Research Article

Changes in plasma steroid hormones and gonadal histology associated with sexual maturation in wild southern hake *(Merluccius australis)*

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ABSTRACT. A detailed study of gametes development and characterization of plasma sex steroid hormones during the maturation cycle was performed for the first time in the southern hake (*Merluccius australi*s). Fish were caught in the inland waters of the Reloncaví Sound, Interior Sea of Chiloé, Chile. Samples of gonads and blood were collected for histology and sex steroid hormone (17 β-estradiol, 11-ketotestosterone and 17,20 β-dihydroxy-4-pregnen-3-one) analysis, respectively. Sex steroid hormone quantification was performed using enzyme-immunoassay (ELISA). Results showed that *M. australi*s males and females have asynchronous development of testicles and ovaries, in all stages of maturation. Most spawning fish were found during the spring months. Regarding the sex steroid hormones, serological fluctuations of 17 β-estradiol and 11-ketotestosterone were found during gonadal maturation of *M. australis*. These hormones are the main hormones responsible for vitelogenesis and spermatogenesis processes, respectively. Conversely, 17,20 β-dihydroxy-4-pregnen-3-one did not show any serological fluctuation in females and males. Further studies involving gonadotropins, 17,20 β,21-trihydroxy-4-pregnen-3-one and vitellogenin quantification are required in order to obtain a more complete description of the reproductive physiology of wild and farmed *M. australis*.

Keywords: *Merluccius australis*, southern hake, gonad maturation, sex steroids, southern Chile.

Cambios en las hormonas esteroidales plasmáticas y en la histología gonadal asociados a la maduración sexual a la merluza austral (*Merluccius australis*)

RESUMEN. El presente estudio se realizó para caracterizar el desarrollo de los gametos y el comportamiento de las hormonas esteroidales sexuales en plasma durante el ciclo de maduración de la merluza austral (*Merluccius australis*). Los peces estudiados fueron capturados en las aguas interiores del Seno de Reloncaví, Mar Interior de Chiloé, Chile. Muestras de gónadas y sangre fueron recolectadas para histología y análisis de hormonas esteroides sexuales (17 β-estradiol, 11-cetotestosterona y 17,20 β-dihidroxi-4-pregnen-3-ona), respectivamente. La cuantificación de las hormonas esteroidales sexuales se realizó utilizando la enzima-inmunoensayo (ELISA). Los resultados mostraron que machos y hembras de *M. australis* poseen un desarrollo asincrónico de los testículos y ovarios, en todas las etapas de maduración. La mayoría de los ejemplares en etapa de desove se encontraron durante la primavera. En cuanto a las hormonas esteroides sexuales, fluctuaciones serológicos de 17 β-estradiol y 11-cetotestosterona se encontraron durante la maduración gonadal de *M. australis*. Estas hormonas son las principales responsables de los procesos de vitelogénesis y espermatogénesis, respectivamente. Por el contrario, 17,20 β-dihidroxi-4-pregnen-3-ona no mostró ninguna fluctuación serológica en hembras y machos. Nuevos estudios que incluyan la cuantificación de las hormonas gonadotropinas, 17,20 β,21-trihydroxy-4-pregnen-3-one y vitelogenina son requeridos para obtener una descripción más completa de la fisiología reproductiva de *M. australis* en estado silvestre y cautiverio.

Palabras clave: *Merluccius australis*, merluza austral, maduración gonadal, esteroides sexuales, sur de Chile.

Corresponding editor: Guido Plaza

INTRODUCTION

Southern hake (*Merluccius australis*) is a demersal gadiform fish species found in the southern hemisphere between Argentina in the Atlantic Ocean (Tingley *et al.*, 1995) and New Zealand in the Pacific Ocean (Aguayo-Hernandez, 1995; Colman, 1995). This species supports important industrial and artisanal fisheries in Chile, Argentina, and New Zealand, which supply local and mainly overseas market of Japan, USA, Spain and Portugal (Sylvia, 1995).

Global southern hake landings had historical peaks of about 65,000 ton between 1987 and 1989 (Sylvia, 1995), but there were dramatically declining catches in later years due to smaller fishing quotas to protect this resource from overexploitation. Nowadays, global southern hake landings are steady at just over 30,000 ton per year with prices around US$10 per kilo. Nevertheless, the global demand for southern hake is growing and the wild capture is declining, creating an undersupplied market for this fish. Indeed, the extent of this dependence has prompted the development of southern hake farming in Chile.

