peptide that were uniquely present in GQ or PQ eggs. Quantitative differences in protein abundance were assessed as normalized spectral counts (N-SC) by searching the spectra against a concatenated database generated from ENSEMBLE zfPDB (Zv9) with the output transferred to ProteoIQ. Differential expression of proteins common to both egg quality groups was assessed as both the numerical difference and the fold change (expressed as lLog(2)l) in N-SC values between GQ and PQ eggs. Only proteins with a difference of N-SC values of ≥10 and a fold change value of ≥1 between the two sample types were considered. Multiple protein isoforms always exhibited the same pattern in fold change between sample types; only the isoform with the highest N-SC value was considered. Protein annotations and enrichment analyses were made using DAVID, Gene Ontology (GO), or manual literature searches taking into account terms for biological process, molecular function and cellular component.

RESULTS

Of 2719 total proteins detected, and considering only those represented by more than one returned peptide, 103 were unique to GQ eggs and 124 were unique to PQ eggs. It was possible to annotate only 47% and 41% of these unique proteins in GQ and PQ eggs, respectively. Of annotated proteins unique to GQ eggs, 39% are involved in protein synthesis (PS), 31% are involved in cell cycle, division, growth, proliferation or fate (CC), 10% are involved in energy metabolism (EM), 10% are involved in lipid metabolism (LM), 4% are involved in protein degradation or synthesis inhibition (PD), and 6% are involved in other biological processes. Of the annotated proteins unique to PQ eggs, 31% are involved in PS, 26% are involved in CC, 12% are involved in PD, 12% are involved in apoptosis, 6% are oncogene products, only 4% are involved in EM, and only 2% are involved in LM,

with the remaining 8% being involved in other biological processes. In the quantitative analysis of protein abundance, only 2.7% of proteins common to both sample types exhibited an N-SC difference or fold change above the cutoff values ($\geq$ 10 N-SC and lLog(2) foldl $\geq$ 1) indicating differential abundance between GQ and PQ eggs, with 57% being more abundant in PQ eggs and the remainder being more abundant in GQ eggs. At the highest stringency level, DAVID indicated that the pool of proteins more abundant in PQ eggs was enriched with nucleotide binding (NB) proteins and ion binding (IB) proteins with enrichment scores of 0.27, and 0.04, respectively. The pool of proteins more abundant in GQ eggs was enriched for protein folding-related, NB, and IB proteins with enrichment scores of 4.82, 2.29 and 1.84, respectively. Scrutiny of the entire panel of differentially expressed proteins revealed that almost half of the proteins more abundant in PQ eggs were uncharacterized proteins bearing various types of lectin domains or were zona pellucida (ZP) proteins, whereas most of the proteins more abundant in GQ eggs were in the same categories noted above for proteins uniquely present in GQ eggs.

DISCUSSION AND CONCLUSIONS

The pattern of proteins uniquely detected in good versus PQ eggs differed markedly between the two sample types. Specifically, PQ eggs uniquely exhibited several proteins involved in apoptosis and a number of oncogene products, as well as markedly fewer unique proteins involved in energy metabolism and lipid or cholesterol metabolism, with slightly lower numbers of unique proteins involved in protein synthesis or (aside from oncogene products) cell division and differentiation. With regard to proteins differentially abundant between the two sample types, PQ eggs exhibited markedly more ZP proteins and proteins bearing lectin domains whereas GQ eggs exhibited more proteins involved in anabolic and cell division processes noted above. It can be tentatively concluded that PQ zebrafish eggs are characterized by protein deficiencies likely underlying dysfunctional cell division, apoptosis and disrupted energy metabolism and that they also likely bear protein markers (lectins, ZP proteins) indicative of poor quality on their surface (chorion and oolemma). In conclusion, the zebrafish egg proteome appears to be linked to the developmental potential of the egg, a phenomenon that begs further investigation.

ACKNOWLEDGEMENTS

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O15

SEX DETERMINATION AND EGG QUALITY EVALUATION IN THE EURASIAN PERCH, *PERCA FLUVIATILIS*, USING ULTRASONOGRAPHY.

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INTRODUCTION

Among the temperate freshwater fish species, the flesh of percids is very appreciated by consumers. As a consequence percid breeding becomes an interesting market and several fish farming focus on pike-perch and Eurasian perch production. Perch reproduction cycle last for 9 months and is under the control of the temperature and the photoperiod (Abdulfatah *et al.*, 2011). Even if female oogenesis is induced, the reproduction success remains variable. In addition, as for many fish species, there is no sexual dimorphism for the Eurasian perch. As a consequence, scientific studies investigating either oogenesis or reproduction success and fish farmers wishing to check perch oogenesis need to sample some fish in their tank. This is also true with other fish species. In order to find a way to make early sex determination and check oogenesis process, we used ultrasonography on perch. This technique as used with success with several other fish species (for review Novelo and Tierch 2012) to study oogenesis process. This technique has the advantage to be non invasive and to allow accurate study of fish gonadogenesis. The present study aimed at presenting first data obtained to (i) to determine the sex of Eurasian perch individuals, (ii) to define correlations between parameters obtained using ultrasonography and regular methods and (iii) to predict potential egg quality.

MATERIALS AND METHODS

Two populations of Eurasian perch (wild and domesticated) were used in this study and all individuals had been furnished by the fishfarm “Lucas Perches” (Hampont, France). In total, around 300 fish have been reared in our facilities from the larval stages. Their reproduction was induced using a photo-thermo-periodic program previously developed in our laboratory (Abdulfatah *et al.*, 2011). Three and five months after the beginning of the induction, ultrasonography experiments have been performed to study fish gonadogenesis on a sample of 30 females. Experiments have been performed using a MyLab30 Vet gold apparatus (Esaote-Piemedical) using a LA332 probe (Hospimedi France) 3-10 Mhz. The frequency was low (6 MHz) to allow a penetration of the sound waves for at least 5 cm. The area and the perimeters of the gonads were measured on ultrasonography picture. Every fish was put to death, weight and measured. Their gonads were dissected, weighted, photographed and fixed in Bouin’s solution to perform histological studies. The gonado-somatic index was calculated and a comparison of data obtained with the two techniques.

RESULTS

The experiment was first performed three months after the beginning of the induction. It appeared that the sex determination was already possible at that time with an accuracy of around 94 %. Two months later we almost obtained 99% of success. The ovaries appeared as mass punctuated by light dots corresponding to the oocytes (figure 1A,B). On the contrary, testes were less evident to discern and appeared mainly as two black lobes separated by a white line (figure 1C,D).

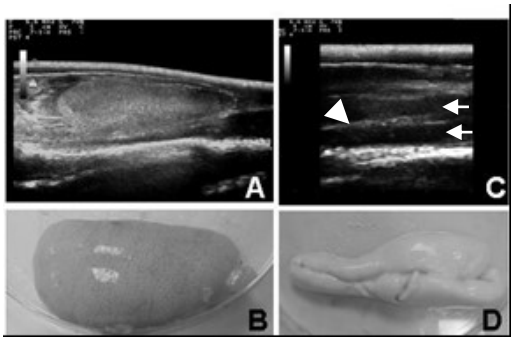

Figure 1: Comparison of ultrasonography images (A,C) with pictures below of the corresponding dissected ovary (B) and testis (D). On the picture A, the perimeter of the ovary is drawn. On the picture C the two dark lobes of the testis (arrows) are separated by a white line (arrowhead).

We measured the ovary area and perimeter for 30 females by ultrasonography and compared it with the gonad weight and the GSI. It appears that these parameters are poorly correlated indicating that we need to accumulate other information to better evaluate oogenesis progression.
Finally, we observed on the echography pictures several pockets of liquid for some fish. These observations were confirmed after dissection and were not present for every fish. Moreover, the egg density in the ovary was also observed and showed that it was not homogenous in every case. Finally, we observed a scale from white to dark gray colors for the oocytes, suggesting that the lipid content may be variable between oocytes. Indeed the gray scale obtained depends on the lipid/water relative composition. These information are important because it could preset reproduction impairment. Indeed, the accumulation of lipid is one of the key elements to confer a good quality to the ova.

DISCUSSION AND CONCLUSIONS

Several studies investigated the possibility to identify the sex of fish that don't present any external sexual dimorphism. For the Eurasian perch this determination was successful 3 months after the beginning of the gonadogenesis as observed in other studies (Novelo and Tierch 2012). However, our measurements failed to characterize efficiently the oogenesis progression. In other studies, several calculations were proposed to make this characterization (Novelo and Tierch 2012). Those reproductive indexes all seem reliable but a comparative study needs to be performed to better characterize them. Finally, few studies tried to find criteria presetting ova quality and reproduction success. We observed that the internal organization of the ovary (liquid pocket formation, oocytes density) and the oocyte lipid/water content may be easily observable. The comparison with histological study of the gonads (still under investigation) will help us to better characterize this point. Ultrasonography appears as new methodology to study fish gonadogenesis because it is non-invasive and easy to perform. This new approach already gives good results for sex identification. It also opens new avenue o research especially for the early determination of ova quality.

AKNOWLEDGEMENTS

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O16

RIBOGENESIS MOLECULAR MARKERS OF OOCYTE DIFFERENTIATION IN EUROPEAN EEL *ANGUILLA ANGUILLA*: TRANSCRIPTIONAL REGULATION DURING ARTIFICIALLY INDUCED OOGENESIS

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INTRODUCTION

European eels (*Anguilla anguilla*) present a complex life cycle, with a blockade of sexual maturation at prepubertal stage in continental waters that is removed during their reproductive oceanic migration to the Sargasso Sea.
It is known that during teleost oogenesis, oocytes undergo diverse molecular changes leading to oocyte maturation and spawning. Such changes include variations in the level and nature of gene expression and the accumulation of reserve molecules necessary, if fertilization occurs, for early embryonic development. This is the case of 5S rRNA which is transcribed in the nucleus by RNA polymerase III (Pol-III) and stockpiled in oocytes during growth and differentiation (Diaz de Cerio et al., 2012). With the onset of vitellogenesis, strong 18S & 28S rRNA expression begins in the nucleolus, under the regulation of RNA polymerase I (Pol-I). Understanding the mechanisms controlling the production of ribosomal building blocks in the oocytes of eels is of particular interest, considering the dramatic decline of populations in European continental waters and the present impossibility to reproduce European eels in captivity. Besides, due to their sexual maturation blockade, eels represent a powerful experimental model to study the process of oocyte differentiation in teleosts.
In this way, the fluctuations in 5S and 18S rRNA production, as well as the changes in transcription levels of target genes associated with ribogenesis, could be used as markers to monitor puberty, oocyte growth, maturation and individual responsiveness during artificially induced oogenesis.

MATERIALS AND METHODS

Silver eels were captured in the Albufera Lagoon during their downstream migration to the sea. After two weeks of acclimatisation in seawater, they were treated with weekly intraperitoneal injections of 20 mg/kg carp pituitary extract (CPE). Groups of 6 eels were sampled after 0, 4, 8 & 12 weeks of treatment. A portion of each ovary was stored in RNAlater for molecular analysis and the rest was fixed in 10% formalin and paraffin embedded for histological staging. The ovarian development was classified according to Pérez et al. (2011). Total RNA was extracted using TRIzol® and RNA quality assessed by absorbance and by capillary electrophoresis (Bionalyzer 2100, Agilent). The concentration of 5S rRNA and 18S rRNA bands were quantified for each sample in the electropherograms, and the 5S/18S rRNA ratio calculated. Then, the transcription levels of TFIIIA, UBTF1, 42sp43, RPL5 and RPL11 were quantified by RT-qPCR. The results were normalized with the exact cDNA amount, as quantified by the Oligreen fluorescence method.

RESULTS

Non-injected females only showed previtellogenic oocytes (PV), while after 4 CPE injections, oocytes in early-vitellogenesis (EV) could be observed. With 8 and 12 injections, oocytes in mid- and late-vitellogenesis (MV and LV) and with migratory nuclei (MN) developed. Some individuals did not respond, even after 12 weeks of CPE treatment, still displaying PV oocytes (non-responders; NR).
5S rRNA, and all the ribogenesis genes tested, were highly transcribed in PV oocytes. As oocytes developed with the hormonal treatment, transcriptional levels of all the genes

and the 5S/18S rRNA ratio decreased in ovaries, following this trend: PV>EV>MV>LV>NM (Figure1). All the transcripts reached their lowest in MN phase, with the exception of 18S rRNA that was at its highest. In contrast, NR females injected for 12 weeks showed high 5S rRNA and nearly non-detectable 18S rRNA expression, as in PV.

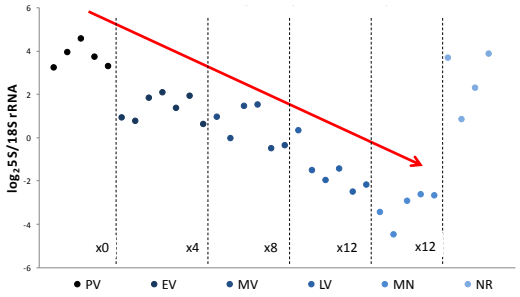

Figure1. 5S/18S rRNA index during oocyte development in eels.

DISCUSSION AND CONCLUSIONS

In all adult teleost species studied to date, presence of perinucleolar oocytes during each reproductive annual cycle is characterised by a massive transcription of 5S rRNA in ovary. On the other hand, initiation of vitellogenesis is marked by the beginning of 18S rRNA transcription. The results obtained show that 5S rRNA expression in European eel ovaries is also at its highest in previtellogenesis. Thus, 5S rRNA transcription could be considered a molecular biomarker of pre-puberty in female eels. With subsequent hormonal injections, the ovary initiates vitellogenesis; process that is accompanied by an up-regulation of 18S and 28S rRNA expression in oocytes. NR females fail to express the largest rRNA molecules, which could thus be taken as markers of successful progression of vitellogenesis.

The molecular mechanism of ribosome production in the ovaries of eels was also analysed by studying the transcriptional regulation of genes that participate in 5S rRNA handling. Transcription factor IIIA (TFIIIA), necessary for Pol-III activation (and 5S rRNA production); 42sp43, necessary to stockpile 5S rRNA in the cytosol; and ribosomal proteins 5 and 11 (RPL5 and RPL11), necessary to import 5S rRNA to the nucleolus from the cytosol to form the large ribosomal subunit, resembled the expression pattern of 5S rRNA through oocyte differentiation. The upstream binding transcription factor 1 (UBTF1), necessary for Pol-I activation and 18S rRNA production, showed the same transcriptional pattern as the previous genes. Therefore, UBTF1 does not follow the increasing transcriptional trend of 18S rRNA. This could reflect that UBTF1 is postranscriptionally regulated through activating phosphorylation events and not transcriptionally.

In conclusion, we have been able to prove that the transcriptional dynamics of ribosomal genes provide useful molecular tools to characterize the process of artificially induced ovary development in European eels. In the same way, these markers can be used for the early identification of NR females.

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O17
OPTIMIZED SPAWNING INDUCTION PROTOCOL FOR MEAGRE (*ARGYROSOMUS REGIUS*) USING WEEKLY INJECTIONS OF GnRHa

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INTRODUCTION
Reproduction of meagre (*Argyrosomus* regius) still remains a problem, as females do not mature and spawn readily in captivity (Duncan et al., 2013; Mylonas et al., 2013a), and exogenous hormones are used to induce spawning. Here we follow recent studies (Mylonas et al., 2015; Mylonas et al., 2013b; Mylonas et al., 2013a) on the reproduction of meagre broodstocks and describe an optimized protocol for the induction of spawning for commercial operations, based on the use of weekly GnRHa injections. The objective was to examine (a) how long females would continue spawning in response to consecutive, weekly GnRHa injections, and (b) obtain egg production and quality data.

MATERIALS AND METHODS
GnRHa were given every week from 7 May to 28 August 2014 to four pairs of fish (n=4). Females (mean ±SD body weight 9.7±1.0 kg) were treated with a GnRHa injection of 15 μg kg^{-1}. The males were treated at the start of the experiment with a GnRHa implant for an effective dose of 43–57 μg kg^{-1} GnRHa, and later on as needed to enhance spermiation. When a cumulative total of two females (*i.e.*, 50%) failed to spawn in response to 2 consecutive injections, the experiment was concluded, and no further injections were given. Monitoring embryo and larval survival was done using 96-well microtiter plates.

RESULTS
The GnRHa injected females spawned for 17 consecutive weeks, both on the 2nd and 3rd day after each injection (Fig. 1). The spawns obtained 2 d after each injection had significantly higher fecundity compared to the spawns obtained 3 d after injection (ANOVA, $P < 0.001$). There was no significant effect of injection number on mean fecundity after each injection (ANOVA, P=0.83), but there was a negative linear correlation (n=32, R^2=0.38, $P < 0.01$, not shown) between 2nd spawn fecundity and GnRHa injection number. Fertilization success was high during the experiment (Fig. 1), without any significant effect of either GnRHa injection week (ANOVA, P= 0.16) or spawn number after each injection (ANOVA, P=0.21). There were no significant differences over the course of the study in response to the consecutive GnRHa injections, in terms of 24-h embryo survival (ANOVA, P=0.99), hatching (ANOVA, P=0.88) or 5-d larval survival (ANOVA, P=0.33), even after 17 weekly injections of GnRHa (data not shown).

DISCUSSION AND CONCLUSIONS
The study demonstrates that multiple GnRHa injections are physiologically an appropriate spawning induction therapy for meager resulting in a very large number of spawns of high fecundity and egg quality. Based on histological evaluations, the annual fecundity of meagre has been estimated to be around 1 million eggs kg^{-1} body weight (Gil et al., 2013). In the present study, mean fecundity of the fish that spawned for 17 weeks was 1,415,000 ± 150,000 eggs kg^{-1}, indicating that meagre may have indeterminate fecundity and that the use of GnRHa injections can result in a higher recruitment of vitellogenic oocytes, thus increasing the potential annual fecundity of the species.

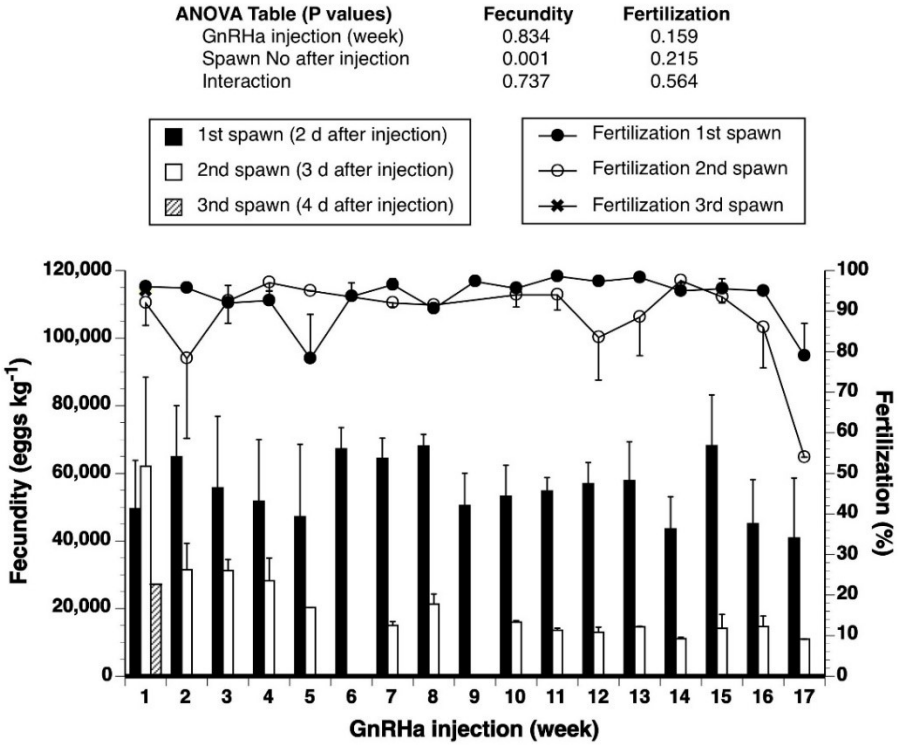

Figure 1. Mean (±SEM) daily fecundity and fertilization of individual meagre females (n=1-4) induced to spawn with multiple GnRHa injections (n=17, once every week) beginning on 8 May 2014. The two-way ANOVA indicated the existence of a significant interaction in fecundity only, while the two main factors did not have any significant effect (either in fecundity or fertilization).

ACKNOWLEDGEMENTS

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O18

GAMETE BIOCHEMISTRY AND GENETIC ARCHITECTURE ON THE RATE OF EYED EMBRYOS IN A EXTERNALLY FERTILIZING FRESHWATER FISH, IDE *LEUCISCUS IDUS*

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INTRODUCTION

Understanding the role of gamete biochemistry in a fertilization event, more specifically the effects of physico-chemical properties of the seminal plasma on the sperm traits and the relationships between ovarian fluid biochemical traits are crucial for artificial reproduction of fish. Limited studies have examined the contribution of additive genetic variance, non additive genetic variance, as well as maternal effects on the rate of eyed embryos in externally fertilizing fishes. Therefore, the objectives of this study were to (i) determine the effects of physico-chemical properties of seminal plasma and ovarian fluid on gamete quality, and (ii) understand the effects of genetic architecture on the rate of eyed embryos in Ide *Leuciscus idus*.

MATERIALS AND METHODS

Five sires and five dams were used in this study. CASA parameters of sperm, from each sire were analyzed. Seminal plasma and ovarian fluid were obtained by centrifugation at 3000 rpm for 15 min at 20 °C. Osmolality and pH of the seminal plasma and ovarian fluid were measured by vapor pressure osmometer (Model: 5600, Wescor. Inc., USA) and microprocessor pH meter (Model: WTW 320, Germany), respectively. Ionic composition (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-), glucose, cholesterol and total protein of the seminal plasma and ovarian fluid were analysed by an auto-analyser (ADVIA 1800 Chemistry System, Siemens, Germany). Moreover, these fish were crossed using a North Carolina II breeding design (5 sires $\times$ 5 dams) with 3 replicate crosses per family combination. Data were then analyzed using a full-factorial ANOVA and variance components were estimated by Restricted maximum likelihood (REML) model using the lme4 package of R. Following fertilization, embryos were kept in a 300 L incubation tank at 15.6 °C with continuous water flow for 5 days. At the eyed-egg stage the percentage of viable eyed embryos was later calculated for each family combination.

RESULTS

Total protein content and K^+ of seminal plasma were significantly correlated with sperm velocity ($p < 0.01$ and $p < 0.05$, respectively). For ovarian fluid, total protein was significantly correlated with K^+ ($p < 0.01$), glucose ($p < 0.05$) and cholesterol ($p < 0.01$). Eyed embryo rate ranged from 8.69 to 91.15% for the 25 family crosses (Fig 1A). The full-factorial ANOVA model showed that the sire, dam, and sire $\times$ dam interaction term were highly significant ($p < 0.0001$) (Fig. 1B); this highly signficant interaction term at fertilization confirms that certain family combinations were more compatible than others. Here, the sire, dam, and sire $\times$ dam random effects explained 20.24%, 10.73%, and 4.83% of the total variance, respectively (Fig 1B).

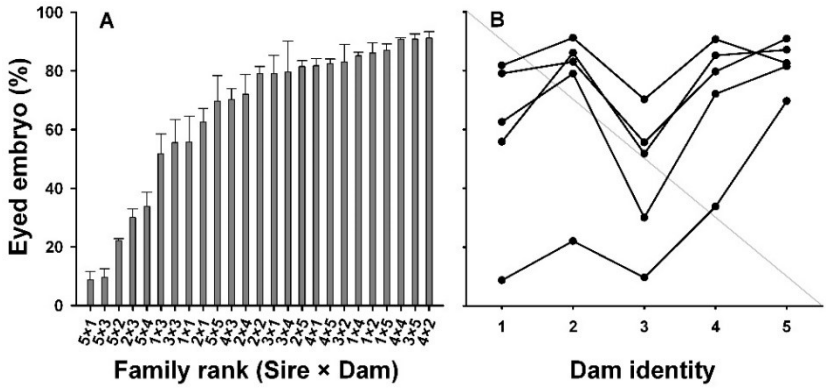

Fig. 1: Eyed embryo rate for Ide, *Leuciscus idus.* (A) Rate of eyed embryo for 25 family crosses; (B) Interaction between sire and dam at the eyed embryo stage.

DISCUSSION AND CONCLUSIONS

Physico-chemical properties of seminal plasma and ovarian fluid play a key role in gamete quality traits in teleost species (Lahnsteiner et al. 2004). Here, the mean concentration of K^+ and Ca^{2+} found in seminal plasma of Ide was lower compared to other cyprinids (Alavi & Cosson, 2006). Additionally, we showed that K^+ and total protein of seminal plasma were key factors for sperm motility traits (i.e. velocity traits) for Ide. Total protein in ovarian fluid was higher than the seminal plasma and significantly correlated with K+, glucose and cholesterol. In the literature it has been shown that the protein content of ovarian fluid plays an important role in egg swelling and hardening of egg membranes after activation and also during fertilization by changing of protein structure (Iuchi et al., 1991).
In this study, we carried out a multi-factorial breeding design to assess the genetic components of eyed embryos on the Ide. This design allowed us to calculate sire, dam and family interaction effects, and thus estimate the contribution of additive, maternal, dominance and environmental components to the total phenotypic variance (Lynch and Walsh, 1998). Our results showed that sire and dam identity both had significant effects on the rate of eyed embryos. Sire effects explained 20.24% of the total variance, which was two fold higher than the effects of dam (10.73%). In conclusion, this study suggested that along with gamete biochemistry, genetic compatibility between sires and dams plays an important role in eyed embryo stage on the Ide and thus should be incorporated into breeding and/or restocking programs for this econimocally important species of freshwater fish.

ACKNOWLEDGEMENTS

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O19

THE EFFECT OF HORMONAL TREATMENT ON EMBRYONIC SURVIVAL, PROXIMATE COMPOSITION AND FATTY ACID PROFILE OF EURASIAN PERCH, *PERCA FLUVIATILIS* L., EGGS

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INTRODUCTION

Hormonal stimulation of ovulation in percids aquaculture is still an indispensable method of synchronization of spawning. This is especially important during the out-of-season (OOS) spawning allowing year-round commercial production. However, during OOS spawning hormonal treatment affect the entire process of final oocyte maturation during which a number of morphological changes occurs together with changes of composition (Żarski et al. 2012a). It is well known that the composition of the eggs (mainly fatty acids [FA] profile) reflect the egg quality (Henrotte et al. 2010). However, it is little known how hormonal treatment affect the composition of the eggs in perch. And this knowledge would allow higher control over the egg quality during the controlled reproduction.

The aim of the study was to evaluate the effectiveness of two hormonal preparation on the egg quality, proximate composition and FA profile while performed out-of and during the reproductive seasons in relation to the eggs collected in the wild.

MATERIALS AND METHODS

Fish (Sasek Wielki Lake population) were obtained OOS and in-season (IS). Before the hormonal treatment the maturation stage of females was determined (as proposed by Żarski et al. 2011) and for experiment were taken fish with stage I and IV during OOS and IS, respectively. During both, OOS and IS three groups were created. Each group was treated either with hCG (500 IU kg^{-1}), GnRHa (100 µg kg^{-1}) or 0.9% NaCl (control group). In this way six groups were distinguished: hCG-1, GnRH-1, C-1 (for OOS spawning), hCG-2, GnRH-2 and C-2 (for IS spawning). Additionally, during the spawning season eggs (n=7) from the same lake were collected considered as naturally spawned (NS) group. Such a comparison was possible as the composition of the eggs remains constant during incubation (Abi-Ayad et al. 2000).

Females from each group were kept at 12°C in a separate 300 L tanks together with randomly allocated males (n=15 for each tank) treated with hCG (500 IU kg^{-1}). Fish were left to spawn spontaneously in order to limit the effect of handling stress. Spawned egg-ribbons were collected and incubated separately. Just before the blastula stage an egg sample from each group was frozen for proximate composition and FA profile analysis (as described by Żarski et al. 2012a). At the eyed-egg stage survival rate of embryos was determined.

RESULTS

The eggs obtained OOS were characterized by a lower quality as compared to the NS group. However, only in group GnRH-1 egg quality was comparable to the IS spawning (Tab. 1). The water content was found to be significantly lower in OOS as compared to IS spawned fish (including NS), among which no differences were found. Similar tendency was found for other compounds (total fat, ash and protein contents) were mostly hCG-1 and C-1 were found to differ from the IS spawned groups (Tab. 1). Similar relationship was found for the FA profile, where in most cases hCG-1 and C-1 could be considered as an outliers (Tab.1). GnRH-1 group only in the case of C18:1 was found to differ from NS group. The IS spawned eggs were always similar to NS group (Tab. 1).

Tab. 1. The results (mean ±SD, n=7) of the embryonic survival (%), proximate composition (%) and essential fatty acids profile (% of total fat) of the eggs of Eurasian perch obtained during spontaneous out-of (groups -1) and in season (groups -2) spawning following the hormonal stimulation with different spawning agents (hCG, GnRH) or without the hormonal treatment (groups C). The results are compared to the eggs spawned naturally in the lake (group NS). Data in rows marked with different letter index were statistically different ($p<0.05$).

	hCG-1	GnRH-1	C-1	hCG-2	GnRH-2	C-2	NS
Embryonic survival	46.8 ±23.3^{c}	58.6 ±33.1^{bc}	31.0 ±21.9^{c}	60.6 ±20.8^{ab}	68.3 ±21.7^{ab}	60.1 ±20.4^{ab}	88.4 ±5.4^{a}
Total fat	1.4 ±0.4^{abc}	1.4 ±0.3^{ab}	1.7 ±0.2^{a}	0.9 ±0.3^{c}	1.2 ±0.2^{bc}	1.3 ±0.2^{ab}	1.2 ±0.2^{bc}
Ash	0.4 ±0.1^{a}	0.3 ±0.1^{ab}	0.3 ±0.0^{ab}	0.3 ±0.0^{b}	0.3 ±0.0^{b}	0.2 ±0.0^{b}	0.3 ±0.0^{b}
Water	90.9 ±1.3^{b}	90.7 ±1.8^{b}	90.1 ±2.2^{b}	93.4 ±0.3^{a}	93.6 ±0.3^{a}	94.0 ±0.4^{a}	92.9 ±0.6^{a}
Protein	5.7 ±1.1^{a}	4.7 ±0.3^{ab}	4.8 ±0.6^{ab}	3.3 ±0.6^{c}	3.2 ±1.1^{c}	3.7 ±0.8^{bc}	4.1 ±0.7^{abc}
C16:0	9.5 ±4.1	8.4 ±0.4	7.7 ±1.0	8.4 ±0.5	8.4 ±0.6	8.7 ±0.8	9.3 ±1.7
C16:1	19.5 ±8.0^{a}	16.7 ±2.1^{ab}	13.3 ±1.3^{b}	16.7 ±2.9^{ab}	18.1 ±3.6^{a}	17.0 ±2.7^{ab}	13.9 ±2.3^{ab}
C18:1	23.2 ±6.5^{a}	17.1 ±1.3^{b}	18.9 ±2.7^{b}	19.0 ±4.2^{ab}	19.7 ±2.5^{ab}	20.1 ±4.4^{ab}	21.1 ±6.8^{a}
C18:2(n-6)	13.7 ±2.8^{a}	13.8 ±3.1^{a}	8.7 ±2.4^{b}	11.2 ±2.7^{ab}	12.0 ±1.4^{ab}	11.4 ±2.7^{ab}	14.9 ±1.8^{a}
C20:5(n-3) EPA	4.6 ±2.2	6.0 ±1.0	6.1 ±1.4	7.3 ±4.3	5.7 ±1.1	5.8 ±1.5	3.9 ±0.7
C22:6(n-3) DHA	10.1 ±7.9^{c}	17.4 ±3.1^{ab}	23.1 ±3.9^{a}	15.6 ±1.5^{ab}	15.9 ±2.5^{ab}	15.6 ±2.9^{ab}	14.1 ±1.1^{b}
Total saturated	14.9 ±4.5^{a}	12.2 ±0.6^{b}	11.2 ±1.3^{b}	12.3 ±0.6^{b}	12.2 ±0.8^{b}	12.5 ±1.0^{b}	13.0 ±2.1^{b}
Total monounsaturated	44.7 ±13.1^{a}	35.4 ±3.1^{ab}	33.4 ±2.8^{b}	37.0 ±3.3^{ab}	39.2 ±3.6^{ab}	38.5 ±2.9^{ab}	35.8 ±6.7^{ab}
Total n-6	17.9 ±3.8	19.8 ±2.5	16.6 ±2.5	18.5 ±4.1	18.6 ±1.3	19.0 ±3.8	22.1 ±3.5
Total n-3	22.5 ±12.0^{b}	32.6 ±5.3^{ab}	38.8 ±5.6^{a}	32.2 ±6.4^{ab}	30.1 ±3.8^{ab}	30.0 ±3.3^{ab}	29.1 ±3.7^{ab}

DISCUSSION AND CONCLUSIONS

On the base of the presented results it is very difficult to indicate the parameter (among the studied ones) alteration of which can correspond to lower egg quality. Especially, that huge variation in egg quality (in many cases probably masking significant differences to the NS group) in experimental groups was observed. Nonetheless, the type of hormonal preparation was found to have a significant effect on the egg composition, as well. The presented data suggest that application of GnRH OOS allowed to obtain eggs with composition in many cases similar to that of NS group, although significantly lower egg quality was observed. The only difference was found in water content and C18:1 FA. The latter was suggested by Żarski et al. (2012a) to be possible quality indicator since its level in wild fish was usually almost two fold higher than observed in cultured ones (Henrotte et al. 2010). However, in this case the possible relation with egg quality is not so clear, what requires further studies. The lower water content in OOS spawned eggs suggest lower intensity of cortical reaction (which was previously found to correspond with egg quality in percids – Żarski et al. 2012b) or not well developed gelatinous coat of the egg which could possibly acquire more water.

In conclusion, the present study for the first time proves that the hormonal treatment may alter the egg composition in perch, but only when OOS spawning is performed.

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O20

CONTRIBUTION OF NUCLEOPLASMIN GENES TO EGG DEVELOPMENTAL COMPETENCE IN FISH

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INTRODUCTION

The molecular mechanisms controlling the ability of the egg to successfully support early embryonic development once fertilized (*i.e.* egg developmental competence) remain poorly understood. Previous studies have highlighted the link between egg developmental competence and egg transcriptome. Among candidate genes analyzed in fish, nucleoplasmin 2 (*npm2)* is of particular interest. This gene is known as a maternal-effect gene in mouse, and *Npm2* knock-out (KO) leads to major developmental failures at early stages (Burns et al., 2003). We recently characterized the maternal contribution of *npm2* maternal mRNA to embryonic developmental success in zebrafish *Danio rerio* (Bouleau et al., 2014). A gene closely related to *npm2* and known as *npm2a* is present in the zebrafish genome, but its role and presence in other species remain unclear. The present work aimed at investigating the evolutionary history of *npm2* and *npm2a* and possible roles in egg developmental competence.

MATERIALS AND METHODS

npm2 and *npm2a* expression profiles were analyzed by QPCR analysis during oogenesis and early embryonic development in zebrafish and *Xenopus tropicalis*. Phylogenetic analysis of *npm2* and *npm2a* genes was performed using neighbor-joining method with 1000 replicates (MEGA software). An antisense morpholino-oligonucleotide (ATG-MO) directed against the translation initiation site was used in order to knock-down Npm2 protein expression. Micro-injection was performed in 1-cell zebrafish embryos and phenotype was assessed throughout early development.

RESULTS

Using phylogenetic analysis and synteny, we demonstrate that *npm2a,* which was previously thought to be a zebrafish-specific duplication of *npm2*, is also present in other teleost fish species and in tetrapods but probably lost in mammals.
By QPCR analyses, we also demonstrated, as previously shown for *npm2* in zebrafish, that *npm2a* is predominantly expressed in the ovary. During early development, *npm2a* mRNA decreases and follows a typical pattern of maternally-inherited mRNAs with no or little zygotic expression. QPCR analyses of *npm2a* in a tetrapod species (*Xenopus tropicalis*) show similar expression patterns in tissues as well as during oogenesis and early development for *npm2* and *npm2a* that are also very similar to fish *npm2* and *npm2a*.

DISCUSSION AND CONCLUSIONS

Here we show by phylogeny and synteny analyses that a duplication of *npm2* occurred in the vertebrate ancestor that led to *npm2* and *npm2a*. Both duplicates are found in fish and tetrapods but our data suggest that *npm2a* was lost in mammals.
In zebrafish and *Xenopus*, both genes are found in the oocyte and predominantly expressed in the ovary, a typical feature of maternal effect genes (i.e. genes that encode mRNA and/or protein that are produced in the oocyte during oogenesis and play an essential role during early embryogenesis (Abrams and Mullins, 2009; Ma et al., 2006; Pelegri, 2003)). During early development, the rapid decrease around maternal-to-zygote transition and subsequent lack of zygotic expression observed in both zebrafish and *Xenopus*, suggest an important role during the first

steps of development, in consistency with what is known for Npm2 in zebrafish and mice. Together, these results show that, similarly to *npm2*, *npm2a* mRNA could play a role in the molecular mechanisms that define egg developmental competence not only in fish, but also in tetrapods. Further functional studies are needed to test this hypothesis.

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O21

CORTICOSTEROIDS DEEPLY DEPRESS THE *IN VITRO* STEROIDOGENIC CAPACITY OF PERCH OVARY AT THE END OF THE REPRODUCTIVE CYCLE

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INTRODUCTION

It has been reported that stress-induced corticosteroid modulation has negative impact on the endocrine stimulation of steroidogenesis in fish but positive effects have been reported for some fish species such as Nile tilapia depending on the oocyte maturation stage (Gennotte et al, 2012). In percid fishes, the interaction between sex hormones and corticosteroids is not yet clarified. Therefore, the objectives of the study were (1) to determine to what extent glucocorticoid (cortisol) or mineralocorticoid (11-deoxycorticosterone, DOC) hormones interfere with the *in vitro* steroidogenesis capacity of Eurasian perch follicles; (2) to test whether this interference may be mitigated by metabolic endocrine factors, namely insulin-like growth factor-1 (IGF-1), human chorionic gonadotropin (HCG) and thyroid hormones (T3, T4).

MATERIALS AND METHODS

A group of 50 females were reared in outdoor ponds under natural conditions to induce ovarian maturation. Then, six sexually mature fish were selected and dissected twice, during late vitellogenesis (early March) and before the final oocyte meiotic maturation (early April). Just after dissection, pieces of ovarian tissues (12-15 oocytes) were incubated during 6 h at 12°C in control Cortland medium or in medium containing various endocrine compounds as follows:

- Control (medium only): CT; Control + ethanol: CTet
- Metabolic factors: IGF-1 (100nM), HCG (50UI/ml), T3/T4 (20,100ng/ml)
- Corticosteroids: cortisol (10, 100, 1000ng/ml), DOC (1, 10, 100ng/ml)
- Mixture of corticosteroids and metabolic factors at the same doses

Twenty nine endocrine treatments were compared to controls with six replicates per treatment. The *in vitro* follicle steroidogenesis was evaluated in terms of aromatase activity (AA) in the cultured ovarian tissues, and of sex-steroid (testosterone-T, estradiol-E2, 11-ketotestosterone-11KT, and 17α-20ß-dihydroxy-4-pregnen-3-one, DHP) production in the culture medium. For each period, the proportions of advanced oocyte developmental stages were checked by histological examination.

RESULTS

Proportions of advanced oocyte developmental stages were lower in March than in April (49-22 and 16-79 for late vitellogenic oocytes - final meiotic oocytes, respectively, $P<0.05$). AA did not differ in controls over the two periods, but HCG and IGF induced significantly higher response in April not in March. T3 and T4 had no marked effect on AA whatever the dose and the period (Fig. 1a). Both corticosteroids suppressed AA ($P<0.05$), except for DOC at 1 ng/ml (Fig. 1b). HCG prevented the AA suppression induced with low doses of cortisol but failed when it was combined with the same doses of DOC (Fig. 1c). IGF was ineffective in preventing AA suppression by cortisol. HCG was more effective than IGF in stimulating T, E2 and DHP production ($P<0.05$) while T3 or T4 were ineffective. Both corticosteroids decreased sex-steroid productions in a dose-dependent manner ($P<0.05$). For low doses of cortisol, such negative effect was prevented by HCG only for T and E2 production not for DHP and 11KT.

DISCUSSION AND CONCLUSIONS

In terms of aromatase activity, data showed an increase in oocyte sensitivity to HCG or IGF over the time as previously reported for Eurasian perch and other fish

species (Chourasia and Joy, 2007; Milla et al, 2009). Such increase of sensitivity to endocrine factors in April may be related to changes in the oocyte developmental maturation stage since the proportions of final meiotic oocytes were higher in April than in March. But despite the differences in oocyte sensitivity to HCG or IGF, the capacity of E2 secretion seemed comparable since the E2 production induced by these compounds did not significantly differ between March and April as did AA in controls. Both cortisol and DOC induced a dose-related inhibition of the steroidogenesis capacity in contrast to the positive effect on ovarian maturation reported for cortisol in Nile tilapia (Gennotte et al., 2012). Only HCG seemed effective in contracting the negative effects of cortisol at low doses on steroidogenesis capacity; but not in the case of DOC.

The results suggested that high corticosteroid release inhibits steroidogenesis capacity in percid fish by hampering sex-steroid productions and/or aromatase activity. They also indicated that gonadotropin stimulation may mitigate the negative effects of cortisol in case of low release of some corticosteroid.

ACKNOWLEDGEMENTS

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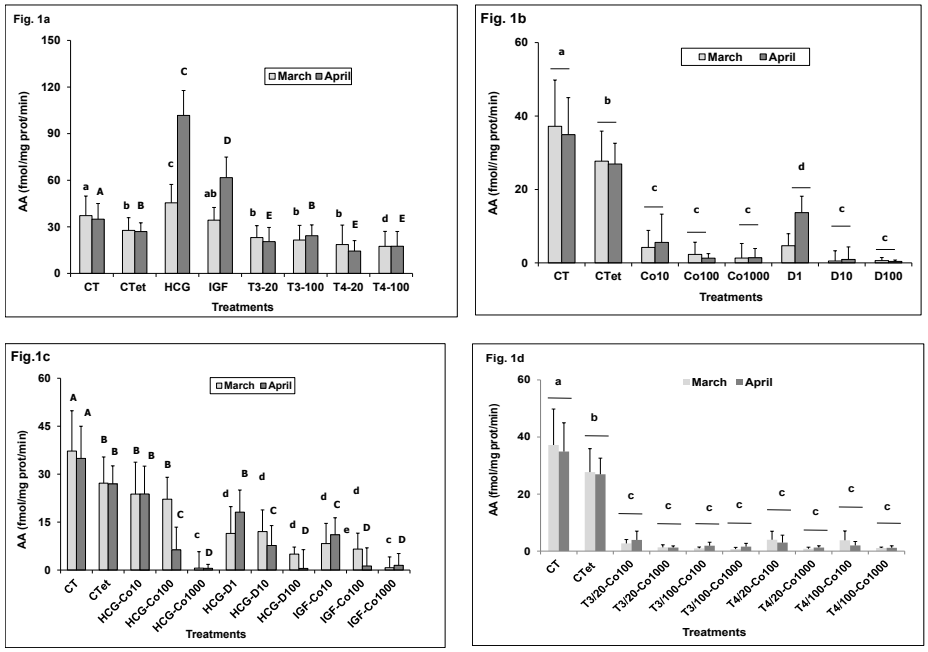

Figure 1a,b,c : *In vitro* ovarian aromatase activity of Eurasian perch oocytes incubated in medium containing metabolic endocrine factors (a), corticosteroids (b), mixtures composed of HCG or IGF + corticosteroids (c) or mixtures composed of T3 or T4 + corticosteroids (d).

O22
SEX STEROID AND ULTRASOUND DIAGNOSIS OF MATURATION IN CAPTIVE CHINESE STURGEON *ACIPENSER SINENSIS*

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INTRODUCTION
Chinese sturgeon *Acipenser sinensis* is one of the critically endangered fish species native to the Yangtze River of China. Biological characteristics such as long lifespan and late maturation make it difficult for establishing the captive populations and realize their successful breeding, which bring handicap for its restocking and conservation. Efforts have been done with raising up the ever largest group of 10-16 years old and more than 100 individuals. During the past four years, most of them were still not mature with stage II gonad, while 5 females and 12 males were observed had been on the processes of maturation. The objectives of this study is trying to establish an combining diagnosis with serum sex steroid and ultrasound images of the gonad development of captive Chinese sturgeon to help our understanding of their final maturation in captive.

MATERIALS AND METHODS
Physical checkings for the older than 10 years captive Chinese sturgeon (more than 100 individuals) were conducted in April and September every year from 2010 to 2015 and 5 mature females and 12 males were confirmed and checked 3-5 times before and after breeding. Ultrasound imaging is conducted with a Terason T3000 portable ultrasonograph and a model 5C2(5-2MHZ) transducer for monitoring the gonad development and health. The incidentally dead specimen were dissected and live gonad tissues were sampled by a self make steel or bronze stick with a hole on the end for identifying the exact gonad stage. Blood samples were collected from the caudal vein and then the serum 17-βestradiol (E2) and testosterone (T) were measured using EIA ELISA kits.

RESULTS
Sex steroid monitorings showed that serum E2 of female Chinese sturgeon developed with stage III and IV ovary increased average 257.5 and 232.9 times than that of sturgeon with stage II ovary respectively. And at the same time, serum T of female Chinese sturgeon also increased dramatically when they developed from stage II ovary to stage III and IV ovary. During the maturation of males from stage II to stage IV, their serum E2 decreased to nearly zero, while serum T increased obviously only from stage III to stage IV testis. After spawning or the gonad degenerated without spawning, sex steroid of both female and male would drop back to the same lever with stage II gonad.

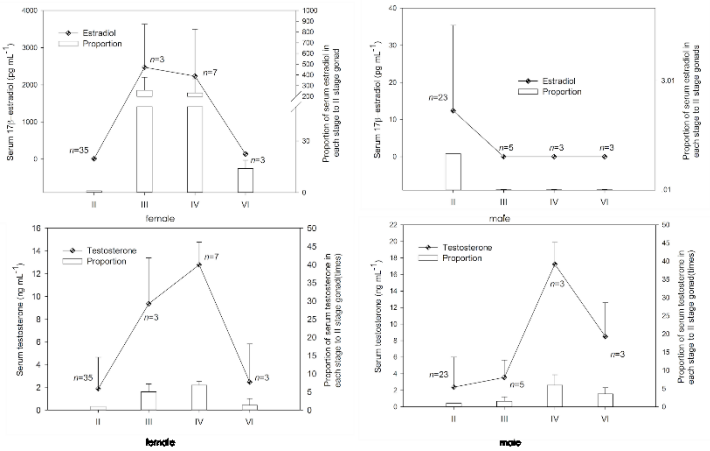

Fig.1 Serum E2 and T of female and male Chinese sturgeon with gonad development from stage II to stage VI. Stage VI means the sturgeon have spawn or the gonad degenerated without spawning.

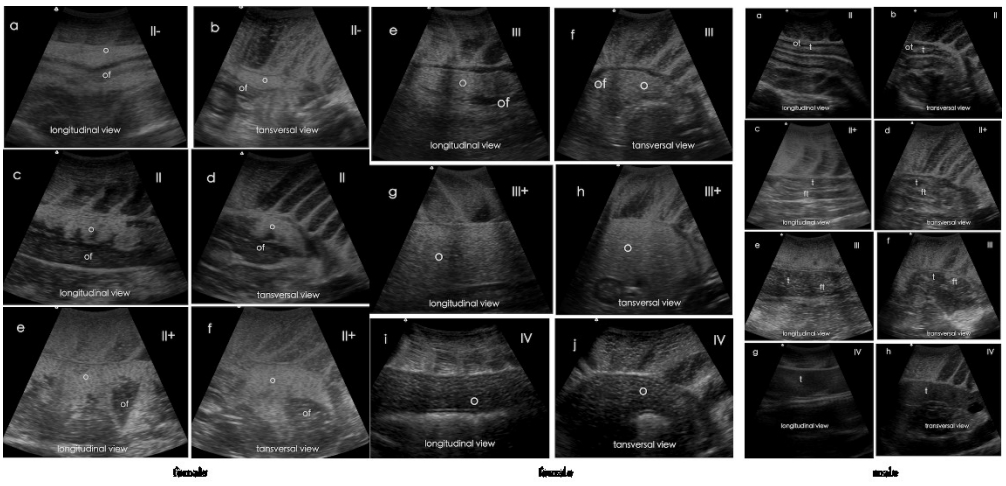

Fig.2 ultrasound images of female and male Chinese sturgeon with different development status. o: ovary; of:ovary fat; t:testis; ft: testis fat

With great changes in concentration of endocrinal sex steroid during gonad maturation, the entity of the gonad of Chinese sturgeon also changed dramatically. Distinguishable ultrasound images showed the morphological changes of gonads in both transversal views and longitudinal views: in females, the thickness of ovary from stage II to IV increased 2-5 times larger than that at stage II ovary(Fig.2 female a-c). At the same time, with the composition and conversion of the ovary fat into oocytes, the echogram of the gonad changed obviously. In the stage II ovary, typical gonad will have unregular fats, which make echogram of the oocytes obviously bright than fat(Fig.2 female-c). Fat almost gone before the female entering stage III that the oocytes can be a macroscopical size with average 1-2 mm in diameter (Fig.2 female-e & g).Oocytes in stage IV ovary can be clearly saw with an average 4 mm in diameter (Fig.2 female-i & j) .In males, echogram of testis is a little bit darker than that of fats, which make almost the whole gonad is darker than that of muscles(Fig.2 male a-h) , which is one of the key elements to identified males from females. With fat transfer into the testis, the gonad will become homogeneous dark than the tissues of muscles (Fig.2 male g & h). That tells the fish is already to sperm even without any hormone inducing. Misdiagnosis usually happen with distinguished from stage II ovary to stage II testis, due to the small gonad with lot of fat. While, as large as 10 years older sturgeon, it can be distinctive using both longitudinal views and transversal views (Fig.2 female a & b vs male a & b; female c & d vs male e & f).
It is easier to make the accurate diagnosis of the maturation of captive Chinese sturgeon combining the sex steroid and ultrasound images together.

DISCUSSION AND CONCLUSIONS

It is the first observations of the changes of the sex steroid and ultrasound images of captive Chinese sturgeon in the whole maturation circle so far, which support a basic data of diagnosis for their maturation and it will essentially benefit for of the future aquaculture, breeding and restocking of the ever-endangered fossil species.

ACKNOWLEDGEMENTS

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Fish germ cell: from basic sciences to applied biotechnologies

SOA4

PRODUCTION OF VIABLE TROUT OFFSPRING DERIVED FROM FROZEN WHOLE FISH

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INTRODUCTION

A number of salmonid species are experiencing rapid population decline and several species are already extinct. Long-term preservation of fish fertility is, therefore, increasingly important for the conservation of endangered fish species. The most common method to preserve genetic resources is raising live individuals in captivity. However, this strategy involves several risks, including facility accidents, infectious disease outbreaks, genetic drift, and the reduced fitness within natural habitats of individuals raised in captivity. Cryopreservation of mature oocytes or embryos would be a valuable preservation tool, but suitable techniques have not yet been developed due to the large size and high lipid content of these materials.

The authors of the present study previously demonstrated a surrogate broodstock technology in which immature germ cells isolated from a target fish species are transplanted into a closely related species, so that the surrogate species can produce the eggs and sperm of target species (Okutsu *et al*., 2007). We also established a method for producing functional eggs and sperm derived from cryopreserved trout spermatogonia using allogeneic surrogates (Lee *et al*., 2013). If frozen-thawed spermatogonia were capable of differentiating into both eggs and sperm in recipients of different species, it would be possible to generate endangered fish species through interspecies transplantation of thawed spermatogonia whenever the need arose. However, previously established protocols are rather complicated and difficult to apply in emergency cases, such as when an endangered fish species maintained in captivity unexpectedly dies. Therefore, a simple and foolproof method to preserve the genetic resources of endangered fish is urgently required.

The simplest procedure we can imagine is freezing whole fish in a freezer without any manipulations. If it was possible to retrieve viable cells from frozen whole fish, we would be able to convert germ cells retrieved from the frozen whole fish into functional eggs and sperm. In the present study, we aimed to produce functional eggs and sperm using type A spermatogonia (ASG) retrieved from whole trout kept in a freezer.

MATERIALS AND METHODS

To determine the viability of GFP (+) ASG after periods of frozen whole fish storage, hemizygous pvasa-*Gfp* transgenic rainbow trout (*Oncorhynchus mykiss*) (GFP/-) were stored at −80°C deep freezer for 1, 7, 30, 94, 191, 251, 372, 556, 735, 846, and 1,113 days. After these periods, they were thawed by shaking in a 10°C water bath for at least 20 min. Testes isolated from these frozen-thawed trout were used to determine the viability of GFP (+) ASG by a flow-cytometer. Testicular cell suspensions containing GFP (+) ASG were prepared from whole trout frozen for 371 days and used for the transplantation study. Approximately 20 nl of the cell suspension, containing approximately 500 GFP (+) ASG, were intraperitoneally transplanted into WT triploid hatchlings of masu salmon (*Oncorhynchus masou*) as previously described by Takecuhi *et al*. (2004). Recipient fish were reared until sexual maturity. To determine the production of sperm derived from frozen whole trout, total genomic DNA was extracted from the milt obtained from male recipients and subjected to PCR with *Gfp*-specific primers. Two-year-old salmon males and females were mated with each other. If F1 offspring were derived from donor frozen trout (GFP/−), they would be expected to exhibit a 75% ratio of donor phenotypes (GFP-positive) following Mendelian inheritance. To identify genotypes of the offspring produced by the salmon

recipients, RAPD analysis was performed according to the method of Takeuchi *et al*. (2004)

RESULTS

The viability of ASG obtained from fish stored in a deep freezer did not change significantly with increasing storage duration for at least 1,113 days. Frozen trout ASG that were transplanted into salmon recipients migrated toward the recipients' genital ridges and were subsequently incorporated into them. The transplanted donor ASG began to proliferate and differentiate into oocytes in female recipients. The colonization and proliferation capacities of ASG frozen for 371 days were not significantly different from those of freshly prepared control ASG.

Two-year-old masu salmon recipients that received ASG retrieved from whole trout frozen for 371 days produced milt (4.0 ± 0.3 ml) containing numbers of sperm ($16.8 \pm 2.8 \times 10^{10}$) that were equivalent to those produced by recipients that received freshly prepared ASG. The genetic background of the sperm obtained from these 2-year-old salmon recipients was examined using PCR with *Gfp*-specific primers. The results showed that all of the milt obtained from male recipients was positive for the presence of the *Gfp* gene. Female salmon recipients that received ASG retrieved from frozen whole fish also produced numbers of eggs similar to those of recipients that received freshly prepared ASG and of WT salmon. Their fertilizability and hatchability were also similar.

In the F1 offspring produced by the recipients at 2 years pt, the donor-derived haplotypes of green fluorescence were also transmitted following Mendelian inheritance. More importantly, the external morphology of the F1 juveniles was considered normal for regular rainbow trout. In addition, RAPD analysis of the F1 offspring produced by the recipients showed that the DNA fingerprinting patterns of the F1 offspring were the same as those of rainbow trout and clearly distinct from those of the recipient masu salmon.

DISCUSSION AND CONCLUSIONS

We could successfully produce viable offspring completely derived from ASG retrieved from frozen whole trout using both allogeneic and xenogeneic recipients. The procedure of simply freezing whole fish can be directly applied to trout hatcheries as well as to field conditions. Indeed, since most of the trout hatcheries do not have the required laboratory apparatus, they can keep the endangered fish in a deep freezer until the endangered fish specimens can be sent to laboratories where spermatogonial transplantation is routinely performed. Therefore, this methodology of whole fish freezing is a convenient emergency tool that can be used to save endangered or even extinct fish species stored in a deep freezer.

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O23

MOLECULAR SIGNATURE AND STEMNESS PROPERTIES OF PAIRED SPERMATOGONIA IN ZEBRAFISH

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INTRODUCTION

The preservation of the genetic resources (mitochondrial and nuclear genomes) and the regeneration of individuals are important economic and ecological issues for the sustainability of the aquaculture sector and conservation of the biodiversity. In fish, the cryopreservation of oocytes and embryos is not possible but the cryopreservation of diploid germ stem cell entities and their transplantation in pre-hatching recipient embryos is a promising approach to obtain sperm and oocytes from the preserved donor (Yoshizaki et al, 2011, for review). However, the rareness and the difficulty in purifying and amplifying *in vitro* the germ stem cells entities from sexually mature animals limit the efficiency and the dissemination of this procedure. There is a need to better characterize the germ stem cells entities in adult fish and to unravel the mechanisms involved in their self-renewal. The present study was aimed to purify and to characterize a subpopulation of A spermatogonia that could participate to the maintenance of the spermatogonial stem cell pool in adult fish.

MATERIALS AND METHODS

Transgenic zebrafish lines were produced using the tol2 transposon-mediated method. The expression pattern of the transgene was investigated using whole mount RNA in situ hybridization and immunocytochemistry techniques. The fluorescent cell population was purified from adult testes using a multi-step procedure. Testicular cells were dissociated using a collagenase and dispase mixture. Post meiotic germ cells were first removed using a 45% percoll cushion followed by a differential plating step on gelatin-coated culture wells. The remaining fluorescent cells were purified using fluorescence assisted cell sorting (FACS). Real time quantitative PCR was carried out to determine the relative abundance of transcripts in the sorted and unsorted cell fractions. A panel of 15 genes representative of different testicular cell types and stem cell biology was analysed. The stemness properties of the sorted cell fraction were investigated using transplantation assays in the abdominal cavity of 17 days old zebrafish fry.

RESULTS

We have produced a transgenic zebrafish line that carries the GFP reporter gene under the control of a short proximal promoter fragment of the *vasa* gene. We observed that transgene expression was first detected in the gonads from 19 days post fertilization onwards. Transgene expression occurred in primary oocytes at stage 1 and gfp transcripts were maintained in ovulating oocytes of mature females. The GFP protein was transmitted to the embryo but did not accumulate in the primordial germ cells (PGCs) of the developing transgenic embryos. In the testis of pubertal fish, high GFP protein expression was observed in doublets of A spermatogonia although gfp transcript accumulation occurred only in one of the two cells. The fluorescent cells were isolated using the FACS technique, and candidate gene expression patterns were investigated using qPCR. We observed that transcripts previously proposed as spermatogonial stem cell markers in mammalian and/or in fish species were also increased in the sorted cells. Moreover, the relative abundance of transcripts restricted to doublets of A

spermatogonia or involved in pluripotency was also increased in the fluorescent cell fraction. In agreement with the molecular signature, the sorted GFP-positive spermatogonia were capable to colonize the gonads after transplantation in the abdominal cavity of 16 day-old recipient fry.

DISCUSSION AND CONCLUSIONS

The stemness properties of purified spermatogonia originating from doublets suggest that these cells should be considered as part of the pool of spermatogonial stem cells in adult male zebrafish. This assumption could also be relevant for other fish because doublets of A spermatogonia have been reported in the testes of numerous species including medaka (Shibata and Hamaguchi, 1988) and trout (Loir, M., 1999, Bellaiche et al., 2014). A deep sequencing of the transcriptome is being initiated to unravel molecular mechanisms involved in the decision of the spermatogonial stem cells to self-renew or to differentiate in gametes.

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O24

CRYOPRESERVATION OF TESTICULAR AND OVARIAN CELL SUSPENTION VS WHOLE TISSUE IN STURGEON

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INTRODUCTION

Several sturgeon species are close to extinct due to human activities. Therefore it is required to establish an efficient reproduction and conservation strategy. Early stages of germ cell can be used for long term storage and restoration of genetic information of target species using surrogate production via close related species.

MATERIALS AND METHODS

The aim of this study was to compare cryopreservation of sturgeon (*Acipenser baerii* and *A. gueldenstaedti*) spermatogonia and oogonia using different procedure and cryoprotectants. The cells were frozen as whole tissue or as dissociated cells; with different cooling rates; and in straws or cryotubes. Cryomedium was PBS containing 0.5 % BSA, 50 mM glucose and different cryoprotectants in concentration 1.5 M: DMSO, glycerol, ethylen glycol, and DMSO together with propandiol. The fresh and thawed cryopreserved cells from the best condition were labelled by PKH26 and transplanted into hatchlings (*Acipenser ruthenus*). The development of transplanted cells was observed under fluorescent microscope one month post transplantation (mo pt). RT-PCR with species specific primers for *vasa* was used to identify development of transplanted cells 3 mo pt.

RESULTS

The results showed the best survival of spermatogonial as well as ovarian cells (75.4 and 72.7 %) in the case of cryopreservation of whole tissue in 1.8 ml cryotubes, using ethylen glycol as cryoprotectant. One month post transplantation, 62% of recipients contained PKH26 positive cells. *vasa* species specific primers revealed positive RT-PCR in more than half fish transplanted either by fresh or cryopreserved spermatogonia or oogonia.

CONCLUSION

The results showed that crypreservation of whole tissue was easier and efficient compare to cell suspension. Moreover dead cells were digested during dissociation step, which simplified the transplantation process. The gonadal tissue after thawing and dissociation is possible to transplant interspecifically and the transplanted cells are proliferated in body of recipient.

ACKNOWLEDGEMENS

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O25

ABNORMAL CLEAVAGE PATTERNS PRODUCE HAPLOID/DIPLOID MOSAICISM IN STURGEON *(ACIPENSER RUTHENUS)*

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INTRODUCTION

The Acipenceriformes, including sturgeons, is the oldest order within the Actinopterygii other than the Polypteriformes (Grande L., *et al.* 1991). Since sturgeons are used for artificial breeding, a study of their development is of practical value (Detlaff T.A., *et al.* 1981). Understanding of embryo development, discovering the reason of embryonic malformation would be of great economic value to hatcheries (Lee CS., 2003).

In normal development, sturgeon eggs divide into multiples of two cells until blastula stage. Fertilization is normally monospermic (Ginsburg A.S., 1953). Several researchers (Detlaff T.A., *et al.* 1981) described abnormal cleavage division, such as 3 cells at the 2-cell stage. Generally it has been believed that this "abnormal" cleavage is caused by polyspermy mainly due to highly concentrated sperm suspension or poor physiological conditions of the eggs, because sturgeon egg has multiple-micropyles. However, there is no report that provides the direct evidence of polyspermic fertilization. Another hypothesis is retention of second polar body in egg. This second polar body can be also involved in development and formed the additional blastomere(s). In addition to this all reports suggested, that such eggs demonstrate malformation and die during embryonic development.

In this study, we tested whether 1) this abnormal cleavage was truly caused by polyspermy and 2) the abnormal embryo could develop after embryonic stage. Furthermore, we studied the elements that may affect the tendency of abnormal cleavage. The results obtained in this research will be useful for producing healthy seeds production.

MATERIALS AND METHODS

Fish gametes were collected according to Psenicka *et al.* (2010). Before insemination spermatozoa concentration and motility were analyzed. 4 groups with different sperm concentrations insemination were performed. After fertilization, abnormal developed embryos (3, 5, 7 cell) were collected at 2 to 4 cell stage and kept separately. This experiment was repeated using Russian sturgeon sperm with sterlet eggs. The species were differ in ploidy level (8n and 4n respectively).The ploidy level of specimens was evaluated using flow cytometry (Paa Partec CCA I; Partec GmbH, Münster, Germany) using 4 ′,6-diamidino-2-phenylindole (DAPI) according to Linhart *et al.* (2006).

To study if there is a differences in the micropyles between the normal and the abnormal eggs, chorions were removed from eggs and stained in 0.1% Methylene blue in distilled water for 30 min. Then, the numbers of micropyles and the distances of the most far located micropyles on chorion were analyzed under a stereomicroscope and compared between normal and abnormal eggs.

RESULTS

We found untypical development of sterlet *(Acipenser ruthenus)* embryos with 3, 5, 7 cells (from 1.7 to 11.25% embryos). The ratio of embryos with abnormal cleavage pattern increased as sperm concentration was increased. These "abnormal" embryos showed 2n/4n mosaicism, although all normal embryos showed 4n ploidy (normal physiological state of the species). In case of hybrid combination, the abnormal embryos showed 4n/6n mosaic ploidy. Analysis of micropyles showed that abnormal embryos have significantly more micropyles and the distance between them was bigger than in control group.

DISCUSSION AND CONCLUSIONS

Our result clearly demonstrated that the abnormal cleavage embryos caused by polyspermy and they could develop further and survive with or without abnormalities. The control of reproduction is a key issue in aquaculture. One of the limiting factors of the reproductive success is the quality of male and female gametes. The relative effects of each factor on gamete quality can be highly variable and are not always well characterized. Some of the factors responsible for the observed variability of gamete quality remain largely unknown or poorly understood (Bobe J., *et al.* 2010).

This fenomenon can cause troubles by contamination of broodstock, so the fate of the blastomeres produced by an additional sperm in the abnormal embryo must be studied in detail.

ACKNOWLEDGEMENTS

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O26

A CANDIDATE GENE APPROACH TO PRODUCE STERILE, GERM CELL-FREE ATLANTIC SALMON BY CRISPR-CAS9 MEDIATED GENOME EDITING

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INTRODUCTION

Introgression of farmed salmon escapees into wild stocks is regarded a major threat to the genetic integrity of wild populations, a threat that could be solved using sterile fish in aquaculture. Our study investigated whether it is possible to develop new sterility models and to produce germ cell-free salmon by knocking out a number of candidate genes using the CRISPR-Cas9 system. CRISPR-Cas9 can induce biallelic mutations in F0 in several fish species, including Atlantic salmon, and is therefore suitable for functional studies in fish with a long generation time (Edvardsen et al., 2014). We used CRISPR-Cas9 induced mutations of *slc45a2* (*albino/alb*) as a visual tracer for successful double allelic mutations.

MATERIALS AND METHODS

We applied the CRISPR-Cas9 system developed by Jao et al., 2013 to induce double strand breaks in candidate genes for knock out studies. Candidate genes were selected based on their exclusive expression in gonad tissues (Kleppe et al., 2015) and on their gonad specificity in *Oncorhynchus mykiss* (Berthelot et al., 2014). We used the Atlantic salmon genome (Acc. No. AGKD00000000.3) to design CRISPR gRNAs for the respective genes for injection into one-cell stage embryos. Fin-clips of pigment-less juvenile fish were screened for mutations by PCR and sequencing and mutated fish were pit-tagged and kept under constant light for early maturation. Representative controls and KO of one-year old male and female fish were investigated for gonad development by histology and gene expression analysis by qPCR.

RESULTS

Our candidate gene screening revealed positive results for one of the sterility candidate genes assayed. Using *alb* as a tracer for successful gene editing resulted in fish with graded mosaic pattern in pigmentation and complete loss of pigmentation which indicated biallelic disruption of *alb* (Edvardsen et al., 2014). Double gene targeted fish showing complete loss of pigmentation were further investigated and histological sections revealed the absence of germ cells in both male and female one-year-old fish. Gonads of these double knockout fish lacked *vasa* expression further confirming their sterility. *Cyp19a1a* expression confirmed a somatic female phenotype in female germ cell-free gonads, while in male germ cell- free gonads *anti müllerian hormone* (*amh*) was overexpressed proving their male identity.

DISCUSSION AND CONCLUSIONS

The CRISPR-Cas9 system is the method of choice for genome editing and analysis already in the F0 generation in (fish) species with long life spans. Furthermore, using *alb* disruption as a visual marker for functional studies of other genes in F0 is an ideal tool. Our observations suggest that germ cell-free female gonads develop an ovarian somatic structure resembling the situation found in loach and goldfish, in contrast to the situation in zebrafish, medaka and tilapia where germ cell-free fish develop somatic testes (Fujimoto et al., 2010; Goto et al., 2012; Slanchev et al., 2005; Kurokawa et al., 2007; Li et al., 2014). The histological and molecular analyses revealed the maintenance of the respective

sexes in germ cell-free fish and thereby demonstrate a germ cell independent sex differentiation in salmon gonads.

