

# Sex differentiation and early sex identification in hatchery produced greater amberjack (*Seriola dumerili*) reared in sea cages

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## Research Article

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# Abstract

The histological process of gonadal differentiation together with the endocrine changes of glucocorticoids and sex steroids was studied in hatchery produced greater amberjack (*Seriola dumerili*) from 101 until 408 days post-hatching. In the samplings, which were conducted every 50 days, no size dimorphism was observed between the sexes and sex ratio was 1:1, suggesting that there was no influence of early rearing in captivity on sex differentiation. Plasma concentrations of cortisol, cortisone, adrenosterone (Ad), androstenedione ( $\Delta 4$ ), 11-ketotestosterone (11KT), testosterone (T), estradiol ( $E_2$ ), progesterone (P4) and 17,20 $\beta$ -dihydroxyprogesterone (DHP) were measured in males and females with the use of liquid chromatography tandem mass spectrometry (LC-MS/MS) to examine their role in the sex differentiation process. From the nine hormones, the only one that exhibited differences between the sexes was 11-KT. However, variations were observed in the mean values of cortisol, Ad,  $\Delta 4$ , 11-KT, T, P4 and DHP over time in one or both sexes, indicating their involvement in the sex differentiation process. Moreover, the 11-KT/ $E_2$  ratio was found to be a useful diagnostic biomarker for greater amberjack sex, from as early as 150 dph.

## 1. Introduction

Greater amberjack (*Seriola dumerili*) is a cosmopolitan fish species with fast growth and good taste, studied for its aquaculture potential since the late 90s (Crespo et al. 1994; Kawabe et al. 1996; Marino et al. 1995a; Marino et al. 1995b; Micale et al. 1998; Micale et al. 1997; Micale et al. 1999). The interest in aquaculture research for greater amberjack has been rekindled in recent years, and this species has been finally produced commercially in the Mediterranean Sea (Corriero et al. 2021; Fakriadis et al. 2020b; Pérez et al. 2020). It is a gonochoristic fish, in which sex-determining genes have been recognized after gonad transcriptome sequencing (Sarropoulou et al. 2017) and females are thought to be the heterogametic sex (Kawase et al. 2018). Gonadal differentiation follows the direct type, as undifferentiated gonads become directly ovaries or testes (Marino et al. 1995b) and sex differentiation is completed at the end of the first year of life in wild-caught cage-reared individuals (Marino et al. 1995b; Micale et al. 1998).

In fish reared in captivity, knowledge of the sex differentiation process and the resulting sex ratio of fish produced entirely under aquaculture conditions is essential in order to ensure that rearing conditions do not lead to deviations from the natural sex ratios occurring in the wild. Naturally occurring skewed sex ratios may be found in sequential hermaphroditic species, such as the protandric gilthead seabream *Sparus aurata* (Mylonas et al. 2011) or the protogynous dusky grouper *Epinephelus marginatus* (Sarter et al. 2006). However, unbalanced sex ratios may also appear in gonochoristic fishes with temperature-dependent sex determination, if exposed to different than natural temperatures during early life (Guiguen et al. 2010; Ospina-Álvarez and Piferrer 2008). In the case of European seabass *Dicentrarchus labrax*, for example, rearing at  $> 17^\circ\text{C}$  during the first days of life favors the production of very high percentages of males (Koumoundouros et al. 2002; Mylonas et al. 2005; Pavlidis et al. 2000), which is undesirable since males grow 30% less than females (Navarro-Martín et al. 2009; Saillant et al. 2001; Sfakianakis et al. 2013) and mature precociously before they reach marketable size (Papadaki et al. 2005). Furthermore, knowing the timing of the sex differentiation process in aquaculture species, where one of the two sexes is more preferable than the other, is important for the development of monosex populations, since hormonal induction of sex reversal is most effective when applied just prior to and/or during the period of sex differentiation (Blázquez et al. 1998; Budd et al. 2015; Chen et al. 2018; Piferrer 2001).

After the hypothesis that sex steroids can affect gonadal differentiation in fish was first proposed (Yamamoto 1969), hormonal induction of sex change, in vitro steroid excretion by the gonads and ultrastructural observations for the presence of sex steroid producing cells have linked sex steroid hormones to the sex differentiation process (Depeche and Sire 1982; Feist et al. 1990; Nakamura 1984; Nakamura and Nagahama 1993; Rothbard et al. 1987; Vizziano et al. 1995). In fish, 17 $\beta$ -estradiol ( $E_2$ ) is the female-specific estrogen (Yamamoto 1969) and 11-ketotestosterone (11-KT) is the male-specific androgen (Borg 1994). Testosterone (T) is the precursor to both androgens and estrogens, and sex steroid synthesis shifts to the production of progestogens, such as progesterone (P4) and 17,20 $\beta$ -dihydroxyprogesterone (DHP) as gametogenesis progresses (Nagahama 1994), with the role of progestogens in sex differentiation being poorly studied so far. Recently, adrenal corticosteroids have been shown to be also involved in the sex differentiation process, with cortisol having been related to male sex differentiation in a number of species (Fernandino et al. 2013; Goikoetxea et al. 2017; Hattori et al. 2009; Yamaguchi et al. 2010). Description of the process of sex differentiation and correlation with the relevant for other species steroid hormones has not been carried out so far in greater amberjack, neither in wild nor in hatchery produced populations. Such information could be very useful, especially as this fish is currently becoming an important aquaculture species.

In addition to providing information on the role of steroid hormones during sex differentiation, the relative ratios of some of these steroids may be useful in sex identification at an early stage (juvenile), before the age of first maturation (puberty) that in this species is around

3–4 years of age (Marino et al. 1995a). For example, the concentration ratio of 11-KT to E<sub>2</sub> has been used as a sex identification tool for a number of fish species, such as the wreckfish *Polyprion oxygeneios* (Kohn et al. 2013) and the Eurasian perch, *Perca fluviatilis* (Rougeot et al. 2007). Identification of sex in prepubertal fish is important to ensure the required sex ratio when implementing selective breeding programmes. Furthermore, in sequentially hermaphroditic fishes, that change sex between reproductive seasons, knowing the sex of the fish during the reproductively inactive period is of great importance. As a result, readjusting the broodstock sex ratio is necessary, in order to (a) ensure optimal sex ratios for reproductive performance and (b) ensure breeding only between selected males and females. Since sexual dimorphism in external morphological characteristics is rare in fishes, sex identification can be made only during the brief spawning season, either using a gonadal biopsy -catheterization of the ovaries and sperm collection by application of gentle abdominal pressure- or by measuring the levels of sexual steroids (androgens in males and estrogens in females) or the levels of vitellogenin in females. However, during the reproductively quiescent period as well as before puberty, plasma sex steroid hormone levels are low and sex identification can only be achieved by killing the fish and examining the gonads macroscopically or microscopically. Greater amberjack presents an additional feature that complicates sex identification in the species: the musculature surrounding its abdominal cavity is very hard, which means that semen cannot be released easily after applying abdominal pressure (Mylonas et al. 2004). Therefore, developing a method to identify sex in prepubertal or reproductively quiescent greater amberjack can be very useful to the aquaculture industry, and may have applications in other fishes as well.

