Morini, MAM.; Peñaranda, D.; Vilchez Olivencia, MC.; Gallego Albiach, V.; Nourizadeh-Lillabadi, R.; Asturiano Nemesio, JF.; Weltzien, F.... (2015). Transcript levels of the soluble sperm factor protein phospholipase C zeta 1 (PLCZ1) increase through induced spermatogenesis in European eel. Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology. 187:168-176. doi:10.1016/j.cpba.2015.05.028.

Transcript levels of the soluble sperm factor protein phospholipase C zeta 1 (PLCζ1) increase through induced spermatogenesis in European eel

Marina Morini[1], David S. Peñaranda[1], María C. Vílchez[1], Víctor Gallego[1]., Rasoul Nourizadeh-Lillabadi[2], Juan F. Asturiano[1], Finn-Arne Weltzien[2], Luz Pérez*[1]

[1]Grupo de Acuicultura y Biodiversidad. Instituto de Ciencia y Tecnología Animal. Universitat Politècnica de València, Camino de Vera s/n. 46022, Valencia, Spain.

[2]Department of Basic Sciences and Aquatic Medicine, Norwegian University of Life Sciences - Campus Adamstuen, P.O. Box 8146 Dep, 0033 Oslo, Norway.

Running title: Expression of PLCζ1 in European eel testis

ms. has 34 pages, 6 figures, 1 table

*Corresponding author:
E-mail: mlpereig@dca.upv.es
Dr. Luz Pérez
Grupo de Acuicultura y Biodiversidad
Instituto de Ciencia y Tecnología Animal. Edificio 7G
Universitat Politècnica de València
Camino de Vera, s/n
46022 Valencia (Spain)

Abstract

Activation at fertilization of the vertebrate egg is triggered by Ca^{2+} waves. Recent studies suggest the phospholipase C zeta (PLCζ), a sperm-specific protein, triggers egg activation by an IP3-mediated Ca^{2+} release and allow Ca^{2+} waves at fertilization.

In the present study we cloned, characterized, and phylogenetically positioned the European eel PLCζ (PLCζ1). It is 1521bp long, with 10 exons encoding an open reading frame of 506 amino acids. The amino acid sequence contains an EF-hand domain, X and Y catalytic domains, and a carboxy-terminal C2 domain, all typical of other PLCζ orthologous. The sequence is truncated not only at the N-terminus of the EF-hand domain, as in all teleost PLCζ, but also in the C-terminal region of the X-domain and in a large part of the N-terminal X/Y linker region.

The tissue distribution was studied, and the gene expression was determined in testis during induced sexual maturation at three different thermal regimes. Also, brain and pituitary expression were studied through sex maturation at constant temperature. *plcζ1* was expressed in brain of male and female, in testis but not in ovaries. By first time in vertebrates, it is reported *plcζ1* expression in the pituitary gland. Testis *plcζ1* expression increased through spermatogenesis under all the thermal regimes, but being significantly elevated at lower temperatures. It was very low when testis contained only spermatogonia or spermatocytes, while maximum expression was found during spermiogenesis. These results support the hypothesis for an eel sperm-specific PLCζ1 inducing egg activation, similarly to mammals and some teleosts, but different from some other teleost species, which express this protein in ovaries, but not in testes.

Keywords:

Teleost, Reproduction, Fertilization, Spermatozoa, *Anguilla anguilla*

1. Introduction

Sperm fusion with the egg induces egg activation in all animals studied so far through a rise in intracellular Ca^{2+} (Stricker, 1999; Tarin, 2000; Kashir et al., 2010; Horner and Wolfner, 2008a). Three models have been proposed for mechanisms by which fertilization-induced Ca^{2+} waves are initiated: a) Ca^{2+} bolus/conduit (Jaffe, 1983, 1991), where the sperm trigger the entering of extracellular Ca^{2+} into the oocyte; b) membrane receptor (Jaffe, 1990; Evans and Kopf, 1998), with an intracellular Ca^{2+} release provoked by the binding of an oocyte surface receptor with a sperm ligand; or c) a soluble sperm factor (Swann et al., 2006; Parrington et al., 2007; Saunders et al., 2007) released into the oocyte after gamete fusion, triggering egg activation. This sperm factor corresponds to a sperm-specific phospholipase C (PLC) called PLCζ (Swann and Lai, 2013; Ito et al., 2011). After fertilization, PLCζ induces a reaction chain by cleaving phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Igarashi et al., 2007; Miao and Williams, 2012). These two metabolites, in turn, cause IP3-mediated Ca^{2+} release from the endoplasmic reticulum, and the activation of such targets as DAG-sensitive protein kinase Cs (PKCs) (Miyazaki et al., 1993; Saunders et al., 2002; Swann and Yu, 2008; Yu et al., 2008).

During the last ten years, several studies have demonstrated the importance of the soluble sperm factor to allow Ca^{2+} waves at fertilization. Injection of recombinant PLCζ cRNA (Saunders et al., 2002) or protein (Kouchi et al., 2004) into mouse eggs leads to Ca^{2+} oscillations at fertilization. Saunders et al. (2002) showed that when endogenous PLCζ was removed by immunodepletion, mouse sperm protein extracts lost their ability to release Ca^{2+}. Moreover, *in vitro* fertilization of mouse eggs with sperm from transgenic mice expressing lower amounts of PLCζ (due to a short hairpin RNAs targeting PLCζ) induced Ca^{2+} oscillations that ended prematurely, negatively affecting egg activation and embryonic development (Knott et al., 2005). Furthermore, infertile men whose sperm failed in egg activation showed abnormal expression and localization of PLCζ in the sperm (Yoon et al., 2008; Heytens et al., 2009). Until now, mammalian PLCζ orthologues have been reported in mice, monkeys,

humans, boars, hamsters, and bulls (Cox et al., 2002; Saunders et al., 2002; Yoneda et al., 2006; Young et al., 2009; Cooney et al., 2010). In non-mammals, PLCζ orthologues were reported in the chicken (Coward et al., 2005), medaka (Ito et al., 2008), quail (Mizushima et al., 2009) and in two pufferfish species *Takifugu rubripes* (Fugu) and *Tetraodon nigroviridis* (Tetraodon) (Coward et al., 2011). In these non-mammalian species, like chicken or medaka, PLCζ mRNA is expressed in the testis, in line with the situation in mammals. In contrast, in two pufferfish species, *plcζ1* is expressed in the ovary, but not in the testis (Coward et al., 2011).

Due to its unique life cycle and its phylogenetical position, the European eel (*Anguilla anguilla*) is a particularly interesting model to investigate the regulatory mechanisms of reproductive physiology and for providing insights into ancestral regulatory functions in teleosts. Prepubertal silver eels migrate across the Atlantic Ocean to reach their probable spawning area in the Sargasso Sea (Tesch, 1977). Gonadal development and maturation probably takes place during the supposedly 6-7 month migration period, at low temperature, whereas the spawning takes place at high temperatures, considered to be around 20 °C (Boëtius and Boëtius, 1967, 1980). However, as detailed information from the field is still lacking, it is difficult to simulate the variable environmental factors which would occur during the migration (temperature, photoperiod, pressure, etc). That is why, in captivity, silver eels are blocked in a pre-pubertal stage (Dufour et al., 2003; Pasqualini et al., 2004; Vidal et al., 2004) and must receive a long-term hormonal treatment to induce sexual maturation and spermiation (Boëtius and Boëtius, 1967; Ohta et al., 1996, 1997; Asturiano et al., 2005; Huang et al., 2009; Pérez et al., 2000; Gallego et al., 2012).

In this study, we characterized and cloned the *Anguilla anguilla plcζ1* mRNA, analysed the structure and investigated the position of this protein among vertebrates by phylogenetic analyses, studied the tissue distribution of this gene and finally, for the first time in teleost, we studied the expression profile of *plcζ1* in the brain and gonad through spermatogenesis. The impact of water temperature on the maturation process of European eel has been highlighted in females (Pérez et al., 2011; Mazzeo et al., 2014) and males (Gallego et al., 2012, 2014; Baeza et al., 2014), and in order to simulate the natural conditions during the reproductive migration and testing its potential effect on *plcζ1* expression, three different thermal regimes were tested for the gene expression profile experiments,

two variable regimes (changing gradually from 10 to 20 °C or from 15 to 20 °C), and one constant regime (20 °C).

2. Material and methods

2.1. Fish maintenance, hormonal and thermic treatments, and sampling

Three hundred and seventeen male European eels (mean body weight 100 ± 2 g) from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) were hormonally matured at the Aquaculture Laboratory at the Polytechnic University of Valencia. They were randomly distributed and kept in six 200-L fiberglass tanks (approximately 50 males per aquaria, 2 aquaria per treatment) equipped with separate recirculation systems, thermostats/coolers, and covered to maintain constant shadow.

The fish were gradually acclimatized for one week to seawater (37±0.3‰ of salinity) and the water temperature was kept at 20 °C or changed to 15°C in one week or to 10°C in two weeks, depending on thermal groups. Starting three weeks after arrival to the Aquaculture Laboratory, the eels were treated with weekly intraperitoneal injections of human chorionic gonadotropin (hCG, Profasi®, Serono, Italy); 1.5 IU g^{-1} fish; during 13 weeks to induce maturation and spermiation, as previously described by Perez et al. (2000).

