Comparative Biochemistry and Physiology, Part A 290 (2024) 111590

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Superoxidase dismutases (SODs) in the European eel: Gene characterization, expression response to temperature combined with hormonal maturation and possible migratory implications

L. Ferrão, M. Blanes-García, L. Pérez, J.F. Asturiano, M. Morini *

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

ARTICLE INFO

Edited by: Michael Hedrick

Keywords:
Antioxidant
Enzymes
Temperature
Migration
Anguilla anguilla

ABSTRACT

Superoxide dismutases (SODs) are antioxidant enzymes that protect cells from oxidation. Three SODs have been identified in mammals, but there is limited information in teleosts. This study investigates SODs in the European eel and their expression patterns during testis maturation. Phylogenetic and synteny analyses revealed SODs paralogs and their evolution in vertebrates. The eel possesses one SOD1 and two SOD2/3 (a and b), indicating SOD2 and SOD3 duplication in elopomorphs. SODs expression were then evaluated in various male and female tissues. SOD1 is more expressed in females, while SOD2a and SOD2b dominate brain-pituitary-gonad tissues in both sexes. SOD3a showed predominant expression in the ovary and the male livers, whereas SOD3b was found in the pituitary and brain of both sexes. The effects of different maturation protocols (standard hormonal treatment vs. same protocol preceded with cold seawater pre-treatment) on SODs expression during testis maturation were evaluated. Salinity increase at the onset of standard treatment at 20 °C, simulating early migration, upregulated SOD1, SOD2a, and SOD2b, coinciding with spermatogonia type A differentiated cells dominance. Thereafter, SOD2a and SOD3a decreased, while SOD2b increased during hormonal treatment-induced spermatogenesis. Pre-treatment with seawater at 10 °C, mimicking the conditions at the beginning of the seawater migration, downregulated SOD1 but increased SOD3a expression. Finally, the standard hormonal treatment, replicating spawning at higher temperatures, downregulated SOD1 in eels without any pre-treatment while SOD2a expression increased in pre-treated eels. This study revealed tissue-specific, sex-dependent, and maturation-related SOD expression patterns, predicting SODs dynamic expression profiles during their reproductive migration.

1. Introduction

The catadromous life cycle of the European eel (*Anguilla anguilla*) is complex. The adult eels undertake an extensive reproductive migration across the Atlantic Ocean, that lasts up to 6–7 months, to reach their spawning site in the Sargasso Sea (Aarestrup et al., 2009). Then, the larvae swim for 8–9 months back to the eastern Atlantic coast, where they transform into glass eels and grow until they reach maturity (Van Ginneken and Maes, 2005). In addition to its complex life cycle, the European eel holds a phylogenetical position at the basis of the teleosts, the largest group of vertebrates (Henkel et al., 2012a, 2012b), making this species an ideal model organism to study ancient regulatory functions. Nevertheless, the European eel survival is threatened as the population of the species has suffered a significant decline and is considered "Critically Endangered" by the International Union for Conservation of Nature (IUCN) (Pike et al., 2020). Despite this, the European eel still supports fisheries and aquaculture industries in Europe, as it is common practice to capture wild glass eels from the wild and grow them until they reach adulthood. Thus, it is crucial to develop innovative protocols to reproduce this species in captivity to reduce the reliance on wild populations for the eel industry and restocking purposes. However, the successful completion of the full life cycle of the European eel in captivity remains a challenge, limiting eel aquaculture production (Asturiano, 2020). One of the major obstacles encountered in this process include the search for alternative methods to induce eel sexual maturation for the acquisition of high-quality gametes which still relies on expensive and long-term hormonal treatments.

Silvering in the European eels marks the end of the growth phase and

* Corresponding author at: Universitat Politècnica de València, Instituto de Ciencia y Tecnología Animal (Edificio 7G), Camino de Vera s/n, 46022, Valencia, Spain.
E-mail address: mamo14@upvnet.upv.es (M. Morini).

https://doi.org/10.1016/j.cbpa.2024.111590
Received 7 November 2023; Received in revised form 25 January 2024; Accepted 25 January 2024
Available online 26 January 2024

the onset of sexual maturation, and it is characterized by the change in skin color as maturing eels, initially yellow or dark green, turn to a silvery-white shade (Rousseau et al., 2009) Other changes in silver eels include enlarged eyes, elongated pectoral fins, increased skin thickness and gonad weight increase. As most teleosts, European eels present a pair of testes with lobular structure, where spermatogonia are found along the lobule (Pérez et al., 2009). To mature the testes in captivity conditions, the standard protocol in male eels consists of first transferring male eels from freshwater to seawater at 20 °C and once acclimatized, eel spermatogenesis is stimulated with weekly injections of recombinant human chorionic gonadotropin (hCGrec) (Gallego et al., 2012; Herranz-Jusdado et al., 2018). However, in the last years, our team focused on understanding the effect of environmental factors, especially temperature, on European eel maturation by carrying out experiments with the application of thermal regimes with or without hormonal treatments (Peñaranda et al., 2016; Rozenfeld et al., 2019a). It has been proposed that temperature plays a crucial role in eel gonad maturation through the modulation of steroidogenic enzymes gene expression. At low temperatures, the androgen synthesis occurs through the upregulation of steroidogenic enzymes, resulting in early spermatogonia (SPG) proliferation. Then, the testis maturation process stops until the water temperature increases, which induces a steroidogenesis process shift from androgen to progesterone synthesis (Peñaranda et al., 2016). Recently, another study with male European eels (without any hormonal treatment) corroborated these results by showing that cold seawater treatment stimulates androgen production in early sexual development, but the effect of androgens on SPG differentiation is suppressed by low temperature. Moreover, the transcriptomic analysis revealed that genes are differentially expressed in the brain-pituitary-gonad (BPG) axis of eels maintained in cold seawater in comparison with males maintained at 20 °C (Rozenfeld et al., 2019a, 2019b). These previous studies support the idea that thermal pre-treatment (before the onset of the standard hormonal treatment) with low temperature replicates the physiological transition of the European eel during the early stages of their oceanic migration. However, further research is required to identify the genes involved in this thermoception process which seems to regulate sexual maturation.

Superoxide dismutases (SODs) are key antioxidant enzymes that scavenge the superoxide radicals ($O2^{-}$) and convert into hydrogen peroxide (H_2O_2), which produces less cellular damage (Wang et al., 2018). However, it is important to mention that although ROS are implicated in damaging processes, they can also be helpful depending on their levels. At concentrations so high that exceed the antioxidant defenses, ROS can lead to cellular damage by oxidative stress (Ghezzi et al., 2017). However, when ROS are generated at controlled levels, they can serve as signaling molecules in cellular mechanisms, mediating crucial physiological functions (Aitken and Drevet, 2020). In mammals, three SOD genes have been identified in different cellular localizations. SOD1 (intracellular copper/zinc SOD) and SOD2 (manganese SOD) are located in the cytoplasm and in the mitochondrial matrix respectively, while SOD3 (extracellular copper/zinc SOD) is mostly found in the extracellular space (Andrés et al., 2022; Zhang et al., 2020). In teleosts, all three SODs have been characterized in the zebrafish (*Danio rerio*) (Matthiesen et al., 2021), fugu (*Takifugu rubripes*) (Bayir, 2020), European sea bass (*Dicentrarchus labrax*) (L'Honoré et al., 2021), Atlantic salmon (*Salmo salar*) (Lazado and Voldvik, 2020) and marbled eel (*Anguilla marmorata*) (Wang et al., 2016a, 2016b). These studies revealed that the expression patterns of SODs can be influenced by various environmental factors (salinity, temperature, starvation, pathogens), suggesting that SODs play a critical role in maintaining homeostatic balance. However, SODs characterization in teleosts is incomplete, as most studies only report the expression of one or two specific SODs. Therefore, a complete description of SODs could provide valuable knowledge about the various mechanisms in which each SOD gene might be involved. Additionally, a few studies explore SODs role in fish spermatogenesis, a dynamic process involving cell divisions with high ROS production (Guerriero et al., 2014). In Japanese eels (*Anguilla japonica*) treated with hCG, Celino et al. (2011) detected oxidative activity throughout the hormonal treatment, indicating a constant ROS generation during sexual maturation. However, in testicular cultured cells matured with androgen, SPG cells found in early spermatogenesis presented high levels of SOD1 which provided them tolerance to ROS damage. However, once SPG developed into more advanced-stage germ cells with androgen treatment, the SOD1 levels decreased (Celino et al., 2011). This study highlighted the importance of SOD1 in early spermatogenesis, but it did not explore how mature germ cells are shielded from upcoming ROS production in the later stages. Celino et al. (2012) also showed in early proliferating SPG cultured cells supplemented with androgen that the total SOD activity decreased under oxidative conditions, leading to ROS production and cell apoptosis. The precise mechanisms behind the balance between the oxidant and the antioxidant response in later development stages behind SPG proliferation remain unknown. Thus, evaluating the specific role of SODs throughout complete sexual maturation achieves great relevance, especially in the European eel as these antioxidant enzymes may be involved in response to different combined external factors.