The aim of the present study was to gain insights on the process of sex differentiation in hatchery produced greater amberjack and use liquid chromatography tandem mass spectrometry (LC-MS/MS) to (a) investigate the sex steroid profiles in the plasma of males and females and (b) examine the possibility of using an androgen/estrogen ratio as a diagnostic biomarker predicting the sex in 0+ age class greater amberjack.

## 2. Materials And Methods

### 2.1 Samplings

Fish used in the present study were produced from eggs obtained in Argosaronikos Fish Farm S.A. (Salamina Island, Greece), after spawning induction of wild-caught breeders with gonadotropin releasing hormone agonist (GnRHa) implants (Fakriadis et al. 2020a). Eggs were transferred to the facilities of the Institute of Marine Biology, Biotechnology and Aquaculture (Hellenic Center for Marine Research, HCMR, Registration No EL91-BIObr-03 and EL91-BIOexp-04) and reared until 50 days post-hatching (dph). Then, fish were moved to the pilot sea cages of HCMR at Souda Bay, Chania, Crete, Greece (GR94FISH0001), where they were maintained until the end of the experiment.

A total of seven samplings were conducted from October until August at intervals of about 50 days, between 101 and 408 dph. During sampling, total length (TL, mm) and wet weight (WW, g) were measured and gonads (n = 17–23) were extracted and fixed in 4% formaldehyde:1% glutaraldehyde (McDowell and Trump 1976) for histological processing. Blood was also collected from all fish at each sampling using a heparinized syringe, it was centrifuged at 6000 rpm and the collected plasma was stored at -80° C until analysis.

### 2.2 Histological analysis

The excised gonads were dehydrated in a 70–95% ethanol series and embedded in glycol methacrylate resin (Technovit 7100, Heraeus Kulzer, Germany). A semi-automatic microtome (Leica RM2245, Germany) was used to obtain serial sections of 3–5 µm using disposable blades. Slides were stained with methylene blue/azure II/basic fuchsin (Bennett et al. 1976), they were examined under a light microscope (50i Eclipse, Nikon, Japan) and photographed using a digital camera (Progres, Jenoptik AG, Germany).

### 2.3 Plasma hormone measurement

#### 2.3.1 Chemicals and reagents

Standards of the nine steroid hormones under investigation, *i.e.* cortisol, cortisone, 11-KT, adrenosterone (Ad), androstenedione (Δ4), E<sub>2</sub>, T, P4, DHP; ≥98% purity) and the internal standard (N,N dimethyl-L-phenylalanine; 99% purity) were purchased from Sigma-Aldrich. Stock solutions of each analyte (125 ng µL<sup>-1</sup>), the working solution of internal standard (2 ng µL<sup>-1</sup>), as well as the calibration standard mixtures of hormones (0.2 to 5000 pg µL<sup>-1</sup>) were prepared in methanol and stored at -20°C until use. All solvents, including methanol, acetonitrile and water, were of HPLC-grade (Chromasolv for HPLC; ≥99.9%), while formic acid was of LC-MS grade (LiChropur for LC-MS; 98–100%) and they were all purchased from Sigma-Aldrich.

#### 2.3.2 Sample extraction and cleanup

Custom-made Solid Phase Extraction (SPE) cartridges were prepared by dry-packing 10 mg of polymer-based C18 sorbent (Strata-X 33 $\mu$ m polymeric reversed phase, Phenomenex) into 1-mL polypropylene pipette tips, the lower end of which were stoppered with a small piece of wool. Packed cartridges were mounted on a vacuum manifold (VM12 12-port vacuum SPE manifold, Phenomenex) and conditioned with 500  $\mu$ L of methanol and 500  $\mu$ L of water. Subsequently, a 200- $\mu$ L aliquot of each plasma sample was diluted 1:1 with water and loaded onto a SPE cartridge. After a two-step washing procedure with 500  $\mu$ L of water and 350  $\mu$ L of methanol 40% v/v, the hormones were selectively eluted using 450  $\mu$ L of pure methanol and collected in amber glass vials. The flow rate during SPE procedure was adjusted to 0.5 drop/sec. The eluates were spiked with 20  $\mu$ L of internal standard solution (2 ng  $\mu$ L<sup>-1</sup>), evaporated to dryness using a Centrivac VR-1 vacuum concentrator (Heraeus, Germany) and finally redissolved in 200  $\mu$ L of methanol.

### 2.3.3 LC-MS/MS analysis

All analyses of hormones were carried out using an Agilent 1260 Infinity binary pump HPLC system coupled to an Agilent 6460C triple quadrupole mass spectrometer equipped with an Agilent Jet Stream Electrospray source (Agilent Technologies). The chromatographic separation of analytes was achieved on a Poroshell 120 column fitted with a guard column (EC-C18, 150 mm x 3 mm, 2.7  $\mu$ m particles; Agilent Technologies) by applying the following binary gradient of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile): from 10–100%B in 20 min, hold at 100%B for 5 min and then back to 10%B in 2 min with a hold of 2 min. The column temperature was set at 35°C and the flow rate was 0.5 mL min<sup>-1</sup>.

The operating parameters of the electrospray ionization source were optimized for hormones analysis and the optimal conditions were as follows: drying gas temperature 150°C; drying gas flow rate 8 L min<sup>-1</sup>; sheath gas temperature 380°C; sheath gas flow rate 12 L min<sup>-1</sup>; nebulizer pressure 25 psi; capillary voltage 4500 V; nozzle voltage 2000 V. The triple quadrupole was operated in the positive ion scan mode using dynamic multiple reaction monitoring (d-MRM) for enhanced selectivity and specificity and the retention time window (Delta RT) for the detection of analytes was set at 2 min. Two MRM transitions (one quantitative and one confirmatory) were acquired for each hormone, the d-MRM parameters of which were optimized and presented in Table 1. Processing of LC-MS/MS data and quantitation of hormones was performed with MassHunter Quantitative Analysis software version B.07.01 (Agilent technologies).

Table 1  
Optimized MRM parameters of the quantifier and qualifier transitions that were used for the LC-MS/MS analysis of hormones.