During the experiment, the animals were maintained in three thermal regimes (2 aquaria per treatment): T10-T20: 10 °C (first 5 weeks, with one week of temperature acclimation), 15 °C (next 3 weeks) and 20 °C (last 6 weeks); T15-T20: 15 °C (first 6 weeks, with two weeks of temperature acclimation) and 20 °C (last 9 weeks); and T20: 20 °C during the whole experimental period. These thermal regimes were previously described by Gallego et al. (2012).

Groups of 5-8 eels per treatment were anaesthetized with benzocaine (60 ppm) and sacrificed by decapitation each week along the hormonal treatment. Morphometric parameters such as total body, gonad weights were recorded to calculate the gonadosomatic index (GSI = (gonad weight/total body weight)*100) for each fish (Pankhurst, 1982). Furthermore, testicular tissue samples were fixed in 10% formalin buffered at pH 7.4 for histological processing and subsequent determination of maturational status. Samples of pituitary, testis, liver, heart, gill, muscle, spleen, fins, and kidney were

collected for analyses of gene expression levels by qPCR. Brains were dissected into five parts: olfactory bulbs, telencephalon, mes-/di-encephalon, cerebellum, and medulla oblongata. All the samples were stored in 0.5 ml of RNAlater (Ambion Inc., Huntingdon, UK) at -20 ºC until extraction of total RNA (Peñaranda et al., 2010).

Because eels stop feeding at the silver stage and throughout sexual maturation, the fish were not fed during the experiment and were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC).

2.2. Gonadal histology

Fixed testis samples were dehydrated in ethanol and embedded in paraffin. Sections of 5-10 µm thickness were cut with a Shandom Hypercut manual microtome and stained with haematoxylin and eosin. Slides were observed with a Nikon Eclipse E-400 microscope, and pictures were taken with a Nikon DS-5M camera attached to the microscope. Stages of spermatogenesis were determined according to the most advanced germ cell type present and their relative abundance, degree of development of the seminal tubules, GSI and sperm production by the male in the same week of the sacrifice. Stage 1 Spermatogonia (SPG): dominance of spermatogonia, in some cases, a few spermatocytes were present in low number, mean GSI = 0.08 (0.0-0.36); Stage 2 Spermatocytes (SPC): spermatocytes were present in proportion ≥50% with spermatogonia, in some cases appeared low number of spermatids, mean GSI = 0.72 (0.27-1.54); Stage 3 spermatids (SD): spermatids were the dominant germ cell, some sperm cells can appear, mean GSI = 3.28; and Stage 4 spermatozoa (SZ): spermatozoa was the dominant germ cell, mean GSI =7.35 (3.41-12.8) (Fig. 1).

2.3. Isolation of PLCζ sequence

2.3.1. European eel genome database analysis.

The TBLASTN algorithm of the CLC DNA Workbench software (CLC bio, Aarhus, Denmark) was used to retrieve the genomic sequence of the PLCζ from the European and Japanese eel genomes (Henkel et al., 2012a, Henkel et al., 2012b)

Exons and splice junctions were predicted using the empirical nucleotidic splicing signatures, i.e.: introns begin with ‘‘GT’’ and end with ‘‘AG’’. The peptidic sequences of *Tetraodon nigroviridis* PLCζ1 sequence (Accession: HQ185299. GI: 322510422. 1,889 bp mRNA) were used as query. Percentage of European eel PLCζ1 identity with other osteichtian PLCζ sequences was calculated with Secuences Identites And Similarities (SIAS) server (imed.med.ucm.es/Tools/sias.html)

2.3.2. Partial cloning of the PLCZ1 gene

cDNA was generated using 1 µg of total RNA. A mixture of cDNA from different tissues of female silver eels were used as template for amplification of PLCζ. Partial PLCζ cDNA was amplified by PCR using specific primers which were designed based on the predicted PLCζ sequence of European eel using Primer3 Software (Whitehead Institute/Massachusetts Institute of Technology, Boston, MA): PLCZ1fw1: GGCTTCCTCCGGTACATGGA; PLCζ1rv1: TGTAGTTGGAGGACAGCGTGC; PLCZ1fw2: AGATTCATCAGCAGGATCTATCC; PLCζ1rv2: TACTGGCCCATGAAGTCGTT. PCR amplification was run in a Hybaid PCR express, using 25 µl of reaction mixture containing 1x PCR buffer (Invitrogen), 200 µM dNTPs (Invitrogen), 0.1 IU of Taq DNA polymerase (Invitrogen), 500 nM of each primer and 1 µl of cDNA template. PCR products were visualized in 2% agarose gel stained with SYBR Safe DNA gel Stain (Invitrogen) and bands of expected size were purified using Qiaquick Gel Extraction kit (Qiagen) and ligated into the pGEM-T easy vector (Promega, WI, USA). Cloning was performed in competent E. coli JM109 cells (Promega). Positive colonies were isolated and plasmids extracted by Qiagen Plasmid Mini Kit (Qiagen). Plasmids with insert were sent to Eurofins Genomics (Germany) for sequencing.

2.3.3. Phylogenetic analysis

Amino-acid sequences of known or predicted sequences of gene coding for the PLCζ from 14 species retrieved from NCBI or ENSEMBL were first aligned using ClustalW (Thompson et al., 1994), then manually adjusted. Human, *Homo sapiens*, and mouse, *Mus musculus* PLCβ1 were used as outgroup. The JTT (Jones, Taylor and Thornton) protein substitution matrix of the resulting alignment was determined using ProTest software (Abascal et al., 2005). Phylogenetic analysis of the PLCζ sequence

alignment was performed using the maximum likelihood method (PhyML software, Stamatakis et Ott, 2008), with 1000 bootstrap replicates.

2.4. Gene expression analyses by quantitative real-time PCR

2.4.1. Primers and reference gene

Acidic ribosomal phosphoprotein P0 (ARP): ARPfw: GTG CCA GCT CAG AAC ACT G; ARPrv: ACA TCG CTC AAG ACT TCA ATG G (Aroua et al., 2007; Weltzien et al., 2006) was used as reference gene in the quantitative real-time Reverse Transcriptase-Polymerase chain reaction (qPCR) because its mRNA expression has been shown to be stable during experimental maturation (Weltzien et al., 2005). The qPCR expression stability of the reference gene was determined using the BestKeeper program (Pfaffl et al., 2004), reporting a standard deviation (SD [±Cq]) lower than 1. In the testis, T10-T20: SD= 0.79 ; T15-T20: SD= 0.97; T20: SD= 0.79; $p<0.05$ with a Cq geometric mean of T10-T20: 24.3±1.73 ; T15-T20: 24.37±1.96; T20: 25.17±1. 73; in the brain and pituitary olfactory bulb: SD= 0.85; telencephalon: SD= 0.56; mes-/di-encephalon: SD= 0.53, pituitary: SD= 0.77; $p<0.05$ and a Cq geometric mean of olfactory bulb: 23.74±1. 8; telencephalon: 22.43±1.48; mes-/di-encephalon: 22.17±1.44; pituitary: 22.77±1.71. The BestKeeper calculated variations in the reference gene are based on the arithmetic mean of the Cq values. Genes with a SD value higher than 1 are defined as unstable. European eel PLCζ specific qPCR primers qPLCζ1fw: GAA GAG CCA CCT GTT TGC AT; qPLCζ1rv: CAG CAG TCG ATC TCC AGA CA; were designed based on the full-length European eel CDS sequences. All the primers were designed on two different exons, in order to avoid amplification of potential genomic contamination, using Primer3 Software (Whitehead Institute/Massachusetts Institute of Technology, Boston, MA, USA). All primers were purchased from Integrate DNA Technology, Inc. (IDT, Coralville, IA).

2.4.2. SYBR Green assay

To quantify gene expression, qPCR assays were performed using a model 7500 unit (Applied Biosystems; Foster City, CA, USA), with PCR protocol previously described by Peñaranda et al. (2013).

The total volume for every PCR reaction was 20 µl, performed from diluted (1:20) DNA template (5 µl), forward and reverse primers (250 nM each), and SYBR Green/ROX Master Mix (12 µl) (Fermentas GMBH). Transcript levels were determined using an efficiency-adjusted relative quantification method as described by Weltzien et al. (2005). Serial dilutions of cDNA pool of gonad tissues were run in duplicate and used as a common standard curve. One of these dilutions was also included in each run as a calibrator. Target and reference genes in unknown samples were run in duplicate PCR reactions. Non-template control (cDNA replaced by water) for each primer pair was run in duplicate on all plates.

2.4.3. PLCζ tissue distribution

In order to investigate the tissue distribution of PLCζ mRNA expression, gonads (testes and ovaries) and somatic tissues (liver, heart, gill, muscle, spleen, fins, kidney, brain, pituitary) were collected from three immature male eels (mean body weight 118 ± 14 g; mean GSI <0.1) from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) and three female eels (mean body weight 608 ± 35 g; mean GSI 0.9 ± 0.3) from the Albufera lagoon (Valencia, Spain). Samples were stored in RNAlater (Ambion, Austin, Texas, USA) immediately after decapitation and stored at 20 °C until RNA extraction. The brain was dissected into five parts: olfactory bulbs, telencephalon, mes-/di-encephalon, cerebellum and medulla oblongata as previously reported by Weltzien et al. (2005).

Total RNA was extracted following the method used by Hildahl et al. (2011). Total RNA was treated with DNase I (Turbo DNA-free; Ambion) at 37°C for 30 min. First-strand cDNA was prepared from 1µL total RNA using superscript III (Invitrogen) according to the manufacturer's protocol. All tissues were analysed by qPCR.

