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Study of reproductive biology of European hake (*Merluccius merluccius*) in Adriatic Sea

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Alla mia famiglia, che mi ha sempre sostenuto e incoraggiato ad andare avanti

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CHAPTER 1

INTRODUCTION

The European hake (*Merluccius merluccius*) is a nectobenthonic species widespread in the Eastern coast of Atlantic Ocean and in Mediterranean Sea. European hake, called in Italian "nasello" or improperly "merluzzo", belongs to Gadiformes Order and to Merlucciidae Family, that includes 13 species spread in worldwide.

Systematic classification of European hake	
Kingdom	Animalia
Phylum	Chordata
Class	Actinopterygii
Order	Gadiformes
Family	Merlucciidae
Genus	*Merluccius*
Species	*M. merluccius*

The European hake has very commercial importance, it is one of the main target species of demersal commercial fishes in the Mediterranean Sea with total landings of 22547 tons in 2011 (Druon et al. 2015) as well as in the Atlantic Ocean, where the stock is an important sustenance of the fisheries (Cerviño et al. 2013). Because of the intense fishing of this stock, FAO included it in the list of overfished species (FAO 2011).

Figure 1. One of the biggest European hake sampled during three years in Adriatic Sea. The total length was 52 cm.

1.1 Area of the study

The European hake is equally distributed in Mediterranean Sea. While the Atlantic and Mediterranean population are considered as two distinct sub-species, the Mediterranean hake is considered a homogeneous stock, in fact the Adriatic stock was genetic coincident with North-western area (Orsi Relini et al. 2002; Lo Brutto et al. 1998). The Adriatic Sea is a semi-enclosed and shallow basin and the deeper areas are only the Jabuka/Pomo Pit and South Adriatic Pit with over 200 m of depth, while the rest of the sea is represented by continental shelf (less 200 m deep). The our sampling area is 80-85 m deep and it is identified by the FAO- Geographical Sub-Area 17 (GSA17) between Northern and Central Adriatic Sea close to Ancona city. According to FAO (2016), also in Adriatic Sea the European hake stock status is in overexploitation with relative intermediate biomass. In this area, it represents an important fishing resource, shared by fisheries of several countries.

Figure 2. Map of Adriatic Sea.

1.2 Biology of European hake

The body of hake is tapered with a grey-silver color.

The bathymetric distribution of hake in the Mediterranean Sea is between 25 to 1000 m depth. However, hake is mainly found at depths ranging from 100 to 400 m (Orsi Relini et al. 2002). The bathymetric distribution of European hake can change in relation to different physiological factors during life (feed availability, reproductive cycle, age, season) (Lloret et al. 2008).

The analysis of stomach contents evidenced that the diet characteristics of European hake change during life. Fishes smaller than 16 cm, feed crustaceans, instead with increase of their size, the

specimens prefer mainly teleosts (*Sardina pilchardus, Sprattus sprattus* and *Engraulis encrasicolus*) and rarely cephalopods (Vrgoč et al. 2004). Occasionally, also cannibalism event were confirmed for this species, when the density of the population is too high to give support to its conspecifics (Orsi Relini et al. 2002).

M. merluccius can live more than 20 years and it can reach 130 cm of total length (Jardas 1996). However, the size composition of exploited stock is constituted mainly of 0+, 1+ and 2+ year-old specimens (Vrgoč et al. 2004), represented by specimens shorter than 20 cm of total length (Zupanovic & Jardas 1986). The growth rate in hake varied with size and sex. Using tagging method and recapture techniques, Mellon-Duval and co-workers (2010) showed that from the second year of life, females grow faster than males and that hake became mature at the age of two years for both sexes, instead of 3 or 4 years as previously accepted. The length at which half population is sexual mature (L_{50}) was estimated by different authors and it slightly changed based on the fishing areas and the sex. In fact, in Moroccan Atlantic coast it was 28.6 and 33.8 cm respectively for males and females respectively (El Habouz et al. 2011), in Iberian Atlantic waters 32.8 for males and 45.4 for females (Pineiro & Sainza 2003), in Alger 21.5 for males and 30.6 for females (Bouaziz et al. 1998), in both Catalan Sea and Tyrrhenian Sea was reported only female measure that was 35.8 and 35.1 respectively (Recasens et al. 2008). Finally, as reviewed by Vrgoc and co-warkers (2004), the length at which half population is sexual mature (L_{50}) was estimated by different authors, in the forty years the range was 22-30 cm for males and 20-28 cm for females, subsequently in the eighty years the range of 20-28 cm for males and 23-33 cm for females is reported. In the low Adriatic areas Ungaro et al. (1993) found the same range of L_{50}, 25-30 cm for both sexes.

European hake is a multiple spawner species with indeterminate fecundity, that spawns different times during reproductive season. Although the spawning females were found all year around (Murua & Motos 2006; Recasens et al. 2008; Recasens et al. 1998), the main reproductive season

was identified from Winter to Summer by many authors. In Bay of Biscay the main spawning season was intensified from January to March (Murua & Motos 2006; Lucio et al. 2000), in Galician Shelf in Winter and in Spring/Summer (Dominguez-Petit et al. 2010), in Tyrrhenian Sea from January to May, while in Catalan Sea from August to December (Recasens et al. 2008), in Eastern central Atlantic Ocean in Winter and in Spring with certain inter-annual variability (El Habouz et al. 2011), in Adriatic Sea, also, two spawning seasons were identified at different bathymetry, one in Winter in deeper waters and one in spring-summer in shallower waters (Zupanovic & Jardas 1986; Ungaro et al. 1993). *M. merluccius* spawns at 70-150 m of depth, at water temperature of 10°-13°C (Coombs & Mitchell 1982). The floating eggs of European hake are transparent, spherical with a single yellow oil globule (Bjelland & Skiftesvik 2006). As reported by Sánchez and co-workers (2012), that for the first time, provide information about spontaneous spawning of European hake, showed that eggs diameter was a range of 0.94–1.03 mm, while the total length of hatched larvae was about 3.0 mm. In rearing condition, the larvae started to feed 6 days after hatching and they approach to the surface (Bjelland & Skiftesvik 2006; Sánchez et al. 2012). In nature, after a first period of pelagic behavior of the larvae (estimated as 40 days post hatching), the animals have metamorphosis and move close to bottom sediments (Arneri & Morales-Nin 2000). The recruitment of young individuals to adult stock change based to reproductive period of the different area, for example in North-Western Mediterranean Sea and in Adriatic Sea is recorded in spring and autumn (Zupanovic & Jardas 1986; Arneri & Morales-Nin 2000; Orsi Relini et al. 2002), while in central Mediterranean Sea is recorded in late summer (Orsi Relini et al. 2002).

The economic importance of European hake is mainly due to the flesh quality (edible portion) that with low amount of fat, it is suitable for any type of human nutrition. Indeed, this species has the ability to accumulate the bulk of lipid (energy) especially in the liver (Lloret et al. 2008) while in

the muscle the lipid percentage is very low and the presence of perivisceral fat has not been found. Some authors studied whether the energy stored in the liver of hake is directly involved in reproduction, as typical for species temperate zones, or not. The results are different, while Lloret and co-workers (2008) reported that the energy reserves in the liver contribute to improve the estimation of the reproductive potential in this species, for Dominguez-Petit and co-workers (2010), the energy reserves stored in liver do not directly influence the egg production but rather the reproductive process can depend on environmental conditions and food availability.

Different fishing tool were used to catch the animals, the long-liners catch large adult female hake and trawlers and gill-netters targeted smaller size of specimens. In the Adriatic Sea, hake is mainly fished with bottom trawlers, while long-lines are less used (Vrgoč et al. 2004). High percentage of landings is represented by species under 20 cm of total length (Ungaro et al. 2003), impacting mainly juvenile hakes. Furthermore, the hake fishing has also negative effects on other species. As reviewed by Tsagarakis and co-workers (2014), in fact, such methods are not selective for hake and lead to produce a discard around 40% in several Mediterranean areas.

Despite the negative impact of fishing that led to a depletion of European hake stock in the world, the commercial aquaculture for this species has not been achieved. In the last years, the interest for this species as potential candidate for aquaculture production is increased, in fact its characteristics, as fast growth rate and the flesh quality which gives it a very high market value, contribute to the high potential value for aquaculture (Groison et al. 2010). Different authors tried to keep these animals in captivity. Bjelland and Skiftesvik (2006) caught live hakes for keeping in captivity but without success. However, the authors obtained eggs through stripping, providing advice about egg incubation, hatching and larvae growth. Afterwards, for the first time, Iglesias and co-workers (2010) bred wild hake for extended period, showing that it is possible to maintain hake specimens

in captivity. Only in 2012, Sánchez and co-workers obtained a spontaneous spawning from a hake broodstock kept in captivity for 2 years. Unfortunately, the larvae died during experiments and to date the complete hake life cycle in breeding was not achieved.

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CHAPTER II

AIM OF THE STUDY

Since the critic status of hake stock in the world and the commercial importance of this species, the protection of the population is a fundamental importance. To preserve the species and to improve fishing management, it is necessary increase the knowledge about the habits of the European hake and its stock conditions. For this purpose, the aim of present doctoral project was to assess the current state of the stock of European hake (*Merluccius merluccius*, L. 1758) in Adriatic Sea and overall to increase the knowledge about its reproductive biology. In addition, toxicological investigations were performed on the hake males to analyze the presence of endocrine disruptors in the environment.

In order to realize the project, a multidisciplinary study was carried out using macroscopic, histological and molecular approaches.

The macroscopic analysis consisted of sex determination and the measure of different body parameters of fish, total body length, the total and gutted weight and the liver and gonad weight of European hake in Adriatic Sea. Such information allowed to calculate the trend of morphometric indices, the Le Cren's condition factor (K_n), the hepatosomatic index (HSI), the gonadosomatic index (GSI) in three years. Also, the size at first maturity (L_{50}) was evaluated in males and in females. Furthermore, for the first time in Adriatic Sea, the fecundity, estimated as batch and relative fecundity, were analyzed in the female specimens and compared with values of other fishing areas in Mediterranean Sea and in Atlantic Ocean.

In addition to macroscopic analysis, also the histological analysis of the ovaries and testis were performed to validate the macroscopic scale that we used.

The molecular approaches were important tool to complete the scenario of the reproductive physiology of European hake. In fact, though the European hake is relevant commercial species, few studies focused on its reproductive physiology. So, the work was focused on studies regarding

the gonadotropin hormones, both the follicle stimulating hormone (Fsh), and the luteinizing hormone, (Lh).

In addition, also their relative receptors were analyzed. The sequences of such hormones and the receptors were sequenced and characterized. For Fsh and Lh, the mRNA expression were measured in pituitary gland in female specimens at different ovarian stages during natural reproductive cycle and their molecular localization was investigated in pituitary gland. Regarding the gonadotropin hormone receptors, their molecular levels were analyzed in oocytes at different maturity stages.

Finally, toxicological studies were performed through the analysis of molecular expression of vitellogenin A and B and estrogen receptor alpha in liver of males captured in the last three years.

CHAPTER III

GONADOTROPIN CHARACTERIZATION, LOCALIZATION AND EXPRESSION IN EUROPEAN HAKE (*MERLUCCIUS MERLUCCIUS*)

Gonadotropin characterization, localization and expression in European hake (*Merluccius merluccius*)

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Abstract

In vertebrates, the regulation of gametogenesis is under the control of gonadotropins (Gth), follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh). In fish, the physiological role of Gths is not fully understood, especially in species with asynchronous ovarian development. In order to elucidate the role of Gths in species with asynchronous ovary, we studied European hake

(*Merluccius merluccius*) during the reproductive season. For this aim, we first cloned and sequenced both hormones. Then, we characterized their amino acid sequence and performed phylogenetic analyses to verify the relationship to their orthologues in other species. In addition, the quantification of gene expression during their natural reproductive season was analyzed in wild-caught female hake. Our results revealed that *fshb* peaked during the vitellogenic phase, remaining high until spawning. This is in contrast to the situation in species with synchronous ovary. *lhb*, on the other hand, peaked during maturation as is common also in species with synchronous ovarian development. Finally, combining double-labeling fluorescent *in situ* hybridization (FISH) for Gth mRNAs with immunofluorescence for Lh protein, we evidenced the specific expression of *fshb* and *lhb* in different cells within the *proximal pars distalis* (PPD) of the pituitary. In addition to gonadotrope cells specific to expression of either *fshb* or *lhb*, some cells showed co-expression of both genes. This suggests either that gonadotropes with co-expression are not yet specified, or they could have a plasticity that permit changes from one cell phenotype to another during certain life stages and in turn during different physiological states.

Keywords: Gonadotropin, European hake, Fsh, Lh, Fluorescence *in situ* hybridization, Immunofluorescence.

Introduction

The pituitary gonadotropins, follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh), are hormones directly involved in regulation of gametogenesis in vertebrates (Nagahama 1994; Swanson et al. 2003; Weltzien et al. 2004). Both gonadotropins are heterodimeric glycoproteins and consist of a common α subunit shared with thyroid-stimulating hormone (Tsh) and a hormone specific β subunit important for their biological specificity.

Fsh and Lh are secreted by gonadotropes present mainly in *proximal pars distalis* (PPD) and some cells of *pars intermedia* (PI) of the pituitary gland in teleosts (Weltzien et al. 2003a). In mammals, the gonadotrope cells produce both gonadotropins (Nakane 1970) and rarely was found an overlapped expression. On the contrary, in teleosts, Fsh and Lh are synthesized by two different cell types (Zohar et al. 2010) and the colocalization of the two mRNAs in the same gonadotrope cell was only occasionally evidenced (García Hernández et al. 2002; Weltzien et al. 2014).

Because of the variety of reproductive strategies in different teleost species, the physiological roles of the two gonadotropins during the ovarian cycle do not seem to be the same for all species. Following Tyler and Sumpter (1996), reproductive strategies can be differentiated on the basis of the dynamics of ovarian development: ovaries can be classified as synchronous or asynchronous, even though such division could be reductive due to the wide number of reproductive strategies. The species with synchronous ovaries spawn eggs once within the reproductive season. These species are characterized by increasing plasma levels of Fsh with ovarian development (Tyler et al. 1997), while Lh surges during final maturation and spawning (Planas and Swanson 1995; Gomez et al. 1999; Yoshiura et al. 1999; Schmitz et al. 2005).

The regulatory mechanism of gonadotropins during gametogenesis is not so clear when we consider the teleosts with asynchronous ovaries, i.e. the species that spawn multiple batches of oocytes during the reproductive season. For instance in chub mackerel (*Scomber japonicus*), *fshb* mRNA levels increase during the first stages of ovarian cycle, peaking at the end of vitellogenesis, whereas *lhb* mRNA levels significantly increase during late vitellogenesis. Finally, the mRNA levels for both hormones significantly decrease during post-spawning (Nyuji et al. 2012). Also in other multiple batch spawners as *Sparus aurata* (Gen et al. 2000), *Dicentrarchus labrax* (Mateos et al. 2003), *Hippoglossus hippoglossus* (Weltzien et al. 2003b), *Anguilla japonica* (Han et al. 2003) and *Paralichthys olivaceus* (Kajimura et al. 2001) both hormones show the same fluctuation during gonadal cycle. Unlike these results, in the Atlantic cod (*Gadus morhua*) - a multiple batch spawner -

two peaks of *fshb* were observed, the highest before spawning and the second during spawning time together with *lhb* (Mittelholzer et al. 2009).

For this purpose the current study was aimed to better understand the functions of Fsh and Lh in the reproduction of European hake, a species with asynchronous ovarian development characterized by different phases of maturation (Sarano 1986; Murua et al. 1998). In the present study, the endocrine control of ovarian maturation of European hake was approached for the first time, by studying gonadotropin molecular characterization, expression and localization in the pituitary gland throughout the seasonal reproductive cycle in wild specimens captured in the Adriatic Sea.

Materials and methods

Monthly Sampling

During 2015, a total number of 129 European hake females were collected on board of bottom trawler fishing vessel in the Northern and Central Adriatic Sea (FAO- Geographical Sub-Area 17, according to GFCM division). The specimens were collected under the guidelines of the Data Collection Framework Regulation (EU Reg.199/2008) that established a Community system for the conservation and sustainable exploitation of fisheries resources under the Common Fisheries Policy (CFP). The procedures did not include animal experimentation and ethics approval are not necessary in accordance with the Italian legislation. We considered fish animals in a length range of

28-35 cm, that were macroscopically and microscopically classified in five different ovarian classes: immature/regenerating (F1); developing (F2); spawning capable (F3A); actively spawning (F3B), and spent, post spawning (F4) (Table 1), following the ovarian classification of Brown-Peterson et al. (2011), but adapted for European hake. The brief post-spawning phase make difficult the sampling of class F4 females, consequently only classes F1, F2, F3A, and F3B are included in this work. For the molecular analysis, we considered the animals sampled when the highest values

of gonadosomatic indices (GSI) were recorded during the year. The pituitary glands for qPCR analyses were immediately preserved in RNAlater (Ambion, USA) and stored at -20°C until further analysis. Pituitary glands for fluorescent *in situ* hybridization (FISH) and immunofluorescence techniques were fixed in 4% Paraformaldehyde (PFA) in PBST (PBS with 0,1% Tween-20, pH=7,4) and then dehydrated in increasing concentrations of ethanol before being preserved in pure methanol at -20°C until further processing. Body and gonad weights were recorded to calculate gonadosomatic indices (GSI): GSI (%) = gonad weight *100/gutted weight. Pieces of ovarian tissue from females collected in different maturity stages were fixed in 4% PFA for histological processing to confirm macroscopic classification of gonad development.

	Phase	Ovarian morphology
F1	Immature/Regenerating (Inactive)	Orange, semi-transparent
F2	Developing	Small pink but some oocytes visible
F3A	Spawning capable	Large ovaries, oocytes visible macroscopically
F3B	Actively spawning	Abundance of hydrated oocytes
F4	Spent, regression, postspawning	Small ovaries, blood vessels reduced but present.

Table 1. Criteria used to determine maturational status of European hake females.

RNA extraction, cloning and sequencing of cDNA for European hake fshb and lhb

Total RNA was extracted from twenty pituitary glands using TRIzol Reagent, following the manufacturer's protocol (Invitrogen Life Technologies, Milan, Italy). One total RNA was used for cDNA synthesis, employing iScript cDNA Synthesis Kit (Bio-Rad laboratories, USA). mRNA sequences for *fshb* and *lhb* were amplified using primers designed by Primer3 (http://bioinfo.ut.ee/primer3/) after alignment (ClustalW2 software, at EMBL-EBI, Cambridge, UK) with sequences from other teleost species to localize well conserved regions (Table 2). PCR amplifications were run using annealing temperatures between 52-62°C and 1 min extension time on an iCycler Thermocycler (Bio-Rad, San Diego, California, USA). Each gene product was cloned into the pGEM T Easy vector system (Promega, Milan, Italy) in accordance with the manufacturer's

protocol. From the obtained partial sequences, 5'-RACE and 3'-RACE (Rapid Amplification of cDNA Ends) primers were designed (Table 2). When a single band was obtained following RACE PCR, it was isolated and cloned. All the cloned inserts were sequenced by BMR Genomics of Padova (Italy).