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O27

STERILIZATION OF STERLET STURGEON (*ACIPENSER RUTHENUS*) EMBRYOS BY ULTRAVIOLET IRRADIATION

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O28

EMBRYOGENESIS AND PRIMORDIAL GERM CELLS DEVELOPMENT IN PIKEPERCH, *SANDER LUCIOPERCA*

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INTRODUCTION

Technique called “surrogate production” in fish is attracting a lot of attention for its possibility of efficient reproduction. This technique can be realized by transplantation of donor germ cells into sterilized recipient fish (Saito et al. 2008). We propose that pikeperch (*Sander lucioperca*) can be a good model and recipient species for establishing this technique in perciformes species, which includes numerous commercially and ecologically important species. First of all, to be able to establish this technique, it is necessary to accumulate more information about the development of germ cells, from primordial germ cells (PGCs) in an embryo to the gamete genesis in the matured gonads. The aims of this study are to describe the embryonic development and to apply manipulation technique to this fish to test if it works or not. In this research, we show 1) early embryonic development of pikeperch, 2) optimal stage for manipulation (Güralp et al. 2015), 3) the late embryogenic development, 4) PGCs development during embryonic stage and 5) PGCs transplantation by blastomeres transplantation (BT) technique.

MATERIALS AND METHODS

Pikeperch embryos were obtained by artificial insemination and cultured as described previously (Güralp et al. 2015). Fertilized eggs and embryos were cultured at 15ºC. The chorion was removed by forceps after treatment of 0.2% trypsin and 0.4% urea in Ringer's solution (128 mM NaCl, 2.8 mM KCl, 1.8 mM $CaCl_2$, buffered with TAPS at pH 8.5). During manipulation, dechorionated eggs were kept in Ringer’s solution containing albumen. After the completion of epiboly, embryos were transferred to a second culture solution (1.8 mM $MgCl_2$ and 1.8 mM $CaCl_2$). Developmental stages were determined according to morphological features. Blastomeres divisions were visualized by 4’-6-diaminido-2-phenylindole (DAPI) staining (Güralp et al. 2015). Embryos were fixed every 15 min in Farmer’s fixative (75% EtOH and 25% acetic acid) at 4°C for overnight. After dechorionation, blastodiscs were stained with 5% DAPI in phosphate-buffered saline (PBS) for 10 min. After washing, embryos were observed under an inverted fluorescence microscope. PGCs were visualized by injecting artificially synthesized mRNA, which fused green fluorescent protein (GFP) with the zebrafish nos3 3’UTR, into the blastodisc of pikeperch embryos at the 1- to 2-cell stage. Blastomeres transplantation (BT) was performed between pikeperch embryos at the blastula stage. The donor embryos were co-labeled with GFP-nos3 3'UTR mRNA and tetramethylrhodamin dextran in order that transplanted PGCs and somatic cells can be visible. PGCs migration was observed by time-lapse imaging using a fluorescence stereomicroscope.

RESULTS

The blastomeres divided synchronously up to 1K-cell stage (13.25 hpf), then synchronization of cell division was ended by observing the anaphase or metaphase cycle in almost all embryos. These results showed that the mid-blastula transition started after 1K-cell stage. Late embryonic development was characterized as gastrula; segmentation period and hatching were observed at 20, 45 and 125 hpf, respectively. Gastrula period was marked by beginning of the epiboly include the formation of the thickened germ ring at the periphery of the blastoderm and the appearance of the embryonic shield. Segmentation period was recognized by appearance of the first somites at 45 hpf. Cephalization forming and differentiate was recognized by the construction of the optic and otic

vesicles in early segmentation period during 2-24 somite stages. Somites began to change their shape into a chevron after the 12-somite. The tail region became separated from the yolk and then elongated toward the posterior and the median fin fold was formed at the 30-somite. Heart beating started around the 34-somite. Embryonic body became straight at the 40-somite. Pigmented cells were observed at the 44-somite Embryos began to hatch at the 50-somite. The yolk sac became smaller and the fins and pectoral fin forming appeared at 149 hpf. Hatching rates were determined as 12%, 44%, 38%, 6% at 50-somite stage (125 hpf), 149 hpf, 173 hpf, 197 hpf, respectively. GFP-positive PGCs were observed in all embryos which were injected with GFP-nos3 3'UTR mRNA from the 100% epiboly stage. The average number of the PGCs in each embryo was 12.6 (SD 8.52, Range = 2 to 35). Time-lapse imaging and analysis revealed that their migration pattern from their appearance to the gonadal ridge could be classified into 5 steps. Twelve BT chimeras were produced and 8 chimeras survived until the hatching stage. All of them had red-labeled (donor-derived) cells in the embryonic body. These red-labeled cells were distributed to the whole embryonic body, including somites, brain, and intestine. Two embryos out of 8 had GFP-labeled PGCs at the gonadal ridge.

DISCUSSION AND CONCLUSIONS

In this study, we described the embryonic development of pikeperch from gastrula till hatching in detail. We established the techniques such as injection, blastomeres transplantation, PGCs visualization, and time-lapse imaging (Güralp et al. 2015). By combining them, we could describe the PGCs migration in the normal embryonic development. These information and techniques can be a basis for more applied technology such as surrogate production via "germ-line chimera." The key step in producing germline chimeras is to make donor germ cells incorporate into the host gonad (Saito et al. 2008). In this study, we have demonstrated that PGCs from donor embryos were able to migrate toward the gonadal region of host embryos. We propose that pikeperch can be used as the recipient species for applying surrogate production technology in the Perciformes. Moreover, the fish belong to this group live not only in freshwater area but also marine water. By using pikeperch as a host, it might be possible to produce gametes of marine fish in freshwater.

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O29

STERILIZATION OF STERLET *ACIPENSER RUTHENUS* BY USING KNOCK DOWN AGENT, ANTISENSE MORPHOLINO OLIGONUCLEOTIDE, AGAINST *DEAD END* GENE

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INTRODUCTION

Sturgeons (Chondrostean, Acipenseridae) are ancient fish species, widely known for their caviar. Nowadays most of them are critically endangered. Sterlet (*Acipenser ruthenus*) is a common Eurasian sturgeon species with a small body size and the fastest reproductive cycle among sturgeons (Detlaff et al., 1993). Such species can be used as a host for surrogate production; application is of value for recovery of critically endangered and huge sturgeon species with an extremely long reproductive cycle. One prerequisite for production of donor´s gametes only is to have a sterile host. Commonly used sterilization techniques in fishes such as triploidization or hybridization, do not guarantee sterility in sturgeon (Havelka et al., 2014). Alternatively, sterilization can be achieved by using a temporary germ cell exclusion specific gene by a knockdown agent, the morpholino antisense oligonucleotide (MO). The targeted gene for the MO is the *dead end* gene (*dnd*) which is a vertebrate-specific gene encoding a RNA-binding protein which is crucial for migration and survival of primordial germ cells (PGCs) (Weidinger et al., 2003). For this purpose, a *dnd* homologue of Russian sturgeon (*Agdnd*) (Hagihara, unpublished data), resulting in same sequence in start codon region with isolated fragments of sterlet *dnd* (*Ardnd*), was used.

MATERIALS AND METHODS

Adult sterlet females and males, 5 to 9 years of age, were transferred from outdoor ponds into indoor recirculating aquaculture system during the spawning season March – June 2014. The ovulated eggs were collected from three females and eggs were inseminated with sperm from two males in dechlorinated water at 15°C. Eggs were dechorionated (outer layer) 1 hour post-fertilization (hpf) using forceps. Embryos were mainly used for injection of fluorescein isothiocyanate-biotin-dextran (FITC) for PGCs labelling, antisense morpholino oligonucleotide for PGCs depletion, reverse transcription polymerase chain reaction (RT-PCR), histology and *in situ* hybridization (ISH).

RESULTS

The full-length of the Russian *Agdnd* sequence was obtained by NGS. *Agdnd* cDNA was 1710 bp long, containing 1290 bp open reading frame, which encoded 430 amino acids. Fragments of *Ardnd* gene were sequenced with specific primers and aligned with *Agdnd*. Similar fragments with ATG region in both *dnd* sequences were identified, resulting in one *dnd*-MO design for both species (sterlet and Russian sturgeon). RT-PCR confirmed tissue-specific expression of *Ardnd* only in the gonads of both sexes. *Dnd*-MO, for depletion of PGCs, together with FITC, for PGCs labelling, were injected into the vegetal region of 1-4-cell stage sterlet embryos. In control groups, only FITC was injected to validate the injection method and labeling of PGCs. After optimization of MO concentration together with volume injection, 250 µM MO was applied for sterilization of sturgeon embryos. PGCs were detected under fluorescent stereomicroscope in genital ridge of FITC-labelled control group only, whereas no PGCs were present in body cavities of morphants at 21 days post-fertilization (dpf). Moreover body cavity of MO-treated and non-treated fish were examined by histology and *in situ*

hybridization (ISH), showing gonads which had no germ cells in morphants at various stages (60, 150 and 210 dpf).

CONCLUSION

In conclusion, we sterilized sterlet by *dnd*-MO. The successful ablation of PGCs was confirmed by counting of FITC-labelled PGCs under fluorescent microscopy, by histological examinations and gene expression analyses. Our results provided very useful tool of sterlet sterilization for future experiments of surrogate reproduction via germ line chimerism, where a sterile host is required. This could be a powerful method for reproduction of these endangered species.

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O30

STAGNATION IN FISH CRYOBIOLOGY RESEARCH? THINK OF DESICCATION!

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A short review will be presented on recent advances towards storage of cells and gametes in a desiccated form, raising the question: is this relevant to fish reproductive biologists?
Preservation of gametes (spermatozoa, ova, eggs) or embryos in liquid nitrogen (LN2) is of importance in medical, life sciences research and for livestock management of farmed animals. Cryopreservation, although useful, requires long-term storage in LN2, which is expensive and uncertain in many locations. The benefits of desiccation are obvious and include storage at room temperature or cooling at convenient or available temperatures. In comparison with the use of LN2, desiccation significantly simplifies use, transportation and storage of samples, especially in remote places. It also lowers the costs for operating and maintaining sperm or genetic banks. This is particularly true for aquaculture, where farms face difficulties for a regular supply of LN2.
There is a challenging endeavor in recent years and successful results were reported for freeze-dried, evaporated or convective dried spermatozoa (Loi et al., 2013). Dried spermatozoa retained viability for at least four years and can be used for intra-oocyte fertilization. Methods for desiccation of mammalian ova are under investigation (Chakraborty et al., 2012).
While methods for cryopreservation of fish spermatozoa were developed for a wide range of species, their use on a large scale is limited to few species and few locations (Tiersch and Green, 2011). One of the main obstacles is the need for preserving sperm on fish farms. Moreover, freezing of oocytes, eggs and embryos has not been consistently successful due to their high water content and the complex structure of yolk proteins (Hagedorn et al., 1996; Zhang et al., 2011).
With this view in mind, two topics will be addressed: a) The advantages and disadvantages in developing methods for desiccation of fish sperm and b) Based on survival after desiccation, of embryos containing yolk proteins of invertebrate species, can we envision desiccation as an option for fish oocytes/eggs/ embryos?

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O31

MATERNAL FACTORS MODULATING *DANIO RERIO* DEVELOPMENT ARE AFFECTED BY BENEFICIAL BACTERIA

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INTRODUCTION

In the very first hours of life, maternal factors are responsible for directing the processes occurring in the embryo (Pelegri, 2003). Among these, autophagy, apoptosis and dorsalization are known to be of absolute importance for ensuring a proper embryonic and larval development.
On the other hand, probiotics have been demonstrated to positively influence many biological systems, including reproduction (Gioacchini et al., 2013) and development (Lamari et al., 2013). Very few information regarding probiotics and their impact on both the quality and quantity of maternal factors loaded by the mother into the oocytes, though, are available.
Herein we report the findings we collected about the expression levels and the localization of some key genes belonging to the above mentioned process as well as to the degradation of maternal transcripts throughout the first 48 hours of development in *Danio rerio*.

MATERIALS AND METHODS

Parental zebrafish kept with temperature and photoperiod parameters of 28°C and 10/14 L/D respectively, were fed with commercial food supplemented with 10^6 CFU/ml water of *L. rhamnosus* IMC 501® (Sinbyotec, Italy). Embryos obtained from natural spawning were collected at 0, 2, 4, 8, 12, 24 and 48 hours post fertilization (hpf). The gene expression patterns of biomarkers of autophagic (*beclin*, *ambra1a*, *ambra1b*, *lc3*), apoptotic (*caspase3*, *bcl2*, *bax*), dorsalizing processes (*goosecoid*, *chordin*), as well as of RNA processing (*drosha*, *dicer*) and pluripotency maintainance (*nanog*), were assessed by means of q-PCR, TUNEL and Whole Mount *in situ* Hybridization.

RESULTS

The administration of the probiotic *Lactobacillus rhamnosus* on zebrafish adult fish produced evident effects on both maternal and zygotic levels of transcripts herein considered.
Real Time q-PCR analyses on autophagic signals revealed the typical pattern of the maternally inherited transcripts throughout the first eight hours of development for both experimental groups. In the majority of the developmental stages ranging from 0 to 4 hpf, except for *beclin1* and *ambra1a* levels, embryos from probiotic-treated fish had a statistically significant lower availability of autophagy-related transcripts. Their abundance in control embryos were higher in the remaining developmental stages (12, 24 and 48 hpf) for *beclin 1* and *ambra.* Instead, *lc3* level was constantly and statistically lower in the treated group. On the other hand, WMISH procedure did not show clear differences between the two groups.
Concerning apoptotis, *caspase3*, *bax* and *bcl2* transcripts were monitored and as well appeared to follow the typical tendency of maternally controlled genes, even though the first two messages were higher in embryos coming from probiotic-treated fish, exception made at 24 hpf. *Bcl2*, being an anti-apoptotic signal, exhibited a trend opposed to that of the *caspase3*. The TUNEL assay performed at 12 and 24 hpf confirmed the q-PCR data, since the treated group had a higher and lower richness of cells undergoing programmed death, respectively.
At a dorsalizing level, *goosecoid* and *chordin* transcripts were tested. At 0 and 2 hpf no changes in their expression levels were found: hence, in this case the probiotic did not influence the maternal storage of transcripts. Anyway, both were

strongly up-regulated in their expression by the probiotic supplementation from 4 hpf. Such changes were appreciated also with the WMISH analyses.
At last, we focused on three signals exerting important functions, such as RNA processing and pluripotency maintenance during gastrulation. These were *drosha*, *dicer* and *nanog*. Overall, they showed a similar trend in their transcription, consisting in a decline of their relative abundance between 0 and 4 hpf, with the PROBIO group showing higher levels of all three signals and differences among groups being statistically significant at all three stages, except for *dicer*, which exhibited significance just at 0 hpf. After the 8 hpf, the situation did not change between groups in any of the three genes analyzed. Noteworthy, as already reported in literature, *nanog* mRNA was exploited in the first hours of development but not replaced by the zygotic-controlled message.

DISCUSSION AND CONCLUSIONS

From the data that were achieved, we found that the administration of the probiotic *Lactobacillus rhamnosus* was able to influence the normal maternal and zygotic control of the progeny, resulting in a differential modulation of transcripts ascribable to important biological process and, ultimately, in a faster and more successful embryonic development.

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Progress and perspectives on fish gametes cryopreservation

SOA5
PROGRESS, CHALLENGES AND PERSPECTIVES ON FISH GAMETE CRYOPRESERVATION

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Progress

Cryopreservation of fish gametes has evolved during the last decades due to the increasing number of potential applications. The most evident is its use for aquaculture purposes, allowing the improvement of broodstock management at hatcheries, preserving the genetically selected strains, or even the original wild genotypes for the recovery of genes in the future. But other uses are also possible, as the storage of genetic resources of the increasing number of fish in the lists of endangered species (biodiversity cryobanking) or new species attracts the interest of cryobiologists and aquaculturists, mainly in South America and Asia. Moreover, the increasing use of aquatic biotechnology models such as zebrafish requires the use of transgenic lines that need adequate storage.

Increasing numbers of studies have described methods to cryopreserve sperm in many species, evidencing the extreme diversity of fish. Spermatozoa have been the objective of most of the studies because of their simple structure in comparison with oocytes, embryos or larvae. In fact, the practical impossibility of freezing oocytes or embryos has led to the search of alternative diploid cell sources for genome preservation, as primordial germ cells (PGCs), spermatogonia, blastomeres or even somatic cells (i.e.: from fin tissue).

Challenges

The main objective has always been maintaining a high fertilizing ability after thawing. However, the difficulties in obtaining reproducible results using sperm cryopreserved using the published methods have limited the use of cryopreserved sperm in production. Thus, efforts must be made on the full description and standardization of protocols for sperm cryopreservation, including a very wide area of topics: determination or estimation of sperm motility, substances used for activation of sperm, details of dilution of sperm with extender and cryoprotectants (new ones as the antifreeze proteins, AFPs, or better combinations of classic ones), use of straws (sealed or unsealed), cooling of samples (dry ice vs. liquid nitrogen, styrofoam box vs. programmable freezer), methods of calculating fertilization and hatch results.

Regardless of the very high number of publications on this topic, few of the published methods have been adapted to aquaculture practice. There can be several reasons for this failure of application; however, one of them is beyond doubt the lack of standardization not only in methodologies but also in reporting them correctly. The difficulties in interpretation and replication of methods lead to a disappointment and ultimately rejection by the aquaculture industry. We also need to understand that in most fish species sperm is not a limiting factor during induced spawning and individual selection is not as advanced in fish as it is in terrestrial livestock.

The cryopreservation of somatic or embryonic cells (including PGCs) is an alternative to the cryobanking of gametes. They can be a good source of diploid genome to reconstruct fish. However, this means to develop a series of complex and specific techniques finishing with the transplantation of the thawed cells into recipient fish (of the same or related species), that must be explored in different fish species.

Another alternative technique is vitrification, that has successfully been applied to the cryopreservation of PGCs in several fish species and, recently, first application to the cryopreservation of sperm has been published, although further research is necessary.

Perspectives

Trying to solve the lack of commercial-scale know-how for scaling-up to industry and practical aquaculture, specialized centers must improve the standardization (definitions, methodologies, reporting), and offer quality assessment and cryobanking services that companies can purchase to develop their own line of products.

Sperm cryopreservation will be achieved commercially in some species where the protocols are better developed, the aquaculture industry has demanded such tools and there are economic interests. For other species, sperm cryopreservation will be used occasionally or in niche areas for solving acute problems.

Techniques dedicated to preserving oocytes, embryos or larvae could be another key area of research, although much effort has been made with little success.

An alternative or complementary way is to improve emerging biotechnological techniques, as the use of PGCs, spermatogonia or alternative diploid cell sources for genome preservation and transplantation, in several technical aspects such as cell isolation, identification, labelling, transplantation, nuclear transference or genome inactivation of the recipient. Moreover, better and simpler methods are required for DNA integrity evaluation, and basic research is required on aspects as cell reprogramming, germ cells pluripotency, types of cryodamage (induced by freezing, thawing or cryoprotectants) on the chromatin and cellular structures and their epigenetics consequences, or regarding the production and effects of reactive oxygen species (ROS).

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O32

INVESTIGATION OF VARIABILITY OF COOLING PROFILES IN TWO FREEZING METHODS APPLIED TO STERLET *ACIPENSER RUTHENUS* SPERM CRYOPRESERVATION

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INTRODUCTION

Cryopreservation technologies now provide opportunities for long-term storage of cells and tissue in cryobanks. This material must be of the highest quality to be usable for genomics, proteomics or managed breeding in conservation programs. Standardization of freezing protocols for fish sperm cryobanking, is required so as to insure post-thaw sperm motility and fertilizing ability homogeneity. Thus, in the present work we tested the effects of freezing methods, which are commonly used in fisheries practice, on post-thaw motility parameters in sterlet (*Acipenser ruthenus*). The goal was to investigate influence of straw number on post–thaw motility for samples frozen by freezing in a polystyrene box (Glogowski et al., 2002) and influence of straw location during freezing by dry-shipping container (Harvey, 2000).

MATERIALS AND METHODS

Semen was collected from sterlet) males by catheterization via the urogenital papilla. Sperm was diluted 1:1 ratio in Tris–sucrose–KCl (30 mM Tris, 23.4 mM sucrose, 0.25 mM KCl, pH 8.0) 10% methanol and cryopreserved in 0.5-ml straws. Freezing was performed by two methods (1) using a raft (3cm thickness, with 1 straw and 60 straws on the raft) floating on the surface of liquid nitrogen in polystyrene box Styrofoam box, and (2) freezing in dry-shipping container with different positions of straws in holder (periphery and center). Freezing rates inside the straws were registered with a thermocouple. Thawing was carried out in a water bath at 40 °C for 6 s. The motility of sperm from each sample was tested before/after freezing using Computer-Assisted Sperm Analysis (CASA) from Integrated System for Semen Analysis (ISAS) software (Proiser; Valencia, Spain). The data were analyzed by Statistica software.

RESULTS

The freezing methods were clearly different (Fig.1): cooling rates before ice nucleation and duration of ice formation at the freezing point are similar only for the raft with one straw and dry-shipper with straws located at periphery of holder. Motility percentage was significantly influenced by freezing method applied.

Table 1. Motility parameters of sperm samples after freeze-thawing by different freezing method.

Sperm parameters after freezing	Freezing method			
	Raft 60straws	Raft 1 straw	Dry-shipper outside	Dry-shipper center
Motility,%	$24\pm14^{a*}$	49 ± 15^{b}	41 ± 18^{b}	29 ± 13^{a}
Motility duration, s	120±36	127±48	120±25	122±20

*-values in row with different superscripts are significantly different ($P<0.05$, multiple comparisons for all groups.

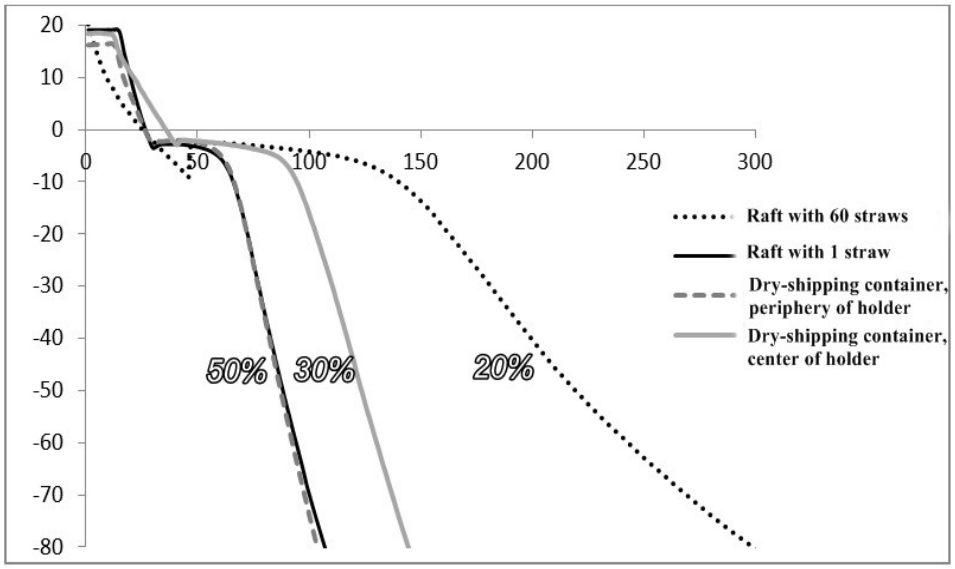

Figure 1. Measured temperatures in straws during cooling by two methods studied.

DISCUSSION AND CONCLUSIONS

In our study we demonstrate, that sterlet sperm could be cryopreserved by both studied methods. Straw number during freezing in polystyrene box and straw location during freezing by dry-shipper influence cooling profiles, which in accordance two-factor hypothesis of cryodamage (Mazur et al., 1972) could be the reason for observed variability of post-thaw sperm motility. We conclude that straw number and location during application of different freezing methods should be controlled to allow standardization of cryopreservation protocols for fish sperm cryobanking.

ACKNOWLEDGEMENTS

This study was financially supported by the Ministry of Education, Youth and Sports of the Czech Republic - projects "CENAKVA" (No. CZ.1.05/2.1.00/01.0024), "CENAKVA II" (No. LO1205 under the NPU I program) and COST (No. LD14119) and COST Office (Food and Agriculture COST Action FA1205: AQUAGAMETE).

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O33

CRYOPRESERVATION OF TENCH *TINCA TINCA* L. ISOLATED TESTICULAR CELLS

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INTRODUCTION

In recent years, the importance of manipulations with primordial germ cells (PGCs) and spermatogonial stem cells (SSCs) has drastically increased since the introduction of transplantation methods by Brinster and Avarbock (1994). Transplantation of SSCs can lead to production of both sperm and eggs in recipients. Since the cryopreservation method has been developed only for spermatozoa so far, the possibility of cryopreservation of germ cells presents a very valuable tool in preserving genetic resources.

Tench *Tinca tinca* (L., 1758) is a fish species with a great potential in aquaculture and with an increasing interest for development of culture technologies, but which is also facing population declines in some parts of Europe (Flajshans et al., 1999; Gela et al., 2006; Pompei et al., 2012). To the best of our knowledge there is only one paper dealing with cryopreservation of tench germ cells so far (Linhartová et al., 2014), thus the aim of this study was to test the application of various cryoprotectants in different concentrations for cryopreservation of all isolated testicular cells.

MATERIALS AND METHODS

A total of eight tench males were used in this study. Fish were sacrificed by an overdose of 2-phenoxyethanol. Testes were excised, pooled and incubated in phosphate buffered saline (PBS, Sigma-Aldrich). Large blood vessels and fatty tissue were removed before digestion. Testes were digested in 0.3% trypsin (Sigma-Aldrich) in PBS for 2.5 hours with gentle mixing at 25 °C. Digestion was stopped by adding 1% BSA to the solution and the cells were filtered through 50 µm filters to eliminate undigested parts of the tissue. Obtained suspension was centrifuged at 30 g for 10 min at 4 °C and the pellet was collected and resuspended in PBS containing 50 mM glucose and 0.5% BSA. The number of cells was calculated in Bürker-Türk hemocytometer at 40× magnification under a Nikon Eclipse E600 microscope.

Three cryoprotectants (dimethyl sulphoxyde – DMSO, methanol and ethylene glycol) at three concentrations (1 M, 2 M and 3 M) were used for cryopreservation. Testicular cells were diluted in extender (PBS + 0.5% BSA + 50 mM glucose) and cryoprotectant solution at a ratio of 1:3 (Linhartová et al., 2014) and placed in cryotubes (1.8 ml) for 15 minutes for equilibration period. Freezing was conducted in controlled rate freezer (IceCube 14s, IceCube Series v. 2.24, Sy-Lab,Neupurkersdorf, Austria). Cryotubes were cooled from 4 °C to -80 °C with a cooling rate of 1 °C/min (Kobayashi et al., 2004; Linhartová et al., 2014) and plunged into liquid nitrogen afterwards. Samples were thawed in a water bath at 38 °C for 40 s. Viability was tested by using SYBR green and propidium iodide as live/dead staining. Moribound cells were not taken into account. All percentage data were arcsine transformed prior to statistical analysis. Two-way ANOVA was used to test the effect of cryoprotectants and their concentrations on cell viability. All statistical analysis were performed in Statistica v12 software (Statsoft Inc., USA).

RESULTS

Cell viability after tissue digestion and before cryopreservation exceeded 99%. Cell viability was reduced following cryopreservation in all groups (Figure 1). Cryopreservation with 3 M DMSO yielded the highest viability with 58.2±4% while cryopreservation with 1 M methanol yielded the lowest viability with 33.7±7.3%.

Two-way ANOVA displayed significant main effect of cryoprotectants ($F_{(2,45)}$=8.18, p<0.01) and their concentrations ($F_{(2,45)}$=29.01, p<0.01), while the interaction was non-significant ($F_{(4,45)}$=1.42, p>0.05).

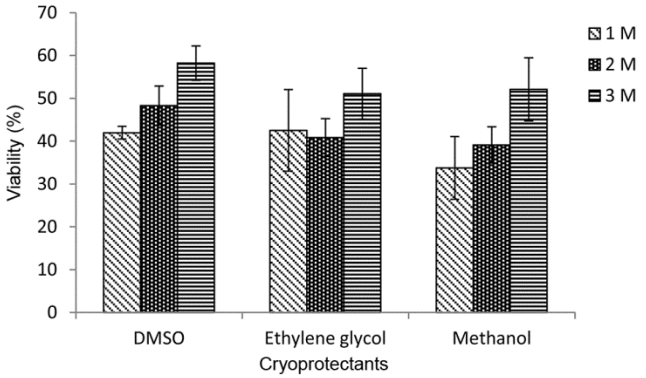

Figure 1. Percentage of viable isolated tench testicular cells following cryopreservation with three cryoprotectants at three concentrations. Data are displayed as mean ± standard deviation.

DISCUSSION AND CONCLUSIONS

In the present study we have demonstrated that cryopreservation of all isolated testicular cells in various cryoprotective media leads to favorable viability post-thaw. We have demonstrated for the first time that cryoprotectant concentrations have a strong influence on cell survivability and that higher cryoprotectant concentrations lead to higher cell viability. As previously mentioned, cryopreservation of germ cells adds a new perspective to the preservation of genetic resources. As these cells can be transplanted following cryopreservation (Kobayashi et al., 2004; Psenicka et al., 2012 cited in Linhartová et al., 2014), further studies are needed for developing even higher post-thaw viability and to assess the possibility of these cells to colonize recipient gonads following transplantation post-thaw.

ACKNOWLEDGEMENTS

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O34

IMPROVEMENT OF COMMON CARP (*CYPRINUS CARPIO*) SPERM CRYOPRESERVATION: THE APPLICABILITY OF A CONTROLLED-RATE FREEZER AS A STANDARD DEVICE

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INTRODUCTION

Common carp (*Cyprinus carpio*) is one of the most important cultured freshwater fish on the world. There is an increasing interest in methodical and practical innovation in induced carp spawning. Cryopreservation is an efficient assisted reproductive technology whereby the effectiveness of farm production can be enhanced (gamete storage and transfer, gamete production synchronization etc.) (Cabrita et al., 2010). This study focused on the optimization of extenders, dilution ratios, activating solutions and equilibration times. The suitable post-thaw storage time of sperm was also measured. We adapted our formerly established cooling protocol using a controlled-rate freezer for common carp sperm.

MATERIALS AND METHODS

Motility parameters [progressive motility (Pmot), curvilinear velocity (VCL), straightness (STR)] of randomly selected carp males (*N*=6) were investigated using a CASA system before and after cryopreservation. Samples were activated in distilled water or in an activating solution for cyprinids (SA, Horváth et al., 2003) with added BSA. For cryopreservation, 10% metanol and the following extenders were used: ”grayling” extender in two preparations [(Regular (Rg): 200 mM glucose, 40 mM KCl, 30 mM Tris, pH 8, Horváth et al., 2012) , (Modified (Mg): 100 mM glucose, 100 mM KCl, 30 mM Tris, pH 8)], carp seminal fluid (Cs) and Hank’s Balanced Salt Solution (HBSS, H9269). Sperm was diluted in a ratio of 1:9 (except in *Experiment 4.*) For the cryopreservation of sperm, 0.5 mL straws were frozen 3 cm above the surface of liquid nitrogen for 3 minutes (polstyrene box (P.box)). A controlled-rate freezer (CRF) was used as an alternative method whereas straws were frozen from 7.5 ºC to -160 ºC with a cooling rate of 56 ºC/min (Bernáth et al., 2015).

Experiment 1: The effect of equlibration time on the motility of carp sperm:
Sperm was frozen with Rg in a styrofoam box 0, 30, and 60 minutes after dilution. Motility of sperm was measured at the end of equilibration and following thawing.

Experiment 2: The comparison of two cryopreservation procedures
Samples were cryopreserved with Rg using a P.box and a CRF.

Experiment 3: The comparison of 3 different extenders for cryopreservation
Sperm was cryopreserved with Rg, Cs, and HBSS using CRF.

Experiment 4: Application of grayling extenders at 4 different dilution ratios
Samples was frozen with CRF in Rg and Mg at ratios of 1:1, 1:5, 1:9 and 1:20.

Experiment 5 : Post-thaw activation time in two different activating solutions
Straws were cryopreserved using CRF following dilution with Rg. Post-thaw motility was measured at 10,20,30,60,90,120 seconds following activation in two activators.

Experiment 6 : Post-thaw storage of carp sperm
Samples were cryopreserved as described in *Experiment 5.* Post-thaw motility were measured separately up for 1 and 6 hours (1 hour: 15 minutes intervals, 6 hours: 2 hours intervals).

RESULTS

Pmot, VCL and STR did not show a significant reduction 60 minutes after dilution. After thawing, a parallel result was observed. Both cryopreservation methods were similarly effective for freezing carp sperm (P.box: Pmot (33±16%) VCL (47±5µm/s) and STR (88±2%), CRF: Pmot(32±13%) VCL (54±10µm/s) and STR

(89±1%). A significantly higher Pmot (42±12%) and VCL (69±4 µm/s) was measured using Rg for cryopreservation compare to Cs (Pmot: 6±2%, VCL: 37±7µm/s) and HBSS (Pmot: 3 ±1%, VCL: 32±9µm/s) whereas STR was similar in all groups. In the course of testing two grayling extenders, highest Pmot, VCL were recorded with Rg at a ratio of 1:9 (Pmot: 52±12%, VCL: 76±9 µm/s), 1:20 (Pmot: 49±8%, VCL: 76±6 µm/s) however the highest STR was measured at a ratio 1:5 (STR: 91±1%). Both using SA and distilled water, Pmot was significantly reduced at 60 seconds post-activation. VCL showed a similar tendency with SA, nevertheless STR did not reduce during 120 seconds. VCL was decreased using distilled water after 20 seconds already whereas STR was significantly lower only at 120 seconds post-activation. Motility parameters of thawed sperm didn't show any reduction both during 1 and 6 hours storage (**Figure A and B**).

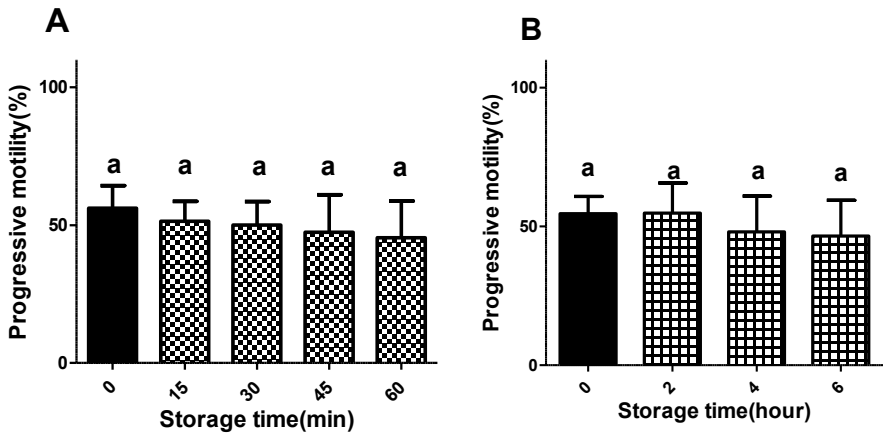

Figure: Post-thaw storage of common carp sperm up for 1(A) and 6(B) hours.

DISCUSSION AND CONCLUSIONS

Equilibration time had no negative effect on the quality of thawed carp sperm. CRF was used most efficiently with Rg at a dilution ratio 1:9. After activation with SA and distilled water, fertilization with thawed carp sperm is recommended within 30 seconds. Storage time for up to 6 hours did not affect the quality of cryopreserved common carp sperm.

ACKNOWLEDGEMENTS

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O35

PROGRESS TOWARDS STANDARDIZATION OF CRYOPRESERVATION METHODS FOR SALMONID SPECIES OF THE ADRIATIC DRAINAGE BASIN

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INTRODUCTION

Application of cryopreservation in the conservation and culture of Adriatic salmonids such as the marble trout (*Salmo marmoratus*) and the Adriatic grayling (a genetically distinct lineage within *Thymallus thymallus*) provides a good opportunity for methodical standardization and optimization of developed protocols. Thus, we have recently reported our results on the optimal sperm-egg ratio in the Adriatic grayling (Horváth et al., 2015). As a continuation of this work, our objective in the present study was to optimize the sperm-egg ratio in the marble trout and to intercalibrate the three protocols that were published for the cryopreservation of grayling sperm (Lanhsteiner et al. 1996, Nynca et al., 2015 and Horváth et al., 2015).

MATERIALS AND METHODS

Sperm was collected from 13 marble trout males and 16 grayling males of which five were selected for cryopreservation in each species. Sperm concentration of each sample was determined at 1000× or 100× dilution in a Bürker-type hemocytometer.

Sperm of marble trout was cryopreserved and thawed according to the protocol by Horváth et al. (2015) described for the sperm of the grayling. For fertilization, egg batches of approximately 200 eggs (20 g) were used and sperm concentration was set to have 50 000, 100 000, 200 000 or 300 000 spermatozoa to one egg.

Sperm of the grayling was cryopreserved according to the protocols by Horváth et al. (2012), Lahnsteiner et al. (1996) and Nynca et al. (2015). Samples were thawed uniformly in a 40 °C water bath for 13 seconds (0.5-ml straws) or 5 seconds (0.25-ml straws). Post-thaw motility was evaluated using a SpermVision 3.7.4 CASA system. Batches of 10 g of eggs (approximately 650 eggs) were fertilized with cryopreserved sperm using a pre-set sperm-egg ratio of 150 000 spermatozoa to one egg.

In both species, fresh sperm was used in excess in the controls to monitor egg quality. Fertilization rates were determined at the eyed stage and at hatching.

RESULTS

In the marble trout, a significant effect of sperm-egg ratios on the percentage of eyed eggs (P=0.0449) was observed (one-way ANOVA), however, Tukey's HSD failed to show any differences among the tested sperm-egg ratios. No significant effect of sperm-egg ratios on the hatch percentages of marble trout eggs was observed (Fig. 1).

In the grayling, the type of the protocol used for cryopreservation had a significant effect on the post-thaw motility (P=0.0103), percentage of eyed eggs (P=0.00174) and hatch (P=0.00184) of cryopreserved grayling sperm (Fig. 2). No significant effect of cryopreservation protocols was detected on other motility parameters (linearity and curvilinear velocity) of grayling sperm.

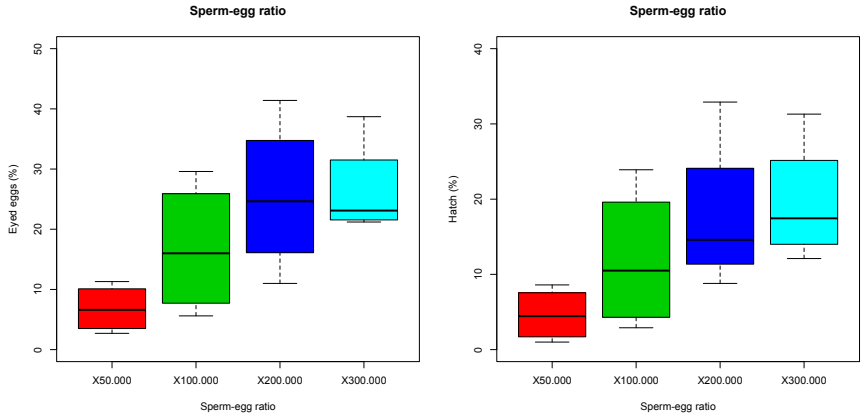

Figure 1. Percentages of eyed eggs and hatched larvae from frozen marble trout sperm at various sperm-egg ratios (Control: 41±3% eyed eggs, 31±2% hatch, N=5).

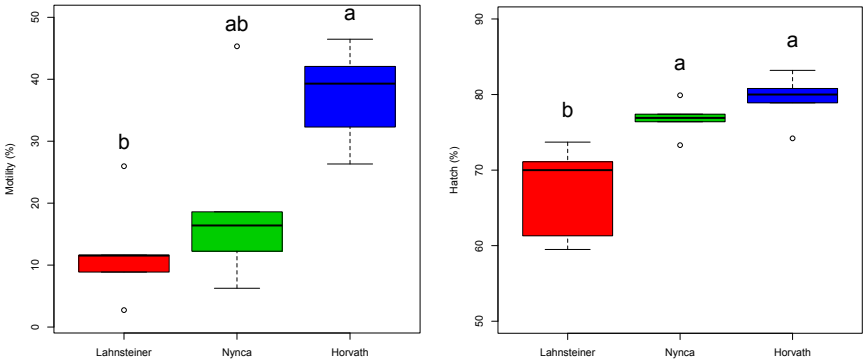

Figure 2. Percentages of motility and hatch following fertilization with grayling sperm frozen according to various protocols (Control: 91±5% motility, 80±2% hatch, N=5).

DISCUSSION AND CONCLUSIONS

In the marble trout, a clear trend was observed with higher sperm-egg ratios resulting in higher fertilization, although, no statistical difference was detected among the individual groups. This can partly be attributed to low egg quality.
In the grayling, the protocols published by Nynca et al. (2015) and Horváth et al. (2015) provided ideal conditions for fertilization with cryopreserved sperm.

ACKNOWLEDGEMENTS

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O36

PRELIMINARY STUDIES ON PORTUGUESE OYSTER (*CRASSOSTREA ANGULATA*) SPERM CRYOPRESERVATION

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INTRODUCTION

Crassostrea angulata, known as the Portuguese oyster, is naturally found all along the southern coast of Portugal and Spain, mainly in river mouths and head of estuaries with suitable hard substrates (Michinina *et al*., 1997). Until 1970s in Europe shellfish industry, *C. angulata* was the major species cultivated (Boudry *et al*., 1998). However, a bad management of the "oyster industry" together with a decrease of water quality exposed the species to several pathologies that almost lead to the Portuguese oyster extinction (Comps *et al*., 1976). Nowadays, pure wild populations of *Crassostrea angulata* are rare to find. Cryopreservation technology could promote alternative techniques to contribute for the resource management of the Portuguese oyster and associated economic activity. Cryopreservation could play an important role both in terms of production by maintaining available seed all-over-the-year and by promoting conservation programs for the conservation of this autochthonous species. There are no reports on *Crassostrea angulata* sperm cryopreservation. Therefore the present work pretends to contribute to the resource management efficiency of the Portuguese oyster by developing technics in broodstock management that could be applied for production and conservation.

MATERIALS AND METHODS

Oysters. Broodstock was acquired in Alvor and Mira bivalve farms and maintained in IPMA, Tavira, Portugal. During conditioning period, oysters were fed daily with a mixture of two microalgae (*Chaetoceros calcitrans* and *Skeletonema costatum*: 50 cells per µL of each microalgae and kept in a recirculation system (RAS) at temperatures between 18 to 22 °C.

Sperm collection and dilution. Sperm was collected by dry method, extracting the sperm directly from the gonad using a micropipette. Gonad area was previously wiped from any contamination. Sperm was immediately diluted 1:10 (v/v) in calcium-free Hank´s balanced salt solution (HBSS) adjusted at 1000 mOsm/kg (0.137 M NaCl, 5.4 mM KCl, 1.0 mM $MgSO_4$, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4 4.2 mM $NaHCO_3$ and 5.55 mM glucose, pH 7.8) or in artificial sea water (Gwo *et al*., 2002). Sperm concentration was 1-2 x 10^9 in all analysed samples.

Sperm cryopreservation. Several conditions were tested 1) Freezing methods: Slow freezing rate: 0.3 ºC/min from 0 to -30 ºC and then plunged directly into liquid nitrogen, using a portable programed biofreezer, Asymptote Grant EF600.
Fast freezing rate: 2 cm over liquid nitrogen surface; 2) Different cryoprotectants (Dimethyl sulfoxide, DMSO, and Polyethylene glycol, PEG) at different concentrations (10 and 20%). According to freezing rates, the highest concentration of cryoprotectants was used in the fast freezing and the lowest concentration was used in slow freezing method; 3) Caffeine supplementation (10 mM) in the dilution of sperm.
All samples were cryopreserved using 0.5 mL French straws. Thawing was performed in a water bath at 37 °C for 10 s and samples were immediately used to evaluate sperm quality. All the experiments were repeated using at least three different males/pools.

***Sperm evaluation*.** For sperm motility assays, 10 µL of thawed sperm were used for CASA evaluation. Motility was assessed in a Makler chamber using a phase-

contrast microscope, a digital camera (25 fps) and a CASA software (ISAS, Proiser R+D, S.L., Spain). Post-thawing spermatozoa viability was determined mixing 15 μL of diluted sperm, 0.5 μL SYBR Green (final concentration 100 nM) and 1.5 μL Propidium iodide (final concentration 12 μM) and observed in a fluorescence microscope. For DNA fragmentation (comet assay), samples were thawed and the protocol described by Cabrita *et al*. (2005) was adapted for oyster sperm. ATP content was determined by bioluminescence (ATP Bioluminescence Assay, Kit CLS II, Boeringer). For all analysis a fresh control was tested.

Statistical analysis. Data collected were treated and analysed using the software SPSS. Percentages were arcsin square-root transformed prior to analysis. One-way ANOVA was performed followed by S-N-K *post hoc* test.

RESULTS AND DISCUSSION

Comparing slow and fast freezing rates, the first revealed higher agglutination levels than the second one after thawing. These results confirmed the previous ones obtained by Dong *et al*. (2007) in a similar species, *Crassostrea gigas*. DMSO produced the best results in terms of cell protection as previously demonstrated by Yang *et al*. (2013) in *Crassostrea virginica* sperm. Although PEG and caffeine supplementation produced good results in terms of low toxicity prior to cryopreservation, after thawing induced sperm agglutination at all freezing rates tested.

CONCLUSIONS

Sperm agglutination is the main problem in Portuguese oyster sperm cryopreservation. This phenomenon can be controlled using a combination of fast freezing, high cryoprotectant concentration and adequate sperm density.

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O37

EFFECT OF DILUTION OF FROZEN/THAWED SPERM OF SEX-REVERSED RAINBOW TROUT ON SPERM MOTILITY AND FERTILIZING ABILITY DURING POST-THAW STORAGE

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INTRODUCTION

In our recent study we have proven that milt of sex-reversed rainbow trout could be successfully cryopreserved in simple glucose–methanol extender (Dietrich et al. 2014). Scaling up of this technique may require optimization of various steps of cryopreservation procedure including post-thawing storage. Thawing process is relatively time-consuming, therefore dilution of post-thaw semen in sperm immobilizing solutions may secure frozen/thawed semen until fertilization trials. The aim of current study was to determine the suitability of sperm immobilizing solutions for post-thaw storage of semen of sex reversed rainbow-trout.

MATERIALS AND METHODS

The cryopreservation procedure followed previously described procedure using glucose-methanol (Dietrich et al. 2014). Immediately after thawing, samples of milt (n=5) were diluted 1:10 in immobilizing solutions (Morisawa and Morisawa 1988; Kobayashi et al. 2004) and glucose-methanol extender. Control samples were not diluted after thawing. Sperm motility was examined using Hobson Sperm Tracker (Hobson Vision Ltd., Baslow, UK). The fertilization success was established by calculating the percentage of hatched larvae obtained after fertilization of eggs with thawed semen with spermatozoa to egg ratios 500 000:1. Data were subjected to repeated measures two-way ANOVA followed by Sidak's post hoc test. All analyses were performed at a significance level of 0.05 using GraphPad Prism software (GraphPad Software Inc. San Diego, CA, USA).

RESULTS

Relatively high sperm motility (about 47%) was reported for samples undiluted or diluted in glucose-methanol during first 15 min of storage. At that time, low sperm

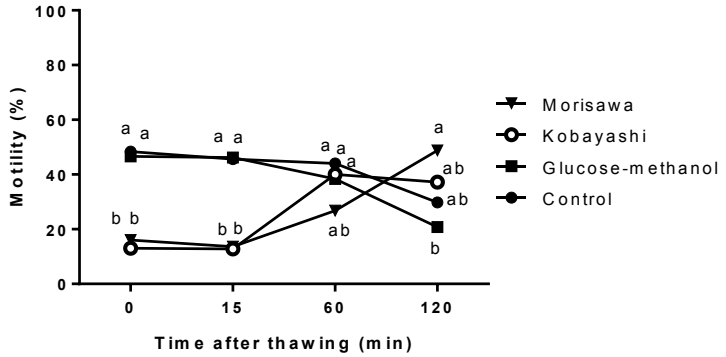

Fig. 1. Effect of post-thaw storage in different media on sperm motility of sex-reversed rainbow trout (n=5). Values (mean ± SD) with different superscripts are significantly different (P < 0.05) among different dilution media.

motility (15%) was observed for both sperm immobilizing solutions. After 60 min of storage, sperm motility of frozen/thawed semen diluted in immobilizing solutions increased up to 38% and 27% (for Kobayashi and Morisawa solutions, respectively). A 120 min storage resulted in significant increase of sperm motility of semen stored in Morisawa solution (49%) followed by Kobayashi solution (37%), while sperm motility significantly decreased in undiluted samples and samples stored in glucose-methanol (30 and 21%, respectively).

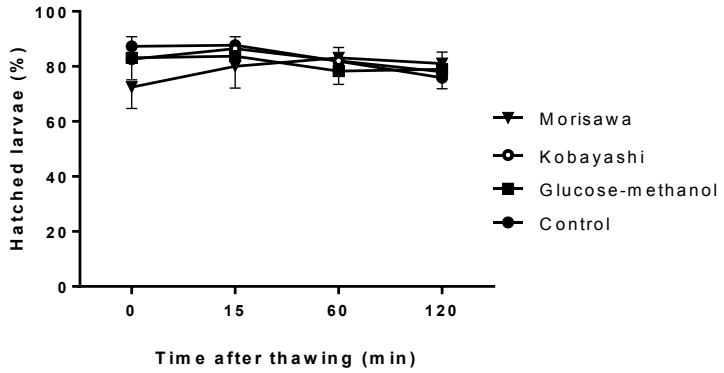

Fig. 2. Effect of post-thaw storage in different media on sperm fertilizing ability measured at hatching (n=5). Values (mean ± SD) were not significantly different ($P < 0.05$) among different dilution media.

Hatching success of larvae fertilized immediately after sperm thawing ranged from 73% (Morisawa solution) to 87% in control. No significant differences between experimental variants were found at any of tested periods of storage. Furthermore, time of storage of frozen/thawed sperm did not affect sperm fertilizing ability.

DISCUSSION AND CONCLUSIONS

Our results indicate that frozen/thawed sperm could be successfully stored after thawing for at least 120 min without loss of fertilization ability. However, special care should be taken in case of immobilizing effect of dilution media on sperm motility during first 15 minutes of storage. Perhaps time-related increase of post-thaw sperm motility in sperm immobilizing solution could be connected with maturation effect of these media on semen of sex-reversed rainbow trout, which effect is known to be different from glucose-methanol (Ciereszko et al. 2015).

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O38

VITRIFICATION OF FISH SPERM

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INTRODUCTION

Vitrification is the solidification of a liquid into an amorphous or glassy state which can be attained at very fast cooling rates (10^6-10^{10} °C/s). Recently, several studies have been published on the vitrification of fish sperm (Cuevas-Uribe et al., 2011/a, b, Figueroa et al., 2013, 2015). These studies report results on different fish species, such as Channel catfish (*Ictalurus punctatus)*, Green Swordtail *(Xiphophorus hellerii)*, Rainbow trout *(Onchorynchus mykiss)*, Atlantic salmon *(Salmo salar)*, but no information is available on the vitrification of sperm in other fish species.

MATERIALS AND METHODS

Vitrification experiments were carried out in 7 species. Eurasian perch *(Perca fluviatilis)*, common carp *(Cyprinus carpio)*, tench *(Tinca tinca)*, goldfish *(Carassius auratus)* and zebrafish *(Danio rerio)* individuals were were maintained at the hatchery of the Department of Aquaculture, Szent Istvan University, Gödöllő, Hungary. European eel *(Anguilla anguilla)* individuals were kept at the Polytechnic University of Valencia, Spain, and grayling *(Thymallus thymallus)* were mainteined in the facility of the Angling Association of Tolmin, Slovenia.
Progressive motility of fresh sperm was evaluated with computer-assisted sperm analysis (CASA, Sperm VisionTM v. 3.7.4., Minitube of America, Venture Court Verona, USA). In case of all investigated species, several dilution ratios, cooling media and cryoprotectants were tested. Three cooling devices were used: straws (250 µl), cryoloops and Cryotops (Kitazato-Dibimed, for 2 µl of solution). Sperm suspension was plunged directly into liquid nitrogen without pre-cooling in its vapour. The efficiency of the vitrification was evaluated with CASA analysis (all species), fertilisation tests (in case of perch) and computer automated sperm head morphomtery analysis (ASMA, Proiser, in case of European eel).

RESULTS

Best results were achieved by vitrifying small volumes of diluted sperm (2 µl) with the use of 30-40% total cryoprotectant content. According to the characteristics of the investigated species, different cooling media and dilution ratios were used. In general, we can conclude that above the cryoprotectant concentration of 40% the toxicity of chemicals is too harmful for the cells, and below 30%, ice formation is not entirely inhibited. The use of 250 µl straws was not efficient in any of the tested species, thus we can conclude that for fish sperm vitrification devices for small amounts of sperm (some microliters) are needed.
With species-specific protocols (table 1.) we were able to recover motile cells in the sperm of all investigated species after thawing (3-20% progressive motility) and developing embryos in case of perch.

Species	Achieved result	Device	Dilution ratio	Media	Cryo-protectant
Eurasian perch *(Perca fluviatilis)*	13,95±1,67% progressive motility, 2,54±1,67% fertilisation	Cryotop	1:5	Tanaka	15% MOH + 15% PG
European eel *(Anguilla anguilla)*	5±0,81% progressive motility	Cryotop	1:1	FBS	20% MOH + 20% PG
Common carp *(Cyprinus carpio)*	7,16±0,62% progressive motility	Cryoloop	1:100	Carp seminal plasma + grayling extender	10% MOH + 10% MTX +10% PG
Tench *(Tinca tinca)*	3,07±0,12% progressive motility	Cryoloop	1:4	Grayling extender	10% MOH + 10% MTX +10% PG
Goldfish *(Carassius auratus)*	3% progressive motility*	Cryoloop	1:4	Grayling extender	10% MOH + 10% MTX +10% PG
Grayling *(Thymallus thymallus)*	20% progressive motility*	Cryotop	1:1	Grayling seminal plasma	15% MOH + 15% PG
Zebrafish *(Danio rerio)*	3% progressive motility*	Cryotop	1:4	HBSS	10% MOH + 10% MTX +10% PG

Table 1.: Most efficient vitrification protocols for differenet fish species. MOH=metanol, PG=propylene-glycol, MTX=2-methoxyethanol. *:preliminary results

DISCUSSION AND CONCLUSIONS

Successful sperm vitrification in 7 fish species was carried out for the first time. Motile spermatozoa were recovered following vitrification of sperm and fertilization of eggs with vitrified sperm resulted in developing embryos in one species (Eurasian perch). Thus, vitrification of sperm is feasible in fish species, although further studies are needed to improve this technique.

ACKNOWLEDGEMENTS

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O39

STORAGE AND TRANSPORTATION OF *PROCHILODUS LINEATUS* (CHARACIFORMES) SPERM PRIOR TO CRYOPRESERVATION

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INTRODUCTION

The streaked prochilod *Prochilodus lineatus* is migratory fish species, native of South America, well adapted to captivity, easily reproduced artificially, and thus used as a model in a number of studies addressing nutrition, health, genetic diversity and reproduction (Viveiros et al 2010). The quality of sperm, and therefore its suitability for cryopreservation, is influenced by a wide variety of factors. Above all, it is important during stripping of sperm to avoid contamination with urine, feces, water or mucus, which activates sperm motility (Donaldson et al 2011). If contamination happens, and it happens rather frequent, freezing of sperm should occur soon after collection. However, freezing sperm in the field under fluctuation of environmental temperature, variation and incidence of sun light and lack of sanitary conditions to manipulate sperm certainly lead to lower results than freezing sperm under standardized conditions at a laboratory. But then storage under optimal conditions and transportation of samples are needed. Thus the aim of this study was to test whether *P. lineatus* sperm could be stored and transported prior to cryopreservation, without losing post-thaw quality. Post-thaw quality of sperm stored during 3 and 6 h prior to cryopreservation and frozen under laboratory conditions was compared to sperm frozen immediately after collection under field conditions.

MATERIALS AND METHODS

Ten *Prochilodus lineatus* males were selected during spawning season at the Fish Culture Station of the Minas Gerais Power Company (CEMIG) in the city of Itutinga, Brazil. Males received two doses of carp pituitary extract at 0.4 and 4 mg/kg BW in a 12-h interval. After 7.5-8 h at 27-28 ºC, the urogenital papilla was dried and about 2 mL of sperm from each male was hand-stripped. Contamination of sperm with water, urine or feces was carefully avoided.

Sperm was frozen immediately after collection (Control) or after storage and transportation by car for ~100 km from the Fish Culture Station to the Laboratory of Semen Technology at Federal University of Lavras. Sperm was stored and transported in a closed 1.5 mL tube in a cooler (9-11 ºC) either *in natura* without dilution (Undiluted) or diluted in a glucose solution at 325 mOsm/kg to a ratio of 1 sperm:4 glucose (Diluted) and frozen 3 or 6 h after collection. Thus the five treatments were: Control, Undiluted-3h, Diluted-3h, Undiluted-6h and Diluted-6h. The freezing medium was composed of a glucose solution at 325 mOsm/kg and methyl glycol (Viveiros et al 2009) to a final ratio of 1 semen: 8 glucose:1 methyl glycol. Sperm was drawn into 150 unsealed 0.25-mL straws (n = 3 replicate straws x 5 treatments x 10 males) and frozen in nitrogen vapor vessel (dry vapor shipper) at approximately -170°C. Final dilution, loading and freezing took 15 min. Within 24 h, straws were transferred to a liquid nitrogen vessel for storage.

Straws were thawed in a water bath at 60°C for 3 s and post-thaw sperm was immediately evaluated for motility rate and velocities (curvilinear = VCL; average path = VAP; straight line = VSL) using a Computer-Assisted Sperm Analyzer (SCA™ software, Microptics, Barcelona).

RESULTS

Post-thaw sperm motility rate was similar (P>0.05) between samples frozen soon after collection (Control = 64%), or after 3 (60%) and 6 h (62%) of storage as long as the samples remained undiluted during storage. However, a decrease on post-thaw motility rate was observed when samples were first diluted and then frozen after 3 (45%) and 6 h (35%) of storage.

Velocities were higher when samples were frozen soon after collection (Control) or when frozen after 3 h of storage without dilution (Undiluted-3h). The mean velocities were: VCL of 265 µm/s, VAP of 244 µm/s and VSL of 147 µm/s for Control samples, and VCL of 254 µm/s, VAP of 219 µm/s and VSL of 134 µm/s for Undiluted-3h samples. Samples stored after dilution as well as samples stored for 6 h prior to cryopreservation yielded lower ($P<0.05$) velocities compared to Control and Undiluted-3h samples (Table 1).

Table 1 Post-thaw motility and velocities (VCL=curvilinear, VSL=straight-line, VAP=average path; mean ± SD) of *Prochilodus lineatus* sperm frozen immediately after collection (Control) or after 3 and 6 h of storage and transportation either undiluted or diluted in a glucose solution.

Treatments	Motility (%)	VCL (µm/s)	VAP (µm/s)	VSL (µm/s)
Control	64 ± 8 [a]	265 ± 28 [a]	244 ± 26 [a]	147 ± 11 [a]
Undiluted-3h	60 ± 13 [a]	254 ± 33 [a]	219 ± 30 [ab]	134 ± 19 [ab]
Diluted-3h	45 ± 9 [b]	221 ± 29 [b]	195 ± 29 [b]	121 ± 17 [bc]
Undiluted-6h	62 ± 15 [a]	198 ± 22 [b]	164 ± 29 [c]	111 ± 20 [bc]
Diluted-6h	35 ± 11 [b]	158 ± 35 [c]	130 ± 40 [d]	103 ± 37 [c]

[a,b] Means followed by different superscript differ ($P<0.05$, SNK test)

DISCUSSION AND CONCLUSIONS

P. lineatus sperm was frozen in the field immediately after collection or in the lab under standardized conditions after 3 or 6 h of storage and transportation without dilution or after dilution in a glucose solution. Although dilution of sperm in an iso- or hyperosmotic medium is a common practice to overcome the loss of sperm quality after collection, it was harmful for *P. lineatus* sperm, even though the glucose solution is recommended for the freezing of sperm of this fish species (Viveiros et al 2009; 2010). The storage of undiluted *P. lineatus* sperm for 3 h prior to freezing did not affect post-thaw sperm motility or velocities, but after 6 h sperm velocities significantly decreased. It suggests that *P. lineatus* sperm should be frozen sooner than 6 h after collection. Similarly, Agarwal (2011) stated that fish sperm can be successfully frozen 4 h after collection.

In conclusion, *P. lineatus* sperm tolerates transportation *in natura* form and should be frozen sooner than 6 h after collection.

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O40

CRYOPRESERVATION OF NILE TILAPIA (*OREOCHROMİS NİLOTİCUS*) SPERMATOZOA: EFFECT OF EXTENDER SUPPLEMENTED WITH DIFFERENT CRYOPROTECTANTS ON POST-THAW SPERM MOTILITY, VIABILITY, DNA DAMAGE AND FERTILIZATION

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INTRODUCTION

The importance of cryogenic storage of gametes is now widely recognized as an effective management tool for conservation of genetic resources in aquaculture. Although spermatozoa cryopreservation is a valuable technique for preservation of genetic material, it can cause mechanical damages to spermatozoa. It is generally accepted that a substantial number (50%) of sperm cells are damaged during cryopreservation (Watson, 2000). The main cause of damage to cells undergoing cryopreservation is the formation of intracellular ice crystals (Mazur, 1984). This damage may be reduced by the incorporation of cryoprotectants (Aman and Pickett, 1987). The composition of the extender and the inclusion of suitable cryoprotectants are important factors in successful sperm cryopreservation (Curry et al., 1994). Addition of optimum amount of cryoprotectant reduces cell damages associated with dehydration, cellular injuries and ice crystal formation (Leung and Jamieson, 1991). Although cryoprotectants help to prevent cryoinjuries during freezing and thawing, they can become toxic to the cells when the exposure time and concentration are more (Tekin et al. 2007). It is therefore necessary to adjust the extender composition, the type of cryoprotectant, the amount of cryoprotectant, and the cryopreservation protocol to meet the specific needs of sperm from each species (Yavaş et al. 2014). To the best of our knowledge, there is limited information regarding protective role of cryoprotectants on DNA damage of Nile tilapia sperm. Thus, this study aims to compare the effect of type and concentrations of various cryoprotectants (CPAs) such as dimethyl sulfoxide (DMSO), methanol (MET) and glycerol (G) on post-thaw motility, duration of motility, viability, DNA damage as well as on fertilization ability of cryopreserved Nile tilapia (*Oreochromis niloticus*) spermatozoa.

MATERIALS AND METHODS

Semen was collected from 20 healthy mature fish after anesthetizing them with 10 ppm quinaldine (Reanal Ltd., Budapest, Hungary). Sperm quality from each male was determined immediately following semen collection. Samples with >80% motility were pooled and used in the experiments. Pooled semen was diluted at ratio of 1:3 with an ionic extender composing 75 mmol/L NaCl, 70 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgSO_4$ and 20 mmol/L Tris (Lahnsteiner et al. 2003). The extender contained one of three cryoprotectants such as dimethyl sulfoxide (DMSO), glycerol (Gly), and methanol (MeOH) at final concentrations of 5%, 10% or 15%. Following dilution of semen with this extender, diluted semen were drawn into 0.25-mL plastic straws (IMV France) and sealed with polyvinil alcohol (PVA). Following equilibration of semen for 5 min at 4°C, the straws were placed on a styrofoam rack floating on the surface of liquid nitrogen in a styrofoam box and the semen were frozen in liquid nitrogen vapour 3 cm above of the liquid nitrogen surface for 10 min (Tekin et al., 2003). Following, frozen sperm were kept in liquid nitrogen container until analyses. Thereafter, the frozen sperm were thawed in a water bath at 30°C for 20 s. Thawed sperm was activated using 3‰ NaCl and observed under microscope for determination of motility, motility durations and viability (three replicates). Fertilization was conducted at ratio of

$1x10^5$ spermatozoa/egg. DNA damage was evaluated by Comet assay using image analysis system.

RESULTS

Supplementation of 10% glycerol and methanol showed better cryoprotective effect for sperm motility, duration of motility and viability against freezing damage ($p<0.05$) when compared to DMSO. The highest fertilization rate (48%) was obtained with extender containing 10% glycerol ($p<0.05$). The results obtained demonstrate that the cryopreservation protocol used to cryopreserve the Nile tilapia sperm cause significantly damage at DNA level. In comet test, glycerol at dose of 10% gave the best score (5%) when compared to the other cryoprotectants reducing DNA damage ($p<0.05$).

DISCUSSION AND CONCLUSIONS

The presence of ions in the extender affected post-thaw sperm quality positively and also methanol and glycerol were determined as suitable CPAs for the Nile tilapia, leading to a good protection of motility and viability during the cryopreservation process. On the other hand, glycerol showed the best protective effect against DNA damage of cryopreserved Nile tilapia sperm. The present study demonstrates that cryopreservation process can induce DNA damage in the Nile tilapia spermatozoa and this fact should be taken into account in the evaluation of freezing/thawing protocols.

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Reproduction and development in ornamental fish and invertebrates

SOA6

MARINE ORNAMENTAL SPECIES CULTURE: THE PAST, THE PRESENT AND THE FUTURE

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Many fish collectors in tropical and subtropical countries still employ destructive fishing methods such as cyanide and dynamite to collect fish, making it easier to collect them, but harming the delicate coral reefs ecosystems. Marine ornamental aquaculture may thus represent an alternative supply of fish as well as generate new insights about the reproductive and development biology of these species.
An overview of data on breeding and rearing protocols for some marine ornamental species will be discussed as well as the possibility to use some of the studied species as laboratory experimental models. In particular, attention will be put on clownfishes (*Amphiprion* sp), dottybacks (*Pseudochromis* sp.), seahorses (*Hippocampus* sp) and angelfishes (*Centropyge* sp.) as well as other species.
Data on spawning, embryo development, first feeding, and tank design will be presented and discussed. Particular emphasis will be provided to the role of different live prey including rotifers, *Artemia,* ciliates and copepods during larval development. In particular, nutritional qualities will be related to growth, stress and development factors in fish larvae and their role in larval survival.
With the increase in popularity of ornamental invertebrates in reef aquariums, as well as their important role in the study of the global change and their primary role as ecological moldels details on the feeding behavior and possible prey-predator interactions will be discussed.
Since the early '900 many corals are known to be both auto and heterotrophs. Studies have demonstrated the importance of heterotrophy for several species as well as in certain stressful situations like bleaching, deep and turbid waters. Corals are known to have multiple heterotrophic inputs, including particulate organic matter bacteria and and phyto- and zooplankton, including diatoms. Since zooxanthellae cannot provide nitrogen, phosphorous, or many other, the coral host must replenish these through heterotrophic means. The ability of different corals species to feed on plankton has been deeply studied using different approaches and methodologies; however, these studies have mainly been performed on mesomacro zooplankton and less frequently on pico- and nanoplankton (<100 microns). Nevertheless, pico- and nanoplankton communities are extremely abundant on coral reefs and play a key role in food webs and in coral feeding.
Different laboratory techniques can be used to assess coral feeding and will be discussed during the presentation.

At date, much effort is still necessary to increase survival in marine ornamentals in order to promote coral reef preservation.

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O41

THE QUALITY OF GREAT SCALLOP (*PECTEN MAXIMUS*) SPERM AFTER THAWING

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INTRODUCTION

Basic elements of a cryopreservation protocol for great scallop sperm were recently defined (Suquet et al., 2014). The aim of the present work is to describe the quality of great scallop sperm cryopreserved and thawed using this protocol, and compare it to the characteristics of fresh sperm.

MATERIALS AND METHODS

After intragonadal serotonin injection, sperm was collected and cryopreserved (n=6 individuals) in 500µl straws, each filled with 125µl sperm and 375µl (DCSB4+10% polyethylene glycol). The straws were maintained 5.5cm above the surface of liquid nitrogen (LN) for 10 min before being plunged in LN. After thawing (water bath: 25°C, 15s) and activation in seawater+5g/l BSA (dilution 1:625), the percentage of motile sperm and Velocity of the Average Path (VAP) were determined using the plugin CASA, developed for Image J. Sperm viability was assessed using flow cytometry (Le Goïc et al., 2013). ATP content was determined by bioluminescence (kit ATP lite). Then, the fertilization capacity of fresh and thawed sperm was compared using triplicates batches of 25 000 oocytes each (sperm to oocyte ratio 500 : 1).