Compound	Retention Time (min)	Precursor ion m/z	Fragmentor	Quantifier			Qualifier		
				Product ion m/z	Collision Energy	Cell Accelerator Voltage	Product ion m/z	Collision Energy	Cell Accelerator Voltage
Cortisol	8.465	363.0	120	121.0	20	3	326.9	12	4
Cortisone	8.59	361.0	140	163.0	21	4	121.0	36	4
11-Ketotestosterone	9.255	303.0	110	121.0	22	3	259.0	19	4
Adrenosterone	10.278	301.0	105	121.0	23	4	257.0	20	3
17 $\beta$ -Estradiol	11.176	255.0	120	159.0	15	2	133.0	15	2
Testosterone	11.517	289.0	115	97.0	21	4	109.0	22	4
17 $\alpha$ ,20 $\beta$ -Dihydroxy-4-pregnen-3-one	11.724	333.0	110	97.0	26	4	109.0	30	4
4-Androstene-3,17-dione	12.415	287.0	110	97.0	21	4	109.0	22	4
Progesterone	15.037	315.0	120	97.0	20	2	109.0	22	2
N,N Dimethyl-L-phenylalanine (I.S.)	3.132	194.0	70	147.9	13	4	133.0	31	4

### 2.4 Statistical analysis

Differences in mean TL, WW and sex steroid hormone concentrations over time were analyzed by one-way Analysis of Variance (ANOVA) for each sex, followed by Tukey HSD test at a minimum significance of P < 0.05, and differences in TL, WW and 11-KT/E2 ratio between

sexes at each sampling were analyzed by one-way ANOVA, followed by Tukey HSD test at a minimum significance of  $P < 0.05$ . To test if the sex ratios (number of males: number of females) at each sampling were different from 1:1, a chi-square test of independence was used, with  $\alpha = 0.05$  as criterion for significance. Unless otherwise mentioned, results are presented as means  $\pm$  S.E.M. Statistical analyses were performed using JMP (SAS Institute Inc., Cary, NC).

## 3. Results

### 3.1 Fish growth

The growth of greater amberjack was similar for both sexes and females had the same size as males at all samplings (one-way ANOVA, Tukey's HSD,  $P < 0.05$ , Figs. 1b and c). Both TL and WW exhibited stable values from the second until the fifth sampling (from November until mid-April), a fact that can be attributed to the low temperatures in the cages during this period (Fig. 1a). When the temperature started to rise, the TL and WW values also started to rise, reaching  $41.22 \pm 3.83$  cm and  $41.7 \pm 1.99$  cm TL and  $809.56 \pm 193.82$  g and  $827.3 \pm 104.22$  g WW in females and males, respectively, at the last sampling in August (Figs. 1b and c).

### 3.2 Sex differentiation

At 101 dph (Fig. 2a,  $14.98 \pm 6.20$  cm TL), in female gonads the ovarian cavity was already formed, and germ cells were visible around the cavity in histological sections. A more developed ovarian cavity with proliferating germ cells was found at 150 dph (Fig. 2b,  $25.5 \pm 1.29$  cm TL), while at 198 dph (Fig. 2c, insert,  $25.8 \pm 0.14$  cm TL) the first primary oocytes were visible. The typical ovarian structure with ovarian lamellae and occasional presence of primary oocytes was apparent at 260 dph (Fig. 2d,  $27.75 \pm 1.89$  cm TL). At 305 dph (Fig. 2e,  $28.41 \pm 1.29$  cm TL) and at 357 dph (Fig. 2f,  $34.86 \pm 2.15$  cm TL) the number of primary oocytes kept increasing, to reach complete ovary differentiation at 408 dph (Fig. 2g,  $41.22 \pm 3.83$  cm TL), when the ovarian lamellae were filled with primary oocytes.

In males, at 101 dph the gonads contained mostly somatic cells and connective tissue and no germ cells were present (Fig. 3a,  $14.47 \pm 6.60$  cm TL). The first germ cells were apparent at 150 dph, when spermatocytes could be found in the gonads (Fig. 3b, insert,  $24.71 \pm 3.09$  cm TL). The number of proliferating and differentiating germ cells increased at 198 and 260 dph (Figs. 3c and d,  $28 \pm 2.39$  cm and  $29.75 \pm 2.82$  cm TL, respectively) and the typical testicular structure featuring all types of male germ cells, was observed at 260 dph (Fig. 3e,  $28.63 \pm 2.85$  cm TL). This structure was obviously maintained in the following samplings (357 and 408 dph, Figs. 3f and g,  $34.25 \pm 2.98$  cm and  $41.7 \pm 1.99$  cm TL, respectively).

As already stated, male and female gonads were found from the first sampling at 101 dph and their relative percentages increased with time (Fig. 4). On the other hand, the percentage of undifferentiated gonads was high at the first three samplings, until 198 dph, and decreased thereafter, with the lowest number of undifferentiated gonads found in the last sampling, at 408 dph (Fig. 4).

### 3.3 Hormonal profile of males and females during sex differentiation and 11-KT/E2 ratio

Of the nine measured hormones in greater amberjack females and males during the sex differentiation process, the ones that exhibited statistically significant changes in time were cortisol, Ad,  $\Delta 4$ , 11-KT, T and P4 in females and Ad, 11-KT, T, P4 and DHP in males (Fig. 5). Cortisol and Ad exhibited lower values as the sex differentiation period was progressing in females; on the other hand, Ad, 11-KT, T and P4 in both sexes and DHP in males presented higher values at the end of the sex differentiation period (Fig. 5).

The 11-KT concentration was significantly different between the two sexes at all samplings, except at 198 dph. The 11-KT/E<sub>2</sub> ratio was significantly different between the sexes from the second sampling at 150 dph until the last sampling at 408 dph (Fig. 6).

## 4. Discussion

In the present study, no sexual growth dimorphism was observed, as greater amberjack females were the same size as males in all the samplings conducted. Moreover, the sex ratio was always found to be around 1:1, in accordance with the sex differentiation pattern of the species, which is gonochoristic. In a study on wild fish in the South-Eastern Adriatic Sea, the sex ratio was also around 1:1 (Kozul et al. 2001), whereas in the Gulf of Mexico the sex-related size differences that were encountered were attributed to age, as females were found to live longer than males (Thompson et al. 1999). The observed absence of sex differences in growth means that both sexes may be equally preferable in aquaculture, whereas the balanced sex ratio demonstrates that the larval and nursery rearing in hatchery conditions did not affect the sex differentiation process, as it has been shown to do in European seabass (Koumoundouros et al. 2002; Mylonas et al. 2005; Pavlidis et al. 2000).

Growth of greater amberjack during on-growing in sea cages was found to be closely related to temperature in the present study, being high until October, stable during the winter and spring months (from November until mid-April) and high again in the summer months (from June-August), when temperature started to rise. In a study on greater amberjack caught from the wild in September and grown in sea cages in the Balearic Islands, the growth results were similar, with the growth rate decreasing in the winter months and rising again in spring. The final weight of these fish at one year of age reached around 1000–1200 g in June (Pastor et al. 2000). In another study on wild-caught greater amberjack reared under natural seawater temperatures, feed intake was also found to decrease at temperatures lower than 12°C (Skaramuca et al. 2001). In accordance to the previous studies, better performance, in terms of growth and feeding parameters, was found in tank-reared greater amberjack at 26°C, with respect to 17 and 22°C in a study conducted in Spain (Fernández-Montero et al. 2017). In the HCMR cage facilities, feeding is reduced at temperatures < 16°C, and the fish return to feeding normally at temperatures > 19°C (personal observations). Temperature effects on growth have been also shown in the congeneric fish, yellowtail kingfish *Seriola lalandi* (Abbink et al. 2012; Bowyer et al. 2014).

