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An overview of the reproductive cycle of cultured specimens of a potential candidate for Mediterranean aquaculture, *Umbrina cirrosa*

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ABSTRACT

The shi drum (*Umbrina cirrosa*), belonging to the Sciaenidae family of fish (Nelson et al., 2016) has attracted attention as it is an excellent diversification species in Mediterranean aquaculture to combat the low prices of the most cultured species due to the appearance of new producing countries in the Mediterranean area. In this manuscript we describe for the first time the reproductive cycle of captive shi drum specimens that are able to spontaneously spawn in captivity. Our data show that spermatogenesis started in one year old shi drum specimens, although the fish were not mature enough to complete the reproductive cycle. Thus, three year old specimens showed multiple spawns with around 65% of the eggs fertilized. In mature males, levels of 11-ketotestosterone (11KT), testosterone (T) and progesterone (P) were detected in serum and gonad, while 17β-estradiol (E_2) was only detected in gonad. Testicular levels of E_2 and P suggest that these hormones might have a role in spawning and in the subsequent spermatogonial proliferation. In mature females, however, P and E_2 were detected in serum and gonad, while T and 11KT were only detected in gonad. The pattern shown by P in the ovary indicates a link between this hormone and ovarian renewal and a delay in vitellogenesis, which would explain why the vitellogenic stage of shi drum is quite short. The level of T and 11KT in the ovary suggests a role in oocyte maturation and spawning. The differences observed between sexes in the prevalence of some infection and the important economic losses produced by some vertically transmitted pathogens; have increase the attention to the interactions between the immune and reproductive systems. In this study we observed differences between sexes in the protease and bactericide activity levels. Thus, this manuscript represents the most complete overview to date of the shi drum reproductive cycle.

1. Introduction

Species diversification in Mediterranean aquaculture has been identified as one way to address the problem of diminishing market prices for the two major cultured species, the European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) due to the appearance of new producing countries in the Mediterranean area (Basurco and Abellán, 1999). Among the species that are being considered for aquaculture, the shi drum (*Umbrina cirrosa*, Linnaeus 1758) has attracted attention due to its high growth rate and market value in all the Mediterranean area.

The shi drum is a species belonging to the Sciaenidae family (Nelson et al., 2016). Its distribution area comprises the Eastern Atlantic, from the Bay of Biscay to Senegal, the Mediterranean and the Black Sea. It is appreciated in the Mediterranean Region where it is consumed regularly (Turkey, Morocco, Greece and Cyprus). It has a good growth rate and, under optimal stocking densities, can reach a mean body weight of 650 g in < 24 months (Mylonas et al., 2004). Together with its high growth rate, its high market value, good flesh quality (Melotti et al., 1995) and its great adaptability to culture conditions point this specie as a promising candidate specie for Mediterranean aquaculture (Basurco and Abellán, 1999). In addition, this species has other significant advantages that make it a strong candidate for aquaculture. It spawns in the summer and, therefore, does not compete for larval

Abbreviations: 11KT, 11-ketotestosterone; E_2, 17β-estradiol; dph, days post hatching; DHA, docosahexaenoic acid; FOM, final oocyte maturation; LSD, Fisher Least significant difference; GSI, gonadosomatic index; GnRH, gonadotropin-releasing hormone; HSI, hepatosomatic index; IEO, Instituto Español de Oceanografía; MB, body mass; MG, gonad mass; ML, liver mass; mAb, monoclonal antibody; OD, optical density; PO, perinucleolar oocytes; PBS, phosphate buffer saline; pPO, preperinucleolar oocytes; P, progesterone; RT, room temperature; SC, spermatocytes; SD, spermatids; SZ, spermatozoa; SEM, standard error to the mean; T, testosterone; TMB, tetramethylbenzidine hydrochloride; TCA, trichloroacetic acid; TSA, tryptic soy agar; TSB, tryptic soy broth; SGA, type A spermatogonia; SGB, type B spermatogonia; SGSC, spermatogonia stem cells; Vh, *Vibrio harveyi*

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rearing facilities with commonly cultured Mediterranean fishes. Shi drum larvae do not exhibit cannibalistic behavior and have a good survival rate: higher than 60% in different rearing conditions (Arizcun et al., 2009; Melotti et al., 1995; Zaiss et al., 2006). Furthermore, because of the rapid development of their digestive system, larvae of shi drum can be weaned on artificial diets very early (Papadakis et al., 2009; Zaiss et al., 2006). They are able to ingest *Artemia* nauplii from day 5 post hatching (Abellán et al., 2014) and could even be reared without using *Artemia*, changing directly from rotifers to artificial diets (Papadakis et al., 2009).

Despite the nascent state of the cultivation of this sciaenid, the results obtained so far suggest that this species is suitable for aquaculture production. In any case, it is necessary to develop studies covering all aspects of their biology to obtain the data to confirm this categorically. The knowledge of their reproductive biology is especially important in order to successfully set up breeding programs under intensive conditions. Early studies of the reproductive biology of the shi drum indicated that this is a multiple-batch group asynchronous spawning fish with a June–August spawning season (Barbaro et al., 1996). Previous studies have shown that the vitellogenesis in shi drum is a rapid process and can process normally in captivity upon gonadotropin-releasing hormone (GnRH) stimulation of spawning (Barbaro et al., 2002; Mylonas et al., 2004).

Reproduction depends on gonadal development, the induction of the gametogenic process and the ability to spawn the gametes. All these processes are under hormonal control, being the sex steroid hormones the last of a hormonal cascade that begin in the brain/pituitary and allow the synchrony of reproduction with the optimal environmental and physiological conditions and between specimens (Zohar et al., 2010). In all vertebrates including fish, a clear connection between endocrine and immune systems have been described, being the prevalence and intensity of infections higher in males than females (Klein, 2004). Moreover, infected fish show a clear alteration of androgen, estrogen, and vitellogenin serum levels (Deane et al., 2001; Hecker and Karbe, 2005). Additionally, the increment of estrogen in serum decreased the survival rates of *Yersinia*-infected rainbow trout (*Oncorhynchus mykiss*) in a dose-dependent manner and increase the susceptibility of goldfish to *Trypanosoma danilewskyi* (Wang and Belosevic, 1994; Wenger et al., 2014). Several studies demonstrated that hormones such as cortisol, growth hormone (GH), prolactin (PRL), some proopiomelanocortin-derived peptides, and reproductive hormones including steroid-derived sexual hormones, regulate leukocyte functions through their specific receptor present in these cell types (Chaves-Pozo et al., 2018; Engelsma et al., 2002; Harris and Bird, 2000; Iwanowicz and Ottinger, 2009; Lutton and Callard, 2006; Segner et al., 2017; Verburg-van Kemenade et al., 2013). Interestingly, seasonal differences on immune activities have been described in several fish species (Hernández and Tort, 2003; Kumari et al., 2006; Valero et al., 2014), although these variations have been attributed usually to photoperiod and temperature without study the probable influence of sex hormones. Other studies have reported that increases on sex-steroid serum levels during sexual maturation or upon hormonal administration, led to the modulation of several humoral immune responses (Cuesta et al., 2007; Hou et al., 1999a, 1999b). The earlier immune responses elicited upon an infection, are cellular and humoral innate responses which in fish are considered a key component in the fight against pathogens (Fearon, 1997; Fearon and Locksley, 1996; Whyte, 2007). Non-specific humoral defense substance, such as proteases, peroxidase (the enzyme involved in the production of reactive oxygen intermediate (ROIs) to kill bacteria) and a wide range of bactericide compounds, represent an immediate defense barrier in mucus and serum against pathogens (Alexander and Ingram, 1992; Whyte, 2007). One of the most threaten infection, nowadays in aquaculture, is produced by nodavirus (NNV), that causes mortalities up to 90% in larvae and juveniles of several species worldwide distributed including gilthead seabream and European sea bass (Dalla Valle et al., 2001; Thiery et al., 2004). Although no mortalities have been reported in shi drum larvae or juveniles, the NNV has been detected in the brain of juveniles with clinical sign of disease and on sperm and ovarian biopsies of wild and farmed specimens without clinical signs (Comps et al., 1996; Dalla Valle et al., 2000; Skliris et al., 2001; Thiery et al., 2004). Interestingly, NNV is able to colonize the gonad to be vertically transmitted and its infection behavior has been related to sex steroid levels (Valero et al., 2015a). Other pathogens, as *Sphaerospora testicularis*, shown higher prevalence during a specific reproductive stage and sex (Sitja-Bobadilla and Alvarez-Pellitero, 1993). Taking all this into account, it is worthy to study some immune responses and their relation with sexual hormones; in order to have a wider overview of the physiological changes occurred during the reproductive cycle.