Name	Sequence (5'-3')	Orientation	Usage
3' RACE universal primer	5'-CGCGGATCCGAATTAATACGACTCACTATAGG-3'	Reverse	3'-RACE primer
5'RACE universal primer	5'-CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG-3'	Forward	5'-RACE primer
bact	5'-GTCATGGACTCCGGTGATGG-3'	forward	qPCR
	5'-GAGGTAGTCTGTGAGGTCGC-3'	reverse	qPCR
fshb	5'-ATGCAGCTGGTTGTCATGG-3'	forward	3'-RACE primer
	5'-TGCTCTGACACAGGGAACAC-3'	reverse	5'-RACE primer
	5'-TCTGTCGCCCAGTCAACTTC-3'	forward	qPCR
	5'-CCCACCGGACAGTCTTCAAA-3'	reverse	qPCR
lhb	5'-GTGGAGACCACCATCTGCA-3'	forward	3'-RACE primer
	5'-CAGCGGACACTGCATCAC-3'	forward	qPCR
	5'-ACAGTCCGGCAGCTCAAA-3'	reverse	qPCR

Table 2**.** Primer sequences of European hake used for 5'- and 3'-RACE and qRT-PCR. RACE, rapid amplification of cDNA ends; *bact*, β-actina; *fshb,* follicle-stimulating hormone beta subunit; *lhb*, luteinizing hormone beta subunit

Protein prediction, identification and multiple alignments

Primary structures were predicted and characterized by web-based bioinformatics tools such as Expasy Translate (http://web.expasy.org/translate/), ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/), the BLAST suite (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The open reading frames were aligned with Clustal Omega v. 1.2.1 (Sievers et al. 2011 - http://www.ebi.ac.uk/Tools/msa/clustalo/) from the Multiple Sequence Alignment tools provided by the EBI website. Gonadotropins were aligned with MUSCLE v. 3.8

(Edgar 2004 - http://www.ebi.ac.uk/Tools/msa/muscle/). The printing and shading of the alignment files was obtained with BOXSHADE v. 3.21 (http://www.ch.embnet.org/software/BOX_form.html), with a consensus threshold set at 0.5 to be represented by symbols.

Real time PCR

qPCRs were performed with the SYBR green method in an iQ5 iCycler thermal cycler (Bio-Rad). Triplicate PCRs were carried out for each sample analyzed following Santangeli et al. (2016). β-actin (b*act*) was used as housekeeping gene to standardize the results by eliminating variation in mRNA and cDNA quantity. As shown in Raingeard et al. (2009), the *bact* was chosen like reference gene because the mRNA levels were close to those of our target genes and the levels did not vary through different stages of reproduction. No amplification product was observed in non-template controls and no primer-dimer formations were observed in the control samples. The data obtained were analyzed using the iQ5 optical system software version 2.0 (Bio-Rad) including GeneEx Macro iQ5 Conversion and Genex Macro iQ5 files.

Fluorescence In Situ Hybridization (FISH)

RNA probes for European hake *fshb* and *lhb* were synthesized using pGEM-T easy vector (Promega, Madison, WI). Antisense and sense RNA probes were labeled with digoxigenin-11-UTP for *fshb* and fluorescein-12-UTP for *lhb* (Roche Diagnostics, Indianapolis, USA). Probes were purified using Nucleospin RNA clean-UP kit (Macherey-Nagel, Hoerdt, France). Regarding the tissue preparation, the pituitary glands were separated from brain. After the samples were rehydrated by a descending series of ethanol solutions (75%, 50% and 25% ethanol), they were placed in 3% agarose and cut (120 µm sagittal sections) with a VT1000S Leica vibratome. FISH was performed according to Fontaine et al. (2013). Briefly, tissues were permeabilized by 45

minutes treatment with proteinase K (10 µg/ml; P6556, Sigma) and prehybridized in hybridization buffer for 4 hours at 65ºC. Hybridization was performed at 65ºC for 18 h in hybridization buffer containing a mixture of probes (150 ng/ml each). Samples were washed with wash buffer composed of 25 ml formamide, 12,5 ml SSC 20x, 50 µL Tween20, 770 µL of 1 M Citric acid and water at different concentrations (50% formamide/50% 2x SSC [saline sodium citrate buffer]; 2x SSC; 0.2x SSC; PBST), treated for 30 minutes with 3% H_2O_2 to inactivate endogenous peroxidases. They were then incubated overnight at 4°C with antibody. For digoxigenin-labeled *fshb* probe, an anti-digoxigenin-peroxidase conjugated antibody (Roche Diagnostics) and a Cy5-conjugated tyramide (Perkin Elmer) were used. Fluorescein-labeled *lhb* probe was recognized by an antifluorescein-peroxidase conjugated antibody (Roche Diagnostics) and revealed by a homemade fluorescein-conjugated tyramide. After extensive washes, samples were mounted between slide and coverslip in Vectashield H-1000 Mounting Medium (Vector, Eurobio/Abcys). The sense probes were used for negative control (data not shown).

Combined FISH and immunofluorescence

In order to identify the phenotype of *lhb*-expressing cells in the hake pituitary gland, a triple labeling protocol was achieved by combining two-color FISH and immunofluorescence. After FISH, the sections were incubated for 2 hours at room temperature in a blocking solution (normal goat serum 4%, dimethylsulfoxide 1%, Triton 0.3% in PBST) and subsequently incubated over night at 4ºC with a rabbit antibody (1/1000) directed against the β subunit of medaka Lh (*Oryzias latipes*). The secondary antibody used was a goat antirabbit IgG coupled to DyLight 549 (Jackson ImmunoResearch Europe Ltd, Newmarket, Suffolk, England). Negative control for the immunofluorescence was verified in section of different part of pituitary gland (data not shown).

Image acquisition and processing

Fluorescent images were acquired using a Zeiss LSM 710 laser scanning confocal microscope. For fluorescent tyramides used in two-color FISH and for fluorophore-coupled secondary antibodies used in immunofluorescence studies, lasers with a wavelength of 488 (FITC; Alexa-488), 555 (DyLight 549), and 633 (Cy5; Alexa-633) nm, respectively, were used. Channels were acquired sequentially to avoid cross talk between the different filters. The focal planes were recorded using Zeiss ZEN 2009 software. Z-projections were obtained using Image J software distributed by Fiji (Schindelin et al. 2012). Composites were assembled using Adobe Illustrator CC (Adobe Systems, San Francisco, California).

Ovarian histological processing

Female gonads were maintained in 4% of PFA for fixation overnight and stored in 70% ethanol until further processing. Analysis of ovarian stages were conducted on histological paraffin sections (6-7 μm thickness) stained by Harris Hematoxylin and Eosin method following Caputo et al. (2001) and examined by Zeiss Axioscop (Oberkochen, Germany) light microscopy with phase contrast optics at a 200x magnification.

Phylogenetic analysis

Alignments and similarity matrices were calculated using Basic Local Alignment Search Tool (BLAST). The structure of deduced amino acid sequences of cloned fragments was predicted by comparison with known structures. Phylogenetic analyses of deduced amino acid sequences of Fshb and Lhb for European hake and other fishes were performed by MEGA version 6.0 (Tamura et al. 2013) using maximum-likelihood and default settings. A rooted consensus phylogenetic tree was generated by means of the Neighbor-Joining algorithm, using as outgroup the glycoprotein hormone

beta subunit-related protein of *Drosophila melanogaster* for Fsh and Lh. The robustness of the nodes was carried out by a bootstrap analysis from thousand data set replicates.

Statistical analysis

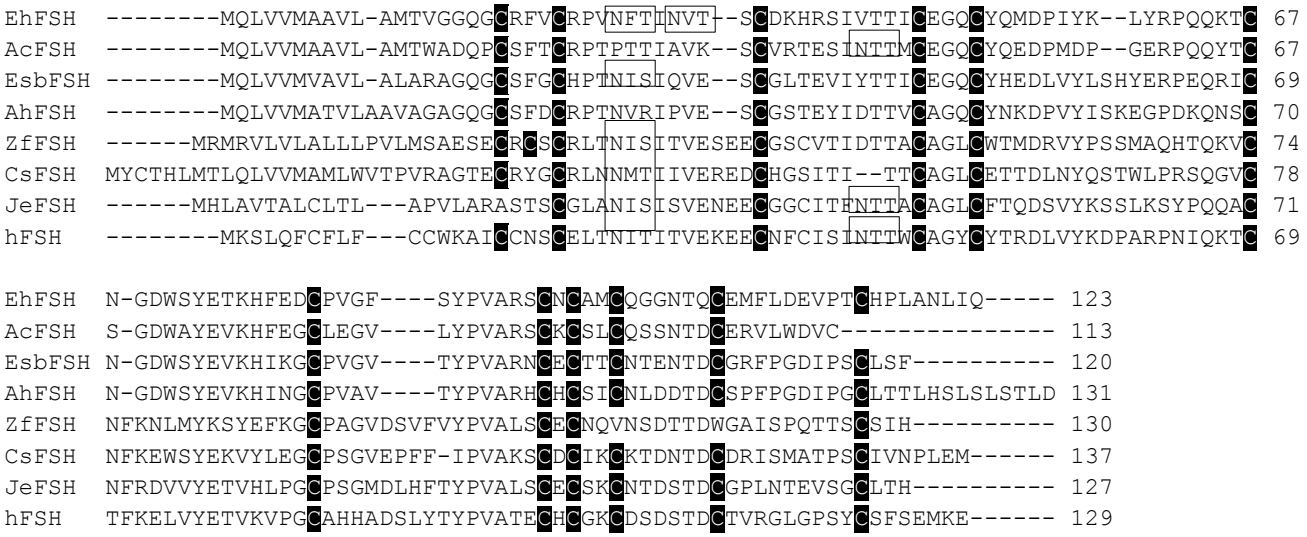
Results were expressed as the mean ± s.d. Statistical differences were determined using one-way ANOVA, followed by Bonferroni's multiple comparison test. All statistical analyses were performed using Prism 6 (GraphPad Software, San Diego, CA, USA). P-values <0.05 were considered to be significant. Exclusively for GSI data, P-values <0.1 were considered significant and the results were expressed as the mean ± SEM.

Results

Molecular characterization of gonadotropin beta subunits

The European hake cDNA encoding the *fshb* consisted of 550 nucleotides with an open reading frame (ORF) of 372 nt and with 5' and 3' untranslated regions (UTRs) of 108 and 70 nucleotides, respectively (Accession number of Genbank, KX377614). The consensus polyadenylation signal (AATAAA) was 15 nt upstream to the poly(A) tail. The similarity of *fshb* nucleotide sequence of European hake to the sequences of other fishes was in a range from 65% to 85%. The deduced amino acid sequence of Fshβ was 123 amino acid long. *In silico* analysis of the mature peptide showed a signal peptide of 18 amino acid, two N-linked glycosylation sites (NFT) and 12 conserved cysteine residues (fig. 1). A fragment of *lhb* of 509 nt was amplified, cloned and sequenced (Accession number of Genbank, KX377615). The nucleotide fragment showed high homology with *lhb* of other teleosts in a range from 68% to 82%. The partial deduced amino acid sequence was 89 aa long and although partial, we recognized some conserved clusters, like 9 cysteine residues highly conserved.

(A)



(B)

Figure 1. Amino acid sequence alignments of beta subunits of follicle-stimulating hormone, Fshb (A) and luteinizing hormone, Lhb (B) of European hake and other fishes. The cysteine residues are highlighted and potential N-glycosylation sites are shaded. The sequences were extracted from GenBank databases and their abbreviation and accession numbers are: *Merluccius merluccius* (Eh KX377614, KX377615), *Gadus morhua* (Ac: ABD62883, ABD62884), *Dicentrarchus labrax* (Es: AAN40506, AAN40507), *Danio rerio* (Zf: AAV31152, AAV31153), *Anguilla japonica* (Je: Q9YGK3, BAD14302), *Oncorhynchus keta* (Cs: AAA49408, AAA49409), *Hippoglossus hippoglossus* (Ah: CAD10501, CAD10502) and human (P01228, P01229).

Phylogenetic analysis of gonadotropins

Phylogenetic analyses showed that the vertebrate Gths were divided into Fsh and Lh clusters in the phylogenetic tree rooted using the glycoprotein hormone beta subunit-related protein of *D. melanogaster* as outgroup (fig. 2). The percentage of trees in which the associated taxa clustered together is shown below the branches. Both gonadotropins of *M. merluccius* are closely associated to gonadotropins of *G. morhua* (fig. 2).

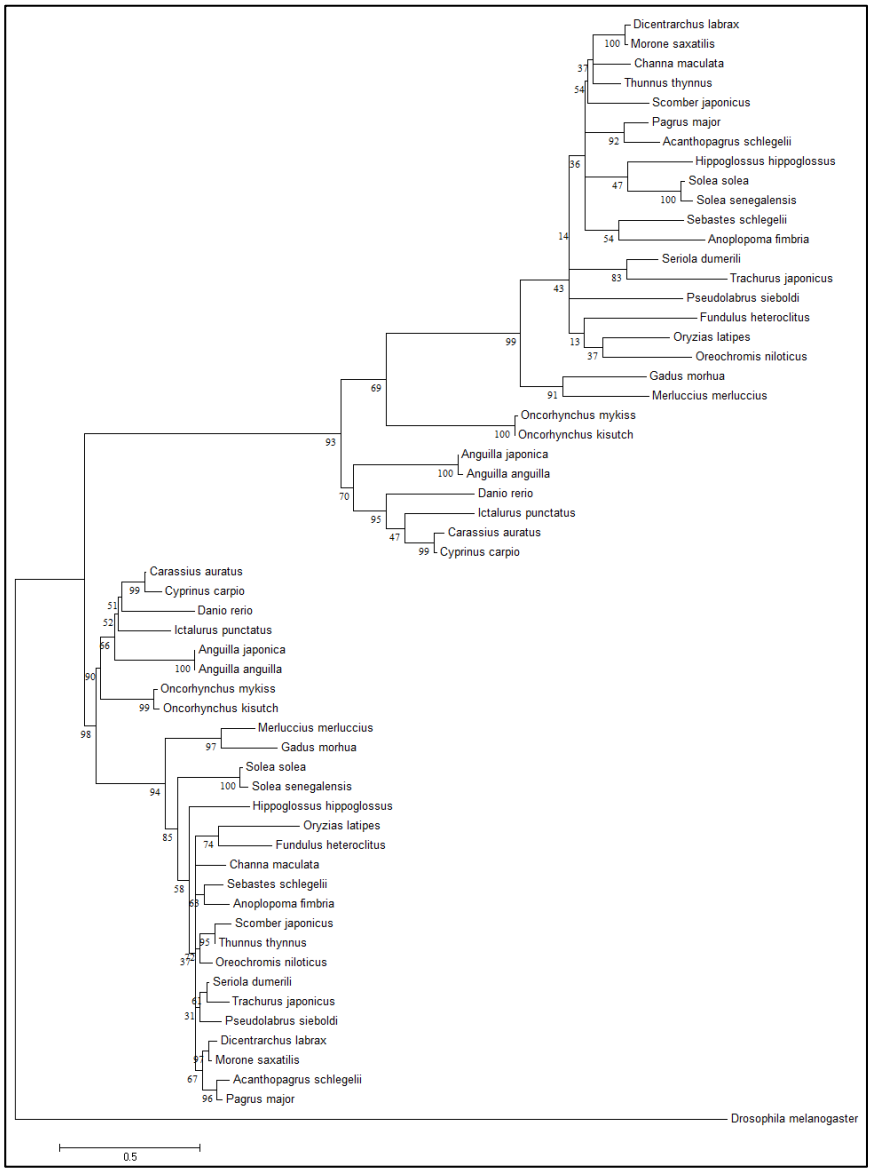


Figure 2. Phylogenetic comparison of fish full-length follicle-stimulating hormone (Fshb) and luteinizing hormone (Lhb) amino acid sequences. The analysis was performed by MEGA6 using maximum likelihood and default settings. A rooted consensus phylogenetic tree generated by means of the Neighbor-Joining algorithm, glycoprotein hormone beta subunit-related protein of *D. melanogaster* as outgroup. Bootstrap values from 1000 replicates are indicated for each tree node.

Ovarian histology and gonadosomatic index (GSI) variation

The analysis of GSI of hake females through the year evidenced a significant increase ($P<0.1$) in June compared to precedent months (fig. 3), suggesting the summer season as reproductive period for this species in Adriatic Sea. While, the GSI peak in December was not significant compared to other months. The histology confirmed the asynchronous development of oocytes for this species (fig. 4), meaning that the ovaries exhibit multiple oocyte stages (fig. 4b,c,d).

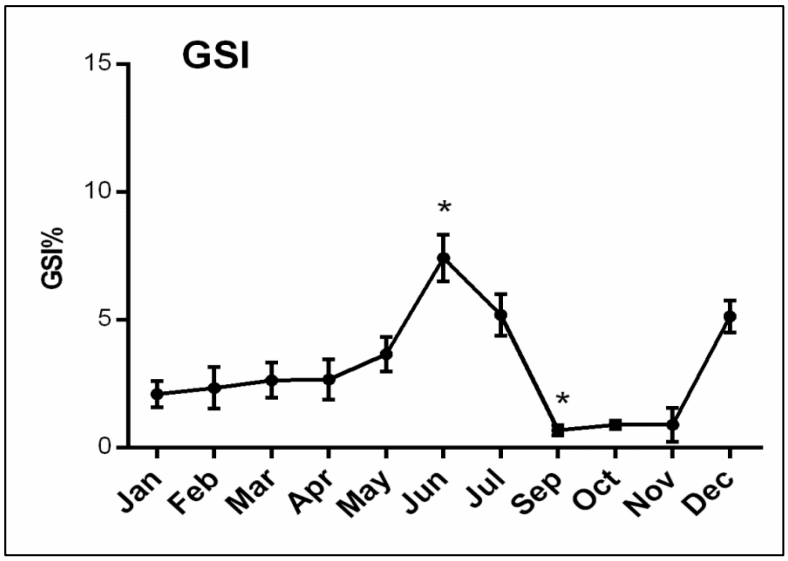


Figure 3. Monthly seasonal variation of European hake females gonadosomatic index (GSI). The asterisks indicate the presence of statistical significance ($P<0.1$) with respect to prevoius month. The values are Mean ± SEM.

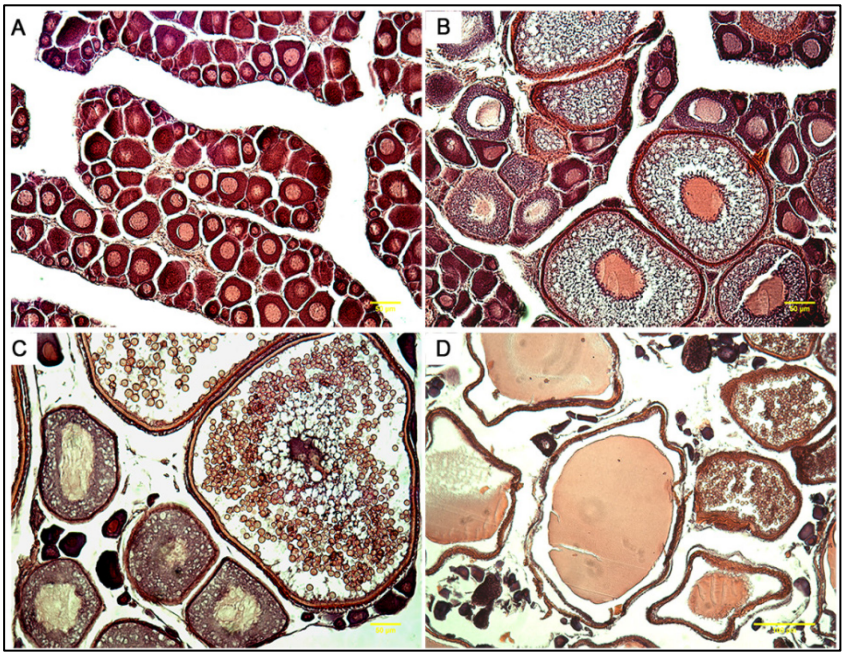


Figure 4. Tissue sections of the ovarian follicles at different developmental stages of European hake females. (A) F1, immature/regenerating; (B) F2, developing; (C) F3A, spawning capable; (D) F3B, actively spawning. Scale bar = 50 µm for A, B, C; scale bar = 100 µm for (D). Different stages of ovarian cells were reported in figure. PO, Primary Oocytes (unyolked oocyte); Vtg1, Vitellogenin 1; Vtg2, Vitellogenin; Vtg3 Vitellogenin 3; Hydrated oocytes (present only in actively spawning individuals); POF (Post-ovulatory follicle).