Even though there is an advanced understanding of the biology of the southern hake (Aguayo-Hernández, 1995; Colman, 1995; Tingley *et al.*, 1995; Bustos *et al.*, 2007; Effer *et al.*, 2013), there are some relevant questions concerning their reproductive biology, particularly the sexual maturation cycle and reproductive endocrinology that still remains unrevealed. To date, it is known that fish reproduction is regulated by a wide variety of abiotic and biotic environmental factors that trigger internal physiological mechanisms responsible for causing sexual maturation of fish (Arcand-Hoy & Benson, 1998).

Wild broodstock fish can spawn naturally in the tank when the environmental conditions are favorable, nevertheless, several fish species exhibit reproductive dysfunctions when they are raised in captivity (Mylonas *et al.*, 2010). Reproductive dysfunctions are usually more seriously in female broodstock and can be associated with final oocyte maturation, ovulation and spawning (Zohar *et al.*, 1988; Peter *et al.*, 1993). These dysfunctions most likely result from the combination of the stress induced by captivity, and the lack of a suitable environment for natural spawning (Schreck *et al.*, 2001; Pankhurst, 2011). Therefore, in the case of the absence or scarcity of natural spawning, several studies have shown that hormone induced spawning is a reliable method of inducing reproduction in these fishes (Zohar & Mylonas, 2001). However, this method has been reported to exhibit negative effects on the quality of gametes and survival rate at later stages of *Salmo salar* (Crim & Glebe, 1984; Crim *et al.*, 1986), *S. trutta*

(Mylonas *et al.*, 1992), *Oncorhynchus nerka* (Slater *et al.*, 1995), and *O. mykiss* (Arabaci *et al.*, 2004). In southern hake farming, one of the most critical aspects is to achieve the spawning of wild broodstock under captive conditions. Wild southern hake broodstock are spawned mainly by the use of hormones, causing a low survival rate of their larvae during weaning from *Artemia* to dry feed in culture conditions.

Considering the above-mentioned problem, the analysis of blood steroid levels has been used to clarify the optimum time to hormone induce spawning in fish, which can help to obtain higher quality gametes and therefore more suitable larvae, and also prevents the occurrence of over-maturation and follicular atresia of the gametes (Donaldson, 1996). Strictly, the use of quantitative analysis of blood steroid hormones, as a method for predicting the maturation stage of southern hake, involves less handling of broodstock compared to current method of gonadal biopsies, which is an invasive method and requires large samples. Moreover, the knowledge of the reproductive management concepts such as maturation cycle, reproductive endocrinology and gonadal development are scarce in southern hake (Bustos *et al.*, 2007; Effer *et al.*, 2013) and therefore, in order to scale up the commercial farming of southern hake is relevant to research in this important area. Hence, the aim of this study was to identify the gamete developmental stages and characterise plasma sex steroid hormones during the maturation cycle of southern hake.

MATERIALS AND METHODS

Sample collection

The specimens were captured by longline gear at 250-300 m depth in the inland waters of the Reloncaví Sound, Interior Sea of Chiloé in the Lagos Region, Chile (41°31'S, 72°44'W). Fishing activities were carried out from September 2011 to January 2012. During this period, fishes were collected every two or three weeks depending on weather conditions. Immediately upon reaching the surface, fishes were sacrificed and the samples of blood and gonads were collected and stored for later analysis.

Gonadal histology

Seventy six samples of gonads in different maturation stages from 40 females and 36 males of *M. australis* were collected for histological analysis. The dissected tissue was fixed in 5% formalin for 24 h and stored in 70% ethanol. The fixed tissue was subsequently dehydrated and embedded in the paraffin wax. The waxed tissue were cut in transverse sections of 6-7 μm thickness (Microtome, Leica Microsystems, model

RM2125, Bannockburn, IL, USA) and then stained with hematoxylineosin. Sample sections were examined under a light microscope (Leica Microsystems model DM750, Leica, Bannockburn, IL, USA) and classified according to their maturation status as immature, proliferation, growth, maturation and spawning. After the maturation stage was determined, the samples were correlated with the levels of serological steroid.

Analysis of hormonal steroids

Blood samples were extracted from each fish by caudal venipuncture and immediately placed on ice, where they were allowed to clot for 3-6 h. Blood samples were later centrifuged for 15 min at 1500 g (MiniSpin Centrifuge, AG 22331, Merck, Hamburg) and serum stored at -80°C for later sex steroid hormones analysis.