Sex differentiation was completed at the end of the first year of age in the present study, although undifferentiated individuals were encountered throughout the whole sex differentiation period. Macroscopical identification of the gonads was possible at 357 dph. In a study on wild-caught individuals reared in sea cages, sex differentiation was also found to be completed at the end of the first year, with simultaneous cytological and anatomical gonadal differentiation (Marino et al. 1995b). In another study on tank-reared wild-caught fish, on the contrary, sex differentiation was considered completed both histologically and macroscopically much later, at the 21st month of age, and undifferentiated gonads were found until the 17th month (Micale et al. 1998). In the latter study, anatomical differentiation preceded the cytological one (Micale et al. 1998), in accordance with the present study, where ovarian cavity formation was the first sex-specific gonadal feature.

Sex steroid hormones have been linked to the sex differentiation procedure and different studies have correlated their concentrations with sex differentiation. Testosterone, 11-KT and  $\Delta 4$ , but not  $E_2$  or DHP concentrations were found to be different between sexes in the coho salmon *Oncorhynchus kisutch* (Feist et al. 1990), whereas between T, 11-KT and  $E_2$ , only T levels were linked to sex differentiation in tilapia (Rothbard et al. 1987). Testosterone and aromatase plasma levels were sexually dimorphic in grey mullet *Mugil cephalus*, whereas  $E_2$  and 11-KT exhibited similar values between the sexes (Chang et al. 1999). In the present study, only 11-KT exhibited different concentrations between the sexes, suggesting that it is the main male-specific hormone in this species. Nevertheless, different hormones exhibited statistically significant differences in time and can be considered related to the sex differentiation procedure. More specifically, cortisol and Ad decreased and  $\Delta 4$ , 11-KT, T and P4 increased in females and  $\Delta 4$ , 11-KT, T, P4 and DHP increased in males during the sex differentiation process.

Cortisol has been shown to play an important role in sex differentiation, as it can induce masculinization in species with temperature-dependent sex determination (Fernandino et al. 2013), such as the pejerrey *Odontesthes bonariensis* (Hattori et al. 2009) and partial masculinization in protogynous species, such as the black seabass *Centropristis striata* (Miller et al. 2019). In the present study, cortisol levels decreased as sex differentiation progressed in females and remained unchanged in males. Cortisone, an active metabolite of cortisol, on the other hand, was unchanged in both sexes. The role of glucocorticoids in sex differentiation of gonochoristic species has not been elucidated yet, however the fact that the plasma cortisol concentrations fluctuate in females during the sex differentiation period suggests a role for this hormone in the process.

Progesterone is a progestin mostly associated with female sex differentiation (Van den Hurk et al. 1982). However, treatment of juvenile zebrafish *Danio rerio* for 40 days with natural (P4) and synthetic (norgestrel) progestins induced sex ratio shifts towards females and males, respectively (Liang et al. 2015), suggesting that progestins can play a significant role in gonadal differentiation of both sexes. Moreover, in the present study P4 and androgen levels rose simultaneously in females and males during the sex differentiation period, in full agreement with the results of another study, on the in vitro P4 metabolism in cultured testicular fragments of the rainbow trout *Oncorhynchus mykiss*, where it was shown that in early testicular maturation, when only spermatogonia are present in the tissue,  $\Delta 4$ , Ad, T, and DHP are produced from P4 (Depeche and Sire 1982).

Another progestin, DHP was shown in the present study to be linked to the sex differentiation procedure of male greater amberjack. Known to be involved mainly in the female oocyte maturation stages (Nagahama and Yamashita 2008), DHP was found in the zebrafish to be linked to male sex differentiation and steroidogenesis (Chen et al. 2010). Moreover, it was found to be connected with cortisol and 11-KT production during early spermatogenesis in testicular cultures of the Japanese eel *Anquilla japonica* (Ozaki et al. 2006), whereas in the rainbow trout, it was detected at very early testicular maturation stages as well (Vizziano et al. 1995), suggesting a role for this hormone in male sex differentiation and early gametogenesis.

In the present study, E<sub>2</sub> was both similar between the sexes and unchanged in time, whereas 11-KT showed different levels and rose in time in both sexes. It has been suggested that only estrogens are essential for fish female differentiation, whereas male differentiation results from down-regulation of female differentiating genes and hormones (Kobayashi et al. 2013; Li et al. 2019). However, the simultaneous androgens rise in both sexes of the present study pinpoints to an important role of androgens in both sexes' gonadal differentiation in greater amberjack. In the rainbow trout, it was shown that the enzyme 11 $\beta$ -hydroxylase was essential for male sex differentiation (Liu et al. 2000), stressing the role of 11-KT in the process. Moreover, using Cytochrome P450 17 A (*cyp17a1*) knockout zebrafish, it was shown that androgens are essential for male brain sex differentiation (Shu et al. 2020). In greater amberjack, 11-KT has been used as a sex-identifying hormone, as its plasma values are a lot higher in males (Aoki et al. 2019). The role of 11-KT in female sex differentiation remains unclear; however, recent studies in different species have revealed a role for this hormone in oocyte size increase and lipid accumulation (Akhavan et al. 2019; Lokman et al. 2007; Wang et al. 2020).

Sex identification in fish is a rather complicated process, as fish do not possess sex-specific external characteristics; identifying fish sex, however, is very useful for aquaculture purposes and different methods for sex recognition have been suggested, with the 11-KT/E<sub>2</sub> ratio being the most common (Baroiller et al. 1999). In the greater amberjack, observation of external urogenital pore characteristics (Smith et al. 2014) and 11-KT concentration (Aoki et al. 2019) have been suggested as non-invasive methods for sex identification. However, the first method is more applicable in fish larger than 50 cm fork length (FL), whereas the latter was conducted in fish older than 412 dph and larger than 39 cm FL. In the present study, it was shown that the 11-KT/E<sub>2</sub> ratio was significantly different between the sexes already at 150 dph, suggesting that only by collecting blood and measuring 11-KT and E<sub>2</sub> levels through LC-MS/MS sex can be identified in this species. Although differences between the sexes in the absolute values of 11-KT levels could also be used for sex identification in the greater amberjack as already suggested (Aoki 2019), using the 11-KT/E<sub>2</sub> ratio is a safer option, as it is commonly used in fish and leads to more independent estimations than absolute values. The advantage of using LC-MS/MS is that simultaneous measurement of a number of hormones is achieved in small amounts of plasma. In small fish, large blood volumes are difficult to collect and, at the same time, their plasma sex steroid levels are quite low, rendering the measurement of more than two hormones very difficult with the use of ELISA. The use of LC-MS/MS for sex steroid hormone measurements in fish plasma has been implemented recently in toxicological and endocrinological studies (Budzinski et al. 2006; Nouri et al. 2020).