2.4.4. PLCζ expression through spermatogenesis

To study PLCζ expression during spermatogenesis, total RNA of gonads, olfactory bulb, telencephalon, mes-/di-encephalon and pituitary was isolated from the RNAlater preserved tissues as described by Peñaranda et al. (2013). Testis RNA of males from thermal groups T10-T20, T15-T20

and T20 was treated and purified with DNase I of NucleoSpin RNA XS kit (Macherey-Nagel, Düren, Germany). First-strand cDNA was synthesized from 500 ng of testis total RNA, using qScript cDNA Synthesis Kit (Quanta Bioscience, MD, USA) with 15 µl RNA used as template.

Total RNA extracted from the olfactory bulb, telencephalon, mes-/di-encephalon and the pituitary of males from the thermal group T20 was treated with deoxyribonuclease (gDNA Wipeout Buffer, Qiagen), using a total volume of 14 µl for 500 ng of total RNA for the olfactory bulb ant pituitary, or 1 µg for the telencephalon and the mes-/di-encephalon. First-strand cDNA was synthesized in 20 µl reactions using Quantiscript Reverse Transcriptase (Qiagen) with 14 µl used as template, which were obtained in the previous step. RNA concentration and quality were evaluated by using a NanoDrop 2000C Spectrophotometer (Fisher Scientific SL, Spain).

2.5. Statistics

Each variable was first checked for normality. If the variables did not have a normal distribution, they were log-transformed and their normality was checked again.

Then, data were analyzed by analysis of variance (One-way ANOVA), using the Student-Newman-Keuls test to compare means. Variance homogeneity was checked with the Bartlett test. Differences were considered significant when $p<0.05$.

Statistical analyses (One-way ANOVA) were also performed to study the evolution of PLCζ expression in one tissue throughout sex development, and to study the differences in expression between thermal regimes in the same developmental stage (SPG, SPC, SD or SZ).

T-test analyses were performed to compare differences between males and females in a same tissue from the data obtained in the study of PLCζ tissue distribution.

All statistical procedures were performed using Statgraphics Plus 5.1 (Statistical Graphics Corp., Rockville, MO, USA). Results are presented as mean ± standard error (SEM).

3. Results

3.1. Characterization of European eel PLCζ1

289 The *plcζ1* gene was identified *in silico* in both the European and Japanese eel genomes. The European
290 eel *plcζ1* predicted sequence differed from the Japanese eel predicted sequence by 30 nucleotides and
291 13 amino acids. From the *Anguilla anguilla plcζ1* predicted sequence, specific primers were designed
292 to clone and confirm this sequence. Two overlapping fragments covering 1167 bp were cloned and
293 sequenced. The European eel *plcζ1* cDNA sequence (Fig. 2) differed from the corresponding partial
294 sequence characterized in the European eel genome by only 2 nucleotides and a gap of 3 nucleotides
295 in position 700 of the European eel *plcζ1* cDNA sequence. This gap led to a lack of 1 amino acid,
296 which did not affect the reading frame. The complete *plcζ1* CDS was 1521 bp long, composed by 10
297 exons giving an open reading frame (ORF) of 506 amino acids (GenBank accession number
298 AFV13732.1) (Fig. 2).
299 European eel *plcζ1* showed a high identity when compared with *plcζ* from other teleosts: from 76.69%
300 for the Fugu *Takifugu rubripes* to 79.32% for the Atlantic cod *Gadus morhua*, with the highest
301 identity. The European eel *plcζ1* share 78% of identity with the non-teleost actinopterygian spotted
302 gar *Lepisosteus oculatus*. When compared with sarcopterygian Plcζ amino acid sequences, European
303 eel Plcζ1 presented 70.44% of sequence identity with the human, 66.82% with the mouse and the
304 lizard, and 69.13% with the chicken. The highest identity with a sarcopterygian Plcζ was found with
305 the coelacanth, with 72.22% of identity. Classical domains of European eel Plcζ1 protein were
306 predicted using Interproscan software (http://www.ebi.ac.uk/interpro/) and revealed a typical Plcζ
307 domain structure with the following conserved domains: EF hand-like domains from position 16 to
308 98, X domain in position 101-240, Y domain in position 243-360, and C2 domain in position 383-467
309
310 **3.2. Phylogeny**
311 We performed phylogenetic analyses on five actinopterygian Plcζ1 amino acid sequences (four Plcζ1
312 from teleost species and one from a non-teleost species, the spotted gar), and five sarcopterygian
313 PLCζ1 amino acid sequences, with the PLCβ1 (phospholipase C, beta1) from two mammalian species
314 as outgroup (Fig. 3). In this phylogenetic analysis, the actinopterygian and sarcopterygian Plcζ1
315 clustered in two monophyletic groups. In the actinopterygian group, the European eel Plcζ1 clustered
316 with the spotted gar at the basis of the teleost clade, constituting an actinopterygian Plcζ1 clade as

317 sister clade of the sarcopterygian Plcζ1. This phylogeny confirmed that European eel Plcζ1 is
318 orthologous with actinopterygian Plcζ1 and sarcopterygian Plcζ1.
319
320 **3.3. *plcζ1* tissue distribution in the European eel**
321 *plcζ1* mRNA expression was compared in various tissues of female and male European eels (Fig. 4).
322 The *plcζ1* showed a differential expression in male and female European eel. In female eels, very low
323 expression of *plcζ1* was detected in peripherical tissues such as liver, heart, gill, muscle, spleen, fins,
324 kidney, ovary or pituitary whereas there was high expression in the different brain parts. In male eels,
325 no expression was found in the muscle and in the kidney. Low expression of male *plcζ1* mRNA was
326 detected in the liver, gill, heart, spleen, fins, medulla oblongata, cerebellum and pituitary. However
327 there was high expression in the testis, olfactory bulb, telencephalon and mes-/di-encephalon. *plcζ1* in
328 the olfactory bulb, telencephalon and gonads was expressed at higher levels in females compared to
329 the males ($p<0.05$).
330
331 **3.4. *plcζ1* expression during spermatogenesis**
332 Once demonstrated the expression of male eel *plcζ1* in the brain, pituitary and testis, we studied the
333 testis *plcζ1* mRNA expression of the males from all the thermal regimes through spermatogenesis; and
334 the brain (olfactory bulb, telencephalon, mes-/di-encephalon) and pituitary *plcζ1* mRNA expression in
335 group T20 (kept at 20 ºC) through spermatogenesis. In the brain and pituitary of the males kept at 20
336 ºC, *plcζ1* expression was stable from spermatogonia to spermatozoa and did not show significant
337 differences throughout maturation. The higher *plcζ1* expression was observed in the telencephalon and
338 in the olfactory bulb (Fig. 5). The mes-/di-encephalon and the pituitary showed the lower *plcζ1*
339 expression levels ($p<0.05$).
340 Testis *plcζ1* expression (Fig. 6) increased through spermatogenesis in all the thermal regimes. The
341 *plcζ1* expression was very low when testis showed only spermatogonia (S1) or spermatocytes (S2)
342 (Fig. 6). Maximum *plcζ1* expression was found between S3 and S4 (spermiogenesis) when it was 75-
343 fold higher than at S1 ($p<0.05$). Furthermore, when comparing thermal regimes for a same stage of

development, testis *plcζ1* was significantly highly expressed at stage spermatozoa in the lower thermal regime (T10-T20) than in the higher thermal regimes (T15-T20 and T20, $p<0.05$).

4. Discussion

European eel *plcζ1* sequence showed a Plcζ typical domain structure but its sequence is shorter when compared with other vertebrate Plcζ, suggesting that eel Plcζ1 could have conserved its activity, but maybe at a lower level. The expression of eel testis *plcζ1* mRNA increase through spermatogenesis reaching maximum levels during spermiogenesis, and its expression is significantly higher at lower temperature compared to higher temperatures, suggesting that temperature may play a role in the regulation for *plcζ1* transcription in the testis, when *plcζ1* seems to acquire its function.

4.1. Molecular structure and function

The European eel *plcζ1* contains an EF-hand domain located in the amino-terminal region of the molecule, X and Y catalytic domains, and a carboxy-terminal C2 domain, all typical of other PLCζ orthologues (for review see Kashir et al, 2013; Ito et al, 2011, Parrington et al, 2007). Similar to Medaka (Ito et al, 2008), and Fugu and Tetraodon *plcζ* (Coward et al, 2011), the European eel *plcζ1* sequence is shorter when compared with mammalian *PLCζ*, showing an EF-hand domain truncated at the N-terminus, like in all the teleosts *plcζ* studied so far (Fig. 2). EF-hands are involved in binding Ca^{2+} and are thought to be important for the oscillatory Ca^{2+} activity of the enzyme (Ito et al, 2011). In teleost species, studies showed that a deletion of a part of the EF-hand domain reduces the Ca^{2+} oscillatory activity of Plcζ (Kouchi et al, 2005; Kuroda et al, 2006). It remains possible that eel Plcζ1 does not trigger Ca^{2+} oscillation, however medaka Plcζ, which is similarly truncated at its N-terminus, can induce Ca^{2+} oscillation in mouse oocytes but at a lower activity than for full-length mammalian PLCζ (Coward et al, 2011; Kuroda et al, 2006; Ito et al, 2008). These results suggest that these domains are involved in the Ca^{2+} signal but are not obligatory to induce Ca^{2+} oscillation. This means eel Plcζ1 could have conserved its activity, but maybe at a lower level. Furthermore, EF-hand domains seem also to play a role in nuclear translocation (Kouchi et al, 2004, 2005; Yoda et al 2004). According to Kuroda et al. (2006), Trp13, Phe14, and Val18, which may be necessary for appropriate

conformation for nuclear translocation, may also be necessary to keep normal Ca^{2+} oscillation-inducing activity as well. Nevertheless, despite the lack of a part of the N-terminal of all teleosts studied so far, at least some of these Plcζs still can trigger Ca^{2+} oscillations.

