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Additional Information

Fish sperm motility assessment as a tool for aquaculture research, a historical approach

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Abstract

Fish sperm motility is nowadays considered the best quality biomarker for fish spermatozoa, and sperm motion parameters from more than 300 fish species have been already reported throughout 1500 scientific articles covering a wide range of topics, from molecular to ecology issues. Within these topics, *i)* sperm storage (involving both the use of chilled-storage protocols for short-term periods, and sperm cryopreservation techniques for long-term storage), *ii)* sperm physiology (fathom in the spermatozoa activation process and the whole propulsion machinery of the sperm cells), and *iii)* broodstock management (covering aspects such as rearing conditions, dietary requirements or hormonal induction treatments), have been the most studied fields through the evaluation of fish sperm motion, enhancing many aspects of management practices in fish farms. In addition, other aquaculture and ecological topics such as *iv)* the knowledge of the breeding cycle of the species, *v)* the phenomenon of the sperm competition, and *vi)* ecotoxicological studies for the evaluation of aquatic environments, have also been approached from the evaluation of sperm motion performance.

To sum up, fish sperm motility assessment can serve as a potential tool for aquaculture and ecological purposes, covering key topics of fundamental and applied research. This review gives an overview of the major research areas in which fish sperm motility have been applied successfully.

Keywords:

Spermatozoa; velocity; sperm quality; kinetic, CASA

1. Introduction

Fish are the most diverse and numerous group of vertebrates, and a wide diversity of reproductive strategies can be found both in freshwater and marine species (Bone *et al.* 1995). However, most of them share a common fertilization mode, the external fertilization, in which gametes both from males and females are released into the aquatic environment (Crowe & Russell 2009). Up to this point, fish spermatozoa remain completely quiescent in the spermiduct, and they became motile once are released to the external medium by an hyper- or hypo-osmotic shock, depending on seawater or freshwater species (Morisawa 2008). In this context, sperm activation will be an essential requirement in the pursuit of female gametes, and a wide range of factors of both external and internal origin will determine the fertilization success.

It is reasonable to imagine that fertilization success will depend on gamete quality and, focusing in males, it makes sense to define and understand what gamete (sperm) quality means. From a biological standpoint, sperm quality could be defined as the ability of the spermatozoa to exploit their swimming ability for reaching and fertilize the oocyte (Fauvel *et al.* 2010). Therefore, any quantifiable sperm trait directly correlated with the fertilization success could be potentially used as a sperm quality biomarker. A relatively high number of sperm quality biomarkers have been reported over the years in several fish species (Rurangwa *et al.* 2004). These sperm biomarkers have so far been documented in scientific papers, and several traits of the fish/sperm itself such as osmolality, pH and chemical composition of the seminal plasma (Alavi *et al.* 2004); enzymatic activity (Burness *et al.* 2005); ATP concentration (Dzyuba & Cosson 2014); spermatocrit and sperm density (Sørensen *et al.* 2013); sperm motility (Ottesen *et al.* 2009); or sperm morphology and ultrastructure (Ciereszko *et al.* 2015) have been linked to the ability of sperm to fertilize the ova. Although some of these parameters are relatively easy to score and commonly used (spermatocrit, viability and subjective motility); others need sophisticated laboratory analyses (biochemical analyses), expensive equipment (objective and quantitative motility) or availability of eggs (fertilisation success).

2. Sperm motility as a sperm quality biomarker

Although it is a set of sperm characteristics that contribute to determining sperm quality, sperm motility is currently considered the best sperm quality biomarker in fish (Suquet *et*

al. 2010; Boryshpolets *et al.* 2013; Mylonas *et al.* 2016). In fact, high correlations have been reported between sperm motility and fertilization and hatching rates in some fish species (Gage *et al.* 2004; Gasparini *et al.* 2010; Gallego *et al.* 2013a). Although sperm motility could loosely defined as the capacity of spermatozoa to move or not once released into the activation media, a deeper comprehensive description would also involve other kinetic features such as spermatozoa velocity, progressiveness, linearity, etc. In this sense, the method or technique chosen by the researcher for assessing the sperm movement will generate the degree of depth of understanding of the motion pattern. Nowadays, sperm motility evaluation can be done by two different ways in the laboratory: i) the subjective way, in which an experienced technician (or not) make an evaluation of sperm motility through a simple observation under the microscope; and *ii)* the objective way, in which sophisticated software integrate the successive positions of the heads of moving spermatozoa in consecutive frames of video records to calculate the trajectories and their characteristics. Subjective evaluation method has been the most used technique to appraise sperm motility over the history, but some problems have emerged from this method. First drawback is focused the own limitation of human eye, through which we can only provide a coarse evaluation of sperm quality by motility criteria assessing classes in terms of percent of motile sperm and motility duration. In addition, this type of evaluation will depend on the observer's experience and several aspects such as sperm density, sperm velocity, drift, etc. can cause over- or underestimated readings. Therefore, the low reproducibility of this subjective motility evaluation, which can result in variations of 30 to 60% from the same sample, often makes it difficult to interpret and compare the results intra- and inter-labs (Verstegen *et al.* 2002; Rosenthal *et al.* 2010).

On the other hand, the gradual appearance of Computer Assisted Sperm Analysis (CASA) systems has made possible to estimate a higher number of sperm motion parameters not given by subjective evaluation, providing the scientific community with new useful tools to be applied in multidisciplinary studies. These systems, which are the evolution of multiple photomicrography exposure and video-micrography techniques for spermatozoa track, represent an objective, sensitive and accurate technique for obtaining sperm kinetic features (Kime *et al.* 2001). CASA systems were first used in the 70´s in mammalian sperm, and only in the 90´s modern CASA systems have been adapted for fish spermatozoa studies (Perchec *et al.* 1995; Toth *et al.* 1995; Christ *et al.* 1996). The differences on the size and the biology of fish and mammalian spermatozoa may explain the delay in the release of adequate tools for the measurement of sperm motility in fish.

To date, these systems have been used and validated in a wide range of animal groups such as marine invertebrates (Riesco *et al.* 2017), birds (Lüpold *et al.* 2009), marine mammals (Montano *et al.* 2012), reptiles (Tourmente *et al.* 2011) or even insects (Al-Lawati *et al.* 2009). It is noteworthy that most of the parameters evaluated by CASA systems have been correlated positively with spermatozoa fertilization potential, thus CASA is a very useful tool for assessing sperm quality in fish reproduction research (Tuset *et al.* 2008; Gallego *et al.* 2013a).

3. Fish sperm motility: a useful tool for multidisciplinary studies

The first scientific reports focused on fish sperm motility date from a century ago (Gee 1916). From this date, new articles about this topic were reported sporadically until 60s, and became continuous but still scarcely until 80s. Nevertheless, a marked and continuous increase of scientific contributions were detected from 90's to present, and we can currently find more than 1500 publications using fish sperm motility as a research tool through a wide range of topics: from ecology to molecular issues (Figure 1 and 2).

To date, sperm motion parameters from 340 fish species belonging to different families have been already studied. However, only a few species (~30) represent more than 50% of published papers, of which salmonids, cyprinids and sturgeons are the most studied families. In this context, scientists have devoted much more time to study freshwater than seawater species, so the flood information is five-fold bigger in freshwater fish. Here we present an overview of the state of the art about the most developed research areas in which sperm motility have been applied successfully.

3.1 Sperm physiology

Sperm physiology has centred the use of spermatozoa motion as a research tool. In fact, first studies using fish sperm motility were carried out on this topic at the beginning of the last century, assessing the sperm behaviour of rainbow trout on different activation media conditions. Since then, this research field has shown a continuous increase over the years, and more than 400 sperm motility-physiology articles have been published (Figure 2).

Fish sperm activation process has been the key subject within this area, and learning about the process by which spermatozoa begin to move has been the main goal of fish physiology. In this context, three general pathways through which sperm becomes motile

have been discovered and reported in teleost fish (Morisawa 2008): *i)* marine fish spermatozoa become motile by an hyperosmotic shock; *ii)* spermatozoa of freshwater fish become motile by an hypoosmotic shock; and *iii)* spermatozoa from salmonids and sturgeons become motile by a low environment K^+ concentration. Although these three spermatozoa activation models are widely accepted by the scientific community and they can be tested even by subjective motility evaluation, in-depth motion analysis through CASA systems have contributed to describe deeply these activation pathways (Gallego *et al.* 2013b; Pérez *et al.* 2016). Nowadays, novel research lines about the effect of absence/presence of certain ions in the activation medium on the sperm kinetics parameters are emerging both in freshwater and seawater species (Dietrich *et al.* 2007; Vílchez *et al.* 2016, 2017)

Another important topic on this research area has focused on the relationship between the composition of the seminal plasma and sperm motility. The literature reveals many data about the ionic composition in different species belonging to different families, but the correlation between these seminal plasma compositions and the sperm the motility has been investigated in only a few species. Regarding cyprinids, a positive relationship between Na^+ and sperm motility was reported both in common carp (*Cyprinus carpio*) and common bleak (*Alburnus alburnus*), while K^+ had a negative correlation with sperm motion parameters (Lahnsteiner *et al.* 1996; Bozkurt *et al.* 2009). Regarding salmonids, Ca^{2+} was significantly correlated with the sperm fertilization capacity in rainbow trout (*Oncorhynchus mykiss*), estimating optimum levels from 0.8 to 1.2 mmol/L for carrying out fertilization trials (Lahnsteiner *et al.* 1998). In Atlantic salmon (*Salmo salar*), Na^+ and K^+ levels were correlated with fertilization rates (Aas *et al.* 1991). Regarding marine fish we can find mixed results: while in the European eel (*Anguilla anguilla*) [K^+] increased and [Ca^{2+}] and [Mg^{2+}] showed a progressive reduction as sperm quality improved (Asturiano *et al.* 2004); in Atlantic cod, [Ca^{2+}] showed significant and positive relationship with sperm motion parameters in several months during the breeding season (Butts *et al.* 2011).

Moreover, the propulsion machinery of spermatozoa has been another research focus within sperm physiology studies. In this regard, scientists have tried to find correlations between sperm motility and other factors such as spermatozoa flagella or its power source, the energetic metabolites. Although several studies have reported a sharp decrease of ATP once motility start in a wide range of teleost species (Perchec *et al.* 1995; Butts *et al.* 2010), it is not an easy task to find significant correlations between ATP content

and spermatozoa velocities or motilities. Sperm ATP levels have been correlated with motility, velocity and⁄or fertilizing ability in two salmonid species like rainbow trout (Lahnsteiner *et al.* 1998) or chinook salmon, *Oncorhynchus tshawytscha* (Bencic *et al.* 1999); and even in some marine species like sea bass, *Dicentrarchus labrax* (Zilli *et al.* 2004). In contrast, no correlations between ATP and sperm motility were found in species as common bleak, *Alburnus alburnus* (Lahnsteiner *et al.* 1996); bluegill, *Lepomis macrochirus* (Burness *et al.* 2005); or Atlantic cod, *Gadus morhua* (Butts *et al.* 2010). Actually, it is known that ATP alone is not a strong indicator of sperm motility by itself, and other metabolites such as ADP or CrP should be taken into account (Dzyuba *et al.* 2017). In this context, the macroenergic phosphates content have been recently proposed as a biomarker for semen quality (Hatef *et al.* 2013; Cabrita *et al.* 2014), but it is noteworthy that this parameter could be only used in a species-specific context.