RESULTS

Significantly lower percentages of motile sperm and VAP ($P<0.001$ for both parameters) were observed for thawed sperm (mean±SEM, respectively: 22±12 %, 32±13 µm s^{-1}), compared to fresh sperm (88±9 %, 221±37 µm s^{-1}). The percentage of dead sperm remained limited but significantly higher in thawed sperm compared to fresh one (6.3±1.3 % and 0.9±0.2 % respectively, $P<0.001$). Intracellular ATP concentration was not different between fresh and thawed sperm. Finally, the D-larval rates resulting from the use of fresh (12.3±6.1 %) and thawed sperm (9.4±5.1 %) were not significantly different.

DISCUSSION AND CONCLUSIONS

Compared to fresh sperm, the quality of thawed great scallop sperm was decreased. However, fertilization rates similar to those recorded using fresh sperm were observed using a non-limiting sperm to oocyte ratio, validating the use of cryopreservation for great scallop sperm cryobanking. However, this study highlights the need for further improvements of artificial reproduction in great scallop.

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O42
SPERM GUIDANCE STRATEGIES IN BROADCAST SPAWNERS

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INTRODUCTION

Evaluation of sperm quality greatly relies on their capacity for motility in a drop of swimming solution as well as the observation by optical microscopy. A complement set of information should be added, that dealing with sperm capacity to approach the egg, a crucial step leading to fertilization. Chemo-attraction of sperm by egg is best understood in a few broadcast-spawning species but is poorly documented in the case of fish gametes. State of the art in this field is presented in this review.

A variety of broadcast-spawning marine organisms require sophisticated sperm guidance mechanisms, named chemotaxis, to locate the egg and fertilize it. In the few species where this is has been described, oocytes release specific molecules that bind to sperm flagellar receptors and trigger a signaling pathway that results in intracellular Ca^{2+} concentration fluctuations. Each transient Ca^{2+} increase leads to a momentary elevation of flagellar bending asymmetry, which results in a pronounced turn essential for chemotaxis. In addition, this process needs a precise spatio-temporal coordination between the Ca^{2+}-dependent turns, the form of chemo-attractant gradient and periods of straighter trajectory.

This review summarizes the sequence of events and known components of the signaling pathway leading to chemotaxis in spermatozoa of aquatic species, with special emphasis on fish gametes and the strategies employed to unravel this fascinating and fundamental process called sperm guidance.

MATERIALS AND METHODS

Most methods are based on physical constitution of a concentration gradient originating from a punctual source. The most relevant quantification uses visualization of the sperm tracks in the vicinity of the attractant source. The source is initially the biological structure from which emanates the chemo attractant: whole egg, egg deprived of surrounding structures (chorion as example), or an egg sub-structure. Then, a crude extract ("egg water") or further purified fractions are included in various kinds of supports creating artificial attractant source; the latter can be a micropipette with small opening (µm diameter), comprising a mixture of extract with agar to minimize molecular diffusion or porous beads (5-10 µm diameter) impregnated with the substances to be tested. Other experimental designs were also used: as example, micro-electrophoresis gels (0.05 x 5 cm) were put in presence of sperm suspensions allowing determination of molecular size or charge; in other cases, multi-chamberered microscopy devices with inter-connecting capillaries were described in combination with video tracking of trajectories of sperm heads. Our understanding of the signaling pathways involved in chemotaxis has notably progressed in the recent years due to significant advances in Ca^{2+} imaging.

RESULTS AND DISCUSSION

The 3 best-described models are sea urchin, ascidian and herring. *Sea urchins* have become the best-characterized system at a molecular level. The egg-jelly, an extracellular matrix surrounding the egg, contains polysaccharides and small peptides that regulate sperm motility (Sperm-Activating Peptides): SAPs diffusion induces rapid changes in sperm cyclic nucleotide levels, membrane potential (Em), pHi, $[Ca^{2+}]_i$ and other ionic permeabilities (Darszon et al., 2008; Kaupp et al., 2008). When sperm swim in a concentration gradient of attractant, Ca^{2+} spikes are evoked in synchrony with the stimulus suggesting that a precise timing of Ca^{2+} spikes controls the directed navigation process. *In ascidians*, a chemo-attractant derived from the eggs of *Ciona* was determined to be a low-molecular-weight

sulfated hydroxysterol. The cAMP level in spermatozoa is increased by the Sperm-Activating and -Attracting Factor (SAAF); this process requires extracellular Ca^{2+}. *Situation in fish gametes:* in the pacific herring, *Clupea pallasi (*marine fish), the micropyle is of conical shape (2.6 x 30 µm). In the vicinity of the micropylar depression, spermatozoa activate and swim at 100-150 µm/sec for several hours, following the chorion surface, When reaching the micropyle opening itself, their velocity decreases down to 30 µm/sec. but sperm cells accumulate in the micropyle funnel. A mild trypsin treatment of eggs as well as DTT prevents sperm cells to be attracted by the micropyle. Ca^{2+} ions are implied in this chemotactic behavior. Two types of molecules released by eggs act cooperatively: the SMIF (Sperm Motility Initiation Factor) and the HSAP (Herring Sperm Activating Peptide). Motility initiation is regulated by a reverse Na^{+}/Ca^{2+} exchanger in combination with HSAP and SMIF. HSAP has a protease inhibitor motifs, which binds to an endo-peptidase of the flagellar membrane. The SMIF is a 105 kDa glycoprotein localized in the chorion part surrounding the micropyle which induces a trajectory switch from linear in Sea Water to helical in the micropylar funnel. Membrane depolarization is the signal next to HSAPs, inducing a Ca^{2+}-dependent and cAMP-independent cell signaling for motility initiation. The HSAPs are a Kazal-type trypsin inhibitor resembling acrosin inhibitor. These findings and the role of trypsin inhibitor-like HSAPs for activation of herring sperm motility suggest that function of protease inhibitor on the regulation sperm motility in the fertilization is conserved over the vertebrate species in the course of evolution. *Other fish species:* in the vicinity of trout egg, sperm swim along the chorion surface and many spermatozoa enter the micropylar opening (Yanamigachi *et al.*, 1992). Absence of Ca^{2+} prevents the attraction behavior but the latter does not occur if eggs are mildly treated by trypsin, leading to suspect a protein nature of the attractant. Recently, Yeates *et al.* (2013) demonstrated the participation of ovarian fluid to the sperm attraction process in salmonids.

CONCLUSIONS

Since the pioneering studies of Lillie in 1914 in sea urchin, chemotactic response was demonstrated by Miller with various degrees of details: in Cnideria, Mollusca, Echinodermata (Asteroidea, Holothuroidia and Ophiuroidea) and in Urochordata chemotaxis is established without ambiguity; in addition, there are some evidences which need better experimental support in Nematoda, Chaetognata, Lophophora (Bryozoa) and Annelida. Some weak evidences are present in Cephallopoda, Calcarea (phyllum Porifera). The most salient examples were briefly presented above only for the few representative species, including fishes, the latter needing investigation in more species.

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O43
qPCR TOMOGRAPHY OF THE *ACIPENSER RUTHENUS* OOCYTE

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INTRODUCTION
Sturgeons (order *Acipenseriformes*) have several features which make them very interesting and a unique object of study, not only because of economic issue but also because of their conservation, evolutionary age, wide distribution, phylogeny and genome evolution (Ludwig, 2001). Besides the listed properties, *Acipenseriformes* in their developmental pattern occupies a special place between amphibians and teleosts (Bolker, 1993; Gilbert, 2000, Takeuchi, 2009). Regulation of gene expression with a precise temporal and spatial control, and subsequently embryo development can be achieved by localization of specific RNAs to subcellular domains (Martin, 2009). The machinery of this process plays a significant role for the spatial setting of gene expression (King, 2005). Thereby, using qPCR tomography to study mRNA localization in complex biological samples two main spatial expression profiles similar to the *Xenopus laevis* distribution pattern were found in *Acipenser ruthenus* oocytes (Sindelka *et. al.*, 2010).

MATERIALS AND METHODS
Ovulation was induced with CPE injected in two steps: the first, 0,5 mg/kg b.w. and the injection second, 4,5 mg/kg b.w., 12 h after the first injection. The ovulated eggs were collected 18–20 h after the second injection. The oocytes were embedded in a drop of optimum cutting temperature (OCT) on a pre-cooled dissection block. The samples were dissected into 80 slices 30 µm along the animal–vegetal (A–V) axis and consecutive slices were pooled into five tubes with sixteen slices in each. RNA extractions were performed for each sample according to the TRIzol manufacturer's protocol. RNA concentrations were measured with the Nanodrop ND1000 quantification system. cDNA was produced starting with 50 ng of total RNA with Superscript III. The quantification was performed using IQ Sybr Green Supermix (BioRad). The qPCR cycling program consisted of 3 min at 95°C for activation of the hot-start enzyme, followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 20 s, and elongation at 72°C for 20 s. Melting curves analysis was performed to validate the formation of expected PCR products.

RESULTS
The performance of qPCR tomography identified spatial localization of selected genes with the similar to *X. laevis* gradient distribution (according to Sindelka *et al.*, 2010). Animal hemisphere mostly includes reference genes, such as Alas1, Beta-actin, Cyp a, Sdha and Yawhz (Fig. 1a). The spatial distribution of mRNA coding germ plasm determinants such as Dead end, Vasa (Fig.1b) and Ziwi (data is not shown) show a gradient toward the vegetal pole , suggesting that these mRNAs are localized in marginal zone and the vegetal hemisphere with highest abundance close to the pole itself, in X.laevis oocyte this gradient has more steep character.

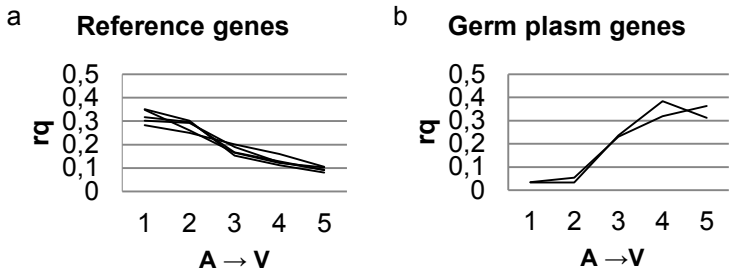

Fig.1. Spatial expression profiles of the reference genes (a) and of the germ line genes (b) in segments along the A-V axis in the *A.ruthenus* oocyte

DISCUSSION AND CONCLUSIONS

In this study, we revealed undeniable resemblance in the localization of mRNA molecules between two unique models *Acipenser ruthenus* and *Xenopus laevis*. Despite the fact that the spatial distribution of genes has the gradient character, it is also worth noting a slightly smoother transition of these genes along the animal-vegetal axis. Method of qPCR tomography established by Sindelka *et al*., has a number of advantages compared with other transcriptome analysis techniques, as a major is quantification of mRNA localization in oocyte, as a single system. Thereby, this technique was successfully optimized and applied for the sturgeon oocytes.

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O44

EFFECTS OF VITAMIN E ON REPRODUCTIVE PERFORMANCE OF PINDANI (*PSEUDOTROPHEUS SOCOLOFI* JOHNSON, 1974)

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INTRODUCTION

Studies on dietary requirements of ornamental fish could improve the success of their hatchery propagation. Successful propagation in hatcheries could reduce the cost of seed production and the pressure generated by aquarium traders on wild stocks for obtaining cheaper seed (Sales and Janssens, 2003). Vitamin E has profound effects on fish reproduction (Izquierdo et al. 2001) and there are limited numbers of studies on vitamin E requirement of ornamental fish. In this study, we investigated the effects of vitamin E on reproductive performance of pindani (*Pseudotropheus scolofi* Johnson, 1974) which has high economical value in aquarium trade.

MATERIALS AND METHODS

For the study, pindani brood candidates (mean weight of 2.96±0.04 g and mean total length of 5.69±0.01 cm, *N*=180) were fed a commercial diet (control) specifically formulated for Malawi cichlids (ArtAkua Inc., Izmir, Turkey) or extra (50, 100, 150 mg/kg) vitamin E added forms of the same diet during gametogenesis. With the initiation of spawning on week 13, triplicate sets of randomly selected 6 female and 2 male fish from each experimental group were stocked into 100 L spawning aquariums and continued to be fed with the same diets. Spawning activity of brood fish were monitored another 12 weeks. During this time, egg or yolk sac fry were collected from the mouth of females, counted and egg diameters were measured to nearest 0.1 mm. Afterwards each egg or yolk sac fry batch was placed into floating type incubators for evaluation of hatching and yolk sac absorption rates. Post larval survival rates for each batch were also evaluated at 10 days post hatch. All growth, feeding, reproduction and survival data were analyzed at α=0.05 significance level by using one-way ANOVA and Duncan multiple range test.

RESULTS

Feeding pindani for 12 weeks prior to or during spawning with a different amounts of vitamin E containing diet had no significant effect on growth, feed consumption and survival. Yet, adding extra vitamin E to the diet which naturally contained 121.33±4.60 mg/kg vitamin E due to its ingredients significantly improved their reproductive performance. Egg diameters, number of spawns and spawn sizes, live egg, pre and post larvae ratios in all brooder groups fed with extra vitamin E added diets were significantly higher than that of control fish. At the highest vitamin E content of 270.00±16.82 mg/kg, however, slight but significant declines in the numbers of spawn and spawn sizes were observed.

DISCUSSION AND CONCLUSIONS

Dietary vitamin E requirement of fish might differ depending on species, lipid content of diet, environmental conditions and developmental stage (NRC, 1993; Izquerdo et al. 2001; Hamre, 2011). Results of this study demonstrate that for pindani diets containing 121.33±4.60 mg/kg or lower vitamin E could yield the best growth, FCR and survival rates both in juvenile and adult stages. Together with this, for a better reproductive performance brood fish should be fed diets containing higher level of vitamin E (219.30±2.89 mg/kg) during gametogenesis and spawning. Nevertheless, increasing vitamin E levels above that might require special attention against possible negative effects of extra vitamin E on lipid metabolism (Tokuda and Takeuchi, 1999).

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O45
FLAGELLAR BEHAVIOR OF FISH SPERM AT MOTILITY INITIATION

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INTRODUCTION
In conventional experiments, an extremely rapid transition of fish spermatozoa from immotile to a fully active state upon the contact with the aquatic medium prevents the investigation of flagellar waveform at the initial stages of sperm activation (Cosson, 2004). By including DMSO in amount of 10% into the swimming medium, we could design an experimental situation where sperm motility initiation was delayed in time up to several seconds, so the earliest and most significant activation steps of sperm flagella became possible to observe and video-record (Prokopchuk et al., 2015). In the present study, taking advantage of generating such an artificial delay in motility activation, we describe the precise chronology of flagella wave development at the motility initiation of sperm from three different fish species (sterlet, carp and Chinook salmon).

MATERIALS AND METHODS
Studies of flagellar wave initiations were carried out on spermatozoa of carp (*Cyprinus carpio*), sterlet sturgeon (*Acipenser ruthenus*) and Chinook salmon (*Oncorhynchus tshawytscha*). Sperm motility was triggered by dilution into either a species specific activating medium (sterlet: 1mM CaCl2, 10mM NaCl, 10mM Tris-HCl, pH 8.5; salmon: 125 mM NaCl, 0.1 mM CaCl2 30mM Tris-HCl, pH 9.2) or distilled water (carp) both supplemented with DMSO at a final concentration of 10%. Bending forms of sperm flagella were observed either by use of a 50X dark field objective or a 100X positive phase contrast objective. Observations were recorded using a high-speed video camera (model i-SPEED TR from Olympus, UK) with spatial resolution of 848×688 pixels and time resolution up to 1000 frames per second in routine records to obtain sharp images of flagella. From several series of images covering one or several successive beat cycles, the detailed analysis of the flagellar beating behavior was performed by use of image analysis software (Olympus Micro Image 4.0.1. for Windows, Japan).

RESULTS
In most cases, wave initiation occurred in the very proximal region of the flagellum close to the head, though the origination of the wave at the middle or tip of the flagella could also be documented occasionally. Starting from fully immotile, sperm flagella showed some brief shivering at low amplitudes, yet without real wave development. In case of sterlet sperm, such shivering process was hardly recognizable as cells twitched irregularly and with extremely low amplitude. In contrast, in salmon or carp spermatozoa the flagella was intensively bending into one large curvature always in the same direction and subsequently unbending for about 250-500 ms. Right after, such trembling behavior evolved into appearance of a first real bend. The first bend, so-called “principal bend”, was initiated at the head-tail junction along 25% to 30% of a flagellum with localization of the wave covering a distance about 5 to 9 μm from the head in case of sterlet and carp spermatozoa, and about 1 to 4 μm in salmon sperm cells. The amplitudes of such first principal bends were 4.28±0.65 μm, 1.39±0.35 μm and 1.19±0.36 μm for sterlet, carp and salmon spermatozoa, respectively. These introductory bends were followed by the “reverse bend,” which presented far lower wave amplitudes: 1.14±0.32 μm, 0.85±0.08 μm and 0.02±0.01 μm in sterlet, carp and salmon, respectively. This couple of bends started to propagate towards the flagellar tip but gradually faded when reaching the mid-flagellum. Consequently, the sperm cell remained still non-progressive. Altogether, the 2 to 5 following waves showed similar behavior until a stage where the amplitude of the reverse bend gradually

reaches a value similar to that of the principal bend. The larger amplitude of this couple of bends finally leads to sustain a real "takeoff" of the sperm cell characterized by a full flagellar wave propagation generating an active forward displacement similar to that occurring during regular steady state motility observed several seconds after activation. Although flagella produced the first successive principal bends in one direction, the wave propagation along the flagellum and formation of new waves in sterlet and carp spermatozoa proceeded in a partly helical manner leading to a 3-dimensional rotation of the whole spermatozoon. By contrast, salmon spermatozoa showed mostly planar wave propagation, though, starting from the earliest period of motility they induced asymmetric beating leading to a circular swimming path.
Eventually, we estimated that the time period needed from the activation signal (contact with fresh water) to full wave propagation ranges from 0.4 to 1.2 seconds. This time period correlates with biochemical results describing activation process.

DISCUSSION AND CONCLUSIONS

To our knowledge, this study is the first one that describes the flagellum waveforms during the successive steps of motility initiation in fish spermatozoa. However, several well-detailed studies on sperm flagella activation have been reported in sea urchin sperm (Goldstein, 1979; Gibbons and Gibbons, 1980; Ohmuro et al., 2004). Similarities occur between the latter authors' results and those in our present work. The process of motility initiation of fish sperm presents several successive phases, although they proceeded slightly differently than in sea urchin. In case of fish spermatozoa, the flagella motility initiation lasted significantly shorter than in sea urchin sperm, which is expected considering the total motility duration of fish sperm. The experimental design described in the present study can provide an opportunity to investigate flagellar motility initiation in spermatozoa of many freshwater fishes and can lead to a basic understanding of their natural sperm activation. In addition to the practical applications, estimation of different parameters specific to flagellar beating may also lead to a better understanding of the internal mechanics which explain how the motion of flagella is controlled.

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Epigenetic programming: from gametes to embryo

O46

ANALYZING MICRORNA REPERTOIRES IN EGGS FROM 4 FISH SPECIES CAN REVEAL SECRETS OF BOTH LIFE HISTORY TRAITS AND EMBRYO VIABILITY/QUALITY

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INTRODUCTION

Egg and embryo viability is a common problem in fish aquaculture, especially in marine species. Numerous factors may influence viability (reviewed in Bobe and Labbé, 2010) such as nutrition, environmental factors, husbandry practices and broodstock stress. In order to produce viable fish eggs, we have to understand what makes a good egg, which means all aspects of how the egg is assembled, and regulated at a molecular level. This study aims at identifying miRNAs connected to specific life history traits in four different species including zebrafish, medaka, cod and salmon. In addition the study aims at identifying miRNAs that can be associated with egg quality and early embryo development in these species.

MATERIALS AND METHODS

Eggs from different females (N≥10) of Atlantic salmon (*Salmo salar)*, Atlantic cod (*Gadus morhua*), zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) were sampled and frozen for RNA extraction. Eggs from the same batches were fertilized and followed until hatching. Scoring of key developmental stages was used to evaluate egg quality and based on this, egg batches were placed in either bad or good quality groups. For both groups, RNA was extracted for all species (n=3-4 per species and condition). Small RNA sequencing (Illumina) and miRNAs differential analysis were performed on all samples (using MiRDeep2 and DESeq algorithms). In order to understand potential gene regulatory role of the candidate miRNAs, putative targets were predicted using miRanda.

RESULTS

Small RNAs from bad and good quality eggs of Atlantic salmon, Atlantic cod, zebrafish and medaka females were subjected to high-throughput sequencing. Seven samples per species were sequenced and each samples produced more than 15 millions reads. Analysis of miRNA repertoire showed large differences in egg miRNA diversity between species. Only a few miRNAs displayed conserved level of expression between species. Those results have been confirmed by RT-qPCR with high correlation (R^2>0.75). Analysis of putative target genes was performed in order to give us a hint of the role of miRNAs in eggs. No clear relationship could be identified between egg quality and miRNAs of the egg in any of the species studied.

DISCUSSION AND CONCLUSIONS

Using comparative biology, we show that expression of miRNAs in eggs is quite different between fish species and that some miRNAs are very specific to one species and therefore may be related to specific traits. A few miRNAs have a conserved expression in the 4 studied species and therefore might be very important for early embryo development. In contrast to a study in rainbow trout were they found few miRNAs differentially expressed between good and bad quality eggs (Ma et al., 2015), miRNAs expression profiles were not clearly related to egg quality in our study. Functional studies are needed to better understand the specific role of candidate miRNAs in the egg.

ACKNOWLEDGEMENTS
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O47

ENVIRONMENTAL DOSES OF BPA INHIBIT ZEBRAFISH REPRODUCTION THROUGH A DEREGULATION OF EPIGENETIC PATTERN

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INTRODUCTION

Bisphenol A, commonly known as BPA, is one of the most produced Endocrine Disruptors Compound worldwide. It interferes with vertebrates' reproduction by binding to nuclear receptors ERα and ERβ and, ultimately, altering gene expression. As an environmental pollutant, BPA is likely able to alter epigenetic mechanisms through methylation of the CpG sites (Dolinoy, Huang, & Jirtle, 2007). Even though the matter lacks a good knowledge, BPA is supposed to alter histone modification and, hence, the structure of chromatin correlated with either activation or repression of transcription (Kundakovic & Champagne, 2011). Therefore, the aim of our project was, firstly, to determine the effects of environmentally realistic BPA concentrations on female reproductive physiology and, secondly, to investigate whether the changes in the expression levels of genes related to the reproductive physiology could be caused by histone modifications. Given the results we obtained, we also tested BPA doses on embryo development.

MATERIALS AND METHODS

The experiments were carried out on adult female zebrafish (*Danio rerio*) treated for a three-week time with three different doses of BPA: 5 µg/L; 10 µg/L; 20 µg/L. Oocyte growth, oocyte maturation, autophagic and apoptotic processes, as well as histone modifications and DNA methylation, were assessed through qPCR, histology and ChIP-qPCR.

RESULTS

Among all tested doses, the lowest one provoked the most severe effects**.** Molecular analyses in the ovary showed that this dose was able to alter reproduction and particularly affected oocyte maturation and apoptotic process, according to significant changes of *esr1*, *esr2a*, *lhcgr* and *pgrmc1* such as *p53* and *caspase3.* These results are supported by the histological analysis, which demonstrated the presence of atresia in almost every mature follicle belonging to the BPA-treated group. In agreement with the molecular outputs are the results obtained by ChIP-qPCR analysis. The enrichment of H3K4me3 and H3K27me3 histone modifications in the TSS of both *star, fshr* and *lhcgr* genes was performed. Molecular analyses on DNMTs additionally showed the changes of *dnmt1* and *dnmt3* with respect to controls, supporting the data obtained by ChIP.

DISCUSSION AND CONCLUSIONS

To conclude, our study demonstrates that the most harmful of the tested BPA doses is the lowest. It interfered with *Danio rerio* oocyte growth and maturation. From the evidences we collected, it is likely that the negative effects of BPA on reproduction are due to an upstream capacity of such pollutant to deregulate the epigenetic mechanism.

ACKNOWLEDGEMENTS

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ABSTRACTS OF SIDE EVENT

SE1

USE OF ZEBRAFISH MODEL FOR BUILDING UP THE KNOWLEDGE TO IMPROVE HUMAN ASSISTED REPRODUCTIVE TECHNOLOGY

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ABSTRACT

To date, the problem of infertility affects about 10-15% of couples and the number of women who need Assisted Reproductive Techniques (ART) to achieve a pregnancy is constantly increasing. However, despite the remarkable progresses obtained by research in the last thirty years, the success rates are still unsatisfactory (25-30%). In this light, basic studies which lead to a deeper understanding and elucidation of molecular mechanisms causing infertility appear essential.

Zebrafish has become a very popular laboratory animal over the past 15 years. Its major application in scientific research has been initially in developmental biology. Rapidly, however, due to the availability of the genome sequence and of the many hundreds of mutants and transgenic lines, zebrafish appeared as a vertebrate model with an impressive range of possible applications. It is now extensively used for research in physiology, cancer, drug discovery, toxicology, endocrinology, for high-throughput screening of drug libraries, and for reproduction studies. Numerous studies have confirmed that zebrafish and mammals are similar in their physiology, development, metabolism and pathways. Zebrafish organs and tissues have been shown to be similar to their mammalian counterparts at the anatomical, physiological, cellular and molecular levels. The zebrafish genome is fully sequenced and shown to share an approximately 85% homology with their human counterparts. In this light zebrafish is one of the current perspectives on the use of alternative species in human health studies. In 2003, the National Institutes of Health (NIH) ranked the zebrafish as the third most important experimental organism after rats and mice.

Recently, starting from many indications obtained from basic studies conducted on zebrafish ovary and gametes, important understanding have been achieved related to injuries caused by aging on human female gametes by applying FPA FTIR Imaging.

ACKNOWLEDGEMENTS

Aquagamete FA1205 COST.

SE2

DO WE NEED TO MAKE SCIENCE QUALITY EVALUATION MORE SCIENTIFIC? THE PROS AND CONS OF VARIOUS COMMONLY USED ASSESSMENT INDICATORS

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ABSTRACT

During the past two decades various assessment methodologies have been more rigorously used by science bureaucrates and granting agencies to value the "quality" of science.

The so-called "Impact Factor" (IF) is presently the most commonly applied indicator by many administrators but also by an increasing number of scientists without questioning its validity. It should be noted that the origin of the impact factor and the scientific working hypothesis behind its development and purpose is mostly unknown by the average science bureaucrate and – more surprisingly – by most of the scientists. Certainly, any numerical accounting on science output and impact on society has pros and cons. The paper presents a number of these shortcomings and urges for a re-assessment of the assessment tools. It is concluded that while the impact factor and other factors (e.g. Hirsch Factor) have their merits, they are primarily an indicator on number of early citations but have no meaning when it comes to quality assessment of scientific work and specfically when judging the "impact" of science in terms of society needs. In fact a high "impact factor" of a paper can also be obtained for a paper that is definitely wrong as it will be initially frequently cited to correct the mistake. It is concluded that while some metrics are useful as supplemental information, nothing can truly replace the peer review process. However, the emphasis has to be placed on a "well-structured" peer review process.

ACKNOWLEDGEMENTS

AQUAGAMETE FA 1205 COST.

SE3

IMPRESS: IMPROVED PRODUCTION STRATEGIES FOR ENDANGERED FRESHWATER SPECIES

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ABSTRACT

As a consequence of human activities such as over-fishing, pollution, and habitat destruction including hydroelectric power dams and turbines, European freshwater fish populations have been under threat during recent decades. In order to reestablish sustainable populations, habitats must be restored and fishing and pollution reduced. In some areas, though, stocking or re-introduction of native species is necessary. Although projects for stocking or re-introduction have been performed at least since the early 1900s, the scientific basis for successful stocking or re-introduction is deficient. Current rearing techniques produce juveniles with low post-release survival and reproductive success due to both physiological and behavioural/cognitive shortcomings that renders them maladapted for life in the wild. This is mainly due to the fact that the employed hatchery techniques were developed for food producing aquaculture, and not suitable for *wildtype conditioning* purposes.

The primary research objective of the recently initiated Marie Sklodowska Curie ITN-project *IMPRESS* is to raise knowledge and develop innovative production strategies for the conservation and management of endangered freshwater fish, focusing on three key European species; the Atlantic salmon (*Salmo salar*), the European eel (*Anguilla anguilla*) and sturgeons (Family *Acipenseridae*). In *IMPRESS*, we want to focus on improved production strategies for stocking purposes, in order to increase survival and improve behavioural adaptation to natural conditions (increased fitness). *IMPRESS* aims to improve current practices by employing a multidisciplinary intersectoral approach based on new experiments and existing knowledge. This includes research to improve basic knowledge of fish reproductive physiology, expansion of available tools in biotechnology and genomics, and hatchery techniques better suited for *wildtype conditioning*. Importantly, implementation of improved rearing systems for *wildtype conditioning* will only be successful if they are accepted and implemented by the stakeholders responsible for stocking actions. Therefore, *IMPRESS* will also focus on the social and economic dimensions of the transfer of improved technology to and between fish farmers and relevant stakeholders as an innovative feature.

ACKNOWLEDGEMENTS

Aquagamete FA1205 COST.

ABSTRACTS OF POSTER PRESENTATIONS

POSTER SESSION I

Tuesday, September 8th

P1

EFFECT OF TRICLOSAN ON SPERM QUALITY PARAMETERS AND VITELLOGENIN LEVELS OF GOLDFISH

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INTRODUCTION

A variety of chemicals end up in aquatic ecosystems and can exhibit endocrine disruptive function in aquatic life forms. An endocrine disruptive chemical (EDC) may act directly on the gonad or may affect several tissues including the hypothalamus, pituitary, gonads or liver with consequences on the rate of gonadal development and the viability of the gametes (Kime and Nash, 1999). Triclosan (TCS) is a widely used antibacterial agent found in a variety of household products and cosmetics. There is some evidence to suggest that TCS is a suspected endocrine disruptor in various animals including fish. Exposure to TCS can impair male gonadal development, induce vittelogenesis and result in decreased sperm count in semen (Raut and Angus, 2010).The aim of the present work was to asses the effect of exposure to TCS on gonadal development and sperm quality parameters of male goldfish.

MATERIALS AND METHODS

Male goldfish were divided into two groups. One group was the control (C) and the other was the Triclosan treated fish (TCS) which was exposed to TCS (100 μg l^{-1}). After a period of four weeks, samples (n=5 from each group) of sperm and blood (500μl) were collected. Sperm density and sperm motility were evaluated according to number of spermatozoa per ml of semen and percentage (%) of forward moving spermatozoa using a commercial sperm analysis system (Chatzaropoulos et al., 2015). The weight of gonads of ten fish from each group was measured and the gonadosomatic index (GSI) was calculated. Blood levels of vitellogenin (Vtg) were estimated using a kit for spectrophotometric determination of Vtg in fish (Biosence laboratories, Norway).

RESULTS

All the measured sperm quality parameters were significantly decreased in goldfish exposed to TCS for 42 days (Table 1). Exposure of male goldfish to TCS for 14 to 42 days, resulted in a gradual increase in plasma levels of Vtg (Figure 1).

DISCUSSION AND CONCLUSIONS

The results indicate that exposure to TCS may have negative consequences on gonadal development of male goldfish. The GSI of fish exposed to TCS was lower than the control group indicating that TCS impaired testicular growth of goldfish. This negative effect of TCS was parralled by the induction of vitellogenesis in male goldfish exposed to TCS. In the same manner, the negative effect of TCS on male goldfish was also observed in the sperm quality parameters which were investigated in this study.

Table 1. Spermatocrit (Scr, %), sperm density (number of spz x 10^{-9} ml^{-1} sperm), duration of spermatozoa motility (seconds after activation) and gonadosomatic index (GSI, %) of untreated goldfish (Control group) or goldfish exposed to triclosan (TCS group, 100 µg L^{-1}). C= control group, TCS=fish exposed to triclosan (100 µg L^{-1}). In parentheses, the standard error (±SE) values are indicated.

	Control	TCS group	t-test
Spermatocrit	39.46 (1.36)	35.43 (1.80)	P<0.001
Sperm density	10.34 (0.43)	8.74 (0.84)	P=0.019
Duration of motility	86.6 (2.95)	76.1 (6.58)	P<0.001
GSI%	3.64 (0.36)	3.13 (0.49)	P<0.05

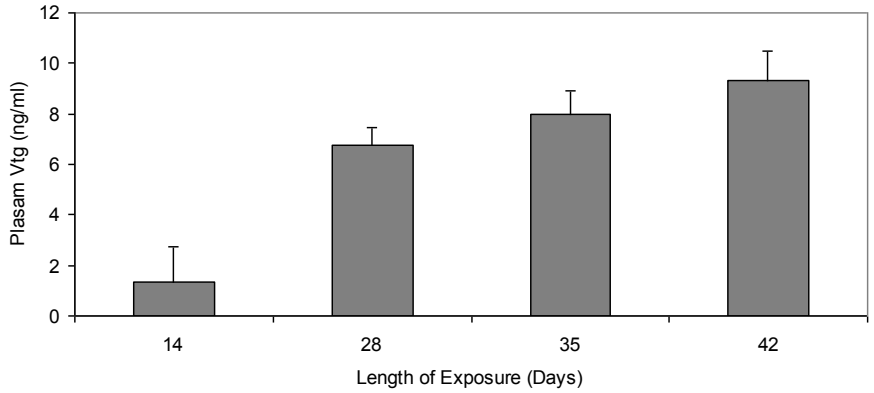

Figure 1. Mean levels of vitellogenin (Vtg, ±SD) in plasma of male goldfish after exposure to Triclosan for 14 to 42 days.

Exposure to TCS resulted in reduced sperm density and motility. The results are in agreement with those of other studies which reported that exposure to EDCs may affect sperm quantity and quality with negative consequences for the reproductive success of aquatic life forms. The concentration of TCS used in the present study is at least 50 times higher than the maximum expected levels reported in surface waters (Mills and Chichester, 2005). Nevertheless, the results of this study indicate that TCS is a potential endocrine disruptor with consequences on gonadal development and sperm quality of fish exposed to this chemical. These results could be used for risk analysis studies and the development of bioassays for the investigation of potential endocrine disruptive effect of this chemical on aquatic ecosystems.

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P2

EFFECTS OF THE EXPOSURE TO POLLUTED RIVER SEDIMENTS ON THE DEVELOPMENT OF THE GONADS OF BARBEL: MORPHOLOGICAL ENDPOINTS

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INTRODUCTION

The continuous input of contaminants makes river sediments a critical route of exposure to many chemical stressors for fresh water fish communities (van Geest et al., 2011). In recent years, a marked decline of native fish species in the River Po, such as the barbel (*Barbus plebejus*), the first species showing intersexuality in this water course (Viganò et al., 2001), has been observed. This appears to be linked to the presence of the complex mixture of chemicals transported by rivers draining densely anthropised sub-basins, such as the River Lambro. The aim of this study was to describe the effects on the histo-morphology of developing gonads in juvenile barbels after chronic exposure to sediments collected in two sites of the River Lambro at different level of contamination.

MATERIALS AND METHODS

Sediment samples were collected from two stretches of the River Lambro, one located upstream and the other downstream the city of Milan, put in glass aquaria (300 L) and kept at 25°C, under flow through conditions with aerated unchlorinated tap water (3 renewals/day). After 48hrs, 300 juvenile barbels (50 days post-hatching) were distributed in upstream (UP), downstream (DO) and control (CR) tanks, respectively and fed three times per day. Immediately (t0) and after 1 (t1), 3 (t2) and 7 (t3) months of exposure, fish were sub-sampled for the assessment of gonad differentiation and histopathology. Whole fish (t0 and t1) and dissected gonads (t2 and t3) were fixed in Bouin's fluid for 24hrs and served in ethanol 70% until inclusion in Bioplast. Five µm thick sections were stained with haematoxylin-eosin (H-E) and Mann Dominici (MD) for the determination of stages of spermatogenesis and oogenesis according to the germ cell types present and their relative abundance. As "intersex" were considered those fish showing simultaneous presence of testicular and ovarian tissue in the gonads. The occurrence of apoptosis was studied using the TUNEL assay. Alterations were assessed in three sections in three different area per each gonad.

RESULTS

After 1 month exposure, there were no differences in the development and morphology of the gonads between groups CR, UP and DO. They consisted by thin structures containing primordial germ cells (PGC) within somatic cells (Fig. 1A). After 3 months of exposure, all specimens showed well differentiated gonads. Testes were all well organized in seminiferous lobules and the predominant cell type consisted of spermatogonia (SPGs) (Fig. 1B). Males from the DO exposed groups showed more numerous spermatocyte (SPCs) cysts along the lobule wall. Females had ovaries with short ovigerous folds along which oocytes (O) and previtellogenic oocytes (PVGs) were organized (Fig. 1C). At the end of the trial, after 7 months of exposure, the testis of CR fish contained still almost only SPGs and only rare cysts of SPCs while UP fish showed testis with rare groups of spermatozoa (SPZ), too (Fig. 1D). About 30% of the individuals analyzed presented in fact a small number of germ cells, single or in clusters, with signs of apoptosis and the presence of syncytial cells germinal epithelium and alteration of the lobule structure (Fig. 1E). Numerous DO fish (> 20%) showed intra-testicular oocytes (Fig. 1F) and therefore were indicated as intersex. No differences were observed in the ovaries, except for the occurrence of some atretic oocytes in group DO.

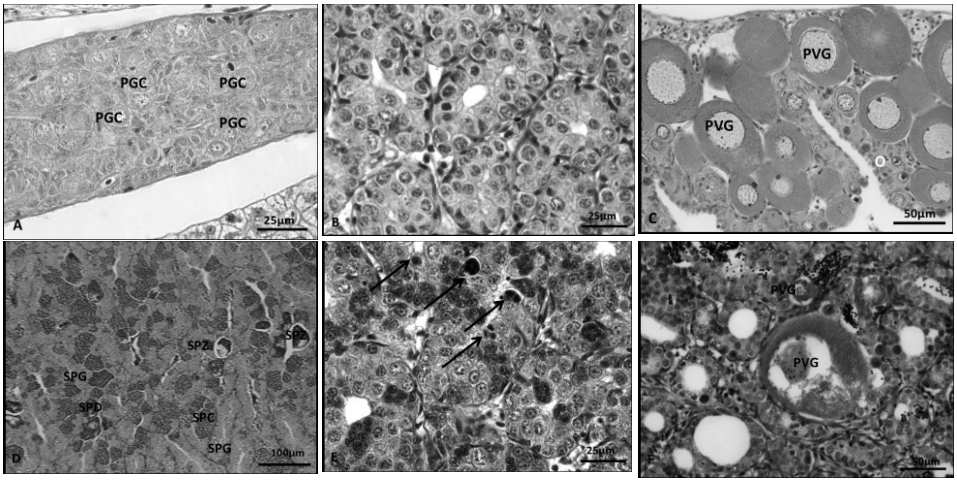

Fig. 1 A: Gonads of barbel juveniles at t1; B and C: differentiated testis and ovary, respectively, at t2; D: testis of an UP barbel at t3; E: altered germ cells (arrow) in UP barbell at t3; F: occurrence of intra-testicular PVG in the gonad of a DO barbell at t3. A) and C) MD; B)-D) E) and F) H&E.

DISCUSSION AND CONCLUSIONS

Alterations such as those found in the testis of the UP exposed barbels can be indicative of environmental mixtures in which predominates a component of polluting effect de-masculinizing (Pieterse et al., 2010). Although this alteration in testicular structure in about 13% of DO fish, in this group the parameter that appeared to be most important was the presence, in about 20% of specimens, of intra-testicular oocytes indicating exposure to a mixture in which predominates an estrogenic component. This value is similar to that observed in juvenile carp exposed to Lambro sediment (personnel data) in which, alongside a reduction in the number of spermatic cysts have also been observed PVGs. In conclusion, these results confirm that chronic exposure to polluted river sediments can induce germ cell development alteration in the gonads of juveniles of a native species such as barbel.

ACKNOWLEDGEMENTS

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P3

EFFECTS OF BISPHENOL A OR TETRABROMOBISPHENOL A EXPOSURE ON SPERMATOGENESIS AND STEROIDOGENESIS IN A ZEBRAFISH (*DANIO RERIO*) *EX VIVO* TESTICULAR MODEL

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INTRODUCTION

Central to male reproduction is spermatogenesis, the process during which undifferentiated spermatogonial stem cells give rise to sperm via several proliferation and differentiation steps. This process is regulated by the gonadotropic hormones released from the pituitary, androgen production in Leydig cells and paracrine or juxtacrine communication between testicular somatic cells, in particular Sertoli cells and germ cell. Bisphenol A (BPA) and its derivate tetrabromobisphenol A (TBBPA) are known as endocrine disruptors (EDs), being capable of interfering with hormonal homeostasis. Both BPA and TBBPA can be found in common household and industrial products, and large quantities are being released into the environment following their synthesis and manufacturing. As these EDs can mimic estrogenic activity, which might affect the reproductive health of fish, as well as that of other vertebrates, we wanted to investigate their possible effects on steroidogenesis and spermatogenesis in zebrafish (Danio rerio). Unlike in mammals, the complete process of spermatogenesis can be studied ex vivo in a testicular organ culture in fish (Anguilla japonica[1]; Danio rerio[2]). This provides a simple tool to study the possible effects of EDs on the spermatogenic process, without systemic interference and gonadotropin regulation.

MATERIALS AND METHODS

To investigate possible effects on spermatogenesis, testes from 28 sexually mature zebrafish were cultured using the whole organ culture technique and exposed to either 10^{-5} M BPA or 10^{-6} M TBBPA in the presence of 10^{-8} M 11-KT. Cultured testes were harvested at day 7, fixed, dehydrated, mounted in Technovit resin, sectioned and stained, before one section from each testis was analyzed, both in terms of spermatogenic active tissue area and relative presence of different germ cell stages. For measurements of steroidogenic activity, i.e 11-ketotestosterone (11-KT) and testosterone (T) production, testes from another 84 fish were submerged in L-15 media containing 0.5 µM forskolin, w/wo 10^{-5} M BPA or 10^{-6} M TBBPA and incubated overnight, before the testis were weighed and the media collected and analyzed via LC-MS/MS. In both experiments, one testis from each fish was exposed, while the other served as control. Statistics were performed using the paired t-test.

RESULTS

One week of TBBPA exposure did not affect the total number of germ cell cysts per mm^2 tissue (Figure 1A), but affected the germ cell composition with both a significant increase in type A differentiated spermatogonia and a decrease in leptotene/zygotene primary spermatocytes cysts as compared to control (Figure 1B). No effects from BPA exposure was detected.

Acute (24h) TBBPA exposure negatively affected the 11-KT production, but did not

interfere with T production. No effects from BPA exposure was detected (Figure 2).

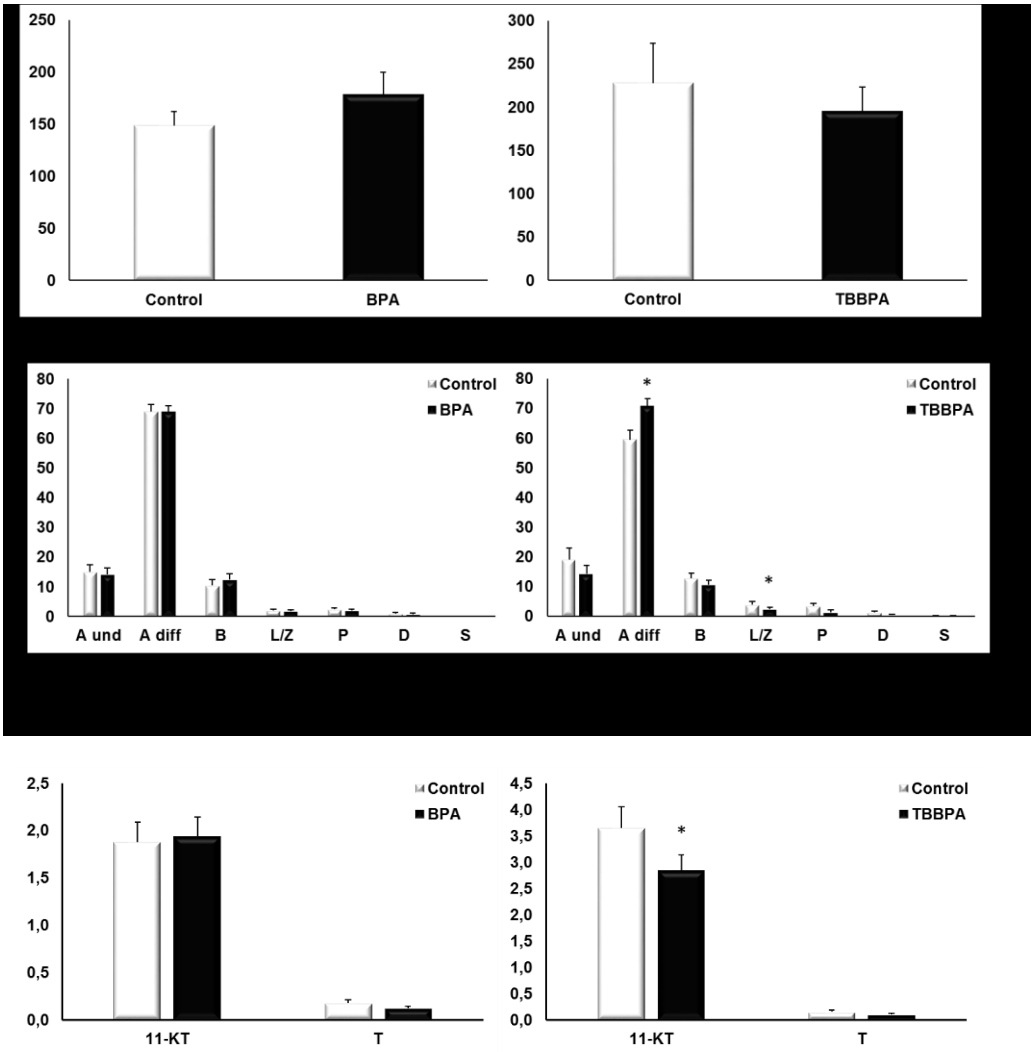

Figure 2: Androgen production (ng/mg tissue) after 24h of BPA or TBBPA exposure, presented as mean±SEM (n=42 (11-KT) and 30 (T))

DISCUSSION AND CONCLUSIONS

The continued release of EDs into nature is of great concern, as possible effects from many of these compounds are not yet mapped. Our data suggests that acute exposure to TBBPA affect testicular steroidogenesis by inhibiting 11-KT production. As 11-KT stimulates spermatogenesis, a reduced level likely downregulate the spermatogenic process. This was indeed observed after TBBPA exposure, with a greater number of germ cell cysts pausing as type A differentiated spermatogonia and not continuing the differentiation into type B spermatogonia and primary spermatocytes. No effects from 10-5 M BPA exposure was detected in this experiment, however, some studies indicate that BPA can be more potent at low doses. We are currently investigating this possibility by repeating the experiment with low doses of both BPA and TBBPA (10-10 and 10-9 M, respectively).

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P4

DINP AS ENDOCRINE DISRUPTOR: THE CASE OF ENDOCANNABINOID SIGNALING IN REPRODUCTIVE SYSTEM OF *DANIO RERIO*

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INTRODUCTION

Phthalates, commonly used as plasticizers, whose production accounts for about 6.0 million tons, have become an ubiquitous anthropogenic contaminant and have been reported to potentially induce toxicity effects on a wild range of organisms (Oehlmann et al., 2009, Carnevali et al, 2010). Among them is the Di-isononyl phthalate (DiNP), which can interfere as endocrine disruptor through an enhanced-estrogenic activity in *Danio rerio* (Chen et al., 2014). Related to the endocrine disruptor activity, the Endocannabinoid system has been suggested to participate and modulate reproductive functions in teleosts (Cottone et al., 2005, Cottone et al., 2013) and mammals, being critical for gonadotropin release and sex steroid biosynthesis (Meccariello et al, 2014).
For these reasons, the aim of our study was to assess for the first time the effects of such endocrine disruptor, DiNP, on the Endocannabinoid system of zebrafish reproductive system and the possible interaction with the quality of gametes and reproductive events.

MATERIALS AND METHODS

Adult zebrafish were exposed to a range of different concentrations of DiNP (0.42; 4.2; 42; 420 and 4200 µg/L) during three weeks.
For estimation of fecundity, the total amount of fertilized eggs belonging to both control and exposed fish were counted under the stereomicroscope Nikon Wild M3B.
Regarding gene expression, total RNA was extracted from 5 ovaries and 5 testis of each treatment using RNAzol RT (Sigma-Aldrich). cDNA was synthetized and real time PCR assay was performed using SYBR green. Quantification of gene expression of CB1, CB2, FAAH, NAPE-PLD, Magl, Daglα and Trpv1 was normalized using ARP and EF1α as house-keeping genes. Statistical analysis were performed using GraphPad Prism 6 software.
Morphological approach by histology was also performed using the standard Eosin-Hematoxylin staining.

RESULTS

The exposed zebrafish display a significant decrease in the number of fertilized eggs, showing the lowest fecundity at highest and lowest pollutant concentration. The potential activity of DiNP was also analyzed in the Endocannabinoid signaling in both ovary and testis.
Regarding the ovary, all DiNP doses led to a significant decrease of the expression of the endocannabinoid receptors CB1 and CB2, as well as Trvp1, which is significant lowered at the doses of 0.42 µg/L and 420 µg/L. The expression of Daglα and NAPE-PLD, biosynthetic enzymes for 2-AG and AEA, respectively, was also significantly lowered by all doses of DiNP.
The pollutant also led to a significant decrease of Magl at 0.42 µg/L, 42 µg/L and 420 µg/L, while FAAH is significantly affected only by 4.2 and 4200µg/L dose.
All genes considered in the ovary, after exposure do DiNP, were significantly decreased in the testis as well.

DISCUSSION AND CONCLUSIONS

In the past years, endocannabinoids system have emerged as essential players in some aspects of human reproduction (Macarrone, 2009). An endocannabinoid balanced signaling has an essential role in sperm quality and successful

pregnancy, where aberrant tone of EC may produce abnormal embryo development and male infertility (Macarrone et al, 2015).
On the other hand, some studies have demonstrated phthalates to act as endocrine disruptors targeting the reproductive system as well as gametes quality in mammals and no mammalian vertebrates (Carnevali et al, 2010; Corradetti et al, 2013; Hannon et al, 2015; Golshan et al, 2015). Few information on the relation between endocannabinoid system and endocrine disruptors are available for teleosts, though, and for the first time the present study showed the alteration of the endocannabinoid signaling in *Danio rerio* reproductive system after exposure to DiNP. Furthermore, as some Authors pointed out (e.g. Meccariello et al, 2014), the interaction between endocannabinoid signaling and reproduction is essential, so, we suggest the possible relationship between endocannabinoid system and a successful reproductive event in the freshwater teleost *Danio rerio*.
This suggestion is supported by the reduction of the fertilized eggs associated to endocannabinoid system alteration in both ovaries and testis of fish exposed to DiNP. Further studies are necessary to elucidate the relationship between endocrine disruptors, endocannabinoid signaling and reproduction.

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P5

EFFECT OF STRAW VOLUMES AND THAWING RATES ON MOTILITY, VIABILTY AND FERTILIZATION CAPACITY OF CRYOPRESERVED COMMON CARP (*CYPRİNUS CARPİO*) SPERM

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INTRODUCTION

Despite large number of studies on gamete preservation and important advances obtained in small-scale laboratory experiments, the transfer of this method to the commercial-scale applications still have many practical problems. One of the major problem is the handling of large quantities of sperm. Packaging of sperm in traditional 0.25 ml straws has been successfully applied to cryopreserve semen in several fish species and to fertilize small egg batches (Cruz-Casallas et al., 2004). Packaging of sperm in small straws is useful for the laboratory purposes such as gene banking that small quantities of sperm are required (Bozkurt et al., 2012). On the other hand, it makes frozen semen impractical for the commercial-scale applications where large amount of eggs and sperm are necessary (Horvath et al., 2007). From this point of view, the objective of the present study was to evaluate the influence of different cryo-straw volumes (0.25, 0.5 and 1.5 ml) at different thawing rates (30°C / 10 s, 20 s and 30 s) on post-thaw motility, motility duration, viability and fertilization ability of cryopreserved common carp (*Cyprinus carpio*) sperm.

MATERIALS AND METHODS

Before stripping, broodfish were anesthetized in a 15 ppm solution of Quinaldine (Reanal Ltd., Budapest, Hungary). Spermiation and ovulation were induced with an intraperitoneally injection of carp pituitary extract (CPE). Semen volume was measured and expressed as ml. The spermatozoa concentration was calculated by Thoma haemocytometer using x400 magnification. Percent motile spermatozoa (%) were determined after triggering of motility under dark field microscopy (Olympus BX 50, Japan) (x20 objective magnification). For cryopreservation experiments, samples containing below 80% motile spermatozoa were discarded. Duration of sperm motility was determined using a sensitive chronometer (sensitivity:1/100 s) by recording the time following addition of the activation solution to the sperm samples. A pool of suitable sperm sample was diluted (1:5) in an extender (75 mM NaCl, 70 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM Tris and pH was 8.0) (Lahnsteiner et al. 1998) containing 10% methanol and packed into different straw volumes such as 0.25 ml, 0.5ml and 1.5 ml and were sealed with polyvinyl alcohol (PVA). Following, sperm straws were frozen for 10 min in liquid nitrogen vapour, 4 cm above of the surface of liquid nitrogen, using an insulated box with an adjustable tray. After freezing, straws were plunged into liquid nitrogen container for storage. The frozen straws were thawed by plunging them into water bath at 30ºC for 10 s, 20 s and 30 s. Thawed sperm was activated using 0.3% NaCl and motility, movement durations were tested and recorded again. Fertilization was carried out after thawing of frozen semen at spermatozoa:egg ratio of 1×10^5:1. Differences between parameters were analyzed by repeated analysis of variance (ANOVA) followed by Duncan post-hoc test ($P<0.05$). All the data expressed in percentage were subjected to the arcsine transformation before their statistical analysis.

RESULTS

Sperm frozen in 1.5 ml straws showed the highest post-thaw motility regardless of the thawing rates. The highest post-thaw motility duration was determined as 46.2±4.2 s with cryopreserved sperm thawed at 30 second at 30ºC in 0.5 ml straws. On the other hand, thawed sperm in 1.5 ml straws for 10 second at 30ºC

showed the lowest duration of motility as 10.4±2.5 s. The overall mean fertilization rate was determined as 93.9±1.42% while the best fertilization rate was determined as mean 98.6±2.3% with thawed sperm at 30ºC for 30 second in 0.5 ml straws. Motility and fertilization rates of cryopreserved common carp sperm was statistically different between the experimental groups ($p<0.05$).

DISCUSSION AND CONCLUSIONS

The use of 0.25 and 0.5 ml straws for semen cryopreservation has been extensively used for experimental purposes. However, large straws facilitate the artificial fertilization when it is necessary to fertilize large quantities of eggs (Richardson et al., 1999). On the other hand, there is a lack of available data in regards to thawing conditions. Generally, thawing rates should be high to avoid recrystallization (Yavas and Bozkurt, 2011). The rate of temperature change should allow movement of water and cryoprotectant while preventing intracellular ice recrystallization and irreversible membrane damage. Although, Lahnsteiner et al. (1999) reported that optimal thawing temperature was 25ºC for the cyprinid spermatozoa, successful post-thaw motility and fertilization results were determined in the present study when frozen semen was thawed at 30ºC. Based on the results obtained, it is possible to suggest for the large scale insemination that sperm cryopreserved in 10% methanol, packed in large-volume straw (1.5 ml) and thawing at 30ºC for 30 s are suitable to cryopreserve common carp sperm as the fertilization capacity of sperm frozen is similar to that of spem frozen in 0.25 and 0.5 ml straws.

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P6

EFFECT OF DOCOSAHEXAENOIC ACID (DHA) ON MOTILITY, VIABILITY AND FERTILIZATION ABILITY OF CRYOPRESERVED BROWN TROUT (*SALMO TRUTTA MACROSTİGMA*) SEMEN

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INTRODUCTION

Docosahexaenoic Acid (DHA) has been used in the cryopreservation of sperm from different animal species and successfully improves resistance to damage arising from the formation of ice crystals and also spermatological properties. It was claimed that DHA is an essential component of healthy sperm cells, enhancing membrane integrity and tail flexibility, as well as increasing output (El-Razek et al., 2009). There is also evidence that the lipid and fatty acid compositions of chicken sperm play important role to maintain semen quality (Cerolini et al., 1997). Also, Kaeoket et al (2010) reported that supplement of DHA enriched yolk in egg yolk-based extender could improve the sperm quality in boar following cryopreservation. On the other hand, there is no study usage of DHA on fish sperm cryopreservation. From this point of view, the aim of the present study was to investigate the effect of docosahexaenoic acid (DHA) on the cryopreservation of brown trout (*Salmo trutta macrostigma*) spermatozoa.

MATERIALS AND METHODS

Semen was collected from 20 male broodfish by abdominal massage. In the present study, the semen was divided into six experiment groups according to the composition of freezing extender. The freezing extender contained 350 mM glucose, 30 mM Tris, 10% DMSO and 20 ml egg yolk without supplementation of fish oil. To prepare experimental groups, the extender supplemented with 0.5, 1, 2.5, 5 and 10 g fish oil (Fish oil 1000, Solgar Inc., Leonia, NJ, USA, containing DHA 100 mg⁄g fish oil) per ml of egg yolk respectively. Thus, we had six groups of semen: control group (without added fish oil) and the groups I, II, III, IV and V supplemented with fish oil. Semen samples diluted at the ratio of 1:3 with these diluents were subjected to cryopreservation process. The final sperm concentration was $1.0x10^6$ spermatozoa⁄ml. The semen was cooled at 4°C for 15 min and loaded into 0.25 mL straws. After, the straws were placed on a rack 3 cm above of the liquid nitrogen in a styrofoam box. Sperm freezing was performed in liquid nitrogen vapour during 15 min and after that time the straws were immersed into liquid nitrogen (−196°C). The frozen semen was stored in a liquid nitrogen container until analyses. After thawing in a water bath at 30°C for 20 s, post-thaw spermatozoa characteristics such as progressive motility percentage (%), progressive motility duration (s), viability (%) and also fertilization rate (%) were evaluated and compared with control and fresh semen.

RESULTS

The extenders containing DHA (fish oil) exhibited higher percentage motility and motility duration than control group ($p<0.05$). There were significant increases in progressive motility percentage, progressive motility duration and viability at the group IV (5 g fish oil) and the group V (10 g fish oil) ($p<0.05$). The post-thawed progressive spermatozoa motility (62.4±0.6 s) at the treatment group V was significantly greater than that of the other experiment groups ($p<0.05$). The fertilization rates were also determined higher at the experimental groups IV and V.

DISCUSSION AND CONCLUSIONS

To our knowledge, this is the first study showing that adding of DHA (fish oil) directly into freezing extender could improve frozen–thawed brown trout (*Salmo trutta macrostigma*) semen quality. The results of the present study showed that the DHA (fish oil) maintained the integrity of the spermatozoa during cryopreservation process. We concluded that supplementation of extender with 5 or 10 g fish oil can be beneficial for the cryopreservation of brown trout spermatozoa.

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P7

COMPOSITION AND OSMOLALITY OF THE ACTIVATING AGENT ON FRESH SPERM QUALITY OF *PROCHILODUS LINEATUS*

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INTRODUCTION

The streaked prochilod (*Prochilodus lineatus*) has great potential for aquaculture and has been used in restocking programs through artificial propagation. Like the majority of teleosts, sperm of *P. lineatus* is immotile in the male genital tract and motility is triggered when sperm is in contact with the aqueous environment during natural reproduction or with the activating agent during artificial propagation. In our previous study (Gonçalves et al., 2013), the initiation of *P. lineatus* fresh sperm motility was evaluated in different solutions (NaCl, Glucose and BTS™) prepared at osmolalities ranging from 45 to 450 mOsm/Kg. It was observed that maximum motility was achieved when solution osmolality ranged from 135 to 225 mOsm/kg, regardless of solution composition. Motility features (rate and velocity score), however, was evaluated only subjectively. Thus, the aim of this study was to reassure the effects of composition and osmolality of the activating agent on fresh sperm motility and curvilinear velocity of *P. lineatus,* using a Computer-Assisted Sperm Analyzer (CASA).

MATERIALS AND METHODS

Prochilodus lineatus males (n=10) were selected during spawning season at the Fish Culture Station of the Minas Gerais Power Company (CEMIG) in the city of Itutinga, MG, Brazil. Males with detectable running sperm under soft abdominal pressure received two doses of carp pituitary extract (Argent Chemical Laboratories) at 0.4 and 4 mg/kg BW in a 12-h interval. After 7.5-8 h at 27-28ºC, the urogenital papilla was dried and about 2 mL of sperm from each male was hand-stripped directly into test tubes. Contamination of sperm with water, urine or feces was carefully avoided. The samples were transported in a closed 1.5-mL tubes in a cooler (9-11ºC) by car for ~50 km to the Laboratory of Semen Technology at University Federal of Lavras where motility rate and curvilinear velocity (VCL) were estimated using a Computer-Assisted Sperm Analyzer (SCA™ software, Microptics, Barcelona). The activating agents tested were NaCl and glucose solutions prepared at the following osmolalities: ~0 (distilled water), 50, 100, 150, 200 and 250 mOsm/kg. In order to track the decrease of sperm motility post-activation, the video was recorded at 10, 30 and 50 s post-activation (spa).

RESULTS

There was no effect of activating agent composition on sperm motility, when osmolality ranged from 0 to 150 mOsm/kg. However, at higher osmolalities of 200 and 250 mOsm/kg, samples activated in glucose yielded higher values (91-92%) than those activated in NaCl solution (74-80%). Curvilinear velocity was always higher in samples activated in glucose (322-356 µm/s) than in NaCl solution (192-283 µm/s) or in distilled water (298 µm/s), regardless of osmolality (Table 1). When motility was evaluated during 50 spa, the rate decreased more strongly when samples were activated in distilled water, from 91% at 10 spa to 24% at 50 spa. When NaCl solution was tested, motility decreased faster at 50, 200 and 250 mOsm/kg (36-47%), compared to 100 and 150 mOsm/kg (59-62%), at 50 spa. When glucose solution was tested, motility remained at high values (80-83%) at osmolalities from 100 to 250 mOsm/kg, even after 50 s (Figure 1).

DISCUSSION AND CONCLUSIONS

In the present study, glucose solution was a better activating agent than NaCl, in terms of motility rate at higher osmolalities and of VCL in all osmolalities tested.

Differently, in our previous study (Gonçalves et al., 2013) motility rate and velocity scores were not affected by activating agent composition using sperm of the same fish species. The method of sperm motility evaluation was not the same, and the use of CASA system is more accurate to detect small differences among samples than the subjective analysis. Furthermore, the evaluation of VCL and other velocities (data not shown) clearly demonstrated that glucose solution induces faster motility than NaCl solution. Osmolality plays an important role on the process of suppressing and activating fish sperm motility. In *P. lineatus*, the effect of osmolality was less intense in glucose solution than it was in NaCl solution. In glucose at 100-250 mOsm/kg, motility rate was still very high (above 80%) after 50 spa. Thus, a large range of osmolalities was adequate to trigger sperm motility. On the other hand, samples activated in NaCl were more sensitive to both low and high osmolalities. Glucose solution at 100-250 mOsm/kg is recommended as activating agent of sperm motility of *P. lineatus*.

Table 1 Motility and curvilinear velocity (VCL) of *Prochilodus lineatus* fresh sperm after activation in glucose or NaCl solutions at different osmolalities.

(mOsm/kg)	Motility rate (%)		VCL (µm/s)	
	NaCl	Glucose	NaCl	Glucose
0		91 ± 7 ^{a}		298 ± 25 ^{a}
50	91 ± 7 ^{aA}	91 ± 8 ^{aA}	247 ± 38 ^{bB}	322 ± 47 ^{aA}
100	88 ± 7 ^{aA}	92 ± 6 ^{aA}	283 ± 29 ^{aB}	357 ± 34 ^{bA}
150	89 ± 6 ^{aA}	90 ± 6 ^{aA}	256 ± 24 ^{bB}	356 ± 43 ^{bA}
200	80 ± 6 ^{bB}	91 ± 7 ^{aA}	231 ± 24 ^{cB}	348 ± 6 ^{bA}
250	74 ± 14 ^{bB}	92 ± 4 ^{aA}	192 ± 24 ^{dB}	335 ± 36 ^{bA}

Different lowercase in the same column or uppercase in the same row are different (P<0.05, Scott-Knott). Mean ± SD.

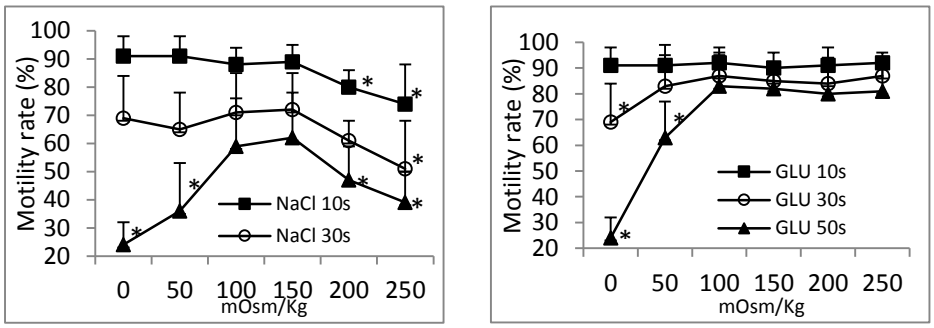

Fig 1. Motility rate of *P. lineatus* sperm activated in NaCl (left) or glucose solution (right) at different osmolalities and evaluated at 10, 30 and 50 s post-activation. Means within the same evaluation time followed by the symbol (*) are significantly lower (P<0.05, Scott-Knott). Y bar = SD.

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P8

PRELIMINARY RESULTS OF CRYOPRESERVATION EFFECT ON THE SEMINAL PROTEIN PROFILE OF *COLOSSOMA MACROPOMUM*

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INTRODUCTION

The use of proteomics in recent years has contributed to the improvement in the interpretation of the problems in fish breeding. The study of seminal protein profile before and after cryopreservation, can help in understanding the causes behind cell damage , assisting in the development of mechanisms to mitigate the negative impacts. The objective of this study was to analyze the effects of cryopreservation of semen in protein profile of *Colossoma macropomum,* a Brazilian native species.

MATERIAL AND METHODS

Semen was collected from *C. macropomum* during the breeding season and samples of each animal were divided in two. One portion of the sample was centrifuged at 7.500 G, 2min at 4ºC and seminal plasma was separated from the cells, and subsequently frozen in liquid nitrogen. The second part of the sample was diluted 1:4 in the extender solution BTS® containing10% dimethylformamide (DMF) and placed into 250 µL straws. The straws were placed in a dry shypper for 12 hours, and then stored in liquid nitrogen until the time of analysis. Thawing was performed at 45°C for 8 seconds. The thawed semen was centrifuged, as mentioned above, to obtain the plasma after cryopreservation. Samples of fresh and cryopreserved seminal plasma were analyzed spectrophotometrically for protein quantification using the kit BCA Protein Assay (Thermo Scientific). SDS-PAGE was performed to measure proteins molecular weight and an aliquot of 10 µL of plasma was diluted in 15 µL of loading buffer. After this step, proteins were denaturationed at a temperature of 100 °C for 10 min. Electrophoresis was performed using 5% polyacrylamide gel for stacking and 12.5% for race. Each race course contained 25 µL of final sample (plasma + buffer). Samples were concentrated to 70V for about 30 min and the separation was performed at 120V for approximately 150 minutes. The gels were stained with Comacie Blue R250.