The present study aimed to characterize SODs in the European eel, and further analyze their expression profiles during sexual maturation induced with standard hormonal treatment or using cold seawater as a pre-treatment, previous to hormonal induction. This is the first approach to describe all SODs in the European eel and to understand their specific role in their reproduction.

2. Material and methods

2.1. Identification of SOD sequences

2.1.1. Phylogenetic analyses of SOD gene family in vertebrates

SOD gene sequences from vertebrate species representing different groups were obtained by searching in the NCBI (https://www.ncbi.nlm.nih.gov/) or Ensembl (Ensembl release 103, https://www.ensembl.org/index.html) genome databases using the TBLASTN algorithm.

For each SOD gene, phylogenetic analyses were conducted using species that hold representative phylogenetic positions in the vertebrate phylogenetic tree. These included two chondrichthyans (elephant shark, *Callorhinchus milii*; spotted catshark, *Scyliorhinus canicula*), an early diverging sarcopterygian (coelacanth, *Latimeria chalumnae*), various tetrapods such as sauropsids (green anole, *Anolis carolinensis*; common snapping turtle, *Chelydra serpentina*; Australian seawater crocodile, *Crocodylus porosus*; duck, *Anas platyrhynchos*), an amphibian (tropical clawed frog, *Xenopus tropicalis*) and mammals (human, *Homo sapiens*; platypus, *Ornithorhynchus anatinus*). Additionally, a non-teleost actinopterygian (spotted gar, *Lepisosteus oculatus*), two basal teleosts (Asian bonytongue, *Scleropages formosus*; European eel, *Anguilla anguilla*) and several teleosts (Atlantic herring, *Clupea harengus*; zebrafish, *Danio rerio*; Atlantic cod, *Gadus morhua*; medaka, *Oryzias latipes*; pufferfish, *Takifugu rubripes*; turbot, *Scophthalmus maximus*; Northern pike, *Esox lucius*; Atlantic salmon, *Salmo salar*; rainbow trout, *Onchorhynchus mykiss*) were included.

Phylogenetic trees were structured based on the amino acid sequences of each SOD gene (Supplementary Table S1) with the chondrichthyan SOD sequences serving as the root. The sequence alignment was carried out using Clustal Omega (Sievers et al., 2011) with Seaview 5.0.1 software (http://doua.prabi.fr/software/seaview) and adjustments were made manually. The JTT (Jones, Taylor, and Thornton) protein substitution matrix of the aligned sequences was defined using ProTest software (Abascal et al., 2005). The phylogenetic trees for each SOD gene were constructed using the RAXML program (Randomized Accelerated Maximum Likelihood) from CIPRES Science Gateway (Miller et al., 2010) with 1000 bootstrap replicates, and the resulting trees were visualized using Figtree 1.4.4 (http://tree.bio.ed.ac.uk/).

2.1.2. Synteny analyses of the SOD genes in vertebrates

For each SOD gene, synteny analyses of SODs genomic regions were conducted using species that hold representative phylogenetic positions in the vertebrate phylogenetic tree. These included one chondrichthyan (spotted catshark), two sarcopterygians (human and tropical clawed frog), and actinopterygians (spotted gar and various teleosts, such as Asian bonytongue, European eel, zebrafish, Atlantic cod, pufferfish, Japanese medaka, northern pike, and Atlantic salmon). For the synteny analyses, the neighboring genes of each SODs gene were identified using Genomicus PhyloView of Genomicus v100.01, with the spotted gar serving as the starting template. BLAST analyses were subsequently executed within the ENSEMBL and/or NCBI databases to detect potential paralogs among the neighboring genes, or to undercover any non-annotated neighboring genes that might be present in the considered genome species. Due to the European eel genome unavailability in the Ensembl and Genomicus databases, the European eel genome (GCF_013347855.1, fAngAng1 genome, Future Genomics Technologies B.V., Leiden, Netherland) was used as our primary reference for characterizing the surrounding genomic regions, which was conducted in NCBI. SODs neighboring gene references and specific locations for each species provided in Supplementary Table S2.

2.2. Experimental design

2.2.1. Fish maintenance and handling

Sixty-eight male eels (mean body weight = 89.80 ± 17.01 g) maintained in freshwater at the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia) were transferred to the Fish Reproduction Laboratory in the Polytechnic University of Valencia (Valencia, Spain). After arriving, fish were randomly distributed in 200-L tanks filled with fresh water at 20 °C (at similar conditions to those used in the farm) and equipped with separated recirculation systems and temperature control systems (with heaters and coolers). Then, fish were maintained in freshwater conditions for 3–4 days before being gradually acclimatized over one week to seawater (37 ± 0.3‰ of salinity). The tanks were covered to maintain constant shadow and reduce stress. The fish were not fed during the experiment.

2.2.2. Ethical comittee

This study was carried out in strict accordance with the recommendations given in the Guide for the Care and Use of Laboratory Animals of the Spanish Royal Decree 53/2013 regarding the protection of animals used for scientific purposes (BOE 2013). The protocol was approved by the Experimental Animal Ethics Committee from the Universitat Politècnica de València (UPV) and final permission was given by the local government (Generalitat Valenciana, Permit Number: 2019/VSC/PEA/0073). All efforts were made to minimize suffering and fish were sacrificed with benzocaine overdose followed by decapitation.

2.2.3. SODs expression in different tissues in male and female European eel

To describe the SODs expression among sex and tissues, the previously identified SOD genes were analyzed in male and female European eels, in the gonadal (testis and ovaries) and somatic tissues (heart, eye, muscle, spleen, nose, kidney, skin, lateral line, intestine, liver, gills, fat, pituitary, anterior and posterior brain). For brain sampling, dissection was performed as described by Weltzien et al. (2005). Tissues were collected from 6 males (mean body weight = 89.80 ± 17.01 g) from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia) and 6 wild female eels (mean body weight = 473.75 ± 129.32 g) fished by local fishermen in the Albufera lagoon (Valencia, Spain). Total RNA from all tissues was extracted, reverse-transcripted, and analyzed by real-time qPCR.

2.2.4. SODs expression in male European eel under different maturation conditions

2.2.4.1. Experiment 1. Standard hormonal maturation experiment. Once eels arrived from the fish farm in fresh water (FW) conditions, a group of 8 eels were sacrificed. The water salinity of the remaining males was gradually increased (salinity 37 ± 3 g/L) at 20 °C for 7 days (Fig. 1A). Then, another group of 8 eels maintained in seawater (SW) was sacrificed before starting the hormonal treatment. The maturation induction of the remaining eels was carried out by injecting intraperitoneally hCGrec (Ovitrelle®, Madrid, Spain, 1.5 IU/g fish) weekly as described by Gallego et al. (2012), and groups of 8 eel males were sacrificed after 2 and 4 weeks of hormonal treatment in seawater conditions (2 W and 4 W SW hCGrec).

2.2.4.2. Experiment 2. Cold seawater pre-treatment experiment. The effect of cold seawater pre-treatment was analyzed by comparison with the group previously described in experiment 1 that received standard treatment protocol, serving as a control group in this experiment. For the cold seawater treatment, two groups of male eels were acclimatized to seawater at 10 °C and maintained in these temperature conditions for 2 and 4 weeks (2 W and 4 W Cold SW), respectively (Fig. 1B). Once finished each pre-treatment, groups of 8 eels were sacrificed. Afterward, the water temperature of the remaining eels progressively increased to 20 °C before starting the standard maturation protocol which proceeded as explained above. After 4 weeks of hormonal treatment, another group (one per time of pre-treatment) of 8 eel males was sacrificed.

2.3. Testis sampling

Samples of testicular tissue were collected from sacrificed male eels and were processed for histological and gene expression evaluation, following the same procedures described by Morini et al. (2017). Part of the testis was fixed in 10% paraformaldehyde and adjusted to 7.4 for histological analysis. Other testis samples were stored in 0.5 mL of RNAlater (Ambion Inc., Huntingdon, IK) at −20 °C until total RNA extraction for gene expression analysis.

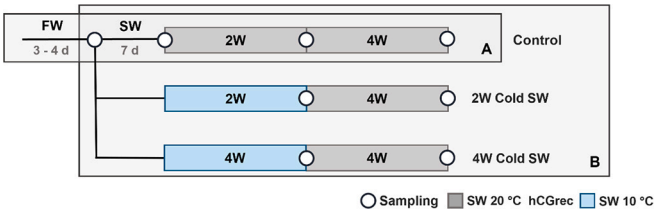

Fig. 1. Representation of experimental design. (A) Standard maturation protocol. Fish were maintained in fresh water (FW, $n = 8$) for 3–4 days and the testis were sampled afterward. The remaining eels were transferred to seawater at 20 °C (SW, n = 8) for 7 days. After this acclimatization period, testis samples were collected after hormonal treatment with recombinant human chorionic gonadotropin (hCGrec). Testis samplings were performed after 2 weeks (2W, n = 8) and after 4 weeks (4 W, n = 8) of hormonal treatment in seawater; (B) Cold seawater pre-treatment protocol. Pre-treated fish without any hormonal treatment were maintained in cold seawater (approximately at 10 °C) for 2 weeks (2 W Cold SW, n = 8) or 4 weeks (4 W Cold SW, n = 8). These pre-treated groups were compared with the control group (without pre-treatment) after the seawater acclimatization period (SW, n = 8) and after hormonal treatment in seawater (SW hCGrec) for 4 weeks (4 W, n = 8). Once each pre-treatment finished, testis samples were collected, and the seawater temperature was increased to 20 °C before receiving hormonal treatment in seawater (SW hCGrec) for 4 weeks (4 W, n = 8). After this period, testis tissue from each pre-treated group was collected.