In conclusion, it was shown in the present study that hatchery produced greater amberjack shows no sexual size dimorphism and the sex ratio in the cultured population was always 1:1, underlining that the early life rearing method did not have any influence on the process of sex differentiation. Sex identification could be conducted in juvenile fish from 150 dph, with the use of the 11-KT/E<sub>2</sub> ratio. Moreover, using LCMS/MS, with just 200  $\mu$ L of plasma, a large number of steroid hormones of the cholesterol metabolism pathway could be detected, enabling the study of the biochemical pathway involved in the teleost sex differentiation process. More studies are needed in order to decipher the exact role of each separate hormone in sex differentiation, both of greater amberjack and of other species.

## 5. Author Declarations

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**Conflicts of interest/Competing interests:** The authors declare no conflict of interest.

**Availability of data and material:** The original data of the study are available on request.

**Code availability:** Not applicable

**Authors' Contributions:** MPapadaki and CCMylonas designed the experiment. The fish husbandry was carried out by MA and sample collection were performed by MPapadaki, MA, NP and PK. Hormonal analyses were carried out by MM and TIA. Histological evaluations were carried out by MPapadaki and MPouli. Data analysis was performed by MPapadaki, TIA and MM. The manuscript was written by MPapadaki, MM and CCM.

**Ethics approval:** Ethical approval for the study was obtained by the relevant Greek authorities (National Veterinary Services) under the license No 255332 (ΑΔΑ: ΩΨ2Κ7ΛΚ-Η7Ξ). All procedures involving animals were conducted in accordance to the "Guidelines for the treatment of animals in behavioral research and teaching" (Anonymous 1998), the Ethical justification for the use and treatment of fishes in research: an update (Metcalf and Craig 2011) and the "Directive 2010/63/EU of the European parliament and the council of 22 September 2010 on the protection of animals used for scientific purposes" (EU 2010).

**Consent to participate:** All authors have agreed to participate in the manuscript.

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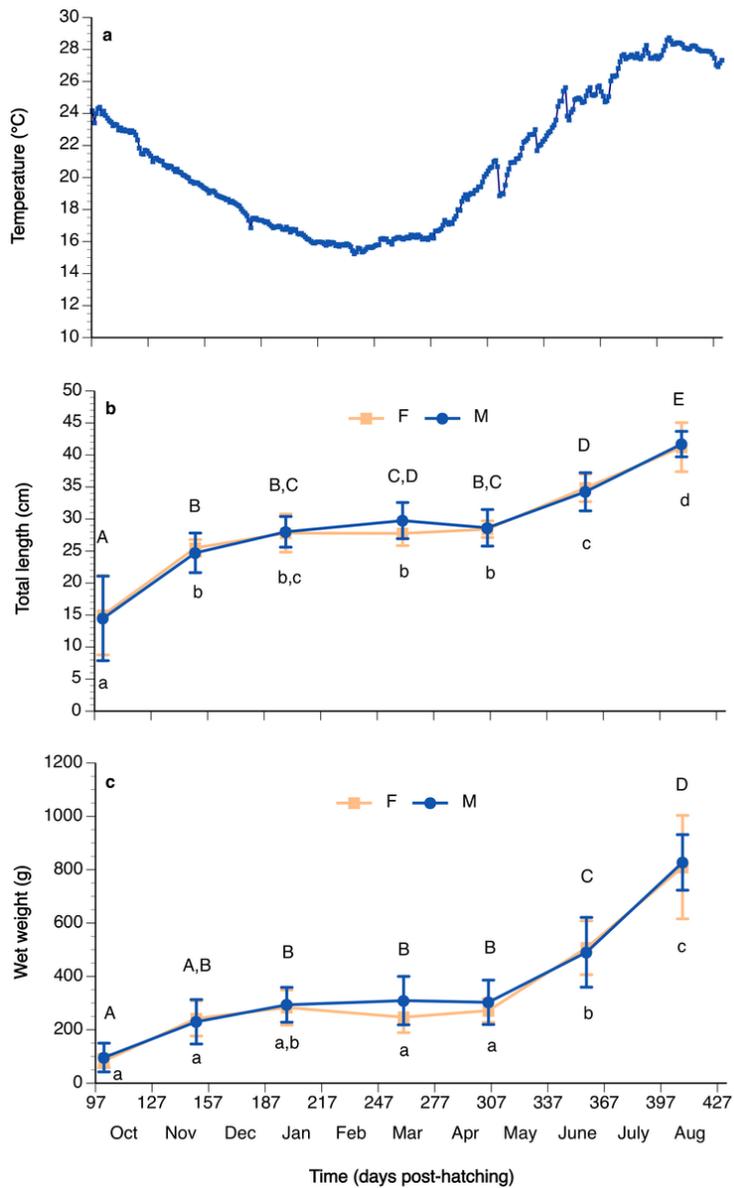
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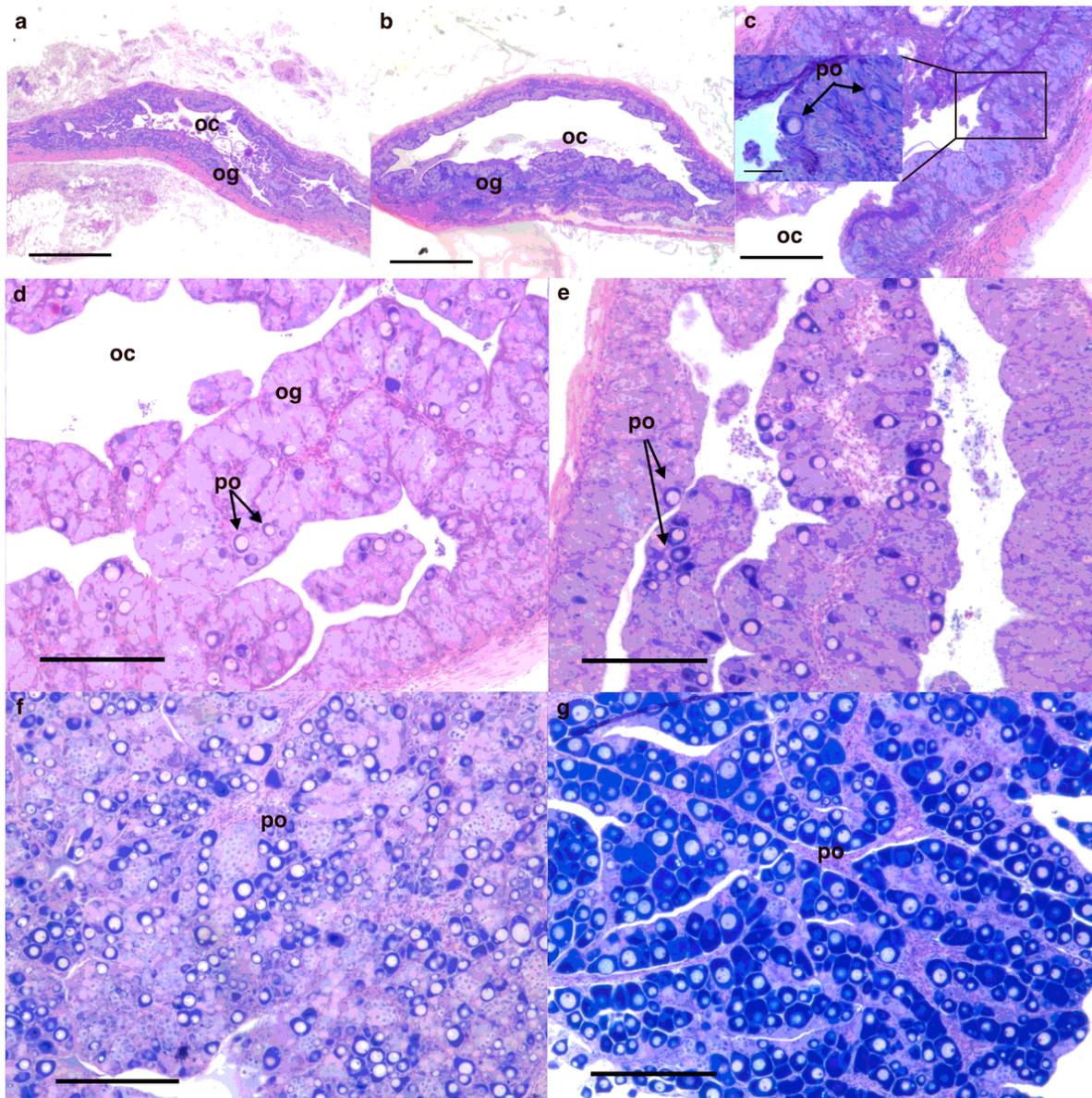
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## Figures