The shi drum's reproductive cycle has been scarcely characterized as all studies performed until now had been focused on induce the spawning (Barbaro et al., 2002; Mylonas et al., 2004). In Spain, spawning in captivity with hormonal treatment was achieved in 2009 and the first spontaneous spawning from fish born in captivity (without any hormonal treatment) was achieved in 2013 (Arizcun et al., 2014, 2009). In this manuscript we report on one hand the spontaneous spawning of the broodstock (born in captivity in 2010) during four consecutive years from 2013 onwards; and on the other hand, the gonadal development of shi drum specimens under rearing conditions in terms of sex hormone levels in serum and gonad, gonad morphology as gametogenesis progresses during a complete year in both sexes and several humoral immune activities. Two different cohorts of shi drum have been studied: immature juveniles (born in captivity in 2013) and sexually mature adults (born in captivity in 2011).

2. Material and methods

2.1. Animals

Specimens of shi drum were bred at the *Centro Oceanográfico de Murcia*, *Instituto Español de Oceanografía* (IEO) facilities from spawnings of culture broodstock (Arizcun et al., 2014). For a month before the spawning period, the breeders were fed with special commercial broodstock diets (Vitalis Repro diet from Skretting, Burgos, Spain). Fertilized eggs were harvested from superficial water, counted and placed in 0.5 m^3 cylinder-conical tanks where they were incubated and hatching took place. The larval rearing was carried out in a 1 m^3 tank under natural photoperiod and temperature which progressively raise from 23 °C to 27 °C during the rearing period, according to previous study (Arizcun et al., 2009). Briefly, microalgae *Nannochloropsis* sp. were used for green water culture from the second day up to 17 days post hatching (dph). Larvae were fed with rotifers from 2 to 17 dph (5–20 rot/mL), *Artemia* nauplii from 11 to 17 dph (1–3 nauplii/mL) and *Artemia* metanauplii with docosahexaenoic acid (DHA) Selco (INVE Aquaculture, Belgium) from 16 dph (3–5 metanauplii/mL) to 25 dph. Weaning started at 20 dph and ended at 25 dph, when the concentration of *Artemia* was fully replaced with the artificial diet (200–300 μm). The juveniles were reared in 9 m^3 tanks and fish were fed with a commercial diet for *Argyrosomus regius* (Skretting, Burgos, Spain), since there is no specific diet for shi drum yet.

Two different cohorts of shi drum, hatched in two different years (2011 and 2013) with a body weight at the beginning of the sampling of 20.6 ± 5.7 g and 539.4 ± 43.7 g, respectively, were sampled (n = 10 specimens/sampling) once a month during a year, except in June and July (the spawning season) where the sampling was performed every 15 days. The fish were anesthetized with 40 μL/L of clove oil during the morning, weighed, decapitated, and blood, gonad and liver were removed. The serum samples were obtained from trunk blood. They were centrifuged (10,000 × *g*, 1 min, 4 °C) and immediately frozen and stored at −80 °C. The gonad were weighed and processed for light microscopy and sex steroid serum levels analysis as described below. The liver was weighed to calculate de hepatosomatic index.

A broodstock group of 8 females and 16 males was established in a tank of 15 m^3 with specimens hatched in 2010 and kept in the same conditions as the sampled specimens in order to determine their spawning behavior over several years (2013–2016). The mean body weight of the broodstock specimens were 985 ± 43 g and 2800 ± 60 g in 2013 and 2016, respectively. The sex of the specimens were identify at the beginning of the first spawning season by means of abdominal massages and all individuals were identify using an intramuscular implanted magnetic-tag. Water temperature ranged between 21 and 29 °C (June–July). The fish spawned at night and the eggs were collected early in the morning.

All specimens studied were handled in accordance with the Guidelines of the European Union Council (2010/63/EU) and the Bioethical Committee of the IEO (reference REGA ES300261040017).

2.2. Light microscopy analysis of the gonad

Fragments of gonad (n = 10 fish/group and time) were fixed in Bouin's solution for 16 h at 4 °C, dehydrated with increasing concentration of ethanol in water (70% 90 min, 96% 60 min and two bath of 100% during 60 min each), washed in two bath of n-isoamyl-acetate during 30 min each and embedded in paraffin (Paraplast Plus; Sherwood Medical) overnight. The sections were performed at 5 μm. After de-waxing and rehydration, some sections were stained with hematoxylin–eosin in order to determine morphological changes and development throughout the reproductive cycle of the gonad.

2.3. Analytical techniques

2.3.1. Sex steroid hormones levels

Serum and tissue levels of testosterone (T), 11-ketotestosterone (11KT), 17β-estradiol (E_2) and progesterone (P) were quantified by ELISA following the method previously used in gilthead seabream and sea bass (Rodríguez et al., 2000; Valero et al., 2015a). Steroids were extracted from 25 μL of serum or 20 μL of tissue (1 g/mL) homogenized in lysis buffer [50 mM phosphate buffer with 10 mM DTT (Sigma) and 250 mM sucrose (Sigma)] in 1.3 mL of methanol (Panreac). Then, the methanol was evaporated at 35 °C and the steroids were re-suspended in 250 or 200 μL of reaction buffer [0.1 M phosphate buffer with 1 mM EDTA (Sigma), 0.4 M NaCl (Sigma), 1.5 mM NaN_3 (Sigma) and 0.1% albumin from bovine serum (Sigma)], respectively. Then, 50 μL were used in each well so that 5 μL of plasma or homogenized tissue were used in each well for all the assays. T, 11KT, E_2 and P standards, mouse anti-rabbit IgG monoclonal antibody (mAb), and specific anti-steroid antibodies and enzymatic tracers (steroid acetylcholinesterase conjugates) were obtained from Cayman Chemical. Microtiter plates (MaxiSorp) were purchased from Nunc. A standard curve from 6.13×10^{-4} to 5 ng/mL (0.03–250 pg/well), a blank and a non-specific binding control (negative control) were established in all the assays. Standards and extracted plasma and tissue homogenate samples were run in duplicate and all the measures were corrected with the blank and negative control. The lower limit of detection for all the assays was 7.6 pg/mL for plasma and tissue samples. The intra-assay coefficients of variation (calculated from sample duplicates) were 4.6 ± 1.1% for T, 5.6 ± 1.3% for 11KT, 4.3 ± 1.0% for E_2 and 3.6 ± 0.6% for P assays in plasma and 3.4 ± 0.5% for T, 4.8 ± 0.9% for 11KT, 5.9 ± 1.0% for E_2 and 3.2 ± 0.5% for P assays in tissue homogenate analysis. The inter-assay coefficients of variation at 50% of binding were 11% for T (n = 4), 8% for 11-KT (n = 7), 48% for E_2 (n = 7) and 12% for P (n = 4) for plasma samples and 11% for T (n = 3), 3% for 11-KT (n = 3), 18% for E_2 (n = 3) and 5% for P (n = 3) for tissue homogenates. Details on cross-reactivity for specific antibodies were provided by the supplier (0.01% of anti-11KT reacts with T; 2.2% of anti-T reacts with 11KT; 0.1% of anti-E_2 reacts with T and 7.2% of anti-P reacts with E_2).

2.4. Protease activity

Protease activity in serum of three year old specimens was determined as the percentage of hydrolysis of azocasein using a modified previously described protocol (Charney and Tomarelli, 1947). Briefly, 10 μL of serum were incubated with 100 μL of phosphate buffer saline (PBS) and 125 μL of 2% azocasein (Sigma) dissolved in PBS for 24 h at room temperature (RT). The reaction was stopped by adding 10% trichloroacetic acid (TCA, Sigma) and incubating for 30 min at RT. The mixture was centrifuged at 6000 × g for 5 min after which 100 μL of the supernatants were transferred to a flat-bottomed 96-well plate in duplicate. One hundred μL of 1 N NaOH (Panreac) were then added and the optical density (OD) was read at 450 nm using a plate reader (MultiskanGo, ThermoFisher Scientific). For a positive control, 10 μL of 2 mg/mL proteinase K (AppliChem) in PBS replaced the sample (100% of activity), and for a negative control, PBS replaced the sample (0% of activity). Samples were run in duplicates. The percentage of protease activity for each sample was calculated as a % of the activity of the positive control. Results were expressed as a % of activity in serum.

2.5. Peroxidase activity

The peroxidase activity in serum was measured according to a previously described protocol (Quade and Roth, 1997). Briefly, 5 μL of serum were diluted with 45 μL of Hank's buffer (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates (Nunc). As a substrate, 100 μL of 10 mM 3.3′,5.5′-tetramethylbenzidine hydrochloride (TMB, Sigma) solution containing 0.015% H_2O_2 (Sigma) was added. The colour-change reaction was stopped after 15 min by adding 50 μL of 2 M sulphuric acid (Sigma) and the OD was read at 450 nm using a plate reader (MultiskanGo, Thermo Fisher Scientific). Wells with HBSS but no sample were used as blanks. Samples were run in triplicates. One unit was defined as the amount of activity producing an absorbance change of 1 and the activity was expressed as U/mL serum.

2.6. Bactericidal activity

The pathogenic marine bacteria *Vibrio harveyi* (Vh) (strain Lg 16/100) was grown in agar plates at 25 °C in tryptic soy agar (TSA, Sigma). Then, fresh single colonies of 1–2 mm were diluted in 5 mL of tryptic soy broth (TSB; Sigma), cultured for 16 h at 25 °C on an orbital incubator at 2700 × g and adjusted to 10^8 bacteria/mL of TSB.