3.2 Sperm storage

Sperm storage, by either short-, medium- or long-term period, has been the most investigated field using sperm motility as a research tool. More than 500 scientific publications reporting kinetic spermatozoa parameters have contributed for discover and improving sperm storage protocols in a large number of fish species (Figure 2). Nowadays, these techniques show a high number of potential applications, ranging from ecology to aquaculture perspective.

3.2.1 Chilled storage

Short- and medium-term storage methods, also known as chilled- or cold-storage protocols, aim to preserve the sperm integrity and quality over several days, weeks or even months. The main applications of these protocols are focused in aquaculture issues, allowing the improvement of broodstock management mainly through the synchronization in the gamete production of male and females. Temperatures of 0 °C (melting ice) to 4 °C are the most widely used as they are easy to reach and easy to regulate. Besides, these low temperatures reduce bacteria growth, so this may explain why temperatures below 6 °C are always reported as better than higher temperatures (Bobe & Labbe 2009). In addition to the temperature, other elements such as the type of extender, the dilution ratio (sperm:extender), or the environmental conditions become essential factors in order to achieve a successful sperm storage. Given that these factors are usually species-specific, a large number of storage protocols can be found through the

literature. The Table 1 summarizes studies on short- and medium-term storage protocols applied to several fish species during the last 30 years, and showing the best results obtained in terms of sperm motility.

Regarding short-term storage, great results have been obtained in the key aquaculture families such as Salmonidae, with more than 50% of motility after 7 and 14 days in rainbow trout (Ubilla *et al.* 2015) and Atlantic salmon (Parodi *et al.* 2017), respectively; Cyprinidae, keeping good quality samples (>70%) in common carp and perch (*Perca fluviatilis*) during the first week of storage; Acipenseridae, with more than 50% of motility after 7 days in Siberian sturgeon (Shaliutina *et al.* 2013); and marine fish species, where spermatozoa still retained some motility after 30 days storage both in Atlantic cod (*G. morhua*) and haddock (*Melanogrammus aeglefinus*) (DeGraaf & Berlinsky 2004).

In addition to chilled storage, sperm super cooling method (-2 to -5 °C) has been sparingly used in fish species. Within this technique, the use of different substances like cryoprotectants avoid the ice crystallization, keeping a proper membrane integrity on spermatozoa. There are only a few reports of just sub-zero storage on fish sperm: rainbow trout semen was stored at -2 ºC for at least 23 days without loss of fertilizing power (Stoss & Refstie 1983), and Atlantic salmon sperm stored for 21-28 days at -4.5 ºC was able to fertilize 80-90% of eggs (Truscott & Idler 1968). However, this method requires a very strict control of the temperature to avoid ice crystallization if temperature decreases or cryoprotectant toxicity if temperature increases (Bobe & Labbe 2009).

3.2.2 Cryopreservation

Cryopreservation is a long-term storage technique that apply extreme temperatures for keeping viable spermatozoa, most common is -196 °C in liquid nitrogen. At these low temperatures all biological activity stops, so this process is able to preserve and store sperm cells over long periods, from days to years. Therefore, enforcements from this technique evolved from aquaculture purposes (broodstock management, genetic improvement programs, species-specific reproductive problems, etc.) to ecology goals, as cryobanking of genetic resources from endangered species

To date, sperm of more than 200 fish species haven been successfully cryopreserved and techniques of thawed sperm management have been established for freshwater and marine fish species (Tsai & Lin 2012). Although some previous manuscripts have reviewed fish sperm cryopreservation subject (Suquet *et al.* 2000; Kopeika *et al.* 2007; Cabrita *et al.* 2010; Asturiano *et al.* 2017; Martínez-Páramo *et al.* 2017), Table 2

255 summarizes the best results reached on fish sperm cryopreservation on the most important
256 fish families for aquaculture. This table includes the cryoprotectants used (and their
257 concentrations), focusing on pre- and post-thaw motility values obtained for each species.
258 Freshwater species has been the most studied group, and specific protocols have been
259 established for salmonids, sturgeons, carps and catfishes. Regarding Salmonidae, great
260 results have been obtained in the key aquaculture species such as rainbow trout (*O.*
261 *mykiis*), brown trout (*S. trutta*), and Atlantic salmon (*S. salar*); and post-thaw motilities
262 higher than 60% have been reported using methanol and sugars as a rule for
263 cryopreservation protocols on this fish family (Horváth *et al.* 2015).
264 In sturgeons, whose spermatozoa have an acrosome, cryopreservation process can induce
265 some deleterious effects on this structure (Billard *et al.* 2004), and post-thaw motility can
266 be compromised in species such as beluga (*Huso huso*), starlet (*Acipenser ruthenus*), or
267 Siberian sturgeon (*Acipenser baeri*). However, the addition of amino acids to the
268 cryopreservation medium (usually 10% methanol) can noticeably improve post-thaw
269 sperm quality, reaching motility values around 80% in Persian sturgeon (*A. persicus*)
270 (Aramli *et al.* 2016a).
271 In relation to cyprinids, different types of cryoprotectants such as methanol, dimethyl-
272 sulfoxide (DMSO) or glycerol have been successfully applied. DMSO provided great
273 results in silver carp (*Hypophthalmichthys molitrix*) and java barb (*Barbus gonionoutus*),
274 showing post-thaw motilities higher than 80%; while methanol and glycerol provided
275 good results in tench (*P. fluviatilis*) and grass carp (*Ctenopharyngodon idella*),
276 respectively. Surprisingly, the best results in cyprinids have been reported in common
277 carp (*C. carpio*) using only 15% egg yolk as external cryoprotectant, and reaching post-
278 thaw motility values close to fresh sperm values (~90%).
279 Marine species have received much less attention than freshwater species for the
280 development of cryopreservation protocols, and many of these research efforts have been
281 made during the present century. Although DMSO has been the most used cryoprotectant
282 in marine fish, providing remarkable results in sparids and flatfish species (see Table 2),
283 other cryoprotectants such as methanol, glycerol or egg yolk have been successfully and
284 recently applied in other marine species in which DMSO was the common cryoprotectant
285 (Asturiano *et al.* 2017). In this context, new trials using methanol have provided better
286 results than DMSO-protocols in European eel (*A. anguilla*), and notably post-thaw
287 motility values close to 50% have been currently reported in this species (Herranz-
288 Jusdado, pers. comm., 2017).

Finally, successful results have been also published in fish species with internal fertilization. In green swordtail (*Xiphophorus helleri*), a simply and useful protocol based on glycerol (14%) as cryoprotectant provided post-thaw motilities as high as 77% at 10 min after thawing. Moreover, if sperm was immediately diluted after thawing, protocol was be able to retained motility values for as long as 8 days when stored at 4 °C (Huang *et al.* 2004). Lower post-thaw motilities have been reported in other close species such as *Xiphophorus couchianus* and *Xiphophorus variatus* (35 and 37%, respectively) (Yang *et al.* 2009, 2012).

To sum up, methods for sperm freezing have progressed during the last decades, and the assessment of fish sperm motility has been consolidated as a very useful tool for evaluating the validity of cryopreservation protocols. However, new techniques are emerging in order to provide in-depth information on the negative effects of freezing-thawing processes (DNA fragmentation, changes in protein profile, etc.), so fish sperm cryopreservation becomes an interesting research for studying the impact of cryopreservation process through new emerging tools of sperm quality analysis (Cabrita *et al.* 2014; Martínez-Páramo *et al.* 2017).

3.3 Broodstock management

Broodstock management involves a large number of topics that have the common goal of enabling a captive group of fish to undergo reproductive maturation and fertilization success. In this context, spermatozoa motion will have an essential role to achieve this goal, and the effect of different factors such as the environmental conditions, diet composition, type of gamete collection or the use of hormonal treatments, could be tested through sperm motility assessment.

3.3.1 Environmental conditions

In many cases, reproduction of fish in captivity can be controlled or modified exclusively by the use of environmental factors: *i)* water temperature, *ii)* photoperiod and/or *iii)* salinity. When these environmental factors are not optimal, reproductive dysfunctions compromise male gametogenesis and, therefore, sperm quality (Mylonas *et al.* 2010).

There are little reports on sperm quality changes in response to broodstock rearing temperature. In river lamprey (*Lamprea fluviatilis*), male reproductive performance was compared under three temperatures (7, 10 and 14 °C). Temperature had a significant effect on the quantity and quality of sperm produced: 70% of males held at 10 °C and

14 °C did not spermiate, while males held at 7 ºC produced samples with more than 80% of sperm motility (Cejko *et al.* 2016). In Siberian sturgeon (*A. baeri*), sperm production performance was tested at four temperatures (10, 12.5, 15 and 17.5 °C), and the significantly highest spermatozoa motilities (>65%) were also obtained with the lowest one (Williot *et al.* 2000). However, regarding European eel (*A. anguilla*), in which three temperatures were tested (10, 15 and 20 °C), the warmest thermal treatment (20 ºC) showed the best results in all the sperm production parameters (volume, density) as well as the maximum values total motility (>75%) (Gallego *et al.* 2012). All these data remark that temperature seems to be a species-specific factor: while cold-water species need low temperatures for showing the good quality sperm, warm-water species need high temperatures in order to achieve proper sperm motility values. In fact, temperatures above or below the optimum range can adversely reduce gamete quality, or even stop the onset and progression of spermiation (Migaud *et al.* 2013).

Photoperiod is involved on the regulation of annual reproductive rhythms in many teleost fish, and photothermal programs are commonly used in fish farms in order to advance or delay the gamete production (Bromage *et al.* 1993). In rainbow trout (*O. mykiss*), the combination of a long photoperiod (18L:6D, 4 months) followed by a short-one (18D:6L, 3 months) was able to generate a high percentage of spermiating males (~80%) during the out-of spawning season. However, the sperm motility in this experimental group was lower than in the control group maintained with natural photoperiod (70 and 83%, respectively) (Atasever & Bozkurt 2015). In turbot (*S. maximus*), sperm collected in males submitted to a contracted cycle (compressed to 6 months) presented a significantly higher sperm motion parameters at first stripping than that recorded in males submitted to natural photoperiod (Suquet *et al.* 1992). In shortfin silverside fish (*Chirostoma humboldtianum*), males were induced to reproduction through a 81-day artificial photothermal compressed cycle, showing similar sperm motility values (81±7%) than in natural conditions (Blancas-Arroyo *et al.* 2004). Therefore, artificial photoperiods become a useful tool in commercial hatcheries, advancing the sperm production and causing similar motility values than those reached using natural photo-cycles.

Water salinity is the less studied environmental factor, and several trials suggest that gamete maturation and final spawning can take place across a wide range of rearing salinities in several species (Lee *et al.* 1992; Bani *et al.* 2016). Although the effect of salinity has been mainly studied regarding sperm activation process, there are only a few reports about the effect or rearing salinity on breeders and the quality of gametes that they

produce. In this context, motility parameters of sperm activated with two activation media (seawater and a sucrose solution) were compared in blackchin tilapia (*Sarotherodon melanotheron heudelotii*) reared in freshwater (FW; 0‰), seawater (SW; 35‰), and hypersaline water (HW; 70‰). Results showed that for FW fish, sperm motility was high and varied little between individual fish for both activation media. In contrast, spermatozoa of SW and HW fish were significantly less active in sucrose solution than in synthetic SW, with substantial differences between individual fish (Legendre *et al.* 2016). In Mozambique tilapia (*Oreochromis mossambicus*), regulation of sperm motility seems to be modulated during acclimation of the fish from freshwater (FW) to seawater (SW), being independent of extracellular Ca^{2+} in FW and dependent in SW. In this sense, sperm of SW tilapia showed motility even in a hypertonic environment, whereas sperm of FW tilapia were not motile (Morita *et al.* 2004). In another euryhaline species, such as European eel (*A. anguilla*), spermatozoa produced from males induced both in freshwater and seawater could be activated (10–90% motility) in SW solution. Since there is no significant difference between motility of freshwater and seawater spermatozoa, authors assume that the freshwater rearing of males is no limiting factor in the artificial propagation of this endangered species (Müller *et al.* 2005). All these results show that in euryhaline species, spermatozoa can present a wide plasticity regarding activation media, reaching suitable motility values regardless of medium in which they are released.