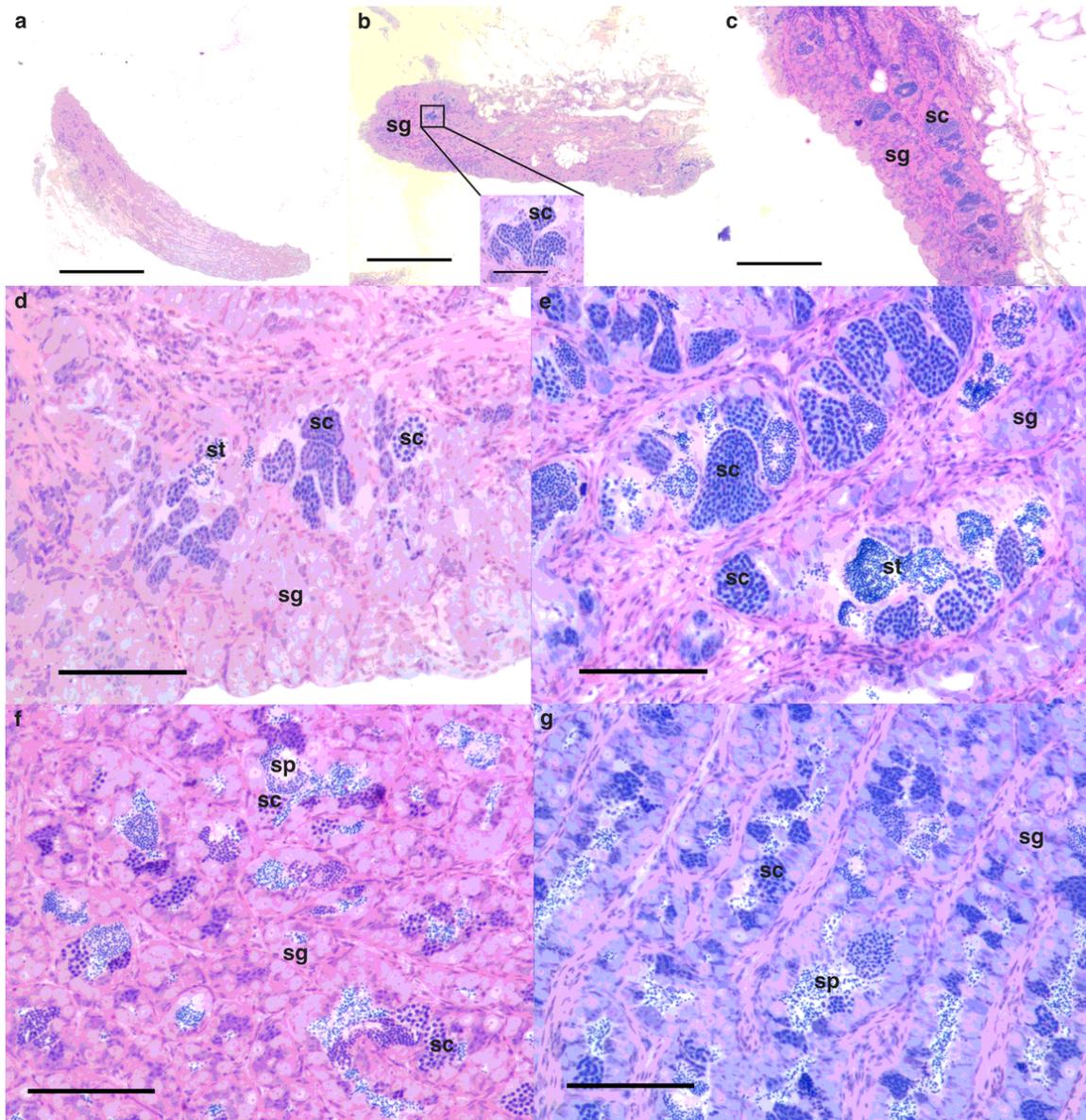
**Figure 1**

a. Water temperature (°C) in the sea cages where the hatchery produced greater amberjack were kept, from 101 until 408 days post-hatching. b. Mean ( $\pm$  S.E.M) growth in total length (cm) of greater amberjack from 101 until 408 days post-hatching, in females and males. c. Mean ( $\pm$  S.E.M) growth in wet weight (g) of greater amberjack from 101 until 408 days post-hatching, in females and males. Different capital letter superscripts above the total length and the body weight values indicate statistically significant differences in the growth of males in time (one-way ANOVA, Tukey HSD,  $P < 0.05$ ), whereas different letter superscripts below the body weight and the total length means indicate significant differences in the growth of females in time (one-way ANOVA, Tukey HSD,  $P < 0.05$ ). There were no significant differences in total length and body weight between the sexes during the monitoring period (one-way ANOVA, Tukey HSD,  $P < 0.05$ )