Gene expression profile analysis

In this study, the expression of *fshb* and *lhb* in the pituitary of females during oocyte maturation were examined by qPCR (fig. 5). Four stages were analyzed, from immature to actively spawning stages. The mRNA level of *fshb* was low in immature phase (F1) and raised in developing phase (F2) ($P<0.05$). It remained at an high level in spawning capable phase (F3A) and significantly decreased in the last phase considered (F3B) ($P<0.05$). The levels of *lhb* showed a significant increasing trend with a significant peak at beginning of maturation (F3A) ($P<0.05$). The *lhb* mRNA levels were declined during actively spawning stage (F3B) ($P<0.05$) as *fhsb*.

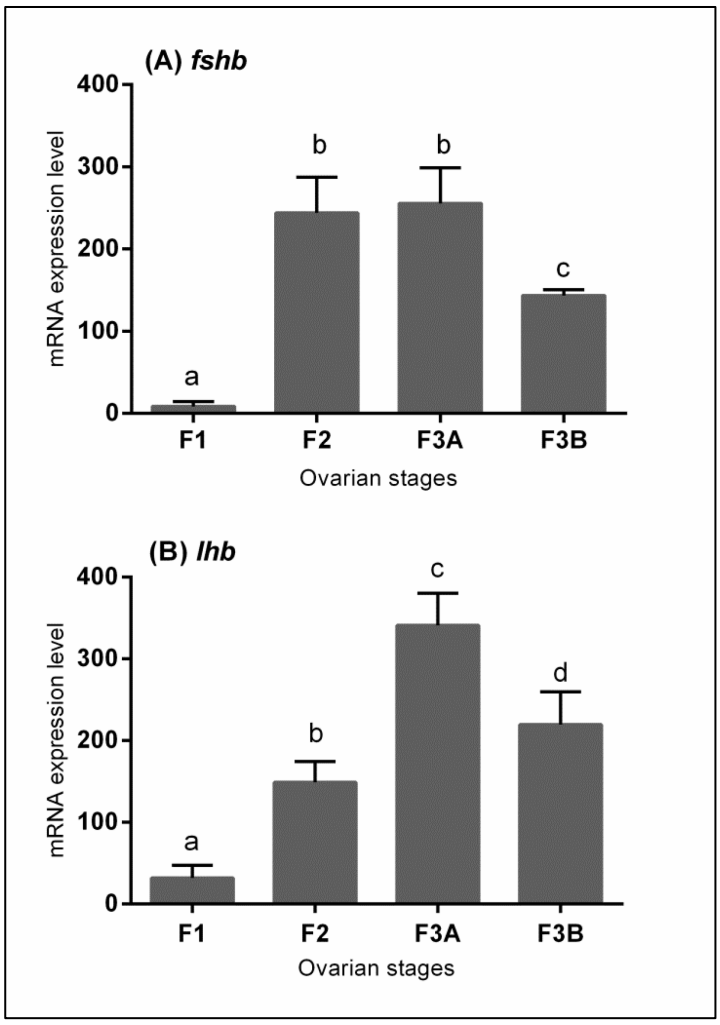


Figure 5. Relative mRNA expression levels of (A) *fhsb* and (B) *lhb* in pituitary gland of European hake in specimens with different ovarian stages. Abundance of *fshb* and *lhb* transcript was determined by qRT-PCR and normalized with b-actin. The values are Mean ± SD.

Localization of fshb and lhb mRNA and Lhb protein

The double-labeling FISH and immunofluorescence techniques were coupled to detect the localization of *fshb* and *lhb* expression simultaneously and Lhβ protein on female adult pituitary tissues during reproductive cycle. Contrary to sense probes (data not shown), antisense probes revealed the expression of *fshb* and *lhb* mRNA in distinct cells in *proximal pars distalis* (PPD) and in the edge of *pars intermedia* (PI). *fshb* and *lhb* were expressed mostly in adjacent pituitary cells, but some cells showed co-expression of both hormones (fig. 6d). Comparing the gene expression of both gonadotropin β subunits with that of Lhb protein, we observed that the anti-Lhb serum merged completely with the cells positive to *lhb* mRNA expression. Also, anti-Lhb stained the cells expressing both hormones, in addition to few cells expressing only *fshb* (fig 7d). During the reproductive cycle the expression of gonadotropins mRNAs and Lhb protein was always detectable.

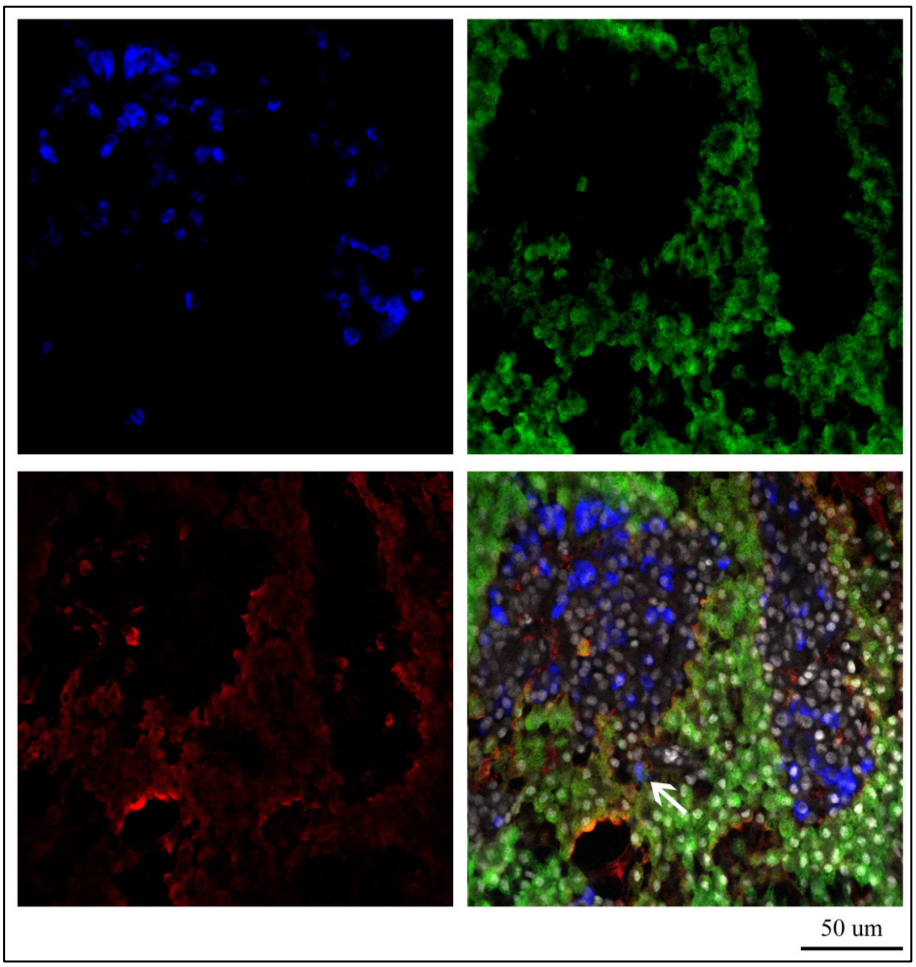


Figure 6. Differential expression of the *fshb* (A), *lhb* (B) transcripts and Lh protein (C) in adult European hake pituitary gland. The picture 6D shows the merging of three pics and the arrow evidences the *fshb* and *lhb* co-expression and the presence of Lh protein in a cell.

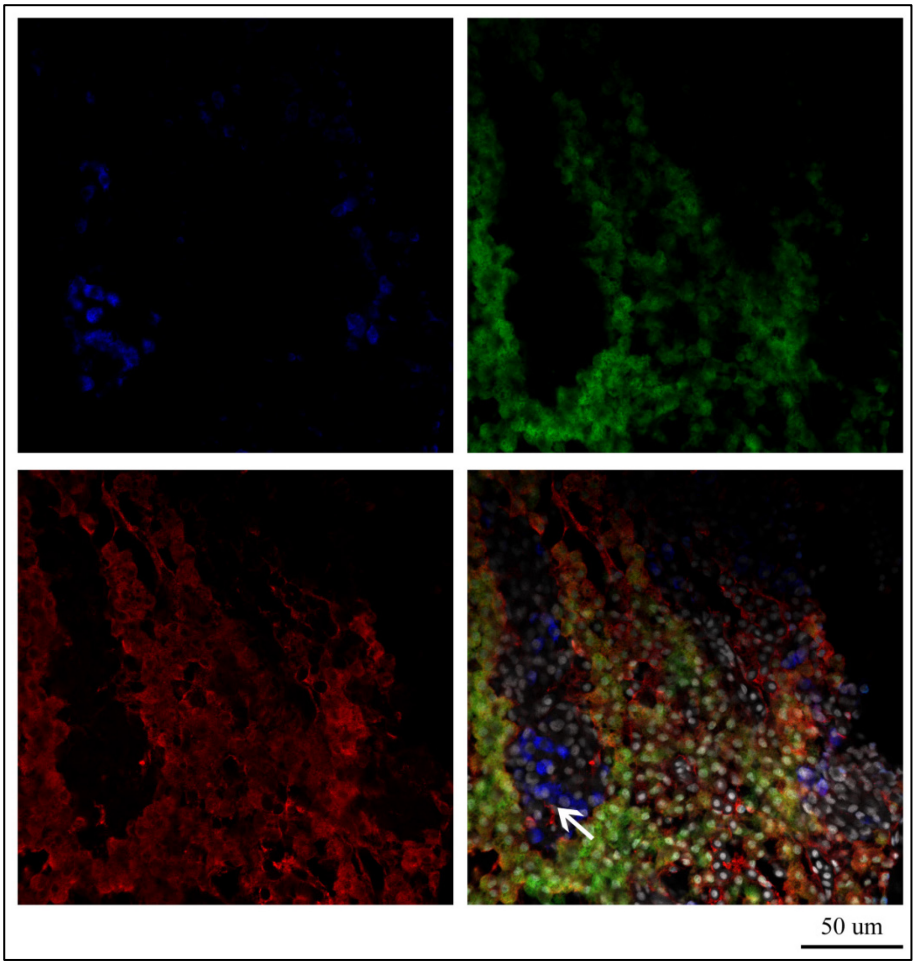


Figure 7. Differential expression of the *fshb* (A), *lhb* (B) transcripts and Lh protein (C) in adult European hake pituitary gland. The picture 7D shows the merging of three pics and the arrow evidences the presence of Lh protein in the *fshb* cells.

Discussion

The characterization and expression studies of gonadotropin genes have been investigated in several teleost species. As already reported in the Introduction, the mRNA expression of gonadotropins during the reproductive cycle has not been well defined in teleosts. The main matter is definitely linked to the wide variety of reproductive strategies of different species. For this purpose the current study was aimed to better understand the functions of Fsh and Lh in the reproduction of European hake, a species with asynchronous ovarian development characterized by different phases of maturation (Sarano 1986; Murua et al. 1998). In the present study, the endocrine control of ovarian

maturation of European hake was approached for the first time, by studying gonadotropin molecular characterization, expression and localization in the pituitary gland throughout the seasonal reproductive cycle in wild specimens captured in the Adriatic Sea.

The *fshb* and *lhb* nucleotide sequences were obtained by the RACE method. While the approach was successfully performed for *fshb*, same approach failed to amplify the 5' terminal part of *lhb*, obtaining a partial sequence of 509 nt. The two nucleotide sequences were used to predict the relative amino acid sequences. Analyzing the Fshb deduced polypeptide, it had 63% sequence homology to another gadoid fish, Atlantic cod (*Gadus morhua*) and 35-59% identity range to other teleosts. It is notable that the N-glycosylation site conserved the position through teleosts, though in the most of fishes the specific consensus sequence of N-linked glycosylation consists in Asn-X-Ser whereas in hake was Asn-X-Thr as in chum salmon (Sekine et al. 1989) and in tetrapods. Also like tetrapods and ancient teleosts (Yoshiura et al. 1999; Quérat et al. 2001), the European hake showed two potential N-glycosylation sites in contrast to other modern teleosts. The presence of two sites ensures the biological potencies of the hormone and stabilize the interactions with its receptor (Bousfield et al. 1994). Also as tetrapods and ancient teleosts, European hake showed 12 cysteine residues associated with 'seatbelt' region. The same numbers of cysteine residues were found also in other teleosts, except for siluriformes and cypriniformes, that present an additional residue at the N-terminus, the Cys3 (according to the numbering in tetrapods). The lack of Cys3 could increase the stability on the tertiary structure of the heterodimer (Xing et al. 2004; Levavi-Sivan et al. 2010). Regarding the partial amino acid sequence of Lhb, it had 77% homology with Atlantic cod and 57-70% identity range to other fishes. We found nine of the expected twelve cysteine residues that are typical for teleosts, and their position was highly conserved. Together with Fshb protein, the Lhb amino acid sequence was used for the construction of the phylogenetic tree that was inferred from various teleost Fshb and Lhb protein sequences. Both hormones were distinctly separated in

independent order and both showed high similarity to respective homolog hormone genes of Atlantic cod, confirming that they belong to Gadiformes order.

The monthly trend of the mean GSI showed two different peaks, one in December and the highest in June, where the reproductive activity was concentrated. The increase of GSI, excluding the spawning period, confirmed that hake spawns continuously throughout the year (Murua and Motos 2006).

Considering the expression profiles of gonadotropin genes in the pituitary gland of European hake during their reproductive cycle, the novel finding is consistent with detectable levels of *fshb* along all the reproductive phases - from Immature/Regenerating to Actively Spawning period as classified by Brown-Peterson et al. (2011). Previous studies concerning the expression pattern of *fshb* during gonadal maturation suggested that the hormone is mainly involved in the first phases of the gonadal cycle and the temporal profile of mRNA expression was distinct from that of *lhb*, which was expressed especially during the last phases of reproduction (Planas and Swanson 1995; Tyler et al. 1997; Gomez et al. 1999; Yoshiura et al. 1999; Schmitz et al. 2005). Unlike species with synchronous ovarian development, the European hake displayed high expression of *fshb* during F2 and F3A phase, suggesting a continues role of this hormone due to the presence of different oocyte stages into the ovary at the same time. Indeed, while some oocytes are involved in final maturation and ovulation, other oocytes in the same ovary are still in vitellogenesis. A similar trend was detected in other multiple spawners (Gen et al. 2000; Kajimura et al. 2001; Mateos et al. 2003; Weltzien et al. 2003b). Remarking the typical reproductive pattern reported and consistent with other teleost species, the gene expression of *lhb* in the pituitary showed a significant peak in F3A as in all teleosts. The low *fshb* and *lhb* expression in actively spawning stage females (F3B) may be caused by a negative feedback exerted on the gonadotropic cells by the high levels of gonadal steroid hormones (Kajimura et al. 2001), though other authors (Asturiano et al. 2002) evidenced that changes in gonadal hormones could lead to the spermiation and ovulation.

Finally, the present study provided for the first time the information about the specificity of hormone production by gonadotropic cells by combining double-labeling fluorescent *in situ* hybridization of *fshb* and *lhb* with Lhβ protein immunofluorescence. The distribution of gonadotropic cells evidenced several differences with other teleosts. Differently to So et al. (2005) where the cells expressing *fshb* were arranged as small cell clusters or single cells and *lhb* like large clusters in zebrafish, there was no apparent difference in the distribution pattern between *fshb*- and *lhb*-cells in European hake, indeed both gonadotrope cell types were arranged like large clusters throughout PPD during all ovarian stages. Although it was difficult to quantitate accurately the numbers of the two types of gonadotropic cells, they seemed to be similar. In addition Cao et al. (2009) detected *fshb* cells in the middle area of PPD and *lhb* in the middle and in periphery of PPD and in PI, whereas in European hake the *fshb* and *lhb* cells were homogenously distributed through the whole PPD and in PI. Previous studies in Senegalese sole and Atlantic halibut (Cerdà et al. 2008; Weltzien et al. 2003b) evidenced mainly the *fshb* and *lhb* expression in distinct gonadotropes of the PPD, although the authors did not exclude the possible presence of the co-expression of both gonadotropins in a single gonadotropic cell. The use of confocal imaging associated with fluorescent labeling allowed us to establish that a limited number of cells co-express both hormones. It is still unclear whether the cells that produced both hormones have always presented such ability or, instead, if this is an acquired function during reproductive cycle. From previous results, Golan et al. (2014) found few cells that showed co-localization of Fshb and Lhb proteins in tilapia and zebrafish, hypothesizing that these gonadotropes are bipotent cells that subsequently undergo the differentiation into either *fshb*- or *lhb*- cells. In addition to these hypotheses, we suggest that the gonadotropes could have a plasticity that permit changes during lifetime and in turn during different physiological states. The presence of Lhb protein merged mainly with *lhb*-cells and also in cells expressing both hormones. Surprisingly, it was also recorded in a few *fshb* cells. Since we could not investigate the presence of Fshb protein due to the lack of suitable antibodies, it remains

difficult to clarify if the presence of Lhb protein in those few cells is the result of a changing of phenotype or those cells are not completely differentiated.

In conclusion, we have used the European hake, an important commercial species, like model to elucidate the role of Fsh and Lh in species with asynchronous ovary. We report the sequences and molecular information on gonadotropin subunits in European hake. In addition, the specific expression of *fshb* and *lhb* in different cells in pituitary gland confirmed the gonadotropin localization similar to other teleost fish, but the additional small number of cells that showed co-expression could provide important bases for further investigations on *fsh* and *lh* cell differentiation.

Declaration of interest

The authors declare no conflict of interests.

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Scomber japonicus (AEN14604; AEN14605); *Thunnus thynnus* (ABP04057; ABP04050); *Sebastes schlegelii* (AAU14141; AAU14142); *Anoplopoma fimbria* (AGS55583; AGS55584); *Acanthopagrus schlegelii* (AAX18926; ABQ96864); *Solea solea* (ABW81403; AHZ13200); *Oryzias latipes* (BAK61761; BAK61762); *Oreochromis niloticus* (AAP49575; AAP49576); *Pseudolabrus sieboldi* (BAF81900; BAF81901); *Trachurus japonicus* (AGO59024; AGO59025); *Oncorhynchus mykiss* (BAB17686; BAB17687); *Oncorhynchus kisutch* (AAO72299; AAO72300); *Carassius auratus* (BAA13530; BAA13531); *Anguilla japonica* (Q9YGK3; BAD14302); *Fundulus heteroclitus* (P30971; P30972); *Cyprinus carpio* (CAA42542; CAA42543); *Danio rerio* (AAV31152; AAV31153); *Ictalurus punctatus* (AAG32155; AAG32156); *Morone saxatilis* (AAC38035; AAC38019); *Drosophila melanogaster* (AAM53262).