RESULTS

The preliminary results showed an alteration between protein profile of seminal plasma, before and after cryopreservation (Fig. 1). It is possible to note that the technique of cryopreservation cause harmful effects to seminal plasma, presenting a visual decrease in the molecular weight of proteins. The protein profile of fresh plasma (A1, A2, A3, A4 and A5) showed higher protein expression of about 40 and 50 kDa. After cryopreservation (C1, C2, C3, C4 and C5) there was an increase in the expression of protein between 15 and 20 kDa.

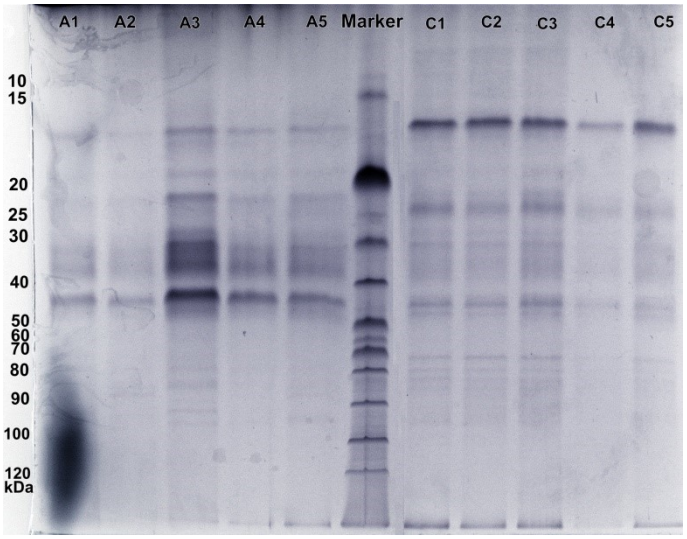

Fig. 1 – Molecular weight of proteins expressed in seminal plasma of *C. macropomum* in polyacrylamide gel (12.5%).

DISCUSSION AND CONCLUSIONS

This is the first study on the evaluation of cryopreservation effects on seminal plasma *C. macropomum* proteins profile. Changes in the protein profiles of seminal plasma were also observed in the species *Dicentrarchus labrax* (Zilli et al., 2005), where there was a decrease in the expression of some proteins, and even the absence of expression of other after cryopreservation. The migration of sperm cell proteins for the seminal plasma was reported by Loir et al. (1990) in *Oncorhynchus mykiss*, and Li et al. (2010), studying *Cyprinus carpio*, reported the degradation of some seminal proteins after cryopreservation. The increased incidence of some proteins can also be associated with the sperm membrane disruption caused by cryo-injury. Knowledge of the protein profile of seminal plasma may help optimize the cryopreservation technique (Ciereszko et al., 2012).

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P9

EFFECT OF CRYOPROTECTANTS COMBINED IN SPERM CELLS OF *PROCHILODUS LINEATUS*

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INTRODUCTION

Curimba (*Prochilodus lineatus*) is a teleost fish native of South America, widely used in assisted reproduction. In addition to offering great advantages to fish farming due, this species readily responds to hormonal induction. Therefore, the interest in preserving curimba has considerably increased, making the development of techniques for this purpose becomes essential.

Cryopreservation is a technique used to preserve the genetic material, storing them in liquid nitrogen (-196 ºC). During cooling, the sperm cells are exposed to external conditions that damage them, interfering directly in their survival. To prevent this damage, the use of cryoprotectants becomes important in the process of cryopreservation.

Therefore, the main objective of this work was to study the effect of the interaction of different internal and external cryoprotectants in cryopreservation of curimba sperm cells.

MATERIALS AND METHODS

19 males of curimba were weighed and received intramuscular injections of 0.5 and 5.0 mg kg^{-1} of crude carp pituitary extract (CCPE), per body weight. After 7 hours, the semen was collected, and the rate of sperm motility was measured in semen *in natura* based on the average percentage of mobile spermatozoa, and duration (in seconds) was measured from mixing with distilled water until only 10% of the cells they met in motion.

Semen samples were diluted 1:5 (100µL of semen : 500µL of extender solution), and four combinations of intra and extracellular cryoprotectants were tested: DMSO+egg yolk, DMSO+lactose, methanol+egg yolk and methanol+lactose.

The samples were were packaged in 0.50 ml straws and were placed in a tank of vapor of liquid nitrogen for 24 hours. After this period, samples were immersed directly into cylinder of liquid nitrogen at -196 °C for 10 days.

After 10 days, the individual straws were thawed by immersion in water bath to 60°C for 8 seconds. The duration and motility rate of sperm cells were estimated and the morphological analysis was performed to evaluate abnormalities in the head, tail and intermediate part.

RESULTS

The highest rates of sperm motility ($p<0,05$) were found in treatments using lactose as extracellular cryoprotectant, combined with DMSO (55,29%) or methanol (57,35%). The solutions containing egg yolk promoted lower rates of motility ($p<0.05$) independently of the intracellular cryoprotectant used.

In this study, the rate of motility after thawing decreased 48% when compared to fresh semen and 38% when compared to diluted semen.

In relation to the duration of sperm motility, the highest values ($p<0,05$) were found in treatments using lactose combined with DMSO (73,94 seconds) or methanol (74,18 seconds). Our data demonstrate a significant reduction in the rate and duration of sperm motility ($p<0.05$) after thawing when using egg yolk as extracellular cryoprotectant.

There was no significant difference between treatments for the analysis of sperm morphology in semen thawed ($p>0.05$).

DISCUSSION AND CONCLUSIONS

In tests using 7.5% methanol and DMSO, Miliorini et al. (2011) obtained, respectively, 87 and 90% of motility rate in semen diluted of curimba, being similar to the values found in this study, 88.24 and 90.29%, using 10% methanol and DMSO. The use of cryoprotectants reduces the deleterious effect of the technique on sperm cells, and the positive effect of methanol and DMSO in the variables of motility may be related to its osmolality (Harvey & Kelley, 1984) and the rapid intracellular penetration (Jamieson, 1991).

According Murgas *et al*. (2014), the high concentration of cryoprotectants can cause significant reduction in sperm quality of fish, but our data show that the use of intra and extracellular cryoprotectants showed no relevant toxicity in any of the combinations tested.

Ribeiro and Godinho (2003) did not consider satisfactory the use of egg yolk in semen cryopreservation of *Leporinus macrocephalus*, and attribute this to the lack of synergy between the cryoprotectants. In cryopreservation trials, Miliorini *et al*. (2011) also observed that the use of egg yolk, showed some toxicity to the curimba semen and our data also demonstrate a reduction of the rate and duration of sperm motility after thawing, when using this substance as an external cryoprotectant.

Freezing and thawing processes can cause changes in spermatozoa protein profiles that may lead to a decreased in semen quality and affect DNA integrity (Li *et al.,* 2010), compromising the ability of fertilization. This negative effect on sperm could be observed in the present research, which shows a reduction of almost 50% in the rate of motility compared to fresh semen.

Lactose combined with DMSO or methanol may have had a better protection of the mitochondrial membrane system, reducing damage during the process of freezing and thawing. This protection will be able to promote a longer beat the sperm flagellum (Miliorini, 2006), increasing the chances of fertilization.

The concentrations of intracellular and extracellular cryoprotectants used in this study promoted a protective effect on the biochemical characteristics of sperm, whose protection reflected in the physical aspects of the cells after thawing, allowing the occurrence of only 25% of the total changes.

All solutions tested in this study were effective for cryopreservation of curimba semen and not detected differences in the morphological sperm changes. However, lactose stood out as extracellular cryoprotectant, with higher rates and durations for motility of thawed semen, independent of its combination with methanol or DMSO.

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P10

OPTIMAL GLUCOSE CONCENTRATION IN EXTENDER IS CRUCIAL FOR SUCCESSFUL CRYOPRESERVATION OF STERLET (*ACIPENSER RUTHENUS* L.) SPERM

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INTRODUCTION

Several sturgeon species, including sterlet are close to extinction due to stock exploitation for meat and caviar as well as habitat destruction (Billard and Lecointre, 2001). Sperm cryopreservation has particular meaning for gene banking of valuable strains and endangered populations to maintain genetic diversity, for synchronization of artificial reproduction and efficient utilization of semen during artificial reproduction (Lahnsteiner et al. 2004).

MATERIALS AND METHODS

Semen of sterlet was cryopreserved in 0.25-ml straws in glucose-methanol extender at a ratio of 1:1 (semen : extender). In the first experiment the effect of glucose at concentrations 0, 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30 M in 15% methanol on sperm motility in fresh-equilibrated and cryopreserved semen (n=7) was tested. The extender consisting of 0.05 M glucose and 15% methanol was chosen for the further experiments. Results are expressed as mean ± SD.

RESULTS

The increasing glucose concentration in extender led to decrease of sperm motility in equilibrated sperm (Fig. 1). Cryopreservation of sterlet semen in glucose-methanol extender caused significant decrease in sperm motility in all glucose concentrations except 0.10 and 0.30 M. The extender consisting of 0.05 and 0.10 M glucose and 15% methanol provided high and consistent results of post-thaw sperm motility (68.3 and 65.1%) and the first extender was arbitrarily selected to the subsequent experiments.

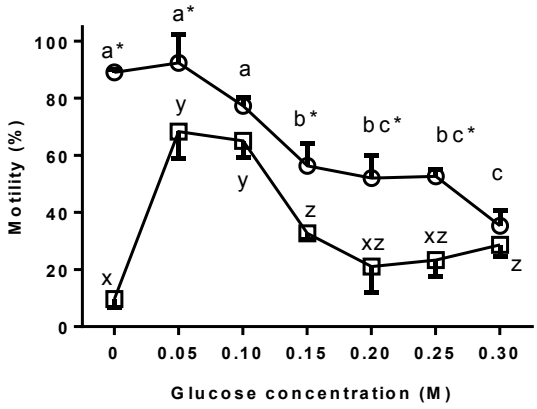

Fig. 1. The effect of glucose concentrations in extender on fresh-equilibrated (O) and frozen/thawed (□) sperm motility parameters of sterlet (n=7, mean±SD). Different superscripts indicate statistical differences (P<0.05) among fresh-equilibrated (a, b, c) and frozen/thawed (x, y, z) semen. Asterisks indicate significant differences between the parameters for fresh-equilibrated and cryopreserved semen (P<0.05).

Cryopreservation of sterlet sperm in 0.05 M glucose in 15% methanol let to obtain very high percentage of sperm motility after freezing/thawing (range 56-83%) (Tab. 1).

Table 1. Sperm motility characteristics of fresh and frozen/thawed sterlet milt in glucose-methanol extender (n=9; mean ± SD). Asterisks indicate significant differences between the parameters for fresh and frozen/thawed semen (P<0.05).

	Fresh	Frozen/thawed
MOT (%)	88.0 ± 12.8	68.8 ± 8.6 *
VCL (μm s^{-1})	159.2 ± 20.2	111.9 ± 18.9 *
VAP (μm s^{-1})	131.4 ± 11.0	95.2 ± 20.0 *
VSL (μm s^{-1})	97.5 ± 11.3	66.7 ± 21.4 *
BCF (Hz)	6.2 ±1.5	4.2 ± 1.7 *
ALH (μm)	13.8 ± 6.8	6.7 ± 3.2 *
LIN (%)	60.4 ± 6.9	47.4 ± 11.7 *

DISCUSSION AND CONCLUSIONS

Our data demonstrated that sterlet semen could be successfully cryopreserved using the simple glucose-methanol extender. Recently Judycka et al. (2015) in their study used similar extender for cryopreservation of Siberian sturgeon sperm. The concentration of glucose is important for cryopreservation efficiency of sturgeons. As such, this new protocol can be alternative method to previously described by Glogowski et al. (2002) for Siberian sturgeon sperm. Further studies should be focused on scaling up this efficient cryopreservation technique for facilitation of controlled spawning of sterlet.

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P11

EFFECTS OF COMPOSITION AND OSMOLALITY OF THE ACTIVATING AGENT ON POST-THAW SPERM QUALITY OF *PROCHILODUS LINEATUS*

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INTRODUCTION

The streaked prochilod (*Prochilodus lineatus*) is a freshwater fish species, native of South America, well adapted to captivity, easily reproduced artificially, and thus used as a model in a wide number of studies. Like the majority of teleosts, sperm of *P. lineatus* is immotile in the male genital tract and motility is triggered when sperm is released into the water. In our previous study (Gonçalves et al., 2013) it was observed that fresh sperm motility of *P. lineatus* reaches maximum values when activated in solutions (glucose, NaCl and BTS™) ranging from 135 to 225 mOsm/kg. It is possible that frozen-thawed sperm motility is highly activated within the same range of osmolality. In the present study we want to determine motility rate and curvilinear velocity of post-thaw sperm after activation in hypo-osmotic solutions, using a Computer-Assisted Sperm Analyzer (CASA).

MATERIALS AND METHODS

Ten *Prochilodus lineatus* males were selected during spawning season at the Fish Culture Station of the Minas Gerais Power Company (CEMIG) in the city of Itutinga, Brazil. Males received two doses of carp pituitary extract at 0.4 and 4 mg/kg BW in a 12-h interval. After 7.5-8 h at 27-28ºC, the urogenital papilla was dried and about 2 mL of sperm from each male was hand-stripped. Contamination of sperm with water, urine or feces was carefully avoided.

The samples were transported in a closed 1.5-mL tubes in a cooler (9-11ºC) by car for ~50 km to the Laboratory of Semen Technology at Federal University of Lavras where freezing took place. Sperm was diluted in a freezing medium containing glucose solution at 325 mOsm/kg and methyl glycol to a final ratio of 1 semen: 8 glucose: 1 methyl glycol (Viveiros et al., 2009). Sperm was drawn into unsealed 0.25-mL straws (n= 4 replicate x 10 males) frozen in nitrogen vapor vessel (dry vapor shipper) and stored in liquid nitrogen vessel. Straws were thawed in a water bath at 60°C for 3 s, and sperm motility rate and curvilinear velocity (VCL) were determined using a Computer-Assisted Sperm Analyzer (SCA™ software, Microptics, Barcelona). The activating agents tested were glucose and NaCl solutions prepared at the following osmolalities: ~0 (distilled water), 50, 100, 150, 200 and 250 mOsm/kg.

RESULTS

Post-thaw sperm motility and VCL were not affected (P>0.05) by the activating agent composition (glucose or NaCl solution), within a given osmolality (Table 1). However, motility rate and VCL were higher when triggered at osmolalities ranging from 0 to 150 mOsm/kg, for both glucose (67-75% and 176-208 µm/s) and NaCl solutions (69-75% and 163-187 µm/s). Samples activated in osmolalities of 200 and 250 mOsm/kg yielded significantly lower motility rate and VCL, regardless activating agent composition.

Table 1 Post-thaw motility and curvilinear velocity (VCL) (mean ± SD) of *Prochilodus lineatus* sperm after activation in glucose or NaCl solutions at different osmolalities.

mOsm/kg	Motility rate (%)		VCL (µm/s)	
	Glucose	NaCl	Glucose	NaCl
0	75 ± 14 a		176 ± 31 a	
50	70 ± 20 [a]	74 ± 16 [a]	189 ± 37 [a]	163 ± 35 [a]
100	67 ± 18 [a]	72 ± 13 [a]	190 ± 38 [a]	168 ± 33 [a]
150	73 ± 19 [a]	69 ± 19 [a]	208 ± 33 [a]	187 ± 36 [a]
200	57 ± 18 [b]	49 ± 10 [b]	165 ± 32 [b]	146 ± 31 [b]
250	43 ± 22 [c]	38 ± 15 [c]	144 ± 53 [b]	128 ± 27 [b]

[a,b] Means followed by different superscript differ (P<0.05, Scott-Knot test)

DISCUSSION AND CONCLUSIONS

In the present study, motility and VCL were not affected by the activating agent composition, but were higher in osmolalities ranging from 0 to 150 mOsm/kg. Similarly, in our previous study (Gonçalves et al., 2013), osmolality but not composition of the activating agent affected sperm motility. However, the highest values (83-93% motility) were observed at 135-225 mOsm/kg. Possibly, the difference between these two studies concerning the interval of osmolality where the highest motility was observed may be due to the type of sperm (fresh or post-thaw) and the evaluation method (subjective or CASA). It has been reported that distilled water (~0 mOsm/kg) triggers motility of *Esox lucius* sperm, but both motility rate and velocity decrease very fast as a function of time (Alavi et al., 2009). Thus, to be on the safe side, we recommend the use of NaCl or glucose solution at 50-150 mOsm/kg as activating agent for *P. lineatus* sperm after freezing and thawing processes.

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P12

VIABILITY OF ZEBRAFISH (*DANIO RERIO*) OVARIAN FOLLICLES AFTER VITRIFICATION

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INTRODUCTION

Cryopreservation of ovarian tissue has been studied for female germline preservation of farm animals and endangered mammalian species. However, there are relatively few reports on cryopreservation of fish ovarian tissue and especially using vitrification protocols. Therefore, the aim of this study was to assess the effects of four different vitrification solutions on ovarian follicle viability at different stages.

MATERIALS AND METHODS

Ovaries were collected from zebrafish adult females, euthanized in a lethal dose of tricaine methane sulfonate (0.6 mg/mL). Ovaries were divided into four different vitrification solution (VS): VS1 (1.5 M of methanol and 4.5 M of propylene glycol), VS2 (1.5 M of methanol and 5.5 M of Me_2SO), VS3 (1.5 M of methanol, 4.5 M of propylene glycol and 0.5 M of sucrose) and VS4 (1.5 M of methanol, 5.5 M of Me_2SO and 0.5 M of sucrose). The ovaries were kept in vitrification solutions for 90 sec and then transferred to the metal container and immediately plunged in liquid nitrogen. After a few days, the metal container was removed from the liquid nitrogen for rewarming. The metal container was immersed into a water bath at 28°C for 30 sec and then opened. Ovaries were removed and transferred to the first warming solution containing 1 M sucrose for 1 min, then to the second solution containing 0.5 M sucrose for 3 min and finally to the third solution of 0.25 M sucrose for 5 min. Cryopreserved and control follicles were isolated by gentle pipetting in Leibovitz L-15 medium (pH 9.0). Five developmental stages were selected: Stage I (primary growth); Stage II (cortical alveolus); Stage III (vitellogenic); Stage IV (maturation) and Stage V (mature) according to Selman et al. (1993). Ovarian follicle viability was assessed by the method live/dead staining with fluorescein diacetate (FDA) and propidium iodide (PI) described by Jones and Senft (1995). Cell viability assessment was performed on an inverted fluorescence microscope. The bright green fluorescent follicles were considered viable and the bright red stained follicles were considered non-viable. Experimental and control groups were composed by at least 100 ovarian follicles in each group, and the experiments were repeated three times. Variables between groups were analyzed using the statistical package SAS 9.2 (2009), passing the normality test, followed by analysis of variance, with mean comparison by Duncan test ($P<0.05$).

RESULTS

Stage I follicles showed the highest cell viability in all the experimental groups after rewarming (Table 1). Stage II follicular viability was higher than observed in Stages III, IV and V in groups VS1, VS3 and VS4 ($P<0.05$).

Table 1. Follicular viability (%) of the five development stages in each group after vitrification.

Stage	Control	VS1	VS2	VS3	VS4
I	95.74±2.44	76.84^{a}±2.28	49.40^{a}±36.46	54.62^{a}±13.30	64.17^{a}±16.34
II	96.66±3.05	43.14^{b}±12.05	20.27^{ab}±16.34	40.50^{a}±4.76	45.66^{a}±19.33
III	91.82±11.70	8.68^{c}±4.25	6.67^{b}±4.89	9.36^{b}±8.16	13.24^{b}±10.13
IV	90.95±5.34	1.29^{c}±1.52	7.06^{b}±36.46	2.30^{b}±3.18	0.98^{b}±0.98
V	88.98±3.32	0.30^{c}±0.52	0.00^{b}	0.59^{b}±1.03	0.30^{b}±0.52

Control (fresh ovaries), VS1 (1.5 M of methanol and 4.5 M of propylene glycol), VS2 (1.5 M of methanol and 5.5 M of Me_2SO), VS3 (1.5 M of methanol, 4.5 M of propylene glycol and 0.5 M of sucrose) and VS4 (1.5 M of methanol, 5.5 M of Me_2SO and 0.5 M of sucrose). Different letters in the same column indicate significant difference. Duncan ($P<0.05$).

DISCUSSION AND CONCLUSIONS

In this study, Stage I follicles showed the highest viability percentage in VS1 after rewarming. Seki et al. (2011) had already suggested that the combination of methanol and propylene glycol might be more effective in reducing damage caused by cryopreservation on zebrafish immature follicles. In addition, in an extensive survey on the toxicity of several cryoprotectants, it was found that methanol and propylene glycol were the least toxic agents for zebrafish follicular survival after cryopreservation (Godoy et al., 2013). The high rates of primary growth (Stage I) follicular viability were obtained after vitrification of ovaries using the metal container. These results are in agreement with other studies that used metal devices to vitrify ovarian tissue of mouse, caprine, human and bovine and reported a good rate of primordial and primary follicle recovery after rewarming (Aquino et al., 2014; Carvalho et al., 2013; Khosravi et al., 2013). Fish ovarian follicles, as well as mammalian follicles have a smaller size in early stages, which results in a higher surface / volume ratio. Therefore, early follicles are likely to be more permeable to water and solutes, increasing survival chances after cryopreservation. Our results on zebrafish follicular viability after rewarming suggest that ovarian tissue vitrification is a promising technology to preserve the maternal genome in fish.

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P13

CRYOPRESERVATION OF ZEBRAFISH SPERM, FIRST TRIALS AND RESULTS

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INTRODUCTION

The zebrafish (*Danio rerio*) is an important model organism used in many research areas. This species has a high number of mutant strains (with reproductive limitations in some cases) and transgenic lines are constantly created. The preservation of this valuable genetic material is required, but their small body size and the small amounts of produced sperm (microliters) makes difficult the handling of the sperm and the cryopreservation protocols are scarcely repetitive. In this study we made some preliminary trials using several cryoprotectants.

MATERIALS AND METHODS

Fish. Young zebrafish adults (AB) were raised in a recirculating system (Tecniplast) under standard conditions at 28 ºC and 14h L/10h D photoperiod. Fish were fed twice a day ad libitum with dry pellets and Artemia nauplii.

Media. All solutions were made in a chilled Hank´s balanced salt solution (HBSS; Sigma Aldrich, Germany) at 300 mOsm/kg (0.137 M NaCl, 5.4 mM KCl, 1.3 mM CaCl, 1.0 mM $MgSO_4$, 0.25 mM Na_2HPO_4, 4.2 mM $NaHCO_3$ and 5.55 mM glucose, pH 7.2; Hagedorn et al., 2011).

Sperm sampling and dilution. Fish were anesthetized with MS-222 (200 ppm) and sperm samples (0.5-1.5 μl) were collected by abdominal pressure using 30 μl capillary straws. A volume of approximately 10 μl was reached adding extender (HBSS without cryoprotectant).

Sperm cryopreservation. From these 10 μl, volumes of 3 μl were placed in 3 cryovials. Then, 7 μl of the different freezing media containing 10% of cryoprotectant (final concentration) were added and gently mixed. The cryovials were placed into empty Falcon tubes that were crushed in dry ice for 20 min (all these steps in less than 30 s). Finally, cryovials were removed from the Falcon tubes and put into liquid nitrogen.

Sperm motility evaluation. The remaining volume of diluted sperm (approx. 1 μl) was used for CASA evaluation (fresh sample), mixing with 5 μl of system water, or, in the case of thawed samples, mixing 1 μl of thawed sperm with 3 μl of system water. Motility was assessed in a Makler chamber using a phase-contrast microscope, a camera (50 fps) and a CASA software (ISAS, Proiser R+D, S.L., Spain) 10 s after the sperm activation.

Thawing. Cryovials were taken out from liquid nitrogen and cap removed. Open vials were partially immersed into a 33 °C water bath for 20 s and immediately used to evaluate sperm motility.

Cell viability. Post-thawing spermatozoa viability was determined mixing 5 μl sperm, 10 μl HBSS, 0.1 μl SYBR Green and 0.2 μl Propidium iodide (1:20) and observed in a fluorescence microscope.

Trial 1. Test of three cryoprotectants. Considering the previous results on the effects of several cryoprotectants (reported by Hagedorn et al. (2011) and Kollár et al. 2014), methanol, dimethyl-formamide (DMF) and dimethyl-sulfoxide (DMSO) were chosen and tested at 10% of final concentration (freezing medium + sperm volume). A total of 11 sperm samples were considered.

Trial 2. Test of different concentrations of DMF. A similar method was used testing three concentrations of DMF: 5, 10 and 20% of final concentration. Samples were discarded when the fresh sperm motility was lower than 40% of motile cells. A total of 10 sperm samples were considered.

RESULTS

Trial 1. The cryopreservation process, independently of the cryoprotectant assayed, induced a reduction of sperm motility (Fig. 1A). Samples frozen with methanol showed a significantly lower motility than those cryopreserved with DMSO or DMF. Samples frozen with DMF showed the highest cell viability (there were no significant differences, but P-value was 0.051).

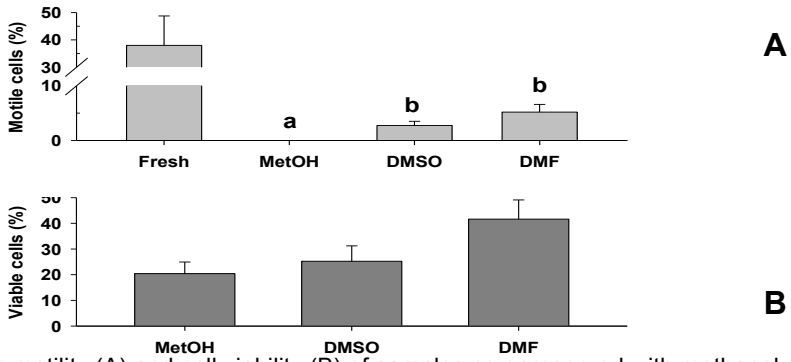

Figure 1. Sperm motility (A) and cell viability (B) of samples cryopreserved with methanol, DMSO or DMF.

Trial 2. The cryopreservation process, independently of the concentrations of DMF, induced a reduction of sperm motility (Fig. 2A). Higher doses of DMF induced higher (but not significant) motility but significant lower viability (Fig. 2B).

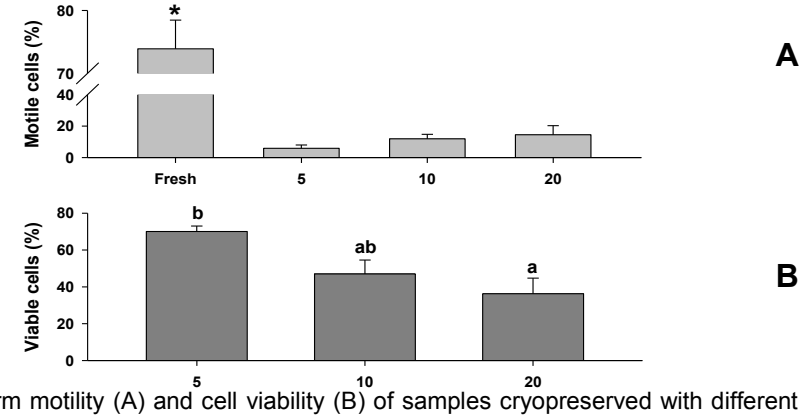

Figure 2. Sperm motility (A) and cell viability (B) of samples cryopreserved with different concentrations of DMF.

DISCUSSION AND CONCLUSIONS

Methanol caused very low motility and viability post-thawing, coinciding with previous reports (Hagedorn et al., 2011 Kollár et al., 2014). Although DMF induced the best results of the tested cryoprotectants, the maximum post-thawing motility was lower than 20% and there was a dose-dependent negative effect on the cell viability.

ACKNOWLEDGEMENTS

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P14

EFFECTS OF CRYOPROTECTANTS IN DIFFERENT CONCENTRATIONS ON SPERM MOTILITY AND FERTILIZING CAPACITY OF TENCH *TINCA TINCA* SPERM

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INTRODUCTION

Tench *Tinca tinca* (L., 1758) is a fish species with a great potential in aquaculture (Flajshans et al., 1999; Gela et al., 2006; Wang et al., 2006; Rodina et al., 2007) and development of methods to improve and facilitate rearing and production of this species is needed. Sperm cryopreservation as a technique that enables storage of sperm and a continuous supply of sperm (Irawan et al., 2010; Horváth et al., 2012) has a significant role in the present development of aquaculture. Because cryopreservation protocols for tench sperm are scarce, the aim of this study was to test the effect of application of two different cryoprotectants (methanol and DMSO) in different concentrations (5%, 10% and 15%) and in combination with a sugar-based extender on motility parameters and fertilization success, as well as post-thaw storage of cryopreserved sperm at 4 °C.

MATERIALS AND METHODS

Tench broodstock was maintained at the Department of Aquaculture, Szent István University. Fish were injected with carp pituitary (25 mg kg^{-1} bw) and anaesthetized with 2-Phenoxyethanol before stripping. Grayling solution (glucose 200mM, KCl 40mM, Tris 30mM, pH 8) was used as an extender, while cryoprotectants used were methanol and dimethyl suphoxide (DMSO) in different concentrations (5%, 10% and 15%). Dilution ratio of sperm with extender was 1:9 in experiment 1, and 1:1 or 1:4 (depending on sperm concentration) in experiment 2. After storage all samples were thawed in a 40 °C water bath for 13 seconds. Parameters values are presented as mean±standard deviation. Statistical significance was accepted when $p<0.05$.

Experiment 1: Effects of different concentrations of cryoprotectants on fertilization and hatching rate. Five males and two females were used in this experiment. Samples were prepared for cryopreservation with Grayling extender in six test groups depending on cryoprotectant and its concentration. Pooled eggs from both females were used. Fertilization was conducted in ratio 1:100,000 spermatozoa per egg and eggs were subsequently incubated on 24 °C. Eggs fertilized by fresh sperm were used as control. Fertilization rate was calculated 24 h after fertilization, while hatching rate was counted 48 h after fertilization.

Experiment 2: Effect of cryoprotectants type and concentration on sperm motility during post-thaw storage. Five males were used in this experiment. Test groups and freezing procedure were the same as in the previous experiment. Following thawing samples were kept at 4 °C during motility analyses which were conducted every 1 hour up to 4 hours in the CASA system. Motility parameters used were progressive motility, curvilinear velocity - VCL and linearity - LIN. Motility parameters of fresh sperm were used as controls.

RESULTS

Experiment 1: In the control, 91.04 ± 7.30% fertilization rate and 90.95 ± 7.05% hatching rate were observed. Among the experimental groups, sperm frozen with 10% methanol yielded the highest fertilization (85.30 ± 15.01%) and hatching

(79.85 ± 13.19%) rates, significantly higher than all other groups (Tukey's HSD, $p < 0.05$). Other groups had rates mostly around 5%.

Experiment 2: 5% methanol, 10% methanol and 5% DMSO groups had significantly higher motility parameters than other groups (Tukey's HSD, $p < 0.05$). During the experiment, progressive motility declined, however the decline was mostly non significant (Tukey's HSD, $p > 0.05$). VCL and LIN displayed little declined. Three-factor ANOVA displayed a significant main effect of cryoprotectants, cryoprotectant concentrations and post-thaw storage time as well as a significant interaction between cryoprotectants and their concentrations for all motility parameters.

DISCUSSION AND CONCLUSIONS

In this study, we demonstrated that using a sugar-based extender and methanol as cryoprotectant for the cryopreservation of tench, sperm can result in high fertilization and post-thaw sperm motility. Results also demonstrate that fertilization success and post-thaw motility are not always in correlation as the highest post-thaw motility was observed with 5% DMSO. On the other hand, the 10% methanol group displayed the highest VCL which may have contributed to the highest fertilization success according to Gallego et al. (2013). However, the fact that there was no difference between the VCL of 5%, 10% methanol and 5% DMSO groups indicates that there might be other factors contributing to fertilization success.

Overall, we found that a sugar-based extender in combination with methanol as cryoprotectant is suitable for the cryopreservation of tench sperm and allows storage of cryopreserved sperm for up to 4 hours post-thaw.

ACKNOWLEDGEMENTS

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P15

DEVELOPMENT OF CRYOPRESERVATION OF ZEBRAFISH (*DANIO RERIO*) SPERM

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INTRODUCTION

Zebrafish has more than 20 000 wild-type, transgenic and mutant strains, which are used in human disease, toxicology, genetics, embryology and drug development research mainly (Knight & Abbot 2000). Therefore conservation of these lines is an important issue. Cryopreservation is a suitable way to maintain the genetic material. Four cryopreservation protocols are made in zebrafish currently (Harvey et al. 1982, Walker & Streisinger 2000, Draper et al. 2004, Yang et al. 2007), but these methods are not standardized. Subsequently, our target was the development of a standardized cryopreservation technique based on the best sperm collection method, extender and cooling rate.

MATERIALS AND METHODS

Exp1: The quality of individual and pooled stripped sperm was compared. *Exp2:* The effect of different extenders during a 3-hour storage was tested: Grayling extender (200 mM glucose, 40 mM KCl, 30 mM Tris, pH=8.0), Hanks' Balanced Salt Solution (HBSS) with 300 and 400 mOsm/kg osmolality, and Leibovitz-15 (L-15). *Exp3:* Cryopreservation with and without a "holding" period in the cooling profile was tested: the protocol by Yang et al. (2007) was used for cryopreservation (10 ^{o}C/min cooling rate) with a 6-minute holding period applied after the crystallization (at -8.5 ^{o}C), compared with non-held samples. *Exp4:* The protocol by Draper et al. (2004) was also tested: Ginsburg Fish Ringer, powdered skim milk and 8% methanol were used for cryopreservation and the samples were split in 2 straws (10 □l final volume) to monitor individual differences between straws. *Exp5:* The effect of splitting the samples into two straws on the fertilizing capacity of sperm was tested using the Grayling extender and the HBSS at 300 mOsm/kg (as described by Yang et al. 2007). Endpoints of experiments were as follows: Experiments 1 and 2: motility, Experiments 3 and 4: motility and fertilization, Experiment 5: fertilization.

RESULTS

Exp1: In case of the different sperm collection method, the pooled samples gave 31±21% motility (N=9), in contrast with the individual samples, where it was 61±24% (N=18; Fig.1.). *Exp2:* A significant main effect of both the type of extender ($P<0.0001$) and storage time ($P=0.0001$) on sperm motility was detected. Motility was significantly higher in the Grayling extender than in L-15 both at 30 min (63±17 vs. 17±16%) and at 180 min (47±18 vs. 4±3%, N=10; Fig.2.). *Exp3:* Application of a holding period to the cooling profile did not have a significant effect on the post-thaw motility (6±6% with holding vs. 4±5% without holding) or fertilizing capacity (15±23% with holding vs. 10±12% without holding) of cryopreserved sperm. *Exp4:* Using the protocol by Draper et al. (2004), the motility after thawing was under 5% (N=4) and the fertilization was 0% (N=4) in each case. *Exp5:* Splitting the samples into two straws did not have a significant effect on the fertilizing capacity of cryopreserved sperm (3±7% in split samples and 15±23% without splitting in HBSS300 vs. 9±9% with splitting and 15±17% without splitting in the Grayling extender).

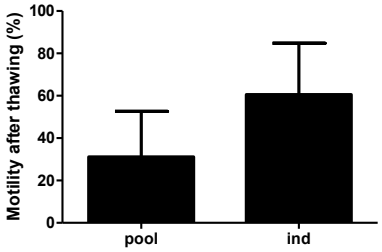

Fig.1.: The average motility after thawing with HBSS300 in case of pooled and individually collected sperm (p=0.0049)

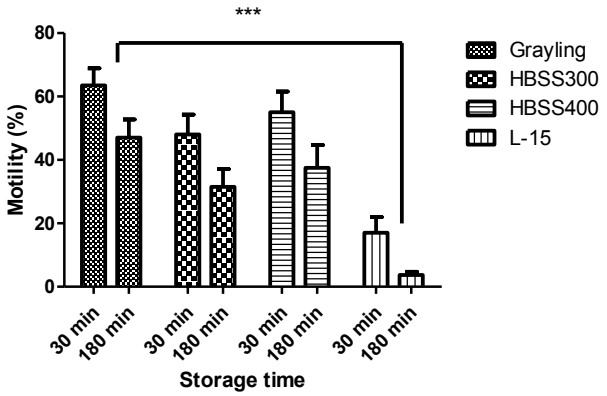

Fig.2.: The average motility in different extenders in the 30th and 180th minutes of storage (p≤0.0001)

DISCUSSION AND CONCLUSIONS

We found, that pooling samples does not have an advantage over using individual samples during collection of zebrafish sperm. The use of L-15 medium for sperm storage was observed to result in a rapid decrease of sperm quality, therefore the use of other extender is recommended for zebrafish sperm. Cryopreservation of zebrafish sperm remains a problematic issue with low post-thaw motility and fertilization rates. Incorporation of a holding period into the cooling profile, the use of various extenders and splitting the samples into smaller aliquots did not improve these parameters.

ACKNOWLEDGEMENTS

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P16

FERTILIZATION OF *STEINDACHNERIDION PARAHYBAE* OOCYTES WITH CRYOPRESERVED SPERM

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INTRODUCTION

Procedures of fish semen cryopreservation were performed in native fish species of South American (Carolsfeld et al., 2003), in which adaptations of cryoprotectant solutions and methodologies used in mammals were made. In fish there are cases where they can be observed high rates of motility after thawing, indicating that these sperm have great chances of fertilizing an oocyte (Viveiros and Godinho, 2009). The success of cryopreservation in fish can be obtained from fertilization of oocytes by sperm that have been subjected to freezing and thawing procedure (Cabrita et al., 2010). However, the protocols need to be standardized for different species of fish (Viveiros and Godinho, 2009) mainly for species that are endangered, such as the Surubim-do-Paraíba, *Steindachneridion parahybae* (Siluriformes: Pimelodidae).

MATERIALS AND METHODS

We used three (a, b and c) semen mixing (four males each - 1.5±0.7kg) diluted in two cryoprotectant solutions: *G5*= 5% of glucose, 7.5% methanol, 5% milk powder; *G8.5*= 8.5% of glucose, 10% methanol, 0.25% milk powder. After dilution (1:3 - semen:solution), the semen were bottled in 0.25 mL straws and subsequently subjected to freezing (Dry-shipper – 18h and liquid nitrogen – 27 days). Analyses were performed of fresh semen (control) and after thawing (15s in water at 25°C): motility rate (MOT), curvilinear velocity (VCL), average path velocity (VAP) and straight line velocity (VSL). For fertilization, was used fresh sperm from three other males (2.7±0.3kg). We used 0.5g (183±10 oocytes) of pool of oocytes from three females (1.2±0.3kg) and 1,000,000 motile sperm oocyte^{-1}. We evaluated the fertilization, hatching and normal larvae rates. The variance analysis and Duncan test were performed.

RESULTS

The pools of semen presented osmolality of 272, 283 and 278 mOsm kg^{-1} for pool *a b* and *c*, respectively. Was observed effect of different solutions and sperm mixing (pool) on fertilization rates (P<0.01) and hatching rates (P<0.05) (Figure1).

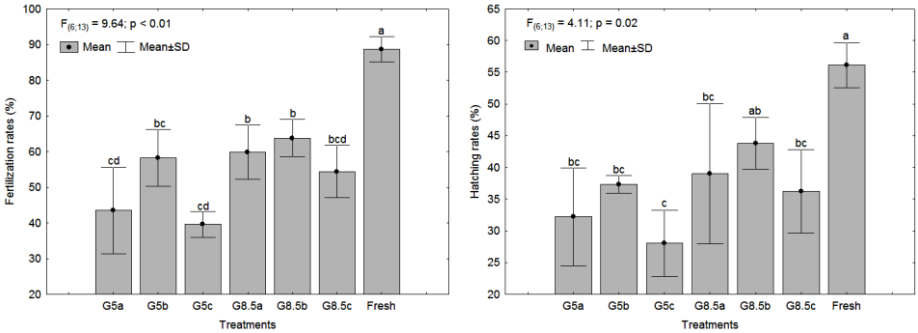

Figure 1. Fertilization rates (right) and hatching rates (left) of *Steindachneridion parahybae* employed fresh sperm (Fresh) and thawing sperm in two solutions (G5 and G8.5) at three semen pool (a, b and c). Different letters indicate significant difference according Duncan Test.

The better results of thawing were verify on the solution contained 8.5% of glucose, 10% methanol, 0.25% milk powder for the pool b, conforming hatching values. The normal larvae rates not were influenced (P>0.05) and presented

means 86.4±5.6%. The motility parameters evaluated were lesser (P<0,01) than control sperm (Figure 2). However, the thawing sperm diluted on the solution 8.5% of glucose, 10% methanol, 0.25% milk powder for the pool b present the better values.

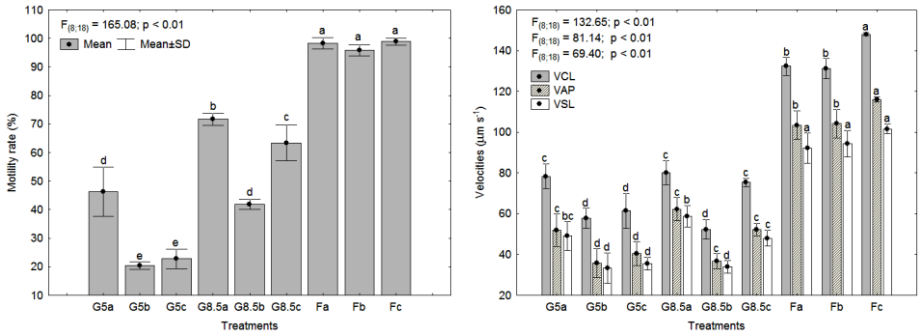

Figure 2. Motility rate (right) and velocities (left) of *Steindachneridion parahybae* sperm after thawing in two solutions (G5 and G8.5), and fresh sperm (F) at three semen pool (a, b and c). Different letters indicate significant difference according Duncan Test.

DISCUSSION AND CONCLUSIONS

The reduction of sperm quality and fertilization rates observed in this experiment were similar to those found by Araújo (2011). The author found for *S. parahybae* that although frozen sperm motility have shown high rates, the fertilization capacity are lower than fresh sperm. This was due to the reduction of key parameter values for fertilization, such as sperm velocity, that directly influenced the fertilization rates. Reduction of sperm quality freshwater native fish is generally checked after sperm cryopreservation and second Viveiros and Godinho (2009), various aspects influenced to this reduction, among them stand out from the cryopreservation protocols applied, such as freezing curves and thawing. Another key parameter is the composition of the used cryoprotectant solution, as this should not activate the sperm and should be able to penetrate inside the sperm cell, without being toxic, and not allow the formation of ice crystals during freezing (Viveiros and Godinho , 2009). The hatching rates with sperm thawed on solution of 8,5% of glucose, 10% of methanol and 0.25% milk powder for "b" semen mixing were statistically equal at control, however, despite this results to be good, differences were verified in the utilization of different pools of males and should be tested in futures works with the same cryoprotectant solution.

ACKNOWLEDGEMENTS

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P17

EFFECT OF ACID PHOSPHATASE, LACTATE DEHYDROGENASE AND β-N-ACETYLOGLUCOSAMINIDASE INHIBITORS ON FERTILIZATION SUCCES IN SANDER (*SANDER LUCIOPERCA* L.)

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INTRODUCTION

Despite there is many information regarding artificial reproduction of sander, literature concerning enzymes present in the sperm of this fish are scarce. Additionally there is no data describing the role of such enzymes during the fertilization process. In this paper, we determined effect of the selected sperm enzymes inhibitors on the sander reproduction success. Additionally we used mannitol as a reagent mimics solely effect of osmolality.

MATERIALS AND METHODS

In the first experiment it was determined how the enzyme inhibition influences sperm motility parameters (CASA analysis). Sperm was mixed in a 1:1 ratio with Kobayashi immobilizing buffer which contained 0.25; 1; 4mM ammonium molybdate (AcP inhibitor), 6.25; 25; 100μM gossypol (LDH inhibitor), 0.03; 0.125; 0.5M acetamide (β-NAGase inhibitor) and 0.03; 0.125; 0.5M mannitol (n=4). In the second experiment, the enzyme inhibitors (in concentrations given above) were added to an activation buffer (80mM NaCl, 20mM KCl, 10mM Tris, pH 8,0) in which the fertilization was conducted (n=4). Fertilization success was determined at eyed-egg stage.

RESULTS

The inhibitors addition did not decreased the value of motility parameters. The addition of ammonium molybdate caused a significant decrease in the percentage of fertilized eggs, from 84% in control to 10% with 4mM ammonium molybdate. The addition of 100μM gossypol caused a decrease in the percentage of fertilization success to 26%. The percentage of fertilization after acetamide inhibition was 52% while mannitol caused dramatic decreased of fertilization where only 4% of eggs were fertilized. Acetamide also lowered the percentage of fertilized eggs, but the highest dose lowered the percentage to 52% in contrast to mannitol, which decreased the fertilization to 4%.

DISSCUSSION

AcP and LDH appear to fulfill an essential function in the fertilization success of sander. The β-NAGase in sander sperm does not seem to be very important for the fertilization process. Mannitol was detrimental for the sander fertilization success.

ACKNOWLEDGMENTS

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P18

SERINE-LIKE PROTEOLYTIC ENZYMES FROM COMMON CARP *CYPRINUS CARPIO* L. SEMINAL PLASMA AND ABLITY TO DEGRADE SPERM PROTEINS

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INTRODUCTION

Serine proteases and metalloproteases are the main proteases noted in the seminal plasma of teleost. Activity of these enzymes was also detected in common carp *Cyprinus carpo* L. seminal plasma with gelatin and casein being used as substrate. In contrast to metalloproteases, serine proteases of common carp displayed a wider range of substrate specificities. The physiological function of metalloproteases and serine proteases is still unclear. The aim of the study was to determine the possibility of degradation of sperm proteins by seminal plasma common carp proteases. The effect of incubation time, pH and temperature on the proteolytic activity of sperm extract proteins was determined.

MATERIALS AND METHODS

The seminal plasma was separated from sperm by centrifugation at 10,000 x g for 10 min. Sperm was then thawed in extraction buffer (50 mM Tris-HCl at pH 7.6) and stored for 1h at 4°C, and then centrifuged twice. Protein concentration was determined by the method devised by Lowry et al. (1951). The seminal plasma samples (10 µl diluted with 10 µl of 0.7% NaCl and 7 µl of double-concentrated stain with SDS) were subjected to electrophoresis in sperm proteins containing (0.1% protein) polyacrylamide (10% acrylamide) gels in the presence of SDS under non-reducing conditions (Siegel & Polakoski 1985). The molecular weight was estimated using prestained SDS-PAGE standards (Bio-Rad, Hoefer, San Francisco, CA, USA) such as Myosin (Mr 198 kDa), ß-galactosidase (Mr 110 kDa), Bovine serum albumin (Mr 64 kDa), Ovalbumin (Mr 44 kDa), Carbonic anhydrase (Mr 31 kDa), Soybean trypsin inhibitor (Mr 28 kDa), Lysozyme (Mr 19 kDa) and Aprotinin (Mr 6 kDa). The molecular weight of protease bands was estimated with the use of the Kodak1D program (Eastman Kodak Company, New Haven, USA). Time of gel incubation in development solution (4, 8, 12 and 24h), pH of development solution (5.5; 6.5; 7.5; 8.5 and 9.5) and temperature of incubation (4, 22 and 37°C) were analyzed. The development solution also containing either 10 mM of benzamidine or 5 mM EDTA was applied.

RESULTS

Two bands of molecular weights of 76 and 163 kDa were able to digest sperm proteins (Fig. 1). They were classified as serine proteases since the addition of benzamidine inhibited they activity (Fig. 1B) whereas the addition of EDTA did not affect their activity (Fig. 1C). Serine proteases activity were observed in a wide range of temperatures (Fig. 2) and pH (Fig. 3) of incubation.

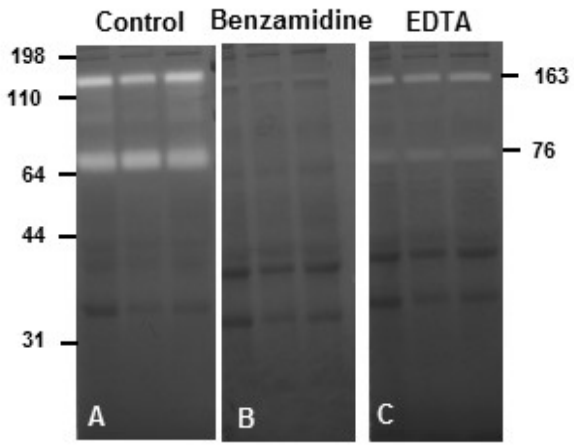

Fig. 1. Effect of inhibitors on sperm proteins activities of seminal plasma of common carp. The incubation was performed during 24h at pH of 7.5 and in 37°C with $CaCl_2$ (A-Control), with benzamidine (B) and with EDTA (C).

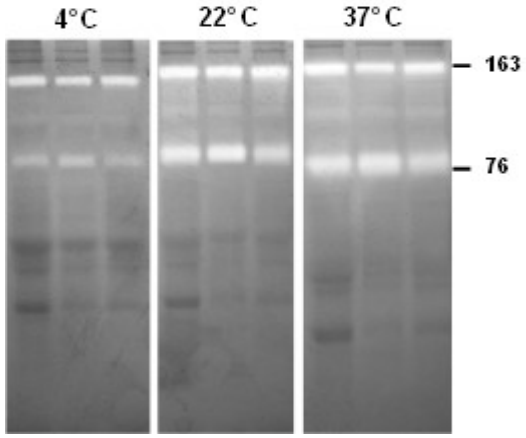

Fig. 2. Zymography of seminal plasma using sperm proteins as a substrate at a different temperature of incubation. The incubation was performed with addition of $CaCl_2$ during 24h and in 7.5 pH.

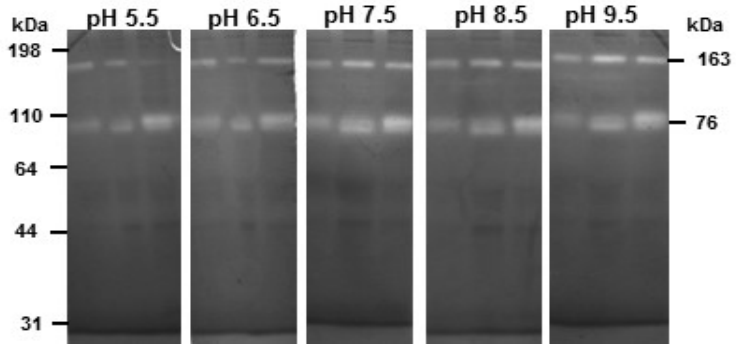

Fig. 3. Zymography of seminal plasma using sperm extract proteins as a substrate at a different pH of incubation. The incubation was performed with addition of $CaCl_2$ during 24h and in 37°C.

DISCUSSION AND CONCLUSIONS

The application of zymography with sperm extract proteins as a substrate allowed for the first time the visualization of serine protease (76 and 163 kDa) activity of common carp seminal plasma. This indicates that serine proteinases possibly degrade sperm during the aging process.

ACKNOWLEDGEMENTS

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P19

CHANGES ON SPERM QUALITY OVER THE SPAWNING SEASON OF *PROCHILODUS LINEATUS* AND *BRYCON ORBIGNYANUS*

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INTRODUCTION

The streaked prochilod *Prochilodus lineatus* and the piracanjuba *Brycon orbignyanus* are fish from the order Characiformes and are native to South America. These species have great potential for aquaculture and have been used in restocking programs through artificial propagation (Viveiros et al., 2009; Lopez et al 2015). During the spawning season (November to February), when photoperiod, temperature and rainfall are adequate, these species migrate to spawning sites in a phenomenon known as piracema. The environmental changes, thus, regulate gonadotrophin production and delimit the period of reproductive success of these species. Climate changes are increasingly evident and the spawning season is losing its striking features, which may result in changes on the time of gonad maturation and affect gamete quality from the beginning through the end of the season as well as the freezing capacity of their gametes. Knowledge on the gamete quality of these species over the spawning season is scarce. Thus the aim of this study was to determine fresh and post-thaw sperm quality evaluated during the 1st and 2nd half of spawning season in *Prochilodus lineatus* and *Brycon orbignyanus*.

MATERIALS AND METHODS

The spawning season was divided in two periods: the first period from mid-November to mid-December with 43 *P. lineatus* and 14 *B. orbignyanus*, and the second period during January with 64 *P. lineatus* and 20 *B. orbignyanus* males. *P. lineatus* males received two doses of carp pituitary extract at 0.4 and 4 mg/kg BW in a 12-h interval and *B. orbignyanus* males received a single dose at 1mg/kg BW, which is the routine method currently used to induce spermiation in the Fish Culture Station of the Minas Gerais Power Company (CEMIG) in the city of Itutinga, Brazil. Eight hours later at approximately 27°C, the urogenital papilla was dried and sperm were hand-stripped directly into glass tubes. Contamination of sperm with water, urine or feces was carefully avoided. After collection, sperm was transported in a cooler (9-11ºC) from CEMIG to the Laboratory of Semen Technology at Federal University of Lavras, in the city of Lavras (~60 km), where cryopreservation and sperm analysis took place. As freezing medium, glucose solution at 325 mOsm/kg and methyl glycol was used for *P. lineatus* sperm (Viveiros et al., 2009) and NaCl solution at 325 mOsm/kg and methyl glycol for *B. orbignyanus* sperm (Lopez et al 2015). Sperm was diluted to a final ratio of 1 sperm: 8 extender: 1 methyl glycol, drawn into unsealed 0.25-mL straws (n = 2 replicate straws per male), frozen in nitrogen vapor vessel (dry vapor shipper) and stored in a liquid nitrogen vessel. Straws were thawed in a water bath at 60°C for 3 s before sperm analysis. Sperm (both fresh and post-thaw) was evaluated for motility rate and curvilinear velocity (VCL) using a Computer-Assisted Sperm Analyzer (SCA™ software, Microptics, Barcelona). Statistical analyses were conducted with the R Development Core Team software program. Data were tested for significant differences using MANOVA. The level of significance for all statistical tests was set at 0.05.

RESULTS

No significant differences were observed for any of fresh and post-thaw sperm features when the 1st period of the spawning season was compared to the 2nd one, in both species. However, fresh sperm yielded higher motility rate and VCL when compared with post-thaw sperm, within the same period (Table 1).

Table 1 Fresh and post-thaw motility rate and curvilinear velocity (VCL) of sperm (mean ± SD) evaluated during the spawning season of *Prochilodus lineatus* and *Brycon orbignyanus*.

	Motility rate (%)		VCL(µm/s)	
P. lineatus	1st period	2nd period	1st period	2nd period
Fresh	95 ± 3 [a]	94 ± 8 [a]	288 ± 40 [a]	285 ± 56 [a]
Post-thaw	76 ± 14 [b]	80 ± 12 [b]	186 ± 35 [b]	189 ± 40 [b]
B. orbignyanus	1st period	2nd period	1st period	2nd period
Fresh	92 ± 8 [a]	94 ± 6 [a]	243 ± 58 [a]	239 ± 54 [a]
Post-thaw	65 ± 21 [b]	67 ± 12 [b]	146 ± 37 [b]	149 ± 30 [b]

[a,b] Means within the same column followed by different superscript differ ($P<0.05$, MANOVA). There was no effect ($P>0.05$) of the period of season on motility rate or VCL within the same type of sperm (fresh or post-thaw) in both fish species.

DISCUSSION AND CONCLUSIONS

The motility rate and velocity are factors of great importance because increment the viability of the gametes and favor fertilization. Changes in sperm quality over the reproductive period have been reported in some fish species, such as rainbow trout (Munkittrick and Moccia 1987), common carp (Christ et al., 1996), and haddock (Rideout et al. 2004), among others. The seasons are losing their remarkable characteristics, due to the intensification of the anthropological actions, as greenhouse effect, deforestation and the destruction of the ozone layer. This could result in the advance of the final maturation of gametes in fish reproduction period. However, this phenomenon was not observed in *P. lineatus* or in *B. orbignyanus*. It suggests that sperm of both species, and possibly of other Neotropical species as well, are not that sensitive to climate variations, including the freezing capacity. Although difference on sperm quality was not observed over the spawning season, we recommend evaluating reproductive parameters in the females as well, as oocyte quality, mainly egg yolk quality, is more vulnerable to climate changes, and strongly depends on the female nutrition.

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P20

HORMONAL INDUCTION OF *BRYCON CEPHALUS* (CHARACIFORMES, CHARACIDAE) TO SPERMIATION USING D-ALA6, PRO9NET-MGNRH + METOCLOPRAMIDE

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INTRODUCTION

Rheophilic fish require a variety of abiotic environmental stimuli and reproductive migration to occur gonadal development and the effective reproduction. However, when hatchery-reared, these species are private of their migratory movements and all water abiotic parameters are controlled. So, it is necessary to apply techniques of hormonal induction to reproduction. (Carneiro & Mikos, 2008; Mylonas et al., 2010). In males, the hormonal induction application leads to the seminal volume increasing, which is important because it makes techniques such as semen cryopreservation easier, as well as reducing the need for a large number of males in the artificial reproduction process (Carneiro & Mikos, 2008).

The most commonly used hormonal inducers for stimulating rheophilic species spermiation are: Carp pituitary extract (CPE) and Analogues of mammalian gonadotropin-releasing hormones (D-Ala6, Pro9 Net-mGnRH) associated with a dopamine inhibitor.

Use of CPE to induce breeding in rheophilic species presents some disadvantages, including: no standardization of gonadotrophic hormonal concentration; not possess gonadotrophic hormones only (FSH and LH), but also growth hormones and osmoregulators, among others, which can cause irregular results, breeding stress and reducing the lifespan (Mylonas et al., 2010).

Therefore, this study aimed at the standardization of a protocol to induce seminal production for *Brycon cephalus* males by mGnRH (Ovopel®), in order to increase seminal volume while maintaining the quality of semen.

MATERIALS AND METHODS

Two-year-old adult specimens of *Brycon cephalus*, weighing 0.37±0.06 kg and with an average size of 30.4±3.2, belonging to the Centro Nacional de Pesquisa e Conservação de Peixes Continentais – CEPTA/ICMBIO, Pirassununga, São Paulo, Brazil, were used. The males were separated into four tanks, each one with five exemplars (n=5). As hormonal inductor was used Ovopel® product (Interfish, Hungary), which is composed of pellets weighing approximately 3 mg and containing 18–20 µg of [(D-Ala^6, Pro^{9}Net)-mGnRH] mammalian analogue + 8–10 mg of metoclopramide. Three different hormonal doses were established as treatments: 1/3 pellet/kg (T1), 2/3 pellet/kg (T2) and 1 1/3pellet/kg (T3). There was also a control group (CO), in which only a physiological solution was applied (0.9% NaCl) as a placebo. To dilute the hormonal doses, 0.5 mL/kg of physiological solution was used (0.9% NaCl). Every treatment was applied in a single dose.

After approximately 165 hours-degree (t= 6 hours; T °C media = 27 °C) from the induction moment, a semen sample was obtained by gentle abdominal massage and sampled in a 10 mL graduated plastic tube to verify of the total volume released by each male. Hereafter, in each sample other seminal parameter were such as subjective spermatic motility, duration of sperm motility, pH, osmolality and spermatic concentration. To estimate the subjective spermatic motility the method of Ninhaus-Silveira et al. (2006) was used, which set up an

arbitrary value scale that varies from 0 to 5. Sperm motility duration was measured in seconds from the activation time until observation be only 10% of motile cells. Seminal pH was evaluated with a pH meter (CHECKER by HANNA Instruments®, Porto Alegre, Brazil). The osmolality was assessed by centrifuging the semen at 3000 rpm for 20 minutes (Quimis®, model 222TM208, Brazil). The supernatant was collected and analyzed using a cryoscopic osmometer (OSMOMAT® model 030, Berlin, Germany). To determine the spermatic concentration, semen was diluted in a formal-saline solution (1:1000 (semen:solution)), which was the count made in a Neubauer hematimetric chamber (Spermatozoa/mL); [*Sptz/mL = [(∑$_n$((∑$_2$grid cells: 2) ∑$_3$ repetition p/sample: 3)): n* 50.000]*1000*], n = males quantity.

The repetition of the treatments was the number of fish. For data, normality (Shapiro-Wilk) and homogeneity (Bartlett) tests were applied. The Kruskal-Wallis test was applied to nonparametric data, and the same to the analysis of the seminal volume with corporal weight ratio. Results that showed significant differences ($p<0.05$), ANOVA and Tukey tests were applied.

RESULTS

Treatment 3 (T3) resulted a higher production of semen (4.66±1.52mL) and was significantly different in comparison to the volume produced by the animals submitted to treatments T1 (2±0.9mL) and T2 (3.5±1.3mL), and by CO (2.3±1.2mL) (Fig. 1A). In relation to the spermatic motility, T2 and T3 showed significantly higher levels [5, (81–100%)] than T1 and CO [4, (61–80%)]. T3 showed a significantly lower average duration of sperm motility (22±5s) than T1, T2 and CO (30±7 s; 28±6 s; 32±8s, respectively). In relation to the seminal parameters of spermatic concentration, pH and osmolality, no statistical difference was observed among the treatments. In relation to the analysis of the volume/body weight there was no difference among the treatments and control.

DISCUSSION AND CONCLUSIONS

The value of the ratio between semen volume and the fish body weight indicates that the two variables are independent. Thus, when compared between treatments, as there was no statistical difference, we can infer that the increase in semen volume obtained after induction is not related to the body weight of the fish, but the effect of the hormone dosage applied. In conclusion, a dosage of 2/3 pellet/kg of Ovopel® (D-Ala6, Pro9Net-mGnRH + metoclopramide) proved to be the most adequate for hormonal induction of *Brycon cephalus* males, by increasing the seminal volume produced, spermatic motility and duration of sperm motility and maintaining semen quality.

ACKNOWLEDGEMENTS

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P21

STANDARDIZATION OF THE SDS-PAGE TECHNIQUE TO SEMINAL PLASMA OF *COLOSSOMA MACROPOMUM* BREEDING TO VERIFY THE SEMINAL PROTEIN PROFILE

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INTRODUCTION

In recent years, studies in the field of proteomics have shown a significant increase because the information generated by the technique can help to solve and improve numerous causes in fish breeding. The use of proteomic is related to the search for knowledge of the seminal profile to assist in improving the reproductive quality of fish, and more complex subjects aimed at discovering the functions of certain proteins in the reproductive system (Ciereszko et al., 2012). In this study we aimed to determine the appropriate methodology to assess the seminal protein profile of *Colossoma macropomum* by SDS-PAGE, comparing breeding profiles.

MATERIALS AND METHODS

Semen used in this experiment was collected from *Colossoma macropomum* breeding in the experimental station Boa Esperança Fish Farm. For the separation of seminal plasma and cells, semen was collected in 2 ml conical tubes and placed in a centrifuge to be processed at 7500 G, 2 min. The supernatant (the seminal plasma) was separated from cells and then stored in liquid nitrogen for further analysis. In the laboratory, seminal plasma was thawed and analyzed by spectrophotometry to quantify protein using the BCA Protein Assay Kit (Thermo Scientific). Subsequently, to determine the molecular weight of proteins, seminal plasma samples were subjected to one-dimensional electrophoresis with polyacrylamide gel (SDS-PAGE). The polyacrylamide gel for stacking, used as a standard was 5%, but the running gel occurred in 12.5 and 15% concentrations. To determine the amount of seminal plasma to be used in the observation of the bands on the gel, 5, 10 and 15 µL of seminal plasma, diluted or not in 10 µL of Milli-Q water were added to 15 µL of loading buffer. After homogenization, the samples were heated at 100°C, 10 min to assist in the denaturation of proteins. Samples were concentrated to stacking between 40 to 70V, with 30 minutes of running time, and the separation was performed between 120 to 150V for approximately 150 minutes. Each run contained 25 µL final sample. The gels were stained with Comacie Blue R250.

RESULTS

The preliminary results indicated that the best concentration which showed bands in the gel was 12.5%. The use of water was not necessary to improve the race and the separation of bands, and the data showed that the best amount of seminal plasma was 10 µL, mixed with 15 µL of loading buffer. The best voltage for stacking was 70V. The best bands separation was observed at 120V. The use of 5 µL plasma was insufficient for a well defined separation of the bands, and using 15 µL of seminal plasma was considered high, causing smudges on race tracks (Fig. 1).

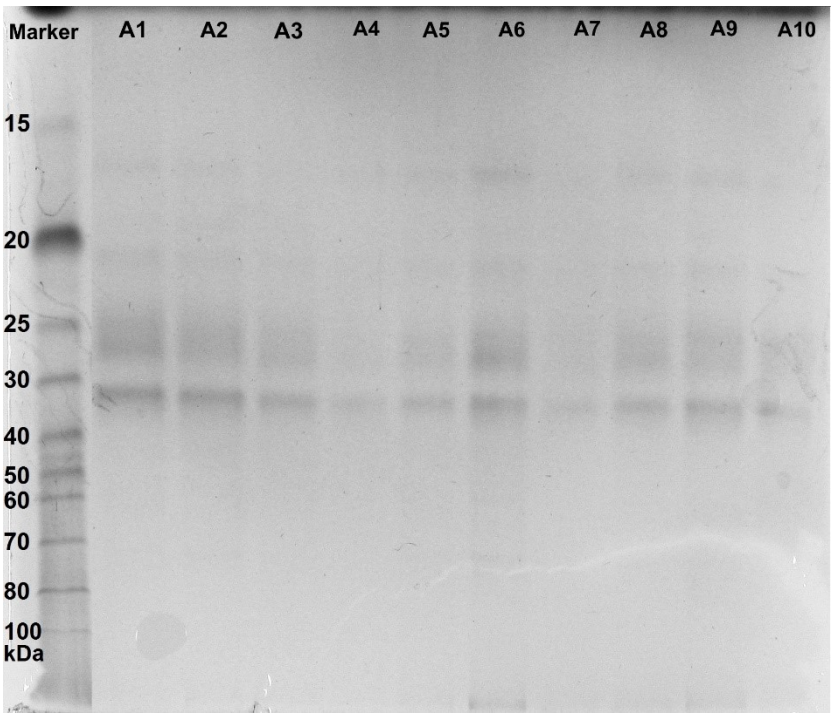

Fig. 1 – Best conditions obtained for SDS-PAGE that allowed a clear separation of *C. macropomum* seminal plasma proteins in a polyacrylamide gel (12.5%). A1 – A10: animals used.

DISCUSSION AND CONCLUSIONS

This study reports the first results obtained in the evaluation of seminal plasma proteins of *C. macropomum*. The results showed no difference in the protein profiles of seminal plasma between breeding, and the molecular weights of proteins can vary from 15 to 50 kDa. Studies with *Solea senegalensis* reported the difference in protein expression between wild and F1 breending (Forné et al., 2009), which was not observed for species studied so far. However, the protein profile of seminal plasma of *Oncorhynchus mykiss* (Lahnsteiner et al., 2004; Asadpour et al., 2013) resembles the *C. macropomum*. So far, we can say that proteins with molecular weight below 50 kDa, are correlated with the physiology of sperm cells.

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P22

EFFECT OF TIME AFTER HORMONAL STIMULATION ON SPERMATOZOA MOTILITY AND SHORT-TERM STORAGE OF SEMEN GOLDFISH *CARASSIUS AURATUS AURATUS* (L.)

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INTRODUCTION

Goldfish *Carassius auratus auratus* (L.) is one of the most common ornamental species in the world. Due to the ever growing demand of goldfish, there is a need to increase its production. Reproduction success is affected by the quality of gametes produced by both gender of the fish. Based on spermatozoa motility parameters we can evaluate the quality of milt and its ability for fertilization. The type of hormonal agents and the time between the stimulation performed and obtaining the milt, significantly affect sperm quality (Cejko et al. 2010). Short-term semen storage at low temperature enables artificial fertilization when the spermiation precedes ovulation; this is especially important for Cyprinid breeding lines (Sarosiek et al. 2012). The aim of this study was to determine the influence of the time after hormonal stimulation on selected CASA parameters of milt of goldfish. Effect of stripping time on this parameters after short-term storage of sperm goldfish was also estimated.