3.3.2 Dietary requirements

Broodstock nutrition is a key factor controlling gonadal development and gamete quality in fish, and diet composition can affect reproduction success and offspring survival (Izquierdo *et al.* 2001). Although there are many publications linking up the dietary with reproduction success, scarce reports are able to link directly the broodstock diet with the kinetic characteristic of spermatozoa. In this sense, Table 3 summarizes studies on this topic over the last years.

Fatty acid composition of broodstock diet has been identified as major dietary factor that determine sperm quality, mainly due to carnivore fish are not able to synthesize certain fatty acids. In general, both freshwater and seawater species need PUFA (polyunsaturated fatty acids) or HUFA in the diet (Izquierdo *et al.* 2001), and the enrichment of dietary broodstock with these fatty acids can generate a substantial improvement in sperm motility parameters (see Table 3). Beirão et al. (2015) reported that sperm quality of Senegalese sole (*Solea senegalensis*) improved through the enriched docosahexaenoic

391 acid (DHA) diet, specifically the sperm velocity (VCL) and the percentage of progressive
392 sperm. In European eel (Butts *et al.* 2015; Baeza *et al.* 2015), diets with high levels of
393 arachidonic acid (ARA) induced medium milt volumes and high sperm motilities, while
394 diets with higher percentage of eicosapentaenoic acid (EPA) induce remarkable volumes
395 of milt and also high sperm motilities. In freshwater fish like rainbow trout (*O. mykiss*),
396 some breeders fed with a diet deficient in essential fatty acids (n-3) showed a lower sperm
397 motility than breeders fed with a control diet (Vassallo-Agius *et al.* 2001); while in other
398 trial carried out in rainbow trout, fish fed with a properly HUFA/PUFA ratio showed the
399 highest semen motility percentage and duration than other treatments (Hajiahmadian *et*
400 *al.* 2016). In aquarium species such as zebrafish (*Danio rerio*) and guppy (*Poecilia*
401 *reticulata*), fatty acid composition of broodstock diet also provided an improvement on
402 sperm quality parameters. In guppy, Rahman et al. (2015) reported significant main
403 effects of PUFAs on sperm viability and weak but significant interacting effects of both
404 nutrients on sperm motility time, evidencing PUFAs as critical determinants of sperm
405 quality. In zebrafish (*D. rerio*), the addition of phospholipids (fatty acids linked to
406 phosphate group) in the diet caused great results in sperm quality of breeders, which was
407 revealed by higher total and progressive motility and higher velocities than sperm from
408 males fed with control diet (Diogo *et al.* 2015).
409 Regarding vitamins, which are also components that the fish cannot synthesize, their
410 addition to the diet often means an improvement of gamete quality. Vitamin C (ascorbic
411 acid) and D have been the most used vitamins for improving broodstock diet, and positive
412 effect on sperm motility have been reported in several species. In Nile tilapia, animals fed
413 with vitamin C-diet showed higher motility values (54.9 ± 8.9%) than fish from control
414 group (22.3 ± 19.4%) (Sarmento *et al.* 2017). In rainbow trout (*O. mykiss*), the highest
415 motility rate was recorded in fish fed with a vitamin E enriched diet (94.5%), while the
416 lowest motility was detected in the control group (62.2%) (Ciereszko & Dabrowski 1995,
417 2000). The addition of vitamin C and /or E to the diet also enhanced sperm motility in
418 goldfish (Kashani *et al.* 2011; Kashani & Imanpoor 2012), African catfish (Dada 2012)
419 and Senegalese sole (Beirão *et al.* 2015).
420
421 *3.3.3 Hormonal induction*
422 Hormonal therapies for the enhancement of spermiation and sperm production have been
423 tried and employed in fish research and aquaculture. These hormonal protocols are often
424 used in two scenarios: *i*) in some species in which sperm production exist during rearing

conditions, but sperm performance (volume, motility density, etc.) is not good enough for hatchery operations; and *ii*) in some fish species in which it is impractical or even impossible to simulate the environmental factors of the breeding process (i.e., spawning migration, depth, etc.), so gonadal maturation does not occur in captivity (Mylonas *et al.* 2016). In both situations, hormonal stimulation could provide several advantages for the aquaculture industry, and breeding males can be able to produce more sperm of higher quality for a longer period, avoiding hatchery problems such as the gamete synchronization or limitation.

The injection of pituitary extracts (PE) from mature fish into breeders was the first method used to control reproductive function in aquaculture handling, and has been used widely in a variety of species, especially cyprinids (Mylonas *et al.* 2010). For example, in common bream (*Abramis brama*), males treated with bream PE (2.5 mg kg^{-1}) or carp PE (2 mg kg^{-1}) showed higher sperm volume and motility than control males (Kucharczyk *et al.* 1997). In common carp (*C. carpio*), CPE (2 mg kg^{-1}) treatment led to 100% spermiation males compared to only 25% in the control group, and sperm quality were also improved by the hormonal treatment (Vazirzadeh *et al.* 2016). In pikeperch (*Sander luciperca*), males treated with CPE produced sperm with higher motility (67.5–86.7%) than control group (Falahatkar & Poursaeid 2014). However, in other cyprinid species such as in dace (*Leuciscus leuciscus*), crucian carp (*Carassius carassius*), or even common carp (*C. carpio*), there were no statistical differences between control and hormone-treated groups (Cejko *et al.* 2012, 2013). In South American fishes, a single or multiple injection of CPE usually did not improve the spermatozoa motion performance, but CPE treatment was able to increase sperm volume and decrease sperm density, facilitating the sperm handling steps over cryopreservation protocols in these species (Viveiros & Godinho 2009). Similar results have been reported in Siluridae, and different catfish species such as European catfish (*Silurus glanis*), African catfish (*Clarias gariepinus*), or Amazon catfish (*Leiarius marmoratus*), that did not show statistical differences in sperm quality parameters between control and hormone-treated groups (Linhart *et al.* 2004; Araújo *et al.* 2014).

Gonadotropin (GTH) preparations of mammalian origin (ovine, mare or human) stage another technique for inducing or enhancing spermiation in some fish species belonging from different families. In cyprinids, notable results have been reported in several species using these hormonal therapies. In pikeperch (*S. luciperca*), males treated with hCG (human chorionic gonadotropin) produced sperm with higher motility (67.5–86.7%) than

control group (Falahatkar & Poursaeid 2014). In goldfish (*Carassius auratus*), hCG treatment was able for inducing 100% of spermiation males (n=10), which showed a motility about 80% (Targońska & Kucharczyk 2011). In common bream (*A. brama*), males treated with hCG showed higher motility (54%) than control males (22%) (Kucharczyk *et al.* 1997). However, if there is a genus in which this hormone has generated great results, this is the genus *Anguilla*. In European eel (*A. anguilla*), three different GTHs (hCG, hCG_{rec} and PMSG) were tested on the induction of maturation on eel males. Regarding motion performance, hCG_{rec} treatment generated the highest values throughout most weeks of treatment, reaching maximums of 70% of total motility, and keeping spermiating males until the 20^{th} week of the treatment (Gallego *et al.* 2012). In relation to Japanese eel (*Anguilla japonica*), repeated weekly injections of hCG provide the onset of spermiation at 5^{th} injection, and the percent motility of spermatozoa remained at approximately 70% from 9 to 14^{th} injection (Ohta *et al.* 1996). It is important to note that the results obtained by GTHs in these species are particularly relevant for the scientific community, mainly due to eels (*Anguilla* spp.) are not able to mature spontaneously in captivity.

Recently, studies have examined the production and use of recombinant (re) GtHs of piscine origin, which have been successfully produced for zebrafish (*D. rerio*), channel catfish (*Ictalurus punctatus*), goldfish (*C. auratus*), Japanese eel (*A. japonica*), European seabass (*D. labrax*), Senegalese sole (*S. senegalensis*), cinnamon clownfish (*Amphiprion melanopus*), and European eel (*A. anguilla*) (reviewed by Mylonas et al., 2017). However, although the *in vitro* effect of these hormones was relatively good in most of these species (stimulating both FSH and LH receptors, steroids production, etc.), specific reGtHs had little *in vivo* effect. In fact, first full spermatogenesis and spermiation has only been achieved in one species: the European eel (Peñaranda, pers. comm., 2015). Although the sperm quality was variable and not all the spermiating males produced samples with high sperm quality, some sperm samples reached motilities ≥50%, densities around 7×10^9 cells ml^{-1} and sperm volumes of approximately 0.4 ml (Peñaranda, pers. comm., 2015).

Finally, gonadotropin-releasing hormone agonists (GnRHa), administered by injections or controlled-release delivery systems, become the last technique for inducing or enhancing spermiation in some fish species. GnRHa treatments offers some important advantages in comparison to GTH treatments, such as *i)* GnRHa are less species-specific due to the high structural similarity of native GnRHs among fishes, and *ii)* this technique

493 decrease considerably the handling stress generated by the repetitive manipulations of
494 breeders. In cyprinids such as barbel (*Barbus barbus*), common carp (*C. carpio*), or
495 crucian carp (*C. carassius*), hormonal stimulation by GnRHa did not have a significant
496 influence on the CASA parameters (motility and velocity indicators), which were shown
497 to be similar in hormonally stimulated groups and control groups (Cejko *et al.* 2014, 2015;
498 Cejko & Kucharczyk 2015). However, in marine species GnRHa implants have provided
499 great results in some species such as Atlantic halibut (*Hippoglossus hippoglossus*), where
500 sperm motility was enhanced in males treated with a high dose of GnRHa (25 μg/kg)
501 compared to controls (Vermeirssen *et al.* 2004); in Senegalese sole (*S. senegalensis*),
502 where sperm motility produced by GnRHa-treated males was enhanced by 2-fold with
503 respect to controls; or in yellowtail flounder (*Pleuronectes ferrugineus*), where
504 percentage of motile sperm activated was higher in the high dose GnRHa treatment (90%)
505 than the control fish (20%). However, GnRHa implants did not have any effect on sperm
506 motility in other seawater species such as meagre (*Argyrosomus regius*), European
507 seabass (*D. labrax*) or bluefin tuna (*Thunnus thynnus*) (Rainis *et al.* 2003; Mylonas *et al.*
508 2016).
509 To sum up, even when hormonal treatments (CPEs, GTHs or GnRHs) can be useful to
510 enhance sperm production (overall in terms of volume) in aquaculture fish, hormonal
511 therapies usually do not affect sperm motion performance. In general, hormonal
512 treatments only provide successful results just in cases where fish species fail to spermiate
513 naturally or produce very small volumes of high-density sperm.
514
515 *3.3.4 Gamete collection*
516 Once the gametes have been produced naturally or thanks to the application of hormonal
517 or environmental treatments, it is time to gamete collection. Although at first glance it
518 may seem like a simple process, gamete collection often become a delicate task that can
519 affect negatively the gamete quality.
520 First step for recollecting gametes is usually the anesthetizing of breeders, which
521 obviously involves the use of different types of anaesthetics for minimizing fish stress.
522 Although most of aquaculture species does not present negative effect of anaesthetics at
523 gamete level, some exceptions can be found on the literature. First report in brook trout
524 (*Salvelinus fortinalis*) showed that tricaine (MS222) affected the motility duration of trout
525 sperm at concentrations as low as 19 mg/L (Allison 1961). In rainbow trout (*O. mykiss*),
526 despite the percentage of motile spermatozoa was also unaffected by the type of

anaesthetic or concentration used, the duration of motility decreased as anaesthetic concentration increased (Wagner *et al.* 2002; Dietrich *et al.* 2005). These results suggested anaesthesia have a moderate effect on total sperm motility values but, by contrast, can affect significantly the duration of sperm movement.