CHAPTER IV

REPRODUCTIVE BIOLOGY OF EUROPEAN HAKE (*MERLUCCIUS MERLUCCIUS*): A MULTIDISCIPLINARY APPROACH

Reproductive biology of European hake (*Merluccius merluccius*): a multidisciplinary approach

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Abstract

The fecundity estimation in fish and the study of the size at first maturity are important factors involved in the evaluation of reproductive potential of the stock. In addition, studies regarding the spawning season of a species, the reproductive physiology and the physiological indicators involved in the energy state are other fundamental tools that allow to improve the knowledge of the state of

the fish stock and to increase the methodologies for the assessment of overexploited species. There are many commercial species involved in this issue and the European hake (*Merluccius merluccius*) definitely deserves special attention, because of FAO included it in overfished species category. Our studies focused on the European hake females in Adriatic Sea, where it plays an important commercial role. For the first time we analyzed the fecundity of the stock in this area evidencing that the Adriatic stock spawn less eggs compared to the bigger hakes of the Mediterranean Sea and the Atlantic Ocean. Also, the gonadosomatic index, the hepatosomatic index and the Le Cren's condition factor were analyzed. The GSI analysis identified the spawning peak in summer, while HSI and K_n indicate Autumn as the period of greatest accumulation of the energy. In addition, the histological analysis were performed to validate the macroscopic observation. Finally, for the first time, the gonadotropin receptors were characterized and their gene expression was quantified in oocytes at different maturity stages. Such studies pave the way for future studies on the stock assessment and reproductive physiology for this overexploited species.

Key words: Adriatic Sea, European hake, Fecundity, Gonadotropin receptors, *Merluccius merluccius*

Introduction

In the last decades, the excessive fishing effort together with the increase of pollution, poor fishing management and impairment of marine ecosystem caused the depletion of worldwide fish stocks. As reported by the Food and Agriculture Organization the 57.4% of fish stocks assessed were fully exploited, the 29.9% were overexploited and only the 12.7% were not-fully exploited in 2009 (FAO, 2011). Mullon and co-workers (2005) have analyzed the FAO data set of world fisheries catches from 1950 to 2000, observing that the main fisheries collapse was verified in demersal fishes. Among demersal commercial species, the European hake (*Merluccius merluccius*, Merlucciidae) had a particular attention because it is one of the main target fishing species in the

Mediterranean Sea with total landings of 22547 tons in 2011 (Druon et al., 2015). Also in Northeast Atlantic Ocean, the population of the European hake is an important sustenance of the fisheries (Cerviño et al., 2013). FAO included European hake in the list of overfished species and the management measures are important specially to reduce the catch of juveniles (<30 cm) that mainly exploit by bottom trawling method (Ligas et al., 2015).

For its commercial importance and the critical status of the stock, several studies have been on European hake in different areas of the Mediterranean sea, concerning the stock assessment, spawning cycle and fecundity estimation (Bouaziz et al., 1998; Nannini et al., 2001; Recasens et al., 2008; Al-Absawy, 2010; El Habouz et al., 2011;), the spatial distribution (Recasens et al., 1998; Roldán et al., 1998; Orsi Relini et al., 2002; Abella et al., 2005), feeding habits (Carpentieri et al., 2005), toxicology studies (Raingeard et al., 2009), analysis of lipid reserves (Lloret et al., 2008), nursery area (Druon et al., 2015), juvenile recruitment (Ligas et al., 2015), fisheries management (Colloca et al., 2013; Tsagarakis et al., 2014; Angelini et al., 2016;). Other studies have been focused on the state of stock in Adriatic Sea (Zupanovic & Jardas, 1986; Frattini & Paolini, 1995; Arneri & Morales-Nin, 2000; Ungaro et al., 2001; Ungaro et al., 2003; Šantić et al., 2011), but none of them investigated on fecundity estimation and reproductive physiology in Adriatic Sea, where this species is overexploited as the rest of Mediterranean Sea.

The aim of this study is to increase the knowledge of the reproductive physiology of European hake and consequently to elucidate the current state of the stock in Adriatic Sea. In order to establish the assessment of this species, the trend of morphometric indices, the Le Cren's condition factor (K_n), the hepatosomatic index (HSI) and gonadosomatic index (GSI) were calculated for three years and the size at first maturity was evaluated. Furthermore, for the first time in the Adriatic Sea, the fecundity, considered as batch and relative fecundity, was estimated. In addition to macroscopic analysis, also the histological analysis of the ovaries was performed to validate the macroscopic scale used in present study. Finally, to complete the scenario of the reproductive physiology of this

relevant commercial species, the gonadotropin receptors were characterized and their expression was analyzed in different class of oocytes. All these information allowed to define the status of female hake stock.

Materials and methods

Sampling

A total number of 976 European hake females, in a total length range from 13 to 64 cm, were collected on board of bottom trawler fishing vessel of Ancona in the Northern and Central Adriatic Sea (FAO- Geographical Sub-Area 17, according to GFCM division) from January of 2013 to December of 2015. No samples were fished in August because the Italian legislation banned the fishing in that months. The animals were collected under the guidelines of the Data Collection Framework Regulation (EU Reg.199/2008) that established a Community system for the conservation and sustainable exploitation of fisheries resources under the Common Fisheries Policy (CFP). The procedures did not include animal experimentation and ethics approval are not necessary in accordance with the Italian legislation. The following parameters were recorded for each fish analyzed: total weight (TW), gutted weight (GW) total length (TL), sex and macroscopic maturity stage of gonads, gonad and liver weights. The animals were macroscopically and microscopically classified in five different ovarian classes: immature/regenerating (F1); developing (F2); spawning capable (F3A); actively spawning (F3B), and spent, post spawning (F4) (Table 1), following the ovarian classification of Brown-Peterson et al. (2011), but adapted for European hake. The ovaries for qPCR analyses were immediately preserved in RNAlater (Ambion, USA) and stored at -20°C for analysis.

	Phase	Ovarian morphology
F1	Immature/Regenerating (Inactive)	Orange, semi-transparent
F2	Developing	Small pink but some oocytes visible
F3A	Spawning capable	Large ovaries, oocytes visible macroscopically
F3B	Actively spawning	Abundance of hydrated oocytes
F4	Post spawning	Ovaries are flaccid and red. Atresia (any stage) and POFs are present.

Table 3: Criteria used to determine maturational status of female European hake

Morphometric and Physiological indices

The specimens were grouped in seven classes of length (cm): ≤15; 16-20; 21-25; 26-30; 31-35; 36-40; ≥41; our studies were focused on adult female specimens and for this reason only the females from 26 cm of length were considered. The somatic parameters were used to calculate the monthly evolution of gonadosomatic index (GSI), hepatosomatic index (HSI) and Le Cren's relative condition factor (K_n) following (Froese, 2006).

$$GSI\ (\%) = \text{gonad weight}*100/\text{gutted weight body}$$

$$HSI\ (\%) = \text{liver weight}*100/\text{gutted weight body}$$

$$K_n = W/a\ L^b$$

where a and b are the regression parameter of the length – weight relationship, W is gutted weight and T is the total length. For logistic problems, in the first quarter of 2013 no livers were sampled.

The estimation of length at first maturation (L_{50}), representing the size at which 50% of the specimens were mature, was determined by fitting the logistic equation (Prager et al., 1989):

$$p = [1 + e - r\ (x - x50)]\text{-}1$$

(where p is the estimated proportion in size class, r is a fitted parameter, x is the total length, x_{50} is the length at which 50% of the specimens was mature), to the proportion of fish in each size class. The females starting from the F2 stage were considered for the estimation. For fecundity, 28 females were used in actively spawning stage. The fecundity was considered like batch and relative fecundity. The batch fecundity was calculated in relation to the total weight of the gonads by gravimetric method following El Habouz and co-workers (2011). Based on results reported by Murua et al. (2006), the distribution of hydrated oocytes in the ovaries was not significant different and only a lobe of hydrated ovary was used for analysis. Three subsamples of 0.5 g (±0.001g) were taken from the anterior, the middle and the posterior part of lobe. The hydrated oocytes of fresh subsamples were manually divided and counted using a stereomicroscope (Optika, Italy). The batch fecundity was estimated as the average of the hydrated oocyte number of the three subsamples

multiplied by ovary mass for each specimens with hydrated oocyte. The relative fecundity is calculated as batch fecundity · gutted weight^{-1} of each specimen.

RNA extraction, cloning and sequencing of cDNA for European hake fsh and lh receptors

Total RNA was extracted from oocytes at different stages using TRIzol Reagent, following manufacturer's protocol (Invitrogen Life Technologies, Milan, Italy). Total RNA was treated with DNAse (10 IU at 37 °C for 10 min, MBI Fermentas). A total amount of 1 µg of RNA was used for cDNA synthesis, employing iScript cDNA Synthesis Kit (Bio-Rad laboratories, USA). Conserved *fshr* and *lhr* mRNA sequences were amplified using primers designed by Primer3 (http://bioinfo.ut.ee/primer3/) after aligning the cDNA sequences of these genes from known teleost (ClustalW2 software, at EMBL-EBI, Cambridge, UK). The teleost sequences were taken from the National Center for Biotechnology Information (NCBI, USA). Primer sequences are reported in Table 2. PCR amplifications were run between 52-62°C annealing temperature and 1 min extension time using an iCycler Thermocycler (Bio-Rad, San Diego, California, USA). Each gene product was cloned into the pGEM T Easy Vector System (Promega, Milan, Italy) in accordance with the manufacturer's protocol. From the obtained sequences, specific primers for 5'-RACE and 3'-RACE (Rapid Amplification of cDNA Ends) were designed using manufactory protocol of FirstChoice RLM-RACE Kit (Thermo Fisher Scientific, Paisley, UK). The single bands obtained were agarose-gel purified and cloned. All the cloned inserts were sequenced by BMR Genomics of Padova (Italy).

Name	Sequence (5'-3')	Orientation	Usage
3' RACE universal primer	5'-CGCGGATCCGAATTAATACGACTCACTATAGG-3'	Reverse	3'-RACE primer
5'RACE universal primer	5'-CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG-3'	Forward	5'-RACE primer
βactin	5'- GTCATGGACTCCGGTGATGG -3'	forward	qPCR
	5'- GAGGTAGTCTGTGAGGTCGC -3'	reverse	qPCR
18S	5'- GAGGCCCTGTAATTGGAATG -3'	forward	qPCR
	5'- CGCAAGACACTCAACCAAGA -3'	reverse	qPCR
fshr	5'- CAGCAGCTACGACAAGGTG -3'	forward	3'-RACE primer
	5'- GTGACGCCTTCAACGACAC -3'	forward	Inside primer regular PCR

	5’- CTGAAGTAGCAGACGCAGAC -3’	reverse	Inside primer regular PCR
	5’- GTGAGGGTCAGTGGCTGA -3’	reverse	5’-RACE primer
	5’- CATGGCCGTGCTCATCTTC -3’	forward	qPCR
	5’- ATGAAGAGGAAGGGGTTGGC -3’	reverse	qPCR
lhr	5’- GCAAAGCGCATGGCTGTGCTC -3’	forward	3’-RACE primer
	5’- GCATTCCTCTCCCTGCACTG -3’	forward	Inside primer regular PCR
	5’- AGCAACAGGTAGAGCCCCAT -3’	reverse	Inside primer regular PCR
	5’- GAATTGGCAGGGATAGAGTC -3’	reverse	5’-RACE primer
	5’- GTCAGCGAGTTGGACATGGA -3’	forward	qPCR
	5’- ATGACCCAGGTGAGAAAGCG -3’	reverse	qPCR

Table 4: Primers and probes used for 5’- and 3’-RACE and for real-time quantitative RT-PCR in

Real time PCR

PCRs were performed with the SYBR green method in an iQ5 iCycler thermal cycler (Bio-Rad laboratories). The used primers are reported in Table 2. Triplicate PCRs were carried out for each sample analyzed following (Santangeli et al., 2016). *β-actin* (b*act*) and 18S were used as housekeeping genes to standardize the results by eliminating variation in mRNA and cDNA quantity. As shown in Raingeard et al. (2009), the *bact* and 18S were chosen like reference gene because the mRNA levels were close to those of our target genes and the levels did not vary through different stages of reproduction. No amplification product was observed in non-template controls and no primer-dimer formations were observed in the control samples. The data obtained were analyzed using the iQ5 optical system software version 2.0 (Bio-Rad) including GeneEx Macro iQ5 Conversion and Genex Macro iQ5 files.

Protein prediction, identification and multiple alignments

The encoded proteins primary structures were predicted and characterized by web-based bioinformatics tools such as Expasy Translate (http://web.expasy.org/translate/), ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/) the BLAST suite (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and SignalP (Petersen et al. 2011 - http://www.cbs.dtu.dk/services/SignalP/). The open reading frames were aligned with Clustal

Omega v. 1.2.1 (Sievers et al. 2011 http://www.ebi.ac.uk/Tools/msa/clustalo/) from the Multiple Sequence Alignment tools provided by the EBI website. The printing and shading of the alignment files was obtained with BOXSHADE v. 3.21 (http://www.ch.embnet.org/software/BOX_form.html), with a consensus threshold set at 0.5 to be represented by symbols.

Phylogenetic analysis

Alignments and similarity matrices were calculated using Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi). The structure of deduced amino acid sequences of cloned fragments was predicted by comparison with known structures. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 56 amino acid sequences. All positions with less than 95% site coverage were eliminated, corresponding to fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. Phylogenetic analyses of deduced amino acid sequences of Fsh and Lh receptors for European hake and other fishes were performed by MEGA version 6.0 (Tamura et al., 2013) using maximum-likelihood and default settings. A rooted consensus phylogenetic tree was generated by means of the Neighbor-Joining algorithm, using as outgroup the FSH-TSH of Drosophila melanogaster (Drosophilidae) and testicular glycoprotein hormone receptor I precursor of Petromyzon marinus (Petromyzontinae). The robustness of the nodes was carried out by a bootstrap analysis from thousand data set replicates.

Ovarian histological processing

173 female specimens in different maturity stages and length classes were chosen for histological analysis to confirm macroscopic classification of gonadal development. A sub-sample of ovarian tissue were fixed at 4° C overnight in 4% paraformaldehyde (PFA) prepared in phosphate-buffered

saline (PBS, 0.1 M, pH 7.4) and stored in 70% ethanol until use. Successively, samples were dehydrated through a graded series of ethanol and embedded in paraffin. Consecutive sections were cut at a thickness of 4 μm using a microtome and stained by Harris Hematoxylin and Eosin for oocyte analysis.

Statistical analysis

Statistical differences were determined using one-way ANOVA, followed by Tukey's multiple comparison test. All statistical analyses were performed using Prism 6 (GraphPad Software, San Diego, CA, USA). P-values <0.05 were considered significant. The results were expressed as the mean ± SD.

Results

Morphometric and Physiological indices

During three years, the European hake females in a range length of 13 to 64 cm were sampled for a total of 976 animals. Each individual was included in one of the following length classes (cm): ≤15; 16-20; 21-25; 26-30; 31-35; 36-40; ≥41 (fig.1).

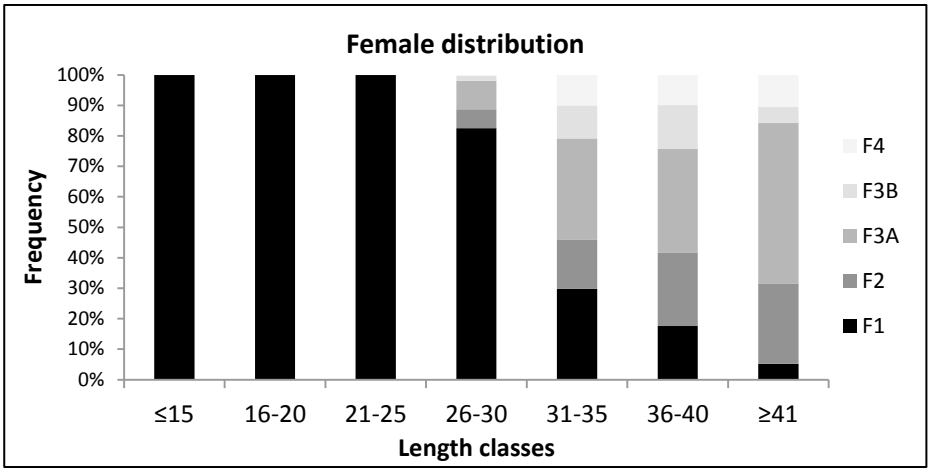


Figure 1: Percentage of females of European hake in the different maturity stages for length classes

The specimens less than 26 of length were immature and the adult specimens, considered as the females that have started the gonadal sexual maturation, were found from length class of 26-30 cm. The GSI, HSI and K_n analysis were performed in the adult specimens. The GSI trend was similar

between 2014 and 2015, with the highest significant peak (P<0,05) in June and lowest in September (fig. 2).

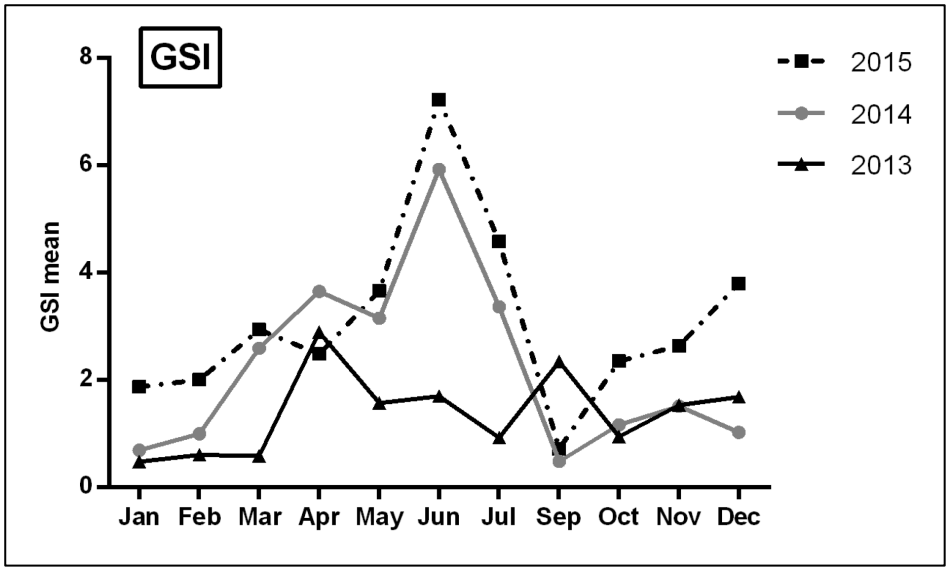


Figure 2. Monthly variation of GSI during 2013, 2014 and 2015 in European hake females.

A slight increase of GSI value was observed also in the last months of 2015, but it was not significant compared to the rest of the year. In 2013 no significant peak was observed in GSI trend and the monthly values were lower than the other two years under study. Also the HSI trend varied in 2014 and 2015 (fig. 3). In 2014, the two highest significant peaks were recorded in September and December (P<0,05), while in 2015 HSI surged in significant way only in September. In 2013, the index was constant throughout the year.