Quantification of sex steroid hormones was performed by enzyme-immunoassay technique (ELISA) using commercial kits protocols; 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) (Cayman Chemicals Company, MI, USA) and 11-ketotestosterone (11-TK) and 17 β-estradiol (E2) (Mybiosource, Beijing, China).

Statistical analysis

The results were analyzed using the programme SPSS Statistics 8.0 for Windows (SPSS Inc. Chicago, IL, USA). Normality and homoscedasticity were assessed using the Kolmogorov-Smirnov and Bartlett's test respectively. An analysis of variance (ANOVA) was performed to determine the existence of significant differences among sex hormones levels of each stage of gonadal maturation. Differences in mean values were determined by Tukey's test. The probability level for all statistical tests was set at 0.05.

RESULTS

Gonad morphology and histology
Male

The testicles of *M. australis* are paired organs of similar size and are white. They are composed of several lobes with similar morphology, which are joined to form a U-shaped structure. These organs are located ventral to the swim bladder of the fish.

Histological analysis of the testicles reported the presence of individuals in all maturation states (Table 1). Five individuals were found in an immature stage, which showed absolute dominance of germ cells (Fig. 1a). Eight individuals were found in the stage of proliferation (spermatogenesis), which had a high presence of spermatogonia and spermatocytes and fewer spermatids (Fig. 1b). Ten individuals were found

Table 1. Histological classification of gonadal maturity stage of wild southern hake (*M. australis*).

Gonadal maturity stage	Males (n = 36)	Females (n = 40)
Immature	5	0
Proliferation	8	15
Growth	10	9
Maturation	9	11
Spawning	4	5

in the growth stage (spermiogenesis), which showed a dominance of spermatids and a considerable amount of sperm, spermatocytes and spermatogonia (Fig. 1c). Nine individuals were found in the stage of maturation (spermiation), which had the exclusive presence of free sperm in the testis lobular lumen (Fig. 1d). Finally, ten individuals were found in the stage of spawning, which showed the presence of a few free spermatozoa and occasionally spermatogonia in the testicles lumen.

Females

The ovaries of *M. australis* are paired organs in the shape of elongated and bilobed sacs, which are located ventral to the swim bladder. The ovarian wall is transparent and thin, allowing oocytes in advanced stages of maturity to be visible to the naked eye. In the posterior region (caudal) is observed the fusion of the ovaries that extend into a short oviduct, which opens in the urogenital pore.

In the early developing stages, the ovaries showed a light orange colour which becomes more intense with advancing sexual maturity. Histological analysis of the ovaries reported the presence of individuals in all maturation stages except the immature stage (Table 1).

Fifteen individuals were found in the proliferation stage (primary growth), showing the presence of chromatin-nucleolar and perinuclear oocytes (Fig. 2a). Nine individuals were found in the growth stage (vitellogenesis), which had got oocytes in cortical alveoli and early vitellogenic stage. Similarly, the presence of oocytes in earlier stages of oogenesis (Fig. 2b) was also observed.

Eleven individuals were found in the maturation stage, which showed the presence of oocytes with nucleus migration and a noticeable size increase due to hydration (Fig. 2c).