2.4. Testis histological analyses

Testis samples were dehydrated in increasing percentages of ethanol and embedded in resin (Technovit 7100) according to the instructions of the manufacturer. Sections of 5 μm thickness were cut with a Microm HM325 microtome and stained with toluidine blue 1%. The slides were observed with a Nikon Eclipse E-400 microscope, and pictures were taken with a Nikon DS-5 M camera attached to the microscope (Nikon, Tokyo, Japan). Cell types were categorized as described by Rozenfeld et al. (2019a) for the European eel. The undifferentiated spermatogonia type A cells (SPGAund) showed irregular nuclear membranes and were found individually. Differentiated SPG type A cells (SPGAdiff) formed groups of 2–8 cells within Sertoli cells surroundings, featuring regular nuclear envelopes, one nucleolus and a cytoplasm darker than in SPGAund. SPG type B cells (SPGB) were smaller in size cells with small amount of cytoplasm and nuclei with large amounts of heterochromatin. Spermatocytes (SPC) were rounder and with larger nuclei in comparison with SPGB and contained clear heterochromatin. Spermatids (SPD) were small, rounded cells with highly condensed heterochromatin, mainly located in the inner part of the cyst. Spermatozoa (SPZ) had the characteristic half-moon spermatozoa head shape. FIJI/ImageJ software was used to count the number of each cell type from 5 microscope fields per sample of each experimental group. From these counts, cell types were transformed into percentage data (sum of each cell type found in each slide/sum of total cell counts * 100). The relative abundance percentage of each cell type was registered.

2.5. Gene expression analyses by quantitative real-time PCR

2.5.1. RNA extraction and cDNA synthesis

Total RNA was extracted from samples previously stored in RNA later using Trizol reagent (Life Technologies, Inc., Carlsbad, CA) as described by Morini et al. (2017). The RNA was diluted in 20 μL of DEPC water. To ensure RNA suitability for cDNA synthesis, RNA quantity was determined using a NanoDrop 2000C Spectrophotometer (Fisher Scientific SL, Spain) at 260 nm, and only high-purity RNA (A260/280 > 1.8) was used. For cDNA synthesis, a QuantiTect Reverse Transcription Kit (Qiagen Hilden, Germany) was employed and 20 μL of cDNA were synthesized from 500 ng of total RNA, which was stored at −20 °C until further analysis.

2.5.2. Primer and reference gene

Quantitative real-time Polymerase Chain Reactions (qPCR) were conducted using acid ribosomal phosphoprotein (ARP) and beta-actin (β-ACT) as reference genes. To enable the specific targeting of European eel SOD-specific genes, qPCR primers (Table 1) were designed based on the complete coding sequences of European eel. The primer pairs were designed to span two different exons, preventing the amplification of potential genomic contamination. Primers were designed using Primer3 Software (Whitehead Institute/Massachusetts Institute of Technology, Boston, MA, USA) and acquired from Integrate DNA Technology, Inc. (IDT, Coralville, Inc., IA, USA).

Table 1
Quantitative PCR primer sequences for SOD genes.

Name	Sequence (5′ - 3′)	Orientation	Efficiency
SOD1	CAA TCG ATC ATC GGG CGA AC	Forward	Eff 2.02
	GTT GCC GGT TTT CAG GCT TT	Reverse	
SOD2a	CTG GGG TTC GAG AAG GAG AG	Forward	Eff 1.99
	GTT CAC CAC GTT CCA GAT CG	Reverse	
SOD2b	CAT CAG TGC GGA GAT CAT GC	Forward	Eff 2.00
	CCT GTG CAG TAA CAT CAC CC	Reverse	
SOD3a	TGG AGA TCG TTT ATG TTC AAT CT	Forward	Eff 1.97
	GAC ACT GGG AGC AGC AGT AT	Reverse	
SOD3b	GCA GCC GAA TAT CCA GCA C	Forward	Eff 2.03
	CCC GTT AAC ACC AGG AGC A	Reverse	
b-ACT	CAG CCT TCC TTC CTG GGT	Forward	Eff 2.09
	AGT ATT TGC GCT CGG GTG	Reverse	
ARP	GTG CCA GCT CAG AAC ACT G	Forward	Eff 2.07
	ACA TCG CTC AAG ACT TCA ATG G	Reverse	

2.5.3. Gene expression analysis

qPCR assays were conducted using a model 7500 unit (Applied Biosystems; Foster City, CA, USA) and following the same procedure described by Morini et al. (2017). The qPCR cycling protocol began with an initial activation of Taq polymerase at 95 °C for 10 min, followed by 40 PCR cycles under the following conditions: 95 °C for 15 s and 60 °C for 30 s. Each qPCR mix contained 5 μL of diluted (1:20) DNA template, forward and reverse primers (250 nM each primer), and SYBR Green/ROX Master Mix (12 μL) and DEPC water to a final volume of 20 μL. To evaluate primer efficiencies, serial dilutions (1:20) of the cDNA pool derived from testis tissues were used to set linear regression slopes. A calibrator was included in each run for the corresponding gene using one of these dilutions. Absolute expression of mRNA levels was quantified for tissue screening analysis by using an efficiency-corrected expression without the reference gene Ct (threshold cycles) value (Pflaffl, 2001). Relative expression of normalized mRNA levels was quantified for testis analysis by using efficiency-corrected expression with the multiple reference genes Ct value (Pflaffl, 2001, Hellemans et al., 2007). Both target and reference genes were subjected to duplicate PCR reactions, while a non-template control (cDNA replaced by water) was also included in duplicate PCR reactions for each primer pair.

2.6. Statistical analysis

The mean ± standard error (SEM) was calculated for SOD gene expression data. Shapiro-Wilk and Levene's tests were used to check the normality of data distribution and variance homogeneity, respectively.

Independent Student's *t*-test was used to compare the relative expression in testis sampled from FW and SW individuals. One-way ANOVA or Kruskal-Wallis tests (for non-parametric data) were used to compare the absolute expression from screening tissues and relative expression in testis sampled from SW (no hCGrec) and hCGrec individuals treated for 2 and 4 weeks.

Differences between expression levels in the control group and low-temperature pre-treated groups (SW at 10 °C) before and after hCGrec treatment for 4 weeks were analyzed using One-way ANOVA or Kruskal-Wallis tests (for non-parametric data). Differences between untreated (No hCGrec) and 4-week hCGrec treated (4 W hCGrec) groups within each experimental group were detected using an Independent Student's t-test.

Differences between cell proportions within each experimental group were detected using One-way ANOVA or Kruskal-Wallis tests (for non-parametric data).

In all the cases, significant differences were detected when p-value<0.05. All statistical analyses were performed using the statistical package SPSS version 24.0 for Windows software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Phylogenetic analysis of SOD gene family in vertebrates

Concerning SOD1 phylogeny (Supplementary Fig. 1), two SOD1 genes were found in both chondrichthyan species, the elephant shark, and the spotted catshark. SOD1 sequence positions agree with their established phylogenetic positions. A single SOD1 sequence of sarcopterygian coelacanth clustered at the basis of the monophyletic group of tetrapods SOD1 sequences. All teleosts studied possess a single SOD1 gene, except the Atlantic salmon and the rainbow trout, which exhibit two SOD1 genes.

Concerning SOD2 phylogeny (Supplementary Fig. 2), the two chondrichthyan SOD2 sequences diverged basally from the osteichthyes

SOD2 sequences. In similarity to the SOD1 phylogeny, the coelacanth SOD2 sequence clustered at the basis of the monophyletic tetrapod SOD2 group, while SOD2 sequences of the actinopterygian spotted gar clustered at the base of all teleost SOD2 group. In this clade, most species presented only one SOD2 gene, except the European eel which exhibited two SOD2 genes.

In SOD3 phylogeny (Supplementary Fig. 3), the elephant shark presented three SOD3 genes, while the spotted catshark exhibited one SOD3 gene. The chondrichthyan sequences clustered at the basis of both actinopterygian and sarcopterygian SOD3 clades. One SOD3 gene was found in the coelacanth, which clustered at the base of the tetrapod SOD3 clade. A single SOD3 gene of the spotted gar clustered in the basis of the teleost SOD3 clade. The teleost species studied exhibited a single SOD3 gene, except the European eel and the zebrafish which presented duplicated SOD3 genes.