The antibacterial activity of serum was determined by evaluating its effects on the bacterial growth of Vh curves using a previously described method (Sunyer and Tort, 1995). Aliquots of 10 μL of the bacterial dilution of Vh (1/10) were placed in flat-bottomed 96-well plates and cultured with 10 μL of serum. The absorbance of the samples was measured at 620 nm every 30 min during 36 h at 25 °C in a plate reader. Samples replacing bacteria by culture medium were used as blanks (negative control). Samples replacing serum by culture medium were used as positive controls (100% growth or 0% antibacterial activity). Samples and controls were run in duplicates. Bactericidal activity was expressed as [100-(% of bacterial growth)]. Results were corrected with the absorbance measured in each sample at the initial time point and expressed as a % of activity in serum.

2.7. Statistical analysis

The Gonadosomatix index (GSI) and the Hepatosomatix index (HSI) were calculated as 100·[MG/MB] (%) and 100*[ML/MB] (%), respectively, where MG is gonad mass (in grams), ML is liver mass (in grams) and MB is body mass (in grams).

All data were analyzed by one-way ANOVA and a post hoc test (Fisher Least significant difference, LSD) to determine differences between groups ($P \leq 0.05$) and by two-way ANOVA to determine differences in the pattern of the parameters analyzed through time

between males and females. The normality of the data was previously assessed using a Shapiro–Wilk test and homogeneity of variance was also verified using the Levene test. Non-normally distributed data were log-transformed prior to analysis. In scarce cases, the transformed data did not meet the parametric assumptions, and then the non-transformed data were analyzed by a non-parametric Kruskal–Wallis test, followed by a pairwise Mann–Whitney test. Statistical analyses were conducted using Statgraphics 15.0 (StatPoint, Inc) and SPSS software. All data are presented as a mean of standard error to the mean (SEM). Significance level (P) was fixed at 0.05.

The nuclear and cytoplasmic diameters of the spermatogonia stem cells (SGSC), type A spermatogonia (SGA) included in cyst with low number of them (2–4), oogonias and different types of oocytes were drawn manually and measured by image analysis using a Nikon eclipse E600 light microscope, an Olympus SC30 digital camera (Olympus soft imaging solutions GMBH) and the image J 1.46r software. The number of cells measured (n = 390) was always higher than the number obtained by the formula (standard deviation·0.83/mean·0.05)^2 for each cell type. The different cell types were determined accordingly with their morphological features previously reported in other fish species (Chaves-Pozo et al., 2005; Liarte et al., 2007).

3. Results

3.1. Eggs spawned

The egg spawning by a brookstock group formed by specimens hatched in 2010 in our facilities was spontaneous.

Thus, fertilized eggs without hormonal induction were observed for the first time in 2013 (three year old fish), although the percentage of fertilized eggs in the total amount of eggs spawned in that year was the lowest level recorded (Fig. 1A). Then, from the spawning stage of 2014 onwards, the percentage of fertilized eggs was very similar in all the years, although the amount of total spawning eggs was variable being the lowest in 2016 (Fig. 1A). In all the spawning seasons recorded, the patterns of spawning were similar showing two main peaks in the middle of the season (Fig. 1B). The range of temperature during the spawning seasons was between 22 and 29 °C (Fig. 1C).

3.2. The reproductive cycle of males and females

The reproductive cycle of males and females was divided into VI stages in accordance with the morphological features of the gonad and the GSI index (Tables 1 and 2).

One year old males showed a constant GSI through the reproductive cycle (Fig. 2A). However, three year old males showed the normal pattern of a functional male cycle with a peak at spawning (stage III; Fig. 2A). The GSI of one year old females sharply decreased between stages I and II reached its lowest levels at stage IV and slightly increase there after (Fig. 2B). However three year old females GSI showed a normal pattern of a functional female cycle with a constant increase from stage I to III while oocytes matured and subsequently decreased at stage IV upon spawning (Fig. 2B).

Regarding the HSI, one year old males showed a decrease from stage I to V and an increase later on at stage VI, while no changes were observed in the HSI of three year old males (Fig. 2C). Similarly, the HSI of one year old females decreased from stage III to V and increased later on at stage VI, while three year old females showed a constant HSI (Fig. 2D).

3.3. Gonad morphology of 1 and 3 years old males

Both cohorts of males developed a spermatogenetic process, although differences were observed between them (Table 1 and Fig. 3). Thus, in three year old males a typical reproductive cycle with four stages (spermatogenesis, spawning, post-spawning and resting) was

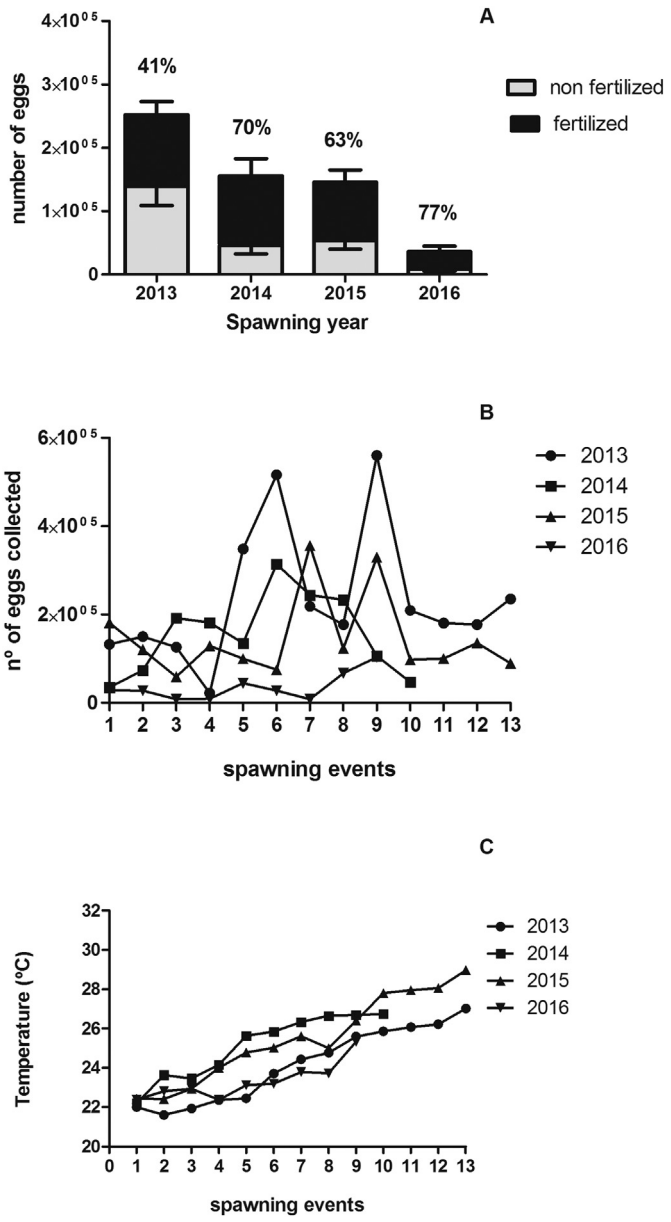

Fig. 1. The total number of eggs and fertilization percentage (A), the pattern of spawning (B) and the water temperature (C) during four consecutive reproductive cycles of cultured *Umbrina cirrosa* broodstock.

observed (Fig. 3D–I), while the morphological features of spawning, post-spawning and resting were not observed in one year old males (Fig. 3A–C). One year old males testis went to continuous waves of spermatogenesis as the germinal epithelium showed all type of cysts of type A spermatogonia (SGA), type B spermatogonia (SGB), spermatocytes (SC), spermatids (SD) and spermatozoa (SZ) and a moderated lumen in the tubules with few spermatozoa throughout the whole year (Fig. 3A–C).

In three year old males, spermatogenesis started in December where cysts of SGB were observed in a germinal epithelium mainly consisting of the SGSC and SGA and continuing over several months leading to the accumulation of free spermatozoa in the lumen of the tubules and in the efferent duct until June when spawning took place. At early spermatogenesis (December–March; Fig. 3D) some tubules showed some spermatozoa remaining from the previous year. At late spermatogenesis (April–May; Fig. 3E) the germinal epithelium was mainly formed of cysts of SC, SD and SZ with a low relative percentage of SG and the

Table 1
Feature of the testis of one and three years old males of *Umbrina cirrosa* during the reproductive cycle.