Moreover, the method of obtaining gametes is also of great importance in order to avoid urine contamination, which can negatively affect sperm characteristics and quality (Lavens *et al.* 1996). Collection of fish sperm can be carried out by different techniques such as *i)* the traditional procedure (manual stripping), *ii)* using a catheter, or *iii)* taking out the testes, which involves killing the animal. Although traditional stripping procedure has been the most widely technique for collecting sperm, it also presents a high risk of urine contamination. In this context, some recent studies carried out in salmonids and cyprinids report excellent sperm motility results collecting sperm samples with a catheter. For example, in Caspian brown trout (*S. trutta caspius*), sperm samples collected with a catheter were characterized by higher spermatozoa motility (~80%) than the sperm collected via stripping (~60%) (Aramli *et al.* 2016b). In pikeperch (*S. lucioperca*), the results were even more conclusive, and motility rate of sperm collected with a catheter was 73%, whereas the motility rate of sperm collected with a syringe (manual stripping) did not exceed 35% (Sarosiek *et al.* 2016). Therefore, in both species, catheter was proven to effectively reduce the contamination of sperm with urine and was the best technique to collect sperm samples.

Last method involves the collection of sperm samples directly from testes (post-mortem samples), and can be applied both in aquaculture, research or field topics (Rosenberg 1983; Aoki *et al.* 1997). In this context, Dietrich et al. (2005) reported that sperm collected from testes of rainbow trout (*O. mykiss*) at different post-mortem times did not show significant differences respect the control groups in sperm motility values (>90%) over the first hour. In dace (*L. leuciscus*), spermatozoa collected from testicles showed same motility values and lower initial velocities than sperm collected from the sperm duct (Kowalski *et al.* 2012). In Indian catfish (*Heteropneustes fossilis*), sperm samples collected from specimens stored during 240 days at –20 °C showed an incredible motility of 96.4% (Koteeswaran & Pandian 2002). However, even this post-mortem sperm reached fertilization rates about 93%, hatching rates were extremely low (~2%) in comparison to the control group (~98%).

In order to maximize the amount of available sperm produced by broodstock males, the possibility of repeat sperm collections (sequential stripping) in a short-time period has

been extensively studied is several species. In Persian sturgeon (*A. persicus*), Alavi et al. (2006) reported multiple collections (x3) at different times within the 48 h after hormone injection. Despite total volume collected over multiple stripping was remarkable, significant differences were found in the percentage of motile spermatozoa between the two first collections (80-90%) and the 3rd collection. In sterlet (*A. ruthenus*), multiple stripping method (every 3 h; from 12 to 66 h after hormone injection; 9 collections in total) yielded larger volumes (>80 mL) than a single collection did. In addition, except for the 1st and 7th stripping, sperm motility was extremely high during all the stripping process, with values closed to 95%.

3.3.5 Biotechnology and genetic engineering

In the last few years, biotechnology and genetic engineering have contributed greatly to fish culture through the application of novel techniques such as chromosome manipulation, transgenesis, etc. (Foresti 2000). Thanks to these methods, it has been possible to produce triploid, tetraploid, haploid, gynogenetic or androgenetic fish for improving the production on fish farms. However, these types of techniques involves from small to large changes in the genetic material of cells, which can produce a negative impact on gamete quality (Pandian & Koteeswaran 1998).

Gynogenesis consist in the production of offspring with the genes of the mother only, and has been successfully applied in a large number of fish species. Due to this technique requires the inactivation of the male genome by exposure the spermatozoa to either ultraviolet (UV) or gamma (Γ) rays, sperm motility assessment should be an essential step after irradiation process. Regarding sturgeons, UV exposure has significant impacts on the sperm motility. In Siberian sturgeon (*A. baerii*), spermatozoa revealed high sensitivity to UV irradiation, with complete loss of motility after homogeneous UV irradiation at doses above 200 mJ/cm^2 (Lebeda *et al.* 2014). Zhang et al. (2011) reported similar results in this species, with significant effects of UV exposure on sperm kinetic parameters such as total motility, velocity, and motility time. With regard to Salmonidae, irradiated sperm of rainbow trout (*O. mykiss*) showed approximately 60% of motility after a 20 minutes exposure to UV irradiation, became activated and maintained progressive movements for at least 15 seconds duration (Goryczko *et al.* 1991). Reduction in spermatozoon activation with UV exposure has also been described for other freshwater species such as silver barb (*P. gonionotus*), catfish (*I. punctatus)* or common tench (*Tinca tinca*) (Goudie *et al.* 1995; Pongthana *et al.* 1995; Nowosad *et al.* 2014).

In marine fish, gynogenesis is widely extended in several aquaculture species, in which female production become more profitable than males. In European seabass (*D. labrax*), exposure of sperm to UV light (≥15000 erg mm^{-2}) reduced the amount of motile spermatozoa, without affecting the duration of motility in the spermatozoa that remained motile (Felip *et al.* 1999). In turbot (*S. maximus*), a dose-dependent effect of UV light on sperm motility was found. The dose at which both the amount of motile sperm and the duration of sperm motility was reduced to 50% of the original value (ID-50) was 28000 erg/mm^2 (Piferrer *et al.* 2004). UV exposure also generated a decreased of sperm motility values in several finfish species such as Atlantic halibut (*H. hipoglossus*), Southern flounder (*P. lethostigma*), or Japanese halibut (*P. olivaceus*) (Luckenbach *et al.* 2004; You *et al.* 2008).

Polyploidy can be defined as the condition for having one or more additional chromosome sets with respect to the number most frequently found in nature for a given species (Piferrer *et al.* 2009). Although polyploidy can be easily induced in some relevant aquaculture species, polyploid organisms can also spontaneously appear in both wild and cultured populations. Some reports have shown the sperm performance of polyploid fish (mainly triploids), and Table 4 summarizes these results. Within triploidy, disparate results about sperm quality have been reported in several species. To begin with, no spermatozoa production has been reported in triploid males of European sea bass (*D. labrax*), turbot (*S. maximus*), gilthead sea bream (*Sparus aurata*), and Arctic charr (*S. alpinus*) (reviewed by Piferrer *et al.* 2009) . Sperm production, but with sperm motility values low or close to zero has been reported in Prussian carp (*C. gibello*), yellowtail flounder (*L. ferruginea*) and pond loach (*Misgurnus anguillicaudatus*) (Manning *et al.* 2004; Flajšhans *et al.* 2008; Fujimoto *et al.* 2008). However, triploid specimens of some fish species are able to produce spermatozoa with high motility and velocity rates, as occur in rosy bitterling (*R. ocellatus*), common tench (*T. tinca*) or cod (*G. morhua*) (Kawamura *et al.* 1999; Peruzzi *et al.* 2009; Pšenička *et al.* 2010). Although usually the triploidy confers genetic sterility, in some species spermatozoa from triploid males could carry out egg activation leading to non-viable aneuploid embryos, generating a genetic impact in fish population (Piferrer *et al.* 2009).

3.4 Breeding cycle

Sperm motility assessment has become a useful tool for studying several aspects of fish ecology. To date, more than 100 scientific publications reporting kinetic spermatozoa

parameters have contributed to explore numerous ecology issues of different fish species belonging to different taxa (Figure 2). During this section, a wide range of topics such as breeder ageing, seasonal changes, characterization of populations, etc. are going to be addressed through the sperm quality perspective.

In fish species with an annual reproductive cycle, the quality of sperm usually oscillates across the spawning season both in the wild and in captivity individuals. Most of these differences may be due to physiological changes and environmental cues related to fish reproduction, and sperm motility assessment will reveal the optimal period in which sperm should be collected in the hatchery (captive fish), or will simply supply information about the breeding cycle of the species (wild fish). Table 6 summarizes the seasonal changes in sperm motility on several fish, and over the data different sequential patterns (depending on the timing of sperm quality peak) can be found among species. Type I pattern includes species whose sperm production (quality and quantity) is higher at the beginning of the spawning season, to subsequently decrease. Species such as Russian sturgeon (*A. gueldenstaedtii*), halibut (*H. hippoglossus*), brook trout (*S. trutta*) or turbot (*S. maximus*) show this breeding pattern. Type II species show the highest sperm motility peak right in the middle of the breeding season, so sperm motion performance is maximum during the central months. Many marine species such as European sea bass (*D. labrax*), cod (*G. morhua*), red porgy (*P. pagrus*) or the ocean pout (*Macrozoarces americanus*) present this sperm quality pattern. Finally, type III pattern includes species whose sperm quality is higher at the end of the spawning season, achieving the peak values of sperm motility at the final months of the breeding season. Key species in freshwater aquaculture such as common carp (*C. carpio*) or the European perch (*P. fluviatilis*) show this motility pattern. Lastly, there are species such as Persian sturgeon (*A. persicus*), common barbel (*B. barbus*) or South American catfish (*Rhamdia quelen*) that not present significant differences in the percentage of motile spermatozoa throughout the breeding season, so sperm motility usually remains high and constant during this period.

Moreover, sperm motility can be also applied to inquire inter-populations differences or even in the kinetic characteristics between related species belonging to the same genus. Concerning inter-populations studies, some authors have reported notably differences studying the same species between different populations and environments. In lake minnow (*Eupallasella percnurus*), the two populations analyzed were markedly different in several sperm quality biomarkers such as milt volume, sperm concentration, and sperm

motility (Dietrich *et al.* 2014). As a result, authors estimated 134 million motile spermatozoa per ejaculate (volume × concentration × percentage of motility) in the "Siedliszcze" population compared to 480 million motile sperm in males from "Guzy" population.

In Atlantic salmon (*S. salar*), two wild populations (belonging from Wiezpra and Vistula river) were compared in the terms of sperm motility and motility time (Biernaczyk *et al.* 2012). Authors reported that both parameters were dependent on the origin of fish: salmons ascending the River Wieprza showed the highest sperm quality values (both in sperm motility and motility time), while animals caught in the Vistula river showed lowest values. In this case, sperm quality was largely dependent on environmental conditions, which were able to explain the 75% of sperm variability. Other authors have studied in depth the sperm trait differences between wild and farmed animals, pondering the possible impacts of escaped farmed fish on wild fish populations. In this sense, Lehnert *et al*. (2012) reported that Chinook salmon (*O. tshawytscha*) farmed males had significantly greater percentage of motile spermatozoa compared to wild males, suggesting that farming practices may lead to increased sperm performance from selective pressure on the aquaculture environment. Authors also reported that these results do highlight the potential for substantial introgression resulting from male-male competition between farmed and wild Chinook salmon in the wild. However, other studies focused on this topic showed different outcomes: Skjæraasen *et al*. (2010) and Butts *et al*. (2011) reported that wild male cod (*G. morhua*) had greater sperm performance compared to farmed cod; whereas Rideout et al., (2004) observed no difference in sperm traits between wild and farmed haddock (*H. hippoglossus*).