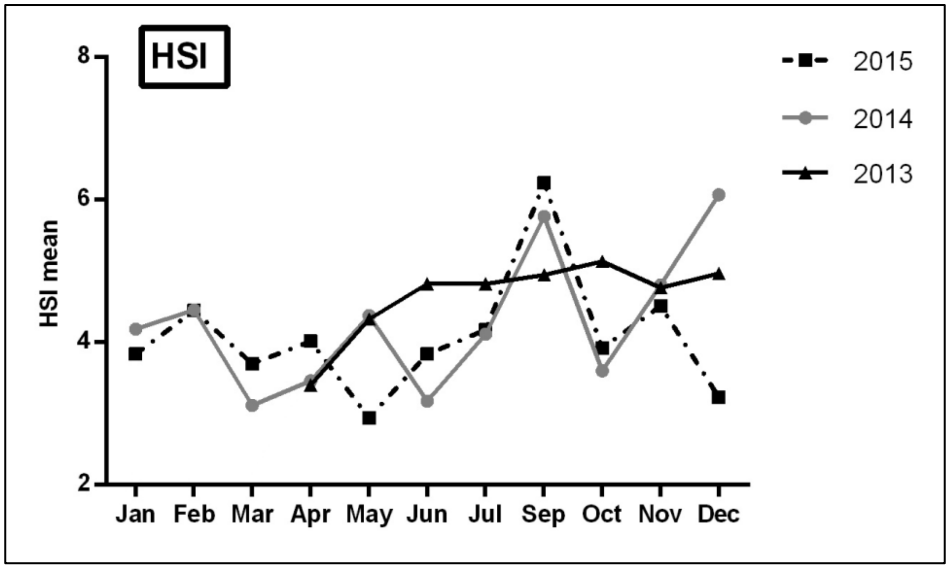


Figure 3. Monthly variation of HSI during 2013, 2014 and 2015 in European hake females.

As the GSI and the HSI, the trend of K_n varied mainly in 2014 and 2015, with a common highest peak recorded in September (fig. 4). In 2014, also December showed a significant difference, but only compared to June. In 2013 the K_n values were lower than the values recorded in the following two years and a lack of peak in September was observed, while a slight increase was recorded in July, only significant respect to April.

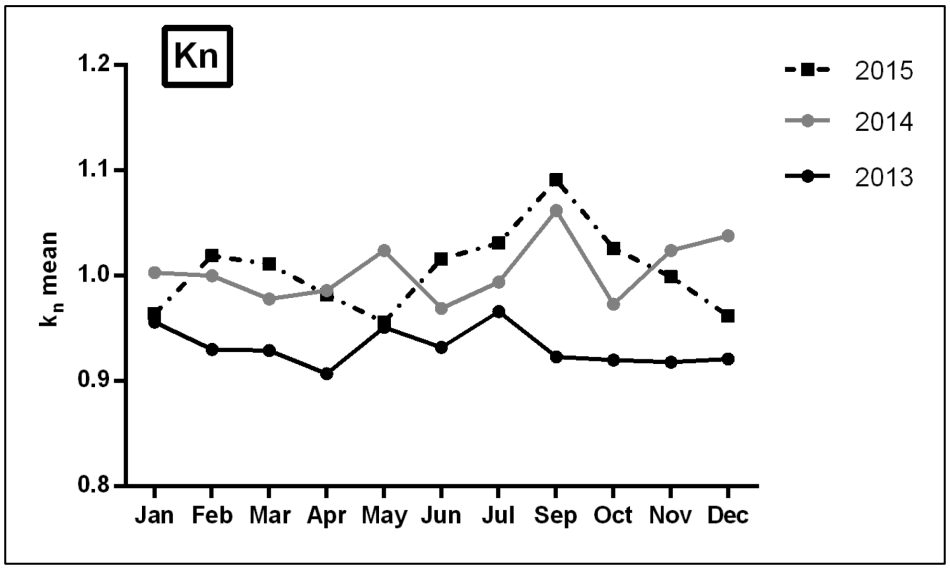


Figure 4. Monthly variation of K_n during 2013, 2014 and 2015 in European hake females.

The L_{50} was estimated as 30,96 cm (fig. 5). The size at which all the females matured at least once was 39 cm. The shortest length at which the female specimens started the ovarian development was 26 cm.

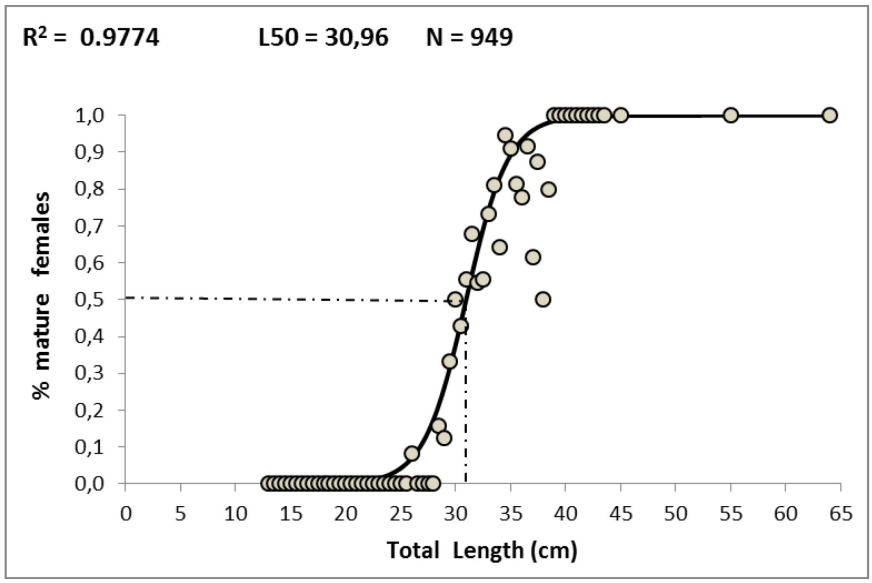


Figure 5: Logistic curve fitted to the proportion of mature females (maturity stages from 2 to 4) in relation to fish length, based on macroscopic data. Dashed line indicates the length at first maturity estimate (L_{50}).

The estimation of batch fecundity ranged between 7771 to 137256. The minimum total length of specimen with hydrated eggs was 29 cm with batch fecundity of 45004 and the maximum was 42 cm with 70519. The mean value of relative batch fecundity was 205 eggs.g^{-1}.

Ovarian histology

The histological analysis evidenced multiple oocyte stages at the same time during reproductive cycle (fig. 6), confirming the asynchronous development of oocytes for this species. Furthermore, the histological investigation showed 68,7% of similarity with macroscopic maturity stage classification of the females.

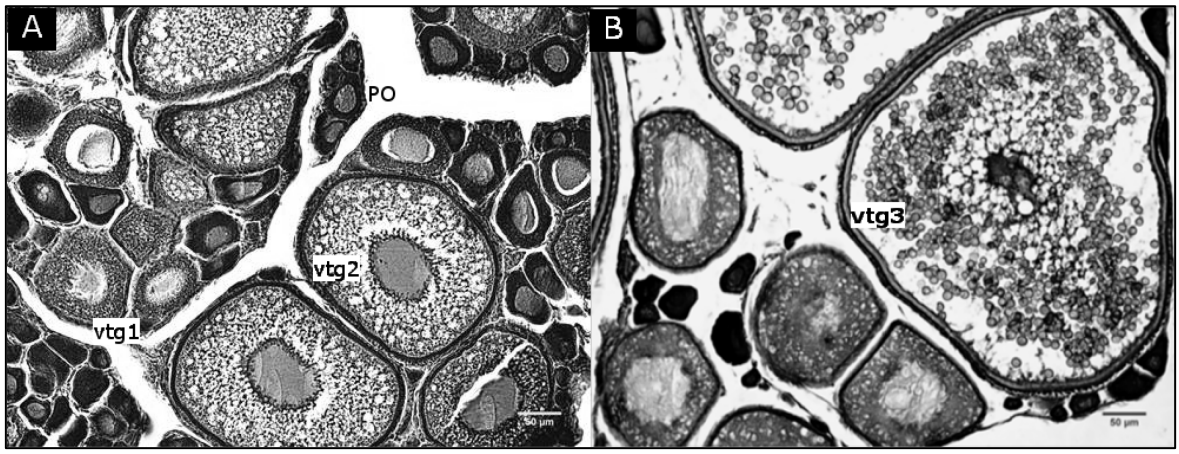


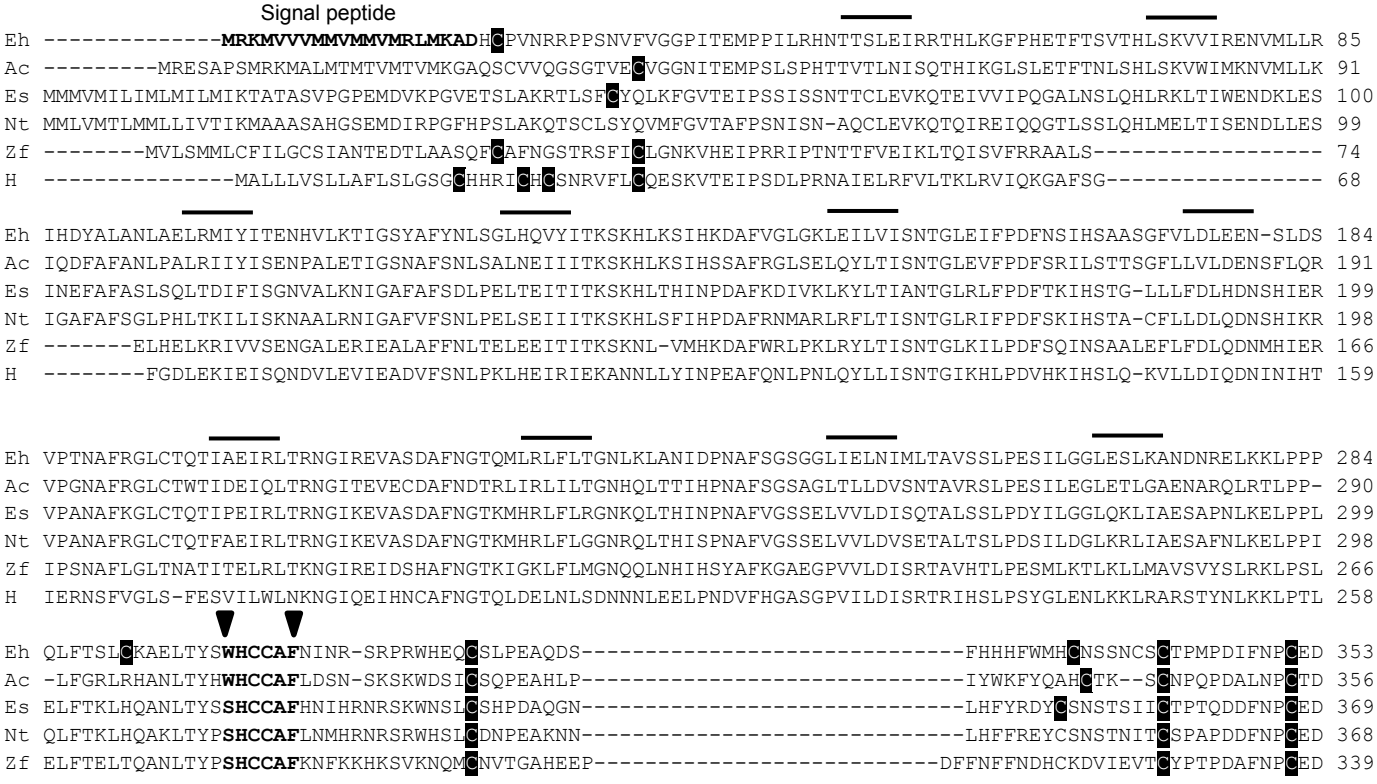
Figure 6. Different oocyte stages in asynchronous ovary of European hake. PO, Primary Oocytes (unyolked oocyte); vtg1, vitellogenin 1; vtg2, vitellogenin; vtg3 vitellogenin 3. Scale bar = 50 μm

Molecular characterization of deduced amino acid sequence of fsh and lh receptors

The complete open reading frame (ORF) of European hake follicle-stimulating hormone receptor (*fshr*) and luteinizing hormone receptor (*lhr*) were obtained together with 5'-untranslated region (UTR) and part of 3'-UTR (Accession numbers: KY178270, KY178271 respectively). The ORF of *fsh* receptor consisted of 2043 bp, with a 5'-UTR region of 137 nucleotides and partial 3'-UTR of 174 nt. The obtained *lhr* sequence consisted of 2281 bp, with a coding region of 1959 bp and 5'-UTR of 232 bp and a partial 3'-UTR of 90 nt. The ORF of both receptors were used to obtain

predicted amino acid sequences. The predicted proteins belong to Glycoprotein Hormone Receptor family (GpHR). The predicted protein of Fshr showed a high sequence identity with Fshr of other teleosts (62-70%). It consisted of 680 amino acids with predicted molecular weight of 75.97 kDa (fig. 7A). The *in silico* analysis of this protein identified an extracellular domain (ECD) containing a predicted signal peptide of 19 amino acids followed by ten imperfect leucine-rich repeats (LRRs), and ending with the cysteine-rich clusters at the C-terminal, present also in the Fshr of other species. The deduced amino acid sequence of Lhr contained 652 residues, with predicted molecular weight of 78.31kDa, and a deduced signal peptide of 26 amino acids. Analyzing the homology with other teleosts, the putative Lhr protein displayed a range 52-79% of identity. Within the ECD there are nine imperfect leucine-rich repeats (LRRs) flanked by conserved N- and C-terminal cysteine-rich regions (fig. 7B). The putative amino acid sequence of two hormone receptors is characterized by other regions typical of GpHR family, important for receptor function and conformation: N-glycosilation sites, the seven-transmembrane domains (7TM) and the "hinge" region of the receptors (SHCCAF) (fig. 8).

(A)



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Ac IMETSPLRVLIWVISLLALLGNATVLLVLLASRAKLTVPRFLMCHLAFADLCMGVYLVVIAMGDMVTRGQYFNHAIAWQMDYGCNIAGFFTVFASELSVF 456
Es IMSAVPLRVLIWIISILALLGNTVVLLVLLGSRTKLTVPRFLMCHLAFSDLCMGIYLVVIATVDMLTQGQYYNHAIDWQTGLGCSVAGFFTVFASELSVF 469
Nt IMSATPLRILIWIISVLALLGNAVVLLVLLGSRYKLTVPRFLMCHLAFADLCMGIYLVVIATVDMLTRGRYYNYAIDWQMGLGCNAAGFFTVFASELSVF 468
Zf IMGFTFLRVLIWFISVLAIVGNTVVLLVLLTSRYKLTVPRFLMCHLAFADLCMGIYLLLIAAVDIHTQSRYYNYGIDWQTGAGCHVAGFFTVFSSELSVY 439
H  IMGYNILRVLIWFISILAITGNIIVLVILTTSQYKLTVPRFLMCNLAFADLCIGIYLLLIASVDIHTKSQYHNYAIDWQTGAGCDAAGFFTVFASELSVY 458

Eh TLTAITLERWHTITHALRLDRKLRLRHACVVMSVGWGFAFCPPCCPLSGVSSYGKVSICLPMDVEKLKSQVYVVTLLLLNVLAFLTVCVCYFSIYLTVRN 553
Ac TLTAITLERWHTITNALRLDRKIRLRHACVVMAAGWVFALLAAALPTLGVSSYDKVSICLPMDVDSLLSQVYVVGLLLLNVLAFLVVCVCYFSIYLAVRK 556
Es TLTAITLERWHTITHALRLDRKLRLRHACIVMTAGWIFSAVAALLPTVGVSSYGKVSICLPMDVEFLGSQVYVVSLLLLNILAFFCVCGCYLSIYLTVRN 569
Nt TLTAITVERWHTITHALRLDRKLRLRHACIIMTIGWIFSLLAALLPTVGISSYGKVSICLPMDVESLVSQFYVVCLLLLNILAFFCVCGCYLSIYLTFRK 568
Zf TLTAITLERWHTITYAMQLERQMRLRHACLVMATGWLFSLLTALTPMFGVSSYSKTSICLPMDVETLLSQGYVVLLLLLNAAAFLVVCVCYTLIYLTVRN 539
H  TLTAITLERWHTITHAMQLDCKVQLRHAASVMVMGWIFAFAAALFPIFGISSYMKVSICLPMDIDSPLSQLYVMSLLVLNVLAFVVICGCYIHIYLTVRN 558

Eh PSSAPAHAADTDVAKRMAVLIFTDFLCMAPISFFAIPAALNRPLINVTQAKLLLIFYP-INSCANPFLYAFFTRTFRQDFFLLTARFGLFKAQAQIYRTE 652
Ac PSSTPAHAADTDVAQRMAVLIFTDFLCMAPISFFAISAALKLPLITMWHAKLLLVLFYPINSCANPFLYAFFTRTFRRDFVLLAARFGLFKAQAQIYRTE 656
Es PSSAPAHADTR-VAQRMAVLIFTDFVCMAPISFFAISAALKLPLITVSDAKLLLVLFYPINSCSNPFLYAFFTRTFRRDFFLLAARFGLFKTRAQIYRTE 668
Nt PSSAAAHADTR-VAQRMAVLIFTDFICMAPISFFAISAALKLPLITVSDSKLLLVLFYPINSCSNPFLYAFFTRNFRRDFFLLAARFGLFKTRAQIYRTE 667
Zf PAFVPANADMR-IAKRMAVLIFTDFLCMAPISFFAISAAFKLPLITVSHAKVLLVLFYPINSCSNPFLYAFFTKTFKRDFFILTSRFGCFKRRAHIYRTE 638
H  PNIVSSSSDTR-IAKRMAMLIFTDFLCMAPISFFAISASLKVPLITVSKAKILLVLFHPINSCANPFLYAIFTKNFRRDFFILLSKCGCYEMQAQIYRTE 657

Eh SSSCQQPAWTS-------PKSSHGTLYSLGKISH------- 679
Ac SSTCQQPAWAS-------PKSSHGTLYSLANISH------- 683
Es SSSCQQPAWTS-------PKSSHVMLYSLANALSLEGKPEF 702
Nt SSSCQQPTWTS-------PKNSRVILYSLVNTLSLDGKQEC 701
Zf ISSGQNGAVVPS------PKTSDGTLYSLVHIAQVH----- 668
H  TSSTVHNTHPRNGHCSSAPRVTNGSTYILVPLSHLAQN--- 695
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(B)

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                Signal peptide
Eh ---MAPQGSSRLLLAPLVLVLVLVPGSLAYSCPRICRCRAD-TFQCTRETQLGSRARPLSVSKLKLSHLSVEIVPSHAFRDIN-VTRIEISQSFSITKIQ 95
Ac MTHMAPQGSLRLLFALSCVVNTCFG--LTYICPAICRCKVE-TFRCNKETQG-IHESGLSFRPLRLSHLSVKIVPRHAFKDIN-VTRIEISQSVSVTEIQ 95
Es -----MWTSLPALLFLSVLGFYGCKCAPGFGCPRICRCFSN-TIRCNNVTQGSALMMDHRDKRLFFYHLSLNTISSHSFDGLKGVQRIEIAQSVTLETIE 94
Nt ------MALREVWLLFALSGVLNARSCCAYTCPAICRCTAD-SFQCSKETQLASRTGPTSVLRLRLTHLPLKRVPSHAFKELINITIIEISQSDCITHIQ 93
Zf --------MWRSALLLVFLLLTSFCCGVCFECPEICRCSQK-SITCNSATE-----SQKSLSRLVLNYISVKTISSRSFDGLKGVRRIEIAQSSSVETIE 86
H  --MKQRFSALQLLKLLLLLQPPLPRALREALCPEPCNCVPDGALRCPGPTAG--------LTRLSLAYLPVKVIPSQAFRGLNEVIKIEISQIDSLERIE 90