		1 year old		3 years old		
Stage	Months	Type of cell present in the germinal epithelium:	Lumen of the tubules	Type of cell present in the germinal epithelium:	Lumen of the tubules	efferent duct
I (Early spermatogenesis)	December–March	Mainly SGSC and cyst of SGA and SGB	No lumen	SGSC and cysts of SGA and SGB and some of SC	Scarce lumen with few remaining spermatozoa	
II (Late spermatogenesis)	April–May	Mainly cysts of SG, SC, SD and SZ	Some lumen in the tubules with free spermatozoa	Mainly cysts of SG, SC, SD and SZ	Some lumen in the tubules with free spermatozoa	Free spermatozoa
III (Spawning)	Early June	Cysts of SGA, SGB, SC, SD and SZ	Some lumen in the tubules with free spermatozoa	Mainly cysts of SC and SD	A wide lumen, plenty of free spermatozoa	A wide lumen, plenty of free spermatozoa
	Late June			Mainly SGSC and cysts of SGA and some cysts of SC, SD and SZ still remaining	Few free spermatozoa	A wide lumen, plenty of free spermatozoa
IV (Post-spawning)	July	Cysts of SGA, SGB, SC, SD and SZ	Some free spermatozoa	Mainly SGSC and cysts of SGA	A narrow lumen in the tubules with scattered free spermatozoa	Free spermatozoa
V (Resting)	August–September	Mainly cysts of SGA and SGB, also some of SD and SZ	A narrow lumen in the tubules	Mainly SGSC and cysts of SGA	Scarce lumen	
VI (Resting)	October–November					

Table 2
Features of the ovary of three year old females of *Umbrina cirrosa* during the reproductive cycle.

Stage	Months	Type of cells present in the ovary:	Cytoplasm features	Nucleus	Follicle related structures
I	December–March	Nest of oogonias (NO)	Slightly basophilic (13.48 ± 3.01 μm)	A nucleolus (7.82 ± 1.60 μm)	Not yet developed
		Pre-perinucleolar oocytes (pPO)	Highly basophilic (22.53 ± 14.91 μm)	Several centrally located nucleoli (13.60 ± 6.36 μm)	
		Perinucleolar oocytes (PO)	Highly basophilic (41.42 ± 15.20 μm)	Numerous nucleolus located next to nuclear membrane (24.17 ± 6.27 μm)	
II	April–May	Vitellogenic oocytes at primary yolk stage (VOPY)[a]	Basophilic with cytoplasmic granules randomly distributed (97.45 ± 18.66 μm)	Plenty of nucleolus located next to the nuclear membrane (52.46 ± 11.07 μm)	Granulosa Zone radiate
III	June–July (1st fortnight)	Vitellogenic oocytes at secondary yolk stage (VOSY)	Acidophilic granules peripherally located and lipidic dropped located next to the nucleus (181.84 ± 75.68 μm)	Many nucleolus located next to the nuclear membrane (75.68 ± 19.75 μm)	Granulosa Theca cell layer Zone radiate
		Vitellogenic oocytes at tertiary yolk stage (VOTY)	Many acidophilic granules (313.34 ± 41.52 μm)	Many nucleolus located next to the nuclear membrane (95.43 ± 12.73 μm)	Granulosa Theca cell layer Zone radiate
		Atretic follicles (AF)[a]	Lipidic dropped and acidophilc globules. Irregular in shape	Highly condensed	Flattened cell monolayer
IV	July (2nd fortnight)	Post-ovulatory follicle (POF)[b]	No ovocyte		Clusters of flattened cells and disorganized material
V	August–September	[a]			
VI	October–November	[a]			

[a] From June to November the germ cell types present in the ovary in stage I also appeared.
[b] In July, the germ cells types present in the ovary in stage I and atretic follicles also appeared.

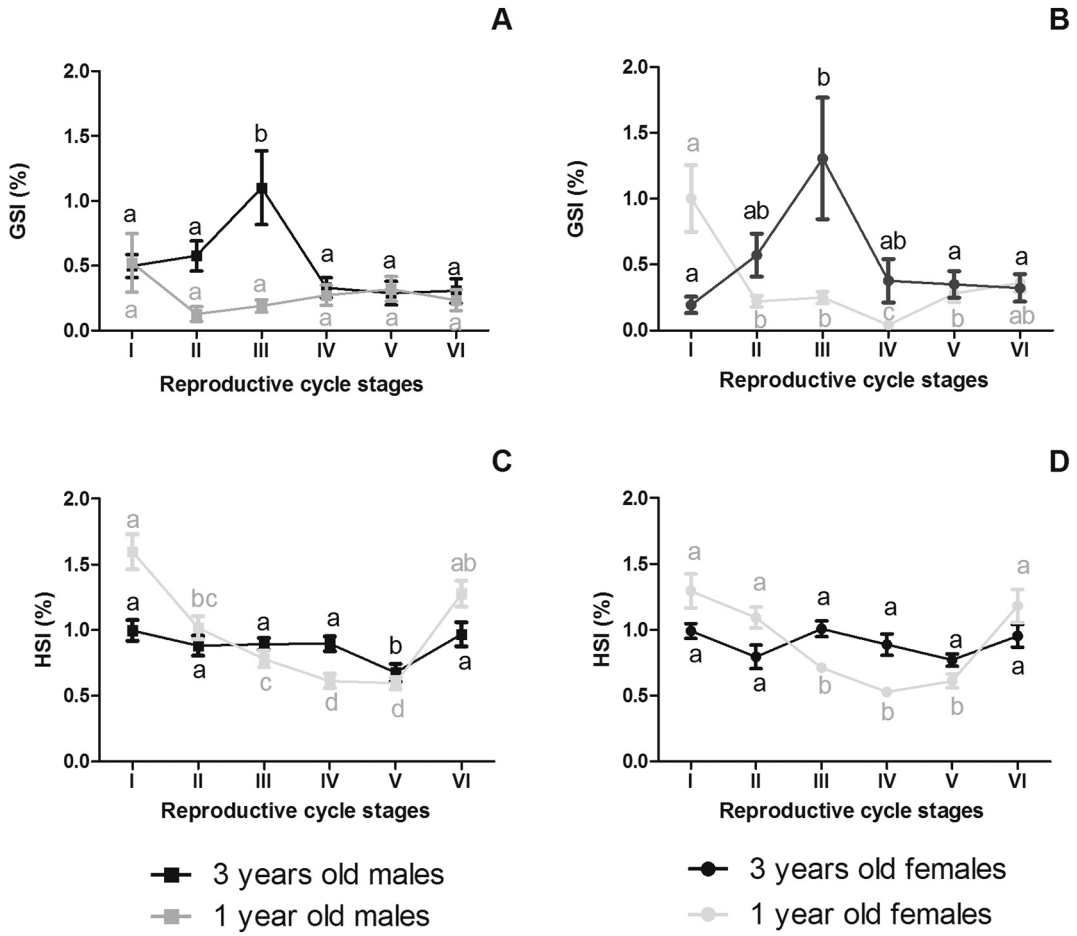

Fig. 2. The gonadosomatic (A,B) and hepatosomatic (C, D) indexes of one and three years old males (A, C) and females (B, D) of *Umbrina cirrosa* specimens with 1 or 3 years of age. Letters denote statistical differences between reproductive stages. Reproductive stages of both sexes are denoted from I to VI as described in Tables 1 and 2.

efferent duct was full of free spermatozoa. The spermatogenesis in this species is an asynchronous process in which all type of cysts (SGA, SGB, SC, SD and SZ) were observed in the same section of the tubules (Fig. 3D, E). The SGSC were identified as isolated spermatogonia with the bigger nuclear and cytoplasmic size (7.2 ± 1.2 and 11.7 ± 1.7 μm, respectively). The SGA showed a smaller nuclear size (5.47 ± 0.10 μm) and their cytoplasm is difficult to be estimated as they are interconnected by cytoplasmic bridges with the rest of SGA of the same cyst. The spawning season was in June and at this stage the continuous waves of spermatogenesis ceased. At the beginning of June, the germinal epithelium was full of cysts of SC and SD and the lumen of the tubules and the efferent duct were full of free spermatozoa (Fig. 3F). However, at the end of June the amount of cysts of SC, SD and SZ decreased and the relative percentage of cysts of SGA increased together with a decrease in the amount of free spermatozoa present in the lumen of the tubules (Fig. 3G). The post-spawning stage, defined as the stage in which the relative amount of SGSC and SGA increased in the germinal epithelium and the lumen of most of the tubules collapsed, was observed in July (Fig. 3H). At resting stage (from August to November) the testis was formed by a dense tissue mainly formed by SGSC and cyst of SGA. The lumen of the tubules was small with remaining spermatozoa in some of them (Fig. 3I).

3.4. Gonad morphology of 1 and 3 years old females

One year old females showed an immature ovary throughout the whole year. Thus, their ovary consisted of nests of oogonias, pre-perinucleolar oocytes (pPO) and perinucleolar oocytes (PO) (Fig. 4A, C). However, three year old females went through a functional reproductive cycle (Table 2 and Fig. 4B–H). From December to March, the ovary was immature (Fig. 4B) but started to develop in April when vitellogenetic oocytes at primary yolk stage were firstly observed (Fig. 4D). Vitellogenic oocytes at secondary (Fig. 4E) and tertiary (Fig. 4F) yolk stage were observed from June to the first fortnight of July (stage III) together with some atretic follicles (Fig. 4G). The spawning was performed in June and the first fortnight of July (stage III) and later on in the second fortnight of July, post-ovulatory follicles were observed (Fig. 4H). Strikingly, we observed oocytes at the early final oocyte maturation (FOM)-defined as oocytes with lipid droplets accumulated around the nucleus-, but not more advanced FOM stages (data not shown). We also observed that the spawning events were performed during the night as the eggs were always collected very early in the morning.