Finally, five individuals were found in the spawning stage, which showed the presence of large amount of post-ovulatory follicles, atretic oocytes and also oocytes in earlier stages of oogenesis (Fig. 2d).
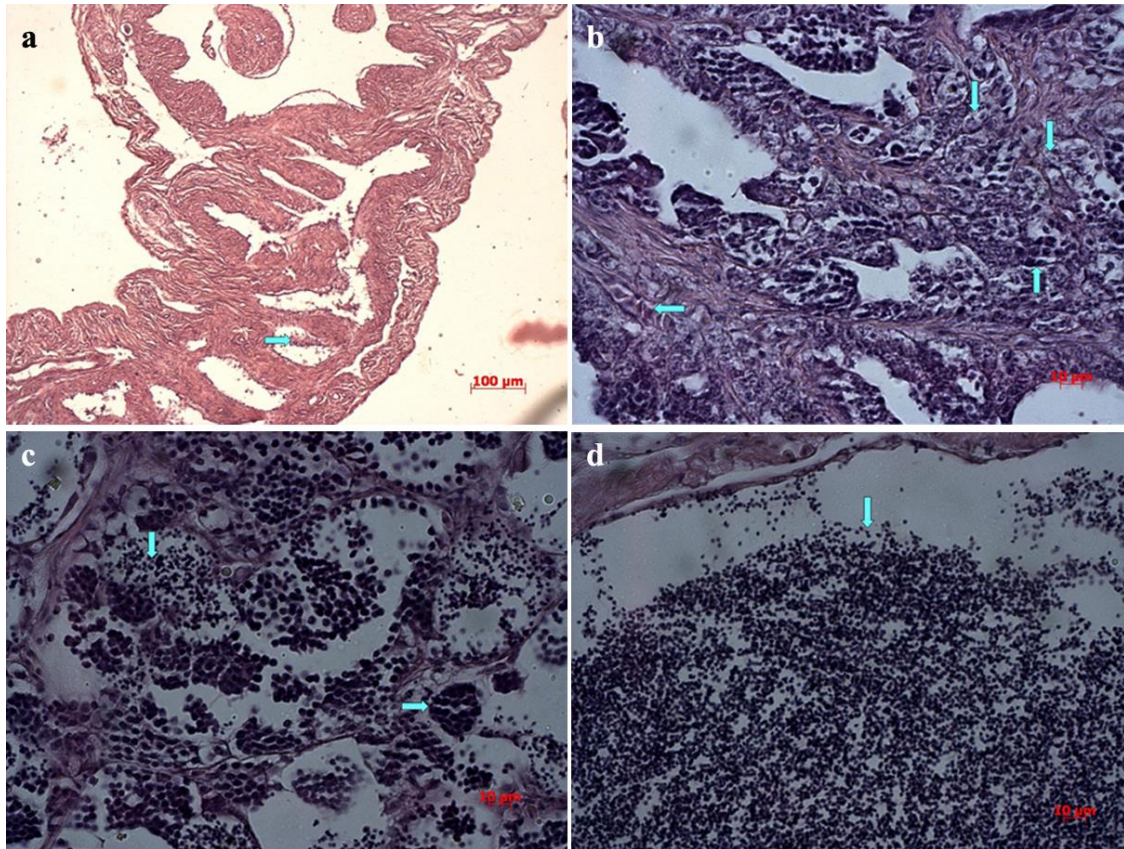
Figure 1. Cross sections of *M. australis* testes showing different maturity stages. a) Testis in immature stage (10x), b) testis in proliferation stage (40x), b) testis in the growth stage (40x), d) testis in the maturation stage (40x). The blue arrows indicate cells in spermatogenesis.

Plasma sex steroid hormonal profile 11-ketotes-tosterone

Plasma concentrations of 11-KT showed significant differences ($P < 0.05$) between the males found in the stage of proliferation and those found in the others maturity stages.

In the immature stage, the individuals reached an average plasma 11-KT concentration of 0.23 ± 0.03 ng mL^{-1}. However, during the proliferation stage, the levels of this hormones in the individuals increased significantly ($P < 0.05$), reaching the highest levels at maturational with an average of 1.04 ± 0.45 ng mL^{-1}. As maturation progressed, the concentrations of 11-KT present in individuals began to decrease, progressively reaching averages of 0.32 ± 0.24, 0.18 ± 0.07 and 0.15 ± 0.04 ng mL^{-1} in the stage of growth, maturation and spawning respectively.

Plasma levels of 11-KT in each of stages of testicular maturity of male southern hake described by the histological analysis are shown in (Fig. 3).

17β-estradiol

Plasma concentrations of E2 in *M. australis* females exhibited significant differences among the different stages of gonadal development ($P < 0.05$). These differences were showed among individuals in the growth phase (vitellogenesis) and those in the late stages of development.

In the proliferation stage (primary growth), the females reached an average plasma E2 concentration of 0.29 ± 0.06 ng mL^{-1}. Afterwards, the concentration of E2 increased significantly ($P < 0.05$) during the growth stage (vitellogenesis), achieving a mean maximum concentration of 0.62 ± 0.14 ng mL^{-1}.