The XY domain, known to form together the active site responsible for PIP2 cleavage (Parrington et al, 2007), is highly conserved. On the contrary, the X/Y linker region, between the two catalytic domains, is a poorly conserved domain with a high diversity of amino acid residues among vertebrate. In the C terminus of the X domain and in the X/Y linker region, PLCζ possesses a cluster of basic amino acid residues (lysine and arginine), which is found in many nuclear proteins (Kuroda et al, 2006, Jones and Nixon, 2000). Eel PLCζ1 is truncated in the C-terminal region of the X-domain and in a large part of the N-terminal X/Y linker region, on approximately 85 amino acids, when compared with the other osteichthyan PLCζ sequences. This loss of protein part leads to a change in the protein conformation (data not shown) which may affect the protein function. Furthermore, due to its loss, eel PLCζ1 misses these two nuclear targeting regions localised in the lost part, which may affect the nuclear translocation of the protein. According to Kuroda et al. (2006), in the mouse, nuclear targeting was absent for point mutation of Lys299 and/or Lys301 in the C terminus of X domain, and nuclear translocation was lost when the residues from the NLS were replaced by glutamate. Nevertheless, these substitutions did not affect PLCζ ability to induce the Ca^{2+} oscillation. Furthermore, European eel PLCζ1 still possesses region for enzymatic catalisis and substrate/Ca^{2+} binding, which are very well conserved residues among osteichthyans, so European eel PLCζ1 catalytic function could be preserved. Further studies to confirm the PLCζ1 function for initiating the Ca^{2+} oscillation after fertilization in eel are necessary.

To better understand the evolutionary history for the PLCζ family, we performed phylogenetic analyses on osteichthyans of key-phylogenetical positions: the human and the mouse, representative of mammalians; the anole lizard and the chicken, representative of sauropsids; the coelacanth, a representative of early sarcopterygians; the spotted gar, a non-teleost actinopterygian; the European eel, a member of an early group of teleosts (elopomorphs), and three members of teleosts (*Medaka*, *Takifugu* and *Tetraodon*). The *Anguilla anguilla* PLCζ1 branch with the spotted gar at the basis of the teleost PLCζ group. Each species exhibits only one PLCζ, which seem to suggest that this protein has

not been affected by the teleost-specific third whole-genome duplication. The duplicated gene must have been lost during evolution.

4.2. Sex-specific and species-specific tissue distribution of *plcζ1*

PLCζ is known to be sperm-specific, but eel *plcζ1* mRNA was highly expressed in different brain parts, also showing low expression in the pituitary and peripherical tissues of male and female eels. Tissue distribution of eel *plcζ1* mRNA revealed a differential expression in male and female European eel, with high *plcζ1* expression in testis, and very low in ovary, like in every *plcζ* orthologous from mammals (Cox et al, 2002; Saunders et al, 2002; Yoneda et al, 2006; Young et al, 2009), birds (Coward et al, 2005; Mizushima et al, 2009), and some teleosts like medaka (Ito et al, 2008) and eel, but different to other fish like in the two pufferfish species *Takifugu rubripes* and *Tetraodon nigroviridis* (Coward et al, 2011). While *plcζ* mRNA is thought to be only expressed in male gametes, eel *plcζ1* also expressed in the brain and pituitary of male and female. Yoshida et al. (2007) found expression of *Plcζ* mRNA in brains of both male and female mice, and Coward et al. (2011) found expression of *plcζ* in *Tetraodon* brain, but its function in the brain is unknown. Nevertheless, it is the first evidence of pituitary expression of PLCζ in vertebrates. These results showed different tissue specific patterns of expression in *plcζ* mRNA, which is not only expressed in fish testis, but also in the brain or in the ovary. PLCζ function is well documented in sperm vertebrates, nevertheless further studies of PLCζ expression and functions in somatic tissues are necessary.

4.3. *plcζ1* expression is stable in brain but increase in testis through spermatogenesis

This is the first study of the effect of the eel sexual maturation on the expression of brain and pituitary *plcζ1* mRNA. In the European eel, *plcζ1* mRNA expression is stable in the pituitary and in the brain through the spermatogenesis. The significance of *plcζ1* mRNA expression in the brain and in the pituitary is unknown, further studies of Plcζ1 protein synthesis in the brain-pituitary-gonad axis should be performed to clarify the role of this protein in the reproductive function.

According to our results, *plcζ1* mRNA expression increases in the testis through spermatogenesis regardless of thermal regime, reaching maximum levels during spermiogenesis. Mizushima et al.

(2009) searched the *PLCζ* mRNA expression in quail sperm cells and found expression in elongate spermatids but not in spermatocytes or in round spermatids. Furthermore, they demonstrated that injection of chicken or quail elongated spermatids lead to successful fertilization and development of mouse and quail eggs, but none of the round spermatids alone induced blastodermal development. These results of *PLCζ* mRNA expression and spermatogenic cell injection support the evidence that the egg activation potency of PLCζ during spermatogenesis is acquired in elongated spermatids in quail. This is in accordance with our results showing a European eel *plcζ1* mRNA expression 75-fold higher at the spermatozoa stage than at the spermatogonia stage, suggesting that eel Plcζ1 function is acquired during the stage of spermiogenesis.

PLCζ function in the process of fertilization is known, but it seems to play further roles in spermatogenesis. For instance, Ito et al. (2010) observed that PLCζ knock-out mice was unable to complete spermatogenesis with spermatocytes failing to proceed beyond elongation, underlying the involvement of PLCζ in spermatogenesis.

The observed increase in eel *plcζ1* mRNA expression during spermiogenesis regardless of thermal regimes clearly indicates that this increase is independent of temperature. However, at the final step of spermatogenesis (stage spermatozoa) European eel *plcζ1* mRNA expression in the testis was significantly higher for the males subjected to the temperature T10-T20 compared to T15-T20 and T20, suggesting that temperature may play a role in the regulation for *plcζ1* transcription in the testis, especially during the process of spermiogenesis, precisely when *plcζ1* seems to acquire its function.

The present study shows that *plcζ1* mRNA synthesis in the eel testis starts after the onset of spermatogenesis. Our results support the hypothesis of a sperm-specific Plcζ1 egg activation in the European eel, similar to many other vertebrates. However, expression of *plcζ1* mRNA showed different tissue specific patterns, expressing in the brain or in the ovary like the two pufferfish species *Takifugu rubripes* and *Tetraodon nigroviridis*. Further studies of the function of PLCζ in the Brain-Pituitary-Gonad axis are necessary to clarify the physiologic processes which control sexual maturation and fertilization. Due to its phylogenetical position and its complex life cycle, the

European eel may be a very useful model to explore the evolutionary origins of PLCζ and its functional role in the egg activation.

In conclusion, the Plcζ1 expression pattern found in the European eel suggests an important function of this protein in the spermatozoa of this species.

Acknowledgements

Funded from the SPERMOT project (Spanish Ministry of Science and Innovation, MICINN; AGL2010-16009). M.C. Vílchez has a predoctoral grant from UPV PAID Programme (2011-S2-02-6521), Marina Morini has a predoctoral grant from Generalitat Valenciana (Programa Grisolía), Victor Gallego has a posdoctoral grant (UPV; PAID-10-14), and David S. Peñaranda was supported by MICINN and UPV (PTA2011-4948-I). Grants to attend meetings from COST Office (Food and Agriculture COST Action FA1205: AQUAGAMETE).

References

Abascal, F., Zardoya, R., Posada, D., 2005. ProtTest: selection of best-fit models of protein evolution. Bioinformatics 21, 2104-2105.

Aroua, S., Weltzien, F.A., Le Belle, N., Dufour, S., 2007. Development of real-time RT-PCR assay for eel gonadotropins and their application to the comparison of in vivo and in vitro effects of sex steroids. Gen. Comp. Endocrinol. 153, 333-343.

Asturiano, J.F., Pérez, L., Garzón, D.L., Peñaranda, D.S., Marco-Jiménez, F., Martínez-Llorens, S., Tomás, A., Jover, M., 2005. Effect of different methods for the induction of spermiation on semen quality in European eel. Aquaculture Research 36, 1480-1487.

Baeza, R., Mazzeo, I., Vílchez, M.C., Gallego, V., Peñaranda, D.S., Pérez, L., Asturiano, J.F., 2014. Effect of thermal regime on fatty acid dynamics in male European eels (*Anguilla anguilla*) during hormonally-induced spermatogenesis. Aquaculture 430, 86-97.

Bedford, S.J., Kurokawa, M., Hinrichs, K., Fissore, R.A., 2003. Intracellular calcium oscillations and activation in horse oocytes injected with stallion sperm extracts or spermatozoa. Reproduction 126, 489-499.

Boetius, I., Boetius, J., 1967. Studies in the European eel, *Anguilla anguilla* (L.). Experimental induction of the male sexual cycle, its relation to temperature and other factors. Meddelser fra Danmarks Fiskeri- og Havunderogelser 4, 339-405.