3.2. Syntenic analysis of the SOD genes in vertebrates

Eleven SOD1 neighboring genes were explored: USP16, CCT8, MAP3K7CL, GRIK1, BACH1, TIAM1, SCAF4, HUNK, URB1, EVA1C and CFAP289, found located in the SOD1 genomic region. A single SOD1 paralog has been found in all vertebrates, including in the spotted gar and among the teleosts, but except for the Atlantic salmon, which presented two SOD1 paralogs, that we name SOD1α and SOD1β, corresponding to the "α/β" nomenclature for salmonid 4R-paralogs (Supplementary Fig. 4). In the remaining teleosts, some SOD1 neighboring genes (BACH1, GRIK1, TIAM1, and SCAF4) were present as duplicated paralogs. For other neighboring SOD1 genes (UPS16, CCT8, MAP3K7CL, HUNK, URB1, EVAC1, and CFAP289), a single paralog gene has been identified, except for the European eel which presented two CCT8 paralogs. In the Atlantic salmon, four copies of MAP3K7CL and GRIK1 and three copies of TIAM1 and SCAF4 have been identified.

Thirteen SOD2 neighboring genes have been retrieved: ASXL2, SELENOI, DRC1, OTOF, MAPRE3, AGBL5, OST4, AHI1, PPP1R14C, IYD, PLEKHG1, FNDC1 and WTAP (Supplementary Fig. 5). This genomic region has been largely rearranged in the chondrichthyan elephant shark and the tetrapods (human and tropical clawed frog), in comparison with the actinopterygian genomic region. Only OTOF and MAPRE3 neighboring genes were duplicated in the considered teleosts, except in the medaka which presented only one MAPRE3 gene. One SOD2 paralog has been identified in the studied vertebrates, except in the European eel, which presented duplicated SOD2 genes, which we named SOD2a and SOD2b, corresponding to the nomenclature for teleost 3R-paralogs. Single ASXL2, SELENOI, DRC1, AGBL5, OST4, AHI1, PPP1R14C, IYD, PLEKHG1, FNDC1, and WTAP have been conserved in the same paralogon as SOD2 In all teleosts, except in the Atlantic salmon. There was a large reorganization of these neighboring genes since some of them were lost or translocated to other chromosomic regions. The Atlantic salmon presented three copies of ASXL2, OFTOF, and MAPRE3.

Twelve SOD3 neighboring genes have been retrieved: GABRA4, GABRB1, ADAGRA3, GBA3, PPARGC1, DHX15, CCDC149, LGI2, MTTP, TRMT10, CYP2U1 and HADH (Supplementary Fig. 6). All these genes were found to be in the same genomic region in both human and spotted gar, whereas some genes were translocated in frog and spotted catshark. In teleosts, CCDC149 and LGI2 genes were duplicated in all the species studied. However, other neighboring SOD3 genes were present as duplicated paralogs depending on the species: GABRB1 in the European eel; PPARGC1 in the European eel and Asian bonytongue; DHX15 in the European eel, Asian bonytongue and Japanese medaka. Only a single paralog SOD3 gene was found in the chondrichthyans, in both tetrapods and all actinopterygians, except in the European eel and the zebrafish which conserved two duplicated paralogs, which we named SOD3a and SOD3b according to the Official Zebrafish Nomenclature Guidelines (http://zfin.org). Single GABRA4, GABRB1, ADAGRA3, GBA3, PPARGC1, DHX15, TRMT10, CYP2U1 and HADH have been conserved in the same paralogon as SOD3 in all the considered teleosts, except in the Atlantic salmon where three copies of ADGRA3, DHX15 and CCDC149 were identified.

3.3. SODs expression in different tissues in male and female European eel

The tissue distribution of SOD transcripts revealed a differential expression in male and female European eels (Fig. 2). SOD1 (Fig. 2.1 A and 2.2 A), was expressed in all the male tissues, but at higher levels in the liver. In females, SOD1 was expressed at higher levels than those found in males, being highly expressed in the ovary.

Concerning SOD2a and SOD2b genes, these revealed similar expression patterns (Fig. 2.1B-C and 2.2B-C). In male eels, SOD2a expression was higher in the testis than the in peripheral tissues (e.g., heart, eye, muscle, intestine), the pituitary, and in all the brain parts. Regarding female eels, both SOD2 genes were detected in peripheral tissues (e.g., heart, muscle, liver, fat), but the ovary showed the highest expression levels.were highly expressed in the ovary.

SOD3a levels were higher in the liver than in the remaining tissues (Fig. 2.1D), whereas in female eels SOD3a was higher in the ovary (Fig. 2.2D). Finally, SOD3b level was the highest in the gills (Fig. 2.1E) and it was widely expressed in female tissues such as the heart, nose, skin, and mostly in the brain parts and pituitary, but no significant differences were found(Fig. 2.2E).

3.4. SODs expression in male European eel under different maturation conditions

3.4.1. Experiment 1. Standard hormonal maturation experiment

Different SOD expression profiles were observed in the testis of eels in experiment 1 (Fig. 3A). The expression levels of SOD1, SOD2a, and SOD2b increased significantly when males were changed from FW to SW conditions, becoming 15-, 4-, and 2-fold higher respectively. In contrast, neither SOD3a nor SOD3b expression levels altered significantly with salinity increase.

As standard treatment progressed, SOD1 mRNA levels showed a decreasing pattern, but no significant differences were found. SOD2a expression levels decreased significantly during hCGrec treatment in SW conditions, contrasting with the SOD2b expression profile which increased through hormonal treatment. SOD3a expression levels showed higher values at the beginning and decreased sharply after 4 weeks of standard hormonal treatment. SOD3b revealed the inverse expression pattern although with high individual variations that probably explain the lack of significant differences.

3.4.2. Experiment 2. Cold seawater pre-treatment experiment

Different SOD expression profiles were studied during experiment 2 (Fig. 3B). Before hCGrec treatment (no hCGrec), the low-temperature pre-treatment significantly decreased SOD1 expression levels. However, control eels exhibited significantly lower SOD1 levels after 4 weeks of standard hormonal treatment. No significant differences were found between SOD1 levels in the pre-treated groups before or after hCGrec treatment.

SOD2a expression levels were lower than SOD1 ones, and no significant differences were observed between control and pre-treated groups before and after the hormonal treatment. In SOD2b, no significant differences were found between experimental groups without hCGrec treatment, indicating that cold seawater pre-treatment did not affect SOD2b expression levels. Nevertheless, 2-week pre-treated eels after hCGrec treatment expressed significantly higher SOD2b levels than pre-treated eels without hormonal treatment, while 4-week pretreated males showed a certain increase but did not reach significant differences.

Concerning SOD3a, 4-week pre-treated eels showed significantly higher expression levels in comparison with control and 2-week pre-treated groups, showing an effect of the low-temperature pre-treatment. After 4 weeks of hCGrec treatment, no significant differences were