Eh RHAFLSLHRLSEISVQNIVSLRAIEKGAFTDLPRLEYLSICNTGMIHFPDFTSVSSLMSNFMLEMADNTKIDSIPANSFKGIAEEYIFMKLFRNGFKEIQ 195
Ac RHAFLSLHCLSEISLQNIISLRYIEKGAFTDLPRLEYLSILNTGMVHFPDFTSVSSLMSNFIIEMADNTKIDSIPANSFNGFAEDYIFMNLYRNGFKEIH 195
Es ALAFNNLLNLSEISVQNTRSLMHIGRRTFNNLPKLHYLSISNTGITLFPDITYINSLESEFILDICDNLYLLEIPPNAFIGLTKEYVTMNLYNNGIREIH 194
Nt THAFLSLYSLAQISVQNINSLRFIEKGAFADLPKLEYLSISNTGIAHFPDFTTISSLSPNIILEMADNMEIDIIPANSFQGITEEYVDMNLVRNGFKEIK 193
Zf SEAFNNLPNVSEISIQNTRNLVHIQQRAFNQLPKLRYLSISNTGISVFPDLTSIFSLEAHFILDICDNLNLRSVPSNAFTGMTSEYATMNLFNNGFQEIE 186
H  ANAFDNLLNLSEILIQNTKNLRYIEPGAFINLPRLKYLSICNTGIRKFPDVTKVFSSESNFILEICDNLHITTIPGNAFQGMNNESVTLKLYGNGFEEVQ 190

Eh SHAFNGTKINSLILRNNEHLYKIHEGAFDGALGPMTLDVSSTALSSFPAKGLTQVKFLTAVSAYTLKVLPPLESFTELLEANLTYPSHCCAFHTWQRKQR 295
Ac SHAFNGTKINSLDLRDNKHLYHIHEGAFDGALGPMVLDISSTALSSFPAEGLTQVRFLTAVSAYTLKVLPSLESFTELREANLTYPSHCCAFQ---RKQR 292
Es DYAFNGTKIDKLVLKNNRNLRVIHRYAFEGATGPGVLDVSATALTKLPPQGLESVLVLFAQSAYALKSLPPLQGLWSLREAHLTYNSHCCALLSWNTHRD 294
Nt SHAFNGTKLNTLVLRDNWYLRNIQEDAFEGATGPTLLDVSSTALRSLPPNGLRHVKFLKASHAYALKSLPLLESLAELLEAELTYPSHCCAFHTWRRKQR 293
Zf SHAFNGTKIDKLVLKNSRDLRVIHEDAFKGALGPTVLGVSSTALETLPSHGMESVLMLTARSAFALKKLPPLKSLKSLREAQLTFPSHCCALINWDNSRD 286
H  SHAFNGTTLTSLELKENVHLEKMHNGAFRGATGPKTLDISSTKLQALPSYGLESIQRLIATSSYSLKKLPSRETFVNLLEATLTYPSHCCAFRNLPTKEQ 290

Eh ENTMKNLTRLCDVSELDMEPTGDGLDVIDYIAFHYHYLELDCLN-------------------------SPFIQCTPKPDAFNPCEDLLGYTVLRFLTWVI 371
Ac ESALKNLTRLCDVNEADMEPADDGLYGIDYITSQYPDMELDCLN-------------------------SSFIKCTPKPDAFNPCEDLLGYSFLRSITWVI 368
Es FSINPAYNNDSTYCDESDQLARVQRVIGGSADTTLVMDMPFFSDVDLSEDEGFGDVNFHYPELDFCQTRPTLVCTPEADAFNPCEDIAGFSFLRVAIWFI 394
Nt ESALKNLTKFCDLMNTEIDPTADDTSLINDINFQYPDLEFDCFS-------------------------NPFVKCSPKPDAFNPCEDLLGFSFLRCLTWII 369
Zf GSVNSALRNRSSYCGDNSSPADLSAISSDDTLESDVIGS-------SSVEDTFGSIDFHYPDLDLCQQRQALQCSPEADAFNPCEDIAGFSFLRVAIWFI 379
H  NFSHSISENFSKQCESTVRKVNNKTLYSSMLAESELSGWDYEYG----------------------FCLPKTPRCAPEPDAFNPCEDIMGYDFLRVLIWLI 369

Eh TVCAVTGNLAVLVVLLVSHQKLTISRFLICNLAFADLCMGLYLLLIAAMDSYSSHQYYNHATDWQTGAGCGIAGFLTVFASELSVYTLTVISLERWHTIT 471
Ac TLSAVAGNLGVLVILLVSHQKLTISRFLICNLAFADLCMGLYLLLIAAMDSYSSPQYYNHAVDWQTGPGCSIAGFLTMFASELSVYTLTVISLERWHTIT 468
Es NILAITGNLTVLLVSFTSRNKLTVPRFLMCHLAFADLCIGIYLLMIATVDFRTQGHYSQHAIEWQTGSGCSAAGFLSVFGGELSVYTLSTITLERWHTIT 494
Nt MVFAVAGNLAVLVILLIGHHKLTVSRFLMCNLAFADLCMGLYLILIAFMDYHSHHEYYNHATDWQTGPGCGIAGFLTVFSSELSVYTLTVISLERWHTIT 469
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Figure 7: Amino acid sequence alignment of Fshr (A) and Lhr (B) mature peptides of European hake and other fishes. The signal peptides are in bold and the cysteine residues are highlighted in black and the LRRs are indicated by upper black line. Seven-transmembrane domains (7TM) are boxed in gray and the "hinge region" (SHCCAF) by black arrowheads. The sequences were extracted from GenBank databases and their abbreviation and accession numbers are: Merluccius merluccius (Eh: European hake KY178270, KY178271), Gadus morhua (Ac: Atlantic cod ABD62885, ABD62886), Dicentrarchus labrax (Es: European sea bass AAN40506, AAN40507), Danio rerio (Zf: Zebrafish AAR84280, AAR84281), Oreochromis niloticus (Nt: Nile tilapia NP_ 001266517, NP_001266524) and human (H: AAB32225, AAA59515).

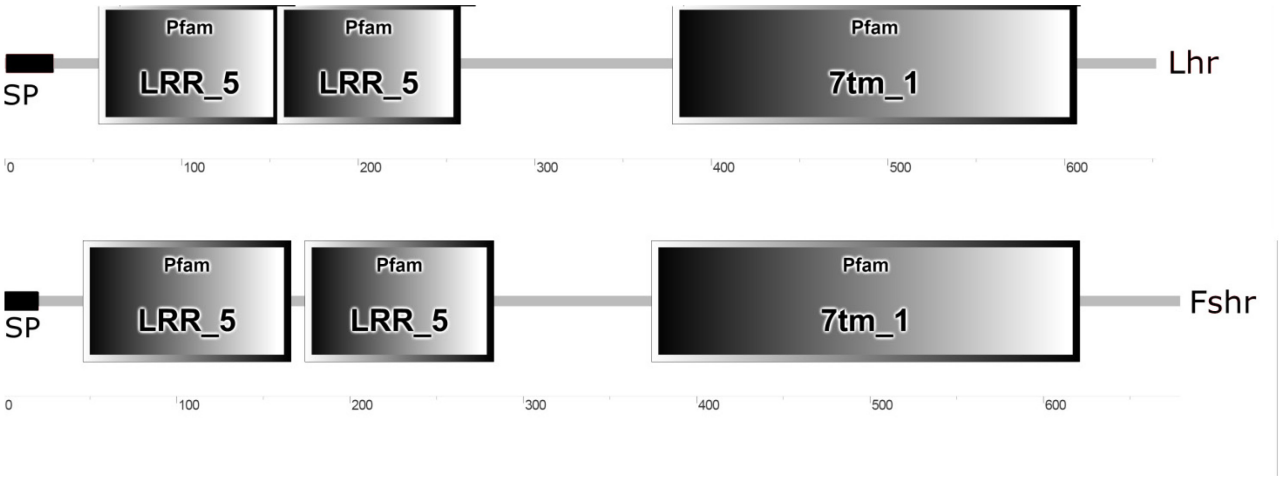

Figure 8. Schematic representation of Fshr and Lhr protein sequences obtained with the SMART tool. SP = Signal Peptide; LRR = Leucine-rich repeats; 7tm= seven transmembrane domain.

Phylogenetic analysis of gonadotropin receptors

Phylogenetic analyses showed that fish Fsh and Lh receptors were divided into clusters in the phylogenetic tree rooted using the glycoprotein hormone beta subunit-related protein of *D. melanogaster* and testicular glycoprotein hormone receptor I precursor of *Petromyzon marinus* as outgroups (fig. 9). The percentage of trees in which the associated taxa clustered together is shown below the branches. Both gonadotropin receptors of *M. merluccius* are closely associated to Fshr Lhr of Atlantic cod (*Gadus morhua,* Gadidae) and confirming they belong to the Order Gadiformes.

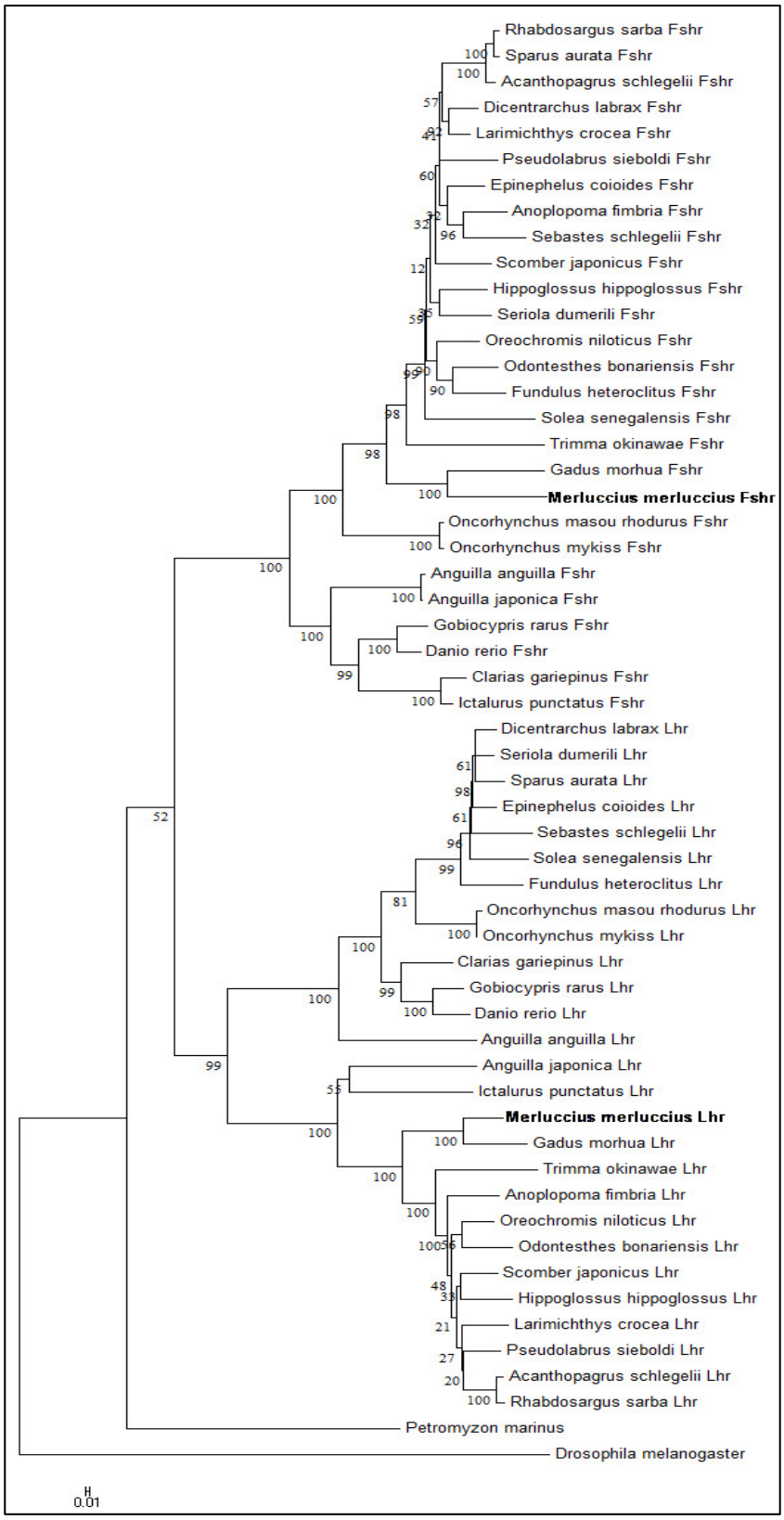

Figure 9. Phylogenetic comparison of fish amino acid sequences of Fsh and Lh receptors analyzed by MEGA6 using maximum likelihood and default settings. A rooted consensus phylogenetic tree generated by means of the Neighbor-Joining algorithm, glycoprotein hormone beta subunit-related protein of *D. melanogaster* and testicular glycoprotein hormone receptor I precursor of *P. marinus* as outgroup. Bootstrap values from 1000 replicates are indicated for each tree node. The Genbank accession number of species are reported in table 3, at the end of this chapter.

Gene expression profile analysis

Four stages of maturation were identified in the hake ovary based on the oocyte morphology: Primary oocytes (unyolked oocyte PO), Vitellogenin 1 and 2 oocytes (Vtg1-2 present in developmental ovaries), Vitellogenin 3 (Vtg3 present in spawning capable ovaries) and Hydration oocytes (Hyd, present in actively spawning individuals). The expression levels of both receptors were detected in primary growth stage and they significantly (P<0.05) increased in Vtg1-2 class (fig. 10), corresponding to developing phase at macroscopic level of the gonads. In Vtg3 stage, the *fshr* expression level started to decrease, but the difference was not significant compared to other stages. The Hyd stage showed a significant decrease compared only to Vtg1-2 (P<0,05). Unlike *fshr*, *lh* receptor significantly raised (P<0.05) in Vtg3 and declined in hydrated oocytes (P<0.05).

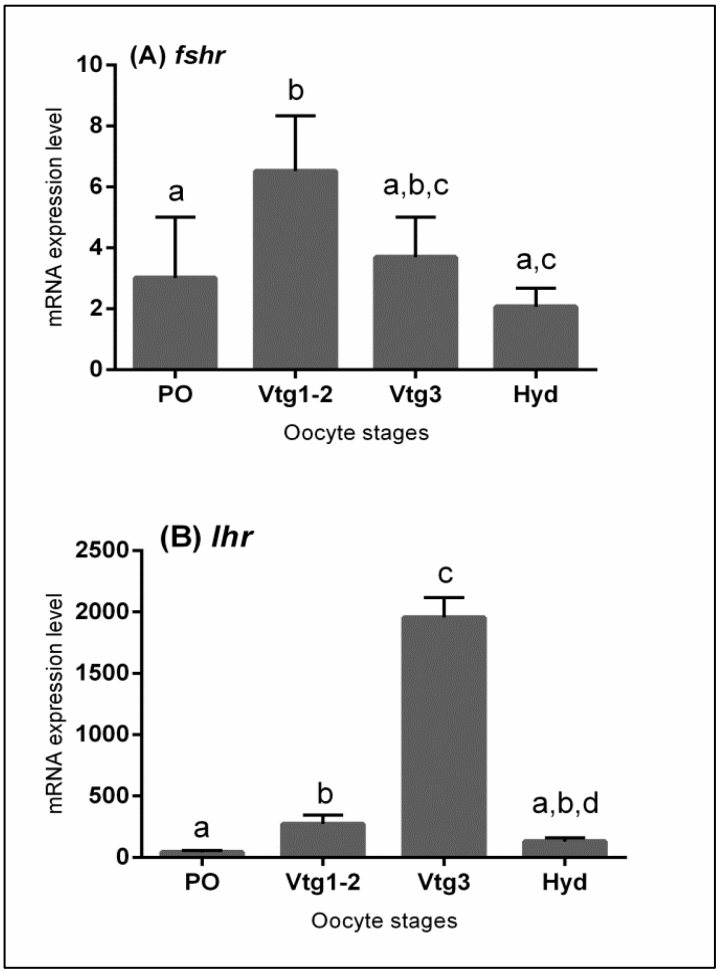

Figure 10. Relative mRNA expression levels of (A) *fhsr* and (B) *lhr* in oocytes at different ovarian stages of European hake. Abundance of *fshr* and *lhr* transcript was determined by qRT-PCR and normalized with *b-actin* and 18S. The values are Mean ± SD.

Discussion

An easiest and a rapid approach to investigate the state of fish stocks or the health of specimens is to analyze the condition indicators (Lloret et al., 2014). For the stock assessment of European hake, we investigated different indices, the gonadosomatic index (GSI), the hepatosomatic index (HSI), the Le Cren's condition factor (K_n) during three consecutive years in female hake stock. The trend of GSI allowed to determine the reproductive season of hake stock in GSA17 from April to July, even though a limited presence of spawning females was observed during all year, confirming a protracted spawning period for this species (Murua & Motos, 2006; Recasens et al., 1998; Recasens et al., 2008). As reported in literature, European hake stocks in the Mediterranean Sea and the Atlantic Ocean have different period for the main spawning activity. In Bay of Biscay, the reproductive activity was intensified from January to March (Lucio et al., 2000; Murua & Motos, 2006), in Galician Shelf in Winter and in Spring/Summer (Dominguez-Petit et al., 2010), in Tyrrhenian Sea from January to May, while in Catalan Sea from August to December (Recasens et al., 2008), in Eastern central Atlantic Ocean in Winter and in Spring with certain inter-annual variability (El Habouz et al., 2011), suggesting that the spawning activity of *M. merluccius* could be independent of the temperature. Unlike 2014 and 2015, in 2013 an unusual trend of GSI was observed, with a lack of the peak typical of active reproductive season. The possible reason of the altered reproductive events observed in 2013 could be related to an exceptional event of dense water occurred in Winter 2012 in Adriatic shelf (Mihanović et al., 2013). Such phenomenon was characterized by currents to high speed that caused a cascading event leading to move more than 50% of water volumes from Northern basin to Southern area of the Adriatic Sea. (Mihanović et al., 2013; Benetazzo et al., 2014; Janekovic et al., 2014). This phenomenon could have caused directly or indirectly unfavorable conditions for the hake reproduction as previously suggested by Recasens and co-workers (2008). These authors proposed that intense cascading events due to a dense water formation could shift several months the hake reproduction in Catalan Sea. Focusing on the liver

index and condition factor trends, they showed highest values in September 2014 and 2015. However, these high levels were not followed by GSI peak and no peak for both the indices was documented before spawning period, probably because this species uses the stored energy to survive to the winter, like for hake stocks in Galician shelf (Dominguez-Petit et al., 2010); this result confirms that the HSI and K_n could not be considered good proxy to assessment of the stock reproductive potential of European hake (Dominguez-Petit & Saborido-Rey 2010). In 2013 the HSI and K_n were constant without relevant changes reflecting the peculiar trend of GSI and suggesting a negative role of the intense dense water occurred in Winter 2012.