Boetius, I., Boetius, J., 1980. Experimental maturation of female silver eels, *Anguilla anguilla*. Estimates of fecundity and energy reserves for migration and spawning, Dana 1, 1–28.

Cooney, M.A., Malcuit, C., Cheon, B., Holland, M.K., Fissore, R.A., D'Cruz, N.T., 2010. Species-specific differences in the activity and nuclear localization of murine and bovine phospholipase C zeta 1. Biol. Reprod. 83, 92-101.

Coward, K., Ponting, C.P., Chang, H.Y., Hibbitt, O., Savolainen, P., Jones, K.T., Parrington, J., 2005. Phospholipase Cζ, the trigger of egg activation in mammals, is present in a non-mammalian species. Reproduction 130, 157-163.

Coward, K., Ponting, C.P., Zhang, N., Young, C., Huang, C.J., Chou, C.M., Kashir, J., Fissore, R.A., Parrington, J., 2011. Identification and functional analysis of an ovarian form of the egg activation factor phospholipase C zeta (PLCζ) in pufferfish. Mol. Reprod. Dev. 78, 48-56.

Cox, L.J., Larman, M.G., Saunders, C.M., Hashimoto, K., Swann, K., Lai, F.A., 2002. Sperm phospholipase Czeta from humans and cynomolgus monkeys triggers Ca^{2+} oscillations, activation and development of mouse oocytes. Reproduction 124, 611-623.

Dufour, S., Burzawa-Gerard, E., Le Belle, N., Sbaihi, M., Vidal, B., 2003. Reproductive endocrinology of the European eel, *Anguilla anguilla*. In: Aida, K., Tsukamoto, K., Yamauchi, K. (Eds.), Eel Biology, Springer, Tokyo, pp. 373-383.

Evans, J.P., Kopf, G.S., 1998. Molecular mechanisms of sperm-egg interactions and egg activation. Andrologia 30, 297-307.

Gallego, V., Mazzeo, I., Vílchez, M.C., Peñaranda, D.S., Carneiro, P.C.F., Pérez, L., Asturiano, J.F., 2012. Study of the effects of thermal regime and alternative hormonal treatments on the reproductive performance of European eel males (*Anguilla anguilla*) during induced sexual maturation. Aquaculture 354-355, 7-16.

Gallego, V., Vílchez, M.C., Peñaranda, D.S., Pérez, L., Herráez, M.P., Asturiano, J.F., Martínez-Pastor, F., 2014. The subpopulation pattern of eel sperm is affected by post-activation time, hormonal treatment and thermal regimen. Reprod. Fertil. Dev. 27, 529-543

Henkel, C.V., Burgerhout, E., de Wijze, D.L., Dirks, R.P., Minegishi, Y., Jansen, H.J., Spaink, H.P., Dufour, S., Weltzien, F.A., Tsukamoto, K., van den Thillart, G.E., 2012a. Primitive duplicate Hox clusters in the European eel's genome. PLoS One 7:e32231.

Henkel, C.V., Dirks, R.P., de Wijze, D.L., Minegishi, Y., Aoyama, J., Jansen, H.J., Turner, B., Knudsen, B., Bundgaard, M., Hvam, K.L., Boetzer, M., Pirovano, W., Weltzien, F.A., Dufour, S., Tsukamoto, K., Spaink, H.P., van den Thillart, G.E., 2012b. First draft genome sequence of the Japanese eel, *Anguilla japonica*. Gene 511, 195-201

Heytens, E., Parrington, J., Coward, K., Young, C., Lambrecht, S., Yoon, S.Y., Fissore, R.A., Hamer, R., Deane, C.M., Ruas, M., Grasa, P., Soleimani, R., Cuvelier, C.A., Gerris, J., Dhont, M., Deforce, D., Leybaert, L., De Sutter, P., 2009. Reduced amounts and abnormal forms of phospholipase C zeta (PLCζ) in spermatozoa from infertile men. Hum. Reprod. 24, 2417-2428.

Hildahl, J., Sandvik, G.K., Edvardsen, R.B., Fagernes, C., Norberg, B., Haug, T.M., Weltzien, F.A., 2011. Identification and gene expression analysis of three GnRH genes in female Atlantic cod during puberty provides insight into GnRH variant gene loss in fish. Gen. Comp. Endocrinol. 172, 458-467.

Horner, V.L., Wolfner, M.F., 2008. Transitioning from egg to embryo: Triggers and mechanisms of egg activation. Dev. Dyn. 237, 527-544.

Huang, H., Zhang, Y., Huang,W.R., Li, S.S., Zhu, P., Liu, Y., Yin, S.W., Liu, X.C., Lin, H.R., 2009. Molecular characterization of marbled eel (*Anguilla marmorata*) gonadotropin subunits and their mRNA expression profiles during artificially induced gonadal development. Gen. Comp. Endocrinol. 162, 192-202.

Igarashi, H., Knott, J.G., Schultz, R.M., Williams, C.J., 2007. Alterations of PLCβ1 in mouse eggs change calcium oscillatory behaviour following fertilization. Dev. Biol. 312, 321-330.

Ito, M., Shikano, T., Oda, S., Horiguchi, T., Tanimoto, S., Awaji, T., Mitani, H., Miyazaki, S., 2008. Difference in Ca^{2+} oscillation-inducing activity and nuclear translocation ability of PLCZ1, an egg activating sperm factor candidate, between mouse, rat, human, and medaka fish. Biol. Reprod. 78, 1081-1090.

Ito, M., Nagaoka, K., Kuroda, K., Kawano, N., Yoshida, K., Harada, Y., Shikano, T., Miyado, M., Oda, S., Toshimori, K., Mizukami, Y., Murata, T., Umezawa, A., Miyazaki, S., Miyado, K., 2010. Arrest of spermatogenesis at round spermatids in PLCZ1-deficient mice. 11th International symposium on Spermatology (abstract). Japan.

Ito, J., Parrington, J., Fissore, RA., 2011. PLCζ and its role as a trigger of development in vertebrates. Mol. Reprod. Dev. 78, 846-853.

Jaffe, L.F., 1983. Sources of calcium in egg activation: A review and hypothesis. Dev. Biol. 99, 265-276.

Jaffe, L.F., 1991. The path of calcium in cytosolic calcium oscillations: A unifying hypothesis. Proc. Natl. Acad. Sci. USA 88, 9883-9887.

Jaffe, L.A., 1990. First messengers at fertilization. J. Reprod. Fertil. Suppl. 42,107-116.

Jaillon, O., Aury, J.M., Brunet, F., Petit, J.L., Stange-Thomann, N., Mauceli, E., Bouneau, L., Fischer, C., Ozouf Costaz, C., Bernot, A., Nicaud, S., Jaffe, D., Fisher, S., Lutfalla, G., Dossat, C., Segurens, B., Dasilva, C., Salanoubat, M., Levy, M., Boudet, N., Castellano, S., Anthouard, V., Jubin, C., Castelli, V., Katinka, M., Vacherie, B., Biémont, C., Skalli, Z., Cattolico, L., Poulain, J., de Berardinis, V., Cruaud, C., Duprat, S., Brottier, P., Coutanceau, J.P., Gouzy, J., Parra, G., Lardier, G., Chapple, C., McKernan, K.J., McEwan, P., Bosak, S., Kellis, M., Volff, J.N., Guigó, R., Zody, M.C., Mesirov, J., Lindblad-Toh, K., Birre, B., Nusbaum, C., Kahn, D., Robinson-Rechavi, M., Laudet, V., Schachter, V., Quétier, F., Saurin, W., Scarpelli, C., Wincker, P., Lander, E.S., Weissenbach, J., Roest Crollius, H., 2004. Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype. Nature 431, 946-957.

Jones, K. T., Nixon, V. L., 2000. Sperm-Induced Ca^{2+} Oscillations in Mouse Oocytes and Eggs Can Be Mimicked by Photolysis of Caged Inositol 1,4,5-Trisphosphate: Evidence to Support a Continuous Low Level Production of Inositol 1,4,5-Trisphosphate during Mammalian Fertilization. Dev. Biol. 225, 1–12

Kashir, J., Heindryckx, B., Jones, C., De Sutter, P., Parrington, J., Coward, K., 2010. Oocyte activation, phospholipase C zeta and human infertility. Hum. Reprod. Update 16, 690-703.

Kashir, J., Deguchi, R., Jones, C., Coward, K., Stricker, SA., 2013. Comparative biology of sperm factors and fertilization-induced calcium signals across the animal kingdom. Mol. Reprod. Dev. 80, 787-815

Knott, J.G., Kurokawa, M., Fissore, R.A., Schultz, R.M., Williams, C.J., 2005. Transgenic RNA interference reveals role for mouse sperm phospholipase Cζ in triggering Ca^{2+} oscillations during fertilization. Biol. Reprod. 72, 992-996.

Kouchi, Z., Fukami, K., Shikano, T., Oda, S., Nakamura, Y., Takenawa, T., Miyazaki, S., 2004. Recombinant phospholipase Cζ has high Ca^{2+} sensitivity and induces Ca^{2+} oscillations in mouse eggs. J. Biol. Chem. 279, 10408-10412.

Kouchi, Z., Shikano, T., Nakamura, Y., Shirakawa, H., Fukami, K., Miyazaki, S., 2005. The role of EF-hand domains and C2 domain in regulation of enzymatic activity of phospholipase Cζ. J. Biol. Chem. 280, 21015-21021.