3.5. Sex steroids hormone levels of 1 and 3 years old males

In the first year of life, the method used only detected 11KT serum levels, and they were low and constant throughout the year (Table 3).

However, in three years old males the 11KT serum levels progressively increased during spermatogenesis (stages I and II) and reached the highest levels at spawning (stage III). After spawning, the 11KT serum levels decreased until the lowest levels (0.024 ± 0.003 ng/mL) which were recorded at resting (stages V and VI) (Fig. 5A). However, the highest T serum levels were recorded during late spermatogenesis and spawning (stages II and III), while the levels were similar at early spermatogenesis, post-spawning and resting (stages I, IV, V and VI)

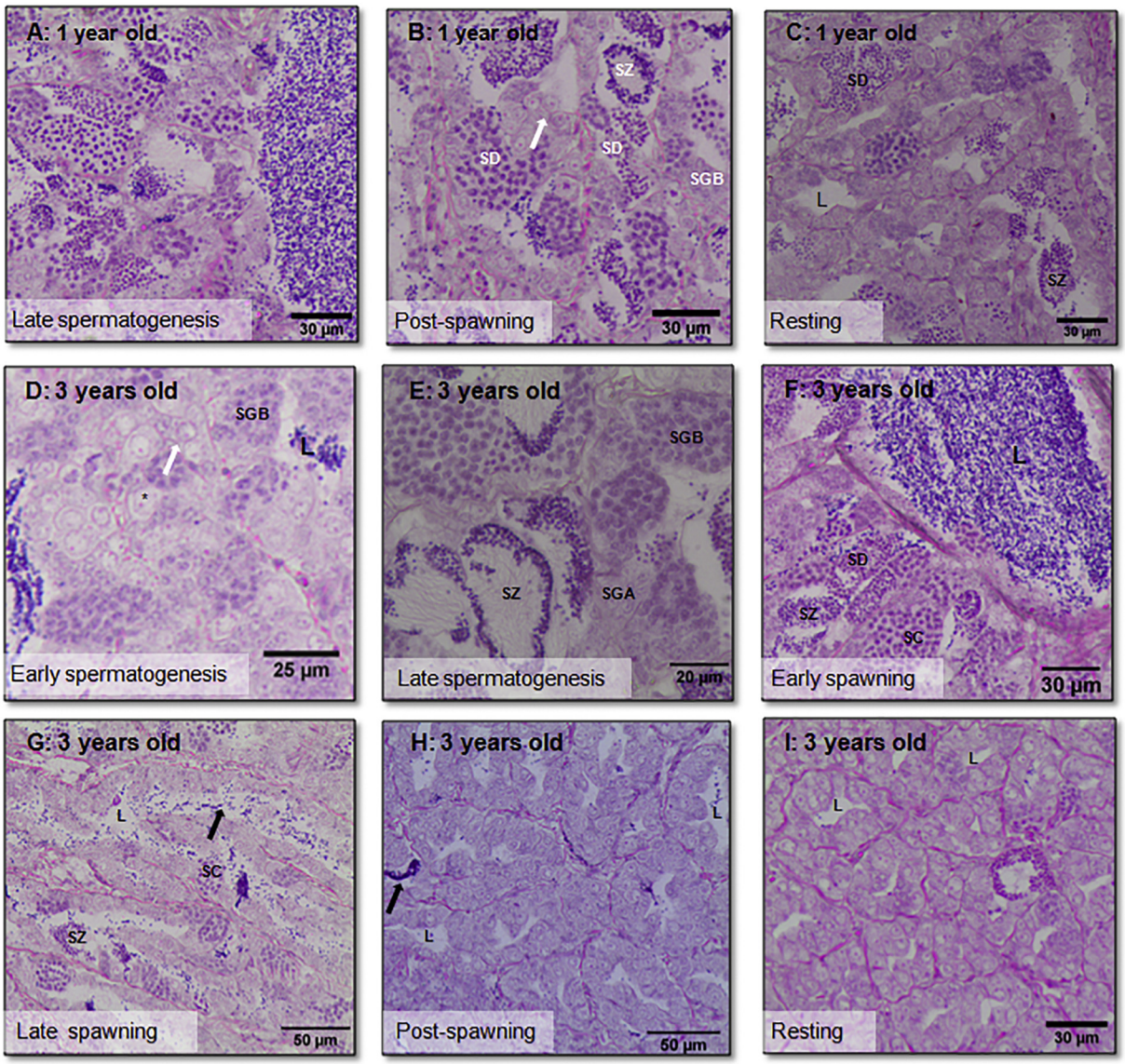

Fig. 3. Sections of the testis of 1 (A–C) and 3 (D–I) years old *Umbrina cirrosa* specimens stained with hematoxylin-eosin and showing the morphological organization of the testes at the different stages described in Table 1. Notably, 1 year old males underwent constant waves of spermatogenesis as the testis is always formed by ameiotic and post-meiotic cyst independently of the season of the year. In 3 year old males the testicular morphology changes in accordance with the season. Asterisk denotes spermatogonia stem cell; white arrows, cyst of SGA; SGB, cysts of spermatogonia B; SC, cysts of spermatocytes; SD, cysts of spermatids; SZ, spermatozoa; L, lumen of the tubules; black arrows, free spermatozoa in the lumen of the tubules. Scale bars A–C, F, I 30 µm, D 25 µm, E 20 µm and G–H 50 µm.

(Fig. 5B). P were detected in the serum of three year old males at all the stages, but remained steady throughout the reproductive cycle (Fig. 5C).

In the gonad of three year old males, the pattern of 11KT and T levels were similar to in serum, with the exception that at the resting stage the 11KT level in gonad did not decrease and was similar to the ones recorded at late spermatogenesis (stage II) (Fig. 6A, B). Interestingly, E_2 was detected in gonad and peaked during spawning, as did 11KT and T (Fig. 6C). However, P levels in the gonad progressively increased from late spermatogenesis (stage II) to resting (stages V and VI) (Fig. 6D).

3.6. Sex steroids hormone levels of 1 and 3 years old females

One year old females showed undetectable levels of 11KT in serum, while E_2 serum levels were very low at the beginning and progressively increased throughout the year (Table 3). In three year old females 11KT serum levels were very low at stage I and II (0.02 ± 0.01 and 0.04 ± 0.03 ng/mL respectively) or undetectable during the rest of the reproductive cycle as were T serum levels during the complete reproductive cycle (data not shown). The serum levels of E_2 and P increased at stage III, when spawning occurs. However, E_2 progressively decreased until reaching the lowest levels at stage VI, while P serum levels remained steady throughout the rest of the reproductive cycle (Fig. 7A, B).

In the female's gonad, 11KT and T levels were detected and they were high during the first stages of the reproductive cycle and they decreased at stages VI and V-VI, respectively (Fig. 7B, C). The levels of E_2 in the gonad were higher than in serum and remained steady throughout the year, while P progressively increased from stage II to stage V and then sharply decreased at stage VI (Fig. 7F).