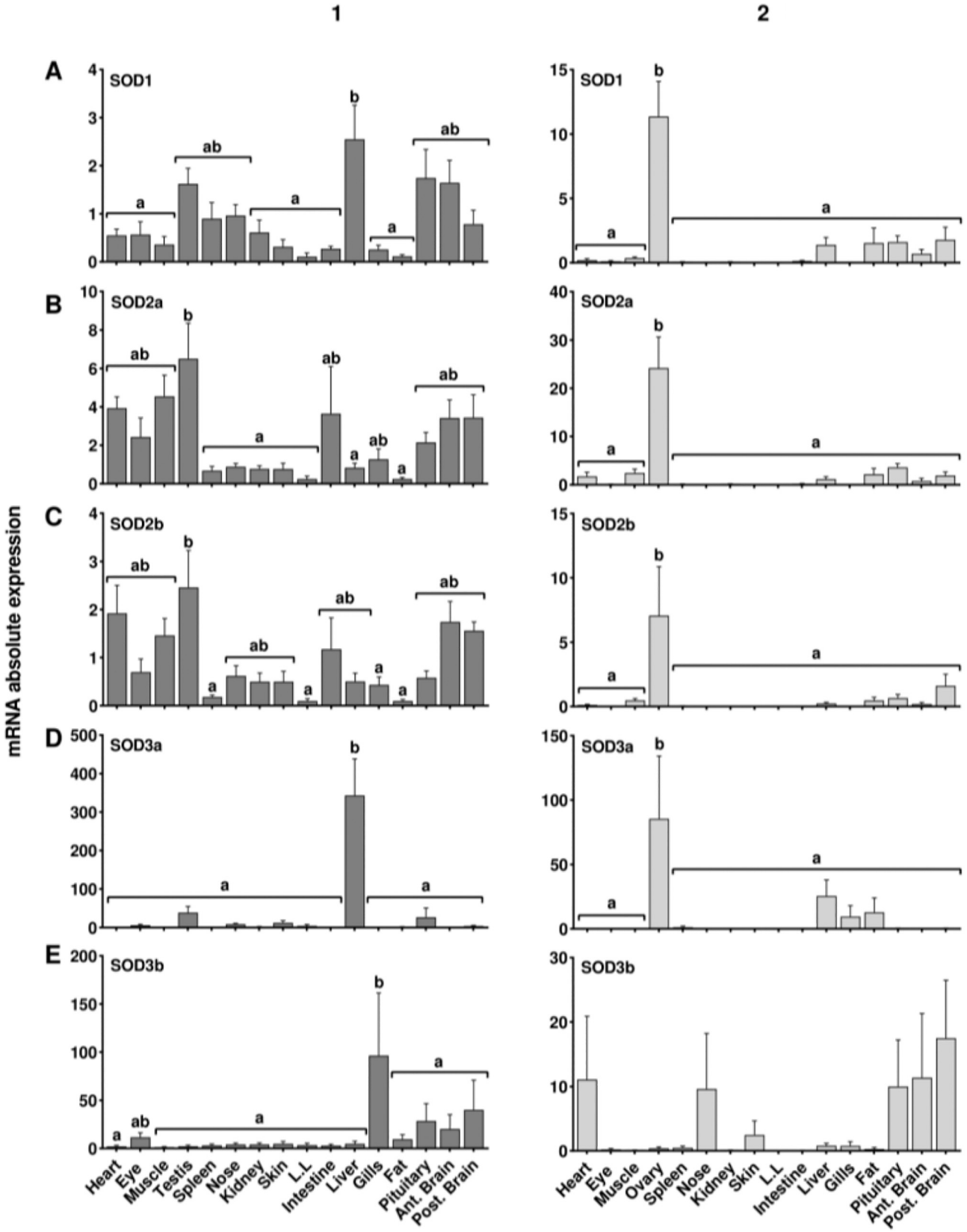

Fig. 2. Absolute expression of tissue distribution of SOD genes in European eel. Relative mRNA expression in males (1) and in females (2) of SOD1 (A), SOD2a (B), SOD2b (C), SOD3a (D) and SOD3b I. Values are presented as means ± SEM ($n = 6$). L.L.: lateral line, Ant. Brain: anterior brain, Post. Brain: posterior brain. Lowercase letters indicate values significantly different between each tissue (one-way ANOVA, post hoc Tukey's $p < 0.05$).

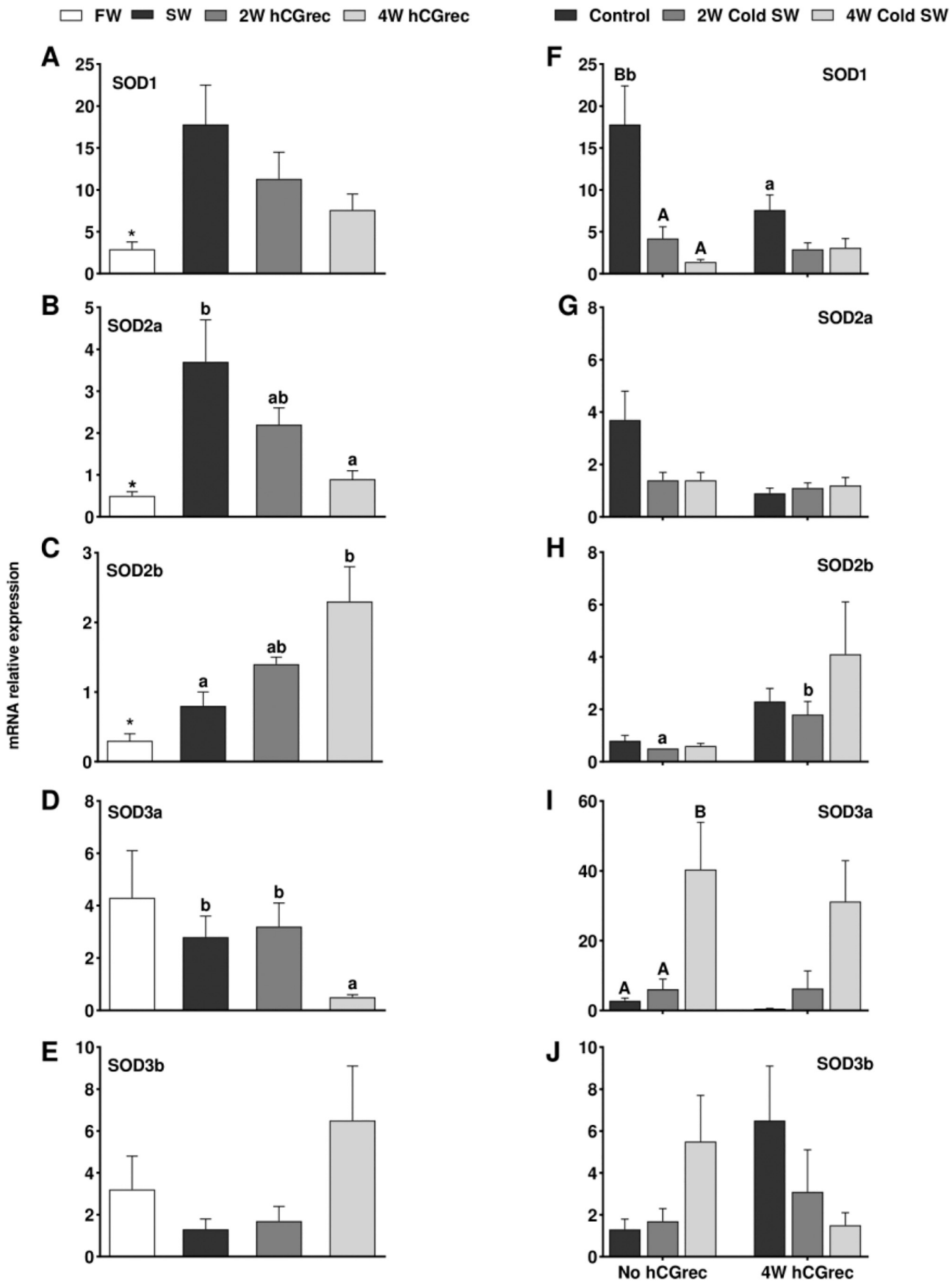

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Fig. 3. Relative expression of European eel SOD genes in the testis with different maturation protocols. Relative mRNA levels of SOD1 (A), SOD2a (B), SOD2b (C), SOD3a (D) and SOD3b I using standard maturation protocol with fresh water (FW, n = 8), seawater (SW, n = 8), after 2-weeks (2 W hCGrec, n = 8) and 4-weeks (4 W hCGrec, n = 8) of hormonal treatment with hCGrec. Asterisks indicate values significantly different between FW and SW conditions (*t*-test, $p < 0.05$); Lowercase letters indicate significant differences between SW, 2 W hCGrec, and 4 W hCGrec conditions (Kruskal-Wallis test, $p < 0.05$). Relative mRNA levels of SOD1 (F), SOD2a (G), SOD2b (H), SOD3a (I), and SOD3b (J) using cold seawater pre-treatment in eels kept in seawater (Control, n = 8) and pre-treated with cold seawater for 2 weeks (2 W Cold SW, n = 8) and for 4-weeks (4 W Cold SW, n = 8) before (No hCGrec) and after 4-weeks of hormonal treatment (4 W hCGrec). Results are expressed as means ± SEM. Data are normalized to ARP and b-ACT genes using relative expressions with multiple reference genes. Uppercase letters indicate values significantly different between control, 2 W and 4 W cold seawater before and after hormonal treatment (one-way ANOVA, post hoc Tukey's $p < 0.05$). Lowercase letters indicate values significantly different before and after hormonal treatment within each experimental group (one-way ANOVA, post hoc Tukey's $p < 0.05$).

found. SOD3b relative expression did not reveal significant differences between experimental groups neither without nor with standard hormonal treatment, and in general, their values were lower than SOD3a ones.

3.5. Gonadal histology

Among the histology samples, three types of cells were registered (Fig. 4A). In experiment 1 (Fig. 4B), FW and SW eel testis exhibited a significantly higher proportion of SPGAdiff cells in comparison with the rest of the cell types. However, 2 weeks of hCGrec treatment resulted in SPGAdiff differentiation as eel testis contained SPGAdiff cells but also SPGB cells in similar proportions. After 4 weeks of hormonal treatment, eel testis contained a significantly higher average proportion of SPC cells, although others such as SPD and SPZ were also detected.

In experiment 2 (Fig. 4C), before hormonal treatment, eel testis from all experimental groups exhibited a majority of SPGAdiff cells. After 4 weeks of hCGrec treatment, SPC cells were the predominant type of cells in the testis of all the experimental groups, showing significantly higher proportions in comparison with other cell types. Moreover, although no significant differences were found between groups, in 2-week and 4-week pre-treated testis sections SPZ were found.