Kuroda, K., Ito, M., Shikano, T., Awaji, T., Yoda, A., Takeuchi, H., Kinoshita, K., Miyazaki, S., 2006. The role of X/Y linker region and N-terminal EF-hand domain in nuclear translocation and Ca^{2+} oscillation inducing activities of phospholipase Cζ, a mammalian egg activating factor. J. Biol. Chem. 281, 27794-27805.

Kyozuka, K., Deguchi, R., Mohri, T., Miyazaki, S., 1998. Injection of sperm extract mimics spatiotemporal dynamics of Ca^{2+} responses and progression of meiosis at fertilization of ascidian oocytes. Development 125, 4099-4105.

Mazzeo, I., Peñaranda, D.S., Gallego, V., Baloche, S., Nourizadeh-Lillabadi, R., Tveiten, H., Dufour, S., Asturiano, J.F., Weltzien, F.A., Pérez, L., 2014. Temperature modulates the progression of vitellogenesis in European eel. Aquaculture 434, 38-47.

Miao, Y.L., Williams, C.J., 2012. Calcium signaling in mammalian egg activation and embryo development: The influence of subcellular localization. Mol. Reprod. Dev. 79, 742-756.

Miyazaki, S., Shirakawa, H., Nakada, K., Honda, Y., 1993. Essential role of the inositol 1,4,5-trisphosphate receptor/Ca^{2+} release channel in Ca^{2+} waves and Ca^{2+} oscillations at fertilization of mammalian eggs. Dev. Biol. 158, 62-78.

Mizushima, S., Takagi, S., Ono, T., Atsumi, Y., Tsukada, A., Saito, N., Shimada, K., 2009. Phospholipase Cζ mRNA expression and its potency during spermatogenesis for activation of quail oocyte as a sperm factor. Mol. Reprod. Dev. 76, 1200-1207.

Ohta, H., Kagawa, H., Tanaka, H., Okuzawa, K., Hirose, K., 1996. Milt production in the Japanese eel *Anguilla japonica* induced by repeated injections of human chorionic gonadotropin. Fish. Sci. 62, 44-49.

Ohta, H., Kagawa, H., Tanaka, H., Okuzawa, K., Iinuma, N., Hirose, K., 1997. Artificial induction of maturation and fertilization in the Japanese eel, *Anguilla japonica*. Fish Physiol. Biochem. 17, 163-169.

Pankhurst, N.W., 1982. Relation of visual changes to the onset of sexual maturation in the European eel *Anguilla anguilla* (L.). J. Fish Biol. 21, 127-140

Parrington, J., Davis, L.C., Galione, A., Wessel, G., 2007. Flipping the switch: How a sperm activates the egg at fertilization. Dev. Dyn. 236, 2027-2038.

Pasqualini, C., Vidal, B., Belle, N.L.E., Sbaihi, M., Weltzien, F.A., Vernier, P., Zohar, Y., Dufour, S., 2004. An antagonist to GnRH in the control of reproduction in teleost fish: dopaminergic inhibition. Ancestral origin and differential conservation within vertebrates. J. Soc. Biol. 198, 61-67.

Peñaranda, D.S., Pérez, L., Gallego, V., Jover, M., Tveiten, H., Baloche, S., Dufour, S., Asturiano, J.F., 2010. Molecular and physiological study of the artificial maturation process in European eel males: from brain to testis. Gen. Comp. Endocrinol. 166, 160-171.

Peñaranda, D.S., Mazzeo, I., Hildahl, J., Gallego, V., Nourizadeh-Lillabadi, R., Pérez, L., Asturiano, J.F., Weltzien, F.A., 2013. Molecular characterization of three GnRH receptors in the European eel, *Anguilla anguilla*: tissue-distribution and changes in transcript abundance during artificially induced sexual development. Mol. Cell. Endocrinol. 369, 1-14.

Pérez, L., Asturiano, J.F., Tomás, A., Zegrari, S., Barrera, R., Espinós, J.F., Navarro, J.C., Jover, M., 2000. Induction of maturation and spermation in the male European eel: assessment of sperm quality throughout treatment. J. Fish Biol. 57, 1488-1504.

Pérez, L., Peñaranda, D.S., Dufour, S., Baloche, S., Palstra, A.P., van Den Thillart, G.E.E.J.M., Asturiano, J.F., 2011. Influence of temperature regime on endocrine parameters and vitellogenesis

during experimental maturation of European eel (*Anguilla anguilla*) females. Gen. Comp. Endocrinol. 174, 51-59.

Pfaffl, M.W., Tichopad, A., Prgomet, C., Neuvians, T.P., 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. Biotechnol. Lett. 26, 509-515.

Runft, L.L., Jaffe, L.A., Mehlmann, L.M., 2002. Egg activation at fertilization: where it all begins. Dev. Biol. 245, 237-254

Saunders, C.M., Larman, M.G., Parrington, J., Cox, L.J., Royse, J., Blayney, L.M., Swann, K., Lai, F.A., 2002. PLCζ: A sperm-specific trigger of Ca^{2+} oscillations in eggs and embryo development. Development 129, 3533-3544.

Saunders, C.M., Swann, K., Lai, F.A., 2007. PLCζ, a sperm-specific PLC and its potential role in fertilization. Biochem. Soc. Symp. 74, 23-36.

Stamatakis, A., Ott, M., 2008. Efficient computation of the phylogenetic likelihood function on multi-gene alignments and multi-core architectures. Phil. Trans. R. Soc. B 363, 3977-3984.

Stricker, S.A., 1999. Comparative biology of calcium signalling during fertilization and egg activation in animals. Dev. Biol. 211, 157-176.

Swann, K., 1990. A cytosolic sperm factor stimulates repetitive calcium increases and mimics fertilization in hamster eggs. Development 110, 1295-1302.

Swann, K., Lai, F.A., 2013. PLCζ and the initiation of Ca^{2+} oscillations in fertilizing mammalian eggs. Cell Calcium 53, 55-62.

Swann, K., Yu, Y., 2008. The dynamics of calcium oscillations that activate mammalian eggs. Int. J. Dev. Biol. 52, 585-594.

Swann, K., Saunders, C.M., Rogers, N.T., Lai, F.A., 2006. PLCζ (zeta): A sperm protein that triggers Ca^{2+} oscillations and egg activation in mammals. Semin. Cell Dev. Biol. 17, 264-273.

706 Tarin, J.J., 2000. Fertilization in protozoa and metazoan animals: a comparative overview. In: Tarin,
707 J.J., Cano, A. (Eds.), Biochemistry and Molecular Biology of Fishes, Cellular and Molecular Aspects,
708 Springer, Berlin Heidelberg, pp 277-314.
709
710 Tesch, F.W., 1978. Telemetric observations on the spawning migration of the eel (*Anguilla anguilla*)
711 west of the European continental shelf. Environ. Biol. Fishes 3, 203-209.
712
713 Thompson, J.D., Higgins, D.G., Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of
714 progressive multiple sequence alignment through sequence weighting, position-specific gap penalties
715 and weight matrix choice. Nucleic Acids Res. 22, 4673-4680.
716
717 Vidal, B., Pasqualini, C., Le Belle, N., Holland, M.C.H., Sbaihi, M., Vernier, P., Zohar, Y., Dufour,
718 S., 2004. Dopamine inhibits luteinizing hormone synthesis and release in the juvenile European eel:
719 neuroendocrine lock for the onset of puberty. Biol. Reprod. 71, 1491-1500.
720
721 Weltzien, F.A., Pasqualini, C., Sébert, M.E., Vidal, B., Le Belle, N., Kah, O., Vernier, P., Dufour, S.,
722 2006. Androgen-dependent stimulation of brain dopaminergic systems in the female European eel
723 (*Anguilla anguilla*), Endocrinology 147, 2964-2973.
724
725 Weltzien, F.A., Pasqualini, C., Vernier, P., Dufour, S., 2005. A quantitative real-time RT-PCR assay
726 for European eel tyrosine hydroxylase. Gen. Comp. Endocrinol. 142, 134-142.
727
728 Yoda, A., Oda, S., Shikano, T., Kouchi, Z., Awaji, T., Shirakawa, H., Kinoshita, K., Miyazaki, S.,
729 2004. Ca^{2+} oscillation-inducing phospholipase C zeta expressed in mouse eggs is accumulated to the
730 pronucleus during egg activation. Dev. Biol. 268, 245-257.
731
732 Yoneda, A., Kashima, M., Yoshida, S., Terada, K., Nakagawa, S., Sakamoto, A., Hayakawa, K.,
733 Suzuki, K., Ueda, J., Watanabe, T., 2006. Molecular cloning, testicular postnatal expression, and
734 oocyte-activating potential of porcine phospholipase Cζ. Reproduction 132, 393-401.
735
736 Yoon, S.Y., Jellerette, T., Salicioni, A.M., Lee, H.C., Yoo, M.S., Coward, K., Parrington, J., Grow,
737 D., Cibelli, J.B., Visconti, P.E., Mager, J., Fissore, R.A., 2008. Human sperm devoid of PLC, zeta 1
738 fail to induce Ca^{2+} release and are unable to initiate the first step of embryo development. J. Clin.
739 Invest. 118, 3671-3681.
740