Concerning inter-species studies, interesting approaches can be done through sperm motility data. Gallego *et al*. (2014) analyzed the sperm motion parameters of swimmer (pufferfish and European eel) and sessile (sea urchin and ascidian) species, reporting sperm motion patterns totally opposite. In this context, sessile species displayed notably higher values than swimmer species in terms of motility time,, keeping high motility values during a longer time. Authors linked the sperm motion patterns to the species-specific lifestyles, postulating that sessile organisms (which show limited or no movement) need sperm with a capacity to swim for long distances to find the oocytes, while swimmer male organisms can move toward the female and release gametes near it, and therefore the spermatozoa does not need to swim for such a long time.

3.5 Ecotoxicology

Aquatic ecosystems are repositories of substantial quantities of natural and man-made environmental contaminants (EC), and fish sperm motility has become a valuable tool for assessing the EC toxicity (Hatef *et al.* 2013). At present, around 100 scientific publications reporting the impact of ECs on sperm motion performance have contributed to understand the toxicity mechanisms and action sites of ECs, and this knowledge can be nowadays applied for wider range of topics.

ECs are diverse along the natural environment and include a heterogeneous group formed by heavy metals, pesticides, biocides or pharmaceuticals, which can usually lead to diminished reproductive parameters, including sperm production and sperm motility parameters (Segner 2011). It is important to take into account that EC effects are extremely variable, and several factors such as the target species, the EC concentrations and the duration of exposure will be key elements on the impact on the sperm motion performance. In this context, Table 6 summarizes the main ECs affecting sperm motility of fish species, indicating the minimum EC dose at which sperm motion performance was significantly affected.

Within the main ECs, xenoestrogens are part of a group of synthetic and naturally endocrine disruptors that specifically have estrogen-like effects. Bisphenol-A (BPA), a synthetic chemical used in the production of epoxy resins and polycarbonate plastics, has estrogenic potency and several studies have evidenced its effect on fish sperm motility. For example, brown trout (*S. trutta*) males exposed to BPA concentrations of 1.75 and 2.40 µg/L showed low sperm quality (sperm density, motility rate, and swimming velocity) than control males at the beginning of the spawning season (Lahnsteiner *et al.* 2005). In fact, production of high quality sperm was restricted to the end of the spawning season, and delayed for approximately 4 weeks in comparison to the control. In goldfish males (*Carassius auratus*), sperm motility was significantly decreased in the BPA-treated groups after 20 or 30 days of exposure, and significant decrease in sperm velocity was observed at 30, 60 and 90 s post-activation in the BPA-treated groups at all exposure times (10, 20 and 30 days) (Hatef *et al.* 2012).

Estradiol (E2) or ethynylestradiol (EE2) are another xenoestrogens easy to find, which usually comes from oral contraceptives, and its occurrence in surface waters is the result of local sewage discharges (Arcand-Hoy & Benson 1998). Recently, both xenoestrogens have been implicated as the primary contaminants contributing to the estrogenic activity in surface waters, and negative effects about reproduction issues have been reported in

731 both freshwater and seawater species. In fighting fish (*Betta splendens*), an exposure for
732 4 weeks of 100 ng/L reduced significantly sperm swimming velocity and, in rainbow trout
733 (*O. mykiss*), exposures for 12 weeks of 10 ng/L also decreased sperm motility parameters.
734 In pejerrey fish (*Odontesthes bonariensis*), although no significant differences in motility
735 parameters were observed between the control group and E2 and EE2 acting separately,
736 a significant decrease in sperm motility was recorded for combined effect of estrogenic
737 agents (E2 + EE2) (Gárriz *et al.* 2015).
738 Furthermore, heavy metals are considered as the most dangerous pollutants around the
739 world, and the toxicity of accumulated metals is determined not only by the type of metal,
740 but also by the physical and chemical properties of water and the protective mechanisms
741 of fish species (Hatef *et al.* 2013). In this sense, and regarding species-specific effect,
742 Lahnsteiner et al. (2004) studied the impact of different heavy metals (zinc, mercury and
743 cadmium) on sperm motility parameters in four teleosts belonging to the most
744 representative freshwater families (Salmonidae, Cyprinidae, Gadidae and Clariidae), and
745 they concluded that toxic concentrations of all pollutants differed markedly for each
746 species. In this sense, African catfish (*C. gariepinus*) spermatozoa were the most resistant,
747 European chub (*L. cephalus*) and burbot (*Lota lota*) spermatozoa showed medium
748 resistance and brown trout (*S. trutta*) spermatozoa were the most sensitive to the heavy
749 metals used. The impact of heavy metals has been also reported in other freshwater
750 species such as common carp (*C. carpio*), in which zinc and cadmium affected
751 significantly sperm motility at 50 mg/L after 24 h of incubation (Chyb & Kime 2000;
752 Dietrich *et al.* 2011); or in rainbow trout (*O. mykiss*), where cadmium and mercury
753 decreased the percentage of motile spermatozoa after 4 h of incubation at 10 mg/L
754 (Dietrich *et al.* 2010). By contrast, scarce studies have been carried out in marine species.
755 In this context, some data in European sea bass indicate that cupper ($CuCl_2 \cdot 2H_2O$) and
756 lead ($PbCl_2$) did not affect sperm motility when the activating media contained up to 100
757 ppm of these metal salts. In contrast, mercury ($HgCl_2$) was able to completely arrest the
758 spermatozoa motion at concentrations as low as 0.1 mg/L (Abascal *et al.* 2007).
759 Finally, some pesticides and biocides can also interfere with normal biological processes
760 causing deleterious effects on the reproductive axes of species from aquatic ecosystems.
761 Within this class of chemicals, dichlorodiphenyltrichloroethane (DDT) and tributyltin
762 (TBT) has been the most studied biocides. Regarding DDT, although banned from use
763 many years ago, it is still widely used in some developing countries. Some studies have
764 reported that large exposures (~40 days) of DDT in stinging catfish (*H. fossilis*) can affect

765 negatively sperm motility (Singh *et al.* 2008). In this sense, non-exposed animals showed
766 80-100% of total motility, whereas DDT-exposed fish showed only 20-40% of motile
767 sperm. In African catfish (*C. gariepinus*) and Mozambique tilapia (*O. mossambicus*),
768 CASA results showed a decrease in motility parameters from the control fish values
769 (Marchand *et al.* 2008, 2010). Specifically, the decrease in motility for *O. mossambicus*
770 was statistically significant (80% against 54%, DDT-area against non-polluted area,
771 respectively). Regarding TBT, widely used as antifouling agent, it is considered another
772 common contaminant that has been shown to be capable of embryotoxicity, genotoxicity
773 and endocrine disruption. Studies in African catfish (*C. gariepinus*) showed that TBT had
774 a significant effect on motility at 0.27 μg/L after 24 h exposure in catfish (Rurangwa *et*
775 *al.* 2002). However, same trials carried out in common carp (*C. carpio*) showed that
776 sperm motility was only significantly affected by TBT exposure at only 2.7 μg/L
777 (Rurangwa *et al.* 2002).
778 To sum up, sperm motility assessment became a valuable tool to check and understand
779 toxicity mechanisms and sites of action of different ECs through *in vitro* and *in vivo*
780 techniques. Although EC effects are extremely variable depending on the target species
781 or on the concentration and the duration of exposure, changes in the sperm motion
782 performance can serve as a useful biomarker for biomonitoring these agents and their
783 potential effects on reproductive function.
784
785 ***3.6 Sperm competition***
786 Sperm competition, which occurs when the sperm from two or more males compete for
787 the fertilization of eggs, is a widespread process that occurs in a wide range of animal
788 taxa, including fish (Stockley *et al.* 1997; Stoltz & Neff 2006). This process usually lead
789 to behavioural, morphological and physiological adaptations both for males
790 characteristics (breeder size, body colour, etc.) and sperm traits (head or flagellum size,
791 total motility, swimming velocity, etc.), so sperm motion assessment become a useful tool
792 for studying this phenomenon (Reinhardt & Otti 2012). In this sense, and despite being a
793 topic of recent interest, about a hundred scientific reports have been published during the
794 last two decades (Figure 2).
795 Dominance hierarchies are common among males living in groups during the mating
796 season, and they have been one of the most studied aspects over sperm competition.
797 Within these hierarchies, males can adopt different reproductive strategies according to
798 their social position (dominant or subordinate), and sperm performance will be often

799 linked to the chosen strategy (Serrano *et al.* 2006). Theoretical models predict that
800 dominant males invest more in "attractiveness" (size, colour, behaviour, etc.) than in
801 gametes (spermatozoa), so sperm quality should be *a priori* lower in dominant than in
802 subordinate males (Ball & Parker 1996). On their hand, subordinate males are not going
803 to invest in "attractiveness", so they will have more resources for enhancing their
804 gametes. This trade-off between social investment and sperm performance has been
805 studied in several fish species, overall salmonids. For example, in Arctic char (*S. alpinus*),
806 sperm velocity was significantly higher in subordinate than in dominant males, suggesting
807 that males with social dominance are unable to maintain high sperm velocity (Serrano *et*
808 *al.* 2006). In Atlantic salmon (*S. salar*), in which there are two alternative reproductive
809 tactics (ARTs; small sneaking "parr" males *versus* large dominant "anadromous" males),
810 parr males compensate their behavioural subordinance by producing physiologically
811 superior spermatozoa. In this sense, the proportions of motile spermatozoa were notably
812 greater in the parr males (Vladić & Järvi 2001). In Chinook salmon, *O. tshawytscha*, parr
813 (jacks) males invested significantly more of their somatic tissue into gonads compared
814 with anadromous (hooknoses) males, and parr males showed higher motility and velocity
815 values (90% and 70 µm/s, respectively) than dominant males (85% and 55 µm/s,
816 respectively). In masu salmon (*Oncorhynchus masou*), sperm velocity and motility were
817 also significantly higher in parr males compared to anadromous males activated by river
818 water (Makiguchi *et al.* 2016). However, in that study, sperm velocity and motility did
819 not differ between the ARTs in the presence of ovarian fluid (OF, produced by females
820 and released with the eggs) on the activation media, so these results could suggest that
821 OF biases paternity in competitive fertilization contexts playing a role in cryptic female
822 choice. In this sense, it is important to note that OF usually plays an important role during
823 fertilization process, and numerous manuscripts have shown an increase of sperm motility
824 and velocity in species such as lake trout, *Salvelinus namaycush* (Butts *et al.* 2012), Arctic
825 char, *Salvelinus alpinus* (Turner & Montgomerie 2002; Urbach *et al.* 2005), Alpine
826 whitefish, *Coregonus sp*. (Urbach *et al.* 2007), three-spined stickleback, *Gasterosteus*
827 *aculeatus* (Elofsson *et al.* 2006), rainbow trout (Wojtczak *et al.* 2007) or Caspian brown
828 trout, *Salmo trutta caspius* (Hatef *et al.* 2009). Therefore, OF effects should be taken into
829 account during the sperm competition studies with the aim of not masking the "true"
830 sperm kinetic parameters obtained by the dominant or non-dominant males.
831 On the contrary, even when theory predicts that dominant males should have lower
832 quality sperm, some studies have shown no effects, or even the opposite situation. In

rainbow trout (*O. mykiss*), no significant differences in sperm quality (motility) or quantity (weight of expressible milt) were evident between fish with different social status (Cardwell *et al.* 1996). In bluegill (*Lepomis macrochirus*), in which males can use three mating tactics ("sneakers", which streak spawn; "satellites", which mimic females; and "parentals", which are territorial), there was no difference in sperm flagellum length, curvilinear swim speed or path linearity among the different mating types (Stoltz & Neff 2006). Regarding the opposite effect, male breeding coloration was positively correlated with sperm velocity in three-spined sticklebacks (*G. aculeatus*), and "attractive" (colourful) males showed the fast spermatozoa (Mehlis *et al.* 2013).