4. Discussion

4.1. SOD1 and duplicated SOD2 and SOD3 genes in the European eel

SODs are considered the oldest enzyme family, whose occurrence dates to the era of primitive Earth evolving in ancient life to protect cells against oxygen toxicity in a reducing environment (Miller, 2012). Moreover, the existence of an initial form of SOD in ancient organisms is widely accepted and it subsequently diverged into modern lineages as an adaptative response to the changing environment (Case, 2017). Therefore, SODs phylogenetic and synteny analysis provides important insights regarding the different types of SOD in vertebrates, particularly in teleosts. These species genome underwent an additional round of duplication, in addition to the two rounds of whole duplication that occurred in vertebrates. This particular evolutionary event, known as teleost-specific whole genome duplication (3R WGD), led to the loss and/or preservation of paralog genes. In addition, the fate of the duplicate genes might disclose the 3R WGD role in speciation and/or evolution of gene functions (Pasquier et al., 2017). Therefore, the evolutionary analysis in teleosts following 3R WDG before gene expression study is important to identify these genes and study their eventual functionalities.

In this study, the chondrichthyans SOD sequences clustered in a clade that diverged at the base of all other Osteichthyes SOD sequences in agreement with these species' phylogeny. In the elephant shark and the spotted catshark, two SOD1 genes were identified, indicating that the chondrichthyans would have a specific duplication of the SOD1 lineage. This contrasted with the SOD2 gene as only one sequence was identified in both species. In the case of SOD3, the phylogenetic analysis revealed the presence of three SOD3 genes in the elephant shark, reflecting a possible local SOD3 species-specific gene duplication in the sarcopterygian group, which includes species from the coelacanth to tetrapods, only one gene each for SOD1, SOD2, and SOD3 was identified, coinciding with previous phylogenetic SOD analyses performed in sarcopterygians (Kim et al., 2021). In the actinopterygian clade, the spotted gar and most teleost species, including early diverging groups like the European eel, presented only one SOD1 gene, which is in accordance with previous studies (Kim et al., 2021; Chatzidimitriou et al., 2020). The synteny analysis highlights a duplication of the genomic region of SOD1 in teleosts, which would come from the 3R WGD, and suggests that the SOD1 duplicate was lost soon after the WGD event. The single SOD1 paralog current in all teleosts studied including the Northern pike would have been inherited by the salmonid lineage where it has been duplicated by 4R WGD, leading to two SOD1 genes that we named SOD1α and SOD1β, corresponding to the "a/b" nomenclature for salmonid 4R-paralogs (Robertson et al., 2017; Mungpakdee et al., 2008). It is noteworthy that previous phylogenetic studies (Chatzidimitriou et al., 2020) that included both the Atlantic salmon and the rainbow trout did not consider the presence of two SOD1 paralogs resulting from 4R as revealed in the present study.

Regarding SOD2 and SOD3, the synteny analysis highlighted the duplication of the genomic region of both genes in teleosts, which would come from the 3R WGD the European eel presented two SOD2 and two SOD3 paralogs and the zebrafish presented two SOD3 paralogs, suggesting that these originated from the 3R. The other teleosts investigated have conserved a single SOD2 and SOD3. These results suggest that SOD2 paralog would have been lost in the teleost lineage, after the emergence of the basal teleost group of elopomorphs, and SOD3 paralog would have been conserved in the European eel and zebrafish and lost in the other species studied. Our study confirmed that the European eel retains more duplicated genes from the whole-genome duplication event compared to other teleost species, as reported in previous studies (Morini et al., 2017; Rozenfeld et al., 2019b). Similarly, the physiological investigation of both SOD3 gene duplicates in zebrafish has recently been considered (Matthiesen et al., 2018, 2021). Nevertheless, the lack of SOD2 and SOD3 duplicates in the Northern pike suggests the loss of these gene paralogs in the common ancestor of Esocidae and Salmonidae. This paralog duplicate absence was maintained in the salmonids that duplicated their genomic region by the salmonid-specific 4R WGD but probably lost these duplicates after the duplication event and this was further confirmed in the synteny analysis of the Atlantic salmon.

4.2. Differential sex and tissue distribution of SODs

Only a limited number of studies concerning SODs explore the expression levels among different sexes and/or focus exclusively on the distribution tissue pattern of one or two SODs (Liu et al., 2015). In the present study, the evaluation of SODs expression in different tissues from individuals of both sexes was performed for the first time in the European eel. It is important to mention that our results could have been affected by the different maturational stages between sexes, as males were probably more immature than females, being in farmed conditions, while females were at the onset of puberty. Some studies reported that SODs can be modulated by aging as different stages require the readjustment of antioxidant systems to support specific cellular signaling and regulation (Lei et al., 2015). Moreover, females might have been submitted to more stress during capture, and therefore it is possible that it influenced the differences observed in Fig. 2. However, there is a lack of literature regarding SODs expression depending on sex and under stress

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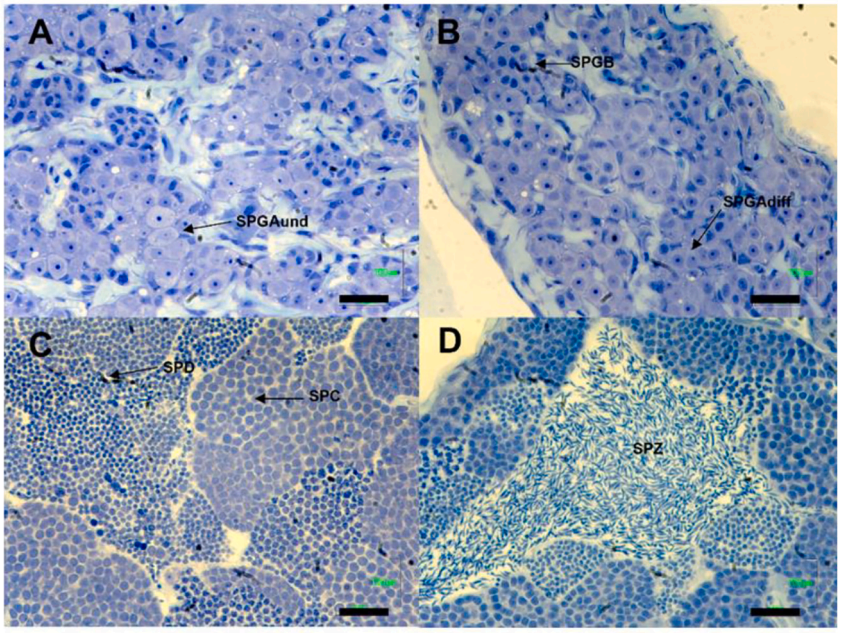

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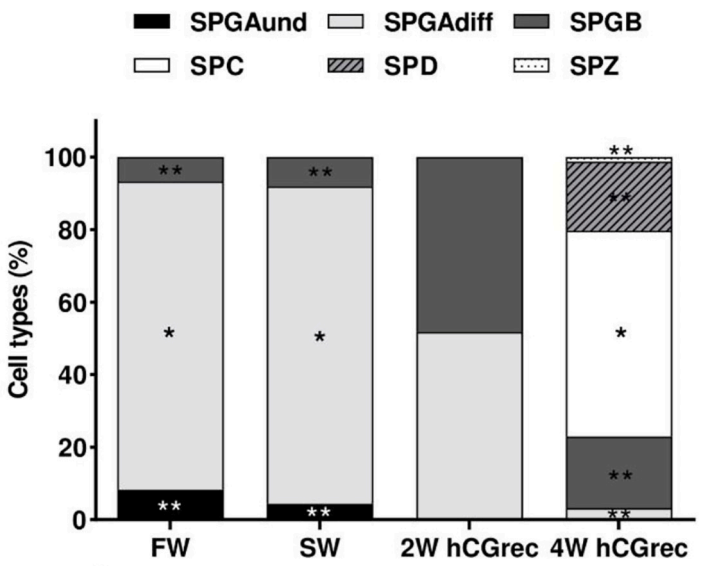

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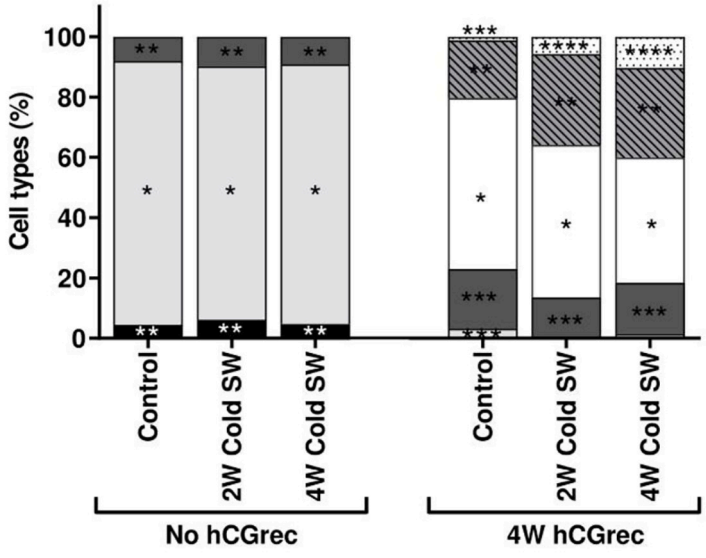

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Fig. 4. Histology results. (1) Cell types in testis samples. A) SPGAund: spermatogonia A undifferentiated; B) SPGAdiff: spermatogonia A differentiated and SPGB: spermatogonia B. C) spermatocytes (SPC) and spermatids (SPD). D) spermatozoa (SPZ). Scale bar = 40 μm. (2) Cell counts in European eel testis from standard maturation protocol experiment. Stacked bars show the percentage proportion of cells in fresh water (FW), seawater (SW), after 2-weeks (2 W hCGrec) and 4-weeks (4 W hCGrec) experimental groups. Asterisks indicate values significantly differences between cell percentages within each group (one-way ANOVA, post hoc Tukey's $p < 0.05$). (3) Cell counts in European eel testis from cold seawater pre-treatment protocol experiment. Stacked bars show the percentage proportion of cells in control (A), 2-weeks (B) and 4-weeks (C) pre-treated experimental groups before (No hCGrec) and after 4-weeks of hormonal treatment (4 W hCGrec). Asterisks indicate values significantly differences between cell percentages within each group (one-way ANOVA, post hoc Tukey's $p < 0.05$).

conditions.