741 Yoshida, N., Amanai, M., Fukui, T., Kajikawa, E., Brahmajosyula, M., Iwahori, A., Nakano, Y.,
742 Shoji, S., Diebold, J., Hessel, H., Huss, R, Perry, A.C.F., 2007. Broad, ectopic expression of the sperm
743 protein PLCZ1 induces parthenogenesis and ovarian tumours in mice. Development 134, 3941-3952.
744
745 Young, C., Grasa, P., Coward, K., Davis, L.C., Parrington, J., 2009. Phospholipase C zeta undergoes
746 dynamic changes in its pattern of localization in sperm during capacitation and the acrosome reaction.
747 Fertil. Steril. 91, 2230-2242.
748
749 Yu, Y., Halet, G., Lai, F.A., Swann, K., 2008. Regulation of diacylglycerol production and protein
750 kinase C stimulation during sperm- and PLCζ-mediated mouse egg activation. Biol. Cell. 100, 633-
751 643.
752
753

754

755 **Table I**

Sequence name	Species name	Accession number
PLCZ1-001	*Mus musculus*	ENSMUSP00000032356
1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase zeta-1-like, partial	*Anolis carolinensis*	XP_008108585
PLCZ1-201	*Gallus gallus*	ENSGALP00000021386
PLCZ1-201	*Oryzias latipes*	ENSORLT00000005752
PLCZ1-201	*Latimeria chalumnae*	ENSLACT00000000957
PLCZ1-201	*Takifugu rubripes*	ENSTRUP00000043591
PLCZ1-201	*Homo sapiens*	ENSP00000402358
PLCZ1-201	*Lepisosteus oculatus*	ENSLOCP00000018988
PLCZ1-201	*Gasterosteus aculeatus*	ENSGACP00000013217
PLCZ1-201	*Tetraodon nigroviridis*	ENSTNIP00000003915
PLCB1-005	*Homo sapiens*	ENSP00000367908
PLCB1-005	*Mus musculus*	ENSMUSP00000105743

756

757

Figure 1

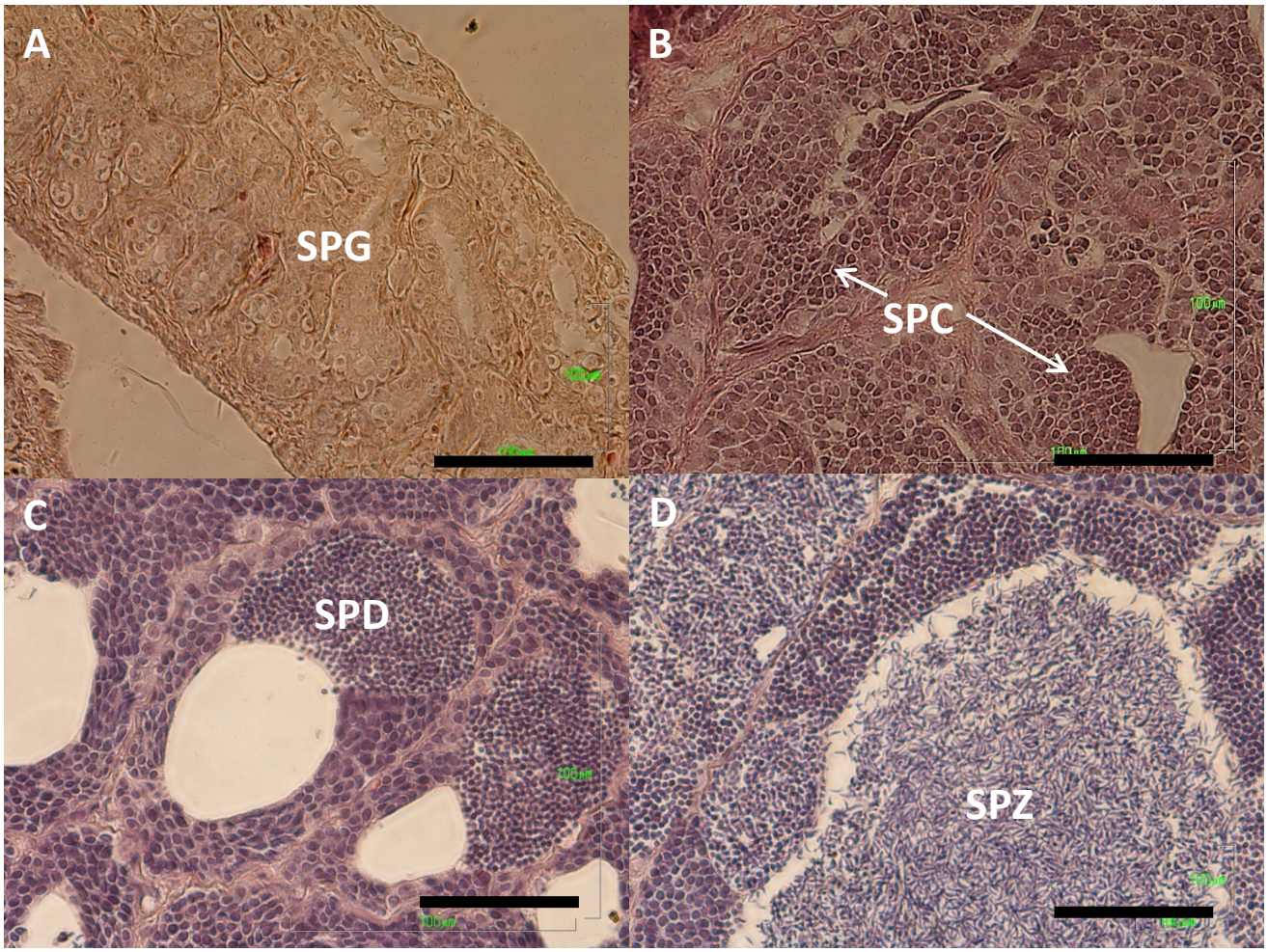
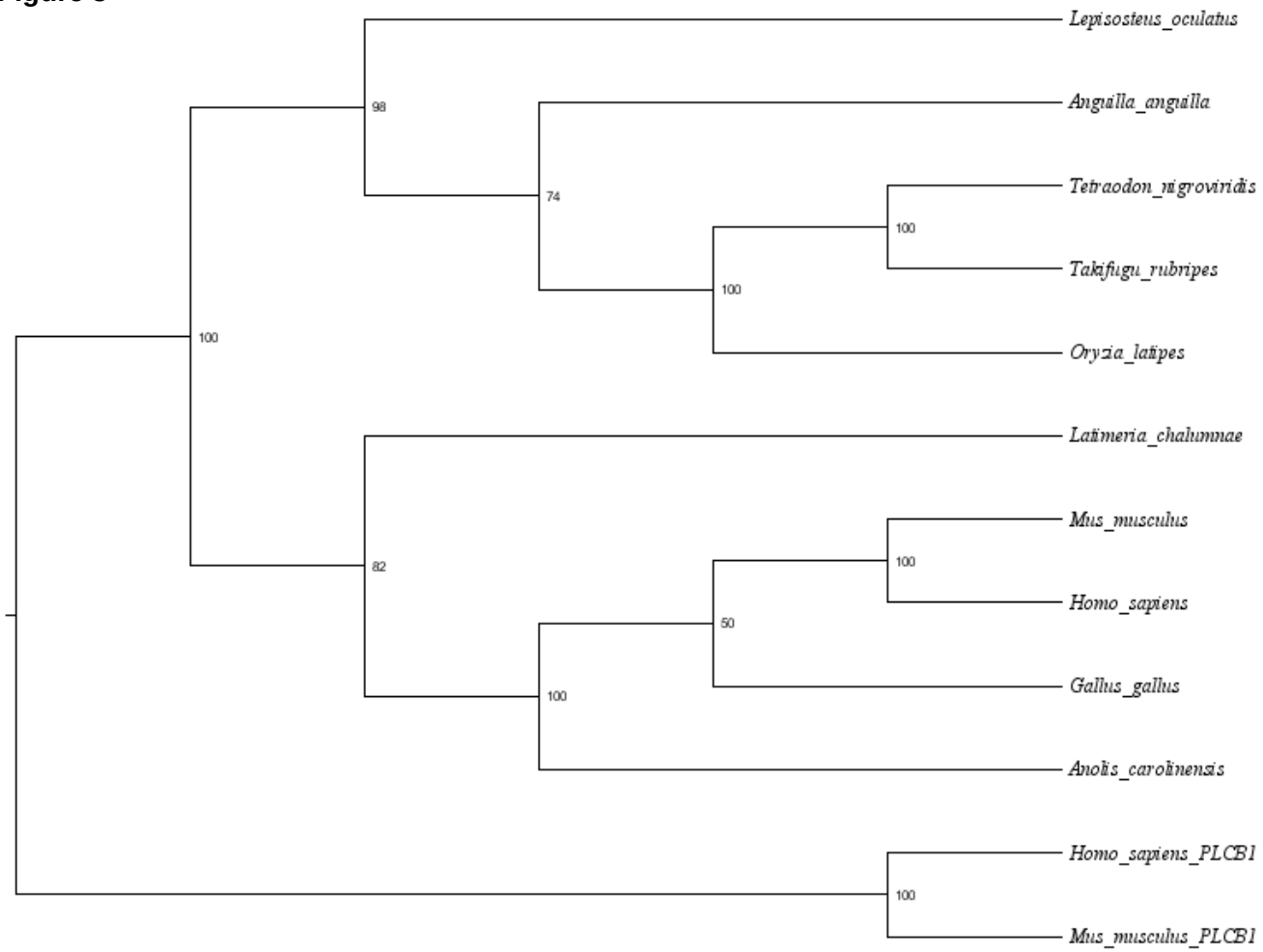
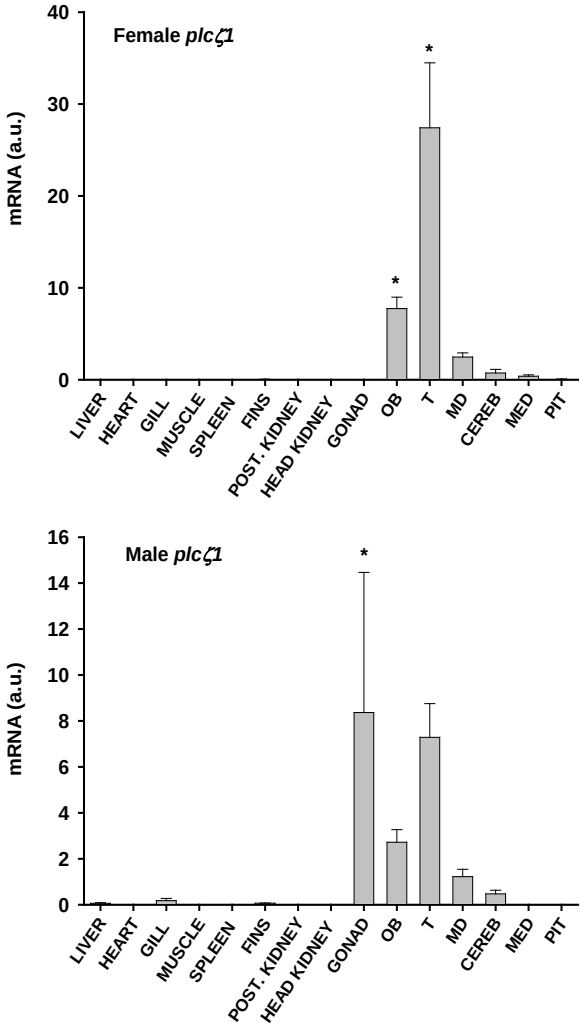