MATERIALS AND METHODS

Adult males of goldfish were hormonally stimulated by intraperitoneal injection using 1 pellet kg^{-1} of Ovopel [(D-Ala6 Pro9 NEt)-mGnRH + metoclopramide] at a 20°C. The sperm was collected 24 h (E) or 48 h (L) after the hormonal stimulation from different males at each time point (n=12/each time point). Sperm motility parameters were determined using the CASA system (Sperm Class Analyzer v. 4.0.0. by Microptic S.L., Barcelona, Spain). For sperm motility activation tap water was used. The dilution was 1:40. Activated sperm was placed on the Leja slide glasses. Recording of sperm movement started eight seconds after activation. For short-term storage, 0.3 ml of semen from each males from both stripping times were placed in new plastic tubes without dilution of any buffer and were stored at 4°C. Sperm motility was analyzed with the CASA system (as described above) after 24 h (E24 and L24) and 48 h (E48 and L48) of semen storage. Sperm motility parameters which were chosen for analysis: MOT– percent of motile sperm (%), VCL – curvilinear velocity (μm s^{-1}), VAP– average path velocity (μm s^{-1}), VSL – straight line velocity (μm s^{-1}), LIN – linearity (VSL VCL^{-1} %), STR– straightness (VSL VAP^{-1} %). The results were statistically analyzed using Statistica software. CASA parameters were compared using one-way ANOVA followed by LSD Fisher post hoc test.

RESULTS

The percentage of motile spermatozoa (MOT %) statistically decreased 48 h after hormonal treatment (Fig. 1). Also, all velocity parameters of spermatozoa were significantly lower at 48 h compared to 24 h following stripping. Between 24 and 48 h following stripping a significant decrease of VCL, VAP and VSL was observed (from 138, 123 and 85 μm s^{-1} to 100, 88 and 64 μm s^{-1} for VCL, VAP and VSL, respectively). Values of other CASA parameters maintained a similar level with no significant differences between stripping time. MOT gradually decreased with time of semen storage (Fig. 1). Differences in MOT parameter between stripping times was still significant after 24 h but not after 48 h of semen storage (Fig. 1). Velocity parameters of spermatozoa also decreased with time of semen storage with significant differences between stripped semen at 24 h and 48 h. After 48 h of storage, in case of semen collected 24 h after hormonal treatment LIN and STR (%) significantly increased from 60.5 and 68.2 to 71.2 and 77.6, respectively.

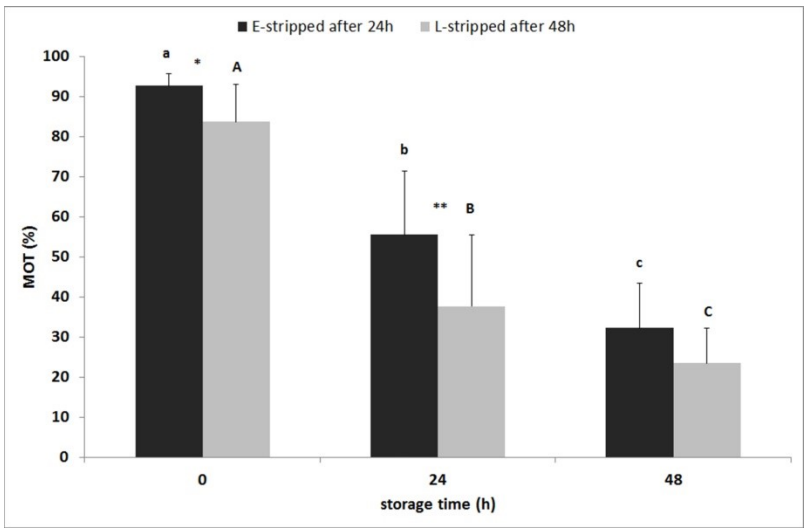

Fig. 1. Sperm motility (MOT%; mean ± SD) in relation to stripping time after hormonal stimulation and short term semen storage for 24 h and 48 h following stripping. Lowercase letters indicate statistical differences among sperm stripped at 24 h following hormonal stimulation between freshly collected and stored (for 24 or 48 h) semen. Capital letters indicate differences among sperm stripped at 48 h following hormonal stimulation between freshly collected and stored (for 24 or 48 h) semen. Asterisks indicate statistically significant differences between times of stripping (E-stripped and L-stripped) semen at the one-time point; p≤0.05.

DISCUSSION AND CONCLUSION

CASA parameters, determining sperm quality, indicate that the 24 h after hormonal stimulation with Ovopel is more suitable for obtaining semen from goldfish than 48 h. Significant decrease ($P<0.05$) in MOT and all velocity parameters (VCL, VAP and VSL) values of sperm stripped 48 h after hormonal treatment indicates a negative impact of time period following stimulation on milt quality. Such changes suggest some aging process in sperm within testis. The similar tendency but without a significance difference in case of semen obtained from barbel *Barbus barbus* (L.) stimulated by Ovopel was observed (Cejko et al. 2012). Opposite, significant increase in MOT and velocity parameters values after 48 h compared to 24 h following stimulation in ide *Leuciscus idus* (L) were observed (Cejko et al. 2010). This may stem from different thermal regimes used. In the case of barbel and goldfish temperature was 19 and 20°C, respectively, whereas in ide the temperature did not exceed 12°C. However, more detailed research is needed to support such speculation. The value of the key parameter (MOT) after 24-hour storage decreased from 93% and 83% to 55% and 38% for milt obtained at 24 h and 48 h after hormonal stimulation, respectively. After another day of storage, most sperm motility parameters were lower than values measured after 24 h of storage. Our study confirms that without dilution with special buffer, semen of Cyprinids quickly loses its quality (Linhart et al. 2006; Sarosiek et al. 2012).

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P23

EFFECT OF URINE CONTAMINATION ON SEMEN QUALITY PARAMETERS IN EURASIAN PERCH *PERCA FLUVIATILIS* L

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INTRODUCTION

Contamination of semen by urine could change the sperm quality of freshwater fish. Such contamination during milt collection by stripping is unavoidable, owing to the proximity of spermatic duct and urinary duct through a single urogenital pore (Linhart et al 2003, Nynca et al 2012). Freshwater fish urine has a low osmolality and contamination of milt with urine could change osmolality of seminal plasma and thus trigger spermatozoa motility. In the present study, changes in Eurasian perch semen quality (osmolality of seminal plasma and sperm motility and velocity) were investigated in response to increasing the amounts of urine in the milt.

MATERIAL AND METHODS

Semen was collected with a catheter from adult perch (n=3) originated from wild stock in Sasek Wielki lake (NW Poland). Urine was collected from the same perch males using a catheter and pooled prior to the experiment. Semen samples from each individual were mixed with 5, 10, 20, 30 and 50% of urine and stored 30 min following mixing prior to motility analysis. The final volume of mixed sample was 300 µl. The control samples were semen from each individual without urine. Sperm motility and velocity for each sperm sample were examined with CASA system using the Sperm Class Analyzer v. 4.0.0. (Microptic S.L., Barcelona, Spain). Sperm samples (1 µl) were activated at a dilution ratio of 1:200 with tap water supplemented with 0.5% bovine serum albumin. Activated sperm was placed on the Leja slide glasses. Recording of sperm movement started ten seconds after activation. Sperm motility parameters which were chosen for analysis: MOT–percent of motile sperm (%), VCL–curvilinear velocity (µm s^{-1}), VAP–average path velocity (µm s^{-1}), VSL–straight line velocity (µm s^{-1}). To obtain seminal plasma, sperm samples were centrifuged at 10000 ×g. Osmolality of seminal plasma was measured using a Vapor Pressure Osmometer 5600 (WESCOR, Logan, USA). The results were statistically analyzed using Statistica software. CASA and osmolality parameters were compared using one-way ANOVA followed by LSD Fisher post hoc test. Data are presented as means ± SD. All analyses were performed at a significance level of 0.05.

RESULTS

The osmolality of pooled urine sample was 42 mOsm kg^{-1}. Contamination of perch milt with urine led to dose-dependent decrease in seminal plasma osmolality, 284 ± 1, 262 ± 2, 259 ± 2, 229 ± 3, 211 ± 2, and 173 ± 1 mOsm kg^{-1}, for 0, 5,10, 20, 30 and 50 % urine contamination, respectively. The equation of linear regression between seminal plasma osmolality and the percentage of urine contamination was calculated as $y = -20.97x + 309.9$; $R^2 = 0.96$; $p<0.05$. The MOT of milt without urine was 80 ± 2%. The addition of urine to semen caused a significant dose-dependent decline in the values of the MOT, 68 ± 5, 57 ± 5, 60 ± 3, 51 ± 5 and 46 ± 4% for 5,10, 20, 30 and 50% urine contamination, respectively. The equation of linear regression between MOT and the percentage of urine contamination was calculated as $y = -6.01x + 81.01$; $R^2 = 0.91$; $p<0.05$. Exposure of milt with urine contamination also resulted in significant dose-dependent decrease in all velocity parameters of perch spermatozoa (Fig. 1). The equations of linear regression between VCL, VSL, VAP and the percentage of urine contamination were also calculated and shown on Fig 1.

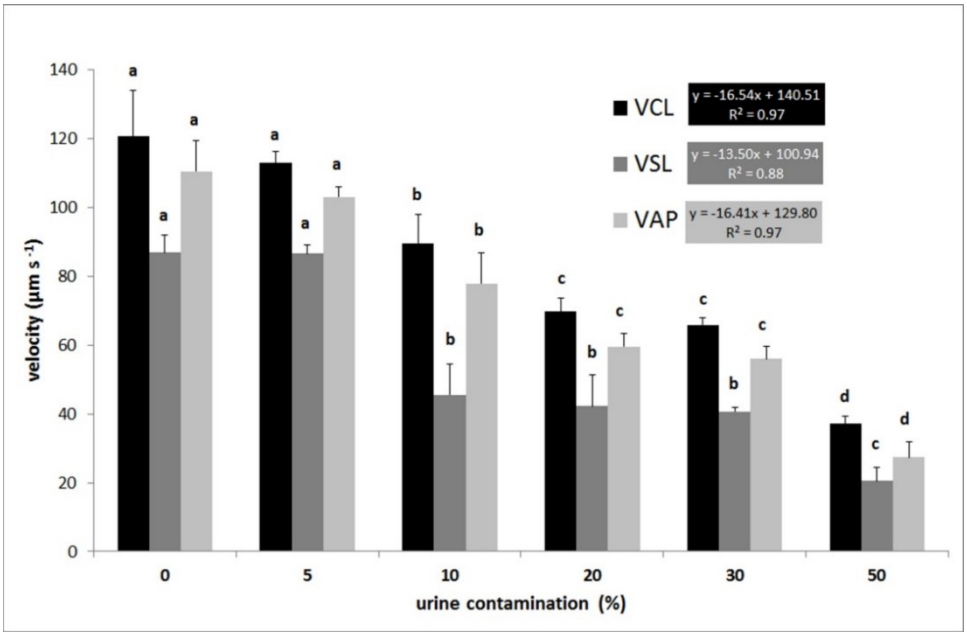

Fig. 1. Curvilinear velocity (VCL), average path velocity (VAP) and straight line velocity (VSL) of perch sperm in relation to urine contamination of milt. Different letters indicate statistically, significant differences between groups $p<0.05$.

DISCUSSION AND CONCLUSION

Osmolality near of 300 mOsm kg^{-1} should be considered as a typical for uncontaminated perch semen and prevents sperm activation in sperm ducts (Alavi et al. 2007). In this study, it has been demonstrated that experimental contamination of perch milt with urine can lead to significant dose-dependent decrease in seminal plasma osmolality. Such contamination significantly reduced percent of perch sperm motility and significantly decreased all velocity parameters of perch spermatozoa. Similar results were reported in other freshwater species: carp (Poupard et al. 1998), tench (Linhart et al. 2003) and rainbow trout (Nynca et al. 2012). In conclusion, it is of important to avoid semen contamination by urine during the stripping procedure in perch, because even a 10% contamination may affect sperm motility negatively and consequently the fertilization capacity of perch sperm. Additionally, the presented results (specifically regression equation between seminal plasma osmolality and the percentage of urine contamination) can be used for determination of the contamination level of perch milt by analysis of osmolality of seminal plasma, when sperm is collected by regular striping procedure (applied widely in the hatchery practice).

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P24

RELATIONSHIP BETWEEN SPERM MOTILITY PARAMETERS, SPERM/EGG RATIO, AND FERTILIZATION RATE IN IDE *LEUCISCUS IDUS* (L.)

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INTRODUCTION

The aquaculture hatcheries have more focused on fish eggs rather than sperm, in spite the use of high quality gametes both from males and females during fertilization is essential in order to achieve suitable reproductive success. Beside quality parameter of both gametes, the sperm/egg ratio is another essential factor which needs to be taken into account during fertilization (Gallego et al 2013). Usually, an excess of sperm is used in experimental fish artificial fertilization. However, in such a case the high number of spermatozoa per oocyte masks the possible negative effect of using low quality sperm, making comparison of different reproductive protocols difficult (Butts et al. 2009). This, in turn, hinders the objective evaluation of the factors having the significant effect on the reproduction outcome. Improvements in this area would allow also a rational use of gametes and more effective broodstock management in terms of number of males needed. Therefore, the main goals of this study were (1) to analyze the effect of the sperm/egg ratio on the fertilization rate and (2) to study the correlations between sperm quality parameters and the fertilization rate in ide, *Leuciscus idus* (L.).

MATERIALS AND METHODS

Reproduction of spawners was carried out according to the methods described by Krejszeff et al. (2009). Sperm concentration was determined by the spectrophotometric method (Ciereszko and Dabrowski, 1993). Sperm motility parameters [MOT– motility rate (%), VCL – curvilinear velocity (μm s^{-1}), VAP– average path velocity (μm s^{-1}), VSL – straight line velocity (μm s^{-1})] were determined using the CASA system (Sperm Class Analyzer v. 4.0.0. by Microptic S.L., Barcelona, Spain). Osmolality of seminal plasma was measured using a Vapor Pressure Osmometer 5600 (WESCOR, Logan, USA). Gametes from six males and three females were separately used for fertilization using all possible combinations (male x female), with eight different sperm/egg ratios (5.0 10^5; 3.5 10^5; 2.5 10^5;1.5 10^5; 10^5; 7.5 10^4; 5.0 10^4 and 2.5 10^4 spermatozoa/egg) for each combination. Eggs from each females were divided into batches of approximately 100 eggs and placed into 60 × 20 mm Petri dishes. A known aliquot of sperm (adjusting the volume according to the calculated sperm/egg ratio) was simultaneously added with 10 ml of water to the corresponding batch of eggs. Eggs were incubated at 15°C in RAS and fertilization rates were evaluated at the eyed-egg-stage (7th day of incubation). All parameters were compared using one-way ANOVA followed by LSD Fisher post-hoc test. Linear regression analysis was used to find the relationship between the different sperm quality parameters and fertilization rate.

RESULTS

Sperm parameters from each male used in the experiment are shown in the Table 1. Despite significant differences in semen parameters between males, no significant correlations between fertilization rate and any sperm motility parameter (MOT, VCL, VSL, VAP), sperm concentration and osmolality of seminal plasma were found. Mean fertilization rate, using ratios ranging from 5.0 10^5 to 2.5 10^4 sperm per egg decreased from 86 to 57%, respectively (Fig. 1). A significant effect of sperm quantity on fertilization rate was noted ($y = -4.4x + 93.6$; $R^2 = 0.97$; $p<0.05$; Fig. 1). Fertilization rate decreased from 10^5 to 2.5 10^4 sperm per egg,

and adding from 1.5 10^5 to 5.0 10^5 sperm per egg had no significant effect on fertilization rate (Fig. 1).

Table 1. Sperm quality parameters from six ide males used in experiment. Values, except sperm concentration, are expressed as mean ± SD (n = 3). Different letters mean significant differences in the same parameter between males ($p<0.05$).

male	MOT (%)	VCL (μm s^{-1})	VAP (μm s^{-1})	VSL (μm s^{-1})	Osmolality (mOsm kg^{-1})	Sperm concentration (10^9 cells ml^{-1})
1	$76±2^{ab}$	$33±3^{ab}$	$27±2^{a}$	$22±1^{a}$	$266±4^{a}$	4.96
2	$79±1^{ab}$	$32±3^{ab}$	$29±3^{ac}$	$25±2^{ac}$	$249±4^{b}$	4.88
3	$71±2^{a}$	$32±4^{b}$	$28±3^{ac}$	$23±3^{ab}$	$234±1^{c}$	4.45
4	$71±5^{ab}$	$39±3^{c}$	$35±3^{b}$	$29±3^{c}$	$263±1^{a}$	6.26
5	$71±4^{ab}$	$36±4^{abc}$	$31±5^{abc}$	$26±5^{abc}$	$233±1^{c}$	7.42
6	$80±6^{b}$	$38±2^{ac}$	$33±1^{bc}$	$28±1^{bc}$	$245±4^{b}$	7.53

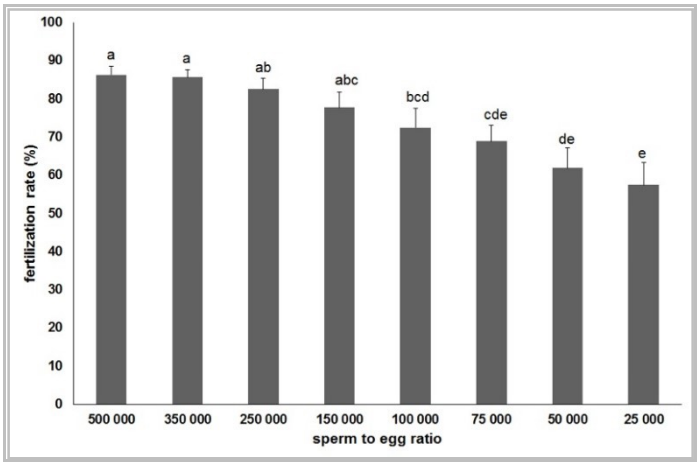

Fig. 1 The effect of sperm to egg ratio on fertilization rate in the ide. Values, are expressed as mean ± SEM (n = 18). Sperm to egg ratios with different letters are significantly different at $p<0.05$

DISCUSSION AND CONCLUSION

Usually, the optimal sperm/egg ratio depends on the sperm quality (Gallego et al. 2013). I our study no significant correlations between fertilization rate and any sperm quality parameters were found. However, this probably stem from the fact that sperm of each of the male used here had similar low velocity parameters. A decrease in fertilization rate was observed down to 1.5 10^5 sperm per egg and adding additional sperm had no significant effect on the dependent variable. Summing up, this gamete ratio is now recommended for studies dealing with ide fertility, as it secured maximal fertilization rate with the use of lowest quantity of sperm possible.

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P25

THE ROLE OF CA^{2+} TRANSPORT AND PLASMA MEMBRANE CA^{2+}-ATPASE (PMCA) ACTIVITY IN MEMBRANE POTENTIAL ALTERATION DURING BESTER SPERM MOTILITY

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INTRODUCTION

Sperm motility in sturgeons is initiated mostly because of rapid alteration of environmental ionic composition (i.e. decrease of K^+, Na^+ and Ca^{2+} concentrations) (Alavi and Cosson, 2006) These changes might results in activation of ion channels and re-equilibration of intracellular ions compositions that are closely related to membrane potential alteration. Among other ions, transport of Ca^{2+} was described to be involved in regulation of membrane potential with further sperm motility activation in trout (Krasznai et al., 2003; Boitano and Omoto 1991). Additionally, Ca^{2+} transport is a well-known participant in sturgeons sperm motility activation. Thus, the two main goals of the present study were to investigate the role of Ca^{2+} in membrane potential alteration during sturgeons sperm motility and to detect the role of plasma membrane Ca^{2+}-ATPase activity in regulation of membrane potential and further sperm motility.

MATERIALS AND METHODS

Sperm from 6 mature bester males (provided by Fujikin Inc. (Tsukuba, Japan)) was collected by routine method and experiments were carried out at the Misaki Marine Biological Station, Japan. Spermatozoa motility parameters (VCL, motility % and duration) were analyzed at 8 s after sperm activation in media of different ionic composition (AM: 1) 25mM NaCl, 2) 25mM NaCl + 2mM EGTA, 3) 50mM Sucr, 4) 50mM Sucr + 2mM EGTA, prepared on 10mM Tris HCl, pH 8.5) by using of CASA system SCA 5.2. For measurement of membrane potential, sperm was incubated with 5 µM Oxanol VI and 1 µM CCCP for 5 min at 20°C. Later on, sperm was suspended 1:50 with activating media and fluorescence was monitored with a fluorescence spectrophotometer (Hitachi 650-10S, Tokyo, Japan) at 523/630 nm excitation/emission wavelength pair. Relative alteration of membrane potential was calculated at 5, 20, 40 and 60 s post activation by formula: $A_{MP}=((I_{IM}/I_{AM})-1)*100\%$, where I_{IM} and I_{AM} are intensities of fluorescence signal in immobilizing (120 mM NaCl, 7 mM KCl, 4 mM $CaCl_2$, 10mM Tris-HCl, pH 8.5) and activating media respectively. For investigation of the Ca^{2+} role, sperm was incubated with 10 µM ionomycin for 5 min at 20°C. For inhibition of PMCA activity, sperm was incubated in 20 µM of carboxyeosin (CBEN) for 5 min at 20°C. Additionally, PMCA was detected by Western Blotting method. For this, proteins were separated on a 12% SDS-PAGE. After electrophoresis, gels were placed on nitrocellulose membranes and electrically transferred. The membranes were blocked and subsequently incubated with anti-PMCA antibodies (5F10) in 5% BSA in TBST (0.1% Tween-20, 20 mM Tris-HCl, 150 mM NaCl [pH 7.6]) (BSA-TBST). Membranes were then washed and incubated with HRP-conjugated Goat Anti-mouse IgG (1:10,000 in 5% BSA-TBST). Reacted proteins were revealed with TMB.

RESULTS

Native sperm was motile in all studied media. Presence of EGTA leads to decrease of motility period but does not affect VCL and motility %. Activation of bester sperm is accompanied by depolarization of membrane in all studied media, but significantly a higher membrane potential was detected in Na^+ containing media. Further motility period is accompanied by decrease of depolarization level,

and relative membrane potential reaches a plateau at 60 s after activation. Ionomycin treatment leads to hyperpolarization of the membrane at 5 s after activation that is accompanied by complete suppress of motility in all studied media. CBEN treatment does not affect VCL and motility %, however it leads to highly increased depolarization level in all studied media. Significant decrease of motility duration was detected in CBEN-treated sperm in comparison to native one. Immuno-blots Incubated with Anti-PMCA ATPase antibody revealed a protein band at 140 kDa MW.

DISCUSSION AND CONCLUSIONS

Our results allow to suggest that depolarization of membrane is a key factor of bester sperm motility activation and that the main participant in this process is transport of Ca^{2+}. Additionally, we propose Na^{+} transport as another participant of depolarization of sperm membrane, since the relative membrane potential in Na^{+} containing media is significantly higher than in Na^{+}-free media (Sucrose). High depolarization level, resulting from inhibition of PMCA, does not affect motility % and VCL, but motility duration: this leads us to hypothesize that PMCA is not directly involved in motility activation of bester spermatozoa.

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P26

ANNUAL CYCLE OF GONAD DEVELOPMENT IN BREAM MALES (*ABRAMIS BRAMA* L.) FROM LOWER ODER RIVER SECTIONS DIFFERING IN THE INFLUENCE OF COOLING WATER

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INTRODUCTION

The temperature of the water inhabited by fish is one of the most significant factors affecting their lives. The factor has also an effect on reproduction characteristics such as sex determination, age of sexual maturity, gametogenesis dynamics, duration of the reproductive season and gamete quality (Breton *et al.* 1980; Billard 1986). In fish living in heated water, accelerated maturation, extension of the reproduction period, as well gonadal malfunctions were observed (Mattheeuws et al. 1981; Lukšjenė et al. 2000). The sexual cycle of female bream from three locations in north-western Poland under varied influence of post-cooling water was described by Domagała et al. (2015). The aim of this study was to analyze the annual cycle of male gonad maturation at those sites.

MATERIALS AND METHODS

Adult males of the bream (*Abramis brama* L.) (Pisces, Cyprinidae) were caught from three aquatic environments: (1) the eastern Oder River, up to 20 km above the Dolna Odra power plant; (2) the Warm Canal with post-cooling water, 200 m from the Dolna Odra power plant, Nowe Czarnowo, near Gryfino, NW Poland (53°11' N, 14°29' E); the canal is open and fish can move freely (3) Lake Dąbie, 20 km below the Dolna Odra power plant. Altogether, material from 288 males was collected and prepared on slides. The evaluation of gonadal maturity stage was done using the 6-grade scale published by Domagała et al. (2014).
The average length of the bream males from the Oder River (34.0 cm) was greater than that of the males from Lake Dąbie (32.2 cm) and Warm Canal (29.4 cm).

RESULTS

The bream males of the thermally natural water above and below the Warm Canal were ready for reproduction from mid-April to the beginning of June. The resorption of the unreleased spermatozoa was quick. In June, the proliferation of spermatogonia in cysts for a new cycle began. In the autumn, gonads with a few cysts containing early primary spermatocytes (early stage 3) appeared in some males. Other males in the winter were at stage 2 and contained cysts with numerous type B spermatogonia. From February, numerous spermatocytes became visible in the gonads. Spermatozoa in gonads at stages from late 3 to 5 were observed in April. Stage 5 was noticed in one male in the first half of June. Some males of size above 28 cm did not mature in that breeding season.
Males from the Warm Canal became sexually mature one month earlier (March) than those caught in the neighboring waters. In this location, males maintained the ability to spawn longer than fish in natural waters. No anomalies in spermatogenesis were determined in the warmed waters.

DISCUSSION AND CONCLUSIONS

Histological studies of the reproductive cycle of bream are based mostly on females (Kopiejewska et al. 1993; Fulga et al 2000). Seldom papers deal with cytological changes in male gonads (Brylińska, Długosz 1973).
In the studied area, the bream males were ready for reproduction in the same period as females, although they were ripe short time before females. The spawning season of the studied bream started at the end of April and lasted through May and June (Domagała et al. 2015), earlier than in other water reservoirs in Poland (Brylińska, Długosz 1970; Kopiejewska et al. 1993). The spawning season of the bream from Costesti-Stânca was shorter, lasting from May to mid-June (Fulga et al. 2009).

In the conditions of the canal with post-cooling water, at a temperature on average 6–8°C higher than that of the water outside the canal, the males became sexually mature one month earlier than those in the neighboring waters and produced spermatozoa for a longer period. A similar offset in the sexual cycle was observed in other species living in water with increased temperature (Mattheeuws et al. 1981).

In the Warm Canal, lower number of fish was caught. Although mature males were found, no ripe bream females were encountered. The conditions in this canal, with faster water current and higher temperature, were unfavorable for the spawning of the species.

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P27

COMPARATIVE CHARACTERISTICS OF SPERMATOZOA MOTILITY TRAITS IN SOME SPECIES OF CYPRINIDS

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INTRODUCTION

It is known that the nature of spermatozoa motility depends on many determinants, such as age of fish, time of sampling during the breeding season, frequency of milt ejaculation, fish nutrition and health. External factors triggering motility have an equally important effect on spermatozoa movement. The main external factor is activation medium composition, including ions, pH, CO_2, organic compounds, attractants and pollutants, and their osmolality (Lahnsteiner et al. 2004; Alavi, Cosson 2005, 2006; Cosson et al. 2008a). Moreover, the temperature of activation medium and glass chamber placed on the microscope table strongly influence movement parameters. Additionally, technical procedures during motility detection, the manner of mixing sperm with activation medium, the time that elapses from activation to the beginning of detection, as well as algorithm settings in the software, affect the final value obtained. These different circumstances set by different authors hinder the comparison of the results obtained in different scientific centers. In view of such background, the aim of this study was to compare spermatozoa movement characteristics of four cyprinid fish species previously belonging to the *Abramis* genus.

MATERIALS AND METHODS

During the middle period of the spawning season, the milt from adult males of the common bream (*Abramis brama* L., 1758), the blue bream (*Ballerus ballerus* L., 1758 syn. *Abramis ballerus*), the white bream (*Blicca bjoerkna* L., 1758 syn. *Abramis bjoerkna*), as well the vimba bream (*Vimba vimba* L., 1758 syn. *Abramis vimba*), was stripped. The analysis of spermatozoa motility parameters was performed in 30 mM NaCl, 20 mM Tris, pH 8.0, with the addition of 0.1% BSA, at a temperature of 15°C. The temperature of the activation solution in the tube was maintained at 15°C using a cooling block (FINEPCR, Korea). The microscope table was equipped with a cooling device (Semic Bioelektronika, Kraków, Poland) set to 15°C. Spermatozoa motility was evaluated using Sperm Class Analyzer (SCA) v. 4.0.0 by Microptic S.L., Barcelona, Spain. Half-second films were recorded at 8–10 s and every 10 s until 150 s of swimming, and then every 30 s until active swimming phase was ceased. The curvilinear velocity > 20 μm s^{-1} and VSL > 10 μm s^{-1} were the criteria of motility. The milt (1–2 μL) was added to 300 μL of the appropriate activating solution in a 1.5 mL polyethylene Eppendorf tube. After intense stirring of the tube contents for 2 to 3 s, 1.2 μL of such diluted solution was immediately placed into a well of a 12-well multi-test glass slide (MP Biomedicals LLC, Eschwege, Germany) and covered with a coverslip. Nine parameters characterizing motility were chosen for the analysis: 1. MOT — percentage of motile spermatozoa; 2. VCL — curvilinear velocity 3. VAP — average path velocity; 4. VSL — straight-line velocity; 5. LIN — linearity; 6. STR — straightness; 7. ALH — amplitude of lateral head displacement 8. BCF — track crossing frequencies 9. motility duration. In order to compare typical spermatozoa motility parameters of the species, milt with the motility of over 80% was selected.

RESULTS

The average duration of the active phase of spermatozoa motility in the blue bream (mean 180 s) was significantly longer than that in the common bream and the white bream (60–70 s). Motility duration in the vimba bream was intermediate (90 s) ($p < 0.001$, Kruskal-Wallis test). At 10 s post-activation, the curvilinear velocity in the blue bream with a mean of 41 μm s^{-1} was significantly lower than VCL of three other species studied ($p < 0.001$, one-way ANOVA). VCL of the common bream, the white bream and the vimba bream was similar, with the range

of mean values from 76 to 83 μm s^{-1}. VAP and VSL developed a similar arrangement. There were no significant differences in LIN and STR between the species. The mean value of these parameters ranged from 80 to 84% and from 87 to 91%, respectively. ALH in the blue bream (0.62 μm) was lower than in the white bream and the vimba bream (0.84 and 0.80 μm, respectively) but similar to that in the common bream (0.75 μm). BCF in the blue bream (14.5 Hz) was similar to that in the white bream and the vimba bream (13.6 and 14.2 Hz), and different from that in the common bream (14.8 Hz).

DISCUSSION AND CONCLUSIONS

Although cyprinids are the most numerous family of fish, little data on the characteristics of sperm movement in these fish can be found in the literature. In the studied species, some analyzed spermatozoa motility parameters were different. The duration of spermatozoa motility in the common bream and the white bream lasted approx. 1 min, which was similar to that observed in the chub, another representative of cyprinids (Lahnsteiner et al. 2004). The duration of motility in other freshwater fish usually does not exceed 1 min (Dziewulska et al. 2011). In the vimba bream and the blue bream, the time in which sperm was moving was longer, while in the latter species, longer than that measured in the carp (Perchec et al. 1995; Ravinder et al. 1997). Only in acipenserids, the motility phase was prolonged (Toth et al. 1997). In marine fish, the duration of motility was extended to a few minutes, but the spermatozoa of these species were introduced into a hyperosmotic environment in which no water absorption is observed (Cosson et al. 2008b). The movement in the blue bream differed from that in the other species in velocity, which was the slowest, even below 50 μm s^{-1}. The spermatozoa of the other studied species moved at a VCL of approx. 76–83 μm s^{-1}. The velocity in the carp was higher but the parameter was determined at a higher temperature (Perchec et al. 1995; Ravinder et al. 1997). The velocity of spermatozoa in some species of salmonids, percids and other families was higher than that in the studied cyprinids (Alavi et al 2007).

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P28

COMPARATIVE ANALYSIS OF SPERM MOTILITY OF THREE SPECIES, *CYPRINUS CARPIO*, *ONCORHYNCHUS MYKISS* AND *ACIPENSER RUTHENUS* AT VARIOUS TEMPERATURES

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INTRODUCTION

Sperm motility is considered as the main parameter that reflects accurately sperm viability and fertilizing ability. One of the major environmental factors involved in the control of reproductive activity is temperature (Emit et al. 1989), the latter greatly influencing sperm motility (Alavi and Cosson, 2005); therefore, understanding function of this crucial factor can be helpful to improve methods of artificial reproduction (Alavi et al., 2007). The effect of temperature variation over optimal ranges on the motility variables has been poorly investigated among fish species. The aim of present study was to investigate how temperature (4, 14 and 24° C) affects the sperm motility variables in *Cyprinus carpio*, *Oncorhynchus mykiss* and *Acipenser ruthenus*, all three being of commercial value and belonging to representative fish families, Salmonidae, Cyprinidae and Acipenseridae. Temperature was investigated in ranges of ecological relevance to natural spawning, i.e. 16–22°C for *C. carpio*, 4–8°C for *O. mykiss* and 13–15°C for *A. ruthenus*.

MATERIALS AND METHODS

Semen of *C. carpio*, *O. mykiss* and *A. ruthenus* was obtained from hatchery of the Research Institute of Fish Culture and Hydrobiology. For motility activation, 10 mM Tris–HCl buffer, pH 8.0, 1mM $CaCl_2$, 0.125 % pluronic acid was used as activation medium (AM). The motility was recorded using a CCD video camera (Sony, SSCDC50AP) mounted on an inverted microscope equipped with a cooling stage (Olympus IX83) and using a X20 NIC contrast objective lens. Recordings were analyzed to estimate curvilinear velocity of spermatozoa (VCL, $\mu m.s^{-1}$), percentage of motile spermatozoa after activation (motility %), and motility duration (s) in triplicate for each fish sample. Temperature of activating medium was adjusted by a thermoblock (HLC BO50/15) and monitored during motility period by copper-constantan thermocouple (Omega, L-044T) via data logger thermometer (Omega, HH127). Data were analyzed by ANOVA (Statistica 10, Statsoft Inc, USA). Statistical significance was considered at $P<0.05$.

RESULTS

The percentage of motile spermatozoa revealed no differences in the temperature ranges investigated in all three species.

Cyprinus carpio

While the duration of motility decreased at 24°C, 10s after activation the velocity was significantly higher at 24°C than at 4°C and 14°C [Fig. 1(a)]. After 30 s, the velocity was higher at 14°C than at 4°C and 24 °C [Fig. 1(a)].

Oncorhynchus mykiss

At 10s post-activation, the motility duration was highest at 4°C, and velocity was highest at 14°C [Fig. 1(b)]. After a motility period of 20 s, velocity was higher at 4°C than at 14°C and 24°C [Fig. 1(b)].

Acipenser ruthenus

The velocity was higher at 24°C compared to 4°C and 14°C till 180s post-activation [Fig.1(c)].

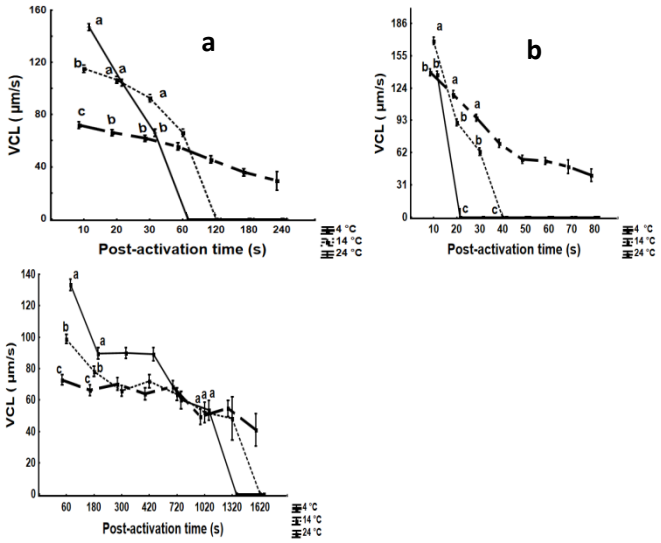

Fig.1. Curvilinear velocity of spermatozoa at different temperature as a function of time after motility activation. a- *Cyprinus carpio*, b-*Oncorhynchus mykiss*, c- *Acipenser ruthenus*. Values with different letters are significantly different (Tukey test, P<0.05).

DISCUSSION AND CONCLUSIONS

This study presents the temperatures effect on sperm motility variables of *C. carpio*, *O. mykiss* and *A. ruthenus*. Variables affected were the motility duration and the swimming velocity. Temperatures yielding the highest sperm motility rates were 4°C for *C. carpio, O. mykiss, A. ruthenus.* They were 4-6°C For *Salmo trutta* and *Lota lota,* 8-16°C for *Thymallus thymallus* (Lahnsteiner and Mansour, 2012) and 12-20°C for *Perca fluviatilis* (Lahnsteiner, 2011). The observation that the duration of sperm motility is temperature dependent with an increase in duration at low temperatures is in conformation with many previous studies (Lahnsteiner, 2011; Lahnsteiner and Mansour, 2012). In the present study, sperm velocity at 10s after activation was increased at 24°C in *C. carpio and A. ruthenus*. Similar results were found for *Solea senegalensis* and sturgeon (Cavaleiro Diogo, 2010; Ginzburg, 1972). In contrast, Lahnsteiner and Mansour (2012) have stated that sperm velocity did not increase and in some cases even decreased with temperature (in range 2-14 °C) in *T. thymallus and S. trutta*. In summary, the duration of motility and velocity of sperm depend on temperature of swimming medium and further investigations are required to identify the underlying mechanism of enzymes activity and ions regulation on how sperm metabolism is affected by temperature.

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P29

ENZYME ACTIVITY IN ENERGY SUPPLY OF SPERMATOZOON MOTILITY IN TWO TAXONOMICALLY DISTANT FISH SPECIES

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INTRODUCTION

As spermatozoon motility duration differs significantly among fish species, the mechanism of ATP generation/regeneration and its distribution along the flagellum may be species-dependent. The relative contribution of adenylate kinase (AK) and creatine kinase (CK) in ATP regeneration and function of the creatine-phosphocreatine (Cr/PCr) shuttle in spermatozoa have been thoroughly investigated only in rainbow trout (Saudrais et al., 1998). The current study compared the role of CK with that of AK in ATP regeneration during motility of demembranated spermatozoa of taxonomically distant fish species, sterlet *Acipenser ruthenus* and common carp *Cyprinus carpio*.

MATERIALS AND METHODS

Experiments were conducted using common carp and sterlet spermatozoa, based on their differing motility duration, modes of motility activation and ultra-structure. The flagellar beat frequency (BF) of demembranated spermatozoa was measured in reactivating media (RM) in presence or absence of ATP, ADP, PCr, fluorodinitrobenzene (FDNB, a CK inhibitor), and P^1,P^5-di(adenosine-5') pentaphosphate (diApp, an AK inhibitor), using stroboscopic illumination and dark field microscopy with reference to calibrated frequency of flash illuminator. AK, CK, and total ATPase activity was measured in Triton-treated sperm extracts.

RESULTS

Beat frequency of demembranated spermatozoa showed positive correlation with ATP level in RM. At 0.05 mM ATP, carp spermatozoa showed slight motility, but it was possible to estimate their BF (27 Hz) only with ATP in RM at 0.1 mM or higher. Carp spermatozoa BF reached a plateau value of 70 Hz with 0.8–1.2 mM ATP. In sterlet, flagella BFs were 38 Hz with 0.1 mM ATP, and reached a plateau (63 – 67 Hz) at 0.6 mM ATP. Apparent Michaelis constant (K_m) for ATP was 180 ± 8 μM for carp and 79 ± 4 μM for sterlet.

For evaluation of the relative contribution of AK and CK to the energy supply of carp and sterlet spermatozoa, flagella BF was measured in RM containing 0.5 mM ADP and 15 mM PCr as well as with either or both diApp and FDNB. In carp spermatozoa, maximal BF reached 60 Hz (ADP + PCr), 63 Hz (ADP + PCr + diApp), 42 Hz (ADP + PCr + FDNB), and 36 Hz (ADP + PCr + diApp + FDNB) (Fig. 1).

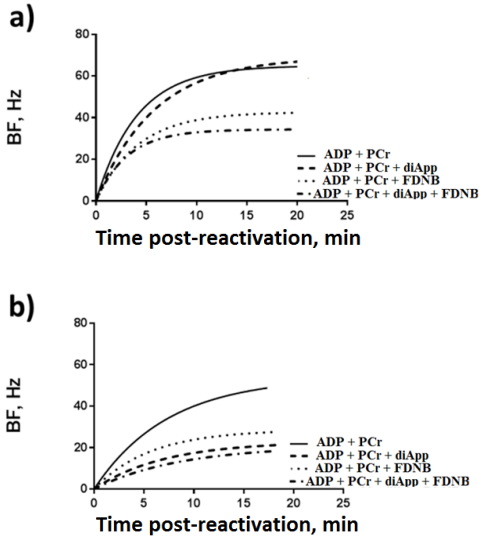

Figure 1. Time vs. beat frequency in demembranated carp (a) and sterlet (b) spermatozoa reactivated in medium with 0.5 mM ADP and 15 mM PCr in presence or absence of FDNB and diApp.

Sterlet BF was sensitive to the presence of inhibitors of both AK and CK, showing BF in the following order: 50 Hz (ADP + PCr), 41 Hz (ADP + PCr + FDNB), 31 Hz (ADP + PCr + diApp), and 17 Hz (ADP + PCr + diApp + FDNB) (Fig. 1).
In sperm extracts, CK activity showed the highest absolute values in both carp and sterlet (Table 1). Sterlet spermatozoon extracts displayed approximately three-fold CK activity and two-fold the ATPase activity observed in carp. Conversely, sterlet spermatozoon extracts showed significantly lower AK activity compared to carp.

Table 1. Enzyme activity in energy supply in demembranated carp and sterlet spermatozoon soluble extracts

Activity	Carp (n = 5)	Sterlet (n = 6)
Adenylate kinase, mU/10^9 spz	6.37 ± 0.69	1.57 ± 0.11*
Creatine kinase, U/10^9 spz	80.0 ± 5.4	223.7 ± 22.4*
ATPase, U/10^9 spz	0.48 ± 0.02	1.03 ± 0.07*

* – within rows indicates significant difference ($P < 0.05$). The data are presented as mean ± SE.

DISCUSSION AND CONCLUSIONS

When demembranated, spermatozoa of both species exhibit dramatically increased flagella BF and motility duration: maximum BF with 1.0 mM ATP was higher than that previously reported in native sterlet and carp spermatozoa (Boryshpolets et al., 2013; Perchec et al., 1995). Demembranated carp and sterlet spermatozoa were motile for more than 10 min, in contrast to 30-40 s in native carp spermatozoa (Billard et al., 1995) and 4 min in sterlet (Lahnsteiner et al., 2004). The lower K_m in sterlet suggests that sterlet axonemal dynein ATPases have a higher degree of affinity to ATP than do those of carp. The presence of a low ATPase activity in soluble extracts could come from mitochondrial- and/or membrane-ATPase.
The obtained results testify that the primary contributor to energy supply in carp was CK, while AK appeared mostly crucial for the maintenance of flagella movement of sterlet spermatozoa. Altogether, our observations indicate that spermatozoa of taxonomically distant fish species utilize similar systems to supply energy for flagella motility, but with different efficacy for their movement.

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P30

AXONEMAL PHOSPHO-PROTEINS AND SIGNALING ENZYMES INVOLVED IN SPERMATOZOA MOTILITY OF STERLET (*ACIPENSER RUTHENUS*)

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INTRODUCTION

Sperm motility represents a very important prerequisite leading to the contact and fusion between male and female gametes at fertilization. It is known that protein phosphorylation / dephosphorylation plays a crucial role in signal transduction in transcriptionally inactive cells, such as spermatozoa. The number of protein structures in spermatozoa axoneme is known to be regulated by phosphorylation / dephosphorylation (Inaba et al., 1998; Itoh et al., 2003). However, most of those proteins in fish are still to be characterized.

The phosphorylated state of proteins is controlled by various kinases, which regulate spermatozoa intracellular signaling. The present study was undertaken in order to provide an essential knowledge about the molecular mechanisms of motility activation, roles of protein phosphorylation and protein kinases in spermatozoa of valuable aquaculture specie, *A. ruthenus*. Specifically, we wish to determine how the protein phosphorylation pattern changed after motility activation in sterlet spermatozoa, and to identify phospho-proteins involved in spermatozoa motility.

MATERIALS AND METHODS

Broodstock handling and collection of gametes

Spermiation in sterlet was stimulated by intramuscular injection of carp pituitary powder dissolved in 0.9% (w/v) NaCl solution at 5 mg kg^{-1} of body weight, 24 hr prior to stripping. Sperm was obtained from adult sterlet males (n = 6; body weight, 0.5-2 kg). Samples were stored on ice (0–4 °C) in closed assay tubes prior to analysis or further processing.

Sample preparation

Separate sperm samples from each experimental male were centrifuged at 300g for 30 min at 4 °C to remove seminal plasma. Sperm pellets were diluted either with immobilizing or activation medium at a dilution ratio of 1:50. Sperm samples were frozen in liquid nitrogen at different time points after motility activation (0, 30, 60, 120 s) and stored at -80 °C. Proteins were extracted from spermatozoa with lysis buffer containing phosphatase inhibitors and protease inhibitors. The BCA assay was used to determine the protein concentration in samples.

Western blotting analysis

Proteins were separated on a 12% gel either with one-dimensional SDS-PAGE or two-dimensional (2D) gel separation. After electrophoresis, gels were placed on PVDF membranes and electrically transferred. The membranes were blocked and subsequently incubated with polyclonal antibodies to phospho-tyrosine, phospho-serine and phospho-threonine or to phospho-(Ser/Thr) PKA and phospho-(Ser) PKC substrates in 5% BSA in TBST (0.1% Tween-20, 20 mM Tris, 150 mM NaCl [pH 7.6]) (BSA-TBST). Membranes were then washed and incubated with HRP-conjugated goat anti-rabbit IgG (1:3000 in 3% BSA-TBST). Reacted proteins were revealed with ECL-plus. Total protein loading was controlled after immunodetection by staining the blot with 0.1% Coomassie Brilliant Blue R-250 in isopropanol.

In-Gel Digestion and Mass Spectrometry
Gels after 2D separation were washed in water, and protein spots (corresponded to phosphorylated spots after 2DE and western blot) were cut from gels. The gel pieces were de-stained and treated with 1 μg of modified sequencing-grade trypsin. After digestion, the peptides were concentrated and desalted using Zip-Tip pipette tips. A 5 mg/ml solution of α-cyano-4-hydroxycinnamic acid (α-CHCA) in 50% acetonitrile-0.1% TFA was used as a matrix. Peptides were analysed by MALDI-TOF MS/MS.

RESULTS

Results of western blotting showed that phosphorylation pattern on three amino acid residues (serine, threonine and tyrosine) changed after motility activation. Incubation with phosphoserine antibodies revealed 10 protein bands of 120, 90, 70, 60, 55, 52, 40, 30, 27 and 25 kDa to be phosphorylated / dephosphorylated after motility activation in sterlet spermatozoa. A total of 8 proteins of 125, 68, 60, 50, 48, 40, 25 and 20 kDa were dephosphorylated on tyrosine residue. In sterlet spermatozoa 5 PKA substrate protein bands with molecular weight of 150, 120, 45, 42 and 27 kDa changed phosphorylation state after motility activation. The antibody to phospho-(Ser) PKC substrates revealed that one protein band of 200 kDa was phosphorylated in immotile spermatozoa and and remained so throughout the motility period.
Results of mass spectrometry identified 18 of 38 selected phosphorylated protein spots in sterlet spermatozoa.

DISCUSSION AND CONCLUSIONS

In the current study results of western blotting with phospho-(Ser/Thr) PKA substrate antibody showed that PKA is involved in motility activation and/or regulation in spermatozoa of *A. ruthenus*. The obtained results suggest that in fish spermatozoa PKA is involved not only in phosphorylation of axonemal proteins as it was shown before (Itoh et al., 2003), but also plays an important role in oxidative phosphorylation and glycolytic pathway. The abundance of metabolic enzymes participating in sterlet sperm motility may explain the relatively long period of their movement (up to 3 min), compared to other freshwater fish species (60 sec in carp), as well as slow ATP decrease in spermatozoa during motility period (Billard et al., 1999). Involvement of other phospho-proteins in sterlet spermatozoa motility implies the complex network of signaling molecules and enzymes involved in sperm motility activation and regulation.

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P31

OPTIMAL SPERM SUBPOPULATION SELECTION IN *SOLEA SENEGALENSIS*

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INTRODUCTION

Solea senegalensis is a promising species for European Aquaculture, however individuals born in captivity display a reproductive failure that hinders the sustainable culture of this species. The reproductive problem has been focused on males reared in captivity. It has been demonstrated that wild males held with captivity breed females produced viable spawns, but non viable spawns were obtained in the opposite situation (Mañanós et al., 2007, Carazo 2013). The absence of courtship is not the only reproductive problem in this species. Sperm quality is in general low, and spermatozoa show low resistance to hyperosmotic shock, high levels of DNA fragmentation, high levels of apoptotic cells and also display different and heterogeneous sperm subpopulations within the same seminal sample (Beirao et al 2009; Beirao et al 2011).

The aim of this study is to implement a sperm selection method for optimal sperm subpopulation recovery. In particular, a method to eliminate apoptotic spermatozoa has been used. Annexin V binds to phosphatidylserine, which is externalized to the outer surface of the sperm membrane in early apoptosis. In this study Annexin V-MACS beads have been used for the separation of apoptotic spermatozoa. This technique has been used in sperm samples from wild and F1 individuals. In order to confirm the efficiency of the technique, apoptotic cell population was studied by flow cytometry using YOPRO-1 and a caspases detection kit.

MATERIALS AND METHODS

Sperm samples from adult Senegalese sole males (wild and F1 broodstocks from el Bocal, IEO, Santander) were obtained by gently pressing the testes on the fish pigmented side. Ejaculates were pooled (3-5 males/pool) attending to similar motility parameters to avoid the influence of the sperm quality, to get volume enough to perform the study and following the routine in Aquaculture companies prior to artificial fertilisation.

Each pooled sample was split into two aliquots. One of them was directly cryopreserved following the only published protocol for this species so far (Rasines et al 2013). Sperm was diluted (1:2 ratio) in Mounib extender with cryoprotectants (10% BSA and 10% DMSO), loaded into 0.5 mL French straws, exposed to liquid nitrogen vapour during 7 min and rinsed into liquid nitrogen until used. This aliquot was considered as control in the experiment.

Magnetic activated cell sorting (MACS) was performed with the other aliquot using a MiniMACS separation unit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) following manufacturer's instructions. Magnetically labelled cells (apoptotic cells) flushed from the MS columns were discarded and the apopototic-reduced elution was cryopreserved following the same protocol as the control.

Samples were thawed (37 ºC, 7 s) and washed prior to cytometer analysis. Two apoptosis detection protocols were carried out: 1) YOPRO-1 (150 nM) (Invitrogen, Leiden, The Netherlands) and 2) CaspGLOW Fluorescein Active Caspase Staining Kit (eBioscience, San Diego, USA) following manufacture's instructions. Both of them were co-stained with PI (2 µg/mL) (Sigma, Madrid, Spain).

After the incubation time samples were analyzed twice in a FACSort Plus Analyzer (Becton–Dickinson, USA) acquiring 10000 events per replicate. The red fluorescence emitted by PI was detected using a 610 nm filter and the green fluorescence emitted by the active caspase detection kit (FITC) and YOPRO-1

with a 516 nm filter.
For each staining, significant differences between the apoptotic cell percentage (green positive/red negative cells for each protocol) between control and MACS samples were evaluated by ANOVA with a SNK (Student- Newman-Kleus) multiple range test ($P < 0.05$). All statistical analyses were conducted with SPSS software (version 20.0).

RESULTS

Results demonstrated that Magnetic Activated Cell sorting eliminated apoptotic cells from *Solea senegalensis* seminal samples. In samples obtained from captured males, apoptotic cell population significantly decreased after the process. This decrease is observed independently of the assessment method used (caspases and YO-PRO-1). However, in F1 individuals, only caspase-positive-cells significantly decreased after the selection. Moreover, the comparative study between seminal samples from captured and F1 individuals showed significant differences only in caspase positive cells, being the percentage of YO-PRO-1 positive cells similar in both cases.

DISCUSSION AND CONCLUSIONS

This study demonstrated that caspase determination is more specific than YO-PRO-1 in the calculation of apoptotic cells in *S. senegalensis* seminal samples. The percentage of apoptotic cells (caspase positive) is, as expected, significantly higher in F1 seminal samples. Magnetic activated cell sorting is applicable in this species for non-apoptotic spermatozoa recovery, but fertility trials must be done to confirm the real potential of the technique.

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P32

INFLUENCE OF CRYOPROTECTANTS ON STERLET *ACIPENSER RUTHENUS* SPERM QUALITY, ANTIOXIDANT RESPONSES AND RESISTANCE TO OXIDATIVE STRESS

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INTRODUCTION

Most of the world's sturgeon populations have experienced significant decline, mainly due to over-fishing, habitat destruction, and pollution (Pikitch et al., 2005). To exceed this limitation, various sperm storage methods are frequently used in fish farms. Cryopreservation methods for sturgeon spermatozoa have been established and modifications of the procedures for species-specific optimization have been proposed. The major factors affecting the results of insemination with frozen/thawed semen are the addition of cryoprotectants. Therefore, the main goal of this study was to investigate the effects of the cryoprotectants methanol (MET), dimethyl sulfoxide (DMSO), dimethylacetamide (DMA), and ethylene glycol (EG) on sperm quality, antioxidant defence, and resistance of sterlet *Acipenser ruthenus* spermatozoa to oxidative stress after cryopreservation, using the xanthine-xanthine oxidase (X-XO) system as a model reactive oxygen species (ROS) inducer.

MATERIALS AND METHODS

Broodstock handling and collection of gametes

Six male sterlet (7 years, 2.5–3.0 kg) were used. Spermiation was stimulated by intramuscular injection of carp pituitary powder dissolved in 0.9% (w/v) NaCl solution at 4 mg/kg of body weight, 36 h before sperm collection.

Experimental protocol

Ten microliters of sperm was diluted in 990 µL immobilizing solution (IS) (20 mM Tris-HCl, 30 mM NaCl, 2 mM KCl, pH 8.5) for a final volume of 1mL. ROS were generated using the X–XO system. Samples were subjected to treatment periods of 5 and 30 min at 4 °C. The experimental groups were: control 10 µL of fresh sperm diluted in 990 µL of IS; Four 10 µL groups of frozen/thawed sperm each containing one of the cryoprotectants (MET, DMA, EG, DMSO) diluted in 990 µL of IS; Four 10 µL groups of frozen/thawed sperm each containing one of the cryoprotectants (MET, DMA, EG, DMSO) plus 0.1 mM X–0.0125 U/mL XO diluted in 986.5 µL of IS; Four 10 µL groups of frozen/thawed sperm each containing one of the cryoprotectants (MET, DMA, EG, DMSO) plus 0.3 mM X–0.025 U/mL XO diluted in 982 µL of IS; Four 10 µL groups of frozen/thawed sperm each containing one of the cryoprotectants (MET, DMA, EG, DMSO) plus 0.6 mM X–0.05 U/mL XO diluted in 974 µL of IS.

Sperm motility and velocity recording

Sperm activity was video recorded using dark-field microscopy (Olympus BX 50; stroboscopic lamp Strobex 9630) to evaluate motility and velocity. Using a CCD video camera (Sony, SSC-DC50AP), the microscopic field was transferred to a video monitor and recorded with a S-VHS system (Sony, SVO-9500 MDP). The strobe frequency was set to automatic register with video frames (50 Hz) for sperm velocity measurement. Motility and velocity were examined at 20x objective magnification. Successive positions of sperm heads were analysed from video frames using Olympus MicroImage software (Version 4.0.1. for Windows with a special macro by Olympus C & S).

Oxidative stress and antioxidant indices analyses
Samples were centrifuged at 5000g at 4°C for 10 min. The spermatozoa pellet was diluted with 50mM potassium phosphate (KPi) buffer and homogenize. The homogenate was divided into two portions: one in which thiobarbituric acid reactive substances (TBARS) and carbonyl derivatives of proteins (CP) was measured and a second that was centrifuged at 12 000g for 30 min at 4°C to obtain the post mitochondrial supernatant for the antioxidant enzyme activity assay. The TBARS method was used to evaluate sperm lipid peroxidation (LPO). Carbonyl derivatives of proteins were detected by reaction with 2,4-dinitrophenylhydrazine (DNPH) according to the method described by Lenz et al. (1989). Total superoxide dismutase (SOD) activity was determined by the method of Marklund and Marklund (1974).

RESULTS

Cryopreservation resulted in a sharp decrease in spermatozoa motility parameters. The highest value recorded for cryopreserved spermatozoa, at 47%, was with 10% DMA, followed by 10% MET and DMSO (45.5 and 43.5% respectively). Post-thaw motility using 10% EG as cryoprotectant ranged from 0-17%. In the presence of the X-XO system, motility rate, and velocity of frozen/thawed spermatozoa decreased in a time- and dose-dependent manner compared to the control. The maximum level of LPO was observed in spermatozoa cryopreserved with 10% EG and exposed to the X–XO system at concentrations of 0.6 mM–0.05 U/mL, with the minimum detected at 0.1 mM X–0.0125 U/mL XO containing 10% MET, 10% DMSO, or 10% DMA and incubated for 5 min. An increase in CP level was observed with increased incubation time in the presence of the X–XO system. SOD activity was significantly enhanced ($P<0.05$) with increasing concentrations of X–XO and duration of incubation in frozen/thawed samples with all tested cryoprotectants.

DISCUSSION AND CONCLUSIONS

Sperm cryopreservation is an important procedure with specific advantages to the aquaculture industry, and freezing/thawing of sperm for artificial insemination is routinely conducted in fish breeding facilities. The data of the present study implied that the quality, and antioxidant responses of thawed sterlet spermatozoa is highly dependent on the cryoprotectant used. Sperm containing 10% MET as cryoprotectant showed higher resistance to oxidative stress than did sperm cryopreserved with 10% DMSO or 10% DMA. EG is not considered a suitable protecting chemical during cryopreservation, and sterlet spermatozoa are highly sensitive to free radicals after cryopreservation using EG.

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P33

EFFECT OF HORMONAL TREATMENTS ON SENEGALESE SOLE SPERM QUALITY

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INTRODUCTION

Senegalese sole (*Solea senegalensis*) F1 reproduction still presents some constraints that difficult the use of these individuals to guarantee further production. F1 breeders do not reproduce naturally in the tank, and therefore, gamete collection and artificial fertilization is a procedure that has been developed (Rasines et al., 2012) and is being adapted to fish farms. One of the principal problems is the difficulty in obtaining sperm due to their low volume and concentration, a characteristic specific in this species (wild males) but more pronounced in F1 males. Some hormonal treatments already proved to be efficient in slightly increasing sperm volume (Guzmán et al., 2011), but there are still some gaps on the evaluation of this contribution. No reports on the quality of sperm has been delivered so far that guarantee a safety use of this material, especially if some management techniques such as cryopreservation will be applied. The main objective of this work is to perform an exhaustive evaluation of sperm quality from Senegalese sole F1 males exposed to different hormonal treatments.

MATERIALS AND METHODS

Fish Maintenance- Fish (n=144) were maintained at the fish farm Sea8, Safiestela, Póvoa de Varzim, Portugal, distributed in 6 tanks with natural photoperiod and temperature. The proportion of 1:1 (males to females) was maintained in all tanks. Fish were feed *ad libitum* with an experimental diet formulated by SPAROS, LDA.

Hormonal treatments- Males were induced hormonally from June to September with hCG (Pregnyl, Merck). Recombinant hCG (Ovitrelle, Merck) or GnRHa (Sigma). hCG treatments were administrated according to Guzmán et al., (2011). GnRHa was administered at doses of 25 µg/Kg on the day previously to the samplings to stimulate spermiation. All injections were applied in the dorsal musculature of the fish.

Sperm quality analysis- Sperm was extracted by abdominal massage, squeezing gently the testis in the fish blind side. Collection was made with a 1ml syringe and samples were introduced in an Eppendorf tube maintained at 4ºC. Male fluency and sperm volume were recorded. Samples were centrifuged (300 g,10 min, 10ºC) to eliminate seminal plasma and any contamination with urine, and sperm mas diluted in Ringer solution and maintained in ice. Sperm quality was assessed in terms of motility using CASA system and DNA fragmentation was assessed using comet assay, both techniques according to the settings previously established by Beirão et al., (2009). Cell viability was recorded using the fluorescent probes PI/SyBR green and flow cytometry. Lipid peroxidation (MDA) was quantified using a colorimetric assay (kit BIOXYTECH LPO-586™, OxisResearch™, USA), following the protocol adapted for fish sperm by Martinez-Páramo et al., (2012). ATP content was determined by bioluminescence (ATP Bioluminescence Assay, Kit CLS II, Boeringer) using the method described by Cabrita et al., (2005). Total antioxidant status (TAS) was assessed in seminal plasma using the kit from RANDOX and adapted for Senegalese sole seminal plasma concentrations.

All the analysis were performed in individual or pooled samples (depending on the analysis) and repeated in consecutive hormonal inductions.

RESULTS AND DISCUSSION

F1 males produced very small amounts of sperm when compared with values reported for wild fish (Cabrita et al., 2006). Hormonal treatments improved sperm volume and sperm quality, being a good alternative for the use of F1 males in sperm management in reproductive strategies.

CONCLUSIONS

The confirmation of sperm quality produced by F1 males induced hormonally open interesting perspectives for the use of this approach to improve sperm production stimulation to be used in techniques such as cryopreservation.

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P34

EFFECT OF IONS Ca^{2+}, K^+ AND Na^+ ON SPERM MOTILITY OF EUROPEAN EEL AND SENEGALESE SOLE

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INTRODUCTION

In teleost fish sperm is immotile into the testis. Releasing to the external medium causes motility activation, due to the osmotic shock (hypo or hyperosmotic, in freshwater or seawater species respectively). However, there is not agreement about the mechanisms that occur during activation. In the case of two marine species, pufferfish (*Takifugu niphobles*) and European eel (*Anguilla anguilla*), an increase of $[K^+]_i$ and $[Ca^{2+}]_i$ has been reported after hyperosmotic sperm activation (Oda & Morisawa 1993, Gallego et al., 2014). Also, we have shown an increase of $[Na^+]_i$ during the European eel sperm activation (Vílchez et al., this meeting).
In the present work, we made a comparative study of the effect of the ions Ca^{2+} , K^+ and Na^+ present in the seminal plasma and in the activation media on sperm motility activation in the Senegalese sole (*Solea senegalensis*) and the European eel.

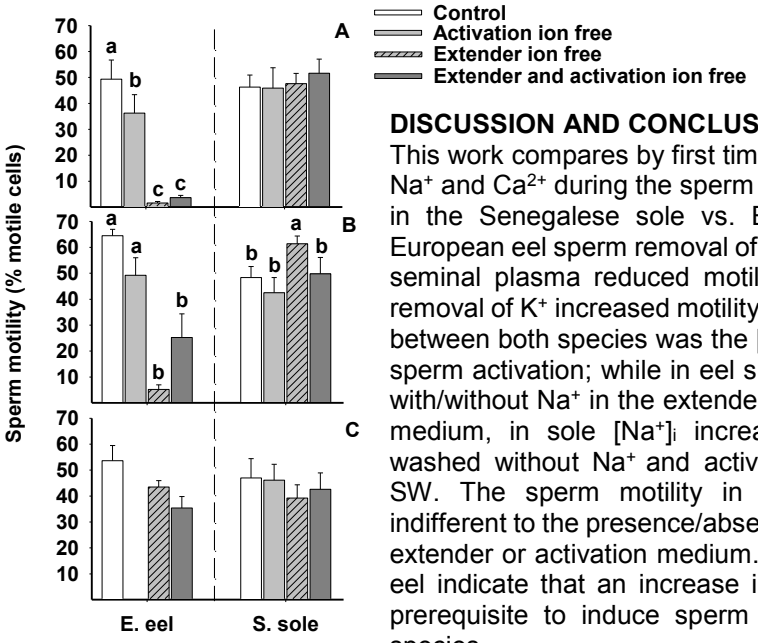
MATERIALS AND METHODS

Sperm samples were obtained from Senegalese sole males maintained at the University of Algarve (Portugal), and from farmed European eel males matured with hCGrec weekly injections (1.5 IU/g) at the Polytechnic University of Valencia (Spain). Sperm samples were selected in terms of motility (>50%). Several trials were conducted:
Trial 1: Role of different ions in sperm motility. Sperm samples were washed in different ion-free extenders (Na^+, K^+, or Ca^{2+} free extenders) or in control extender (species-specific, see Pérez et al., this meeting) containing all the ions studied, and were activated (with seawater, SW) in the presence or absence of different ions (Na^+, K^+, or Ca^{2+} free activators). Sperm motility was evaluated by CASA software (ISAS, Proiser R+D, S.L., Spain).
Trial 2: Determination of intracellular Na^+, and Ca^{2+} by flow cytometry:
Sperm cells washed in ion-free extender or control extender were incubated with CoroNa-Green (for $[Na^+]_i$) or Fluo-4 (for $[Ca^{2+}]_i$) dyes. After incubation (40 min at 4ºC), the fluorescence intensity of the CoroNa-Green or Fluo-4 loaded cells was measured by flow cytometry (Cytomics FC500 and BD FACSCalibur for European eel and Senegalese sole, respectively) in quiescent sperm, and after motility activation (with/without the ion subject of study) in samples washed in control extender or ion-free extender.

RESULTS

Trial 1: The elimination of extracellular Na^+ and K^+ by washing reduced significantly the sperm motility in the eel, but removal of Na^+ in the sole did not cause any effect.
(Fig. 1A,B). In contrast with eel, sole samples washed in K-free extender and activated with SW showed an increase in the sperm motility (Fig. 1B).
In the case of elimination of extracellular Ca^{2+} by washing (Fig. 1C), any effect was found in eel or sole sperm motilities.
Trial 2: The fluorescence intensity (FI) emitted by $[Na^+]_i$ in eel increased significantly after sperm activation (with/without Na^+) compared to the quiescent sperm washed with/without Na^+. In contrast, in sole the FI emitted by $[Na^+]_i$ only increased in sperm samples washed in Na-free extender and activated with SW.

In eel, the $[Ca^{2+}]_i$ from control samples (washed and activated with Ca^{+2}) showed a significant increase after sperm activation. But $[Ca^{2+}]_i$ did not increased in eel sperm samples washed and activated without Ca^{+2}. Contrarily, the $[Ca^{2+}]_i$ from sole samples (washed with/without extracellular Ca^{2+}) showed a significant increase of $[Ca^{2+}]_i$ post-activation with SW or Ca-free activator.



DISCUSSION AND CONCLUSIONS

This work compares by first time the effect of K^+, Na^+ and Ca^{2+} during the sperm motility activation in the Senegalese sole vs. European eel. In European eel sperm removal of Na^+ and K^+ in the seminal plasma reduced motility, while in sole removal of K^+ increased motility. Other difference between both species was the $[Na^+]_i$ response to sperm activation; while in eel sperm it increased with/without Na^+ in the extender or the activation medium, in sole $[Na^+]_i$ increased in samples washed without Na^+ and activated with control SW. The sperm motility in both species is indifferent to the presence/absence of Ca^{2+} in the extender or activation medium. The results from eel indicate that an increase in $[Ca^{2+}]_i$ is not a prerequisite to induce sperm motility in these species.

Fig. 1: Percentage of motile spermatozoa; Control: washed in control extender and activated with SW. Activation ion-free: washed in control extender and activated in ion-free activator;
Extender ion-free: washed in extender ion-free, activated in SW; Extender and activation ion-free: sperm washed in ion-free extender and activated in ion-free activator. **A)** ion Na^+; **B)** ion K^+ and **C)** ion Ca^{2+}. Data are expressed as mean ± SEM (n= 9 in E. eel and n=15 in S. sole) and different letters indicate significant differences ($P<0.05$) with control group (washed in control extender and activated with SW).