Cichlids represent another interesting group to study adaptations resulting from sperm competition, because there is a tremendous diversity in their mating behaviours (Morita *et al.* 2014). Fitzpatrick et al. (2007) tested in *Telmatochromis vittatus*, a small shell-brooding cichlid, the evolution of sperm parameters across four different reproductive tactics present in this species (pirate, territorial, satellite, and sneaker). Because sneakers usually spawn in the presence of another male, sneakers face the highest levels of sperm competition and pirates the lowest, whereas satellites and territorials experience intermediate levels. In accordance with sperm competition theory, sneakers' spermatozoa swam faster (>40 μm/s) than sperm from males adopting the other reproductive tactics (territorial and satellite), whereas sperm from pirates was slowest (<30 μm/s) (Fitzpatrick *et al.* 2007). Fitzpatrick el al. (2009) also provided, after examine sperm characteristics in 29 cichlid species, an evidence that species experiencing greater levels of sperm competition have faster-swimming sperm. In this sense, authors reported that species subject to a high level of competition (polygynous) had relatively larger and longer gonads able to provide faster-swimming and longer-lived spermatozoa compared with species experiencing lower sperm competition (monogamous). However, other study carried out by Morita et al. (2014) among 28 cichlid species showed that sperm velocity was not correlated with sperm competition rank, whereas motility time was considerably longer in bower-building species (high competition rank) compared with species that do not build bowers (low competition rank).

In this context, spermatozoa motion assessment can serve as a useful tool for studying the evolution of alternative reproductive strategies and mating systems in different fish taxa, and several kinetic parameters such as total motility, swimming velocity and/or motility time will provide data to carry out sperm competition studies.

4. Standardization of the procedures for assessing sperm motility

The evaluation of sperm motility and other kinetic parameters have become an essential tool for investigating a wide range of topics in numerous fish species (see section 3), and more than 1500 manuscripts have been already published applying fish sperm motility as a tool research. However, an evident lack of standardization assessing the sperm motion has often provided a low reproducibility between trials, making difficult to interpret and compare the results intra- and inter-labs (Boryshpolets *et al.* 2013; Gallego *et al.* 2013c). Therefore, a serial of biological and technical settings both for subjective (made by a technician) and objective (made by CASA system) assessments should be taken into account before sperm kinetic evaluation.

Biological settings such as sperm collection (see section 3.3.4), the initial dilution in a species-specific extender, the storage temperature before analysis (see section 3.2.1), the sperm:activation medium ratio, or the timing after sperm motility triggering can notably influence the sperm kinetic evaluation (Fauvel *et al.* 2010). In this sense, Billard and Cosson (1992) reported that a relatively high dilution ratio (close to 1/1000) is necessary to initiate simultaneous motility of spermatozoa in cyprinids. At low dilutions, only some of the spermatozoa were activated after mixing with diluent, whereas others became progressively activated afterwards, so sperm dilution became a key issue for assessing sperm motility. On their hand, kinetic characteristics of fish sperm are often species-specific, and the timing in which the technician (both during subjective or objective) assess sperm motility plays a crucial role regarding the species evaluated. For example, spermatozoa from freshwater species usually present a shorter longevity (1-2 min) than marine species, thus freshwater sperm must be evaluated during the first seconds after activation (Billard & Cosson 1992). However, because there is a lot of variability on sperm longevity among marine species, early sperm evaluations (first 15-30 seconds) are often recommended for all teleost fish (Gallego *et al.* 2014).

Technical settings for assessing sperm motility can involve a wide range of factors such as optical contrast, lens magnification, type and depth of the chamber used, etc. (Amann & Waberski 2014). For example, studies carried out in European eel (*A. anguilla*) showed that different magnification lens (10x vs 20x) affected significantly the measurement of sperm kinetic parameters (Gallego *et al.* 2013c). In this case, the number of spermatozoa captured by the 20x magnification lens was much less than those assessed with the 10x lens, therefore the variability coefficients obtained by the wider lens (20x) were much

higher than those obtained with the 10x lens. Thus, the results obtained using the 10x lens should be, *a priori*, more accurate and precise than the results obtained using the 20x lens. CASA settings also play a key role for estimating sperm kinetic parameters, and several factors such as the frame rate of recording (or frames per second; fps), the track sampling time, the focal position of swimming sperm cells inside the open drop, the field of observation location, or even the type of CASA used can notably affect sperm kinetic results (Amann & Waberski 2014; Lu *et al.* 2014). In this sense, Gallego *et al.* (2013) reported for a teleost fish that the number of fps influenced the sperm quality parameters provided by a CASA system. Even the frame rate setting had no effect either on motility or on progressive motility, parameters such as curvilinear velocity, straightness, and beat cross frequency were significantly affected. Authors then demonstrated that low frame rates underestimated the real value of kinetic traits, while a higher fps setting provided a more accurate reconstruction of the sperm trajectories, closely resembling the real trajectory.

In other study, Boryshpolets et al., 2013 examined different CASA systems (CRISMAS; Hobson Sperm Tracker; and Image J) on the same video recordings using three taxonomically different fish species (sterlet: A. *ruthenus*; common carp, *C. carpio*; and rainbow trout, *O. mykiss*). Authors reported that motility parameters were highly affected by the species and the CASA used for analyses, so special care should be taken with regard to CASA settings, recording conditions, and quality of video recordings (Boryshpolets *et al.* 2013).

To sum up, to make it possible to compare the results obtained by different laboratories, all studies using sperm motility assessment must describe its methodology in detail, particularly concerning biological and technical settings. Unfortunately, in most publications, details of these parameters are not provided, thus reducing the possibility of comparing the results intra- and inter-labs. Therefore, it becomes imperative to harmonize common procedures and established protocols to be used in many research groups assessing fish sperm motility for enhancing the reliability, comparability, and applicability of data produced by different laboratories (Rosenthal *et al.* 2010).

5. New emerging tools for sperm quality analysis

Although a relatively high number of sperm quality biomarkers have been successfully applied in several fish species, the new demands on basic research imply the arrival of new techniques for sperm analysis, which in a not so far future will be used by fish

farming companies (Cabrita *et al.* 2014). This new generation of tools, reviewed by Cabrita *et al*. (2014), will improve the knowledge on sperm quality assessment, complementing the information provided by sperm motion assessment.

Genome analysis represent the first emerging tool being a candidate to become a great sperm quality biomarker. In this context, although the evaluation of chromatin damage has been poorly considered in the assessment of sperm quality, some studies have recently related with the fertilization success in fish (Pérez-Cerezales *et al.* 2010a). In addition, it is important to take into account that chromatin modifications could happen even in the absence of measurable effects on other sperm characteristics (like sperm motility), so damage spermatozoa would be able to reach and fertilize the ova, being able to cause harmful effects on the offspring (Pérez-Cerezales *et al.* 2010b). Different methods can be applied to the evaluation of chromatin damage, and most of them related to the detection of fragments or packaging failures. Comet assay or SCGE (single cell gel electrophoresis) is the technique most commonly used and has been validated in numerous fish species (Cabrita *et al.* 2005a; Beirão *et al.* 2008; Pérez-Cerezales *et al.* 2009; Nathanailides *et al.* 2011). This method is based on the different electrophoretic migration patterns of DNA fragments, where cells (spermatozoa) with damaged DNA usually present a comet-like tail structure, being longer is the DNA structure damage is bigger.

Another techniques based on the differential migration of chromatin fragments, such as the SCD (sperm chromatin dispersion test), are nowadays emerging in fish and they need to be set up for different fish species. In fact, this method have been only used in tench (*T. tinca*) sperm, reporting a good correlation with the results obtained simultaneously using the comet assay (López-Fernández *et al.* 2009). Finally, DNA fragmentation can also be assessed more specifically using the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) assay method, based on the addition of a fluorescent nucleotide to the 3′OH end of the strand. So, as more fragmentised is the spermatozoa DNA, higher the fluorescence emit by the nucleus. This technique has been successfully applied in European sea bass (*D. labrax*) and gilthead seabream (*S. aurata*) (Cabrita *et al.* 2011).

Moreover, transcriptomic analysis can represent another tool for predicting gamete quality. Although traditionally the use of microarray had not focused on the evaluation of the RNA profile in breeding males, studies in mammals reporting key roles of residual mRNAs from spermatogenesis (Lalancette *et al.* 2008) have allowed the beginning of the research in this topic in fish species. Guerra *et al*. (2013), for example, reported a different

approach to investigate on the role of mRNAs as quality markers in fish spermatozoa. In this sense, authors were able to define a set of transcripts that had a different profile in testicular cells from good and bad zebrafish breeders, reporting then a correlation between specific transcripts and sperm quality. Although these results were provided using model species such as zebrafish (*D. rerio*), it opened up the possibility of exploring these findings to key species of aquaculture sector (Guerra *et al.* 2013).

To sum up, the great potential of emerging technologies such as genomic, transcriptomic and/or proteomic could establish the first step towards the possibility of selecting fish breeder performance from a molecular point of view (Cabrita *et al.* 2014; Labbé *et al.* 2017; Robles *et al.* 2017). The identification of predictive estimators or markers of sperm quality would have major applications in research, fish farms and biotechnological industries.

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1921 quality. *Biology of Reproduction* **70**: 1679–1684.
1922

Figure legends

Figure 1. Evolution of number of manuscripts published from 1975 to 2016 in SCI journals using fish sperm motility as a research tool (assessed both by subjective or objective method).

Figure 2. Number of manuscripts published by research area (sperm storage, sperm physiology, broodstock management, breeding cycle, ecotoxicology, and sperm competition) in SCI journals using fish sperm motility as a research tool (assessed both by subjective or objective method).

Table legends

Table 1. Studies on short- and medium-term sperm storage protocols applied to several fish species indicating the temperature (ºC) and ratio (sperm:extender) used. Table gives the best results reached for each species in terms on sperm motility (percentage of motile cells). Numbers in brackets indicate the storage days and the absence of brackets means 1-week storage.

Table 2. Studies on sperm cryopreservation of fish species belonging from different groups. Table gives the best results reached for each species in terms on post-thaw motility (percentage of motile cells), indicating the cryo-medium used for each specie.

Table 3. Effect on sperm motility (percentage of motile cells) of different dietary components (carotenoids, lipids, proteins, vitamins, and others) applied on different fish species. Positive effect on sperm motility respect to the control group are represented by "+"; negative effect are represented by "-"; and non-effect are represented by "=".

Table 4. Sperm motility (percentage of motile cells) and velocity (μm/s) of different polyploid fish (2n, 3n, 4n, and 6n) on several teleost species.

Table 5. Seasonal changes in sperm motility (percentage of motile cells) throughout the breeding season on several fish species. Data are structured regarding sequential patterns

of motility: Type I, species whose sperm motility is higher at the beginning of the spawning season; Type II, species whose sperm motility is higher at the middle of the spawning season; Type III, species whose sperm motility is higher at the end of the spawning season. Table also shows if fish belong from fish farms (captive) or in the wild. **Solea senegalensis* spawn naturally in two periods (late spring to the beginning of summer and early autumn, when temperatures are similar)

Table 6. Main environmental contaminants (ECs) affecting sperm motility (percentage of motile cells) on different fish species, indicating the minimum EC dose at which sperm motion performance was significantly affected. Values in brackets indicate the time exposure, *in vivo* or *in vitro*.