Based on our findings, the distribution of SOD1 in male eels was widespread across various tissues. It was detected at high levels in the liver, pituitary gland, all parts of the brain, and the testis. In females, SOD1 exhibited a more restricted pattern, primarily found in the ovary, pituitary gland, and brain regions. In the marbled eel, SOD1 was predominantly detected in the liver (Wang et al., 2016a, 2016b). However, in the crocodile icefish (*Chionodraco hamatus*), it was found in the spleen, gills, muscle, heart, and liver (Chatzidimitriou et al., 2020). In the yellow drum (*Nibea albiflora*), it was recently identified in the kidneys, brain, and liver (Wang et al., 2019). These results indicate that the tissue distribution of SOD1 varies among different teleost species, suggesting species-specific differences in its function. Notably, a study in carp (*Carassius auratus*) found low expression of SOD1 in ovarian tissue (Li et al., 2013). In contrast, SOD1 plays a crucial role in the brain and ovary of female mice, controlling excessive intracellular production of ROS) and modulating steroidogenic pathways (Noda et al., 2012). In female eels, SOD1 expression levels were high in tissues related to the BPG suggesting its involvement in signaling roles in female reproduction.

Both SOD2a and SOD2b exhibited similar tissue-expression patterns in male and female eels, with SOD2a being more highly expressed. These enzymes were prominently detected in the testis, ovary, pituitary gland, and various brain regions in both sexes. In zebrafish, a single paralog of SOD2 was found in all brain regions in both males and females (Pradhan and Olsson, 2015). In female mice, SOD2 deficiency led to excessive oxidative stress and inhibited steroidogenic enzyme genes (Zaidi et al., 2021), suggesting the potential involvement of SOD2a/b, similar to SOD1, in mechanisms related to the BPG axis.

Regarding SOD3 paralogs, both SOD3a and SOD3b exhibited tissue-specific and sex-related expression in eels. In contrast to the other mentioned SODs, two SOD3 paralogs have been identified in the zebrafish and showed differential expression tissues, as SOD3a was predominantly expressed in the liver and intestine, while SOD3b was highly expressed in the cartilaginous tissues (Matthiesen et al., 2018, 2021). Moreover, Ganesan (2020) recently showed that in zebrafish, both male and female individuals showed similar expression patterns of SOD3a and b across different tissues. In male eels, SOD3a was primarily expressed in the liver, whereas in females, it was mainly detected in the ovary. SOD3b, however, exhibited high expression in the pituitary gland and brain regions in both sexes.

4.3. Expression of SODs throughout spermatogenesis with standard maturation protocol

The present study is the first to report the differential and sequential expression of SODs throughout spermatogenesis in the European eel. In the testis, SOD1 and both SOD2 expression levels increased significantly with the transfer of eels from FW to SW, while SOD3a and SOD3b showed no significant change. Our results coincide with those in marbled eel juveniles, where salinity increase altered both SOD1 and SOD2 expression levels in different tissues. In the gills, liver, kidney, and muscle, SOD1 and SOD2 expression levels were inhibited as eels were transferred to SW conditions (Wang et al., 2016a, 2016b). However, SODs response to salinity seems tissue-specific given that SOD1, SOD2a, and SOD2b expression levels in the testis were upregulated in the European eel with SW acclimation, contrasting with the results obtained in the marbled eel in other tissues. On the other hand, a previous study showed that although the increase of salinity did not promote SPGA cell differentiation in the testis, the transfer from FW to SW upregulated the expression of testicular steroidogenic (i.e., aacyp11a1, aacyp17-I, and aacyp19a1) that crucial for the onset of the spermatogenesis (Peñaranda et al., 2016). These results coincide with our study in which eels from both FW and SW groups revealed SPGAdiff cell type dominance in the testis, showing that salinity did not stimulate SPG cell differentiation into more advanced developmental stages than SPGAdiff. However, SOD1, SOD2a, and SOD2b upregulation suggests that, in addition to the steroidogenic enzymes previously mentioned, SODs might play a crucial role in the physiological adaptation to the increase of salinity that occurs during the beginning of the reproductive migration from continental waters to Atlantic conditions (Fig. 5A).

As part of the standard eel maturation procedure, once males were adapted to SW these were treated with weekly injections of hCGrec. We observed different expression patterns of the various SOD genes during this maturation process. SOD1, SOD2a, and SOD3a showed high initial levels of expression when early SPG cells (SPGAdiff and SPGB) were dominant. However, the expression of SOD2b and SOD3b was the opposite, with higher expression occurring as the cells progressed to more advanced developmental stages (SPC and SPD). Our study suggests that the entire process of maturation and sexual development in European eels involves a sequential pattern of SODs expression. In the Japanese eel, in vitro cultured SPGA cells treated with hCG showed high SOD1 levels at the beginning of the spermatogenesis (Celino et al., 2011). However, when the hormonal treatment induced the development of SPG cells into more advanced stages, SOD1 levels decreased. In the present in vivo study, SOD1 showed a similar down-regulation expression profile throughout the hCGrec treatment in SW, confirming that SOD1 may have a crucial role in SPGA cell development at the beginning of spermatogenesis.

Concerning SOD2, Cuenca (2014) showed that SOD2 knockout rats decreased the differentiation of SPG cells in the testis, which resulted in lower SPC and SPD cell proportions. Other studies revealed that SOD2 is highly expressed in the initial stages of spermatogenesis (Yon et al., 2010) and early post-meiotic cells (Esakky et al., 2013) in rodent testis. Moreover, it has been demonstrated that SOD2 knockdown resulted in the inhibition of key steroidogenic gene enzymes implicated in both estradiol and progesterone synthesis in rats (Cao et al., 2014). In our study, the opposite expression patterns of SOD2a and SOD2b through testis maturation suggest that both SOD2 genes are involved in different stages of SPG cell development. It is possible that SOD2a acts in the early maturation stages with renewal and proliferation controlled by estradiol (Fig. 5A), while SOD2b seems to take part in the end of spermatogenesis and spermiation modulated by progestin (Fig. 5C).

Mruk et al. (2002) found that SOD3, primarily detected extracellularly, is produced in the rat testis and that SOD3 expression increases during the early stages of spermatogenesis development, indicating that germ cells may play a role in maintaining the levels of SOD3 in the testis. Additionally, Mruk and Cheng (2000) showed that the increased interaction between Sertoli cells and germ cells during spermatogenesis is responsible for the higher levels of SOD3 in the testis and that the FSH upregulates SOD3 expression, unlike other steroid hormones. These findings may explain our results with the eel as an increase in SOD3a during the early stage of the spermatogenesis was observed and coincided with the testis development phase when the expression levels of FSH receptor are the highest (Peñaranda et al., 2010; Fig. 5B). Moreover, similarly to the observed with SOD2 genes, SOD3a and SOD3b opposite