Subsequent to vitellogenesis, the E2 levels decreased significantly ($P < 0.05$) as the gonadal development progresses. Females in maturation and spawning stages showed average concentrations of 0.32 ± 0.24 and 0.13 ± 0.03 ng mL^{-1} respectively. Plasma levels of E2 related to each of stage of ovarian maturity, described by the histological analysis, are shown in (Fig. 4).
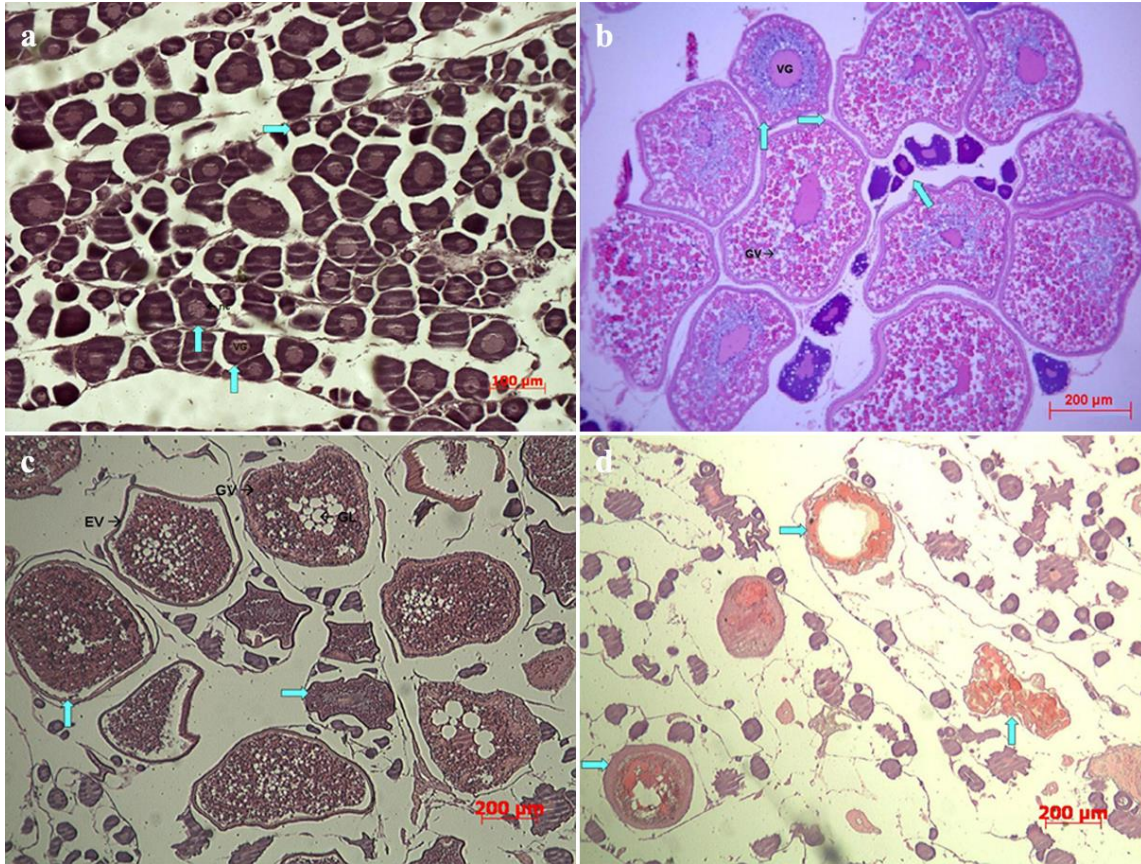
Figure 2. Cross sections of *M. australis* ovaries showing different maturity stages. a) Ovary in proliferation stage (4x), b) ovary in growth stage (4x), b) ovary in the maturation stage (4x), d) ovary in the spawning stage (4x). The blue arrows indicate oocyte cells in oogenesis.

17α,20β-dihydroxy-4-pregnen-3-one

The levels of 17α,20β-DP in the plasma of *M. australis* males showed no significant differences ($P > 0.05$) among different stages of gonadal development. However, the concentration of this hormone reached the maximum average of 0.3 ± 0.1 ng mL^{-1} during the maturation stage (espermiation).

The remaining gonadal development stages had lower levels of 17α,20β-DP, with average values of 0.07 ± 0.03, 0.11 ± 0.03, 0.09 ± 0.03 and 0.04 ± 0.01 ng mL^{-1} in the immature, proliferation, growth and spawning stages respectively.