However, sole showed a significant increase of $[Ca^{2+}]_i$ after activation in presence/absence of Ca^{2+}. Thus, it seems that Ca^{+2} ion have a role in the sperm activation of sole.

The present study suggests different ions fluxes involved on sperm motility in European eel and Senegalese sole, and further studies are necessary to understand the mechanism of sperm activation in both species.

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P35

THE ROLE OF ION POTASSIUM AND pH IN THE EUROPEAN EEL SPERM MOTILITY

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INTRODUCTION

It is known that fluxes of ion K^+ are involved in sperm motility initiation in salmonids and cyprinids, causing a membrane hyperpolarization which in turns, activate the sperm motility. In marine teleosts it has been observed an increase in $[K^+]_i$ during the sperm activation in pufferfish (*Takifugu niphobles*; Krasznai et al. 2003) and European eel (*Anguilla anguilla*; Gallego et al. 2014). Moreover, intracellular pH (pH_i) increase has been suggested as the main factor for sperm motility initiation (Oda & Morisawa, 1993), as the pH_i increase by addition of ammonium salts induced motility in pufferfish sperm in isosmotic conditions. In contrast, in Japanese eel it was suggested that a decrease in pH_i triggers the initiation of sperm motility, and that K^+ transport participates in the H^+ uptake (Tanaka et al. 2004).

In the present work, we studied by first time in European eel the effect of the variations of pH_i on the motility activation, as well as the changes in pH_i produced by modifications in external K^+. Also, a method for the quantitative analysis of pH_i was set up for eel sperm.

MATERIALS AND METHODS

Male eels were matured with hCGrec weekly injections (Ovitrelle®, 1.5 IU/g) and sperm samples were obtained and selected when its motility was >50% (by a CASA system: ISAS, Proiser R+D, S.L., Spain). Several trials were conducted:

Trial 1: SNARF-5 calibration and pH_i measurement by flow cytometry. Sperm cells were washed in standard extender (P1) and incubated in isosmotic high K^+ extenders (100 mM) with known pH concentrations, in presence of nigericin (4 µM) which equals $pH_i=pH_e$, and SNARF-5 as fluorescent dye. This method was based on Balkay et al. (1997)´s and the technical specifications of SNARF-5 (Molecular Probes). Fluorescence intensity (FI) of these cell suspensions was measured by flow cytometry (Cytomics FC500; at two wavelengths), and the calibration equation was obtained as described in the technical specifications of SNARF-5.

Trial 2: Effect of the pH_i on sperm motility. Sperm cells diluted in P1 were activated (mixed) with solutions with different concentrations (25-100 mM) of sodium acetate ($NaC_2H_3O_2$, acidifying the pH_i) and ammonium chloride (NH_4Cl, alkalinizing pH_i) in isosmotic (P1) and hyperosmotic (SW) media, to check by CASA if these internal modifications of pH_i could activate the sperm motility.

Trial 3: Effect of inhibition of K^+ channels in the pH_i. Sperm samples were washed in K^+-free extender, or in control (P1) extender, and incubated with/without 4-AP (4 Aminopyridine, 4 mM, inhibitor of K^+ channels). The pH_i (FI of the SNARF-5 loaded cells) was measured by flow cytometry in quiescent sperm and after motility activation with seawater.

RESULTS

Trial 1. The linear plot calculated, showed a slope of 1.18 and pK_A = 7.16, with high correlation and significance (r = 0.99 and P<0.05, respectively), what agree with the technical specifications from SNARF-5. The pH_i in quiescent stage was 7.2, and was calculated with the following equation: pH_i= -8.53 + 1.18F (where F denotes the fluorescence intensity from SNARF-5).

Trial 2. Sperm samples diluted in isosmotic extender containing 25 and 50 mM of NH_4Cl (alkalinizing pH_i) showed a low (<4%) but significant increase in sperm motility in relation to control (Fig. 1A). In contrast, sperm samples activated in SW with 25-100 mM NH_4Cl (alkalinizing pH_i) showed a strong decrease in motility (Fig.

1B) in comparison with control or with sperm samples activated in SW with 25-100 mM Na-acetate (acidifying the pH_i).

Trial 3. The pH_i in quiescent stage was affected by sperm washing in K-free extender, what caused an increase in pH_i (Fig. 1C). In contrast, pH_i did not change after sperm SW activation.

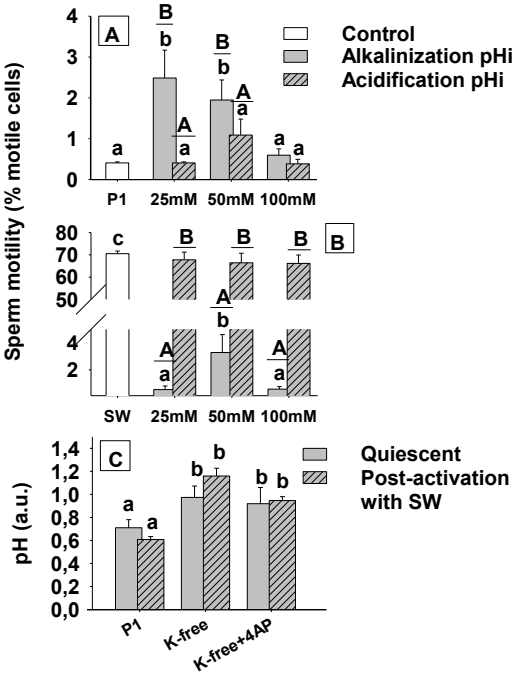

Figure 1. Sperm motility in samples with modified pH_i by adding Na-acetate and ammonium chloride in: A) isosmotic medium (P1) and B) SW. C) Grey bars: FI emitted by pH_i in quiescent sperm washed with P1 (P1), or K-free extender (K-free), or washed in K-free extender and treated with 4-AP. Stripped bars: Fluorescence emitted by pH_i in samples activated with SW and washed with the previous extenders. Data are expressed as mean±SEM (n=8 in A, B and n=5 in C). Small letters indicate significant differences with control group and capital letters indicate significant differences between the modifications of pH_i.

DISCUSSION AND CONCLUSIONS

The pH_i acidification does not cause variations in the sperm motility compared to the control group. In isosmotic conditions, the alkalization of the pH_i increased very weakly the sperm motility, in contrast with the findings of Oda & Morisawa (1993), who found a high increase in sperm motility in isosmotic medium induced only by pH_i alkalinization, in other marine species, the flounder (*Kareius bicoloratus*) and the pufferfish. In the present work, pH_i alkalinization strongly reduced the sperm motility in seawater. Moreover, the elimination of extracellular K^+, which we have previously demonstrated that inhibited the sperm motility, induced an increase in pH_i before and after motility activation. Taken together, these results suggest that the pH_i alkalinization inhibits the sperm motility in European eel, in contrast with the results from Oda & Morisawa (1993) in flounder and pufferfish.

ACKNOWLEDGEMENTS

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P36

IDENTIFICATION OF MAJOR PROTEINS FROM THE SEMINAL PLASMA OF HORMONE-INDUCED SEXUAL MATURED EUROPEAN EELS (*ANGUILLA ANGUILLA*). CORRELATION WITH SPERM QUALITY

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INTRODUCTION

Although a protective effect of seminal plasma on European eel (*Anguilla anguilla*) gametes has been reported (Peñaranda et al. 2010), little is known about its protein composition and its physiological function. They described the protein profile of seminal plasma along induced maturation, having four major electrophoretic bands around 80, 40, 26 and 12 kDa. The bands with 80, 40 and 12 kDa showed higher concentrations in samples showing ≥50% of motile cells, but these bands were not identified, and the results were not concluding.

The introduction of proteomics in the study of male reproduction in fish creates a unique opportunity to unravel, using an integrative approach, the physiological mechanisms important for sperm function, such as motility and fertilizing ability (Ciereszko et al. 2012). It is anticipated that proteomic studies will greatly increase the chances for reliably identifying new biomarkers in male fertility studies. In the current work, the proteomics science has been used to perform a complete description of the seminal plasma protein composition, in order to explore potential correlations between sperm quality parameters and seminal protein profile in the European eel.

MATERIAL AND METHODS

Thirteen European eel males (body weight: 137.6 ± 21.4 g) were hormonally treated with weekly intraperitoneal injections of hCG (1.5 IU g^{-1} fish). Once per week from the 6th week, the fish were sampled 24 h after hormone administration. Sperm samples were kept at 4 °C, until immediate motility evaluation using CASA system (ISAS v1; Proiser R&D, S.L.). Sperm samples were classified into three motility categories depending on the percentage of motile cells found after activation with sea water, I: 0-25%, II: 25-50% and III: >50% and the seminal plasma from each motility was stored at -20 °C.

Protein content in seminal plasma was determined using the Pierce BCA protein assay (Pierce Chemical Company). The protein profile was analyzed by SDS-PAGE and the protein bands were analysed with genetools software (Syngene, IZASA).

Protein composition was analysed in a pool of samples from each motility class by 2DE, using an IPGphor system (Amersham Bioscience). For proteomics analysis, tryptic peptides were separated by nano-Acquity UltraPerformance LC® (UPLC®) using a BEH130 C18 column in-line with a Waters SYNAPT G2 High Definition Mass Spectrometry System. Doubly and triply charged ions were selected for collision-induced dissociation (CID) MS/MS. Fragmentation spectra were interpreted manually (*de novo* sequencing), using the on-line form of the MASCOT program, and processed in Waters Corporation's ProteinLynx Global SERVER 2013 version 2.5.2. Images of gels were obtained with the Image Scanner II (GE Healthcare) using Labscan 5 (GE Healthcare) software. The differential analysis between motility categories was performed by Progénesis Samespots program.

RESULTS

All samples were classified into the category I until the 6th week of treatment. Half of samples reached the category II at 7th week, and 30% the category III at 8th week.

The mean protein content in seminal plasma was 384.75 ± 18.1 mg ml^{-1}, without showing differences along treatment. By SDS-PAGE analysis, 13 bands were identified, which most of them migrated between 20 to 60 kDa. In the last weeks of treatment (12th and 13th week) a significant increase was observed in the percentage of motile spermatozoa, curvilinear velocity and beat cross frecuency. This improvement in sperm quality coincided with a higher amount of proteins located at 19 KDa, while the protein corresponding to 90 Kda decreased. Nevertheless, no differences were observed in both groups of proteins between the categories of sperm motility.

Using high-resolution 2D electrophoresis, five protein families: apolipoproteins, centaurin gamma 2, carbonic anhydrase, serotransferrin and inmunoglobulin were identified in the seminal plasma of European eel. Proteins related to lipids transport (apolipoprotein, Fig. 1A and B) and with immune system (complement C3, Fig. 1C and D) showed significant differences between the sperm motility categories I and III. Apolipoprotein family protein registered higher amounts at lower motilities, unlike complement C3-like family protein was bigger in the samples with the highest percentage of motile cells.

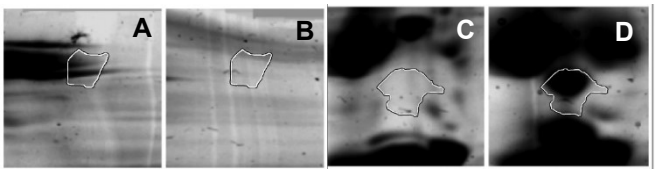

Fig. 1. 2D geles images of presence in seminal plasma of 28kDa-2 apolipoprotein in samples classified into the category I (**A**) and III (**B**), and presence of complement C3-like in samples classified into the category I (**C**) and III (**D**)**.**

DISCUSSION AND CONCLUSIONS

By first time, 2DE protein composition of the European eel in seminal plasma has been described. Different protein profile was observed depending on sperm motility categories, specifically, the proteins related with lipids transport (apolipoprotein) and with immune system (complement C3). Apolipoproteins are important for the stabilization of sperm membranes (Ciereszko et al. 2012) and sperm viability is most likely protected by proteins as complement C3 described by Lahnsteiner et al. (2010). The high presence of lipid transport proteins (apolipoproteins) in samples classified into the motility I category suggests that this family of proteins could have some role in the early phases of sperm production. In contrast, complement C3 showed a high presence in motility III samples, suggesting an immunologic role against microbial infection, especially during the final sperm maturation, when the sperm cell is closed to be released to the external environment. Taken together, these results suggest that the proteins related with lipids transport (apolipoprotein) and with immune system (complement C3) could carry out their functions during different stages of spermatogenesis process.

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P37

EFFECT OF EXTENDERS ON SENEGALESE SOLE (*SOLEA SENEGALENSIS*) SPERM

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INTRODUCTION

The use of extenders for fish sperm has different utilities: it can be used to increase the sperm volume for egg fertilization, for short-term storage, as the basis to develop cryopreservation media, and to handle and study the sperm motility. In Senegalese sole, the extender that has been commonly used is isosmotic sucrose (Beirão et al., 2009. However, the composition of this non-ionic extender is completely different of the seminal plasma, which contains ions as Na^+, HCO_3^-, Ca^{2+}, K^+ and others. Our final objective was to study the role of different ions in Senegalese sole sperm motility, thus we first needed to select a suitable ionic extender to study, one by one, the different ions, as sucrose originated technical problems. Thus, our objective was to define an ionic extender suitable for Senegalese sole sperm.

MATERIALS AND METHODS

Sperm samples were obtained from Senegalese soles males maintained at the University of Algarve from May to June 2014. Immediately after extraction, sperm samples were diluted in three cold extenders at 2 dilution rates (1:2, 1:5), and maintained at 4 ºC until motility analyses.

The extenders tested were P1, designed for European eel sperm (125 mM NaCl, 20 mM $NaHCO_3$, 2.5 mM $MgCl_2$*$6H_20$, 30 mM KCl, 1mM $CaCl_2$, osmolality 310 mOsm, pH adjusted to 8.1; Peñaranda et al., 2010); modified Ringer ROD (112.22 mM NaCl, 2.38 mM $NaHCO_3$, 40.23 mM KCl, 2.7 mM $CaCl_2$, osmolality 250 mOsm, pH adjusted to 8.1, Chereguini et al., 1997), and SUC (300 mM sucrose, osmolality 330 mOsm, pH adjusted to 8.1, Beirão et al., 2009). Twelve sperm samples obtained in 3 different dates were used. Sperm motility (MOT, MP, FA, VCL, VSL and VAP) was evaluated by CASA system after activation with artificial seawater (ASW, 1100 mOsm, pH=8.2). Samples were diluted immediately after collection, and motility was checked 2 and 24 h later.

RESULTS

All the extenders originated better results in motility than undiluted sperm, either 2 h or 24 h after sperm extraction (data not shown).

The dilution rate (1:2 or 1:5) had no significant effect on the sperm motility. The different extenders produced different results (Fig. 1) in sperm motility at 2 and 24 h after sperm dilution. While at 2 h both ROD and SUC extenders originated the highest motilities, 24 h after dilution the best results were obtained by ROD. At that time ROD also induced the highest percentage of fast spermatozoa ($p<0.01$), with values of 37.1±4.5 in ROD vs 20.9±6.0 and 16.1±5.6 in the extenders SUC and P1, respectively.

MOT, MP, FA, VCL, VSL and VAP 15 s post-activation were significantly reduced 24 h after dilution in extender ROD (Table 1), as well as in the other extenders (data not shown).

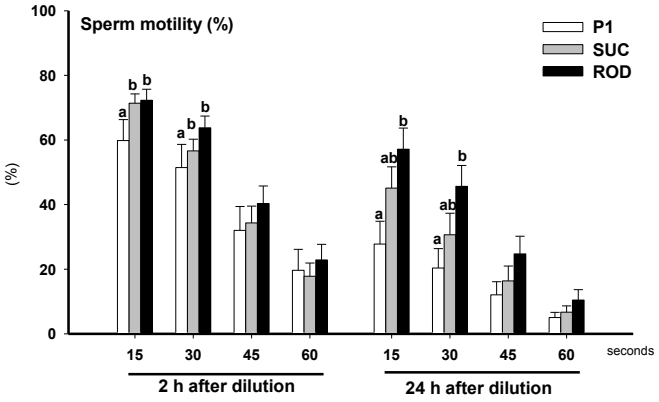

Figure 1. Sperm motility in samples diluted in the different extenders 2 and 24 h after dilution. Different letters means differences between the extenders in the same time post-activation. Values are shown as mean ± SEM.

Table 1. Comparison between sperm kinetic parameters (at 15 s post-activation) in samples diluted in extender ROD, 2 and 24 h after dilution. Values are shown as mean ± SEM. MOT: total motility; MP: progressive motility; FA: percentage of fast spermatozoa; VCL: curvilinear velocity; VSL: straight line velocity; VAP: average velocity.

	MOT	MP	FA	VCL	VSL	VAP
ROD 2 h	72.3 ± 5.2 b	25.8± 2.1 b	52.6± 4.7 b	112.1±7.3 b	68.6± 5.0 b	95.4± 6.6 b
ROD 24 h	57.1± 5.2 a	17.1± 2.1 a	37.1± 4.7 a	88.5± 7.3 a	51.9± 5.0 a	73.8± 6.6 a

DISCUSSION AND CONCLUSIONS

An ionic extender suitable for Senegalese sole short-term sperm preservation has been established, and it can be equally used at dilutions of 1:2 and 1:5, and probably at higher dilution rates. In this way, it would be possible to preserve the sperm quality much better than in undiluted form, in part because the small quantity of sperm obtained from sole males (just some microliters) supposes that very often it dries when it is kept 24 h undiluted.

Although SUC and ROD extenders originated similar results 2 h after dilution, 24 h later ROD originated higher percentage of fast spermatozoa than SUC. This fact, together with the easy bacterial contamination of sugar solutions, makes preferable the use of the extender ROD instead of SUC.

The differences in composition between the best extender, ROD, and the P1, which caused the worst results, were the osmolality, higher in P1 (310 vs 250 mOsm), and the bicarbonate content, almost 10 times higher in P1. Both parameters could have affected the sperm motility.

ACKNOWLEDGEMENTS

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P38

STUDIES ON SPERM MOTILITY ACTIVATION PROCESS IN SEA BASS (*DICENTRARCUS LABRAX*)

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INTRODUCTION

In many marine fish species, the spermatozoa are immotile in the testis and seminal plasma, and motility is induced when they are released in the aqueous environment. It is well known that the drastic change of osmolality faced by the spermatozoa, when discharged into marine environment is the main factor determining sperm motility activation in seawater fish. This extracellular factor (hyperosmolality) controlls sperm motility in marine fish, acting on the axonemal apparatus through signal transduction across the plasma membrane.
The objective of this study is to perform analysis to better understand the mechanisms that determine the initiation of sperm motility in sea bass.

MATERIALS AND METHODS

Fish and sperm collection
Males of sea bass *Dicentrarchus labrax* were obtained from a local fish farm (MARIBRIN, Brindisi, Italy) and used for the experiments. Milt collection was in the winter (December-January). Sperm was collected by applying gentle abdominal pressure to extrude milt that was removed from the gonopore with a syringe. Care was taken to avoid contamination with seawater or urine. The milt was transferred to a glass vial and kept at 4 °C for 60, 120 min, until its use.

Effects of extracellular inhibitors application on sperm motility activation
To determine the effects of different inhibitors the semen was diluted 1:100 in NAM (see above) containing inhibitors (0.5 mM MDL-12330A Hydrochloride or 0.01 mM KT5720 or 0.001, 0.01, 0.1 and 1 mM $HgCl_2$ or 0.02 mM gadolinium). Sperm samples were pre-incubated for 20 minutes with the different inhibitors, or (control) with 0.1% (v/v) DMSO to exclude the effect of DMSO in which the inhibitors were resuspended. Motility was initiated by the addition of seawater (control) or seawater containing different inhibitors (at the same concentration used for pre-incubation).

Western Blot Analysis
Extraction of sperm proteins, electrophoresis and western blot were performed as previously reported (Zilli et al. 2008). The membranes were incubated with specific primary antibody (anti-phosphotyrosine, anti-phosphothreonine, or anti-phosphoserine) at 4°C overnight, followed by a horseradish peroxidase-conjugated secondary antibody for 1 h and 30 min at room temperature. Antibody labeling was detected using enhanced chemiluminescence according to the manufacturer's instructions.

RESULTS

Milt samples were collected from 10 males during the spawning period. To clarify the mechanisms that regulate sperm motility initiation in sea bass we evaluated the effect of different inhibitors on sperm motility. Results obtained demonstrated that 0.01, 0.1 and 1 mM $HgCl_2$ (the well known AQPs inhibitor) completely inhibited sperm motility, while 0.001 mM was ineffective. On the contrary, MDL-12330A Hydrochloride (adenylyl cyclase inhibitor), KT 5720 (PKA inhibitor) and gadolinium (stretch-activated ion channel inhibitor) had no effect on sea bass sperm motility activation (Table 1). In addition, this study investigated by western blot analysis if protein phosphorylation/dephosphorylation has a role in motility initiation process of sea bass sperm and if $HgCl_2$ affects phosphorylation/dephosphorylation

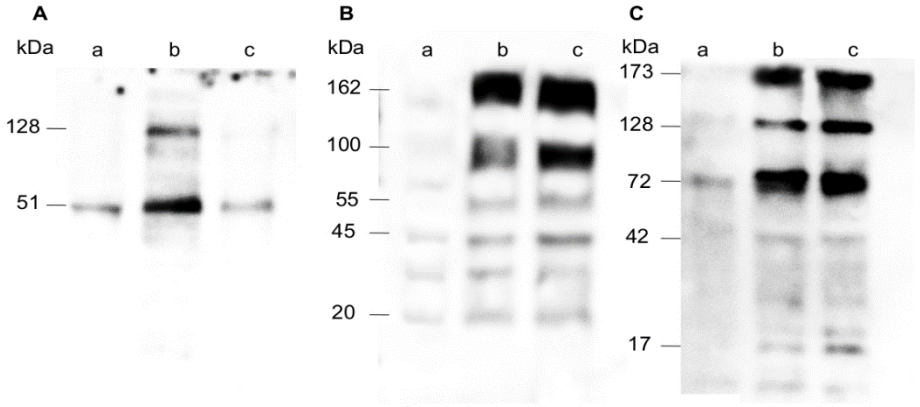
patterns.

Table 1. *Effect of different inhibitors on gilthead sea bream and sea bass sperm motility initiation*

Inhibitors mM	**$HgCl_2$ 0.001**	**$HgCl_2$ 0.01**	**$HgCl_2$ 0.1**	**$HgCl_2$ 1.0**	**MDL-12330A 0.5**	**KT5720 0.01**	**Gadolinium 0.02**
(N=10)	+	-	-	-	+	+	+

Notes: +, Nearly all spermatozoa motile; -, no motility; $HgCl_2$: AQP inhibitor; MDL-12330A: adenylyl cyclase inhibitor; KT5720: PKA inhibitor; Gadolinium: stretch activated ion channel blocker

Results obtained demonstrated that the spermatozoa motility activation process determined the phosphorylation of 2 protein bands at tyrosine level (Fig 1A) with a molecular mass of 128 and 51 kDa, of 5 protein bands at threonine level (Fig 1B) with molecular mass of 162, 100, 55, 45 and 20 kDa and 5 protein bands at serine level (Fig 1C) with molecular mass of 173, 128, 72, 42 and 17 kDa. The presence of $HgCl_2$ affected only the phosphorylation processes at tyrosine level



(Fig 1A).

Figure 1. *Effect of $HgCl_2$ on phosphorylation/dephosphorylation of proteins at tyrosine (A), threonine (B) and serine(C) residues after sperm motility activation of sea bass. Sperm were maintained immotile by dilution in non activating medium (a), either activated in seawater without (b) and with $HgCl_2$ (c).*

DISCUSSION AND CONCLUSIONS

The present work has been carried out to elucidate sperm motility activation process in sea bass, in particular, the intracellular pathway involved, the role of protein phosphorylation/dephosphorylation, and if aquaporin inhibitor ($HgCl_2$) affects the phosphorylation state of these proteins. Data here reported demonstrate that (1) AC/cAMP pathway and stretch-activated ion channels are not involved in sperm motility activation in sea bass, differently from other marine fish (Cosson *et al* 2008, Zilli *et al* 2008), (2) tyrosine, threonine, or serine residues of some sperm proteins are differently phosphorylated following motility activation similarly to other marine fish (Zilli *et al* 2008), and (3) the phosphorylation of proteins at tyrosine level, following the hyper-osmotic shock, was inhibited in the presence of $HgCl_2$, as such as happens in sea bream (Zilli et al 2011). In conclusion, the results showed in this paper suggest that the hyper-osmotic shock and the efflux of water through aquaporins could be the key events leading to activation of motility, but not the only events which cause the change of the phosphorylation state of proteins, that represents the final step of the trigger system of axonemal movement.

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P39

DEVELOPMENT OF SPECIFIC ELISAS FOR MEDAKA (*ORYZIAS LATIPES*) GONADOTROPINS LH (LUTEINIZING HORMONE) AND FSH (FOLLICLE-STIMULATING HORMONE) USING RECOMBINANT PROTEINS

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INTRODUCTION

Vertebrate puberty and sexual maturation is regulated primarily through the endocrine system known as the hypothalamo-pituitary-gonad (HPG) axis, of which Fsh and Lh play integral parts. Fsh and Lh belong to a large family of cysteine knot-forming polypeptide glycoproteins with non-covalently linked heterodimers between a common glycoprotein α-subunit (Gpα), and a β-subunit that is hormone-specific (Fshβ and Lhβ). There are indications that these hormones play important roles also during early development, although detailed functions and underlying mechanisms have not been described.

MATERIALS AND METHODS

As a tool to investigate the function of early pituitary gonadotropin expression, we are developing specific and homologous competitive enzyme-linked immunosorbent assays (ELISA) to quantify Fsh and Lh protein levels in medaka pituitary and plasma, including development of antisera against the two hormones. Plasmids containing Gpa, Lhβ, Fshβ, or single-chain Lhαβ or Fshαβ were expressed using the methylotrophic yeast *Pichia pastoris*.

RESULTS

Hormone production in *P. pastoris* in 1 l cultures over 3 days (large production of recombinant protein) resulted in 2,357 mg of highly purified medaka Lhβ (Figure 1) and 4,168 mg highly purified medaka Fshβ (Figure 2), both based on one-step nickel batch purification. Western blot analysis using his-hrp antibody showed bands with expected sizes of 15 kDa for Lhβ and 12.5 kDa for Fshβ. Specific antisera were raised in rabbits, using three injections (for Fshβ 1 mg/ml first injection, 0,5 mg/ml second and third injection; for Lhβ 0,7 mg/ml first injection, 0,4 mg/ml second and third injection) of purified protein in 0,9 % NaCl and emulsified with complete Freund adjuvant at 3-wk intervals. Gpα will be joined with medaka Lhβ or Fshβ mature protein-coding sequences to form a fusion gene that encodes a “tethered" polypeptide, in which the gonadotropin β-subunit forms the N-terminal part and the α-subunit forms the C-terminal part. We are currently producing Lhαβ and Fshαβ (Figure 3) (25.5 kDa; 3,679 mg) single-chain peptides in *P. pastoris* to be used as standards in the specific ELISAs and for characterization in ligand-Lhr/Fshr receptor-binding studies.

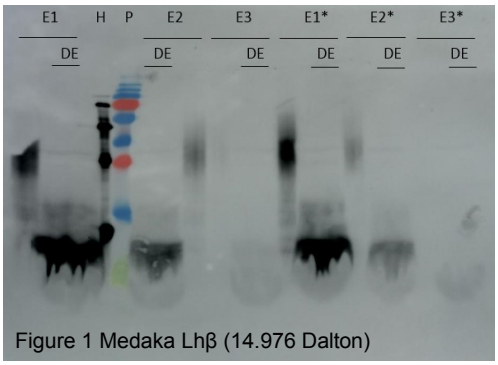

Figure 1 Medaka Lhβ (14.976 Dalton)

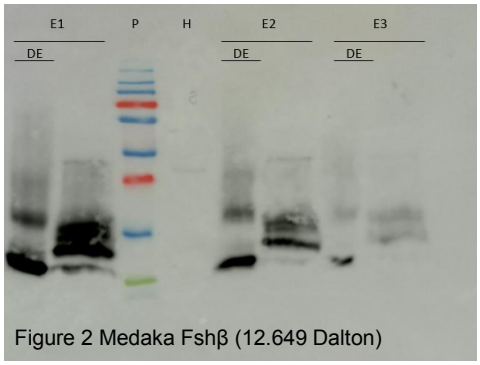

Figure 2 Medaka Fshβ (12.649 Dalton)

Figure 1 (left): Medaka Lhβ (14.976 Dalton). Figure 2 (right) Medaka Fshβ (12.649 Dalton). Regarding the hyperglycosylation trait of the yeast, each sample was deglycosylated using PNgase F [DE] and the correct size of each protein was verified (~ 15 kDa for Lhβ; ~ 12.5 kDa for Fshβ; P PageRuler Plus Prestained Protein Ladder; H 6xHis Protein ladder). The three different elutions of each protein are shown [E1, E2, E3; respectively E1*, E2*, E3* for the second large scale production of Lhβ].

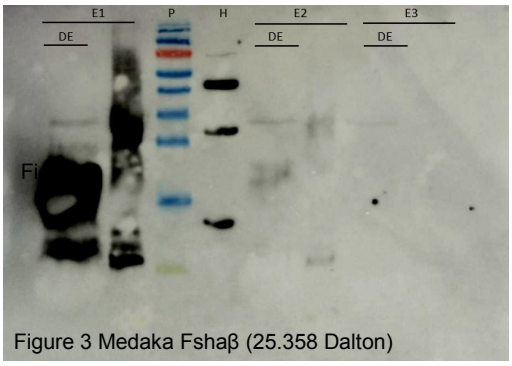

Figure 3 Medaka Fshaβ (25.358 Dalton)

Figure 3 Medaka Fshβ (25.358 Dalton). Production of the "tethered" gonadotropin medaka Fshaβ. Due to the hyperglycosylation trait of the yeast, each sample was deglycosylated using PNgase F [DE], the correct size was verified (~ 25.5 kDa; P PageRuler Prestained Protein Ladder; H 6xHis Protein ladder). The three different elutions are shown [E1, E2, E3]. Hormone production resulted in 3,679 mg highly purified medaka Fshaβ. Currently medaka Lhαβ single-chain peptide in *P. pastoris* is also being produced.

DISCUSSION AND CONCLUSIONS

The validated assays for medaka Fsh and Lh will be important tools to reveal the functional roles of these hormones during different stages of medaka reproductive development.

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POSTER SESSION II

Thursday, September 10th

P40

MOLECULAR ANALYSIS OF SPERM CRYOPRESERVATION DAMAGE IN *ACANTHOPAGRUS SCHLEGELII*

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INTRODUCTION

Cryopreservation is an important technique for a long-term storage of sperm, but it remains unclear if the freeze-thaw procedure leads to genome damages of sperm. The physical injury in the post-thaw sperm has been widely observed, and also studied in detail. However, little is known whether gene expression and regulation is changed by cryopreservation. To gain insight the genome damage of the post-thaw sperm, we investigate the cellular transcriptional profiles between fresh and post-thaw (cryopreserved) sperm. Upon the RNA extraction, we found that fish sperm RNA has the same features as sperm RNA in other species, such as lack of ribosomal RNAs (rRNAs). Thus, the RNA preparation was optimized and the quality was evaluated by Agilent Bioanalyzer. Since good quality sperm RNA was obtained, analysis of gene expression patterns between fresh and post-thaw sperm is being characterized by using Next Generation Sequencing (NGS). The results are expected to further examine if cryopreservation is an issue to change the transcriptional regulation in fresh sperm.

MATERIALS AND METHODS

A modified RNA extraction protocol (Stowe et al., 2014) was applied for total sperm RNA preparation. The integrity and quality of the isolated RNA was evaluated by using Agilent Bioanalyzer. The good quality sperm RNA is being analyzed by using NGS to figure out if gene expression is altered during cryopreservation.

RESULTS

In extracting total RNA from fish sperm, we found that the mature sperm-retained RNAs deplete ribosomal species, while somatic cells have abundant rRNAs that are usually served as RNA quality measurement. The isolated RNA quality is evaluated by two criteria using Agilent Bioanalyzer (Das et al., 2010; Johnson et al., 2011). First, RNA integrity is analyzed by RNA Integrity Number (RIN): the sperm RNA had typical shorter RNA fragments with very low RIN around 1, while total RNA from most body tissues had a higher RIN above 8. Second, the Bioanalyzer profiles also clearly revealed that the sperm RNA is lack of the 18S and 28S rRNAs, indicating no contamination from somatic cells.

DISCUSSION AND CONCLUSIONS

Upon the modified protocol, total cellular RNA, enriched for small RNAs, was isolated from sperm under different cryopreserved conditions. The good quality RNA is being analyzing the transcriptional patterns. The results are expected to examine if cryopreservation changes the gene expression in fresh sperm.

ACKNOWLEDGEMENTS

We thank Hsien-Da Huang and his research group for analysis of Agilent Bioanalyzer profiles. This research was supported by grants from Ministry of Science and Technology, Taiwan, to J.-C. Gwo, under project number 103-2313-B-019-010 (Sustainable management of Taiwan black sea bream genetic resource).

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P41

CRYOPROTECTANTS ALTER DNA METHYLATION PATTERN OF SPERMATOZOA AND DECREASE REPRODUCTIVE SUCCESS IN *COLOSSOMA MACROPOMUM*

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INTRODUCTION

Semen cryopreservation is an important tool in reproductive management of commercial fish species. Different cryoprotectant agents (CPAs) have successfully established in semen cryopreservation protocols, but little is known about DNA alteration caused. CPAs are widely used to preserve the spermatozoa in *Colossoma macropomum* (tambaqui) to improve the resistance of cells and integrity of cell components, like a DNA, during the cryopreservation process. The *C. macropomum* is an important species in South American aquaculture, presenting semen cryopreservation protocols optimized. The semen cryopreservation has been used in *C. macropomum* farming; however, the impact caused by cryopreservation or CPAs on embryonic development and future larvae performance has not yet been studied. Recently, was found that the gene regulation into early embryo development is inherited from the paternal genome (Jiang et al., 2013; Potok et al., 2013). Pattern of methylation into early embryo development to be the same of paternal genome reveals an important role for male until then only attributed to females. So, the aim of this study was to estimate methylation pattern of the semen DNA after cryopreservation from five different CPAs and to assess embryonic development consequences.

MATERIALS AND METHODS

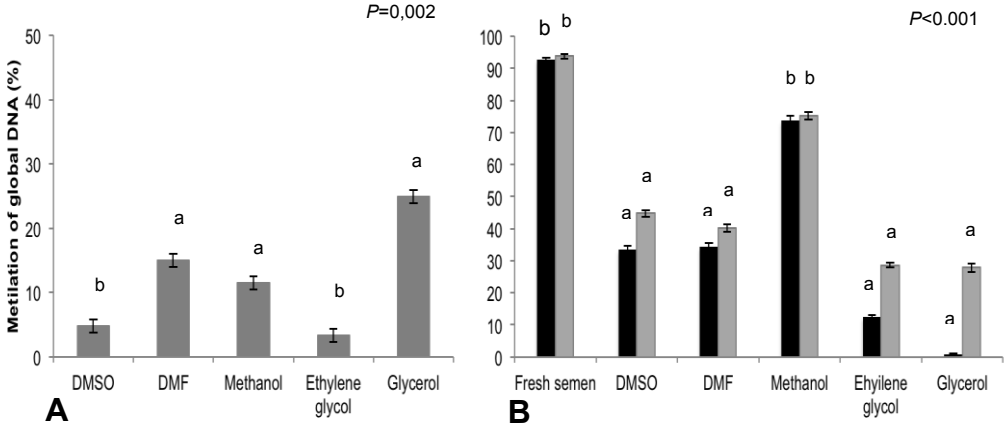
Was used semen collected from three males *C. macropomum* (2.548 kg ± 0.15), which showed at least 90% of motility. Semen cryopreservation was performed through freezing solutions prepared using five CPAs: Dimethylformamide (DMF), dimethylsulfoxide (DMSO), methanol, ethylene glycol and glycerol (all at 10% concentration) and extended in BTS (Beltsville Thawing Solution). Semen was thawed after 24 h and the DNA was isolated from both cryopreserved and fresh semen. Restriction enzymes *MspI* and *HpaII* were used to assess the methylation level estimated by fluorescence intensity using *ImageJ* software (version 1.47). Fertilization was performed using fresh and cryopreserved semen in 1:100.000 ratio (oocyte:spermatozoa) to assess the embryonic development. Were evaluated 120 embryos from each CPAs by qualitative and quantitative parameters throughout embryonic development (Leite et al., 2013).

RESULTS

Different methylation patterns were observed in spermatozoa DNA cryopreserved from different CPAs. Results suggest a hypermethylation in spermatozoa DNA cryopreserved in DMF, methanol and glycerol (Figure 1A). Methylation levels of the spermatozoa DNA cryopreserved using ethylene glycol and DMSO had the lowest levels, 20% less than methanol and DMF; and 40% less than glycerol (P<0.002). Different CPAs showed distinct impact on embryonic development. Rates of viable embryos and hatched larvae from spermatozoa cryopreserved in glycerol were lower (five hours and 13 hours post-fecundation, respectively) when compared to fresh semen (Figure 1B). Higher number of viable embryos and hatched larvae (75 and 74%, respectively), was generated from fertilization using spermatozoa cryopreserved in methanol, only 19% less than fresh semen for the both parameters. In general, methanol showed the better results throughout

embryonic development when comparing to the other CPAs. Embryos generated from semen cryopreserved in methanol showed 66% of normal development (blastocyst stage) at 120 min PF. Differently, embryos generated from semen cryopreserved in DMF and glycerol showed 66% of deformities. Similarly, only 33% of embryos from semen cryopreserved in ethylene glycol were found viable. At 420 min PF, embryos generated from ethylene glycol and glycerol were dead. Glycerol was the cryoprotectant with the highest extent damage to embryos at 690 min PF, where 100% of the embryos sampled were dead.

Figure 1: DNA methylation level of *Colossoma macropomum* semen cryopreserved with different CPAs (**A**); Rate of viable embryos five hours post-fecundation (PF) (gray bars) and hatched larvae (black bars) 13 hours PF by fresh and cryopreserved semen with CPAs (**B**). *P*-values correspond to ANOVA one-



way followed by Tukey test ($P<0.05$). Bars indicate standard error of mean (n = 6-7).

DISCUSSION AND CONCLUSIONS

Glycerol was the CPA that caused the highest methylation ratio and the highest extent of damage on embryos, resulting in less than 1% (±1.74) of hatched larvae. A high methylation pattern seems to be involved in the DNA downregulation, which can explain the low number of viable embryos and hatched larvae (Jiang et al., 2013). However, although some CPAs have showed low rates of methylation, such as DMSO and ethylene glycol, they did not show a high number of viable embryos and hatched larvae. Our results show the importance of alterations in spermatozoa DNA on viability of offspring, which so far was assigned to females; and that the extent of alterations in spermatozoa DNA post-thawing can be minimized by choose of CPA in the freezing solution.

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P42

COMPARATIVE ANALYSIS OF BLOOD AND SEMINAL PLASMA PROTEOME OF RAINBOW TROUT

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INTRODUCTION

Origin of fish seminal plasma proteins is unclear. Loir et al. (1990) indicated that contrary to higher vertebrates, many seminal proteins are antigenically-related to serum proteins. These early findings were confirmed in studies of individual major proteins of fish seminal plasma, including protease inhibitors, proteases, fetuin, and transferrin (Ciereszko et al. 2012). These proteins are suggested to be transported to semen from blood and/or partially synthesized and secreted by reproductive tract cells. On the other hand, a few proteins, such as prostaglandine D synthase (PGDS) was found only in trout seminal plasma (Nynca et al. 2011). Up to date, proteomic comparison of the blood and seminal proteins was studied only for the common carp (Dietrich et al. 2014). The aim of the study was to compare the blood (BP) and seminal plasma (SP) proteome of rainbow trout to identify proteins differentially abundant between these two fluids using two independent methods 2D-DIGE and 1D SDS-PAGE combined with LC-MS/MS. The latter was also used to generate an inventory of the most prominent rainbow trout blood plasma proteins.

MATERIALS AND METHODS

Blood and seminal plasma samples (n=6) were labeled with fluorescent dyes (Cy3, Cy5, Cy2, GE Healthcare) and applied to two-dimensional electrophoresis (2-DE) on 18 cm gel strips (pH 3-10). After isoelectrofocusing, strips were equilibrated with 1% DTE and 2.5% iodoactetamide, and then SDS-PAGE was performed. The gels were analysed using DeCyder software (GE Healthcare), selected protein spots were excised, digested and subjected to MALDI-TOF/TOF (Bruker Daltonics). The MS/MS data were searched with Mascot against the NCBIr fish database. 1-DE prefractionation combined with nano-LC-MS/MS were also applied. The gels were cut into slices and individually reduced, alkylated, digested and subjected to LC-MS/MS. Scaffold software (Proteome Software, USA) was used to compare blood and seminal plasma proteins sets from the same individuals.

RESULTS

Comparative 2D-DIGE analysis revealed that the majority of protein spots are common for BP and SP (about 80%). Applying DeCyder criteria 48 protein spots were found to be more abundant in SP (spots no. 1-48; Fig. 1A) and 49 were more abundant in BP (spots no. 55-103; Fig. 1B). Proteins enriched in SP represented multiple forms of the same protein (7 spots were identified as transferrin, 4 spots as α1-antiproteinase, 5 spots as hemopexin-like protein). Several proteoforms were also identified in BP, e.g. complement C3 (6 spots) fibrinogen beta chain (5 spots) and fibrinogen gamma chain (4 spots). The LC-MS/MS analysis led to the identification of 115 proteins of rainbow trout blood plasma. The LC-MS/MS data set was related to 2D-DIGE results and yields a list of 9 proteins enriched in SP and 34 proteins found exclusively in the SP, including multifunctional 14-3-3 proteins, a lipophilic carrier protein (PGDS; lipocalin precursor), EF-hand family of Ca^{2+}-binding protein, superoxide dismutase, proteins involved in proteolytic degradation (heat shock proteins, proteasomes, ubiquitin), spermatozoal enzymes for energy supply (LDH, creatine kinase,) sperm flagellum proteins (tubulin, cofilin-1) and protein participating in spermatogenesis (gonadal-soma derived growth factor).

Using LC-MS/MS we found 23 more abundant proteins in BP and 19 proteins detected only in BP. The identified proteins represent different forms of complement proteins (C4,C5, C7,C8), fibrinogens (beta, gamma), immunoglobulin M and Fe-transporting proteins (hemoglobin subunits, haptoglobin, heme-binding protein).

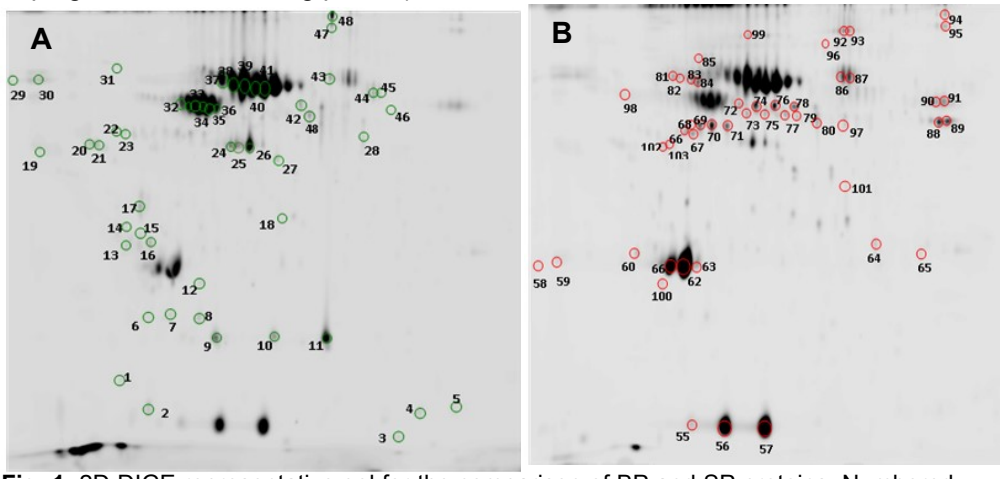

Fig. 1. 2D DIGE representative gel for the comparison of BP and SP proteins. Numbered protein spots (1-48) correspond to differentially abundant proteins in SP and in BP (55-103). A – single channel image (Cy 3) SP proteins; B - single channel image (Cy 5) BP proteins.

DISCUSSION AND CONCLUSIONS

This study provides the first in-depth analysis of the rainbow trout blood plasma proteome, with a total of 115 identified proteins by LC-MS/MS. This study is complementary to our previous results regarding the identification of rainbow trout seminal plasma proteome (Nynca et al. 2014), which enabled the comparison between SP and BP protein complement and identification of differentially abundant proteins in those fluids. Application of comparative proteomic analysis to identify trout SP and BP proteins significantly extends current knowledge regarding the relationship between proteins of SP and BP. The proteins present in higher abundance in seminal plasma can be related to physiology of fish male reproduction including regulation of sperm motility, spermatogenesis, protein turnover and antioxidant protection. In contrast to mammals, in trout most of SP proteins resemble those of BP, which possibly is related to specificity of blood-testis barrier in fish.

ACKNOWLEDGEMENTS

Funded by the National Science Centre granted for research project nr 2011/01/D/NZ9/00619, funds appropriated to the Institute of Animal Reproduction and Food Research, Polish Academy of Sciences. The support of COST Action FA1205 AQUAGAMETE is also acknowledged.

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P43

OPTIMIZATION OF STORAGE OF STURGEON TESTICULAR CELLS IN -80°C

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INTRODUCTION

Cryopreservation of testicular germ cells have recently been performed in many fish species including Senegalese sole, *Solea senegalensis*, (Pacchiarini et al., 2012) and tench *Tinca tinca* (Linhartova et al., 2014). The aim of present study was to compare the viability of testicular cells with same freezing methods for storage which would facilitate shipment of these cells with minimum losing of their efficiency on dried ice or in a deep freezer at approx. -80 °C.

MATERIALS AND METHODS

The testes of three immature sterlet were collected, cut in small pieces, diluted in an cryomedia composed of phosphate buffered saline (pH 8) with 0.5 % BSA, 50 mM glucose and 1.5 M Ethylen Glycol as cryoprotectant (1 volume part of testicular fraction in PBS and 3 volume parts of cryomedia) and subsequently frozen using nitrogen-free programmed biofreezer (PLANER Kryo 10 series III, UK) chilled from 10 to -80°C, while cooling rate was 1°C according to Pšenička et al. (2012). A small fragment of testes from each fish was used as control fresh sample (T1) and for cryopreservation. After freezing by planner, samples were distributed into seven treatments according to their storage condition as follow: two days kept in -80 °C (T2), one week kept in -80 °C (T3); after storage in liquid nitrogen, kept 2 days in -80 ºC (T4), after storage in liquid nitrogen, kept one week in -80 ºC (T5), after 2 days in -80 °C, kept in liquid nitrogen (T6), after one week in -80 °C, kept in liquid nitrogen (T7). To separate testicular cells, thawing was conducted in a water bath at 38 °C for approx. 45 s and immediately washed in phosphate buffered saline (PBS) solution. Then tissues were incubated for 2 hours in 0.3% trypsin in PBS at 22 ºC. The obtained suspension was filtered with 50-µm filter (Partec, Germany), to collect larger sperm cells and debris, and 1% BSA (Sigma–Aldrich A7511) and 40 mg mL^{-1} DNAse (AppliChem A3778) were added. The tubes with gonad cell suspension were centrifuged at 500× g for 20 minutes at 4 ºC. The pellets were resuspended and diluted in 1 ml PBS to obtain homogeneous solution. The numbers of cells were determined microscopically using a Burker cell hemocytometer at 100× magnification under an Olympus BH2 microscope. The number of testicular cells was counted in 20 squares of the Burker cell chamber with two repetitions. The cell viability was evaluated by the addition of 0.4% trypan blue (Sigma).

RESULTS

Cells stored in liquid nitrogen had highest viability regardless of storage time. The results show best survival of testicular cells in the case of storage with preserving cells in T2 and T7, respectively. There was no significant reduction in viability for testicular cells kept common procedure (store on liquid nitrogen) and keeping on -80 for two days before or without liquid nitrogen (Table 1).

Table 1. Total cell viability of *A.ruthenus* testicular tissue with same freezing methods for storage

Groups	Live cells	Dead cells
	Mean ± SD	Mean ± SD
T1	271.60 [a] ± 78.7	25.20 [a] ± 8.3
T2	85.33 [b] ± 38.1	4.33 [a] ± 5.3
T3	19.10 [b] ± 18.7	0 [a] ± 0
T4	86.83 [b] ± 28.04	7 [a] ± 1.3
T5	43.40 [b] ± 7.03	4.83 [a] ± 2.08
T6	19.33 [b] ± 7.1	6.33 [a] ± 2.7
T7	80.83 [b] ± 48.3	20.50 [a] ± 8.3
T8	27.16 [b] ± 16.3	14.50 [a] ± 10.4

DISCUSSION AND CONCLUSIONS

In the present study, we showed that storage of cryopreserved testicular cell in T3 and T6 resulted in highere viability compared with other treatments. In line with our results, Weinberg et al., (2010) showed that Liquid nitrogen (LN)/dry ice (DI) or 70 days storage/shipment on DI for cryopreserved PBMC is associated with a decrease in viability compared with LN/LN. In the present study, our observations demonstrate that preservation of testicular tissue on -80°C for two days before or without liquid nitrogen does not impair cell viability. In summary, our results showed that preservation of testicular tissue at -80°C for 48 h does not impair cell viability. Therefore, testis tissue can be stored or transported for 2 days without loss of potency.

ACKNOWLEDGEMENTS

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P44

PRELIMINARY EVALUATION OF TESTIS STRUCTURE IN MATURE ARAPAIMA *ARAPAIMA GIGAS* (OSTEOGLOSSIFORMES, ARAPAIMIDAE)

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INTRODUCTION

The arapaima, *Arapaima gigas*, is native to the Amazon region, belonging to the most basal branch of extant Teleostei. This species is one of the world's largest scaled freshwater fish species and can reach three meters in length and weigh up to 200 kg. The arapaima is highly prized with wide acceptance in the Amazon fish market, which contributed to overfishing, reducing the number of individuals in the wild. Knowledge on the basic aspects of reproductive biology is of great importance for conservation and management of a given species, thus increasing the reproductive success in breeding programs, both in nature and in captivity. However, there are very few studies on the basic aspects of the arapaima reproduction (Godinho et al. 2005, Arantes et al. 2010, Monteiro et al. 2010), especially those related to the testis structure and function. Thus, the aim of this study was to evaluate the testis structure of *A. gigas* through histological and stereological analyses.

MATERIALS AND METHODS

Four adult *A. gigas* with an average weight of 53 kg were used in this study. The animals were captured in the lowland waters of the Solimões river, Brazilian Amazon region (Latitude 03º17'06" S, Longitude *60º11'09"* W) during the spawning season (December 2014). The animals were euthanized by cervical concussion and the functional testis (left) was dissected out, measured and weighed. The gonadosomatic index (GSI) was obtained by the formula GSI = (testis weight/total body weight) × 100. Testis was fixed by immersion in 4% buffered glutaraldehyde for 24 h and then cut transversally into small fragments obtained from the cranial, medial and caudal areas. Tissue samples measuring 2 to 3 mm in thickness were routinely processed and embedded in plastic (glycol methacrylate). To perform histological and stereological analyses, sections of 4 µm in thickness were obtained and stained with toluidine blue. The percentage of tunica albuginea was obtained from the analyses of ~12 cross sections from the cranial, medial and caudal areas per testis, using ImageJ software. Volume densities (%) of the testis parenchyma components (tunica propria, seminiferous/germinal epithelium, lumen, connective tissue, Leydig cell and blood vessel) were determined by light microscopy using a 540-intersection grid in ImageJ software. Forty-five randomly chosen fields (24,300 points) were scored for each testis per animal at 400x magnification.

RESULTS

As expected, only the left testis was present. It was elongated with the mean length of 33 cm and a mean weight of 7.3 g, providing a gonadosomatic index of 0.014% (Table 1). The testis was covered by a though fibrous capsule or tunica albuginea with a large amount of connective tissue extending inward from the capsule. The mean percentage of tunica albuginea was 9.7%, whereas the volume density of tubular compartment was ~70% and intertubular compartment was 30%. In each compartment, the seminiferous/germinal epithelium occupied ~67% and the Leydig cells 7%. The spermatogenic tubules do not terminate at the testis periphery, but run parallel to the periphery and branch and connect to each other. As expected, the seminiferous/germinal compartment presented

Sertoli cells and germ cells in close association with the presence of spermatids and spermatozoa with round nucleus in the tubular lumen. The interstitial compartment showed a great quantity of collagen fibers, and Leydig cells that were found isolated or forming clusters.

DISCUSSION AND CONCLUSIONS

The preliminary data obtained in the present study indicate that the *A. gigas* presents a testis of anastomosing tubular type, a testis cytoarchitecture characteristic of the primitive teleost taxa (Leal et al. 2009). A testicular germinal epithelium composed of Sertoli cells and germ cells bordering a lumen was present, which is a constant among vertebrates. The presence of interstitial Leydig cells indicates this cell type play a role on the hormonal control of spermatogenesis in this species. The presence of spermatids with different patterns of nuclear chromatin condensation in the lumen suggests the final steps of spermiogenesis also occurs in the tubular lumen as observed in some teleost species such as *Astyanax altiparanae* (Costa et al. 2014). The stereological quantification of the testis components presented in this study can be very helpful to better understand the reproductive cycle of males of *A. gigas*, as well as to compare the testis structure and function with other fish species and vertebrates. However, in order to make our observations more comprehensive testis samples from more animals should be collected in different periods of the year.

Table 1. Biometric and testis stereological data in *A. gigas*.

Parameter (n=4)	Mean ± SD
Body length (cm)	175 ± 14
Body weight (kg)	53 ± 15
Testis weight (g)	7.3 ± 3.2
Gonadosomatic index (%)	0.014 ± 0.002
Tunica albuginea (%)	9.7 ± 2.5
Testis parenchyma volume density (%)	
Tubular compartment	69.9 ± 14
Tunica propria	1.4 ± 0.7
Seminiferous epithelium	46.9 ± 11.4
Lumen	21.6 ± 11.1
Intertubular compartment	30.1 ± 13.8
Conective tissue	25.9 ± 14.4
Leydig cells	2.1 ± 1.3
Blood vessel	2.1 ± 1.3

ACKNOWLEDGEMENTS

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P45

SHORT-TERM *IN VITRO* EXPANSION OF SPERMATOGONIAL STEM CELLS IN CASPIAN BROWN TROUT (*SALMO TRUTTA CASPIUS*)

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INTRODUCTION

Spermatogenesis is a highly organized process that spermatogonial stem cells (SSCs) are at the foundation of this process. These cells have high capacity to self-renew and differentiate into functional germ cells. Although several attempts have been made to culture SSCs (Shikina and Yoshizaki, 2010; Wong and Collodi, 2013), there have been some obstacles in establishing a SSC line that can be used in surrogate broodstock technology especially in fish. Therefore, we aim to evaluate the effect of various feeders on the short-term culture of Caspian brown trout SSCs.

MATERIALS AND METHODS

The testes were obtained from 8 to 16-month-old of Caspian brown trout (*Salmo trutta caspius*) and disaggregated by two-step enzymatic digestion (collagenase type IV and Trypsin). Dissociated testicular cells were seeded at a concentration of 10000 cells /cm^2 on gelatin-coated plates to isolate SSCs by differentially adhesion. Isolated SSCs were co-cultured with Caspian trout ovarian stromal cells (OSCs), Caspian trout testicular stromal cells (TSCs) and/or 0.1% gelatin. To determine the characterization of SSC, PAS staining and immunostaining using mouse anti-Vasa antibody were used 25 days after isolation.

RESULTS

SSCs expanded *In vitro*, however they formed colony morphology on feeders but not on gelatin. The number of colonies was significantly higher on OST on comparison with TSC ($P< 0.05$). The cultured cells were PAS and Vasa positive.

DISCUSSION AND CONCLUSIONS

This study provides evidence that OSC is a suitable feeder for expansion of Caspian brown trout SSCs. This short-term *in vitro* culturing would allow the development of new biotechnologies in fish production.

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P46

CAN THE REPLACEMENT OF CORN BY SORGHUM SUPPLEMENTED WITH PHYTASE AFFECT THE GONADOSOMATIC AND HEPATOSOMATIC INDEXES OF SILVER CATFISH MALES KEPT IN CAGES?

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INTRODUCTION

In recent years, the corn, the main energy food used in fish feed, has been suffering from price fluctuations in the international market. Corn price depends on regional supply and demand conditions which have registered changes with the significant growth of livestock production, human consumption of grains and biofuel production (MATOS et al, 2008; REDDY et al., 2010; CIFUENTES et al., 2014). Among some commercially available substitutes, sorghum stands out for presenting nutritional characteristics similar to corn, market availability and lower price (70-80% of corn price) which can minimize the impacts caused by changes in inputs prices (SELLE et al ., 2010). On the other hand, information on the use of this feed for fish is scarce. Thus, the objective of this study was to evaluate the gonadosomatic and hepatosomatic indexes of male silver catfish *Rhamdia quelen*, fed with diets based on corn and sorghum, supplemented or not with phytase enzyme (0 and 1500UI).

MATERIALS AND METHODS

We used 30 males with 0.134 ± 0.042 kg average weight and 23.34 ± 2.41 cm length were distributed in a completely randomized design in factorial scheme with six treatments and four replications. The fish were kept in cages (20 fish.m^{-3} density) for six months, fed with isoenergetic diets, based on corn:sorghum, supplemented or not with phytase enzyme (0 and 1500UI) (T1: 00:34 without phytase ; T2: 00:34 with phytase; T3: 30:00 without phytase, T4: 30:00 with phytase; T5: 15:17 without phytase; T6: 15:17 with phytase). At the end of the experiment, the fish were selected, taken to the laboratory, euthanized with benzocaine solution at 100 mg L^{-1}, and had their gonads and liver collected to calculate gonadosomatic (GSI%) and hepatosomatic (HSI%) indexes ((organ weight/fish weight)*100). The results were submitted to analysis of variance, and the significant averages were compared through Tukey test at a significance level of 5% by using Statistica 7.0® statistical software.

RESULTS

Gonadosomatic and hepatosomatic indexes presented by silver catfish males show interaction effect ($p<0.05$) among the factors, when fed with corn:sorghum at 00:34 and 30:00 ratio, as seen in Figure 1.

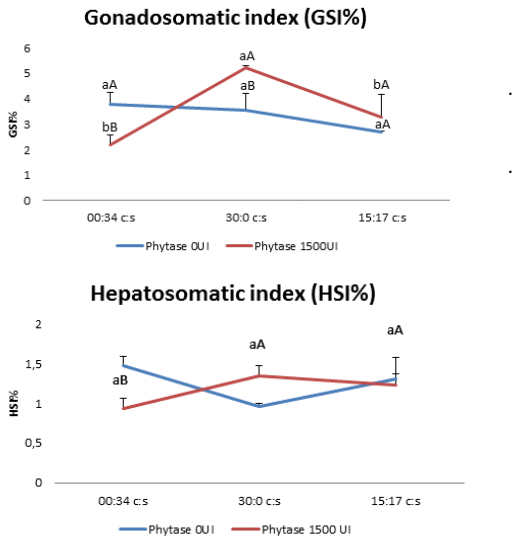

Figure 1. Gonadosomatic (GSI%) and hepatosomatic (HSI%) indexes of silver catfish fed with diets based on corn and sorghum, supplemented or not with phytase enzyme (0 and 1500UI). Averages followed by lowercase letters in the same direction indicate significant differences through Tukey test ($p<0.05$) among treatments with or without phytase supplementation; Averages followed by capital letters between the parallels indicate interaction effect ($p<0.05$) of treatment with or without phytase addition.

DISCUSSION AND CONCLUSIONS

The relative size of organs and tissues under suitable nutritional conditions, depends only on the fish size and its life cycle. The internal organs, except for the visceral lipids, tend to gain weight in proportion to the fish carcass growth (SHEARER, 1994). This growth may be influenced by the proportion of phosphorus available to the animal, since it is an utmost important nutrient for growth, skeletal and reproductive development of fish, mainly when associated with calcium (Kumar et al. 2012). The availability of phosphorus can be achieved by using phytase enzyme concomitantly to growth and size of internal organs, as it may be observed through the interaction of GSI% presented by the fish fed with the ratio of 30:00 and 00: 34 of corn:sorghum, supplemented or not with the enzyme. This same effect was also observed for the HSI% when fed on these same proportions. Thus, the proportions 30:00 and 00:34 corn:sorghum are observed to influence GSI% and HSI% of silver catfish males, as they have interaction among the factors when supplemented with phytase 1500UI. Noting that males fed with corn:sorghum at 30:00 ratio, when supplemented with phytase, present better GSI%.

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P47

CAN ARGININE AFFECT THE REPRODUCTIVE RATES OF *RHAMDIA QUELEN* FEMALES KEPT IN CAGES?

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INTRODUCTION

The arginine is a functional amino acid (Wu, 2009) that acts in the metabolic pathways necessary for maintenance, growth, reproduction and immunity of the organisms (Suenaga et al., 2008). It is a constituent of proteins, and it is also involved in the syntheses of proline and polyamines (Nikolic et al., 2007), which in turn are essential during embryogenesis (Lefèvre et al., 2011). In mammals the arginine benefits the increase of offspring as well as the quality. However, the effects of arginine on fish reproduction are still unknown. Therefore, the present work aimed to evaluate the arginine effect in the reproductive rates of *Rhamdia quelen* females kept in cages.

MATERIALS AND METHODS

We used 120 females distributed in a completely randomized design constituted by five treatments and four replicates. The fish were kept in cages (40 fish.m^{-3}) for eight months (from august 2014 to march 2015) fed with diets containing different levels of arginine (**T1:**1.37; **T2:** 1.67; **T3:** 1.97; **T4:** 2.27 and **T5:** 2.57%). Monthly, three females of each treatment were euthanized with benzocaine solution and the ovaries were removed and weighed so we calculated the gonadosomatic (GSI) and hepatosomatic (HSI) indexes. The results were submitted to repeated measures analysis by Statistica 7.0® software at 5% of significance level.

RESULTS

The GSI's were: T1:3.84; T2: 3.93; T3: 4.95; T4: 4.24; T5:4.38; and they were not different ($P>0.05$) from one another as well as they were not different ($p>0.05$) in the interaction analysis. However, when we analyzed only the months of collection, two peak values related to November and December were observed (4.43±2.18 and 4.55±2.62, respectively) followed by March (6.59±3.53) (Figure 1). Those months showed higher gonadosomatic index and were different from the rest ($p<0.05$). The HSI showed interaction among the levels of arginine and months, being treatment 1 significantly lower than the other treatments during the months from September to December (1.83±0.33; 1.68±0.43; 2.11±0.87; 2.05±1.16), while treatment 4 was superior to the others (5.12±1.06) ($p<0.05$).

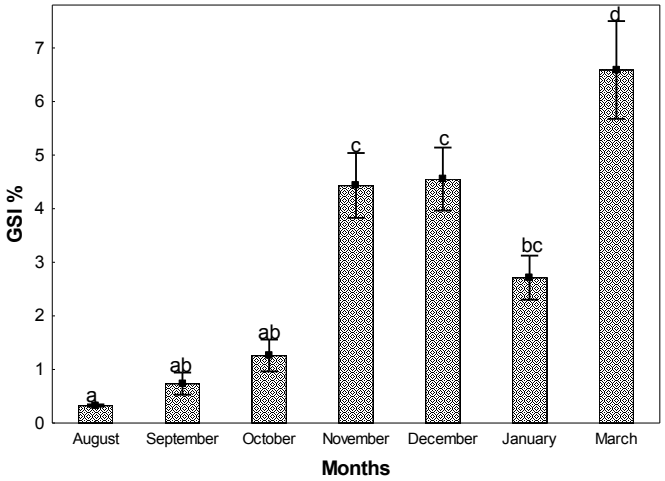

Figure 1. Gonadosomatic index (GSI) of *Rhamdia quelen* females fed with diets containing different levels of arginine kept in cages

DISCUSSION AND CONCLUSIONS

The results found for GSI temporal variation are common for this specie as reported by Ghiraldelli et al. (2007). And the presence of more than one reproduction peak is common for this specie as discribed by Reidel et al. (2010). The catfish energy demand increases during the gonadal maturation period, especially during mobilization in vitellogenic stage, when large amounts of protein, lipid, and glycogen from external (diet) and internal sources are synthesized by the liver (Babin et al., 2007). Arginine does not influence the gonadosomatic and hepatosomatic rates of *Rhamdia quelen* females kept in cages but we can affirm that November, December and March are the months when females are better prepared to reproduce.

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P48

SCREENING FOR STERILITY CANDIDATE GENES IN ATLANTIC SALMON (*SALMO SALAR*)

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INTRODUCTION

Early sexual maturation and genetic interference with wild stocks are major challenges in salmon aquaculture. One way to solve these issues would be to produce sterile salmon through vaccination. To identify suitable vaccine candidates, we need more knowledge about the genes that control germ cell formation. Our aim was to screen for genes specifically expressed in salmon germ cells using a combination of testis trancriptome sequencing together with other tissue transcriptomes in salmonides. This method enables bioinformatic filtering of proteins specifically expressed, in gonads and in areas of the cell which enables vaccine targeting.

MATERIALS AND METHODS

RNA sequencing was performed on salmon testis in immature (n=3), pre-pubertal (n=3) and pubertal (n=3) stages. Using BWA, the sequences were mapped against the salmon genome release and gene expression was obtained accordingly (Wang et al 2014). To filter for gonad specificity, genes expressed in gill and notochord were excluded. To compare with additional tissue transcriptomes, the salmon sequences were blasted against those of trout (Berthelot et al., 2014). Sequences with BLAST score <600 and sequences with more than 10 reads in any extra-gonadal tissues in trout were excluded for further analysis. Functional filtering was performed using KEGG and GO tools developed for the salmon genome (Wang et al., 2014). PCR and ISH (*in situ hybridization*) were used to verify expression of candidate genes.

RESULTS

After filtering, 49 gonad specific candidate genes were identified. 20 genes were selected based on expression level and function in testis and ovary for further PCR and qPCR tissue screens. Ten genes (genes 1-10) were verified to be specifically expressed in gonads. ISH revealed that mRNA from genes 1-7 were exclusively localized to germ cells.

DISCUSSION AND CONCLUSIONS

In this study we have identified novel genes that are specifically expressed in salmon germ cells. This information is valuable in the search for suitable targets in future functional studies, with the main aim to identify vaccine targets to induce sterility.

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P49

GONADAL MORPHOGENESIS IN *BRYCON ORBIGNYANUS* (CHARACIFORMES, CHARACIDAE)

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INTRODUCTION

Brycon orbignyanus is a fish with potential for fish farming and sport fishing because it has a delicious meat and aggressive behavior. However, it is endangered and has problems in induced reproduction, which requires knowledge of the gonadal development to enhance your reproductive management. Thus, the aim of this study was to describe the gonadal development *B. orbignyanus* until 51 days after hatching, in order to provide information that may be useful in developing techniques for the production of the species.

MATERIALS AND METHODS

Thus, aiming at better utilization of the reproductive period of the species, collections were made of larvae and juveniles from the induced spawning in the Estação de Hidrobiologia, CESP, Três Lagoas, MS, Brazil. The larvae and juveniles collected (from the time of hatching until the 51th day of development or 1207 hours after hatching (hah)) were fixed a solution of 4% paraformaldehyde and 2% glutaraldehyde in a Sorensen phosphate buffer 0.1M, pH 7.2, and processed for analysis under light microscope.

RESULTS

In *B. orbignyanus*, the gonadal primordium is already present in coelomic cavity at the time of hatching, remaining at this stage up to 324 hours after hatching (hah). It has few primordial germ cells (CGPs) surrounded by somatic cells (Fig1A,B). The gonadal primordia is identified as a structure pair from 54 hah (Fig1C,D). The CGPS has initially oval in shape, becoming rounded from 79 hah (Fig1B). Somatic cells are squamous up to 103 hah, and irregularly shaped thereafter (Fig1E). From 127 hah, the primordial gonad presents better defined, however until 324 hah, has no blood vessels or mitotic division in the CGPS (Fig1E). After 325 hah, there are blood vessels in the gonadal tissue, which characterizes his transition to undifferentiated gonad (Fig1F,G). However, until 51 days after hatching (1207 hah), the gonads of *B. orbignyanus* remain undifferentiated (Fig1H).