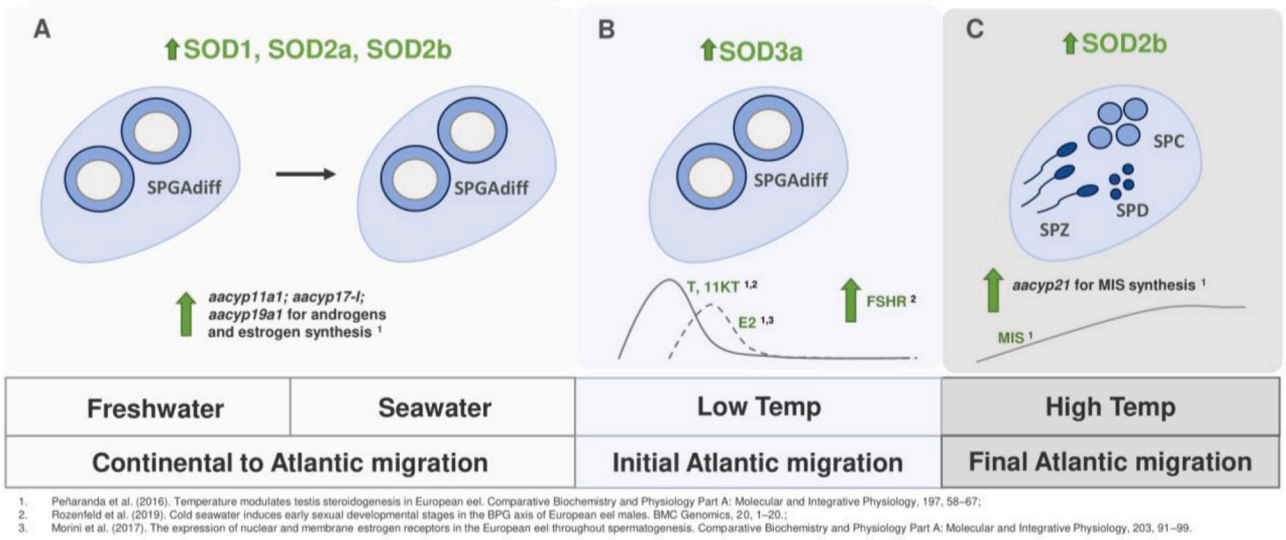

Fig. 5. Hypothesis in SODs dynamics during the European eel migration. (A) Continental freshwater to seawater transition with increase of steroidogenic enzymes (aacyp11a1, aacyp17-I, aacyp19a1) alongside SOD1, SOD2a and SOD2b expression levels in testis containing spermatogonia type A differentiated (SPGAdiff) cells; (B) Initial Atlantic migration - at low temperatures (low Temp) with a peak of androgens (T, testosterone; 11KT, 11-ketosterone) synthesis followed by estrogen (E2, estradiol) production alongside with FSH receptor (FSHR) expression increase, which coincides with SOD3a upregulation in testis composed by SPGAdiff cells; (C) Final Atlantic migration - at higher temperatures (high Temp) with increasing synthesis of DHP (17α,20β-dihydroxy-4-pregnen-3-one) which coincides with SOD2b upregulation in testis containing spermatocytes (SPC), spermatids (SPD) and spermatozoa (SPZ). 1. Peñaranda et al., 2016; 2. Rozenfeld et al., 2019a, 2019b; 3. Morini et al., 2017.

expression patterns throughout the complete testis maturation might support the SPG cells development through spermatogenesis.

4.4. *Expression of SODs throughout spermatogenesis with cold seawater pre-treatment*

In experiment 2, SODs expression levels were evaluated in male eels pre-treated with cold SW, both before and after standard hormonal treatment. Before hCGrec treatment, our histological results revealed that SPGAdiff cells were predominant in the testis of both control and cold SW pre-treated eels. Rozenfeld et al. (2019a) showed in eels without hormonal treatment that low temperature promoted SPG cell proliferation, reporting a higher SPGAdiff cell proportion in comparison with eels maintained at 20 °C. This result was related to the androgens (T and 11KT) production induced by low temperature. However, pre-treated eel testis, similar to control, did not contain cells in more advanced stages than SPGB cells. Our present results showed that cold SW did not promote SPGA differentiation at the beginning of the experiment (Fig. 5B). In teleosts, most studies evaluating salinity and temperature effects on SODs expression only considered one or two SOD types and at the hepatic level (Yu et al., 2017; Cheng et al., 2018). Our findings in the European eel indicate that 4 weeks of cold SW pre-treatment reduces the expression of SOD1 while increases the expression of SOD3a in the testis. This suggests that low temperature and/or salinity conditions lead to these SOD types opposing expression patterns. Concerning SOD1, in the European sea bass, the interaction effects of temperature and salinity showed that temperature did not affect SOD1 expression in the liver, but the increase of salinity upregulated its expression levels (Chang et al., 2021). However, An et al. (2010) demonstrated in black porgy (*Acanthopagrus schlegeli*) liver that low temperature and salinity downregulate SOD1 levels and oxidative stress markers (hydrogen peroxide and malondialdehyde plasma levels). These findings coincide with our results of SOD1 expression in eel testis and support that the combined effects of reduced temperature and salinity may synergistically decrease SOD1 levels during exposure to cold SW treatment. Our findings suggest that these thermal conditions can contribute to the controlled production of ROS during gonadal development, leading to a moderated expression of SOD1 in the testis mainly composed of SPGA cells. On the other hand, the SOD3a expression levels revealed an upregulation with cold SW treatment. As mentioned already, SOD3 synthesis in the testis seems to be modulated by FSH in rats (Mruk and Cheng, 2000). On the other hand, Rozenfeld et al. (2019a) concluded that FSH receptors (FHR) in the testis of the European eel were upregulated by cold SW treatment. Although this should be confirmed, the upregulation of SOD3a expression levels in the testis of pre-treated males might result in the FSHR increased expression upon low temperature, alongside a possible increase of the androgen levels, which is supposed to occur in eels when they begin their Atlantic migration under lower SW temperatures (Fig. 5B).

After 4 weeks of hCGrec treatment at higher temperatures, SOD1, SOD2a, and both SOD3 gene expression levels did not vary in eels previously pre-treated with cold SW, except SOD2b which increased in eels pre-treated. Nevertheless, our histological findings indicated that pre-treated males had a higher proportion of SPZ. This result supports the idea that SOD2b may play a role in progestin synthesis, crucial in final spermatogenesis and spermiation. The upregulation of SOD2b expression with cold seawater coincides with the MIS (maturation inducing steroids) increased production registered previously in eels at higher temperatures (Peñaranda et al., 2016) and could potentially explain the higher percentage of SPZ in pre-treated eel testis upon temperature increase with hormonal treatment, which corresponds with final oceanic migration conditions (Fig. 5C).

In conclusion, our comprehensive analysis of sequence-based phylogenies and synteny in vertebrates has unveiled a greater number of SOD members than previously known, including the discovery of one SOD1 and duplicated SOD2 and SOD3 genes in the European eel. These findings demonstrate the highly dynamic evolution of the SOD gene family, characterized by lineage-specific events that involve local gene duplications, genome duplication, and gene losses. All SODs were expressed in neuroendocrine, gonadal, and non-reproductive tissues in both male and female eels. Further analysis is required to determine the specific functions of each SOD and their potential interactions within the tissues of the BPG axis. Moreover, our findings suggest that these antioxidant enzymes may be involved not only in reproduction but also in

other non-reproductive functions. In the testis, the described SODs showed distinct expression patterns under different environmental conditions, revealing that the European eel has antioxidant mechanisms to support sexual maturation apparently by coordinating the up-and down-regulation of SOD genes throughout the entire process of spermatogenesis.

4.5. SODs dynamics in the European eel migration: a preliminary hypothesis

This study considered two experiments that enabled to formulate a comprehensive hypothesis concerning the dynamics of SODs expression during the reproductive migration of the European eels (Fig. 5). This hypothesis is based on the environmental parameters (i.e., salinity and temperature) encountered by eels during their reproductive migration and considers results from our previous studies with females (Mazzeo et al., 2014) and males (Peñaranda et al., 2010, 2016; Morini et al., 2017; Rozenfeld et al., 2019a). The migration of this species involves a first phase in which eels move from continental freshwater to sea waters which implies a salinity increase (Fig. 5A). During this adaptation to seawater conditions, there is an increase in the testicular expression of enzymes involved in the synthesis of androgen (aacyp11a1; aacyp17-I) and estrogen (aacyp19a1), that onset the steroid synthesis pathways for further testis maturation. Alongside the steroidogenic enzyme expression upregulation with salinity increase, there is also an upregulation of the testicular expression of SOD1, SOD2a, and SOD2b. Once acclimatized to seawater, eels begin their second phase of the transatlantic migration to the Sargasso Sea in deeper waters at low temperatures (Fig. 5B) and no further maturation is observed until temperature increases. During this initial Atlantic migration in cold seawater, there is a peak of androgens (T and 11KT) which is followed by a peak of estrogen (E2). At the same time, low temperature is correlated with an increase in SOD3a expression level coinciding with the increase of expression of FSH receptor (FSHR) (Fig. 5B). As eels arrive at higher temperature conditions in the final phase of their Atlantic migration, the temperature increase induces a shift in the steroidogenic production from androgen and estrogen synthesis to MIS synthesis, due to the upregulation of aacyp21 enzyme expression. This change induces the final testis maturation coinciding with the upregulation of SOD2b (Fig. 5C).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2024.111590.

Funding sources

This study received funding from the Spanish MICIU (Project EELGONIA; RTI2018-096413-B-I00). LF has a predoctoral contract funded by the Generalitat Valenciana, Programa Grisolía (GRISOLIAP/2020/063). MBG has a predoctoral contract from the Universitat Politècnica de València (PAID-01-20). MM had a postdoctoral contract funded by the Generalitat Valenciana (APOSTD/2020/053). Funding for open access charge: CRUE-Universitat Politècnica de València.

CRediT authorship contribution statement

L. Ferrão: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **M. Blanes-García:** Conceptualization, Investigation, Methodology. **L. Pérez:** Conceptualization, Investigation, Methodology, Supervision. **J.F. Asturiano:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **M. Morini:** Conceptualization, Data curation, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing, Validation, Visualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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