Figure 2

Figure 3


Lepisosteus_oculatus
Anguilla_anguilla
Tetraodon_nigroviridis
Takifugu_rubripes
Oryzia_latipes
Latimeria_chalumnae
Mus_musculus
Homo_sapiens
Gallus_gallus
Anolis_carolinensis
Homo_sapiens_PLCB1
Mus_musculus_PLCB1
98
74
100
100
100
82
100
50
100
100

778
779 **Figure 4**
780

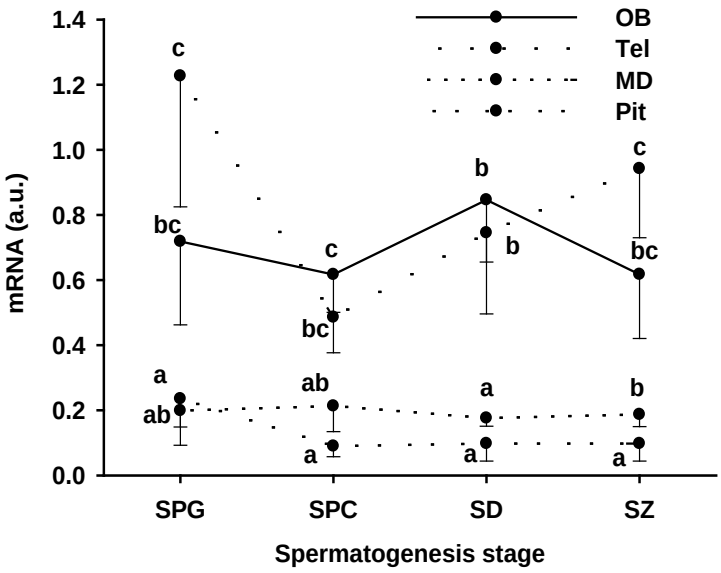


781 **Figure 5**

782

783

784



785 **Figure 6**

786

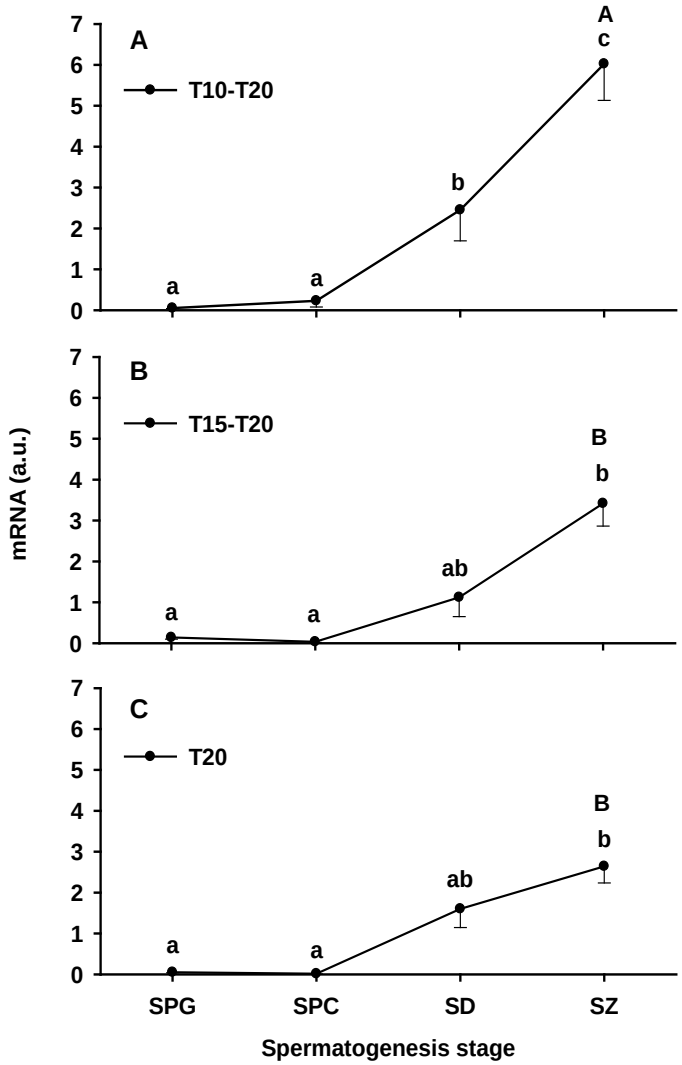


Table legend

Table I. Accession number of the sequences used for phylogenetic analyses

Figure legend

Figure 1. Histological sections of eel testis at different developmental stages during chorionic gonadotropin (hCG) hormonal treatment. A: spermatogonia; B: spermatocyte; C: spermatids, D: spermiation. SPG= spermatogonia; SPC: spermatocytes; SPD: spermatids; SPZ: spermatozoa, Scale bar: A=100µm; B, C, D= 50µm

Figure 2. Multiple sequence alignment of European eel, Mouse and Fugu PLCζ at amino acid level. "*" Conserved residues, ":" conservation between groups of strongly similar properties, "." conservation between groups of weakly similar properties. Residues in red: AVFPMILW, small and hydrophobic. Residues in blue: DE, acidic. Residues in pink : RK, Basic – H. Residues in green: STYHCNGQ, Hydroxyl + sulfhydryl + amine + G. EF-hand like domain, X-domain, Y-domain and C2-domain are shown above the alignment.

Figure 3. Consensus phylogenetic tree of the vertebrate Phospholipase C zeta. This phylogenetic tree was constructed based on the amino-acid sequences of PLCζ (for the references of each sequence see Table I) using the Maximum Likelihood method with 1000 bootstrap replicates. The number shown at each branch node indicates the bootstrap value (%). The tree was rooted using the two sequences of the mouse and human phospholipase beta1.

Figure 4. Tissue distribution of (A) *plcζ1 in female*, (B) *plcζ1* in immature *male* European eel. Data are normalised to eel *arp*. Asterisk indicates significant differences between males and females in a same tissue ($p<0.05$; n=3). Values are presented as means ± SEM ($n = 3$). OB : olfactory bulb, T : Telencephalon, M/D : mes-/di-encephalon, CEREB : cerebellum, MED : medulla oblongata, PIT, pituitary.

Figure 5. European eel *plcζ1* expressions during experimental maturation in 3 brain parts and in the pituitary in fishs kept at 20 degrees. Data are normalised to eel *arp*. Small letters indicate significant differences between the olfactory bulb, the telencephalon, the mes-/di-encephalon and the pituitary, in the same gonad development stage ($p<0.05$; n=6-12). Results are given as mean ± SEM. SPG= Spermatogonia stage, SC= Spermatocyte stage, SD= Spermatid stage, SZ= Spermatozoa stage.

See main text for definition of gonad developmental stages, OB: olfactory bulb, Tel: telencephalon, MD: mes-/di-encephalon, Pit: pituitary.

Figure 6. European eel *plcζ1* expressions during experimental maturation in fish testis kept in different thermal regimes. Data are normalised to eel *arp*. Capital letters indicate significant differences between the thermal treatments in the same gonad development stage ($p<0.05$; n=8-17). Small letters indicate significant differences through spermotogenesis in the same thermal treatment ($p<0.05$; n=6-17). Results are given as mean ± SEM.. SPG= Spermatogonia stage, SC= Spermatocyte stage, SD= Spermatid stage, SZ= Spermatozoa stage. See main text for definition of gonad developmental stages.