3.7. Humoral innate immune activities in 3 year old male and female serums

Protease and peroxidase activities were stable throughout the year

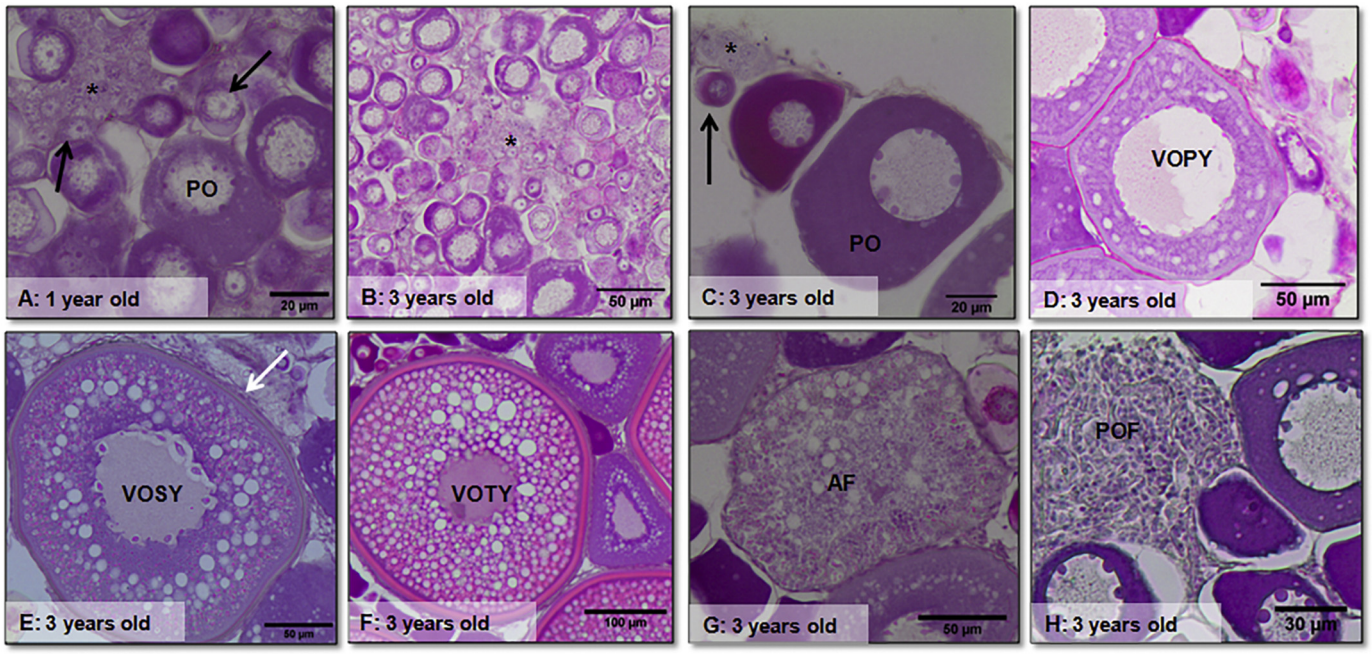

Fig. 4. Section of the ovary of 1 (A) and 3 (B–H) years old *Umbrina cirrosa* specimens stained with hematoxylin-eosin and showing the morphological organization of the ovary at the different stages described in Table 2. The ovary of 1 year old females remains immature. Asterisks denote nests of oogonia; black arrows, pre-perinucleolar oocytes; PO, perinucleolar oocytes; VOPY, vitellogenic oocytes at primary yolk stage; VOSY, vitellogenic oocytes at secondary yolk stage; VOTY, vitellogenic oocytes at tertiary yolk stage; AF, atretic follicles; POF, post-ovulatory follicle. Scale bars A, C 20 µm, B, D, E, G 50 µm, F 100 µm and H 30 µm.

Table 3
Serum levels of 11KT and E_2 in one year old males and females. Letters denote differences between stages.

	Stage	11KT (ng/ml) in males	E_2 (ng/ml) in females
1 year old specimens	I	0.167 ± 0.101	0.069 ± 0.013^{a}
	II	0.118 ± 0.019	0.713 ± 0.073^{a,b}
	III	0.148 ± 0.070	1.086 ± 0.148^{b}
	IV	0.117 ± 0.010	1.160 ± 0.092^{b}
	V	0.104 ± 0.018	0.792 ± 0.075^{b}
	VI	0.074 ± 0.042	1.802 ± 0.299^{c}

in three year old males and females (Fig. 8A, B). Interestingly, protease activity was higher in females than in males (Fig. 8A). The pattern of bactericide activity levels was similar in males and females except at stage V where a statistically significant difference was observed between sexes. A decrease in this activity was observed at stage V in both sexes, but more pronounced in females than in males (Fig. 8C).

4. Discussion

The shi drum belonging to the Sciaenidae family of fish (Nelson et al., 2016) has attracted attention as it is an excellent diversification species in Mediterranean aquaculture. As the whole shi drum life cycle was completed in our facilities by means of spontaneous spawning of shi drum broodstock born in captivity (Arizcun et al., 2014). The spontaneous spawns obtained during four consecutive years of broodstock specimens from their third year of life onwards had been analyzed. It has been found that shi drum broodstock had high fertilization rates from the second reproductive season onwards independently of the amount of spawned eggs, collecting the largest amount of eggs in the middle of the spawning season. The percentage of fertilized eggs during 2014, 2015 and 2016 spawning seasons was around 63–77% quite similar to the one (65%) obtained in previously described GnRH-induced broodstock (Mylonas et al., 2004). However, GnRH induction failed to produce multiple spawns as could be expected in fish species with asynchronous gametogenesis (Mylonas et al., 2004) as occurs when the spawns are spontaneous. Then, the reproductive development at two different ages (1 and 3 years old) of shi drum specimens born in captivity was also analyzed. The GSI of 1 year old specimens was constant throughout the year in both sexes. Regarding males, the 11KT levels remained very low and steady throughout the whole year and this issue may explain why consecutive waves of spermatogenesis were observed in the testis throughout the whole year. However, neither an effective accumulation of spermatozoa nor a renewal of the tissue occurred as it did from June to November in three year old fish. This early spermatogenesis process has also been described in gilthead seabream which have differentiated testes and produce spermatozoa in specimens under one year old (Chaves-Pozo et al., 2009). In the case of 1 year old females, increasing levels of E_2 were recorded throughout the whole year reaching 1.802 ± 0.299 ng/mL of E_2 in serum at the end. However, the vitellogenic process was not developed as the ovary remained immature and was formed only by pre-vitellogenetic oocytes. Increasing levels of E_2 in female serum has been correlated with ovarian growth in several fish species (Liley, 1983; Schulz, 1984). In gilthead seabream, a protandrous hermaphrodite fish species, the E_2 levels progressively increased during the male phase in which the ovarian area is formed by pre-vitellogenic oocytes and cellular proliferation was observed (Chaves-Pozo et al., 2008, 2005).

Three year old specimens showed a normal reproductive cycle in terms of GSI, gonadal morphology and hormone level patterns. Thus, in both sexes GSI increased during gametogenesis and sharply decreased after spawning (stage IV) similarly, as described in other gonochoristic Mediterranean species like the European sea bass (Felip et al., 2001; Valero et al., 2015b). A similar pattern was observed regarding androgens in males and estrogens in females as also occurs in the European sea bass (Felip et al., 2001). In shi drum male serum, only androgens (11KT and T) and P were detected, as previously reported in other fish species (Borg, 1994; Liley, 1983). In some species of teleost low levels of E_2 are present in male serum (Chaves-Pozo et al., 2008; Liley, 1983; Schiavone et al., 2012; Valero et al., 2015b), but not in the shi drum where serum levels of E_2 were undetected. However, E_2 were detected in the testis and showed an increase at spawning (stage III) suggesting that E_2 might be important for the shi drum male's gonadal

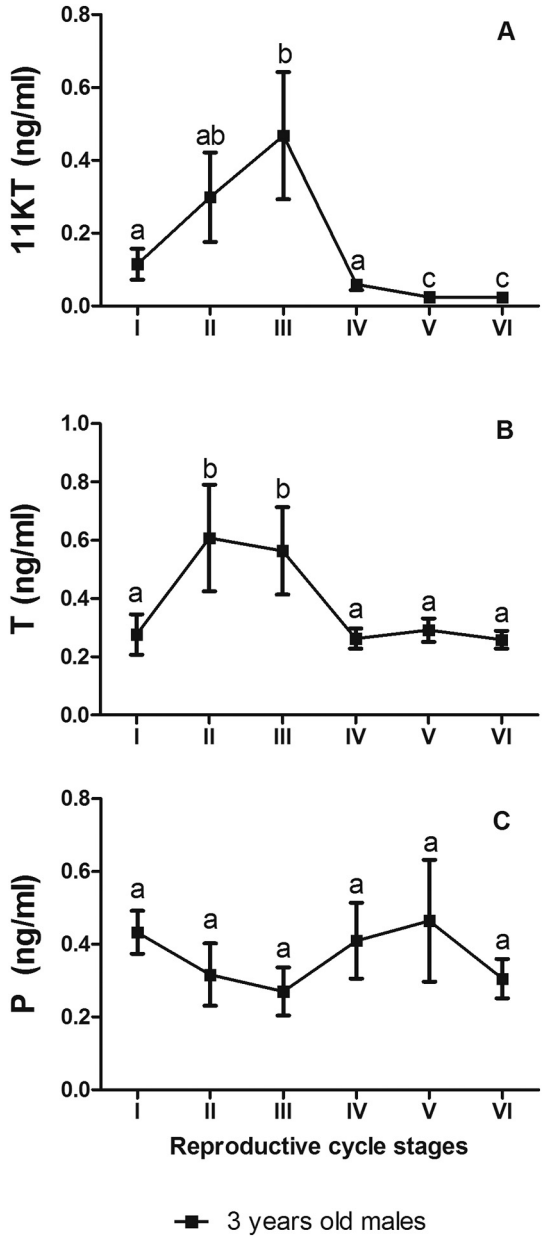

Fig. 5. Serum levels of 11KT (A), T (B), and P (C) in 3 year old *Umbrina cirrosa* males at the different stages of the reproductive cycle described in Table 1. Letters denote statistical differences between stages.

cycle even when E_2 serum levels were undetectable. Higher levels of steroids in the gonad than in serum has been described in several fish species, as this tissue is the main production site of sex steroids (Stocco, 2001). In fact, in gilthead seabream males, the E_2 levels in the gonad are higher than in serum (Sánchez-Hernández et al., 2013). Interestingly, E_2 serum levels increase after spawning and remained high at the later stages of the reproductive cycle in gilthead seabream males (Chaves-Pozo et al., 2008) as also occurs in shi drum, or they have a peak during spawning and then drop to basal levels as in meagre (*Argyrosomus regius*) (Schiavone et al., 2012). Some exogenous administration of estrogen trials have suggested that E_2 orchestrated the regression process of the testis upon spawning or during testicular involution in the gilthead seabream (Chaves-Pozo et al., 2008, 2007; Liarte et al., 2007).