The histological assessment was important to define the correspondence to the macroscopic evaluation (68,7%), evidencing the validity of macroscopic approach. Furthermore, the histological analysis verified an asynchronous ovary and confirmed that European hake is a multiple spawner species. As recently evidenced in our laboratory, in multiple spawner species as European hake, a peculiar trend of gonadotropin expression during reproductive season was found (Candelma et al. 2017). Thus, in order to complete the scenario of the regulatory mechanisms of oocyte development and maturation in this multiple spawner species, the characterization and the molecular expression of gonadotropin receptors were performed in oocytes at different maturity stages. The i*n silico* analysis of the deduced amino acid sequence of both receptors evidenced the typical structure of glycoprotein receptor. They are characterized by an ECD, divided in an N-terminal cysteine-rich region, a series of imperfect leucine-rich repeats, a C-terminal cysteine-rich region known as the "hinge" region of the receptor; the ECD is followed by the TM segments. From alignment of deduced ehFshr with human FSHr and other fish species, it displayed an insertion of 25 aa in ECD also reported in Atlantic cod and other teleost species (Kobayashi & Andersen, 2008; Mittelholzer et al., 2009; Rocha et al., 2007). As evidenced by Vassart et al. (2004) the LRR region of glycoprotein receptors is constituted by nine imperfect leucine-rich repeats and performs the function of recognition and interaction with hormone so the insertion of 25 aa brought the addiction

of one extra LRR as in others teleost species, that may give different specificity with the ligand (Rocha et al. 2007; Mittelholzer et al. 2009). Flanking the LRR region there are the N- and C-terminal cysteine-rich clusters. As reviewed by Levavi-Sivan and co-workers (2010) the N-terminus is different from mammalian FSHRs: in fish, included European hake, there are only one or two cysteine residues, while in mammalian receptors there are four cysteines. The hinge region of CCR is another distinct feature in ehFshr. Usually, it consists in SHCCAF aa both in mammals and fishes, in European hake a substitution of serine (S) with tryptophan (W) was found and also substituted in Atlantic cod (Mittelholzer et al. 2009). Although this results remain to be elucidate, we suggest that this mutation could affect the normal function of ehFshr or in alternative, the activation of a different mechanism for this receptor (Mittelholzer et al. 2009). Unlike Fshr, the NCR of Lhr presents the typical four cysteine residues (^{30}C, ^{34}C, ^{36}C, and ^{43}C) of glycoprotein receptors that together with six cysteine of CCR (^{284}C, ^{285}C, ^{306}C, ^{337}C, ^{345}C, ^{355}C) represent the cap that protects the LRR clusters (Kobe and Kajava, 2001). Also the nine imperfect LRR and SHCCAF clusters in CCR are well conserved in ehLhr. In contrast with Fshr, the deduced amino acid sequence of ehLhr had key regions of ECD more conserved in evolution. In addition, both gonadotropin amino acid sequences were used for the construction of a phylogenetic tree that was inferred from various teleost glycoprotein receptor sequences. Both receptors were distinctly separated in independent clusters and both showed high similarity to respective homolog hormone receptor genes of Atlantic cod, confirming that they belong to Gadiformes order. An important tool to understand how the gonadotropin hormones regulate the gonadal maturation in multiple spawner species is the investigation of the expression pattern of their receptors in gonads during reproductive cycle. The expression levels of both receptors were low in primary oocytes and increased in the following stages, to later decrease in hydrated oocytes, following the trend of their ligands. In fact, as suggested by Candelma and co-workers (2017), the ovarian development and maturation of the European hake could be controlled by synchronous presence of both gonadotropins, as reported in

rainbow trout (*Oncorhynchus mykiss*, Salmonidae) and in European seabass (*Dicentrarchus labrax*, Moronidae) (Bobe et al., 2003; Sambroni et al., 2007; Rocha et al., 2009) and the gonadotropin receptors seem confirm this hypothesis. However, different pattern was evidenced for *fshr* and *lhr* in Atlantic cod and chub mackerel (*Scomber japonicas*, Scombridae) (Mittelholzer et al., 2009; Nyuji et al., 2013), because both species expressed only *lhr* during maturation phase and not *fshr*. These data suggested that the species with asynchronous ovary evolved distinct physiological strategies.

To complete the overview of the stock status of European hake in Adriatic Sea, also the length at which half of the specimens started ovarian development, was estimated. Our results reported the value of 30,96 cm, that was similar to the results obtained by Zupanovic and Jardas (1986), defining 30,5 cm as the length at the end of puberty period in Adriatic Sea. The unchanged value of L_{50} was a good indicator that the fishing effort did not affect the reproductive capacity of the stock in the last years. However, the difference was remarkable when we compare the L_{50} value of Adriatic Sea with other sampling sites of the Mediterranean Sea and the Atlantic Ocean. In Eastern central Moroccan Atlantic coast the L_{50} was 33,8 cm, in Iberian Spanish Atlantic was 45,4 cm, in the Bay of Biscay was 41 cm and in Galician shelf 46,5 cm, in the Catalan Sea was 35,8 cm and 35,1 cm in the northern Tyrrhenian Sea (Pineiro & Sainza, 2003; Domínguez-Petit et al., 2008; Recasens et al., 2008; El Habouz et al., 2011), only in region of Bou-Ismail, in Alger (Bouaziz et al., 1998) the L_{50} was similar to middle Adriatic Sea. As reported by El Habouz and co-workers (2011) about European hake and Hutchings (2005) about Atlantic cod, a reduction of size at first maturity could be a signal the overexploitation of Adriatic hake stock. Finally, the decrease of length at maturity can be linked to a reduction of the number of spawning eggs (Hutchings, 2005). Thus, for the first time we estimated the fecundity in Adriatic Sea, analyzed as batch and relative fecundity.

The value of relative fecundity was similar to the Mediterranean and the Atlantic stock, whereas the values of batch fecundity of the Adriatic stock was lower compared to the other fishing sites (Murua

et al., 1998; Pineiro & Sainza, 2003; Recasens et al., 2008; El Habouz et al., 2011). Therefore, the smaller specimens found in Adriatic Sea will release a lower number of eggs at spawning time with a possible consequent decrease of the European hake Adriatic stock. Furthermore, the L_{50} individuation at 30,96 cm evidenced that the politic choice to allow the European hake fishing from 20 cm, determines the inclusion of many female juvenile individuals, preventing that the female specimens spawn at least once in life and with a consequent decrease of the age of the stock and average body size. As reported by Anderson and co-workers (2008), the decrease of the age of a stock and average body size can reduce the ability of exploited species to survive to annual environmental variation. Thus, the event verified in 2013 in Adriatic Sea can negatively affect the resilience of European hake and other species and the climate change predicted for the Adriatic Sea will presumably increase these particular dense water events.

In conclusion to preserve the fish resources and for sustainable fishing, it is necessary to understand the dangerous signals of the fish population and adopt precautionary politics. To protect the Adriatic Sea European hake stocks, the Italian and European government should take into account appropriate measures, among which continuing monitoring operations on fish population and an increase of the knowledge on reproductive physiology.

Declaration of interest

The authors declare no conflict of interests.

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Table 3. Gene bank accession numbers of used species for phylogenetic analysis

Common Name	Scientific Name	FSHR Acces. No.	LHR Acces. No.
European hake	*Merluccius merluccius*	KY178270	KY178271
Atlantic cod	*Gadus morhua*	ABD62885	ABD62886
European sea bass	*Dicentrarchus labrax*	AAV48628	AAV48629
Gilthead sea bream	*Sparus aurata*	AAT01413	AAT01412

Nile tilapia	*Oreochromis niloticus*	BAB16106	BAB16107
Zebrafish	*Danio rerio*	AAR84280	AAR84281
European eel	*Anguilla anguilla*	BAM33585	CPR30244
Japanese eel	*Anguilla japonica*	BAF79914	ACF35638
Bambooleaf wrasse	*Pseudolabrus sieboldi*	ADE08459	ADE08460
Atlantic halibut	*Hippoglossus hippoglossus*	ACB13177	ACB13176
Killifish	*Fundulus heteroclitus*	BAF48336	BAF48337
Chub muckerel	*Scomber japonicus*	AGG36448	AGG36449
Black porgy	*Acanthopagrus schlegelii*	ABU49599	ABY56689
Sablefish	*Anoplopoma fimbria*	AGS55586	AGS55587
Yellow croaker	*Larimichthys crocea*	KKF21850	KKF19875
Channel catfish	*Ictalurus punctatus*	AAK16067	AAK16066
Rainbow trout	*Oncorhynchus mykiss*	AAQ04551	AAQ04550
Masu salmon	*Oncorhynchus masou*	BAA86898	BAA84638
African catfish	*Clarias gariepinus*	CAB51907	AAN75752
Korean rockfish	*Sebastes schlegelii*	AEJ33654	ADV59773
Senegalese sole	*Solea senegalensis*	ADH51678	ADH51679
Greater amberjack	*Seriola dumerili*	BAR43498	BAR43499
Silver bream	*Rhabdosargus sarba*	ABI93201	ABI93202
Argentinian silverside	*Odontesthes bonariensis*	ACU28776	ACU28775
Okinawa rubble goby	*Trimma okinawae*	BAG56672	BAG56673
Chinese rare minnow	*Gobiocypris rarus*	AGI78913	AGI78914
Orange-spotted grouper	*Epinephelus coioides*	AEG65826	AEG65827
Sea lamprey	*Petromyzon marinus*	AAW80618	
Common fruit fly	*Drosophila melanogaster*	AAB07030	

CHAPTER V

REPRODUCTIVE STUDIES OF MALE EUROPEAN HAKE (*MERLUCCIUS MERLUCCIUS*) IN THE ADRIATIC SEA

Reproductive studies of male European hake (*Merluccius merluccius,* L) in the Adriatic Sea

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Abstract

Since the commercial importance of European hake and poor information about male reproduction, here, we investigated the reproductive biology of this males in Adriatic Sea. The analysis were focused on the study of indices involved in reproduction and energy state, gonadosomatic index (GSI), hepatosomatic index (HSI) and Le Cren's condition factor (K_n) in three years of sampling. For the first time the size at first maturity (L_{50}), the size at which the 50% of specimens were mature, was estimated in Adriatic Sea, showing the lower value than other Mediterranean and Atlantic areas. Together with the macroscopic determination of maturity stage of gonads, testis were used for histological analysis. The cystic structure of testis was identified and the presence of spermatozoa was always evidenced. Finally, because of the inflow of the big rivers in Adriatic Sea, the amount of contaminants is high and their effects on male reproduction was analysed, evidencing the expression of mRNA of *vitellogenin a* and *b* and *estrogen receptor alpha* in liver of males.

The acquired information of European hake males provides a basic data for future studies on the reproduction of this overfished species.

Introduction

The European hake (*Merluccius merluccius*) is an important commercial species widespread in Eastern coast of Atlantic Ocean and in Mediterranean Sea. Hake stocks are declared in overfishing status in many regions of Mediterranean Sea with fishing mortality rate five times higher than other stock (FAO 2014). Also in Adriatic Sea, European hake represent an important fishing resource in endangered condition.

In the present study the reproductive biology of European hake males was investigated in Adriatic Sea. The gonadosomatic and hepatosomatic indices and the Le Cren's condition factor were estimated. The total length at which 50% of specimens of population are mature was determined for the first time in this area.

The Adriatic Sea is a relatively isolated semi-enclosed basin that is connected with the rest of Mediterranean Sea by Otranto's channel. It is characterized by influx of big rivers, like Po river with its polluted water derived from highly industrialized and agricultural areas from Northern Italy. The presence of different contaminants, metal content (Bilandžić et al. 2011), alkylphenols and alkylphenol ethoxylates (Ferrara et al. 2005), Polybrominated Diphenyl Ethers (PBDEs) and Polychlorobiphenyls (PCBs) (Focardi & Renzi 2012; Bodiguel et al. 2009; Garritano et al. 2006) was investigated in European hake both in Adriatic Sea and in other fishing regions of Mediterranean Sea, but no studies regarding the effects of environmental pollutants at molecular level on this species are so far available.

The assessment of the effects of pollutants in marine organisms is fundamental for understanding environmental condition of marine habitat and its repercussions on the reproduction of the populations living in the contaminated areas. To this aim, for the first time, we studied the adverse effects of environmental endocrine disruptors on wild European hake by the analysis of molecular expression of toxicological biomarkers in males. We considered three genes, *two vitellogenins* (*vtg*a, *vtg*b) and the *alpha estrogen receptor* (*er*a) involved in female reproduction and whom their expression should be low or absent in male liver, as biomarker of the presence of endocrine disruptors. The histological analysis on testis were performed to verify eventual intersex presence.

Materials and methods

Sampling

A total number of 427 European hake males, in a total length range from 13 to 33 cm, were collected on board of bottom trawler fishing vessel in the Northern and Central Adriatic Sea (FAO-Geographical Sub-Area 17 (GSA17), according to GFCM division) from January of 2013 to December of 2015. No samples were fished in August because the Italian legislation banned the fishing in that months. The animals were collected under the guidelines of the Data Collection Framework Regulation (EU Reg.199/2008) that established a Community system for the conservation and sustainable exploitation of fisheries resources under the Common Fisheries Policy (CFP). The procedures did not include animal experimentation and ethics approval are not necessary in accordance with the Italian legislation. The following parameters were recorded for each fish analyzed: total weight (TW), total length (TL), sex and macroscopic maturity stage of gonads, gonad and liver weights. The animals were macroscopically and microscopically classified in four different testicular classes: immature/regenerating (M1); developing (M2); spawning capable (M3) and spent, post spawning (M4) (Table 1), following the gonadal classification of

Brown-Peterson et al. (2011). For molecular analysis, we sampled animals when the highest peak of gonadosomatic index (GSI) was recorded (spring-summer season). Because of the difficulty to find males in class M4, only classes M1, M2, M3 are included in molecular experiments. The livers for qPCR analyses were immediately preserved in RNAlater (Ambion, USA) and stored at -20°C for analysis.

	Phase	Macroscopic morphology	Microscopic characteristics
M1	Immature/Regenerating (Inactive)	Small and withish testis shorter than 1/3 of the body cavity.	Only Sg1 present; no lumen in lobules.
M2	Developing	Thin withish testis shorter than 1/2 of the body cavity, but easily identifie	type A and B spermatogonia spermatocytes, spermatids present and spermatozoa can be present in spermatocysts. Sz not present in lumen of lobules or in sperm ducts.
M3	Spawning capable	Whitish-creamy soft testis long about 2/3 of the body cavity. Milt released with light pressure on abdomen.	All stages of spermatogenesis and spermatozoa in lumen of lobules and/or sperm ducts.
M4	Spent, regression, postspawning	Bloodshot and flaccid testis, about 1/2 length of the body cavity. No milt release with pressure.	Residual Spermatozoa present in lumen of lobules and in sperm ducts. Scattered spermatocysts near periphery containing spermatocytes, spermatids, spermatozoa

Table 1. Correspondence between macroscopic and microscopic characteristics of testis stages in European hake.

Morphometric and Physiological indices

The somatic parameters were used to calculate the monthly evaluation of gonadosomatic index (GSI), hepatosomatic index (HSI) and Le Cren's relative condition factor (K_n) in adult males.

$$GSI\ (\%) = \text{gonad weight}*100/\text{gutted body}$$

$$HSI\ (\%) = \text{liver weight}*100/\text{gutted body}$$

$$K_n = W/a\ L\ b$$

where a and b are the regression parameter of the length – weight relationship, W is gutted weight and T is the total length. In January, February and March of 2013 no livers were sampled.

RNA extraction and cDNA synthesis

Total RNA was extracted from livers using TRIzol Reagent, following manufacturer's protocol (Invitrogen Life Technologies, Milan, Italy). Total RNA was treated with DNAse (10 IU at 37 °C for 10 min, MBI Fermentas). A total amount of 1 µg of RNA was used for cDNA synthesis, employing iScript cDNA Synthesis Kit (Bio-Rad laboratories, USA).

Real time RT-PCR of vitellogenin a and b and alpha estrogen receptor

PCRs were performed with the SYBR green method in an iQ5 iCycler thermal cycler (Bio-Rad laboratories). Conserved of *alpha estrogen receptor (er*a), and *vitellogenin a and b* (*vtg*a*, vtg*b, respectively) mRNA sequences were amplified using primers designed by Primer3 (http://bioinfo.ut.ee/primer3/). The hake sequences were taken from the National Center for Biotechnology Information (NCBI, USA). Primer sequences are reported in Table 2. Triplicate PCRs were carried out for each sample analyzed following (Santangeli et al. 2016). *β-actin* (b*act*) and 18S were used as housekeeping genes to standardize the results by eliminating variation in mRNA and cDNA quantity (Bustin et al. 2009). No amplification product was observed in non-template controls and no primer-dimer formations were observed in the control samples. The data obtained were analyzed using the iQ5 optical system software version 2.0 (Bio-Rad) including GeneEx Macro iQ5 Conversion and Genex Macro iQ5 files.

Name	Sequence (5'-3')	Orientation
βactin	5'- GTCATGGACTCCGGTGATGG -3'	forward
	5'- GAGGTAGTCTGTGAGGTCGC -3'	reverse
18S	5'- GAGGCCCTGTAATTGGAATG -3'	forward
	5'- CGCAAGACACTCAACCAAGA - 3'	reverse
vtg a	5' – TGGGTTGAAAGTCAGCAGCA - 3'	forward
	5' – GCCACTGTACTCAAAGGGCT - 3'	reverse
vtg b	5' – TGAGGCTCCTGACACCTTCT – 3'	forward
	5' – GAACACCTTACCCACCACCC – 3'	reverse
er alpha	5' – ATCTGGAGGTCCATCCACTG - 3'	forward
	5' – GCAAGCAGCATGTCGAAGAT - 3'	reverse

Table 5: Primers used for real-time RT-PCR in European hake.

Histological analysis

From total of animals, 72 male specimens in different maturity stages and length classes were chosen for histology to confirm macroscopic classification of gonadal development. A sub-sample of testicular tissue were fixed in 4% PFA overnight and stored in 70% ethanol until use. Analysis of testicular stages were conducted on histological paraffin sections (6-7 μm thickness). Successively, samples were dehydrated through a graded series of ethanol and embedded in paraffin. Consecutive sections were cut at a thickness of 4 μm using a microtome and stained by Harris Hematoxylin and Eosin for oocyte analysis.

Length at first maturity

The estimation of length at first maturation (L50), representing the size at which 50% of the specimens were mature, was determined by fitting the logistic equation (Prager et al. 1989):

$$p = [1 + e - r\,(x - x50)]\text{-}1$$

(where p is the estimated proportion in size class, r is a fitted parameter, x is the total length, x_{50} is the length at which 50 % of the specimens was mature), to the proportion of fish in each size class. The stage M2, M3 and M4 were considered for the estimation. The maturity stage was determined macroscopically.

Statistical analysis

Results were expressed as the mean ± standard deviation (s.d.). Statistical differences were determined using one-way ANOVA, followed by Bonferroni's multiple comparison test. All statistical analyses were performed using Prism 6 (GraphPad Software, San Diego, CA, USA). P-values <0.05 were considered significant and the results were expressed as the mean ± SEM for expression level of *vtg*a, *vtg*b and *er*a, while for GSI, HSI and K_n, the results were expressed as the mean ± SD.

Results

Morphometric and physiological indices and length at first maturity

Several indices were analyzed in male specimens for assessing the health status of the Adriatic Sea stock. For physiological indices, we considered the hepatosomatic and gonadosomatic index, for morphometric measures, we analyzed the Le Cren's condition factor. Because of the complexity to find a valuable number of male samples for each month, the monthly variation was analyzed together for the three years. K_n showed highest peak in October that was significant different only with February and December (fig. 1).

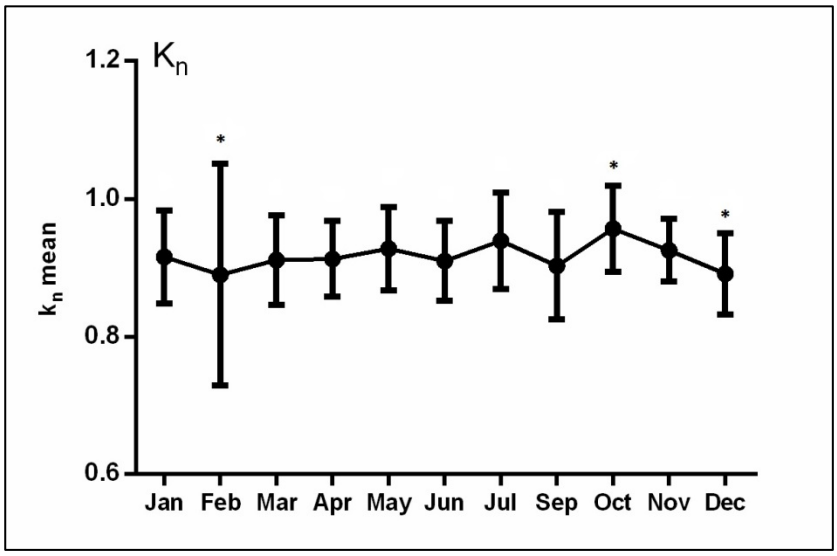

Figure 1. Monthly variation of Kn of European hake males in three years of sampling. The asterisks indicate the significant difference only among October and February and December.