Similar to the results reported in males, levels of 17α,20β-DP in females plasma showed no significant differences ($P > 0.05$) among different stages of gonadal development averaging 17α,20β-DP plasma values of 0.07 ± 0.02, 0.05 ± 0.01, 0.05 ± 0.01, 0.05 ± 0.01 and 0.06 ± 0.02 ng mL^{-1} in immature, proliferation, growth, maturation and spawning stages respectively. Plasma levels of 17α, 20β-DP in each of stages of ovarian and testicular maturity of southern hake

described by the histological analysis are shown in Figures 3 and 4.

DISCUSSION

Studies concerning the anatomy and physiology of the reproductive system are important in order to understand the biology of fish reproduction. This study represents the first attempt at a detailed histological identification of gamete developmental stages and characterization of plasma sex steroid hormones during the maturation cycle of southern hake *(Merluccius australis)*.

Histological evaluation of the gonad development stages in male and female specimens of *M. australis* found cells that exhibit all stages of maturation, showing clearly the type of asynchronous ovarian and testicular development of this species.

Testicular histology showed spermatogonia, spermatocytes and spermatids in the lobular wall, whereas spermatozoa were observed free in the lumen. At the
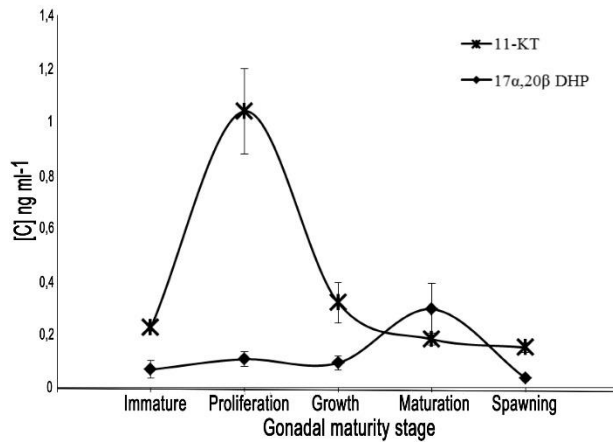
Figure 3. Plasma steroidal profiles of *M. australis* males during maturation cycle (Mean ± Standard Error) ($P <$ 0.05).

Figure 4. Plasma steroidal profiles of *M. australis* females during maturation cycle (Mean ± Standard Error) ($P <$ 0.05).

stage of spermiation, however, the individuals showed almost exclusively spermatozoa, probably due to the extensive spawning period.

According to the observations in this study, ovarian histology is very similar to that reported in studies with *Merluccius hubbsi* (Cornejo, 1998; Honji *et al.*, 2006), and *M. merluccius* (Recasens *et al.,* 2008), exhibiting the same characteristics in each oocyte stage.

Sex steroids concentrations in *M. australis* were at very low levels compared to studies on *Chalcalburnus tarichi* (Ünal *et al.*, 2005), *Perca fluviatilis* (Migaud *et al.*, 2003), *Coregonus clupeamorfis* (Rinchard *et al.*, 2001) and *Pleuronectes americanus* (Harmin *et al.*, 1995). However, studies on fish with asynchronous gonadal development such as *Gobio gudgeon* and *Verasper variegatus* have also reported low steroid concentrations (Rinchard *et al.*, 1993; Koya *et al.*, 2003).

The low concentration of sex steroids present in *M. australis* could be attributed to the fact that this marine fish is a partial spawner and therefore the levels of circulating sex steroids are diluted as consequence of extensive spawning periods.

The androgen 11-ketotestosterone has been identified as the most important steroid hormone in teleost testes (Borg, 1994). In the present study, fluctuations of serological 11-KT levels were found during testicular maturation. The levels of 11-KT were higher in the stage of spermatogenesis compared to other maturational stages, demonstrating the importance of this hormone in the process of spermatogenesis. The observed levels of 11-KT in *M. australis*, are consistent with findings reported by studies with *Hucho perryi* (Amer *et al.*, 2001); *Clupea pallasii* (Koya *et al.*, 2002); *Verasper variegatus* (Koya *et al.*, 2003) and *Solea*
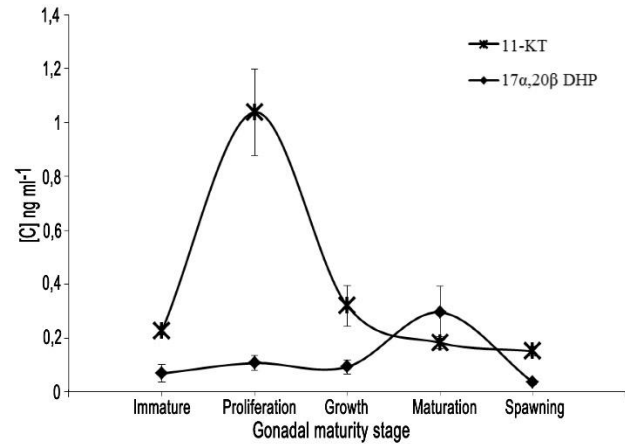
senegalensis (García-López *et al.*, 2006), where 11-KT was the most influential androgen for the spermato-genesis stage.