On the other hand, P was present in the serum of shi drum males but remained steady throughout the reproductive cycle. In the males of several fish species, P serum levels have been correlated with the stimulation of spermatogonial proliferation as a meiotic inducer and in the spermiation process (Fostier et al., 1983; Schulz et al., 2010; Ueda et al., 1985). The serum level of P reported in the males of several fish species is variable and three groups can be established (Scott et al., 2010). Thus, a group in which (i) P peaks at the time of spermiation, (ii) P remained steady and quite low during the complete reproductive cycle or (iii) P serum levels are undetectable (Scott et al., 2010). Our data showed that the P serum levels in shi drum males are steady but not low when compared with 11KT or T serum levels. In fish species that spawn daily and during the night, such as shi drum, the P peak related to spermiation occur during the night and is so tightly regulated that in some cases it is not detected during the day (Scott et al., 2010). This fact could explain why high levels of P in serum were found but were steady throughout the year even when P might have a role in the reproductive cycle of shi drum males. Furthermore, a certain production of P in the testis occurred as the levels inside this organ progressively increased from spermatogenesis onwards and remained high at the resting stage. In salmonids a low peak of P is related to spermatogonial proliferation while a high one occurs at spermiation (Schulz et al., 2010). This would explain why in shi drum testis the P levels progressively increased from spermatogenesis until resting (stages V and VI) when they remained high, although those levels (0.076 ± 0.009 ng/mL) are very low when compared with other sex steroid hormones. Progestins have been described as inducing meiosis in the Japanese eel testis (Ozaki et al., 2006), while in gilthead seabream the action of P through progestin nuclear receptor correlated with spermatogonia stem cell renewal (Chauvigné et al., 2017). Whether progesterone acts as an inducer of spermatogonia renewal in shi drum or not, it would explain why the maintenance of high levels of P after spawning is needed. In any case further studies are mandatory to determine the biological role of P during the resting stage of shi drum males.

Vitellogenesis in shi drum has been reported to be a rapid process compared with other asynchronous fishes (Mylonas et al., 2004). As expected, we demonstrated that vitellogenic oocytes appeared at stage II (April), until spawning (stage III, June-the first fortnight of July), as also suggested previously (Mylonas et al., 2004). Interestingly, at the spawning stage, only oocytes at FOM, were observed but not at more advanced FOM stages. The shi drum spawn during the night, so it is possible that early morning sampling would not catch FOM oocytes in spawning females. In that sense, further studies based on circadian sampling are needed to ascertain the progression of FOM and the late stage of ovulation in this species.

Regarding sex steroid hormone levels, in female fish in general, T serum levels are high and increase during the pre-spawning period and they exceed those of males in some species (Kagawa, 2013; Liley, 1983). High levels of T in the serum of females occur as T is converted to E_2 which orchestrates the vitellogenic process and ovocyte growth (Kagawa, 2013; Nagahama et al., 1995). 11KT is usually non-detectable or present in low concentrations in females. However, there are some species that are exceptional (Liley, 1983). Regarding Mediterranean species, in *Solea solea* and *Solea senegalensis* females, the T and 11KT serum levels were detectable, but low when compared with males, contrary to what occurs with E_2 serum levels in both sexes (Solé et al., 2016). As 11KT has always been considered a male hormone, little information is available regarding this hormone in females. However, some studies point to the 11KT as a regulator of female physiology. Interestingly, in the short-finned eel (*Anguilla australis*), 11KT treatments in vivo increased the female GSI and oocytes diameters (Zadmajid et al., 2015). In vitro experiments using coho salmon ovarian follicle demonstrated that 11KT is involved in the growth of primary follicles and in the development of early cortical alveolus-stage follicles in this specie (Forsgren and Young, 2012). In shi drum females, 11KT was detected in serum at the beginning of the reproductive cycle, although it was produced by the gonad throughout the complete cycle as it was detected in this tissue, reaching the highest levels at stages II and

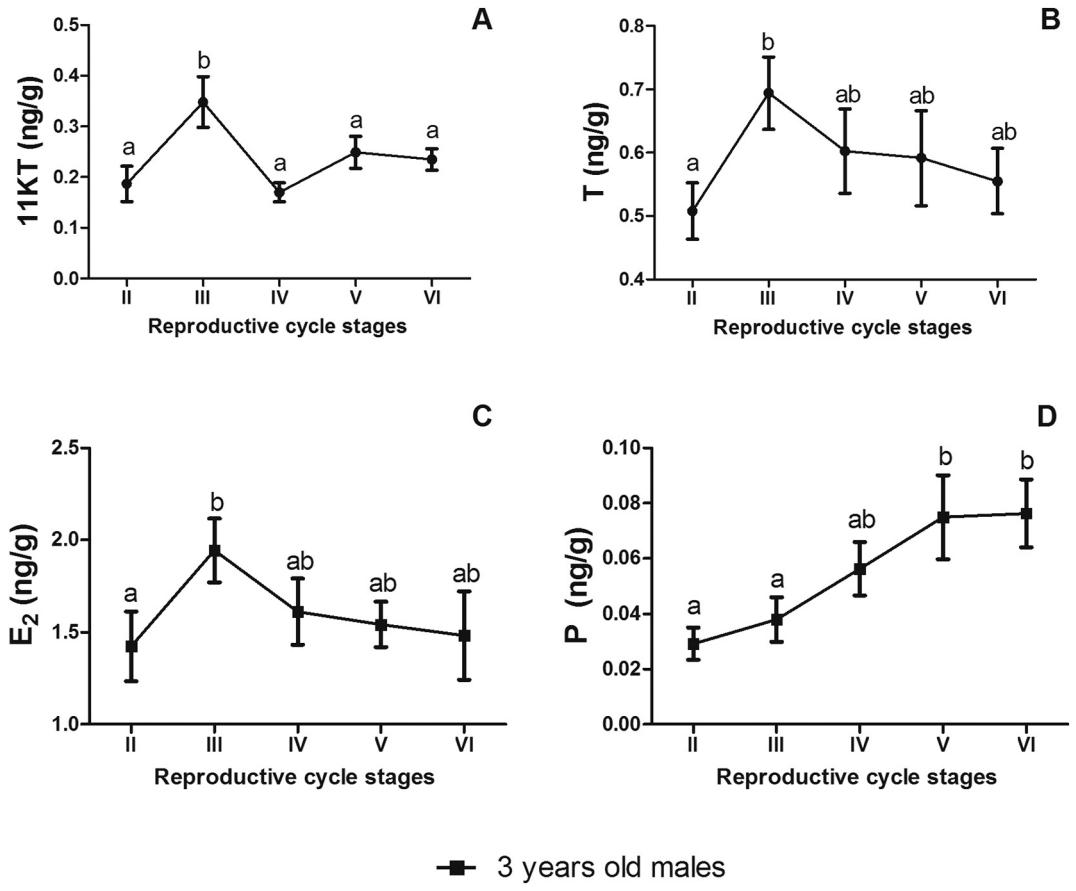

Fig. 6. Gonadal levels of 11KT (A), T (B), E_2 (C) and P (D) in 3 year old *Umbrina cirrosa* males at the different stages of the reproductive cycle described in Table 1. Letters denote statistical differences between stages.

III, during the oocytes growth. In previtellogenic short-finned eels, 11KT regulated the expression of genes that belongs to the transforming growth factor (TGF)-β signalling network such as inhibin and activin that in turn regulated oocytes growth and ovarian functions (Zadmajid et al., 2015). In addition, the initiation of spawning in females of several fish species correlates with an increase in 11KT serum levels (Fostier et al., 1983). Whenever, 11KT's involvement in final oocyte maturation and spawning deserves further studies to clearly establish the role of androgens in female shi drum physiology.

Regarding most of the species studied, the serum levels of E_2 increase during pre-spawning period and drop at ovulation and spawning at the same time as P is elevated (Kagawa, 2013; Liley, 1983). The highest level of this hormone found in the serum of shi drum females was around 0.44 ± 0.09 ng/mL at stage III when the late vitellogenesis, FOM and spawns occurred. The E_2 serum levels recorded were quite low when compared with other fish species, although it showed the same pattern throughout the whole year as other fish species (high during oocytes growth and vitellogenesis and drops after spawning) (Fostier et al., 1983). In the gonad, however, high levels (3.12 ± 0.35 ng/g) were observed during the complete cycle except at stage IV (post-spawning) when the levels dropped. Interestingly, P peaked at stage III (spawning) in serum but at stage V (resting) in the gonad. The different pattern in serum and gonad did not contradict the idea that P is involved in the last events of ovulation and spawning, but included the possibility that P has a role in ovarian renewal during post-spawning and resting stages. Interestingly, in zebra fish (*Danio rerio*) pre-puberal specimens exposure to synthetic progestins have revealed that high levels of these hormones delay the development of the ovary (Hou et al., 2018). This issue might explain why P increased in the gonad of shi drum after spawning (stages IV and V) and later on decreased but remained at similar levels to those recorded at spawning (stage VI), probably in order to delay the beginning of the new vitellogenetic cycle until the following April (around 9 months). The lack of data regarding sex steroid hormone levels in the gonad of several fish species makes it difficult to make comparisons among them. In this sense further studies will be needed. Taking into account that sex steroid hormones regulated immune responses in fish, that some pathogens used the gonad to evade the immune system reaching this tissue probably through the vascular system and that differences in prevalence between sexes have already been observed in some fish species (Valero et al., 2018), some humoral innate activities in males and females have been studied. It was found that protease activity showed higher levels in females than in males throughout the whole reproductive cycle and some differences between sexes were observed in the bactericide activity at the beginning of the resting stage (stage V). However, peroxidase activity patterns were similar in both sexes.