HSI was fluctuating during the year, but the highest values were recorded in Autumn, with peak in November that was significantly different compared to March and July (fig. 2).

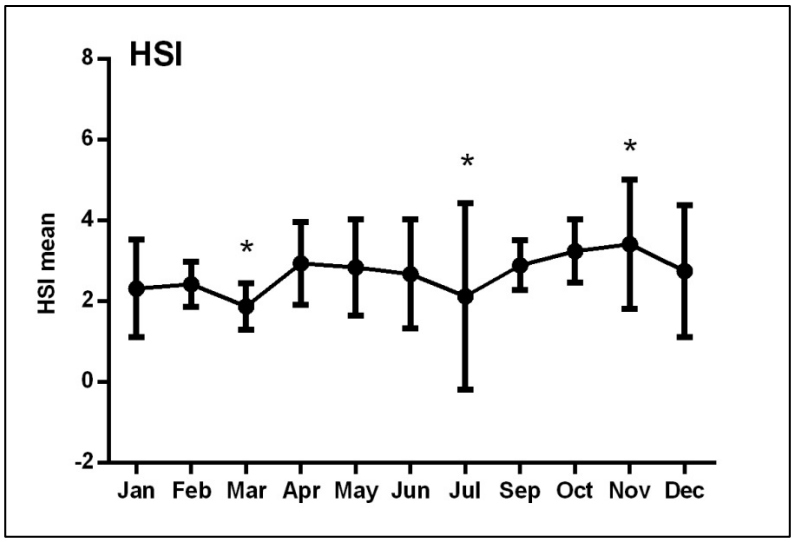

Figure 2. Monthly variation of HSI of European hake males in three years of sampling. The asterisks indicate the significant difference only among November and March and July.

The total GSI trend showed the highest peak in June that was significantly different compared to other months ($P<0.05$) except for May (fig.3). The lowest GSI values were in Autumn in contrast to HSI.

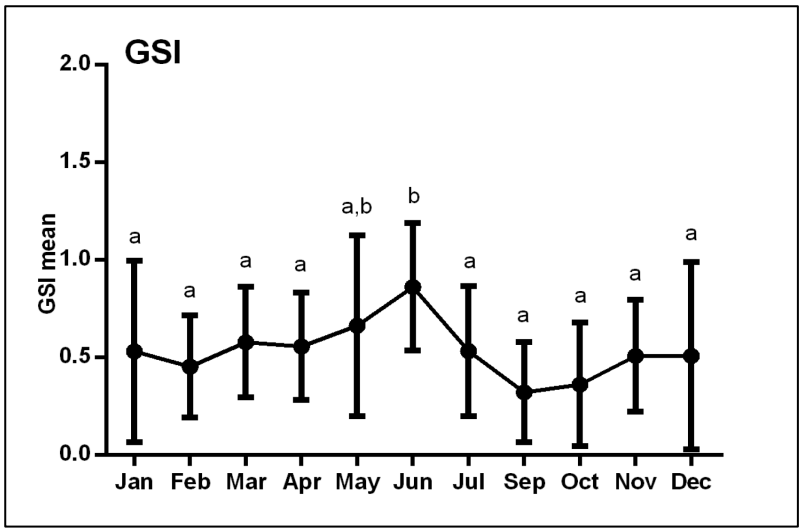

Figure 3. Monthly variation of GSI of European hake males in three years of sampling .

The length at first maturity (L_{50}) was estimated as 21,8 cm of total length (fig. 4). The minimum size at which specimens were mature was 17,5 cm. The specimens over 25,5 cm showed all ripe testis.

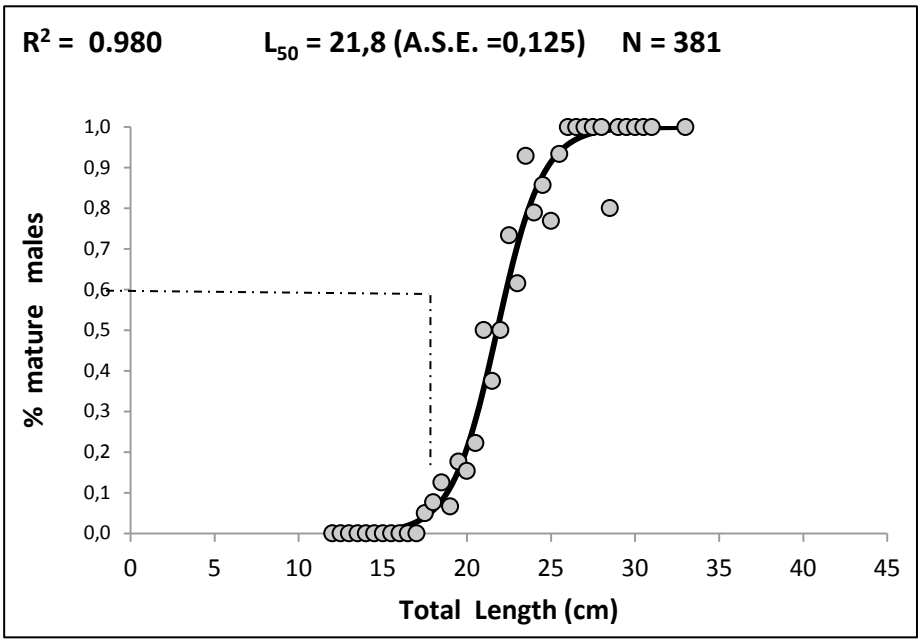

Figure 4: Logistic curve fitted to the proportion of mature males (maturity stages from 2 to 4) in relation to fish length, based on macroscopic data. Dashed line indicates the length at first maturity estimate (L_{50}).

Testicular histology

72 specimens were considered for histological analysis and the range of total length was between 17 and 33 cm. Based on the macroscopic predetermination, all testis stages were considered for the analysis. The histological sections of hake showed a cystic testis (fig. 5), with type A and B spermatogonia, as well as cysts containing spermatocytes, spermatids or spermatozoa.

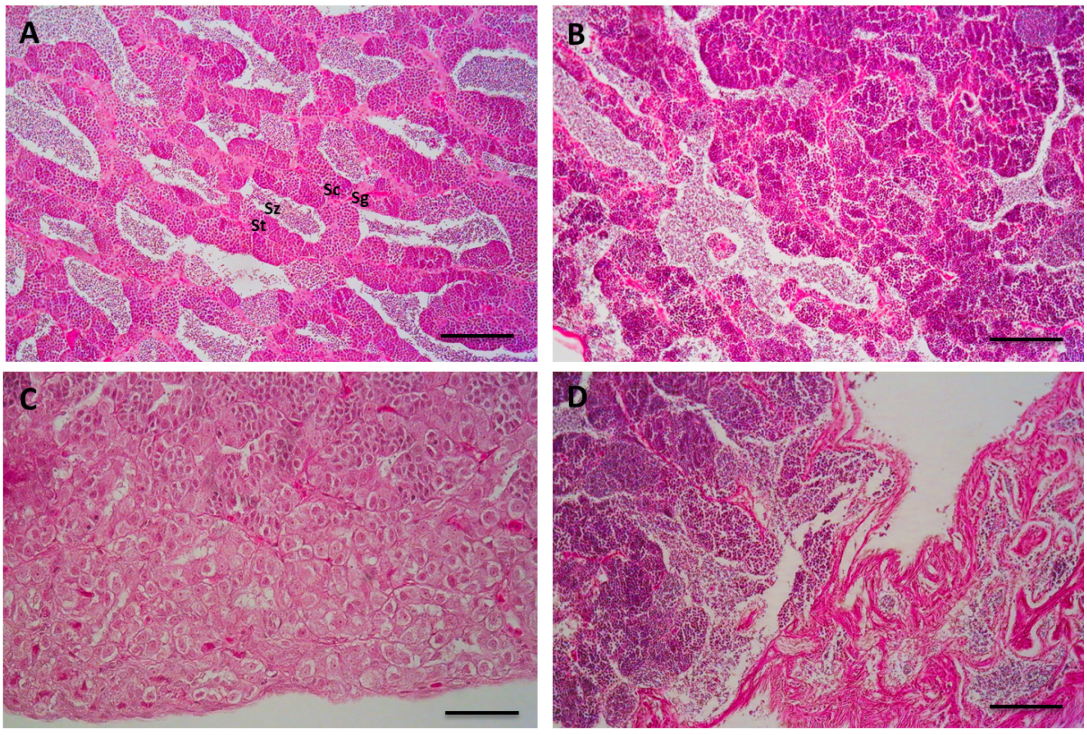

Figure 5. Histological results of different stages of testis in European hake. (A) Testis with less abundant sperm, scale bar 10 µm; (B) Testis with more abundant sperms, scale bar 10 µm; (C) Periphery of testis, scale bar 5 µm; (D) Testis and sperm duct, scale bar 10 µm. Sg: spermatogonia; Sc: spermatocyte; St: spermatid; Sz: spermatozoa.

The interesting results evidenced that the spermatozoa always filled the lumen of the seminiferous lobules in all specimens and throughout the year. The only difference was the amount of spermatozoa in the lumen that were more or less abundant. The sections displayed nor specimens in regressing stage (spent), that consists mainly of spermatogonia and residual spermatozoa in the lumen and nor in immature stage.

Gene expression profile analysis of era, vtga and vtgb in the liver

The experiments, conducted on 28 males in a range length between 20 to 30 cm, regarded the molecular analysis of mRNA expression of three different biomarker, vitellogenin a and b and alpha estrogen receptors, in the liver. The *vtg*b and *er*a were found in all specimens analyzed. The expression levels of *vtg*b was unchanged in different classes of length and no significant difference were found among the samples (fig. 6).

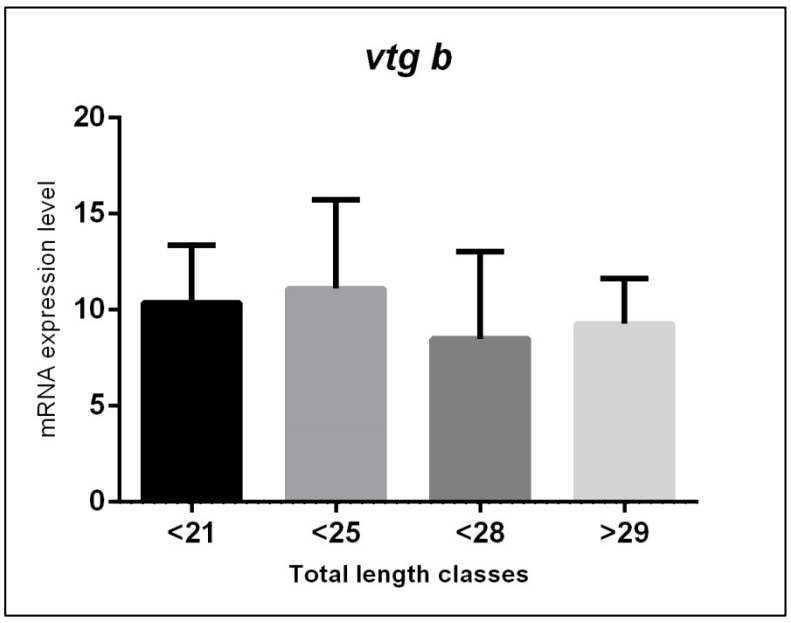

Figure 6. mRNA expression of *vtgb* in male liver of European hake. Mean+SEM.

In contrast to *vtg*b, *er*a displayed a decrease of mRNA expression in the total length class between 26 to 28 cm, but the difference was not significant compared to other length classes (fig.7). Regarding to *vtg*a, only 9 out of 28 samples showed the expression of the molecule and the statistical analysis was not possible.

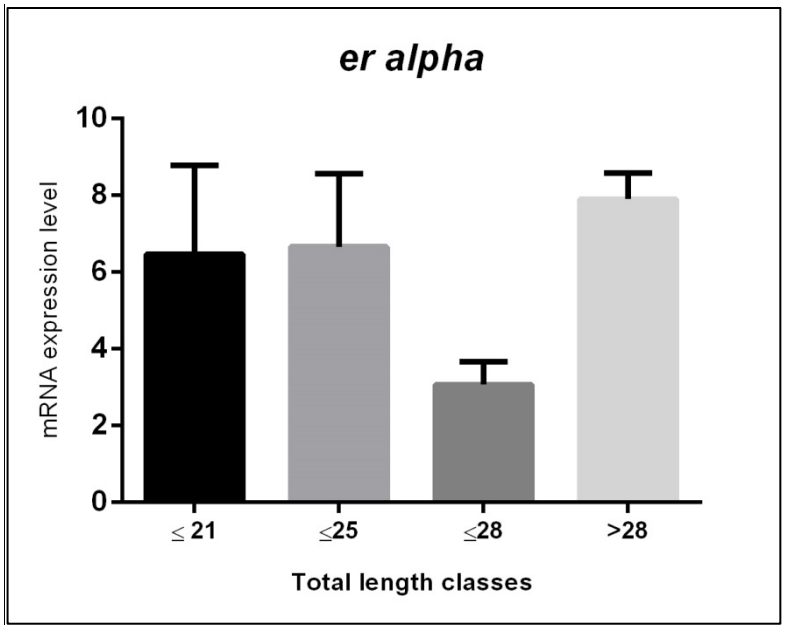

Figure 7. mRNA expression of *er*a in male liver of European hake. Mean+SEM.

Discussion

European hake is important commercial species, but despite its important most of the researches focused on females and information about male are rather limited. Here, the males of European hake were analyzed to clarify some aspects or their reproductive activity in Northern Adriatic Sea. The GSI provided information about the spawning cycle in this area. The examined trend of GSI revealed a spawning peak in summers months, like hake females in this area (Candelma et al., chapter IV). Though Zupanovic & Jardas (1986) demonstrated a spawning event also in winter in deepest water of Northern Adriatic Sea, our results did not evidenced the same situation, probably due to depth of sampling, mainly achieved in shallow waters. After the increase during summer months, the trend of GSI started to decrease achieving the lowest levels in September and October, coinciding with the highest values of HSI. The maximum accumulations of energy in liver in Autumn suggested that the European hake stores reserves independent of reproductive activity. Such results seem to be in accord with the population of European hake of the Galician shelf (Domínguez-Petit et al. 2008). As HSI, also K_n surged in October suggesting that European hake stores the energetic reserves to survive to winter more than reproductive processes.

Different studies in past focused on the length at which 50% of the male specimens of European hake have already matured at least once in Adriatic Sea. As reviewed by Zupanovic & Jardas (1986), the first data reported a range between 22 to 30 cm of L_{50} in male specimens and in the following years, other scientists reduced the range between 20 to 28 cm. Anyway, to date, a specific value calculated with logistic equation does not exist and a comparison of our result with historical data cannot be performed. The L_{50} estimated in other Mediterranean and Atlantic areas indicated higher values compared to the total length of 21.8 cm estimated in Adriatic Sea. In fact, except for Bouaziz et al. (1998) that reported a L_{50} value of 21.5 cm, El Habouz et al. (2011) found a value of 28.6 in Eastern central Moroccan Atlantic coast, Pineiro & Sainza (2003) of 32.8 cm in Iberian Spanish Atlantic and Recasens et al. (1998) of 28.8 cm in Mediterranean Sea Gulf of Lions. All these data showed that hake males in the Adriatic Sea mature earlier and consequently at a smaller size than other regions.

The testis of European hake exhibits the cystic structure typical of the teleosts. Although at macroscopical level, the gonads appear morphologically well differentiated in the different stages of development, the histological analysis of the 72 samples revealed that the testis showed the presence of sperm in all stages, more or less abundant, inducing to consider the presence of only two stages of gonadal development of males, one rich and one with lower number of spermatozoa, however, in all animals including the 17 cm of total length, sperms were present indicating their sexual maturity. In addition, such results support the thesis that European hake are reproductively active throughout the entire year, like observed also in other fishing areas (El Habouz et al. 2011; Recasens et al. 1998; Pineiro & Sainza 2003) and that the macroscopic visual recognition is not sufficient to determine the maturation stages of testis. Unfortunately, there are no remarks on the presence/absence of sperms in sperm ducts or in the lumen of the lobules, elements which in scale, proposed by Brown-Peterson et al. (2011), distinguishes the stadium M2 and M1

The exposure of European hake to endocrine disruptors was abundantly reported in literature (Bodiguel et al. 2009; Ferrara et al. 2005; Ferrara et al. 2008; Bilandžić et al. 2011; Raingeard et al. 2009; Sagratini et al. 2008; Garritano et al. 2006; Barone et al. 2014; Focardi & Renzi 2012). All works focused on the study of amount of contaminants in hake tissues, but their effects on fish healthy were not considered. Here, for the first time, the molecular expression of biomarkers of exposure to estrogen or estrogen-like was examined in male liver of European hake. The toxicological effects of endocrine disruptors implied the transcription of genes typical of female reproduction, like vitellogenin in male liver. Two different isoforms of vitellogenin were investigated, *vtg*a and *vtg*b, but only *vtg*b showed the molecular expression in all samples, whereas the *vtg*a was expressed only in one third of the samples. Such difference could be due to the type of contaminants present in the marine water. Together with the vitellogenins, also the mRNA level of *er*a was analyzed and, like *vtg*b, it was expressed in all samples. The expression level of these genes among samples did not display correlation with the fish length. Probably, the range between 20 to 30 cm is not enough for evidencing a correlation with length of the specimens. The histological analysis did not show cases of intersex in males suggesting better tolerance to endocrine disruptor respect to other species living in the same area (Miccoli et al., submitted).

Declaration of interest

The authors declare no conflict of interests.

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CHAPTER VI

CONCLUSIONS AND FUTURE PERSPECTIVES

The study, performed whithin this doctoral project, provided an overview on the reproductive characteristics of European hake. The results, obtained by different approaches, gave the information on both the stock status of this species in Adriatic Sea and in general, for the reproductive physiology of European hake.

The European hake stock in Northern Adriatic Sea showed the continuously spawning, although the highest number of spawning specimens were found from April to July, considering it as the main spawning season. So, the choise of Italian governament to stop the fish during August and September months has not good effects of overfished species like European hake.

The environmental phenomenon veriefied in Winter 2012 have harmful effects on reproduction causing the absence of spawning peak of hake females in 2013. As reported by Anderson et al. (2008) decrease the age of a stock and average body size can reduce the ability of exploited species to survive to annual environmental variation. Thus, event like that verified in 2012 in Adriatic Sea can negatively affect the resilience of European hake and other species.

Afterwards, the L_{50} individuation at 30,96 cm for females and 21,8 cm for males evidenced that the political choice to permit the fishing of European hake from 20 cm includes many juvenile specimens, preventing their first reproductive process. The precautionary politics are necessary for sustainable fishing and for understanding the dangerous signals of a fish population thereby preserving the fish resources (Mullon et al. 2005), and the Italian and European government should take the appropriate measures into account to protect the European hake stock of Adriatic Sea.

The study of the reproductive physiology at molecular level increases knowledge of the species and provides a good proxy for correct management of the fish.

The analysis performed in this project are rilevant to prevent the collapse of overfished species and they should be included in the annual monitoring programs.

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