In female teleosts, the level of the E2 has been reported to increase gradually during cortical alveoli phase, peaking in the vitellogenesis phase and then declining prior to the ovulation phase (Mayer *et al.*, 1990; Schulz *et al.*, 2010). Overall, these previous findings are consistent with the present results, where the levels of E2 were higher in the stage of vitellogenesis compared to other maturational stages, indicating that this sex hormone is essential to induce the process of vitellogenesis in female *M. australis*. Similar results were reported in *Engraulis ringens* (Cisneros, 2007), *Sardinops melanostictus* (Murayama *et al.*, 1994), *Salvelinus leucomaenis* (Kagawa *et al.*, 1981), *Mugil cephalus* (Tamaru *et al.*, 1991), *Acheilognathus rhombea* (Shimizu *et al.*, 1985), and *Oreochromis mossambicus* (Cornish, 1998), where oocytes development was mediated by increasing 17β-estradiol during vitellogenesis stage.

The 17α,20β-DP has been identified as a maturation-inducing steroid (MIS) in several fish species during final oocyte maturation (Yamauchi *et al.*, 1984; Tamaru *et al.*, 1991; Petrino *et al.*, 1993; Murayama *et al.*, 1994), however, this hormone did not show noticeable fluctuation during any maturation stages in *M. australis* females. Similar findings regarding the consistency in the levels of 17α,20β-DP were reported in *Engraulis ringens* (Cisneros, 2007) and *Dicentrarchus labrax* (Prat *et al.*, 1990). Short-range variations of 17α,20β-DP levels during the process of oocyte final maturation and ovulation in *M. australis* females could be explained by a lack of blood samples at the precise moment of increase in this hormone. A variety of experiments have shown that the

increase in this steroid occurs for a short period of time, just prior to ovulation when the germinal vesicle membrane breaks down (Tamaru *et al.*, 1991; Murayama *et al.*, 1994; Mylonas *et al.*, 1997). Furthermore, *in vitro* studies carried out by Migaud *et al.* (2003) showed that 17α,20β-DP is detectable up to two hours after being synthesized, which could also explain the low concentration of this hormone in the analyzed samples. Another explanation for this phenomenon could be that 17α,20β-DP did not act as MIS in *M. australis* females. Studies carried out with *Micropogonias undulatus* (Trant & Thomas, 1989), *Cynoscion nebulosus* (Thomas & Trant, 1989) and *Halobatrachus didactylus* (Modesto & Canário, 2002) have shown that 17,20β,21-trihydroxy-4-pregnen-3-one (17,20β,21P) act as MIS instead of 17α,20β-DP. However, the role of 17,20β,21P as MIS in *M. australis* females is still unclear and further investigations are needed.

In southern hake males, levels of 17α,20β-DP showed a slight fluctuation during gonadal development. Conversely, studies with other fish species have shown an increase in the levels of this steroid during the espermiation stage (Vermeirssen *et al.*, 1998, 2000; Koya *et al.*, 2002). The difference between our findings and those reported in the literature could be due to the low number of individuals sampled, the continuous process of spermatogenesis, or the short duration of 17α,20β-DP in the bloodstream.

In conclusion, this study reported that there were serological fluctuations of E2 and 11-KT during gonadal maturation of *M. australis*, identifying these hormones as the main hormones responsible for vitellogenesis and spermatogenesis respectively. On the other hand, the levels of 17α,20β-DP did not show fluctuations so, apparently, this hormone is no involved in gonadal maturation of this species. Future research should include the entire maturation cycle of wild and captive *M. australis*, in order to evaluate the physiological effect of captivity conditions on broodstock of this species. Similarly, additional studies regarding the quantification of gonadotropins (FSH and LH), 17,20β,21P and vitellogenin are required for a complete understanding of the reproductive physiology of *M. australis*.

ACKNOWLEDGMENTS

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