DISCUSSION AND CONCLUSIONS

In *Brycon orbignyanus*, migration of germ cells of the endoderm of the yolk sac to the gonadal region happens in the embryonic period, with the gonadal primordium present in the coelomic cavity at the time of hatching, as do *Oryzias latipes* (SATOH and EGAMI, 1972). However in *Pseudobagrus fulvidraco* (PARK et al., 2004) and *Cyprinus carpio* (MAZZONI, 2009), the primordial gonad was visible from three and seven days after hatching, respectively. In addition, the change in shape of primordial germ cells, appears to be the result of cell migration and establishment in the cavity celomática (MAZZONI et al., 2009). According to the classification of Gao et al. (2009), the gonad is considered a gonadal primordium while not present vascularization and mitotic divisions of germ cells. From the moment that present such characteristics, becomes an undifferentiated gonad, it is considered so until the moment they appear the male and female differentiation signals. The results obtained so far allow us to know the gonadal morphogenesis of *B. orbignyanus* until 51th day after hatching (undifferentiated gonads), and such information will serve as subsidies for determining the sexual differentiation period of the species and studies about sex reversal.

ACKNOWLEDGEMENTS
CESP for providing the fish. This research was supported by FAPESP (2010/00988-8; 2014/23379-8).

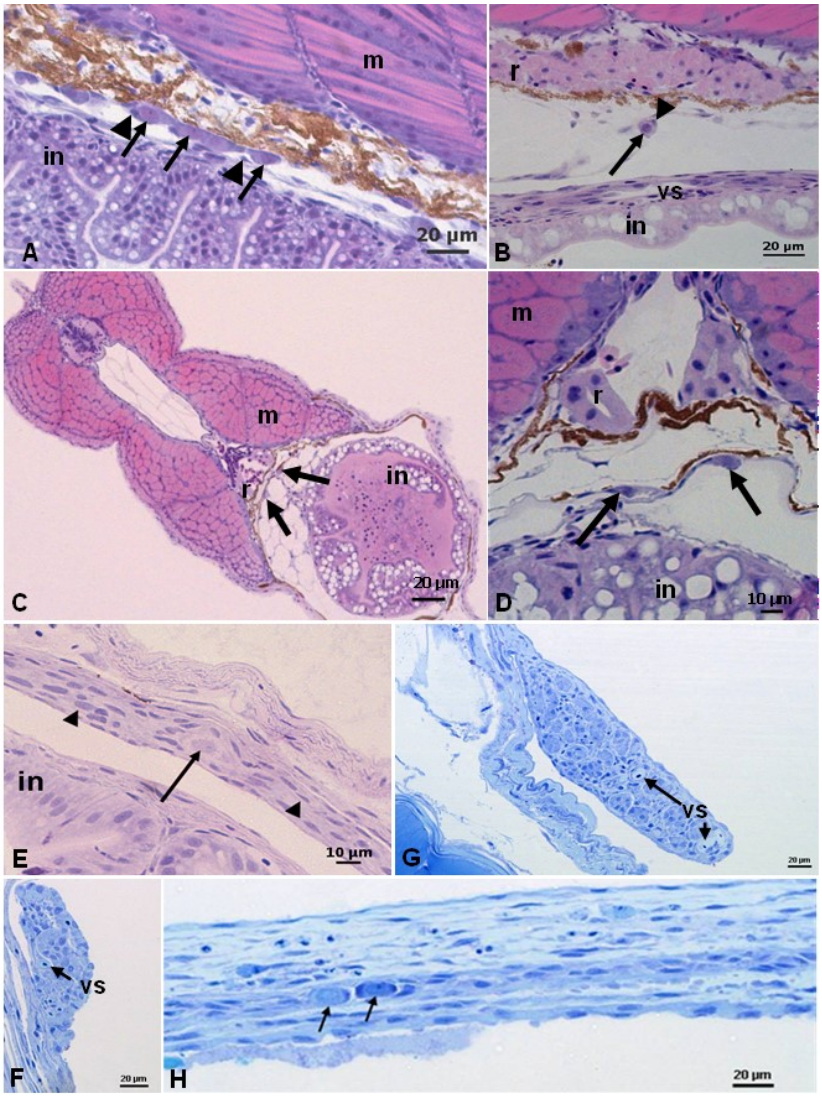

Fig 1 - *Brycon orbignyanus*: larvae and juveniles. **A:** 24 hours after hatching (hah); **B:** 79 hah; **C** e **D**: 54 hah; **E**: 175 hah; **F**: 325 hah; **G**: 511 hah; **H**: **in:** gut; **m:** muscle; **r:** kidney; **vs:** blood vessels; **arrow:** primordial germ cells; **arrowhead:** somatic cells. Stain: A-E: HE.; G-H: Toluidin blue.

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P50

LIPOSOMES: A VEHICLE FOR MOLECULAR/PASSIVE TRANSPORT IN SALMON EGGS

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INTRODUCTION

In order to study the effect of different biomolecules on fish embryogenesis, it is essential to have easy and reliable methods for delivery of the biomolecules (drug) to eggs and the developing embryo. Up to now, delivery of exogenous biomolecules for influencing embryonic development has relied mostly on the microinjection, which is a cumbersome and time consuming method. Thus, new and more efficient methods for the delivery of biomolecules to fish eggs and embryos need to be developed.

Liposomes are hollow spherical assemblies formed by a single-or multiple lipid bilayers. Liposomes can be tailored (via selecting their composition, size, charge, etc.) to efficiently entrap a wide variety of substances (Shi et al., 2010). Because of their biocompatibility, biodegradability, low toxicity, and easy attachment of target moieties for cell or organ specific drug delivery, liposomes has been used widely as a drug delivery vehicle system in higher vertebrates (Akbarzadeh et al., 2013; Allen and Cullis, 2013). In fish, however, application and understanding of the mechanisms determining the *in vivo* uptake and fate of liposomes is still scant. Therefore, in the present study, we used lipophilic fluorescent dye loaded liposomes to develop a delivery model for passive uptake in salmon eggs. The influence of liposome size, charge and incubation time was investigated. This is the first study performed in salmon eggs.

MATERIALS AND METHODS

Liposomes (neutral and negative charge) were prepared using soy phosphatidylcholine, LIPOID S-100 (PC), egg phosphatidylglycerol sodium, Lipoid EPG-Na (PG) and cholesterol (CH). They were mixed in the following molar ratios: PC/CH (9:1) for neutral liposomes and PC/PG/CH (9:1:1) for negatively charged liposomes. Fluorescent dye, rhodhamine B isothiocyanate was added while preparing liposomes at a final molar concentration of 1.0 mol %. Alternately, after the hydration and vortexing, the resulting both neutral and negatively charged liposome suspensions were sonicated to have desired sizes of approximately 50 nm and 160 nm. Unfertilized eggs and rhodamine loaded liposomes (Rh-liposome) were incubated in a modified L-15 media for either 24 or 48 hours at 6 ºC and then fertilized with sperm in excess. Eggs/embryos were sampled for evaluation of internalized liposomes by a fluorescence microscope at different developmental stages until the yolk sac was completely absorbed. Florescence was analyzed using a Zeiss fluorescence microscope.

RESULTS AND DISCUSSION

Liposomes, irrespective of the charge and size, were apparently taken up by the ovulated eggs and also incorporated into the yolk after 24 hrs. The passive uptake of liposomes in the yolk was highest after 48hrs of incubation. This indicates the possibility of successful passive uptake of Rh-liposomes in the salmon eggs. After incubating the eggs with Rh-liposomes, the eggs were fertilized to follow up the uptake distribution or localization. Fertilization was high (approximately 90 %) in all the groups and not influenced by Rh-liposome treatment. Sometimes after hatching (700 day degree, DD), negatively charged liposome with size 160 nm exhibited the highest fluorescence though not so intense in the yolk sac compared to other Rh-liposome groups. The fluorescent signal was maintained in the yolk until all the yolk was absorbed about one month after hatching (i.e. 900-1000 DD post fertilization).

CONCLUSIONS

Since no studies have been done on the liposome based delivery system in salmon, we focused our study on the uptake of entrapped lipophilic dye as a model for the in vitro delivery into salmon eggs.

The present study has provided important insight on the effect of incubation time, liposomal composition, surface charge and vesicle size on uptake properties as well as being a first model for the delivery of lipophilic molecules into salmon eggs. Further studies are required to confirm the observed trends.

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P51

CATHEPSINS EXPRESSION IN FLOATING AND NONFLOATING EARLY EMBRYOS IN THE YELOW-TAIL KINGFISH *SERIOLA LALANDI*

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INTRODUCTION

Seriola lalandi is a marine pelagic fish considered essential to diversify the Chilean aquaculture, however, its full production cycle has been difficult to complete due to high mortality during embryonic and larval stages. Studies performed in others species have shown that the low buoyancy of the eggs is a critical factor to explain this low survival (Carnevali et al., 2001). The egg buoyancy facilitates an efficient oxygen supply for the embryo, the fertilization and the embryo hydration. In pelagic species, an additional processing of yolk proteins generates free amino acids and small peptides which are osmotic effectors that cause water influx into the oocytes during the hydration process, providing buoyancy to eggs and the necessary nutrients for early embryogenesis (Carnevali et al., 1999; Sawaguchi et al., 2006). The main molecules involved in yolk processing are the lysosomal proteases called cathepsins which are essential during oocyte maturation and embryo development (Carnevali et al., 2001; 2008). Thereby, in order to know molecular aspects involved in the buoyancy acquisition process, the aim of this work was to compare the cathepsins expression between floating and non-floating eggs and early embryos in *S. lalandi*. This information would allow proposing the use these genes as potential quality biomarkers in some of these embryonic stages in this specie.

MATERIALS AND METHODS

Newly spawned eggs, early morula, blastula, gastrulae and appearance embryo (Moran et al., 2007) were placed during 5 min in an inverted conical flask (5 Lt), where floating eggs were recovered on the surface and nonfloating from the first two liters evacuated at the bottom of the flask.Total RNA was extracted using the Kit GeneJET™ RNA (Thermo) following the manufacturer's instructions. The RNA concentration was determined using a Qubit® Fluorometer (Invitrogen™). Samples were processed for reverse transcription using the enzyme conjugate SuperScript™ First-Strand (Invitrogen™). The cDNA concentration was determined using the ssDNA Qubit ® Assay. Primers for cathepsin B, D and L, were designed by alignment of Genbank EST sequences from different fish species using the ClustalX program. Primer for □-actin and tubulin, as constitutive genes, were generated from *Seriola quinqueradiata* EST sequences. All primers were tested with Net Primer Software. PCR were performed with an Illumina® Eco Real Time PCR System using the Maxima SYBR Green qPCR Master Mix (2X) (Thermo). After PCR standardization, the expression levels were recorded as Ct values and the relative expression of cathepsins was evaluated (Vandesompele et al., 2002) and expressed as the mean ± standard deviation. Data were analyzed by ANOVA and significant differences by Tukey test considering significantly differences when $P<0.05$.

RESULTS

Nonspecific amplification was evaluated by the shape of the melting curves (Tm) and to validate the qPCR results, the amplification efficiencies of each primers pair were near to 2 (100%). Cathepsin B expression in floating and nonfloating samples showed high mRNA levels in the first three developmental stages. After blastula, the expression decreased significantly. In addition, it was not observed difference between floating and nonfloating samples, at any development stage. The cathepsin D expression level in floating samples was characterized by a progressive increasing until the blastula stage and a drastic decreasing at the appearance embryo stage. In nonfloating eggs, the expression increased in early morula, remained without variation until gastrula. As well as in floating samples, a

drastic decrease was observed in the appearance embryo stage. Differences in the cathepsin D expression, between floating and nonfloating samples, were detected in newly spawned eggs and early morula, which resulted greater in nonfloating eggs. Finally, high expression of cathepsin L was obtained in floating samples from recently spawned eggs and appearance embryos. In these stages, the expression was greater than nonfloating samples, which did not changed during different development stages.

DISCUSSION AND CONCLUSIONS

The persistent presence of the mRNA for these enzymes during early development of *S. lalandi*, indicates that these molecules can execute important function in these stages. However, their expression profiles in the different stages and between floating and nonfloating specimens shown specific patterns. Apparently, cathepsin B is not involved in the buoyancy, since no differences in its expression between floating and nonfloating samples were detected in all stages of this study. Catepsin D expression was higher in not floating samples of newly spawned eggs and early morulae, so this gene could be proposed as a biomarker of poor quality in these development stages. In contrast, the high expression of cathepsin L in floating samples compared to nonfloating, in newly spawned eggs and appearance embryos would indicate that this gene could be considered as good quality indicator in these embryonic stages in *S. lalandi*.

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P52

THE FIRST RESULTS OF HEAT SHOCK TRIPLOIDISATION IN PIKEPERCH (*SANDER LUCIOPERCA*)

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INTRODUCTION

Induced triploidisation in fish is made by inhibiting of the second meiotic cell division and the extrusion of the second polar body by shocking eggs short time post fertilisation (Chourrout, 1988; Malison et al., 1993). Triploid-level has already been induced in several freshwater percid species such as, Yellow Perch (*Perca flavescens*) (Malison et al., 1993), Eurasian Perch (*Perca fluviatilis*) (Rougeot et al., 2003), walleye (*Stizostedion vitreum*) (Malison and Garcia-Abiado, 1996), and saugeye (female walleye *Stizostedion vitreum* x male sauger *S. canadense*) (Garcia-Abiado et al., 2001).
Currently, there is no information about induction of the triploidy in pikeperch. The aim of this study was to find the ideal time of initiation (TI) of treatment (i.e., min post fertilization), intensity and duration of heat shock on ploidy level of pikeperch larvae.

MATERIALS AND METHODS

Eggs from five females were mixed with semen from five males and activated with water at 15-15.5°C to induce fertilization. At TI of 1; 3; 5; 7 and 10 minutes, three batches of 2ml of eggs (1361±70 eggs) were immersed into a water bath at the shock temperature (ST) of 29°C (Experiment 1) and 31°C (Experiment 2) for 20 and 40 minutes. After the shock administration, the eggs were transferred into hatchery with water temperature at 15.4±0.3°C. Twenty four hours post shock treatment, the fertilization rate was determined. Successfully hatched larvae and malformed larvae were counted approximately 48 hours post hatching. Ten larvae were randomly taken from each experimental treatment and their ploidy levels were determined by using the flow cytometry.
To analyze the date from both experiments, a one-way analysis of variance (ANOVA) and Tukey HSD test were used. The level of significance was $P < 0.05$ for all tests.

RESULTS

The TI of 1 minute post fertilization was the worst possible variant to induce triploids or even viable larvae in Experiment 1 (Table1). Also the duration of shock treatment for 40 minutes led to no hatching in most groups in Experiment 1. However TI of 10 minutes for 40 minutes produced the highest number of triploids in Experiment 1 overall (Table1). Much better results and more satisfying data were observed in Experiment 2, where the water bath at the shock temperature of 31°C was applied. There was even observed three groups with 100% triploid larvae (TI of 1 minute for 20 and 40 minutes and at TI of 5 minutes for 20 minutes long shock treatment). The other TIs also induced high number of triploid larvae but no in 100% rate (Table 2). The side effect of ST of 31°C was high occurrence of malformed larvae compare to the ST of 29°C (Table 1 and 2).

	Duration of heat shock treatment 20 min, ST 29°C					Duration of heat shock treatment 40 min, ST 29°C				
Time of initiation (TI) [min post fertilization]	Fert. [%]	Hatch. rate [%]	Inc. time [°d]	Malf. [%]	Trip. [%]	Fert. [%]	Hatch. rate [%]	Inc. time [°d]	Malf. [%]	Trip. [%]
1	18.1±10cd	0c				8.6±3.1c	0b			
3	30.1±3.3c	1.3±0.2c	122.4c	0a	11±19.5a	26±2.1b	0b			
5	24.6±8.8c	3.1±2.5c	114.7b	3.3±5.7a	33.3±23.6a	29.8±2.8b	0b			
7	33±3.9c	8±1.3b	114.7b	0a	16.6±11.5a	22.8±3b	0b			
10	43.1±1.6b	11.5±1b	107.7a	3.3±5.7a	25.3±14.4a	28.1±6.5b	1.3±1.8b	145.3b	0a	75±0a
Control group	76.8±6.8a	59.1±3a	137.7d	2.1±2.4a	0b	76.8±6.8a	59.1±3a	137.7a	0a	0b

Table1. Table shows results of Experiment 1, where the differences in fertilization and hatching rate, incubation time, malformations and triploid percentage among the times of initiation and control group after 20 and 40 minutes of heat shock treatment were observed. Different letters mean significant difference (P<0.05).

	Duration of heat shock treatment 20 min, ST 31°C					Duration of heat shock treatment 40 min, ST 31°C				
Time of initiation (TI) [min post fertilization]	Fert. [%]	Hatch. rate [%]	Inc. time [°d]	Malf. [%]	Trip. [%]	Fert. [%]	Hatch. rate [%]	Inc. time [°d]	Malf. [%]	Trip. [%]
1	50.5±5.6b	2.3±2c	101a	74.1±0a	100±0a	19±3c	3.1±5.4c	101a	0a	100±0a
3	49.6±17.2b	11.8±8.8b	101a	9.6±9c	95.8±7.2a	48±5b	7.1±1.8bc	101a	0a	91±7.7a
5	38.6±20bc	7.8±10.1b	101a	35.5±33.5b	100±0a	48.3±9.5b	8.5±4.3bc	91.8b	0a	88.8±19.2a
7	53.1±9.1b	15.3±5.1b	84.1c	7±2.5c	86.3±15.1a	53.6±10.2b	13.2±1.9bc	91.8b	0a	90±0a
10	55.1±9.1b	20.0±5.2b	91.8b	5.2±1.4c	53.3±5.7b	50.6±12.8b	18.3±7.6b	101a	0.6±0.5a	41.8±19.1b
Control group	74.6±7.3a	61.6±7.3a	91.8b	1±0.8d	0c	74.6±7.3a	61.6±7.3a	91.8b	1±0.8a	0c

Table2. Table shows results of Experiment 2, where the differences in fertilization and hatching rate, incubation time, malformations and triploid percentage among the times of initiation and control group after 20 and 40 minutes of heat shock treatment were observed. Different letters mean significant difference (P<0.05).

DISCUSSION AND CONCLUSIONS

The main reason for carrying out of this experiment was to know, whether some particular heat shock treatment would lead to pikeperch triploid larvae production or hopefully complete triploid population. Heat shock treatment (next to hydrostatic pressure shock) is commonly used for polyploidisation in percid species (Malison et al., 1993; Malison and Garcia-Abiado, 1996; Rougeot et al., 2003) but not always with 100% triploid production. It is a great finding that even three different shock treatments led to 100% triploids production

It is a task for next research to find more efficient way to produce 100% pikeperch triploid population with higher fertilization and especially hatching rate.

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P53

SEXUAL CYCLE OF GONAD IN THE RUDD (*SCARDINIUS ERYTHROPHTHALMUS*) IN RIVER SECTIONS INFLUENCED DIFFERENTLY BY THE HEATED EFFLUENTS FROM A POWER PLANT

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INTRODUCTION

In Poland, the rudd is a common species occurring in lowland, heavily overgrown, slow-flowing rivers and lakes with muddy bottom (Załachowski 2000). Its reproduction is strictly related with sunlight accessibility and temperature (Živkov et. al 2003). This study was undertaken to analyze the annual development of female and male gonads in the rudds from three sites differing in the temperature regime: the Oder river (above the Warm Canal, into which post-cooling water from the Dolna Odra power plant is discharged), Lake Dąbie (which collects water from the East Oder river and Warm Canal) and the Warm Canal at 200 m from the point of post-cooling water discharge (an open channel in which the fish can move freely). The aim of the study was to determine and compare the character and length of the reproductive cycles in both sexes of the rudd from locations differing in the influence of warm water discharged from the power plant.

MATERIALS AND METHODS

The rudd (*Scardinius erythrophthalmus*) individuals to be studied were caught at the three above described sites from July 2009 to June 2010, in the amount of 1–4 samples per month at each site. The total number of studied females was 332, while that of the males was 90, due to female-to-male ratio. Sample preparation was conducted using a standard histological method. The histological evaluation of gonads was done using the 6-grade scale published by Domagała et al. (2013, 2014).

RESULTS

The body length of the rudd females was 22.5 cm in those from the Oder river, 20.5 cm in those from Lake Dąbie and 14.7 cm in those from the Warm Canal. The length of the males was 14.5, 14.7 and 10.8, cm, respectively. The spawning season (stage 5 in females) at the natural sites in the Oder river and Lake Dąbie started at the end of April and lasted through May and June. Spawning was in portions. The regeneration of gonads (stage 3) in the females from the Oder river and Lake Dąbie started in August. From March to April and, in a few females, to May, the gonads were at stage 4. The annual cycle in the rudd females from the above two sites had a similar character. The development of oocytes in previtellogenesis and vitellogenesis was typical of these stages. In post-spawning gonads (stage 6), from May to December, atresia of spent gonads was observed. The fish from the Warm Canal were characterized by the lowest condition coefficient. The gonads of 60% females caught in the Warm Canal were abnormal, with oocytes developed late and degenerated. At that site, females with post-spawning gonads were not harvested.

The rate of the sexual cycle of testes in the two natural sites, the Oder River and Lake Dąbie, was similar. Histological analysis of male gonads showed that males were ready to breed from May to July. The period was a little longer than in the females. After spawning, the gonads entered stage 2 which lasted through the fall and winter until March. Germ cells started meiosis in early April (early stage 3). In April, cells undergoing further stages of spermatogenesis with spermatozoa formation were observed at the end of the month. In the Warm Canal, only a few males were caught and most of them were immature. In April, two mature males were found with gonads at stage 4, with development accelerated by two weeks in relation to the fish from natural sites.

DISCUSSION

The rudd is an important component of lake and river fauna in European waters, which is the foundation of their biodiversity. The spawning season of the studied rudds from the natural sites started at the end of April, and lasted through May and June. In the rudds from Spain, the spawning season was shorter (Garcia-Berthou, Moreno-Amich 2000), but in those from New Zeland, it was longer (Hicks 2003). The analysis of the cycle in the rudd females indicates that during spawning, they lay eggs in portions (Załachowski 2000). Degeneration of oocytes in the gonads of the rudds from the Warm Canal observed in the winter and spring concerned oocytes in previtellogenesis and vitellogenesis. Degeneration in the gonads of bream x rudd hybrids covered most of gonad cross-section and regarded primary and mature oocytes at the stage of migration of the nucleus (Kopiejewska et al., 2007).

In the conditions of the canal with post-cooling water, two of the very few males caught had mature gonads at stage 4. These males became ripe two weeks earlier than those in the natural waters. A similar offset of maturation was observed in other species living in water with increased temperature (Mattheeuws et al. 1981).

The annual cycle of the rudds from the Oder river and Lake Dąbie was typical of this species. The spawning of the rudds occurs at a temperature above 14–18°C (Załachowski 2000). In the Oder River and Lake Dąbie, during the rudd spawning period, it was approx. 17°C (Domagała et al. 2015). From May, water temperature in the Warm Canal was over 23°C. In the Warm Canal, a lower number of fish was caught and most of them were of smaller size and immature. In that location, no ripe rudd females were found. The conditions in this canal were unfavorable for spawning of the species due to the too fast water current and too high temperature.

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P54

EFFECTS OF TEMPERATURE ON SHORT-TERM STORAGE OF STERLET (*ACIPENSER RUTHENUS*) OVA

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INTRODUCTION

Sturgeons (Chondrostean, Acipenseridae) are ancient fish species, and widely known for their caviar. Most sturgeon species have been greatly impacted by anthropogenic conditions, including over-exploitation; many are critically endangered. Artificial propagation of sturgeons is becoming increasingly important for recovery efforts as well as for commercial production. Sterlet *Acipenser ruthenus* is a common Eurasian sturgeon with a small body size and one of the fastest reproductive cycles among the sturgeons. The practical question being addressed in this experiment was whether fertilization of ovulated sterlet eggs can be delayed without reducing the hatching rate, and an ancillary question, under what temperature conditions do eggs retain good quality.

MATERIALS AND METHODS

Males and females were kept in separate tanks (vol. 0.8 m^3) at 15°C. Broodstock were injected with homogenized carp pituitary extract (CPE) at 4 mg kg^{-1} body weight. Milt from three males was selected based on high sperm motility (density around 1.0×10^9 spermatozoa per mL), pooled and stored at 4°C until used. Ovulated eggs from three females were collected by hand stripping and pooled. Eggs (20g) were initially retained in ovarian fluid in small plastic beakers, allocated to various treatment groups for temperature storage (control, 7, 11, 15, & 19°C) until fertilized. Storage times at the regulated temperatures prior to fertilization were for 2.5, 5.0, 7.5 & 10.0 h. After the selected storage time, 4 g of eggs were allocated to each treatment (50-60 eggs/g) in 50- mL plastic beakers, and 200 µL of milt was added to each beaker. The beakers were placed on a shaker platform at a rotation of 250 min^{-1} and then 8 mL of fresh hatchery water was added to each container. After 2 min of mixing, the fertilized eggs were transferred to incubation cages and counted. Three replicates were allocated to each storage period and temperature; aerated and UV-treated water at 16°C was continuously circulated through the cage system. Hatched larvae were counted at 7 d post-fertilization. The percentage of hatched larvae (H_r) was calculated for each treatment from the total number of eggs (E_t) placed in the incubation cage minus dead eggs (E_d) by the following equation:

$H_r = (E_t - E_d)/ E_t * 100$. Hatching success was analyzed using a factorial ANOVA model containing the egg storage time (i.e. 0, 2.5, 5, 7.5, and 10 h) and storage temperature (7, 11, 15, and 19°C) main effects as well as the egg storage time × egg storage temperature. Then, the model was decomposed into a series of lower-order models. Here, the decomposed ANOVA models were run to (i) determine the effect of egg storage time for each egg storage temperature using a series of one-way ANOVA models, and (ii) determine the effect of egg storage temperature for each egg storage time.

RESULTS

The saturated two-way ANOVA model indicated significant effects of storage time and storage temperature on hatching rate ($p > 0.001$), but there was no significant interaction effect of storage time and temperature on hatching ($p < 0.05$). With reference to fertilization and hatching, eggs retained good quality when stored at 7 and 11°C for up to 10 h (Fig. 1 and 2), but egg quality was somewhat reduced after only 2.5 h storage at 19°C compared to cooler temperatures, and after 7.5 h, quality decreased more sharply (Fig. 1).

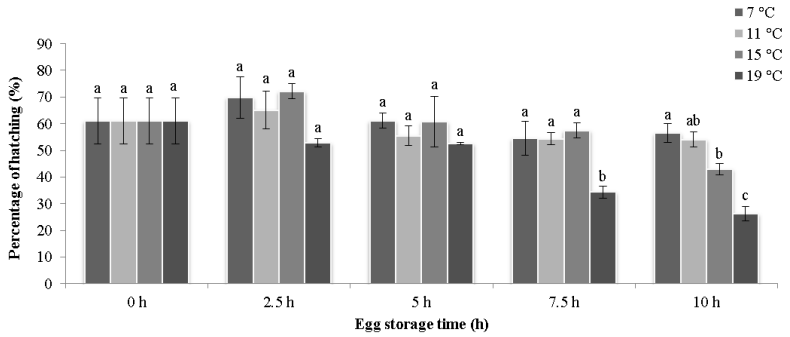

Figure 1: Effects of egg storage time (0, 2.5, 5, 7.5, and 10h) on hatching rate of sterlet *Acipenser ruthenus* eggs. Treatments without a common superscript differ significantly ($P < 0.05$). Error bars represent least-square means standard error.

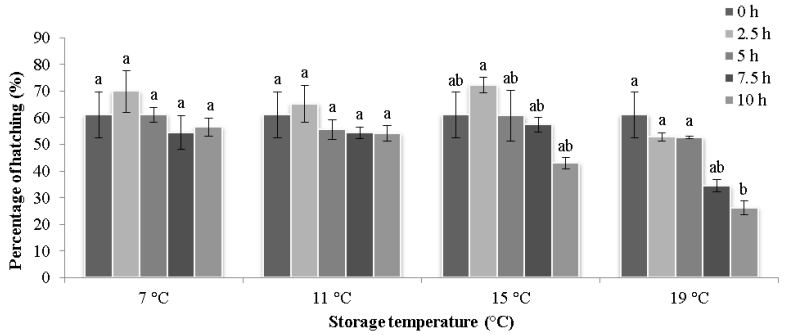

Figure 2: Effects of egg storage temperature (7, 11, 15, and 19°C) on the hatching rate of sterlet *Acipenser ruthenus* eggs. Treatments without a common superscript differ significantly ($P < 0.05$).

DISCUSSION AND CONCLUSIONS

Although basic procedures for artificial fertilization have been described (Dettlaff et al., 1993; Chebanov and Galich, 2011), only a few empirical studies have been conducted with respect to knowledge of sturgeon egg storage (Sohrabnezhad et al., 2006 & Gisbert and Williot 2002). We found that sterlet eggs do not need to be fertilized immediately after collection. Reasonably good quality appears to be retained for several hours if temperature conditions are fairly cool and stable. Uniform temperatures between 7 and 11ºC can be considered as appropriate for storage of eggs in coelomic fluid for up to 10 h.

ACKNOWLEDGEMENTS

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P55

DEVELOPMENT OF STAINING TECHINIQUES TO ASSESS VIABILITY OF TAMBAQUI (*COLOSSOMA MACROPOMUM*) OOCYTES

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INTRODUCTION

Despite its high production potential, Brazilian fish hatcheries still have low productivity. Among the main reasons for the low prolific yield during fish breeding practices is the lack of control on the main causes affecting the gametes viability. This viability in general terms means the gamete's ability to fertilize or being fertilized (Bobe and Labbé 2010), resulting in viable larvae with low mortality up to the post-larval stage. The use of vital dyes could become an alternative on this subject. Its use is already widespread for sperm analysis of some mammal species, and has also been tested on fish. However, for oocytes of aquatic species, especially fish, the use of vital dyes is still incipient. This study aimed to develop a staining protocol to assess the viability of *Colossoma macropomum* oocytes and to correlate it to the fertilization rate.

MATERIALS AND METHODS

Tambaqui (*Colossoma macropomum*) broodstock were randomly sampled at gonadal maturation stage. Selected females were hormonally induced to spawn by injecting a commercial carp pituitary crude extract (CPE). Mature oocytes were stripped into a plastic beaker by gentle abdominal massage and randomly distributed in 6-well plates (≈70 oocytes in each well). To carry out the trypan blue (TB) assay, a 0.4% TB stock solution (Sigma-Aldrich, Dorset, UK) was diluted to the following test concentrations: 0.2, 0.1, 0.05 and 0.025%. Oocytes were stained for 5 min and 1 min at room temperature, and then washed three times in distilled water. Membrane integrity of the oocytes was assessed under a light microscope. Three replicates were used for each TB concentration and experiment was repeated three times using oocytes from three different females.
In order to correlate the viability assessment with fertilization success, after being fertilized eggs from each female were transferred to 250-L open-flow conical incubators at 28 ± 0.1 °C. Six hours later, three samples were taken from each incubator, placed in 3-section Petri dishes and fertilization rate was checked by counting dead and live embryos.

RESULTS

When exposed to TB solution for 5 min, all oocytes were stained at any concentration tested. Likewise, the same pattern was observed when oocytes were exposed to TB solution for 1 min at 0.2 and 0.1%. On the other hand, a high number of viable oocytes (unstained and intact membrane) were observed when exposed to 0.05 (97.09 ± 3.32%) and 0.025 (97.40 ± 3.74%) TB concentrations (%) for 1 min. A high correlation (r = 0.85) was obtained between membrane integrity and fertilization rate from those oocytes exposed for 1 min to 0.05 and 0.025% of TB.

DISCUSSION AND CONCLUSIONS

Colossoma macropomum oocytes showed to be very sensitive to trypan blue dye and a 5 min exposure was too long to such gametes. At this exposure time, the dye was toxic enough to stain every single oocyte, being unable to distinguish between intact and non-intact membrane oocytes. Several reports are found in the literature using TB assay (5 min at 0.2%) to assess zebrafish (*Danio rerio*) oocytes viability after isolation (Guan et al. 2008) and exposure to cryoprotectant solutions (Tsai et al. 2009, Guan et al. 2010 and Godoy et al. 2013) with successful results. However, zebrafish spawns benthophil oocytes whereas *C.*

macropomum spawns pelagophil oocytes, and such spawning behavior probably leads to marked differences in morphological structure of the oocytes (Lubzens et al. 2010). The first results seen to be promising once we found a high correlation between intact membrane oocytes indicated by the staining assay and the fertilization rate. These tests can become an effective tool for assessing the *C. macropomum* spawning quality, as well as having an important approach by reducing costs and time efficiency in broodstock management. We are now trying to improve the protocols by combining dyes that will allow us to check both at morphological and subcellular level at the same time.

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P56

EGGS AND LARVAL QUALITY OF STERLET STURGEON *ACIPENSER RUTHENUS* INJECTED WITH THIAMINE

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INTRODUCTION

The availability of high quality nutrient for broodfish is the main factor affecting the ovarian development and further eggs and larval quality (Palace and Werner, 2006). Micronutrients such as thiamine (vitamin B1) are important in early life stages of fish (Lee et al., 2009). In this regard, some diseases such as early mortality syndrome (EMS) were identified, that is a result of thiamine deficiency and this will lead to major mortality in early stages of larva's life (Tillitt et al., 2005). The present study aimed to investigate the effect of thiamine injection on eggs and larval quality in an ancient fish, Sterlet sturgeon (*Acipenser ruthenus*).

MATERIALS AND METHODS

A total of 45 female fish (698.6 ± 8.9 g) were distributed in 9 fiberglass tanks with three treatments (each with three replicates). Experimental fish were fed practical diets supplemented with 1 g/kg amprolium hydrochloride (as an anti-thiamine) in diet for 5-month before spawning. Thiamine hydrochloride was injected to fish with three doses at 0 (T_0), 5 (T_5) and 50 (T_{50}) mg/kg body weight at three different times (day 30, 90 and 150). At the end of 5 months, feeding was stopped and after that, based on the germinal vesicle index and water temperature, fish were injected by 2.5 ug/kg LHRH-A_2 in two doses with 12 hours interval. During the final maturation and ovulation, the eggs were obtained by cesarean method (Falahatkar and Efatpanah, 2011). Eggs were fertilized with mixing to sperm according to semi-moist method. Then eggs were transferred to small containers (12 × 10 × 10 cm^2) placed in the Yushchenko incubators. At the end of experiment, working fecundity, hatching rate, total thiamine in egg, larval length and weight at 6-day post hatch (dph) were determined and some deformities sings in larval were daily monitored. Data were analyzed by SPSS 16, with one-way ANOVA and Tukey's test at the level of 95%.

RESULTS

Results showed that there were significant increased in hatching rate, total thiamine in eggs and larval weight in T_{50} ($p<0.05$; Table 1). Moreover, the highest working fecundity, larval length and survival rate were observed in T_{50} but not significant ($p>0.05$).

Table 1. Reproductive and larval performance of Sterlet broodstocks fed anti thiamine and injected with different levels of thiamine after 5 months rearing (n=15 broodfish/treatment; mean ± SE).

Treatment	Working fecundity (×10^3/ BW)	Hatching rate (%)	Total thiamine (nmol)	Larval length (mm)	Larval weight (mg)	Larval mortality (%)
T_0	4.5 ± 1.4	71.2 ± 0.12 ^{a}	2.9 ± 0.4 ^{b}	15.33 ± 0.09	21.6 ± 0.00 ^{c}	15.1
T_5	4.6 ± 1.8	45.1 ± 0.15 ^{b}	4.9 ± 0.8 ^{ab}	15.38 ± 0.13	21.7 ± 0.00 ^{b}	nd
T_{50}	6.7 ± 0.6	66.5 ± 0.17 ^{a}	7.3 ± 1.6 ^{a}	15.62 ± 0.14	23.1 ± 0.00 ^{a}	3.9

The data that are shown by dissimilar letters indicate significant differences ($P < 0.05$).

DISCUSSION AND CONCLUSIONS

The results showed that thiamine injection in broodstocks significantly increased the total thiamine in the eggs and larval weight, and improved working fecundity, egg size, and larval length. In addition, some signs of EMS including poor growth, lack of absorption of yolk sac, erratic swimming, loss of equilibrium and deformed yolk sac were observed in fish treated with T_0 at 4 dph. This kind of symptom also was observed by Norrgren et al. (1993) in salmon *(Salmo salar*). Thiamine has a coenzyme role in carbohydrate metabolism and is important in glucose synthesis, nervous and brain functions.

Our results revealed that thiamine injection has a positive effect on growth and larval survival, and injection of thiamine in the sturgeon breeders can reduce the negative impacts of anti thiamine in natural environment.

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P57

NUCLEAR TRANSFER IN STERLET EGGS: A FIRST ATTEMPT

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INTRODUCTION

Nuclear transplantation is a powerful technique to produce clone animals. If this technique does work in sturgeons, which are listed as endangered species; this would provide a valuable tool to produce these fish efficiently. A first key step to be studied for application of this technique to sturgeon is how to keep recipient oocytes inactivate during manipulation. In goldfish, Le Bail et al. (2010) reported that trout coelomic fluid (TCF) could sustain the developmental ability of oocytes for a long term. However, in sterlet sturgeon, an oocyte inactivating media has not been reported. The next important step is how to transplant a cell(s) into oocytes. In general, nuclear transfer protocols include diploid nuclei transplantation into a recipient oocyte, which belong to the same species, although in some researches closely-related species are chosen for a recipient. In addition to this, Yasui et al. (submitted) reported multi-sperm injection into oocytes could produce androogenetic progenies in loach (*Misgurnus anguillicaudatus*). This result implies that there is an unknown mechanism to regulate the embryonic development even after multi-nucleus transplantation into an oocyte.
So, in this study, we explored suitable extender solution in order to keep the oocytes inactivated but viable for receiving the donor diploid nucleus. And then, we performed both intraspecies (albino sterlet's fin cells transferred into wild sterlet's oocyte) and interspecies (russian sturgeon's fin cells transferred into wild sterlet's oocyte) nuclear transplantations. We performed both single and multi-cell injection in both mentioned groups.

MATERIALS AND METHODS

For extender solution Experiment, totally five solutions tested at 15^{o}C: PSACF, Persian sturgeon Artificial Coelomic Fluid (Sohrabnezhad et al. 2006), PBS (Porcine Bovine Serum), PBS plus 1% egg-white, PBS plus 1% BSA (Bovine Serum Albumin) and filtered Water. Firstly, we performed three washing steps in order to remove debris from oocyte's surface (about 25 oocytes per dish) with each solution. Three different batches from different females were used for this experiment. Subsequently in vitro fertilization test performed in 0h, 30min, 1h and 1h30min to monitor oocyte's fertilization ability after remaining in the respective testing solution.
For preparation of donor cells for nuclear transfer, we dissociated Russian or albino sterlet fin cells in 0.5% trypsin in PBS. Oocytes were obtained from wild sterlet females. We transplanted a single-cell or multi-cells into the animal pole of oocytes in PSACF using a micro glass pipette connected with a microinjector (Eppendorf). Oocyte was being hold with a holding capillary during transplantation.

RESULTS

From the extender incubation solution experiment, we observed not significant differences between PSACF, PBS, PBS + Egg-white and PBS + BSA, while the score of filtered Water was lower than the other solutions.
From nuclear transfer experiment, we found developing embryos in all experimental groups. In the experimental group of multi-cell injection either of Russian or Albino species, we observed higher number of developing embryos than from groups from single cell injection (Table 1).

DISCUSSION AND CONCLUSIONS

As far as we know, the present work is the first attempt in cloning sturgeon. Interestingly, embryos could develop not only after intraspecies (albino sterlet - wild sterlet) but also after interspecies (Russian sturgeon/albino sterlet - wild sterlet) nuclear transfer. Furthermore, more importantly, multi-cells transplanted oocytes could also develop, although the mechanism to regulate the development is unknown. This result suggests that relatively rough manipulation can be accepted for producing sturgeon clones, although of course optimization of experimental procedure will be needed in future. This founding is giving us hope for future application in more critically endangered sturgeon species.

Table 1. Single and multi-cell injection of russian sturgeon or albino sterlet into wild sterlet oocytes. All experimental groups gave embryonic developmental rate in observation at 24 hours post activation (hpa) and at 48 hpa.

Experimental group	Total no. of NT-oocytes	The number of embryos develops	
		24 hpf (%)	48 hpf (%)
Single-Albino	33	1 (3.0)	1 (3.0)
Single-Russian	33	1 (3.0)	1 (3.0)
Multi-Albino	18	8 (44.4)	8 (44.4)
Multi-Russian	12	8 (66.7)	7 (58.3)
Control	178	88 (49.4)	80 (44.9)

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P58

CALL TO BUILD A COLLABORATIVE NETWORK FOR THE PROFILING OF FISH GONADAL RIBOSOMAL RNA

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INTRODUCTION

Fish constitute the most diverse group within vertebrates with around 33.000 estimated species. This diversity results in a wide array of biological adaptations and specialized developmental/physiological traits. For instance, this is reflected in the ample spectrum of sex determination and differentiation mechanisms that exist in teleosts. Recently, our team has described a series of ovarian molecular markers that are useful for the identification of sex and of intersex males which, under exposure to environmental estrogens, develop oocytes in their testis (Diaz de Cerio et al, 2012). A simple electrophoresis of total RNA extracted from the gonads, allows identification of high levels of 5S rRNA present in oocytes, diagnostically distinguishing fish ovaries (and intersex testis) from any other somatic tissue, or from testis. This has been proved true in thicklip grey mullets, Atlantic chub and horse mackerel, anchovy, pilchard, European hake, megrim, blue withing, sea bass and zebrafish.

5S rRNA is the prevailing transcript in previtellogenic oocytes. As oogenesis progresses and during vitellogenesis, accumulation of 18S and 28S rRNA begins. Thus, a 5S/18S rRNA index calculated on total RNA eletropherograms allows identifying not only sex, but also ovarian developmental stage, depending on whether previtellogenic, cortical alveoli, vitellogenic or fully mature oocytes predominate in the ovary (Rojo-Bartolomé et al., 2012). In the same way, and as 5S rRNA is in fact an oocyte marker, the 5S/18S rRNA index also works as an objective and quantitative method for the ranking of intersex severity. In this case, and at least in thicklip grey mullets (*Chelon labrosus*), strongly feminized testes (many oocytes) display high index values.

By means of this communication we propose to create a collaborative network of researchers to extend this quantitative and objective molecular approach as a robust method to identify fish sex and female reproductive stage across different laboratory set-ups, fish species and environmental scenarios (seasonal differences, differing food availability, presence of parasites or infections, contaminated *vs* pristine…).

MATERIALS AND METHODS

A Cloud computing based survey will be designed, so researchers can share with us the Agilent 2100 Bioanalyzer files (XAD) where they have assessed the RNA quality of their fish gonads and/or spawned eggs. Access will be open to all scientists using the Agilent platform in fish gonads in the fields of aquaculture, fisheries, or ecotoxicology. The survey will begin using Dropbox file hosting service, for researchers to upload their data files (Email: UserFishOO@gmail.com, Password: UserFishO), and will include a questioner for data characterization and contact details. The submission will not take longer than 10 minutes. Requested information will be: RNA quality files (2100 Bioanalyzer original XAD files), RNA extracting method, fish species and individual characteristics when available; sex, developmental stage, age, gonad histology micrograph, gonad maturation type (synchronous vs asynchronous), sample provenance and date of sampling (if captured in the field), experimental treatment (if the animal was coming from a lab stock). Our research group will calculate the 5S/18S rRNA index on the XAD files and correlate values to the characteristics detailed for each individual (Figure 1). All the results obtained will be sent back to each submitter on due time. Besides, data obtained will allow inter- and intra-species comparisons. Data coming from intersex fish individuals,

either from polluted sites in the field or from laboratory experiments will be mostly welcome, as this will allow establishing threshold values for intersex severity indexes in different species. For instance, in Basque polluted estuaries and harbors only moderate intersex index values (1 to 4, according to the index of Jobling et al. 2006) have been identified in sentinel thicklip grey mullets, oocytes in testis being mostly previtellogenic. It would be very enriching to obtain information of fish with indexes 5 to 7, and displaying vitellogenic oocytes.

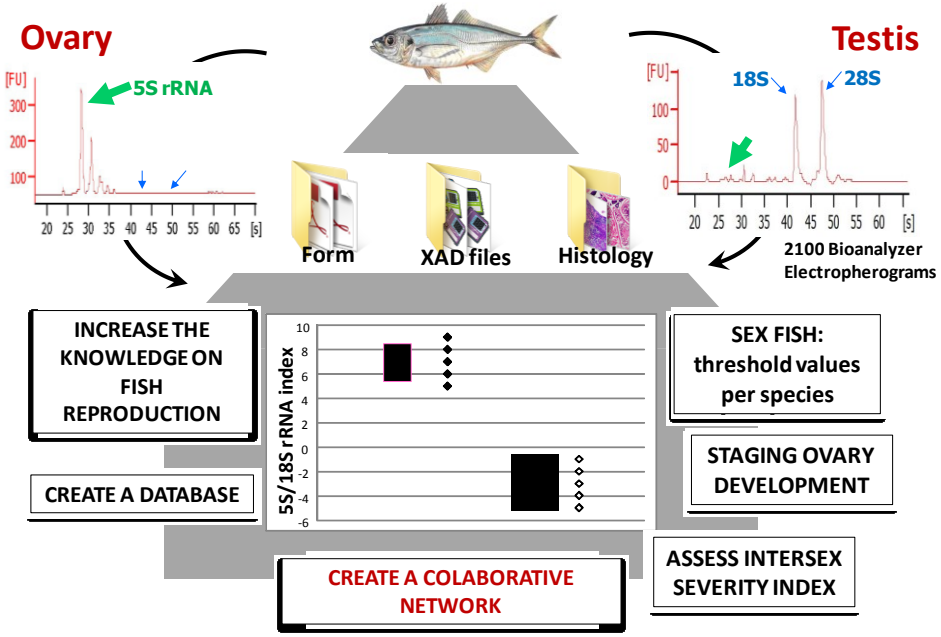

Figure 1. Set-up for collaborative fish gonad ribosomal RNA profiling.

DISCUSSION AND CONCLUSIONS

The fish gonad rRNA profiling database will be made available to collaborators and future investigators, with acknowledgement to all data contributing researchers. We envisage the publication of a position paper, provided that enough information is gathered, in which a member of each contributing laboratory will be invited to sign as co-author.

The metadata generated will offer new research opportunities in the field of fish sex determination/differentiation and will open new avenues for developments and application in fish fecundity analysis.

ACKNOWLEDGEMENTS

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P59

ENZYMATIC ACTIVITY OF LIPASE IN EMBRYONIC DEVELOPMENT AND LARVAE OF NILE TILAPIA *OREOCHROMIS NILOTICUS* WITH BREEDERS FED CRUDE PROTEIN LEVELS

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INTRODUCTION

The activity of digestive enzymes reflects the characteristics of the fish, and has influence on the ability of digesting and absorbing food and on the efficiency of energy input and this being studied in early animal development, most critical phase. This study aimed to evaluate the activity of lipase in embryonic development and larval stages from Nile tilapia, GIFT strain, fed levels of crude protein (CP) diets.

MATERIALS AND METHODS

The experiment was conducted at APTA - UPD, Pirassununga, SP-Brazil, and the experimental design was completely randomized with four treatments and four replications (Tr1: 32%, Tr2: 38%, Tr3: 44% and Tr4: 50% CP). The samples were collected in each stage in embryonic development (T0-cleavage, T1- blastocyst , T2-gastrula and T3- hatching) and larval (T4 - 7th and T5- 10th day after hatching). Then, the analyzes were performed to quantitate the activity of lipase in each stage.

RESULTS

Specific lipase activity were significant differences ($P<0.05$) in the blastula stage, this same treatment (Tr1=0.09 U/mg protein). Furthermore, it was noticed increased levels of this enzyme following the time of hatching, due to this low amount of lipid in eggs and also the first feeding larvae (**Figure 1**).

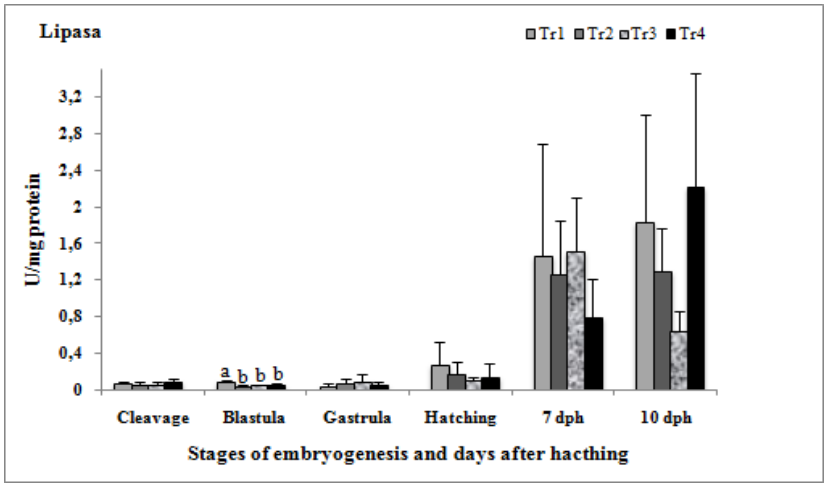

Figure 1 - Digestive enzyme lipase activity specific expressed per milligram of protein on embryonic development and larvae.

DISCUSSION AND CONCLUSIONS

In some cases, high levels of lipase activity were detected in early stages, and then decreased during larval development, which can be attributed to changes in the quality and quantity of food (Walford and Lam 1993). During the larval development of this activity increased until the 10th day post-hatching, unlike what happened with the species California halibut, *Paralichthys californicus* (Alvarez-González, 2006) that the lipase activity increases after 10 days of hatching and so does dentex, *Dentex dentex* (Gisbert et al., 2009) showed increased activity only in the 12th post-hatching day, and in the early development of the species, the activity is constant, that according to the author due to variety of species. However, there was no significant difference between treatments in each gathering day, due to the fact that the diet offered for breeders was not transfer for embryos.

ACKNOWLEDGEMENTS

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P60

MOUTHBROODING NILE TILAPIAS *OREOCHROMIS NILOTICUS* GIFT STRAIN

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INTRODUCTION

The culture of Nile tilapia has been common practice in Brazil in the last few years, mainly due to the economic value of the species. Fish farmers are eager to transform, with maximum efficiency, the high percentage of the total costs of the item “feed” (quality and quantity) into fish production, linked to the low production of eggs by spawning and the lack of synchronicity among the females of this species. To what extent can diet affect the process of oogenesis, the size and number of eggs released by mouthbrooding Nile tilapias, *Oreochromis niloticus* in hapa-based systems?

MATERIALS AND METHODS

This experiment was carried out in APTA, in the town of Pirassununga, Sao Paulo - Brazil, from January to June 2014, where 192 Nile tilapia broodstocks GIFT strain, 144 females (F) and 48 males (M) (350.5±55.0 g) were placed in “hapas” (mesh 1.0 mm, 3.0 x 1.5 x 1.5 m) installed in two masonry tanks with earthen bottom (200 m^2). The fish were electronically identified, weighed (*g*) and measured (*cm*) before being placed in their respective net-cages. The completely randomized design consisted of four treatments (T), using four experimental diets with the following levels of crude protein (CP): T1: 32%; T2: 38%; T3: 44% and T4: 50%, with four replications. Males and females were stocked separately and received the experimental diets for a period of 30 days (December/2014) before the beginning of harvesting. Reproduction occurred spontaneously as couples were paired in the same hapa, at a proportion of 3F:1M, keeping the density of 12 fish per experimental unit (per hapa), for seven days. After that period, males and females were kept separate for 15 days (resting period). All the females were identified as the eggs, if present, were removed from the oropharyngeal cavity. The eggs were then taken to the laboratory, where weight (*g*) and the volume (*mL*) produced by each broodfish were measured, and samples were withdrawn so that they could be counted, and have their diameter measured (µm). Eggs and ovarian fragments were fixed in 10% formalin (monobasic and dibasic buffer), included in historesin and processed according to the routine histological tecniques. All the material was photographed, and the images were analyzed under Zeiss-Eclipse 50 microscope.

RESULTS AND DISCUSSIONS

The eggs collected from all the treatments exhibited yellow color, and there was significant difference with regard to mean egg diameter (T1: 2.03 ± 0.23^{ab}; T2: 2.02 ± 0.19^{c}; T3: 2.06 ± 0.21^{a}; T4: 2.03 ± 0.21^{b}). On the other hand, there was no difference in the number of eggs produced throughout the period sampled (T1: 97,745; T2: 111,210; T3: 97,666; T4: 117,984), which, according to Bhujel (2000), may have occurred because it was the first spawning of the females studied. It is important to point out that the anatomical structure of the oocytes and ovaries of *O. niloticus* presented similar characteristics in all four treatments. Microscopically, the prevalence of oocytes able to spawn, vitellogenic (Vtg) - Mature was observed, indicating the end of the development (Silva, 2014). Fewer previtellogenic oocytes (PV) were observed during the phase of cortical alveoli (CA) and atresia (At). Fertilized eggs at the initial stages of development (cleavage, blastula, gastrula) were also found. The ovaries exhibited

asynchronous development in which the PV oocytes developed, and were then continuously recruited to the stock of Vtg oocytes. In T3 and T4, over 80 to 100 % of signs of atresia (fragmentation of cytoplasm and zona radiata-ZR, appearance of numerous vacuoli, degeneration of nucleus) were observed, and also rare postovulatory follicles - PO (coiled follicles, theca and granulosa cells increase their size and gradually become smaller with pyknotic nuclei scattered within the compact structure). The high levels of CP probably caused the almost total ovarian exhaustion, which corroborates the statement of Gunasekera and Lam (1997), that inadequate levels of protein may result in ovarian recrudescense. However, El-Sayed and Kawanna (2008) reported that an adequate reproductive development for the Nile tilapia reared in a recirculation system was obtained with 40 % protein level. In the present study, treatments T1 and T2 presented ovaries with oocytes at every stage of development. Similar information was described by Oliveira et al., (2014), who observed that the best diet during the reproductive period for females of the same species, Chitralada strain, was 38 % CP and 9.5 g Ed/g CP.

CONCLUSION

The results showed that the performance of mouthbrooding Nile tilapias, using hapa-in-pond system at 1st ovarian maturation was satisfactory for the diets with lower protein level (T1, 32 % and T2, 38 %), whereas diets with higher protein levels (T3, 44 % and T4, 50 %) affected the process of oogenesis and the size of the egg.

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P61

ISOLATING GENES ASSOCIATED WITH CLONAL REPRODUCTION IN CLONE LOACH USING NEXT GENERATION SEQUENCING

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INTRODUCTION

Clonal reproduction has been reported in several fish species composing unisex populations. This reproductive mode is maintained by unreduced gamete production and gynogenesis. Apomixis and premeiotic endomitosis are known as the mechanism of unreduced gamete production. The gametogenesis in the former process does not include meiotic cell division and behave as a mitotic division. Thus, the resultant gametes are genetically equal to their parent. On the other hand, the latter process contains doubling a parental genome before meiosis. The duplicated genome enters meiosis, in which the duplicated homoeologous chromosomes behave as homologous chromosomes, and then, clonal gametes are produced from the gonium.

Loach, *Misgurnus anguillicaudatus*, is freshwater fish including bisexual and clonal reproduction modes. This species consists of two genetically distant populations (A and B groups), and the clonal populations appear at the border between the two groups (Morishima et al., 2008). Clonal individuals reproduce by gynogenesis arisen from unreduced diploid egg produced by premeiotic endomitosis (Arai and Fujimoto, 2013). Furthermore, male clone loach induced by sex reversal treatment also produced unreduced clonal spermatozoa, but the spermatozoa showed lower motility than those from normal diploid loach (Yoshikawa et al., 2007). Though the phenomenon of genome doubling and the genetic feature of the clonal gametes are revealed, the molecular mechanism of the premeiotic endomitosis is still unclear in this species. Thus, we attempted to isolate the genes strongly associated with the premeiotic endomitosis by transcriptome analysis using next generation sequencing (NGS).

MATERIALS AND METHODS

Ovaries and testes were collected from bisexual loach from the two groups and clone loach. Two samples from each group were used for NGS analysis. Total RNA was extracted from each sample and provided for 100 bp paired-end sequencing on illumina HiSeq 2000. Contigs of ovaries or testes were generated by *de novo* assembly using CLC Genomic Workbench (version 7.5). The resultant contig sets from ovaries or testes were annotated by BLASTX and used as a reference data set of expressing genes in ovary or testis. Differentially expressed genes (DEGs) analyses were carried out by mapping the reads obtained from each sample on the reference dataset and the expression value of each contig was normalized and calculated by the fragments per kb million fragments (FPKM method). And then, contigs showing greater than two-fold change between bisexual and clone groups were extracted and a FDR-*p* value <0.05 was used as the threshold. An FPKM filtering cutoff value was 1.0 in terms of detecting expressed transcripts. The functional annotations to the extracted contigs were annotated by BLASTX and the Gene Ontology (GO) analysis was conducted using BLAST2GO.

RESULTS

In assembly of reference data set of ovary, 76,388 contigs (N50: 759 base) were created from total 270,932,994 reads obtained from six females. On the other hand, 142,886 contigs (N50: 697 base) were created from total 242,920,779 reads obtained from six males in assembly of testis reference data set. In the both reference data, genes associated with germ cell specifically expression and meiotic process were detected by homology search using BLASTX.

DEGs analyses revealed the genes (contigs) showing different gene expression pattern specifically in the clone loach (Table 1). DEGs in clone female were larger

than those in clone male. The number of the genes upregulated in the clone loach is larger than those of down-regulated genes in both sex of the clone loach. The genes expressing only in the clone loach occupied large parts of the up-regulated genes in both sex. In contrast, the small number of the down-regulated genes expressing only in clone loach were detected when compared to the other down regulated genes in both sex. In the results of annotation analysis, though about 75% of total DEGs were annotated with at least BLAST results, GO term were annotated in the half of them. Some of them indicated the GO terms associated with cell division, such as chromosome binding, spindle and microtubule formation, etc. Interestingly, a dynein assembly factor did not expressed in the testis of clone male.

Table 1. Genes with up-regulation and down-regulation observed in gonads of clone loach.

	Total	Up-regulation		Down-regulation	
		x2 <	Clone only	x0.5 >	No expression
Ovary	1464	432	914	107	11
Testis	944	356	541	40	7

DISCUSSION AND CONCLUSIONS

In the results of reference data in ovary and testis, almost twice numbers of contigs were created in testis when compared with ovary, though the number of reads used for assembly in each process was almost equal. The difference would be due to varieties of cell types and spermiogenesis in testis. On the other hand, the number of DEGs in ovary was larger than that in testis. This difference might be caused by the difference of component cells among ovaries used for analysis. Although there are differences between ovary and testis, the gene expression patterns showed similar changes between them. Furthermore, similar GO terms were detected in the both DEGs analysis. It suggests that the unreduced gametogenesis via premeiotic endomitosis would be regulated by same molecular mechanism in clone loach. Furthermore, we also found the down-regulated gene, dynein assembly factor, which strongly related with axonemal formation. Thus, this abnormal expression in male clone loach would cause their lower sperm motility. In conclusion, the DEGs results will be useful for finding candidate genes associated with clonal reproduction and other features of clone loach.

ACKNOWLEDGEMENTS

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P62

IMPROVED FERTILIZATION RATE BY TEMPORARY PRESERVATION IN ZEBRAFISH

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INTRODUCTION

Fertilization rate is one of important factors to evaluate embryonic developmental ability. In chromosome manipulation, gametes are needed to preserve *in vitro* until artificial fertilization. It is important to establish a method for preserving gametes appropriately and to keep the quality of gametes during treatment of X ray or UV light in case of androgenesis or gynogenesis. Particularly, eggs immediately activate and take place chorion rising when the eggs are exposed to water. Previously, rainbow trout (*Oncorhynchus mykiss*) ovarian fluid (Gibbs et al., 1994) and Hanks solution with 0.5%Bovine serum albumin (Sakai et al., 1997) were used for preserving zebrafish (*Danio rerio*) eggs. Here, we investigated optimum preserving condition to keep eggs inactivated and to achieve higher fertilization rate after preservation. In addition, we developed a method to improve artificial fertilization efficiency in zebrafish.

MATERIALS AND METHODS

Male and female zebrafish were reared in our laboratory, maintained 14h light and 10h dark photoperiod at 28.5°C. Eggs were collected by squeezing into preserving solution. Sperm were collected by micropipette and diluted with Kurokura's solution (Kurokura et al., 1984). After the following treatments, we inseminated and determined the optimum preserving condition by examining fertilization rate. In experiment 1, eggs were preserved in Hanks + 0.5%BSA (Hanks), rainbow trout ovarian fluid (RTOF) or fetal bovine serum (FBS) for 10 or 30 min. In experiment 2, eggs were preserved in various pH (pH4.0, 7.0, 8.0, 9.0 or 12.0) of Hanks + 0.5%BSA for 30 min. In experiment 3, eggs were preserved in Hanks + 0.5%BSA (pH8.0) with shaking or static condition for 5, 10 or 30min. The fertilization rate was calcurated by the number of cleaved eggs relative to the total eggs used. In addition, the fertilization rate of each treated group was divided by the fertilization rate of the control group. We used this relative fertilization rate in order to compare each experimental group. Control groups were induced by artificial insemination without preserving eggs in the solution.

RESULTS

In experiment 1, the relative fertilization rate (results were presented as means ± SE) of Hanks + 0.5%BSA was higher than those of the other groups regardless of preservation time (preserving time: Hanks / RTOF / FBS, 10min: 1.21±0.05 / 0.83±0.14 / 0.67±0.08, 30min: 1.15±0.18 / 0.70±0.20 / 0.66±0.20). Moreover, in 10 min of preservation in Hanks + 0.5%BSA group, the fertilization rate was significantly improved when compared with the control group (t-test, $P<0.05$) (Figure 1). In experiment 2, the relative fertilization rate of pH8.0 group (0.90±0.09) was higher than those of the other groups (pH7.0: 0.72±0.10, pH9.0: 0.59±0.13). There was no cleaved egg in the pH4.0 and 12.0 groups because all eggs were already activated before artificial insemination (Figure 2). In experiment 3, in each shaking preservation group, the relative fertilization rate were higher than static groups (Shaking / Static, 5min: 1.48±0.08 / 1.37±0.04, 10min: 1.45±0.10 / 1.25±0.11, 30min: 1.30±0.15 / 1.11±0.11). In the 5 and 10 min of shaking preservation groups, the fertilization rate was significantly

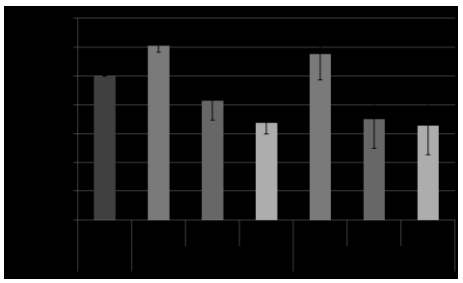

Figure1: Relative fertilization rate after preservation in three kinds of solution

improved compared with the control group (one-way analysis of variance, Tukey test, $P<0.05$) (Figure 3).

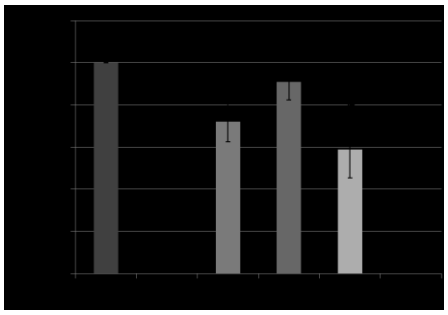

Figure2: Relative fertilization rate after preservation in various pH of solution.

DISCUSSION AND CONCLUSIONS

We concluded that the optimum solution for preserving ovulated eggs was pH8.0 of Hanks + 0.5%BSA. In addition, it was suggested that Hanks + 0.5%BSA was better than salmonid ovarian fluid as a preserving solution. Furthermore the fertilization rate increased when compared with the control group by temporary preservation in Hanks + 0.5%BSA. These results indicated that pH control of preserving condition and washing the surface of ovulated eggs drastically improved the fertilization rate. Therefore, we could expect higher induction efficiency when induced interspecies hybrid or androgenetic haploid by artificial insemination in zebrafish.

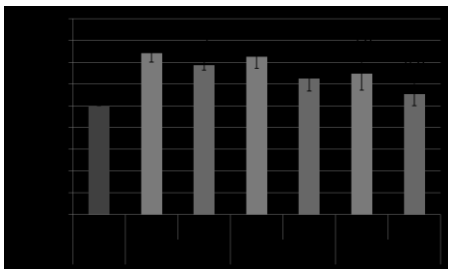

Figure3: Relative fertilization rate after preservation with shaking or static

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P63

FEMALE GONADS DEVELOPMENT IN THE BOGUE *BOOPS BOOPS* (LINNAEUS, 1758) BASED ON HISTOLOGICAL ANALYSIS

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INTRODUCTION

The bogue *Boops boops* is one of the most abundant species of the family Sparidae, commonly distributed throughout the Adriatic Sea. It is a semipelagic species found at depths ranging from 50 to 150 m (Jardas, 1996). Bogue is protogynous hermaphrodite reaching sexual maturity in the second year at total length around 13 cm (Jardas, 1996). Despite the fact that it is commercially important species in the Adriatic, there are no detailed data of its reproduction. Reproductive biology of this species was studied in the Aegean Sea (Soykan et al., 2015), Tyrrhenian Sea (Bottari et al., 2014), Egyptian Mediterranean waters (El-Agamy et al., 2004) and on the Portuguese coast (Gordo, 1995; Monteiro et al., 2006). The aim of this study was to describe female gonads development based on histological analysis.

MATERIALS AND METHODS

Adult specimens were collected monthly from December 2011 to November 2012 in the southern Adriatic Sea. A total of 720 individuals were examined in this study. For each fish, total length (TL) was measured to the nearest 0.1 cm and weighed (W) to the nearest 0.1 g. Fish were gutted, and gonads were removed and weighed with three decimal accuracy. Spawning period was established with monthly variations of the gonadosomatic index (GSI) calculated as: GSI = W gonads (Wg) / W total (Wt) x 100. For histological analysis gonad tissue of 120 females was fixed in 8% buffered formalin. After the standard tissue processing histological slides were used for defining the oocyte development stages following classification proposed by West (1990).

RESULTS

Out of 720 individuals, 365 (50.7%) were females, 311 (43.2%) males and for 44 individuals (6%) it was not possible to macroscopically determine sex. Total length of females ranged from 13.3 to 29.3 cm with a mean value of 18.41 ± 2.0 cm. Hermaphroditism was recorded in a very low percentage of individuals (<1%). The overall sex ratio of males to females was 0.85:1 (x^2=4.314; P < 0.05). Based on monthly values of GSI, spawning extends from December to June. The highest average GSI values of 5.97% for females and 4.21% for males were recorded in February. During August and September oocytes were in primary growth stage and ovaries were composed of oocytes in chromatin nucleolus and perinucleolar stage (Fig. 2a).

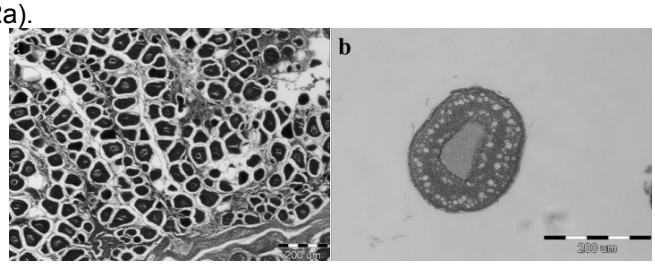

Figure 2. Histological structure of bogue ovaries: (a) primary growth stage; (b) oocyte in cortical alveoli stage (scale bar 200 μm)

Increased ovarian activity was recorded during November associated with a higher frequency of oocytes in cortical alveoli stage (Fig. 2b). In this period the size of ovaries was enlarged due to deposition of yolk and fat. During winter ovaries were in advanced maturation stage containing vitellogenic oocytes (500 μm) with a clearly visible yolk and oil droplets and nucleus situated in the middle of the oocyte (Fig. 3a). High number of oocytes with migrating nucleus and hydration was also visible in this period. Ripe ovaries were found during June and

July containing large number of empty nests and unovulated oocytes undergoing resorption (Fig. 3b).