Figure 1

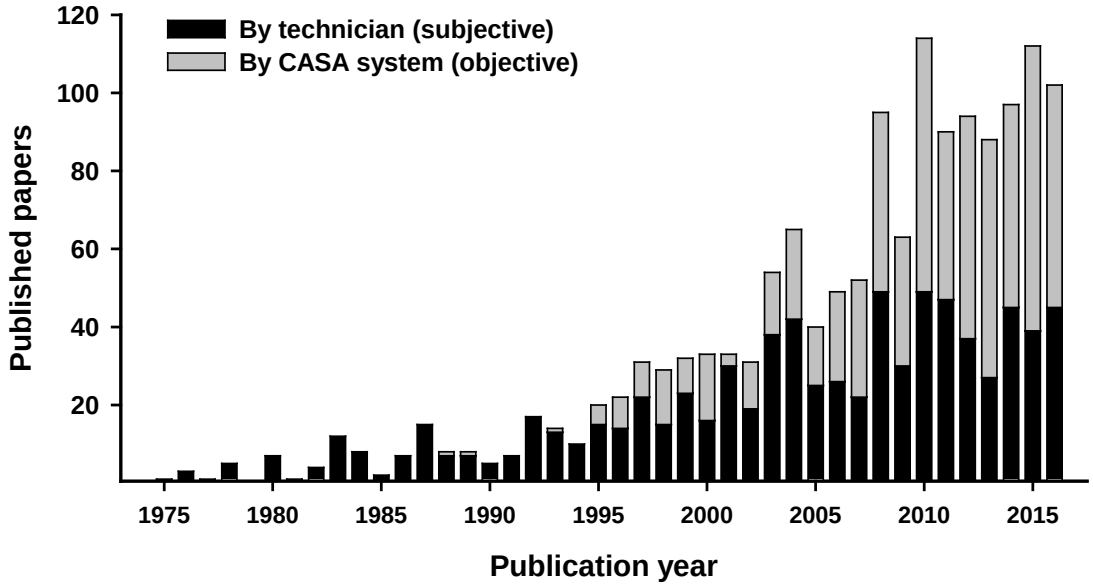
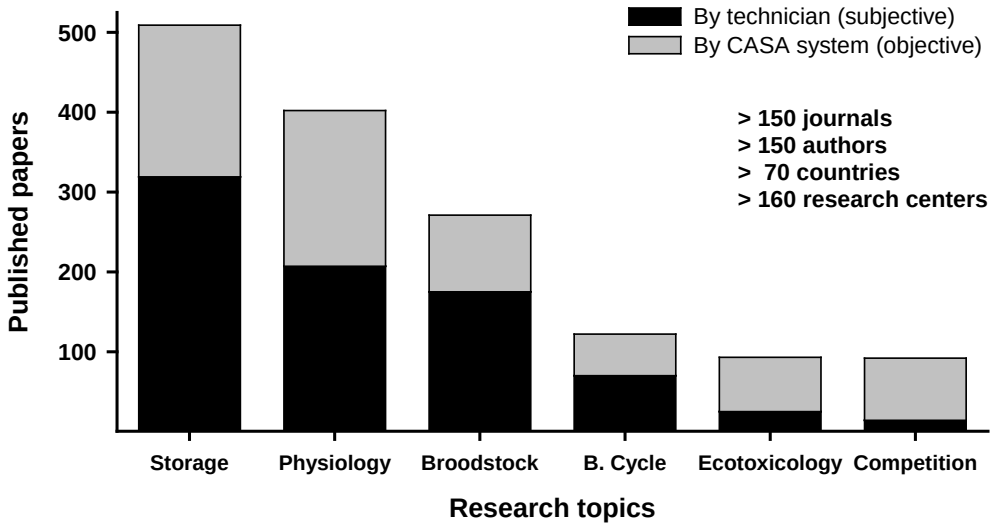

By technician (subjective)
By CASA system (objective)
Published papers
120
100
80
60
40
20
1975
1980
1985
1990
1995
2000
2005
2010
2015
Publication year

Figure 2


By technician (subjective)
By CASA system (objective)
> 150 journals
> 150 authors
> 70 countries
> 160 research centers
Published papers
500
400
300
200
100
Storage
Physiology
Broodstock
B. Cycle
Ecotoxicology
Competition
Research topics

1974 **Table 1**

Species	Motility (%)		Tº	Ratio	Reference
	Short-term	Medium-term			
Acipenser baerii	50-55 (6)	-	4	1:100	Shaliutina *et al.* (2013)
Acipenser gueldenstaedtii	50-55 (6)	-	4	1:100	Shaliutina *et al.* (2013)
Acipenser oxyrinchus	70-75	40-65 (28)	4	1:3	Park and Chapman (2005)
Anguilla anguilla	25-30	-	4	1:50	Peñaranda *et al.* (2010)
Anguilla japonica	80-85	55-60 (21)	3	1:50	Ohta and Izawa (1996)
Brycon orbignyanus	55-60	-	4	1:3	Murgas *et al.* (2004)
Clarias macrocephalus	30-35	15-20 (10)	4	1:4	Vuthiphandchai *et al.* (2009)
Colossoma macropomum	20-25 (5)	-	6	1:4	Garcia *et al.* (2016)
Cyprinus carpio	90-95	25-30 (17)	4	1:100	Saad *et al.* (1988)
Gadus morhua	50-55	15-20 (30)	3	1:3	DeGraaf and Berlinsky (2004)
Hippoglossus hippoglossus	85-90	50-55 (50)	4	1:5	Babiak *et al.* (2006a)
Ictalurus punctatus	25-30	-	4	-	Jenkins and Tiersch (1997)
Melanogrammus aeglefinus	60-65	20-25 (30)	3	1:3	DeGraaf and Berlinsky (2004)
Morone saxatillis	5-10	-	3	1:3	Jenkins-Keeran and Woods (2002)
Nibea albiflora	20-25 (1)	-	4	1:2	He *et al.* (2012)
Oncorhynchus mykiss	70-75	50-55 (14)	4	1:2	Ubilla *et al.* (2015)
Oncorhynchus tshawytscha	40-45	40-45 (14)	4	-	Bencic *et al.* (2001)
Perca fluviatilis	70-75	55-60 (17)	4	1:9	Sarosiek *et al.* (2014)
Poecilia reticulata	20-25	-	4	1:50	Sun *et al.* (2010)
Polyodon spatula	75-100	5-25 (56)	2	1:1	Taylor *et al.* (1995)
Prochilodus lineatus	15-20	-	6-8	1:9	Viveiros *et al.* (2014)
Takifugu niphobles	75-80	15-20 (15)	4	1:50	Gallego *et al.* (2013b)
Rhamdia quelen	45-50	25-30 (12)	6	-	Carneiro *et al.* (2006)
Salmo salar	45-50	35-40	4	1:2	Parodi *et al.* (2017)
Salvelinus alpinus	60-65	10-15 (15)	4	1:10	Sarosiek *et al.* (2013)
Sciaenops ocellatus	35-40	10-15 (10)	4	1:10	Wayman *et al.* (1998)

1975

1976 **Table 2**

Group	Species	Motility (%)		Medium	Reference
		Fresh	Post-thaw		
Salmonids	*Coregonus clupeaformis*	87	52	9% methanol + 0.18 M	Nynca *et al.* (2016)
	Coregonus lavaretus	80	50	7.5% methanol + 0.15 m glucose	Dietrich *et al.* (2016)
	Oncorhynchus mykiss	≥90	≥60	10% DSMO + 7,5% egg yolk	Kutluyer *et al.* (2014)
	Salmo salar	93	62	9% methanol + 0.18 M glucose	Nynca *et al.* (2016)
	Salmo trutta	90	74	7.5% methanol + 0.15 m glucose	Nynca *et al.* (2014)
	Salvelinus alpinus	>70	28	15% methanol	Richardson *et al.* (2011)
	Salvelinus fortinalis	83	57	9% methanol + 0.18 M sucrose	Nynca *et al.* (2016)
Cyprinids	*Barbodes gonionoutus*	91	83	10% DSMO	Vuthiphandchai *et al.* (2015)
	Ctenopharyngodon idella	91	83	5% glycerol + 0.35 M glucose	Yavas and Bozkurt (2011)
	Cyprinus carpio	95	93	15% egg yolk	Yavas *et al.* (2014)
	Hypophthalmichthys molitrix	91	81	10% DSMO	Hossain & Sarder (2013)
	Perca fluviatilis	82	54	10% methanol	Bernáth *et al.* (2015)
	Tinca tinca	>80	45	5% DSMO	Lujić *et al.* (2017)
Sturgeons	*Acipenser baerii*	80	50	10% methanol + glucose	Judycka *et al.* (2015)
	Acipenser persicus	95	80	10% methanol + 10 mM glutamine	Aramli *et al.* (2016a)
	Acipenser ruthenus	92	57	10% methanol	Dzyuba *et al.* (2014)
	Huso huso	82	50	10% methanol + 0.2 M glucose	Aramli *et al.* (2015)
	Polyodon spathula	90	85	10% methanol	Horváth *et al.* (2006)
Characiformes	*Brycon insignis*	98	82	5% BTS + 5% glucose	Viveiros *et al.* (2011)
	Colossoma macropomum	>90	>70	10% methyl-glycol + 5% Egg yolk	Gallego *et al.* (2017)
	Esox lucius	80	60	7.5% methanol + 0.15 M glucose	Dietrich *et al.* (2016)
	Oreochromis niloticus	70	65	10% methanol	Navarro *et al.* (2014)
	Prochilodus lineatus	100	88	10% methyl-glycol + 5% glucose	Viveiros *et al.* (2009)
Model species	*Danio rerio*	84	46	8% DMSO or 4% methanol	Bai *et al.* (2013)
	Oryzias latipes	85	52	10% methanol	Yang *et al.* (2010)

	Poeciliia latipinna	80	50	20% glycerol	Huang *et al.* (2009)
	Poeciliia reticulata	75	60	20% glycerol	Huang *et al.* (2009)
Catfishes	*Clarias gariepinus*	95	71	8% DMSO + 10% egg yolk	Rurangwa *et al.* (2001)
	Ictalurus punctatus	87	48	5% methanol	Christensen and Tiersch (2005)
Marine fish	*Anguilla anguilla*	75	47	10% methanol + 5% egg yolk	Herranz-Jusdado, pers. comm., 2017)
	Anguilla japonica	60	46	10% DMSO	Tanaka (2002)
	Dicentrarchus labrax	>90	>50	10% egg yolk + Na-pyruvate	Sansone *et al.* (2002)
	Gadus morhua	85	70	10% glycerol + 10% egg yolk	Babiak *et al.* (2012)
	Hippoglossus hippoglossus	80	75	10% DMSO	Ding *et al.* (2011)
	Morone saxatillis	88	53	5% DMSO+50 mM glycine	He and Woods (2004)
	Pagrus major	88	78	15% methanol + 100mM threalose	Liu *et al.* (2015)
	Scophthalmus maximus	>80	77	10% DMSO	Chen *et al.* (2004)
	Sparus aurata	95	70	5% DMSO	Cabrita *et al.* (2005b)
Int. fertilization	*Xiphophorus helleri*	95	77	14% glycerol	Huang *et al.* (2004)
	Xiphophorus couchianus	95-90	35-40	14% glycerol	Yang *et al.* (2009)
	Xiphophorus variatus	57	37	10% glycerol	Yang *et al.* (2012)