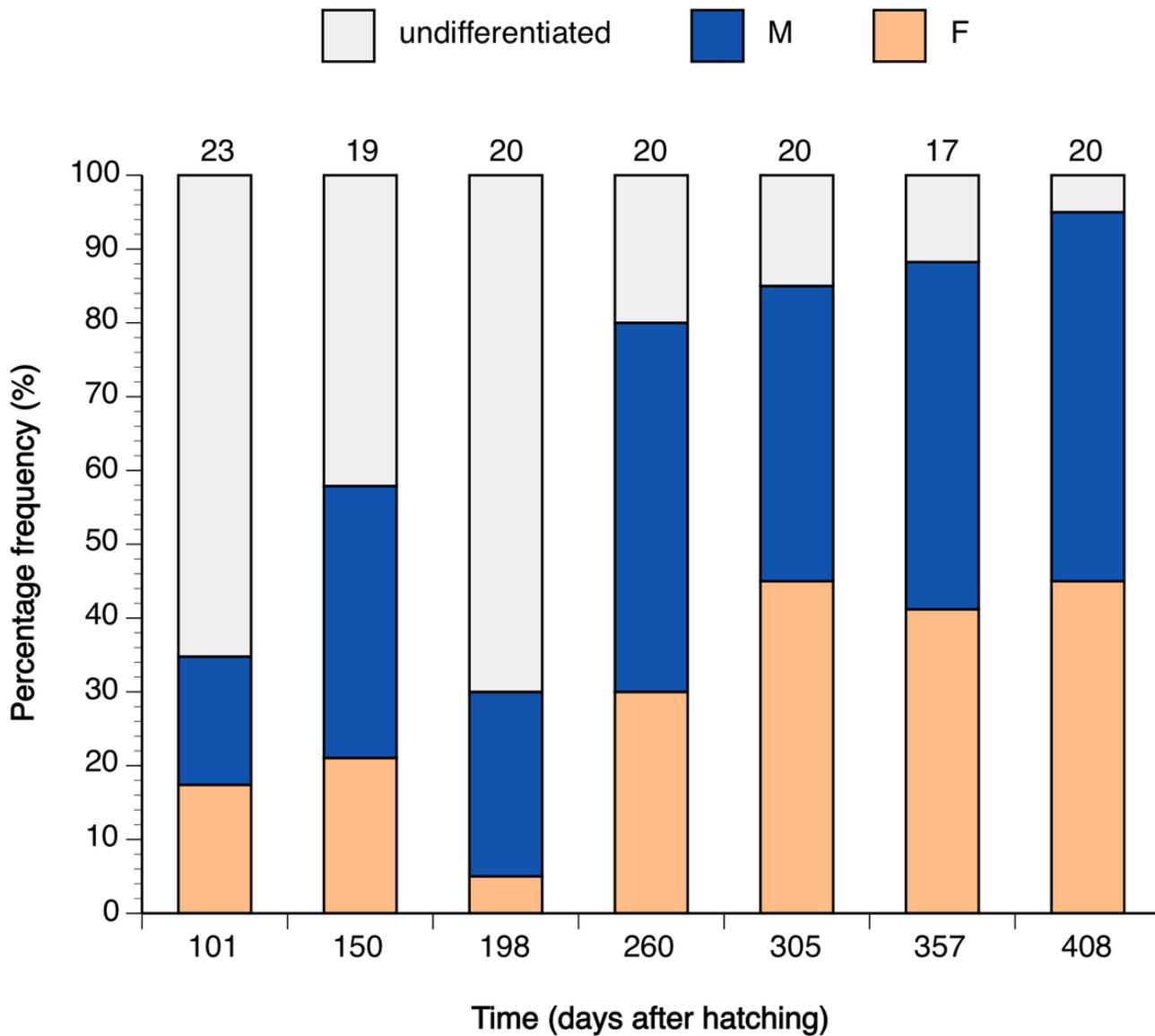
**Figure 2**

Histological sections of ovaries from hatchery produced greater amberjack during the process of sex differentiation. a. Ovary at 101 dph, showing the newly formed ovarian cavity (oc) and the first visible oogonia (og) around the oc. b. Ovary at 150 dph, with a more developed oc, filled with og. c. Ovary at 198 dph, showing isolated primary oocytes (po, insert) among og in the ovarian lamellae. d. Ovary at 260 dph, with the ovarian lamellae mostly filled with og, but also showing po. e. Ovary at 305 dph, with po increasing in number. f. Ovary at 357 dph, when the sex of the sampled fish was first recognized macroscopically, with an increasing number of po. g. Fully differentiated ovary at 408 dph, now filled with po. The scale bars indicate 50  $\mu\text{m}$  (insert), 200  $\mu\text{m}$  (c, d, e, f and g) and 500  $\mu\text{m}$  (a and b)