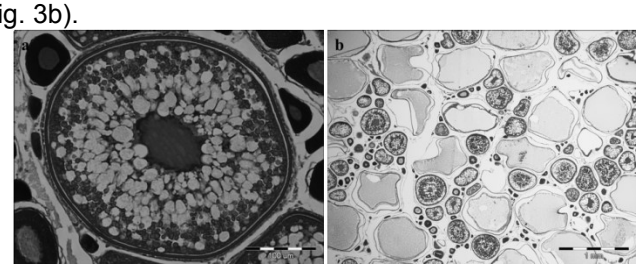

Figure 3. Histological structure of bogue ovaries: (a) oocyte in tertiary vitellogenic stage (scale bar 100 μm); (b) ovary in ripe stage (scale bar 1 mm)

DISCUSSION AND CONCLUSIONS

In this study few criteria were analysed in order to describe oogenesis pattern of bogue (West, 1990; Murua and Saborido-Ray, 2003). Spawning period in the Adriatic Sea is extending from December to June with the peak in February. Similar results, from December to April were reported by Soykan et al. (2015) for the Aegean Sea. Bottari et al. (2014) reported a spawning period from February to April with a peak in May for Tyrrhenian Sea. Different spawning period could be related with the differences in ecological parameters of investigated areas, especially temperature. Low percentage of hermaphrodites in wide range of length classes were recorded in this study supporting the results obtained by Monteiro et al. (2015). Based on the histological sections and oocytes size distribution the ovarian development of the bogue is group-synchronous. Tyler and Sumpter (1996) described two spawning patterns; total and batch spawners. Distribution of the different maturity stages revealed that this species is a total spawner; clutch of yolked oocytes ovulated at once after the maximum peak in February while ovaries from June to August were all in immature stages. High exploitation and low price makes bouge a species with high risk of overexploitation. Knowledge of reproductive biology is vital for rational management of stocks.

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P64

HISTOLOGICAL STUDY OF EMBRYOGENESIS AND CLASSIFICATION OF DEVELOPMENTAL IMPAIRMENTS IN THE EURASIAN PERCH, *PERCA FLUVIATILIS*

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INTRODUCTION

The Eurasian perch is an important candidate to promote diversification of the inland aquaculture in Europe. Indeed, its delicate flesh is appreciated and has a high economical value. Since several years the control of perch reproduction through photo-thermo periodic variations has been unlocked (Abdulfatah et al., 2011). Now, a new bottleneck appears to be the gametes quality and developmental impairments (mortality and/or abnormalities occurrence) during embryogenesis. However, this question is difficult to discern because criteria used to define quality are not always well characterized. In addition, the normal development of the perch is still poorly known. A first description of its embryogenesis remained elusive (Chevey, 1925). We thus choose (i) to better characterize the normal development of *P. fluviatilis* through video-microscopy and histological studies and (ii) to use this developmental table to define most common deformities met in perch development and mortality stages. When possible, those data further allow us to preset spawns 'quality and then do a classification of the spawns.

MATERIALS AND METHODS

To ensure a large diversity of spawn quality experiments have been conducted independently on different broodstock with diverse origins: (i) domesticated fish from Geneva Lake (Lucas Perches, Hampont, France) and (ii) wild perch reared in ponds in Lorraine (GAEC Piscicole du Saulnois, GFA du Kuhweg). Artificial reproductions were performed according to Zarski *et al.* (2012), in order to determine the beginning of development. After fertilization and until the first exogenous feeding, embryos were incubated in our hatchery at 13°C ± 0.5°C. Embryogenesis of 35 spawn was studied by time lapse-video microscopy, using a light upright optical microscope (Nikon Eclipse Ni-U). Embryos were sampled once a day and fixed in Bouin's solution to later perform histological studies and analyze organs ontogeny. In addition, the survival rate was checked daily for 5 days (300 randomly chosen egg/spawn) to determine mortality stages and hatching rates. Abnormal embryos were retrieved and fixed in 4% formaldehyde to perform microscopic studies or in Bouin's solution for histological experiments.

RESULTS

In Eurasian perch, embryogenesis, from ova's activation to the first exogenous feeding lasts for 15 days at 13°C (optimal temperature according to Saat and Veersalu, 1996). It can be divided into 5 main periods: zygote, cell cleavage, gastrulation, organogenesis and free embryo periods from hatching to the first food intake. Some of these periods can further be subdivided. The cell cleavage begins 2.5 hours after fertilization and lasts for 24 hours as the gastrulation. So the third day post-fertilization (dpf), the first tissue differentiation begins with the definition of antero-posterior axis, the notochord and the optic vesicle apparitions before the tailbud closure. We choose to focus a particular attention on the ontogeny of digestive and visual functions because they play an important role in the first exogenous feeding and larval survival. The optic capsule first appears 3 dpf and, the various layers of the eyes develop in parallel. At 7 dpf, the first choroid melanocytes appear and give their color to the eyes. From 8 dpf, the eyes are

histologically mature. The digestive system first develops from 4 dpf with the apparition of intestine anlage. The first peristaltic movements take place at 10 dpf and all embryos first feed 15 days after the fertilization. First hatching occur from 6 dpf and last for 5 days in average within a spawn, whatever the origin of the breeders. The last embryo to hatch was observed 14.5 dpf while the first food intake took place 15 dpf in every tested spawn. It means the free embryo period can elapse from 9 to half a day during which the embryo fast.

Once the normal development described, it was interesting to make an overview of the developmental defects that could be met during perch development. We focused on the characterization of deformities occurrence and the identification of key steps that could lead to a massive lethality. Concerning the later one, three main groups of spawn were found with different survival rates upon the time. The first category groups spawn with hatching rates over than 90%, the second group spawn had high mortality during early embryogenesis and low hatching rate (60 % and 14 %, respectively) and finally the third one corresponds to embryos that never hatch and die during early embryogenesis. An accurate study of these lethalities showed two stock point, 24h and 48h after the fertilization, suggesting that the cell cleavage to gastrulation and gastrulation to organogenesis transitions are key steps for reproduction success. In addition, the study of the deformities occurrences showed that, in our conditions, the deformities rates range from 0 to 20 %. In the meantime, 6 main categories of deformities have been characterized according the abnormal organ (e.g. skeletal, yolk, jaw, eyes or heart). A statistical analysis of these data allows to make a classification of spawn according to the lethality stage and the type of abnormalities.

DISCUSSION AND CONCLUSIONS

Chevey first (1925) investigated *Perca fluviatilis* development. Most of our observations seem similar to his study, and we complete with histological observations. Among the crucial point is the definition of the end of the embryonic development. While some believe that this is hatching, other use the first food intake (Peñaz, 2001). In our study, we clearly observed that the onset the first food intake always takes place 15 dpf while hatching can last over 5 days. In these conditions, this last step better define the end of embryogenesis. Our study of the developmental impairments showed that (i) the end of the cell cleavage and the gastrulation, both constitute crucial stages of defects which could lead to death and (ii) some organs seem particularly touched by potential deformities. These data constitute a solid basis to investigate further the reasons of the developmental defects in perch and their links with the gametes quality.

ACKNOWLEDGEMENTS

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P65

ULTRASOUND ASSISTED DETERMINATION OF THE FINAL MATURATION OF HORMONE INDUCED SICHUAN TAIMEN *HUCHO BLEEKERI* KIMURA, 1934

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INTRODUCTION

Sichuan taimen *Hucho bleekeri*, one of largest salmonids in the world, has nearly been extinction in all its ever-occupied tributaries of the Upper Yangtze River in China. In 2013, an unexpected group of adult *H. bleekeri* was discovered in Shanxi province in Taibai River, one of tributaries of Hanjiang River that merged into the Yangtze River, which bring the hopes for rescuing the species through artificial reproduction for enhancing their population. Due to the limited resource, there are still no any detail information about the artificial production of such species any more. The objectives of this research are to evaluate the possibility of using ultrasonography for sex determining and maturation monitoring and its application as a non-invasive method to assist the artificial reproduction.

MATERIALS AND METHODS

A Terason T3000 portable ultrasonograph with model 12L5(12-5MHZ) transducer was processed during sex determination, hormone inducing and reproduction of 26 adult *H. bleekeri* in 2013 and 2014. Fish were anethesiaed with tricaine methanesulfonate (MS-222) when doing ultrasound scanning. The 4 incidentally died specimen were dissected for confirmation of the ultrasound imaging. Female H. bleekeri were induced twice with the total dose of 20 μ g/kg salmon GnRH (s-GnRH-A) and 4 μ g/kg Domperidone (DOM) and males were induced once with a half dose as females. Based on the final maturation, there will be a supplement inducing for female or males whose gonad was not well developed.

RESULTS

Two scanning areas with two central points were located as easier ways to get the distinguishable ultrasound images for females and males before and after spawning.

Left area covering below the literal line and from the end of pectoral fin to the end of dorsal fin will cover the swimming bladder, which will brings mirror ultrasound images; right area covering below the literal line and from the end of dorsal fin to the begin of anal fin is easier for gonad identification. p1: central point of left area for ultrasound images sampling; p2: central point of right area for ultrasound images sampling(Fig.1 a). Ultrasound imaging was approved with dissection of the dead specimen(Fig.1 c-e).

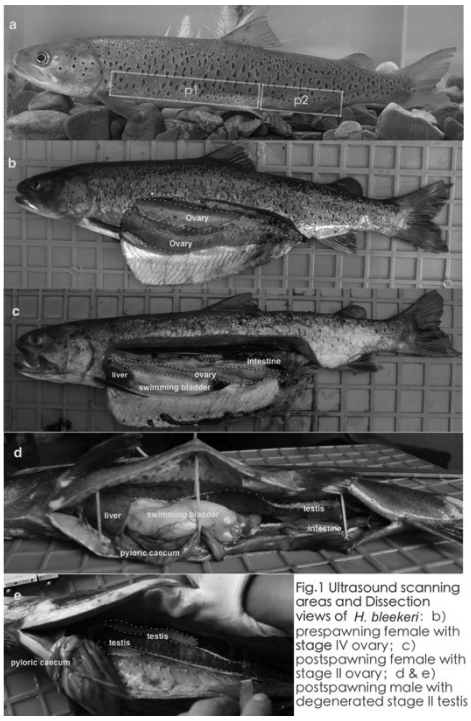

Fig.1 Ultrasound scanning areas and Dissection views of *H. bleekeri*: b) prespawning female with stage IV ovary; c) postspawning female with stage II ovary; d & e) postspawning male with degenerated stage II testis

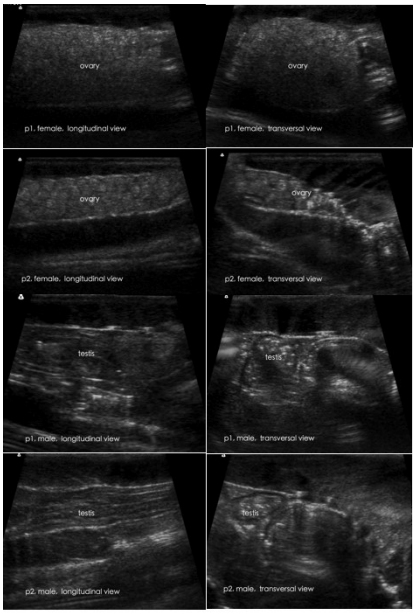

Fig.2 Distinctive ultrasound images of mature female and male *H. bleekeri*.

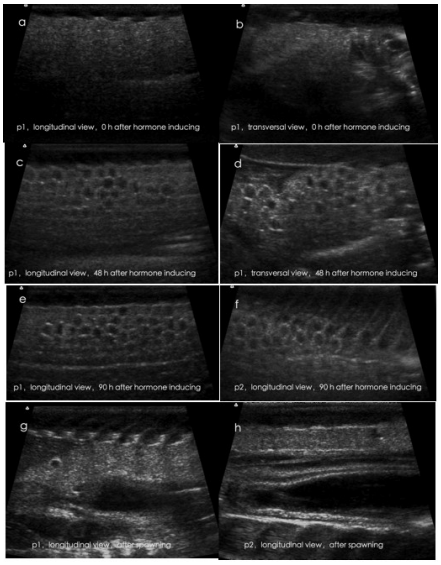

Fig.3 Typical ultrasound images of ovary maturation during hormone inducing.

In mature female, ovary present homogeneous appearance with bright echograms in the p1 and p2 scanning points, while mature testes were usually darker and displayed well defined lobules with distinct margins especially in the transversal views(Fig.2).

One exciting result showed that with the response of ovary to the exogenous GnTH, the vacuolus oocytes appeared(Fig.3 c&d), augmented(Fig.3 e&f), well-distributed and arranged(Fig.3 e&f). Only ovaries with such ultrasound images(Fig.3 e&f) were ready for ovulation and reproduction, which is a key point for making decisions on artificial collection of the eggs.

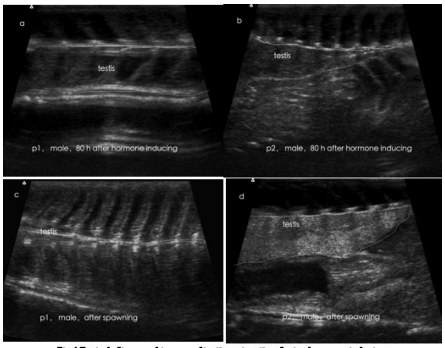

Fig.4 Typical ultrasound images of testis maturation during hormone inducing.

Ultrasound images from p1 and p2 points views of testis before and after spawning were easily distinguishable. At point of p1, after 80h hormone inducing, testis became very dark represent the seminal fluid, which means ready for spermiation. After spawning, testis degenerated to a single line(as shown in Fig.1 d &e). At point of p2, testis were not full of seminal fluid(Fig.4 b) after 80 h hormone inducing. And after spawning, testis at point of p2 still can be imaged with comparative bright echograms(seminal fluid was gone).

DISCUSSION AND CONCLUSIONS

The studies showed the ultrasonography really help for sex determination of adult *H.bleekeri* and their maturation monitoring. The results supported a possible way to easily observe the gonad development to the exogenous hormone, which was seldom mentioned in other salmonides. It helped us adjust the inducing hormone dose and make the right decisions on reproduction of 4 females without well-developed gonads. In summary, with the assisted ultrasonography, the stress during the handling practices of artificial reproduction was alleviated to certain distance, which made contributions to the successful reproduction of *H.bleekeri* in the passed two years. Such practices will also benefit for other endangered fish conservation.

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P66

MOLECULAR CHARACTERIZATION AND RELATIVE MRNA EXPRESSION PROFILES OF GONADOTROPIN RECEPTORS IN EUROPEAN HAKE (*MERLUCCIUS MERLUCCIUS*) DURING PUBERTY

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INTRODUCTION

The gonadotropic hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are the key factors involved in gametogenesis regulation and in the production of gonadal hormones in vertebrates (Swanson et al. 2003). To study the gonadotropic regulation in species with asynchronous ovary, Kitano et al. (2011) and Mittelholzer et al. (2009) have suggested to investigate the expression profiles of their receptors in the ovary. Considering these previous information, this study aimed to better understanding the events regulating the puberty by ovarian morphological changes, molecular characterization and quantification of two key genes, the gonadotropic receptors FSH-R and LH-R in the European hake (*Merluccius merluccius*).

MATERIALS AND METHODS

A total of 20 females were collected during the peak of the spawning season in offshore areas of Ancona (Italy) by commercial fishing vessels. The gonadal stages are divided in different stages following Brown-Peterson et al. (2011): I (immature), II (developing), IIIa (spawning capable), IIIb (actively spawning), IV (regressing). Full-length sequences coding for FSH and LH receptors were performed from European hake by a 5'and 3'RACE-PCR based strategy. The gene expression analysis was investigated by qPCR and only the animals in gonadal stages I, II, IIIa and IIIb were considered, because of trouble to find females in stage IV, this stage was not considered. Part of ovaries were preserved in 4% buffered formaldehyde for histological analysis.

RESULTS

The nucleotide and deduced amino acid sequences showed high homologies with the corresponding sequences of other fish species. The important functional features are identified in deduced amino acid sequences, as N-terminal region, seven transmembrane domains and leucine-rich repeats. The mRNA expression profile of gonadotropin receptors, FSHR and LHR, were investigated in ovary of females to different maturity stages. These expression profiles were analyzed in relation to changes of histological data.

DISCUSSION AND CONCLUSION

Analysis of both nucleotide and deduced amino acid sequences identified them as FSH-R and LH-R, respectively, showing typical structural features of glycoprotein receptors (Mittelholzer et al., 2009). The expression pattern of both gonadotropic receptors showed a similar trend. These results provide the possibility to understand the mechanism of gonadotropic receptors in species with high economic importance.
In conclusion, this study opens new ways to investigate gonadotropic action in this species and gives the possibility to better understand the reproductive mechanism in overfishing species.

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INSIGHTS ON THE REPRODUCTIVE PHYSIOLOGY OF THE EUROPEAN ANCHOVY

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INTRODUCTION

Small pelagic fish are essential elements of marine ecosystems due to their significant biomass at intermediate levels of the food web and to their role in connecting the lower and upper trophic levels (Bakun, 1996). Among them, the European anchovy (*Engraulis encrasicolus*, Linnaeus, 1758) represents one of the most important, commercial coastal pelagic species.
Many aspects of this species' biology have been investigated: feeding ecology (Borme et al. 2009), reproductive biology (Schismenou et al. 2012), the environment's influence on it (Basilone et al. 2013) and its genetics (Montes et al. 2013). However, the reproductive physiology is completely uncharacterized and so are the pivotal genes controlling it.
This study takes advantage of a multidisciplinary approach that includes molecular, bioinformatics and histological tools with the overall aim of reducing the gap of knowledge afflicting this species by providing, for the first time, useful, yet unknown information.

MATERIALS AND METHODS

Male and female anchovy specimens in all sexual maturation stages were collected in the Adriatic sea (FAO Geographical Sub Areas 17 and 18, western side) during the 2014 MEDIAS oceanographic cruises carried out by CNR ISMAR research group. Total RNA was extracted from liver, gonad and whole brain using RNAzol® RT (Sigma-Aldrich) and cDNA was synthesized with the SuperScript™ II kit (Invitrogen, Milan, Italy). Gene sequences were obtained and lengthened with standard and 3' RACE PCRs.
Regarding bioinformatics, the *Engraulis encrasicolus* transcriptome assembled by Dr. Estonba's research group was searched for reads matching with a query database built from sequences of closely related fish species. Gene annotation was performed by means of sequence similarity identified either by BLASTn or tBLASTx searches with e-values cutoff from 0 to 10^{-3}.
The expression levels of important reproductive-related gene levels were achieved through Real Time q-PCR with the SYBR Green method. Primer pair specificity and the lack of primer-dimer formation during each run were confirmed by a single peak in the dissociation curve.

RESULTS

The results of the gene sequencing work consisted in a total of 9 sequences (data not shown). The different isoforms of GnRHs as well as the gonadotropins' alpha subunit and luteinizing hormone's beta subunit were obtained using primer pairs designed on the Japanese counterpart, while the egg envelope proteins' sequences were elongated based on the partial information retrieved from the transcriptome search in order to achieve their complete open reading frame.
The bioinformatic approach yielded a total number of 18 critical putative sequence of interest related to the European anchovy's reproductive physiology. Among them are present genes that i) direct the egg growth in the vitellogenesis step (Vtg, Vtg-R), ii) have a role in the maturational step of egg and/or sperm (m1PR, cdk1/2, Cyclin B1/B2), iii) belong to the egg envelope proteins family (ZPCa, ZPCb, ZPCc, ZPBa, ZPBb), iv) are implicated in embryo's chorion digestion (Hatching enzyme 1/2/3), and v) to be used as housekeeping reference for relative quantification (β-actin, EF-α). Details of the search are reported in table 1.

Table 1. Putative genes annotated from the *Engraulis encrasicolus* transcriptome.

query	subject	%ident	E value	Match
beta-actin	///	///	///	Pers. comm. from Prof. **Yasumasu**
Cdk1	isotig_14043	77,78%	3,00E-73	Cdc2/Cdk1 ***Oryzias latipes***
Cdk2	isotig_14996	97,59%	2,00E-107	[Cdk2] ***Danio rerio***
Cyclin B1	isotig_13097	56,59%	1,00E-43	[CyclinB1] ***Danio rerio***
Cyclin B2	isotig_07868	72,80%	2,00E-84	[Cyclin B2] ***Danio rerio***
m1PR	isotig_18777	83,47%	6,00E-59	m1PR ***Oryzias latipes***
Vtg1	isotig_03059	81,43%	0,00E+00	Vtg1 ***Clupea harengus***
VtgR	isotig_14397	81,18%	1,00E-95	VtgR ***Anguilla japonica***
EIF-alfa	isotig_08653	86,06%	4,00E-97	EIF-alfa ***Clupea harengus***
CatB	isotig_18240	87,96%	8,00E-54	CatB ***Clupea harengus***
Hatching enzyme1	tr_GSG19BM07ILR5M	67,16%	5,00E-39	AcHE3 ***Engraulis japonicus***
Hatching enzyme2	tr_GSG19BM07H2C6Z	93,62%	2,00E-66	AcHE2 ***Engraulis japonicus***
Hatching enzyme3	tr_GSG19BM07ISLSW	90,09%	2,00E-83	AcHE3 ***Engraulis japonicus***
ZPCa	isotig_07186	98,29%	0,00E+00	AcZPCa ***Engraulis japonicus***
ZPCb	isotig_06409	97,52%	0,00E+00	AcZPCb ***Engraulis japonicus***
ZPCc	isotig_07367 isotig_v2_18443	98,45%	2,00E-136	AcZPCc ***Engraulis japonicus***
ZPBa	isotig_v2_11474 isotig_07065	97,04%	8,00E-109	Pers. comm. from Prof. **Yasumasu**
ZPBb	isotig_08979 isotig_v2_20951	98,43%	2,00E-180	AcZPBb ***Engraulis japonicus***

A particular emphasis was given to the study of the anchovy's egg envelope protein. ZPCa, ZPCc, ZPBa and ZPBb were completely identified. They consisted in nucleotide sequences of 1276, 1427, 1677 and 1527 bp, which encoded for 401,445, 480 and 495 AAs, respectively.
AA deduced sequence analyses revealed the presence of a common element in each of the EEP in question, such a conserved characteristic domain known as ZP domain.
Their expression site was evaluated and relatively quantified throughout all sex maturity stages in female specimens. ZPBa, ZPCb and ZPCc were shown to be exclusively expressed in one tissue (liver, ovary and ovary, respectively), while the expression of the remainings appeared in both liver and ovary, in different extent.

DISCUSSION AND CONCLUSIONS

The results collected in this study represent the first preliminary focus in the field of the *Engraulis encrasicolus'* reproductive physiology. Considered the environmental and commercial role, a more complete understanding of its biology would be desiderable.

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P68

QUALITY OF RIVER LAMPREY (LAMPETRA FLUVIATILIS) SPERM COLLECTED FROM MALES KEPT IN DIFFERENT TEMPERATURES UNDER ARTIFICIAL CONDITION

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INTRODUCTION

The river lamprey (Lampetra fluviatilis) is a migratory and diadromous agnathan which spawns only once in a lifetime (Fot. 1.). Formerly, the river lamprey was regarded as a ubiquitous and commercially significant freshwater species, but due environmental pollution, the geographic range of this species has been dramatically reduced. Nowadays, according to the IUCN/WCU classification, the river lamprey is considered an endangered species (EN). Knowledge regarding the biology of lamprey reproduction and gamete quality may contribute to the protection of this endangered species. The aim of this study was to analyze the quality of sperm collected from males in relation to different temperatures of males held in artificial conditions.

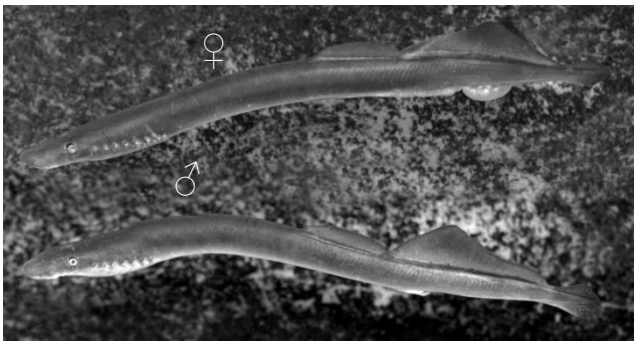

Fig 1. River lamprey (Lampetra fluviatilis) spawners

MATERIALS AND METHODS

The river lampreys were harvested in spring immediately before spawning in the Vistula Lagoon (north Poland) and were transported to the aquarium hall at the University of Warmia and Mazury in Olsztyn (north-eastern Poland). Females and males had distinguishing sex characteristics; the abdomen was visibly enlarged in females, and a distinctive elongated urogenital papilla was noted in males. After sex determination, the males were divided into three group and kept in plastic 1000 dm3 tanks (Kujawa et al., 1999) at a temperature of 7°C (n=10; average BW: 108.1 g and Lt: 39.1 cm), 10°C (n=10; average BW: 95.0 g and Lt: 36.3 cm) and 14°C (n=10; average BW: 87.1 g and Lt: 36.1 cm). After two weeks, the sperm from males kept in different temperature conditions was collected by gently massaging of the abdomen. Immediately after collection, sperm concentration, seminal plasma osmotic pressure and pH were determined. To analyze motility and velocity of the sperm, the CASA system was used. Before analysis, one µl of sperm was activated in 25 µl of solution containing 20 mM Tris and 40 mM NaHCO3 supplemented with 0.5% BSA at pH of 8.5 (Cejko et al. 2015).

RESULTS

There were no differences between sperm concentrations (ranging from 1.9 to 2.3x109 ml-1) collected from males kept in different temperature conditions. The highest seminal plasma osmolality were noted in 7°C (246 mOsm kg-1) and the lowest in 14°C (219 mOsm kg-1) although the values of these parameters did not differ significantly (Fig. 1A; $P>0.05$). At the temperature of 14°C the highest seminal plasma pH (7.6) was noted. Values of this parameter differed significantly from values recorded in 10°C (7.2) and 7°C (7.0), (Fig. 1B). The highest sperm

motility (84.0%) and curvilinear velocity of sperm (152.5 µm s-1) were noted at the lowest temperature (7°C) of males kept under controlled conditions. With the increase in temperature, sperm motility parameters decreased significantly reaching the values of 73.1% and 138.5 µm s-1 at 10°C and values of 34.5% and 100.2 µm s-1 at 14°C (Fig. 2A,B) for MOT and VCL, respectively.

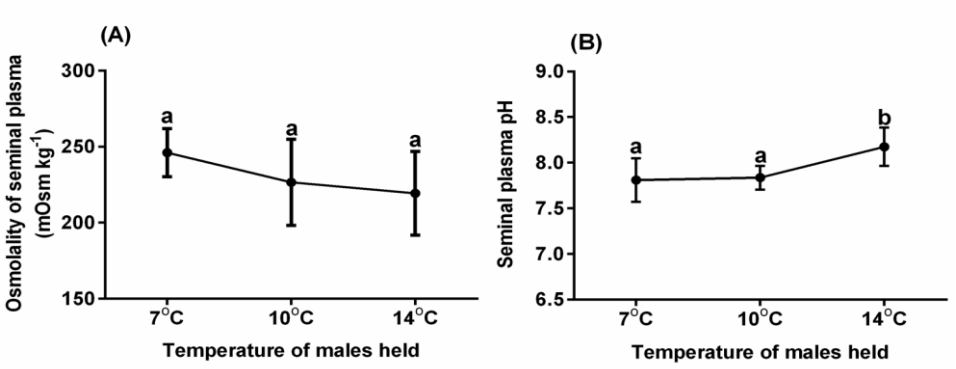

Fig. 1. Osmolality (A) and pH (B) of seminal plasma of river lamprey sperm collected from males kept in different temperatures under artificial conditions (P<0.05).

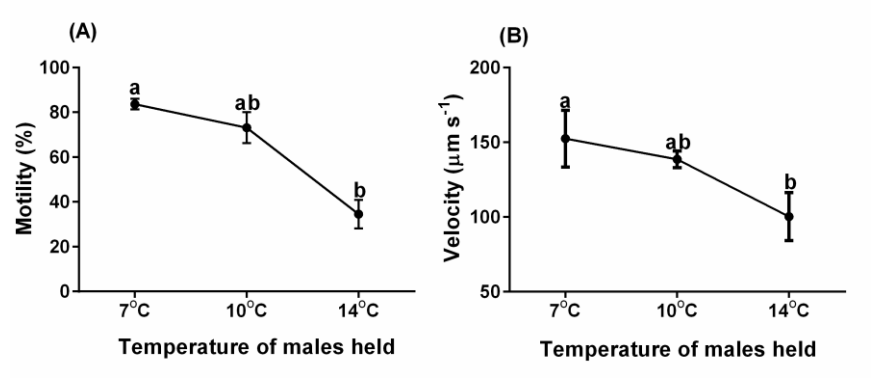

Fig. 2. Motility (A) and velocity (B) of river lamprey sperm collected from males kept in different temperatures under artificial conditions (P<0.05).

DISCUSSION AND CONCLUSIONS

The presented data indicates the influence of different temperatures on the quality of river lamprey sperm collected from males kept under artificial conditions. The increase in temperature over 7°C resulted in a significant reduction in sperm motility and sperm velocity. This indicates that there was no need to change the temperature regime to achieve good quality semen in male lampreys.

ACKNOWLEDGEMENTS

This study was supported by the National Science Center, grant No. DEC-2013/09/B/NZ9/03130 and funds appropriated to the Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Olsztyn, Poland.

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P69

STANDARDIZATION OF A CRYOPRESERVATION PROTOCOL FOR THE GREEN SEA URCHIN *LYTECHINUS VARIEGATUS* SPERMATOZOA: EFFECTS OF CRYOPROTECTANTS ON SPERM MOTILITY

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INTRODUCTION

Gametes and embryos of many aquatic invertebrates are commonly used as biological model in laboratory research (Bellas and Paredes, 2011). The creation of gamete cryobanks may guarantee their continuous supply, and the possibility to use the same batch of semen in different sites or different times (Gwo , 2000; Cabrita et al., 2010). Much research is therefore needed for the standardization of specie-specific cryopreservation protocols for a wide range of aquatic species. Aim of this work has been the evaluation of the effects on sperm motility of different cryoprotectant agents (CPA), in order to develop a specie-specific cryopreservation protocol for the sperm of the green sea urchin *Lytechinus variegatus*, a species living along the Atlantic coast from North Carolina to Brazil, and commonly used in laboratory research, mainly in ecotoxicological tests (Torres et al., 2009).

MATERIALS AND METHODS

Semen samples were collected, after injection of 0.5M KCl, from adult specimens coming from the northern littoral of the State of São Paulo (Brazil); sperm motility was triggered by dilution in filtered seawater 35psu (FSW) and the percentage of progressive vigorous motile spermatozoa (PVM) scored under microscope; samples showing poor motility were discarded. Aliquots of semen were therefore diluted at a rate of 1:6 in four extenders consisting in 1% NaCl containing dimethysulphoxide (DMSO) or glycerol (GlOH) in concentrations of 5 or 10%. After 30min at 22 °C semen samples were activated by dilution in FSW at a rate of 1:200 and the PVM percentages scored. 1% NaCl was used as control. Six independent trials were performed, using different semen samples. The effects on sperm motility of the different cryoprotectant solutions were evaluated by one-way ANOVA and Duncan post hoc test. $P < 0.05$ was considered as significant.

RESULTS

Percentages of PVM spermatozoa after incubation in the tested extenders are reported in Figure 1. A significantly different effect of the various cryoprotectant solutions on %PVM sperm was observed. The highest values were obtained in 5% DMSO, the only extender in which no differences from the control were recorded; significantly lower values were obtained in 10% DMSO. GlOH proved to be more toxic to *L. variegatus* sperm, showing %PVM values significantly lower than DMSO, even around 5% in the 10%-based extender.

DISCUSSION AND CONCLUSIONS

Specie-specific cryopreservation protocols have been developed for sperm of a wide range of fish species; on the contrary only few data are available for echinoderm species (Gwo, 2000), and to our knowledge no reports on cryopreservation of *L. variegatus* sperm can be found in current literature.

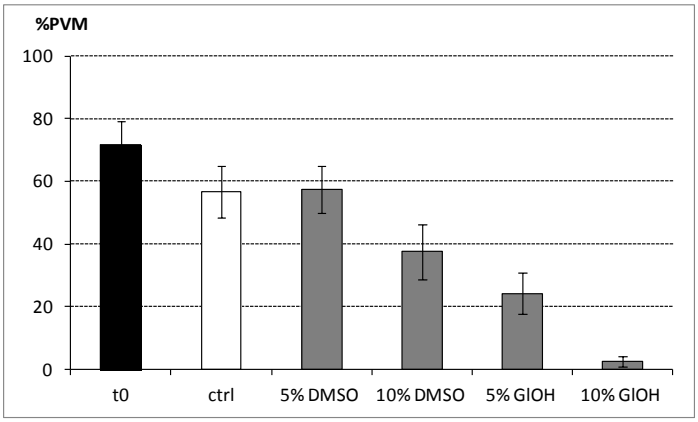

Figure 1. Percentages of progressive and vigorous motile spermatozoa (PVM) on collection (t0) and after 30min in the tested cryoprotectant solutions. Ctrl = 1% NaCl.

Cryoprotectants are known to exert a toxic effect on sperm motility, therefore for the development of a specie-specific cryopreservation protocol, attention has been initially focused on the evaluation of the toxicity of two among the most widely used CPAs (Gwo, 2000; Cabrita et al., 2010). The penetrating CPA DMSO performed significantly better than the not-penetrating GlOH, according to what reported also for other sea urchin species (Asahina and Takahashi, 1978; Adams et al., 2004; Fabbrocini et al., 2014). In particular, no toxic effect in respect of the control was observed after incubation in the 5% DMSO-based extender; similar DMSO concentrations (2.5% to 7%) allowed to restore on thawing both motility and fertilization ability in cryopreserved *E. chloroticus* and *P. lividus* sperm (Adams et al., 2004; Fabbrocini et al., 2014).
On the basis of our results we can conclude that also *L. variegatus* sperm showed a good tolerance to DMSO; extender solutions containing DMSO from 5 to 10% are now being tested, in order to evaluate also its efficiency in protecting sperm cells from this urchin species during the freezing and thawing phases.

ACKNOWLEDGEMENTS
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P70

SHORT-AND LONG-TERM PRESERVATION OF SPERM IN DIFFERENT GOLDFISH TYPES

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INTRODUCTION

Goldfish (*Carassius auratus*) is a well studied model species and its variants have an economically important position among ornamental species. Efficient propagation depends significantly on the quality of spawners and on the synchronization of spermiation and ovulation in both sexes. Short-term and post-thaw storage of sperm are capable to prolong the potential application period during fertilization. A suitable method of freezing sperm could support the long-term preservation of genetic resources of a single individual or in? valuable lines (Cabrita et al., 2010). Information regarding the effect of a suitable short-term or post-thaw storage time and cryopreservation on the quality of sperm in goldfish varieties is notably limited. The aim of our study was to investigate the efficiency of different storage methods and the cryo-sensitivity of sperm in oranda, calico, and black moor types.

MATERIALS AND METHODS

Oranda, calico, and black moor males (*N*=31) and females (*N*=6) were randomly selected from a farmed broodstock located in Nagykarácsony, Hungary. Both sexes were hormonally stimulated with 2 mg/kg bodyweight carp pituitary (in females two doses: 10% 24 hours and 90% 12 hours before) 24 hours before stripping. Motility parameters [progressive motility (Pmot), curvilinear velocity (VCL), straightness (STR)] were investigated using a CASA system before and after cryopreservation and during varied storage time according to the experimental design. Sperm motility was activated with a commonly used cyprinid sperm activator (45 mM NaCl, 5 mM KCl, 3 mM Tris, pH 8, Horváth et al., 2003) combined with 0.1% BSA. Sperm from different varieties was stored at 4 °C for 48 hours and motility of cells was recorded in 6-hour periods. After thawing, samples were stored for 6 hours at 4 °C while motility was measured every 2 hours. The variation in cell concentration among the 3 goldfish varieties was investigated with hemocytometer. Sperm of all types was cryopreserved using a glucose-based extender (20 mM glucose, 4 mM KCl, 3 mM Tris, pH 8, Horváth et al., 2012) in a ratio 1:9. Diluted samples were loaded into 0.5-mL straws and frozen 3 cm above the surface of liquid nitrogen for 3 minutes. After collection of eggs fertilization was implemented both with fresh and cryopreserved sperm in oranda and calico types. Eggs were incubated at a temperature of 23-25 °C. Fertilization rate was calculated in neurulation (3 days post-fertilization).

RESULTS

A significant reduction was detected in Pmot after 18 hours in calico (0h: 92±5%, 18h: 42±26%, **Fig.1A**). VCL was decreased already after 6 hours (0h: 151±6µm/s, 6h: 105±8µm/s). STR didn't changed remarkably through 48 hours. In oranda, Pmot changed significantly only after 42 hours (0h: 84±5%, 42h: 23±2%, **Fig.1B**) however, VCL reduced significantly after 36 hours (0h:150±11 µm/s, 36h: 94±12 µm/s). STR did not show sensitivity for storage time. Pmot, VCL, and STR did not decrease significantly for 48 hours in black moor (**Fig.1C**). A similar cell concentration was calculated among the three varieties (**Fig.1D**). In calico, Pmot decreased significantly 6 hours after thawing (19±11%) compare to 0 hour (55±15%) whereas VCL and STR showed no reduction. Pmot, VCL and STR was similar in oranda through 6 hours of post-thaw storage. A parallel tendency was observed in black moor. Fertilization rate with thawed sperm was significantly

lower compared to fresh control in calico (control: 63±17%, cryopreserved: 23±6%). Analogous difference between cryopreserved and control group was revealed in oranda (control: 72±9%, cryopreserved: 14±2%).

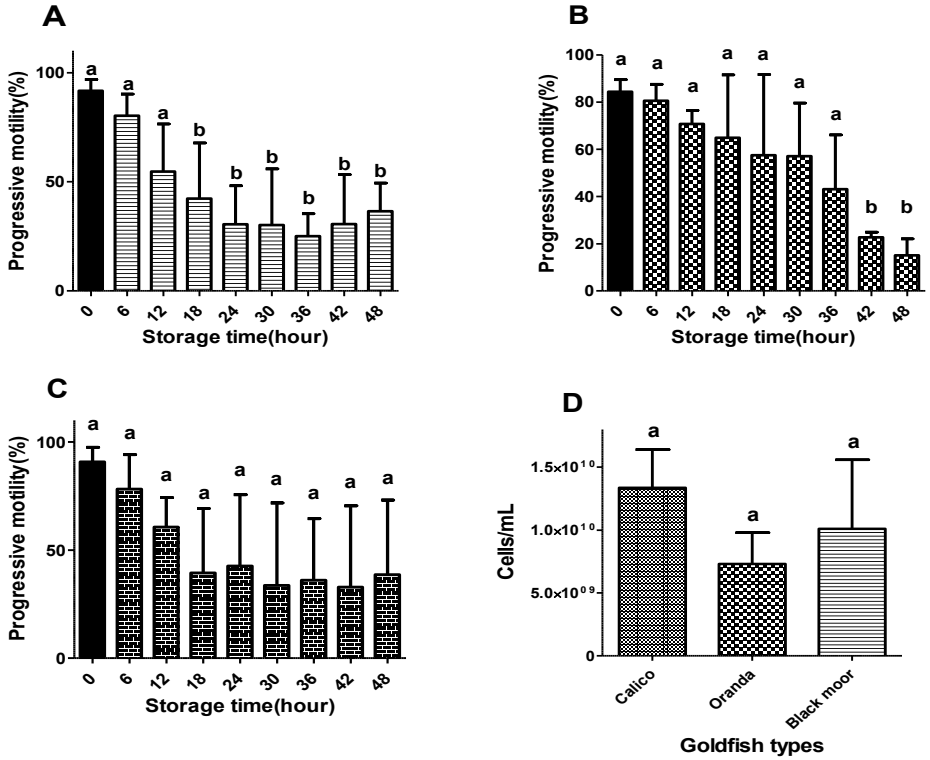

Fig. 1 Progressive motility through 48 hours storage in calico (**A**), oranda (**B**) and black moor types(**C**). Cell concentration in the three goldfish variants (**D**).

DISCUSSION AND CONCLUSIONS

We were able to store fresh sperm for more than 1 day in oranda and blackmoor. It appears, that calico is more sensitive for short-term storage than the two other varieties. Cryopreservation was successfully carried out in all goldfish varieties. A suitable post-thaw storage capacity of cryopreserved sperm was measured in all groups although, calico was more affected by storage time. Preliminary data were recorded on the fertilizing capacity of cryopreserved sperm in calico and oranda varieties.

ACKNOWLEDGEMENTS

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P71

THREE-DIMENSIONAL STRUCTURAL MODELS OF VASA PROTEIN IN SELECTED FISH SPECIES BY HOMOLOGY MODELING

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INTRODUCTION

Vasa is an RNA helicase belonging to the largest RNA helicase family, the DEAD box family which is characterized by a strictly conserved sequence Asp-Glu-Ala-Asp (D-E-A-D). These helicases are involved in many aspects of RNA metabolism such as transcription, splicing, translation, transport and degradation (Linder and Lasko, 2006) and have an essential role in germ cell specification and embryonic patterning, stem biology and chromatin condensation (Lasko, 2013). Furthermore vasa has been found to be expressed characteristically in the germline of many organisms (Lasko, 2013).Identification and expression of vasa protein was observed in several fish species. However, the 3D structure of vasa protein remains unknown in fish. The aim of the present study was to model the 3D structure of vasa orthologs in four fish species by homology modeling and compare these structures to the structures of vasa in *Drosophila* and *Homo sapiens*.

MATERIALS AND METHODS

Primary sequences of vasa protein orthologs from 4 fish species (*Danio rerio* AAI29276, *Oryzias latipes* BAB61047, *Cyprinus carpio* AAL87139 and *Carassius gibelio* AAV70960) were retrieved from the NCBI database (FASTA format) and were used as targets for homology modeling. Structural templates for homology modeling were screened using the BLAST algorithm against the protein databank (PDB). Templates with a sequence similarity of at least 30% are considered valid homology modeling. Sequence alignments were created using clustal w2 (EBI) and edited manually in the program JalView on the basis of agreement between predicted secondary structures of the target proteins and known secondary structures of the template proteins. Target protein secondary structures were predicted using PSI-PRED and results were used to improve alignments between target proteins and their template structures. Based on these refined alignments, homology models were built in the program DeepView (Swiss pdb-viewer v.4.0.3). Resulting models were initially refined by conjugate gradient energy minimization in DeepView using a GROMOS 43B1 force field. Final models were energy minimized using Chiron, which minimizes steric clashes in target protein structures using discrete molecular dynamics simulations (DMD). Model quality was assessed using PROCHECK program on the SAVES server. Resulting models were analyzed in the program PyMol. Structural alignments with corresponding vasa protein orthologs from *Drosophila* and *Homo sapiens* were conducted using the program Dali (pairwise comparison).

RESULTS

Homology models of vasa from *Danio rerio*, *Oryzias latipes*, *Cyprinus carpio* and *Carassius gibelio* were created based on BLAST results and secondary structure prediction. Structural templates were selected based on sequence similarity. Vasa from *Drosophila* (PDB 2DB3) shares high sequence similarity with all four vasa proteins from fish (~63%). Based on these results, a Clustal alignment was created (Figure 1).

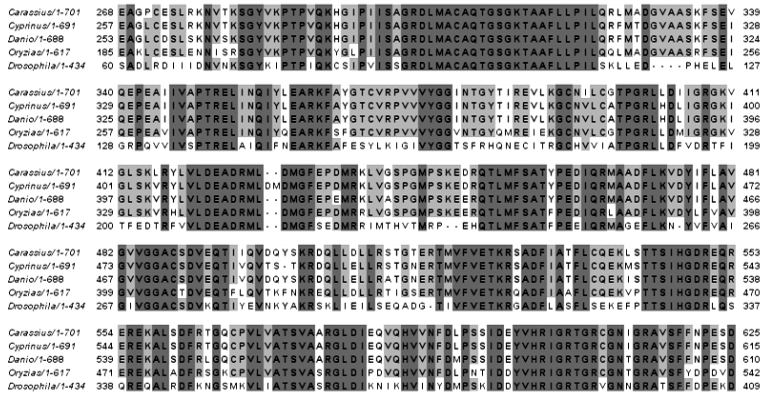

Figure 1. Sequence alignment of vasa from fish and vasa protein from *Drosophila*.

Based on these results, Vasa from *Drosophila* was selected as a structural template for modeling. The structure was solved in complex with an RNA fragment and ATP analog, enabling functional analysis of vasa models from fish. Homology models clearly show the structural/functional conservation of vasa in the selected fish species. Representative homology modeling results are shown in Figure 2.

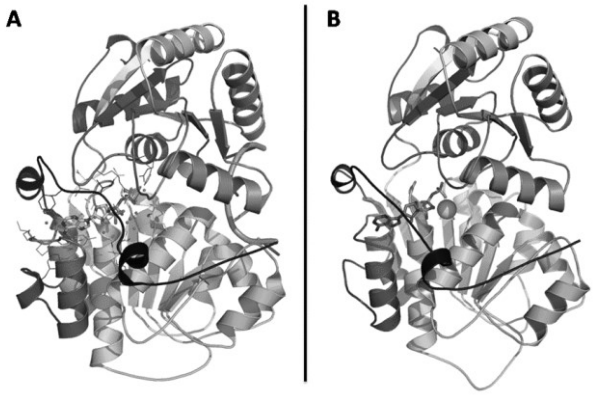

Figure 2. Vasa from *Drosophila* (A). Homology model of vasa from *Danio rerio* (B).

DISCUSSION AND CONCLUSIONS

Using structural bioinformatics and homology modeling, we have created preliminary structural models of vasa protein from four fish species. Predicted structures of vasa from *Danio rerio*, *Oryzias latipes*, *Cyprinus carpio* and *Carassius gibelio* were found to be overall very similar to experimentally determined X-ray crystal structures of vasa from *Drosophila*, *Bombyx Mori* and *Homo sapiens*. In particular, residues involved in ATP binding and interaction with RNA appear to be highly conserved. Due to the high level of sequence conservation among vasa orthologs, these results suggest that structural and functional data available for vasa protein from *Drosophila* (and by extension human vasa) might be applicable to vasa in fish. Thus, structural models of vasa from fish might be used to guide functional studies into the molecular role(s) of this important protein.

ACKNOWLEDGEMENTS

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P72

SPERM CHARACTERISTICS AND ANDROGENS IN THICK LIPPED GREY MULLET *(CHELON LABROSUS)* AFTER ADMINISTRATION OF GNRHA IN THE FORM OF MULTIPLE INJECTIONS OR IMPLANTS

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INTRODUCTION

Milt production in many fishes has been enhanced following treatment with gonadotropin-releasing hormone agonist, GnRHa (Zohar and Mylonas, 2001). A major objective of the present study was to examine the potential of GnRHa administration in enhancing sperm quality of captive thick lipped grey mullet (*Chelon labrosus*) a grey mullet species with a good aquaculture potential (Ben Chemis et al., 2012).

MATERIALS AND METHODS

Three thick lipped grey mullet male broodstocks (n=28) of wild origin (>6 years old, 0.87 kg $fish^{-1}$) maintained at Olympos Fish Farm facilities (Pieria, N. Hellas). Fish were kept in outdoors tanks (3.5m^3) supplied with surface seawater (37-38psu, 13-16ºC, DO: 5.5-6.0 mg l^{-1}) under ambient photoperiod. They fed to satiation once a day, six times a week, with a mixture of a marine grower pelletized diet (Biomar, Greece), frozen squid and deep water rose shrimp. All fish were individually marked with a PIT tag (AVID, UK). Fish were starved 2 days before handling. At each handling, fish were tranquilized initially in their tank (0.08 ml l^{-1} 2-phenoxyethanol) and then transferred to a bath for complete sedation (0.1 ml l^{-1}). Males were treated with saline (control) or gonadotropin-releasing hormone agonist [D-Ala^6, Pro^9, NEt] GnRHa to induce spermiation. GnRHa was administered by intramuscular injection at a dose of 10 μg GnRHa kg^{-1} bw once time a week for a period of 4 weeks or with a single GnRHa-loaded implant (50 μg kg^{-1} bw). Spermiation condition and sperm quality were evaluated on a weekly basis on days 0, 7, 14, 21, 28 post treatment. Spermiation index (SI) was evaluated on a subjective scale. Small volumes of sperm were collected (50–100 μl) for quality evaluation. Sperm density was estimated after a 2500-fold dilution with seawater using a Neubauer haematocytometer (in duplicate). Sperm motility and duration were evaluated on a microscope slide after mixing 1 μl of sperm with a drop of about 50 μl of saltwater (in duplicate). The androgens, Testosterone (T) and 11-Ketotestosterone (11-KT) were quantified in the plasma (200 μl) using enzyme-linked immunoassays (ELISA). Repeated measures analysis of variance (ANOVA) was used to understand the effects of main factors (sampling time and hormonal treatments) and their interactions on each measured parameters. Prior to analysis, data were transformed appropriately in order to meet the ANOVA assumptions. In case of significant effects, l.s.d. post-hoc comparisons were performed. To describe the relationship between measured parameters with sampling time, hormonal treatment and their interaction, a linear regression line was fitted. Physiological relationships between sperm characteristics and androgens were statistically analyzed by Pearson correlation analysis.

RESULTS

Sperm could be obtained from any male at the begging of the experiment (day 0). Spermiation condition significantly decreased ($p<0.001$) only for GnRHa implanted males on day 28 (fourth week). From repeated measures ANOVAs significant effects of sampling time, hormonal treatments and their interactions were observed on sperm density, motility, duration of motility and androgen levels. At day 7 male groups treated with GnRHa in the form of injections or implants showed significantly higher sperm density values than control males. However, at day 14, the males treated with GnRHa implants had lower sperm density values than GnRHa injected or control males. Results of the regression analysis clearly showed that Sampling time had a significant effect on sperm density for GnRHa injected ($R^2=0.21$, $p<0.001$) and control ($R^2=0.22$, $p<0.001$) but not for GnRHa implanted males ($p>0.05$). Sperm motility showed a decreasing pattern in males treated with GnRHa implants and it was significantly decreased at day 21 in males treated with GnRHa compared to control males. Like sperm motility, the duration of motility remained unchanged for males treated with GnRHa multiple injections but it was following a decreasing pattern in males treated with GnRHa implants and control males. Hormonal treatment had no effect on T and 11-KT plasma values but sampling time significantly influenced T and 11-KT plasma levels with T and 11-KT values being decreased at day 7 post treatment when sperm density reached its maximum values in both GnRHa treated and control males ($p<0.01$). Pearson correlation analysis revealed significant relationships ($p<0.05$) between density and duration ($r=-0.36$), motility and duration ($r=0.47$), density and testosterone ($r=-0.46$) or 11-ketotestosterone ($r=-0.25$), duration and testosterone ($r=0.40$) and also testosterone and 11-ketotestosterone ($r=0.45$).

DISCUSSION AND CONCLUSIONS

The GnRHa administered in the form of intramuscularly injections or sustained release delivery systems was effective in enhancing sperm density and spermiation condition. However, the administration of GnRHa in the form of sustained release delivery systems (implants of 50 μg kg^{-1} bw) was shown to be less potent in enhancing spermiation and density than GnRHa administered in the form of multiple intramuscularly injections at low doses. It is possible that the constantly high plasma LH levels induced by sustained-release GnRHa delivery systems may not induce the appropriate endocrine signals to enhance sperm production and density (Mylonas et al., 2003). Similar to various fish species examined so far, the GnRHa treatment did not result in significant changes in spermatozoa motility and motility duration, confirming that GnRHa-treatments may enhance spermiation and sperm density without affecting negatively the fertilizing ability of the sperm. Finally, the GnRHa treatment had no significant effect on shaping T and 11-KT plasma levels but significantly lower values of those hormones came with increased spermatozoa production and density values indicating their involvement in physiological mechanisms regulating spermatozoa production.

ACKNOWLEDGEMENTS

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HORMONAL INDUCTION OF SPAWNING AND EGG PRODUCTION IN THICK LIPPED GREY MULLET, *CHELON LABROSUS*

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INTRODUCTION

Various commercial and environmental attributes make grey mullets important economic resources for fishing communities and important candidates for domestication. However, female mullets fail to undergo final oocyte maturation in captivity and fingerling supplies they have come almost exclusively from the wild (Aizen et al., 2005). Liquid injections and controlled-release delivery systems that release GnRHa for a prolonged period of time (Mylonas and Zohar, 2001) combined with dopamine antagonists have been shown to be effective in inducing maturation of Pacific (Glubokov et al., 1994) and grey mullet (Aizen et al., 2005). The objective of this study was to test the effectiveness of a dopamine antagonist injection and a GnRHa hormone administration via two modes of treatment (implant *vs* multiple injections) in inducing spawning in lipped grey mullet, *Chelon labrosus*.

MATERIALS AND METHODS

Six thick lipped grey mullet broodstocks (n=60, 1F:1M) of wild origin (>6 years old) were kept in outdoors tanks (3.5m^3) supplied with surface seawater (37-38psu, 13-16ºC, DO: 5.5-6.0 mg l^{-1}) under ambient photoperiod. Three groups of females (n=30) were treated (17 March 2010, D_0) once time a week for a period of 4 weeks (D_0, D_7, D_{14}, D_{28}) after they considered as eligible for spawning induction (mean oocyte diameter >500μm with very little atresia present) with: (i) a single injection of dopamine antagonist (metaclopramide, 10μg see aizen et al.,) and an Ethylene–Vinyl acetate (EVAc) GnRHa implant loaded with Des-Gly^{10}, d-Ala^6-Pro-NEth^9-mGnRHa (50 μg GnRHa kg^{-1} bw) or (ii) a single injection of metaclopramide and weekly injections of GnRHa (10 μg GnRHa kg^{-1} bw), or (iii) weekly injections of 0.9% NaCl. Fish fed to satiation once a day, six times a week, and they were starved 2 days before handling. At each handling, they were tranquilized initially in their tank (0.08 ml l^{-1} 2-phenoxyethanol) and then transferred to a bath for complete sedation (0.1 ml l^{-1}). Females were checked for ovulation, a portion of an ovarian biopsies was used to measure oocytes diameter, and a portion was fixed for histological processing. Plasma samples were taken (500μl) to quantify Testosterone (T) and Oestradiol (17β-E_2) using enzyme-linked immunoassays (ELISA). A passive egg collector was placed in the outflow of each spawning tank, to collect the spawned eggs. Eggs were collected every morning into a 10-l bucket and their number (fecundity) was estimated by counting the total number of eggs in a sub-sample of 10 ml (x 3 times), after vigorous agitation. Fertilization success was evaluated at the same time by examining each of the eggs in this 10 ml sample for the presence of a viable embryo using a stereoscope. Repeated measures analysis of variance (ANOVA) was used to understand the effects of main factors (sampling time and hormonal treatment) and their interactions on each measured parameters. Prior to analysis, data were transformed appropriately in order to meet the ANOVA assumptions. In case of significant effects, *l.s.d.* post-hoc comparisons were performed.

RESULTS

A total of 5 spawns occurred in GnRHa implants spawning groups (*GnRHa impl*), 3 spawns in GnRHa injected groups (*GnRHa inj*) and 5 spawns in control groups (C). For both GnRHa treated and Cgroups most of the spawns occurred during the 3rd week post treatment (31 March to 7 April, *GnRHa impl:* 3 of the 5 spawns, one with the highest fecundity and fertilization success, 60,611 eggs bw^{-1}, 50,8%), *GnRHa inj:* all observed spawns, CG: all observed spawns). Females of the *GnRH impl* groups spawned 4 and 7 days post treatment, with low daily relative fecundity and fertilization success (day 4: 4,116, 5,1%, day 7: 2,320, 3,4%). However, females of the *GnRHa inj* group spawned after 3 or 4 injections (24 and 48 hours after the 3rd injection, 24 hours after the 4th injection) achieving the highest (up to an average of 21,446 eggs bw^{-1}) daily relative fecundity and having the highest fertilization success (58%). Although control females spawned as many times as GnRHa implanted females, daily relative fecundity, total fecundity and fertilization success were the lowest estimated in this experiment. Spawns were less intermittent in *GnRHa inj* females but spawns of this group resulted to the highest fecundity (daily and total relative) and fertilization success. Last spawn occurred 24 hrs after the 4th injection in the identical *GnRHa inj* group (on 15th April). T and 17βE_2 plasma values were significantly decreased one week after treatment (D_7, $p<0.001$) and remained in low levels during the experiment. The interaction of treatment and sampling date had no effect on T and 11βE_2 levels.

Table 1. Egg production data from spawning induction experiment with *C. labrosus* induced to spawn during the reproductive season (17 March) with GnRHa in the form of slow release delivery systems (EVACs, *GnRHa impl*) or in the form of multiple injections (GnRHa inj. four weekly injections).

Cl_experiment	**Females** *(n)*	**Spawns** *(n)*	**Relative batch fecundity** *(eggs kg^{-1})*			**Total fecundity** *(eggs kg^{-1})*	**Fertilization** *(mean ± SD %)*
			Min	**Max**	**Mean** ± SD		
GnRHa impl.	10	7	463	60,611	10,646 ± 22,099	74,522	10±10
GnRHa inj.	10	4	10,309	33,458	21,446 ± 11,349	85,783	58±24
Controls	11	5	453	16,168	6,748 ± 5,802	33,738	19±36

DISCUSSION AND CONCLUSIONS

The dopamine antagonist combined with GnRHa were effective in inducing for first time successful spawning and production of fertilized eggs from female thick lipped grey mullet in captivity. GnRHa administration in the form of weekly injections although evoked fewer number of spawns as compared to GnRHa administered via implants resulted to the highest fecundity and fertilization success (total fecundity: 85,800 eggs kw^{-1}, 58% fertilization success). Other studies also demonstrated successful spawning induction protocols consisting of GnRHa and dopamine antagonists given in repeated injections indicating the dopamine's involvement in the regulation of ovulation in mullets (Glubokov et al., 1994, Aizen et al., 2005). The reproductive biology of the thick lipped grey mullet has been poorly studied and almost no information exist on the fecundity of wild spawning females. However, the results on fecundity obtained here and those reported for wild caught spawning females of relative grey mullet species (Albieri and Araujo, 2010) indicate the need for further efforts to design spawning induction protocols aiming at higher fecundity and fertilization rates.

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BROODSTOCK AND SPERM QUALITY DIFFERENCES IN GENETICALLY MODIFIED ZEBRAFISH AND WILD-TYPE ZEBRAFISH (*DANİO RERİO* HAMİLTON-BUCHANAN, 1822)

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INTRODUCTION

Nowadays, Glofish™ and TK coded fish have widespread in the ornemantal fish industries. Glofish™ is including zebrafish (*Danio rerio*), barb (*Puntius tetrazona*), tetra (*Gymnocorymbus ternetzi*) which are produced by Yorktown Technologies; and TK coded fish is including zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) which are produced by Taikong Corp. These fish have a pink, red, orange and green body coloration. Genetically modified (GM) zebrafish is commonly imported fish for ornamental fish sector and also pink zebrafish is the most preferred variety in Turkey. For this reason, we used pink zebrafish in this experiment.

MATERIALS AND METHODS

Genetically modified male pink and non-transgenic zebrafish were perchased from the pet shops in İstanbul. Fish were reared in an aquarium at 28°C. They were fed *Ad libitum* two times per day with commercial food (Sera) and one times per day *Artemia* sp. Male zebrafish testis tissue was removed by dissection and kept in Hank's solution (4°C). After dissecting testis tissue, semen were maintained in Hank's solution. Sperm motion kinematics determined by CEROS II-CASA system (Hamilton-Thorne). Spermatozoa was diluted with distilled water and dilution rate of 1:5 (spermatozoa:distilled water), 0.5 µL and distilled water which was maintained at room temperature for 2.5 based µl were transferred to eppendorf tubes. This example transfered to having a volume of 3 µl micropipette conveyed via one of the slide compartment and motility were measured. Osmolality was measured (305 mOsm/kg) using a micro-osmometer (Fiske-210, Norwood, Massachusetts, USA).

RESULTS

Motility percentage is determined that 60% for a pink zebrafish while it is 95.3% for a non-transgenic zebrafish in 20th sec. But the motility percentages are determined close when two groups are compared in 75th sec. Even though the motility percentage is high for a non-transgenic zebrafish in measurements up to 75th sec., the values of VAP, VSL, VCL and LIN are determined higher in a pink zebrafish. It is observed healty zebra fish couldn't prevent their healty morphological appearance during 45 days of experimental process. It is observed non-transgenic zebrafish that are feed in the same aquarium with pink zebra fish are healty. According to experiments it is not seen any external parasites in fish gill but weakening in muscles and kidney and nematod in intestine are determined. 50% of pink zebra fish are dead as a result.

DISCUSSION AND CONCLUSIONS

Currently, genetically modified fish has growing impact in the ornamental fish sector. For this reason we demonstrated some differences wild-type and genetically modified fish. Snekser et al studied behavioral characteristics in red zebrafish and wild type zebrafish, and showed that red colour is not important to affect social behaviour of the zebrafish (Snekser et al, 2006). Amanuma et al. demonstrated that red and wild-type zebrafish has a same temperature tolerance. In the laboratory studies we observed that pink zebrafish has more stressfull and sensitive than non-transgenic zebrafish. This research contains sperm characteristics of GM zebrafish that is took an important place in aquarium sector

recent years at the same time the accumulated informations at times they are kept in aquariums.

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ANALYSIS OF THE SURVIVAL OF NEWBORN SEAHORSES: PRELIMINARY RESULTS

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INTRODUCTION

The main bottleneck of seahorse aquaculture is the low survival of the juveniles. The reasons associated with this are poorly understood, but disease problems, zootechnical management and feeding issues are considered the most important ones. Furthermore, newborn seahorses have been proposed as completely developed fish, nonetheless there are some general clues that suggest that this could not be completely true. Bearing this in mind, the objective of this study was to analyze the survival of 12 seahorse spp. on the first 120 days post hatch based on the available literature.

MATERIALS AND METHODS

The data were extracted from 31 reports, mainly peer reviewed papers, which have available information from either graphics or numeric data about the seahorse survivorship during the first 120 days of life. The treatments used in each paper were categorized in main factors and then analyzed with a multidimensional logistic regression.

RESULTS AND DISCUSSION

Almost all the seahorse species were characterized by low survivals during the first days of life (Fig. 1). Nonetheless, the survival rates were different among the species. This could be related to the biological differences among the species, particularly the hatching size. Accordingly, the survival of *Hippocampus reidi* had the lowest levels in the earlier ages. Interestingly, the feeding was the factor most related to the seahorse survivorship and the most studied. Ontogenetic development related to the feeding of seahorses and or an incompletely development of newborn seahorses were supported by the results.

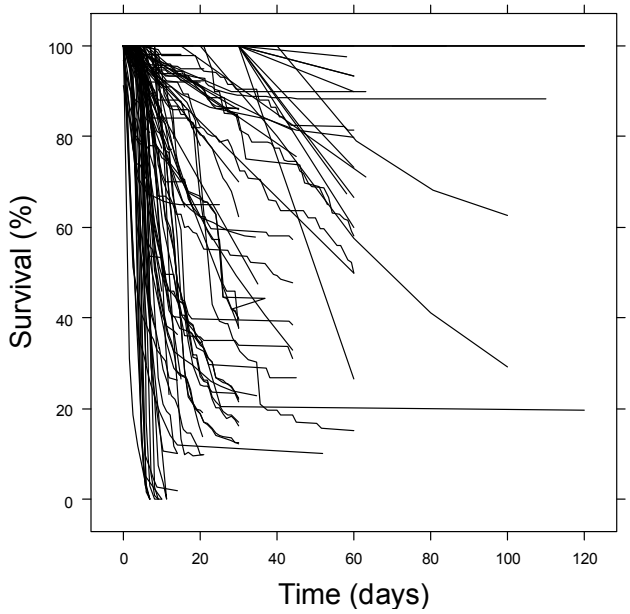

Fig. 1: Survival of 12 seahorse species reared under laboratory conditions reported in 31 studies.

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COPEPODS IN THE DIET OF ORNAMENTAL FISH

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INTRODUCTION

Copepods are the preferred natural prey of most marine fishes during the larval phase. They show a wide range of body size between nauplii and adults, typical movement that stimulate the predatory activity of the larvae, and they also have a high content of highly unsaturated fatty acids (HUFAs). These fatty acids are extremely important for larval fish survival and growth and several studies have demonstrated that they are essential in larval diets. At date, most of the commercial fish species are still reared using rotifers (*B.plicatilis, B. rotundifornis*) and *Artemia* nauplii since they can be cultured in large quantities at high densities. Unfortunately, using rotifers and *Artemia* during this early period in life history, does not always result in optimal larval growth since these live prey may contain an inadequate fatty acid profile and, in some cases, be of an inappropriate size. The use of cultured copepods in intensive fish larviculture has involved both calanoids and harpacticoid but the best results have been obtained using calanoid copepods which have a higher content of HUFAs, are entirely pelagic and usually have very small naupliar stages which are more readily captured by fish larvae with small gapes at first feeding (Holt, 2003; Olivotto et al.,2011). Unfortunately, there are several difficulties in culturing copepods on a continuous basis, since they are usually cultured at very low densities, in large tanks, and need to be fed different algal combinations. As a consequence, the technology for mass scale production of copepods is still in the research stage. In the present study culture techniques for both calanoid and harpacticoid copepods are discussed as well as a new technology able to produce different sized preserved copepods that maintain, through the time, their fatty acid composition.The introduction should include the scope of the problem and state the objectives of the work presented.

MATERIALS AND METHODS

Several phytoplankton species including the microalgae nannochloropsis oculata, Isochrysis galbana and *Rhodomonas baltica* were cultured using F2 media. The same algae were used to growth rotifers (Brachionus plicatilis) Artemia salina and the copepod species *Acartia tonsa, Centropages typicus* and *Parvocalanus crassirostris.*

Feeding studies on ornamental species such as *Centropyge flavissimus, Amphiprion clarkii* and *ocellaris, Meiacanthus grammistes* and *Hippocampus reidi* were performed partially or totally substituting rotifers and *Artemia* with copepods. Survival rates, growth rates and molecular markers such as Insuline like growth factors (IGFs), myoststin (MSTN) and peroxisome proliferator activated receptors (PPARs) were evaluated during the larval development.

RESULTS

While both hapracticoids and preserved copepods improved larval survival and growth when used as supplement to the traditional diet based on rotifers and *Artemia*, the calanoid copepods (*Acartia tonsa, Centropages typicus* and *Parvocalanus crassirostris*), which are entirely pelagic and thus more available for the feeding larvae, were able to improve larval fish survival and growth when used solely. These studies evidenced that all the copepods species analyzed can be considered a valuable source of HUFAs in ornamental fish larviculture and may improve larval fish survival and growth.

DISCUSSION AND CONCLUSIONS

Using rotifers and *Artemia* during the early life history of fish does not always promote optimal larval growth since these live prey may contain an inadequate fatty acid profile and, in some cases, display an inappropriate size (Holt 2003; Olivotto et al. 2011). Because of this, there is a need for identification of alternative food sources that do not have these inadequacies and can promote adequate growth. Adult copepods, along with copepodites and nauplii, are considered good candidates for feeding marine ornamental fish larvae (Holt 2003). The advantages of using copepods in larviculture are mainly related to their wide range of body sizes between nauplii and adults, their typical movement, and their high content of HUFAs. Bottlenecks still remain; the development of a copepod-based commercial production of marine fish still requires the use of large mesocosms. Research should be focused on finding copepod species with short generation times and tolerance to high densities, in addition to gaining a better understanding of the possible involvement of amino acids, protein, pigment and vitamin contents of copepods in larval fish growth and survival.

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