There are evidences in fish that the seasonality associated with immunity is related to several hormone levels including sex steroid hormones. Increases of sex-steroid serum levels during sexual maturation or upon hormonal administration led to the modulation of several humoral immune responses (Cuesta et al., 2007; Hou et al., 1999a, 1999b). This imply that several immune activities in serum and immune tissues depends on the reproductive conditions of the specimens (Chaves-Pozo et al., 2018). In that sense, having information about how the natural reproductive cycle might influence the immune status of some humoral innate immune responses (as the activities we evaluate in this study), will be interesting, especially considering the sex preferences of the prevalence of some infections and the pathogens that are vertically transmitted (Klein, 2004; Valero et al., 2018). Thus, the knowledge of this relationship through the reproductive cycle would be

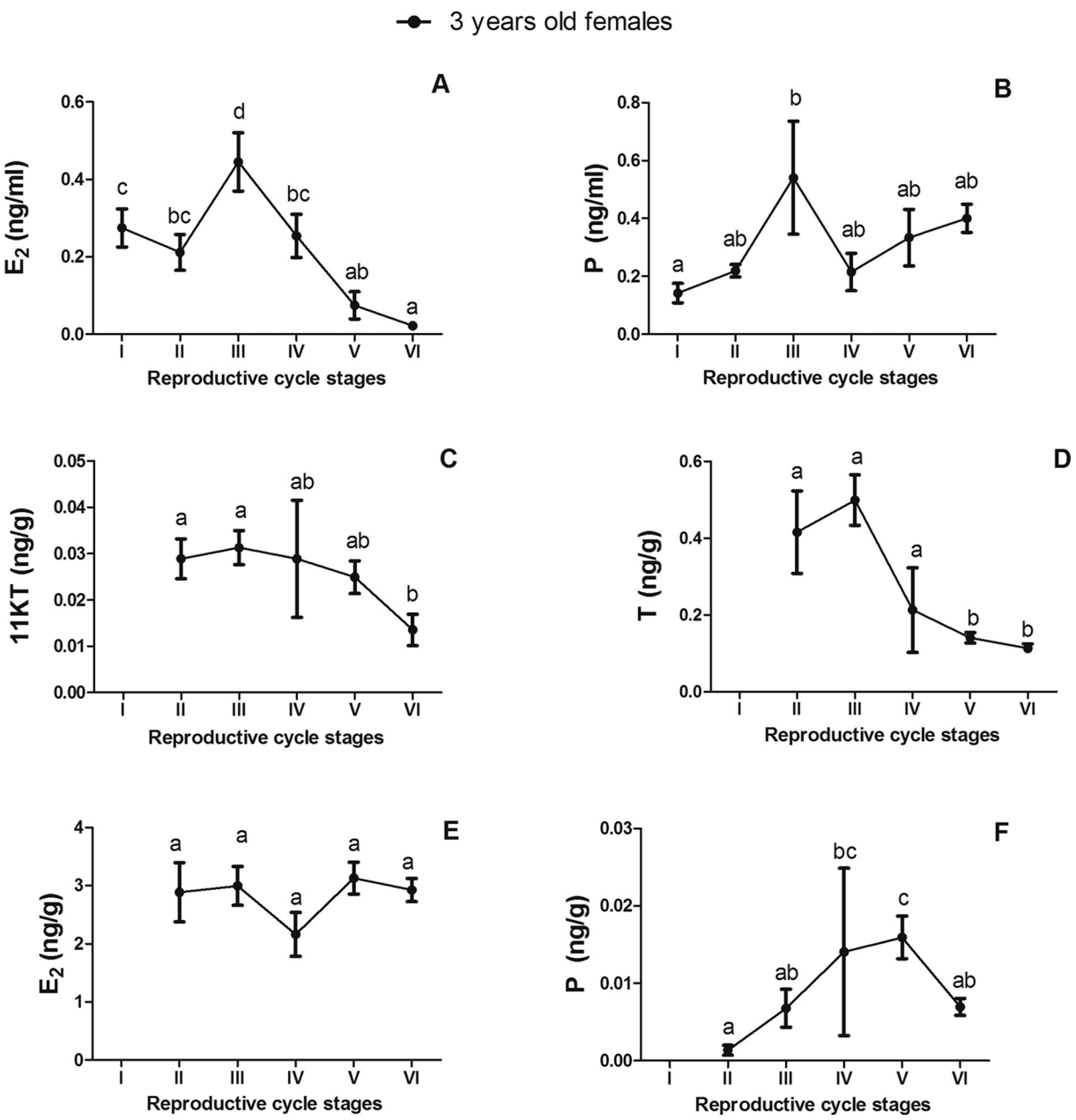

Fig. 7. Serum (A–B) and gonadal (C–F) levels of E_2 (A,E), P (B,F), 11KT (C) and T (D) in 3 year old *Umbrina cirrosa* female at the different stages of the reproductive cycle described in Table 2. Letters denote statistical differences between stages.

worthy to prevent pathogen problems in the maintenance of broodstock fish.

In conclusion, this manuscript represents the most complete overview about the reproductive cycle of shi drum. Until now, some morphological studies on the gonad development of wild and GnRH-induced captive animals have been published (Barbaro et al., 2002; Mylonas et al., 2004), although no data about the sex steroid hormone levels were reported in those studies. So, this is the first time that the reproductive cycle of spontaneously spawning captive shi drum specimens has been described in terms of gonad morphology and sex steroid serum and gonad levels. An attempt has also been made to introduce the study of the immune-reproductive interaction in this new species in a naturally changing physiology.

Authorship of the paper

ECP and MA were involved in the design and execution and interpretation of the data, EA dealt with conception, design and funding acquisition, PB was involved in execution of the analysis performed.

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Declarations of interest

None.

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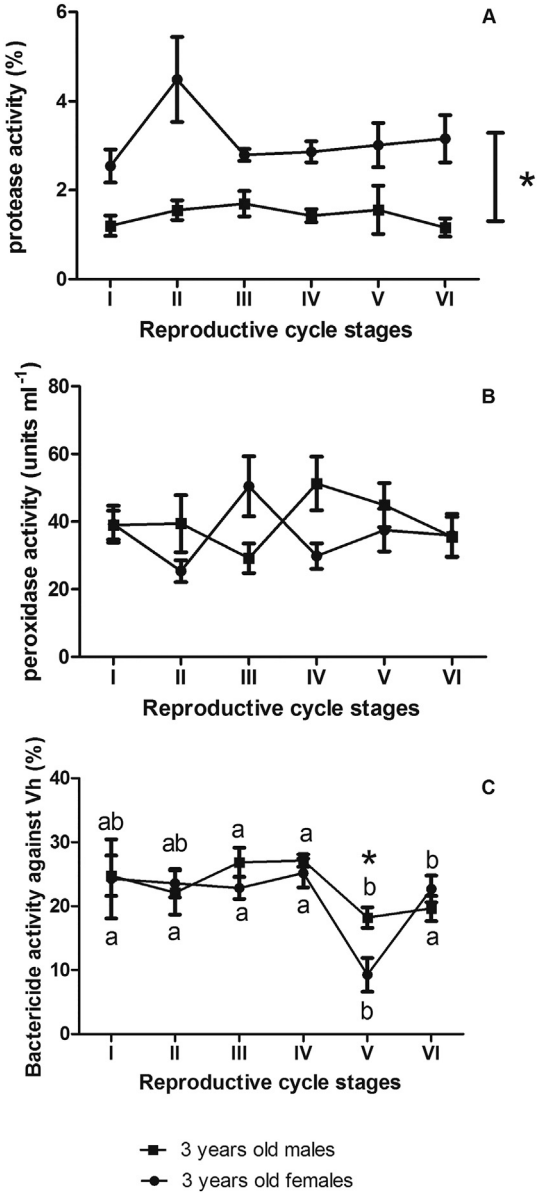

Fig. 8. Protease (A), peroxidase (B) and bactericidal (C) activity in the serum of 3 year old *Umbrina cirrosa* males and females at different stages of the reproductive cycle described in Tables 1 and 2, respectively. Letters denote statistical differences between stages within the same sex. Asterisks denote differences between males and females.

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