1977

1978 **Table 3**

Diet component	Type	Species	Effect on motility	Reference
Carotenoids	Astaxanthin β-carotene	*Amatitlania nigrofasciata*	=	Sullivan *et al.* (2014)
	Astaxanthin β-carotene	*Carassius auratus*	+	Tizkar *et al.* (2015)
	Zeaxanthin, Astaxanthin, β-carotene	*Poecilia reticulata*	=	Rahman *et al.* (2015)
Lipids	Fats	*Rhamdia quelen*	+	Tessaro *et al.* (2012)
	DHA	*Solea senegalensis*	+	Beirão *et al.* (2015)
	EFA	*Oncorhynchus mykiss*	+	Vassallo-Agius *et al.* (2001)
	EPA, DHA, ARA	*Anguilla anguilla*	+	Butts *et al.* (2015)
	HUFAs	*Carassius auratus*	=	Kashani and Imanpoor (2012)
	PUFAs	*Catla catla*	=	Nandi *et al.* (2007)
	PUFAs	*Poecilia reticulata*	+	Rahman *et al.* (2015)
	Phospholipids	*Danio rerio*	+	Diogo *et al.* (2015)
	PUFAs	*Dicentrarchus labrax*	=	Asturiano *et al.* (2001)
	Fish oil	*Fundulus grandis*	=	Patterson and Green (2015)
	HUFAs:SFA	*Oncorhynchus mykiss*	+	Hajiahmadian *et al.* (2016)
	HUFAs ratio	*Perca fluviatilis*	=	Kestemont and Henrotte (2015)
	n-3/n-6 ratio	*Perca fluviatilis*	=	Henrotte *et al.* (2010)
Proteins	Soybean meal (replacement)	*Carassius auratus*	-	Bagheri *et al.* (2013)
	Vegetable meal (replacement)	*Clarias gariepinus*	=	Ajala and Owoyemi (2016)
	Agricultural meal (replacement)	*Clarias gariepinus*	+	Nyina-Wamwiza *et al.* (2012)
	Cottonseed meal (replacement)	*Ictalurus punctatus*	=	Robinson and Tiersch (1995)
	Cottonseed meal (replacement)	*Oncorhynchus mykiss*	=	Rinchard *et al.* (2003)
	Cottonseed meal (replacement)	*Oncorhynchus mykiss*	=	Dabrowski *et al.* (2000)
Vitamins	Biotoin	*Danio rerio*	+	Yossa *et al.* (2015)
	Vitamin C	*Oreochromis niloticus*	+	Sarmento *et al.* (2017)
	Vitamin C	*Clarias gariepinus*	+	Dada (2012)
	Vitamin C	*Oncorhynchus mykiss*	+	Ciereszko and Dabrowski (1995, 2000)

	Vitamin C and E	*Carassius auratus*	+	Kashani *et al.* (2011)
	Vitamin E	*Scophthalmus maximus*	+	Xu *et al.* (2015)
	Vitamin E	*Solea senegalensis*	+	Beirão *et al.* (2015)
	Vitamin E	*Oncorhynchus mykiss*	+	Canyurt and Akhan (2008)
Others	Zinc	*Megalobrama amblycephala*	+	Jiang *et al.* (2016)
	Cadmium	*Oreochromis mossambicus*	-	El-Ebiary *et al.* (2013)
	Genistein	*Oncorhynchus mykiss*	-	Bennetau-Pelissero *et al.* (2002)
	Glycerin	*Oreochromis niloticus*	-	Mewes *et al.* (2016)
	Reservatrol	*Oryzias latipes*	+	Kowalska *et al.* (2017)
	Lactobacillus rhamnosus	*Anguilla anguilla*	+	Vílchez *et al.* (2015)
	Ergosan	*Oncorhynchus mykiss*	+	Sheikhzadeh *et al.* (2010)

1981 **Table 4**

Species	Type	Motility (%)	Velocity (μm/s)	Reference
Acipenser baerii	4n	95	155	Pšenička *et al.* (2011)
	6n	100	181	
Acipenser baerii cierto	2n	95	170	Havelka *et al.* (2014)
	6n	100	152	
Carassius gibelio	2n	69	-	Flajšhans *et al.* (2008)
	3n	23	-	
	4n	45	-	
Gadus morhua	2n	90	12	Peruzzi *et al.* (2009)
	3n	84	11	
	2n	-	55	Feindel *et al.* (2010)
	3n	-	56	
Limanda ferruginea	2n	90-100	-	Manning *et al.* (2004)
	3n	>10	-	
Misgurnus anguillicaudatus	2n	91	-	Zhao *et al.* (2012)
	4n	90	-	
	2n	>80	-	Yoshikawa *et al.* (2007)
	3n	10	-	
	2n	90	-	Fujimoto *et al.* (2008)
	3n	1.5	-	
	2n	91.7	-	Zhao *et al.* (2016)
Rhodeus ocellatus	2n	98	-	Kawamura *et al.* (1999)
	3n	87	-	
Tinca tinca	2n	92-100	90-100	Hulak *et al.* (2010)
	3n	87-96	90-100	

	2n	98	99	Pšenička *et al.* (2010)
	3n	94	91	
	2n	93-100	82-110	Linhart *et al.* (2006)
	3n	37-77	~90	

1982

1983

1984 **Table 5**

	Species	Condition	Motility			Reference
			Early	Middle	Last	
Type I	*Acipenser queldenstaedtii*	Captive	65-70	65-70	40-45	Halimi *et al.* (2015)
	Hippoglossus hippoglossus	Captive	80-85	80-85	20-30	Babiak *et al.* (2006b)
	Salmo trutta	Captive	65-70	55-60	50-55	Hajirezaee *et al.* (2010)
	Scophthalmus maximus	Captive	80-100		60-80	Suquet *et al.* (1998)
	*Solea senegalensis**	Captive	55-60		35-40	Beirão *et al.* (2011)
Type II	*Centropristis striata*	Captive		80-85	40-45	DeGraaf *et al.* 2004)
	Dicentrarchus labrax	Captive		90-100	35-50	Rainis *et al.* (2003)
	Gadus morhua	Captive	40-45	50-55	35-40	Rouxel *et al.* (2008)
	Macrozoarces americanus	Captive	<25	>75	40-50	Wang and Crim (1997)
	Mastacembelus mastacembelus	Wild	45-50	80-85	65-70	Sahinöz E *et al.* (2007)
	Oncorhynchus mykiss	Captive	75-80	80-85	55-60	Munkittrick and Moccia (1987)
	Pagrus pagrus	Captive	45-50	90-95	85-90	Mylonas *et al.* (2003)
	Rutilus rutilus	Wild	60-65	80-85	70-75	Golpour *et al.* (2013)
Type III	*Cyprinus carpio*	Captive	60-60	80-90	80-90	Christ *et al.* (1996)
	Mastacembelus armatus	Captive	0-10	10-20	30-40	Uthayakumar *et al.* (2013)
	Odontesthes bonariensis	Captive	40-45	20-25	60-65	Chalde *et al.* (2016)
	Perca fluviatillis	Captive	80-85	90-95	90-95	Alavi *et al.* (2010)
	Scophthalmus rhombus	Captive	20-40	40-60	60-80	Hachero-Cruzado *et al.* (2013)
	*Solea senegalensis**	Captive	55-60		60-65	Beirão *et al.* (2011)
No diff.	*Acipenser persicus*	Captive	90	80-85	80-85	Aramli *et al.* (2014)
	Barbus barbus	Captive	60-65	60-65	60-65	Hadi-Alavi *et al.* (2008)
	Brycon amazonicus	Wild	100	95		Cruz-Casallas *et al.* (2007)
	Paralichthys orbignyanus	Wild	50-75	50-75	50-75	Lanes *et al.* (2010)
	Pseudoplatystoma metaense	Captive	>90	>95	>90	Ramirez-Merlano *et al.* (2011)
	Rhamdia quelen	Captive	>90		>90	Borges *et al.* (2005)

1985 **Table 6**

ECs	Species	Mode	Dosis (Time exposure)	Reference
Bisphenol A	*Danio rerio*	*In vivo*	0.1 µM (2 months)	Chen *et al.* (2017)
	Perca fluviatilis	*In vitro*	1.5 mM	Hatef *et al.* (2010)
	Carassius auratus	*In vivo*	4.5 µg/L (20 days)	Hatef *et al.* (2012)
	Salmo trutta	*In vivo*	1.75 µg/L (spawning period)	Lahnsteiner *et al.* (2005)
EE2	*Tinca tinca*	*In vivo*	50 µg/kg (30 days, injected)	Oropesa *et al.* (2015)
	Oncorhynchus mykiss	*In vivo*	10 ng/L (12 weeks)	Schultz *et al.* (2000)
	Odontesthes bonariensis	*In vitro*	45 ng/L + 350 ng/L E_2	Gárriz *et al.* (2015)
	Betta splendens	*In vivo*	100 ng/L (4 weeks)	Montgomery *et al.* (2014)
	Sparus aurata	*In vivo*	5 µg/L (28 days, by feeding)	Cabas *et al.* (2013)
Zinc	*Salmo trutta*	*In vitro*	5.9 mg/L	Giardina *et al.* (2009)
	Clarias gariepinus	*In vitro*	2000 mg/L (24h)	Kime *et al.* (1996)
	Cyprinus carpio	*In vitro*	50 mg/L (24h)	Chyb and Kime (2000)
	Lota lota	*In vitro*	75 mg/L	Lahnsteiner *et al.* (2004)
	Leuciscus cephalus	*In vitro*	7.5 mg/L	Lahnsteiner *et al.* (2004)
Cadmium	*Clarias gariepinus*	*In vitro*	100 mg/L (24h)	Kime *et al.* (1996)
	Cyprinus carpio	*In vitro*	10 mg/L (2h)	Chyb *et al.* (2001)
	Oncorhynchus mykiss	*In vitro*	10 mg/L (4h)	Dietrich *et al.* (2010)
	Cyprinus carpio	*In vitro*	50 mg/L (24h)	Dietrich *et al.* (2011)
	Lota lota	*In vitro*	25 mg/L	Lahnsteiner *et al.* (2004)
Mercury	*Oncorhynchus mykiss*	*In vitro*	10 mg/L (4h)	Dietrich *et al.* (2010)
	Perca fluviatilis	*In vitro*	62 µM	Hatef *et al.* (2011)
	Perca fluviatilis	*In vitro*	31 µM (3h)	Hatef *et al.* (2011)
	Clarias gariepinus	*In vitro*	0.001 mg/L	Rurangwa *et al.* (1998)
	Dicentrarchus labrax	*In vitro*	0.1 mg/L (5 min)	Abascal *et al.* (2007)
	Carassius auratus	*In vitro*	>1 mg/L (24 h)	Van Look and Kime (2003)

DDT	*Oreochromis mossambicus*	*In vivo*	>0.01 µg L*	Marchand *et al.* (2008)
	Clarias gariepinus	*In vivo*	>0.01 µg L*	Marchand *et al.* (2008)
	Heteropneustes fossilis	*In vivo*	0.1 mg/L (40 days)	Singh *et al.* (2008)
TBT	*Cyprinus carpio*	*In vitro*	2.7 µg/L	Rurangwa *et al.* (2002)
	Cyprinus carpio	*In vitro*	0.27 µg/L (24h)	Rurangwa *et al.* (2002)
	Clarias gariepinus	*In vitro*	0.27 µg/L (24h)	Rurangwa *et al.* (2002)

1986
1987