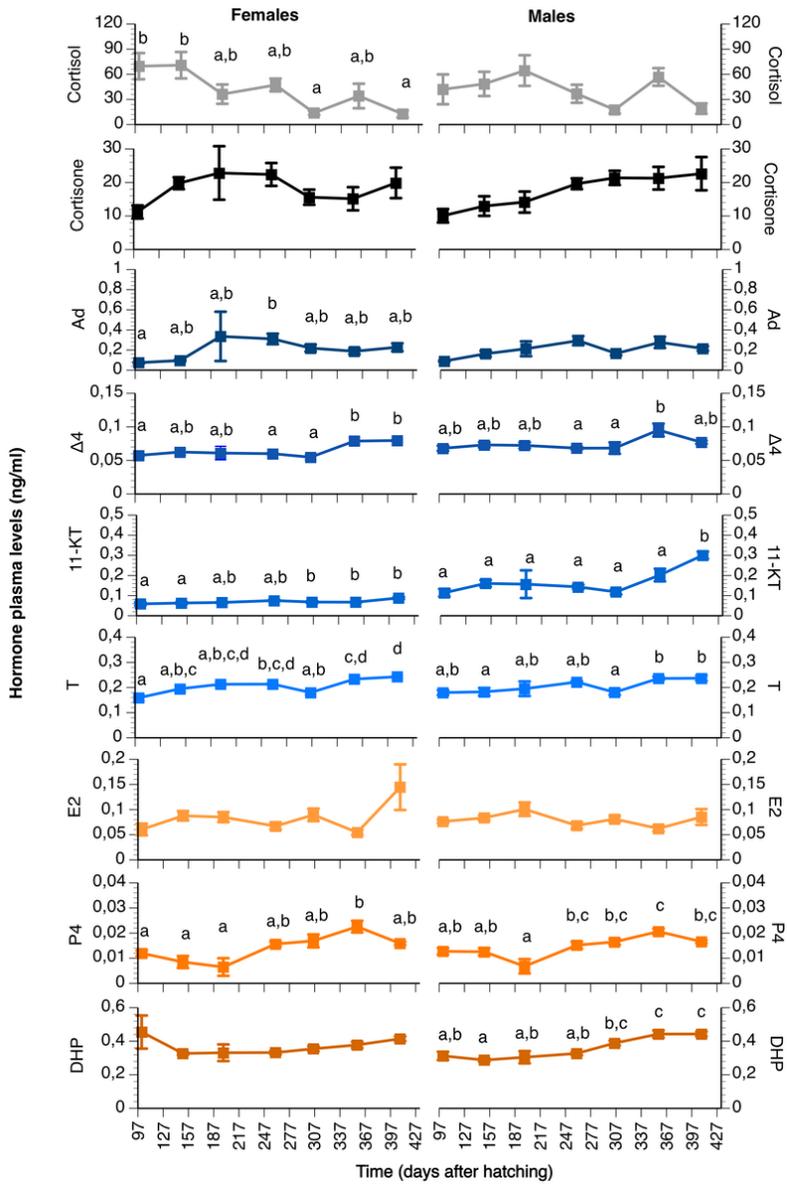
**Figure 3**

Histological sections of testes from hatchery produced greater amberjack during the process of sex differentiation. a. Undifferentiated testis at 101 dph. b. Testis at 150 dph, showing the first identifiable male germ cells, spermatogonia (sg) and isolated spermatocytes (sc, insert). c. Testis at 198 dph, showing a more organized structure, with sg and sc. d. Testis at 260 dph, with the periphery of the tissue filled with sg, but also showing more advanced germ cell types, such as sc and spermatids (st). e. Testis at 305 dph, with fully organized testicular lobules, filled with different germ cell types. f. Testis at 357 dph, when the sex of the sampled fish was first recognized macroscopically and showing even sperm cells (sp) occasionally. g. Fully differentiated testis at 408 dph, showing testicular lobules filled with all the different germ cell stages, from sg to sp. The scale bars indicate 50  $\mu\text{m}$  (insert), 100  $\mu\text{m}$  (d, e, f and g), 200  $\mu\text{m}$  (c) and 500  $\mu\text{m}$  (a and b)



**Figure 4**

The occurrence of hatchery produced greater amberjack with undifferentiated, male and female gonads after histological evaluation, in relation to time. The sample size for each group is shown above the bar. There was no difference from the 1:1 sex ratio in any of the samplings conducted (chi-square test,  $P < 0.05$ )



**Figure 5**

Hormone plasma levels of cortisol, cortisone, adrenosterone (Ad), androstenedione ( $\Delta 4$ ), 11-ketotestosterone (11-KT), testosterone (T), estradiol (E2), progesterone (P4) and 17 $\alpha$ ,20 $\beta$ -dihydroxyprogesterone (DHP) in hatchery produced greater amberjack in relation to time. Different letter superscripts indicate differences between steroid hormones in time (one-way ANOVA, Tukey HSD,  $P < 0.05$ ). Between the sexes, only 11-KT exhibited differences (one-way ANOVA, Tukey HSD,  $P < 0.05$ )

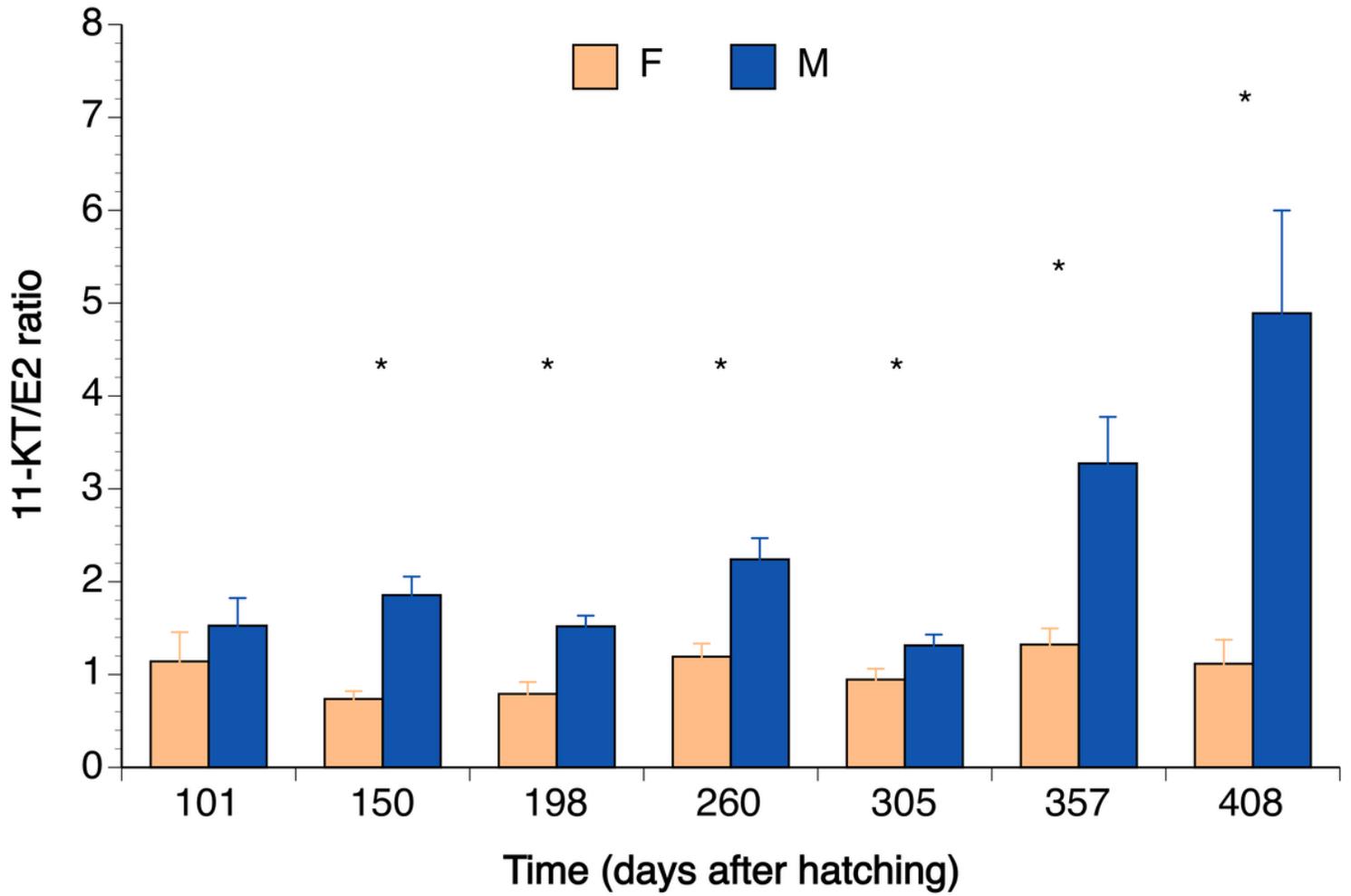


Figure 6

Evolution of the 11-ketotestosterone (11-KT)/estradiol (E2) ratio in hatchery produced greater amberjack in relation to time. Differences in the ratio between males and females are indicated with an asterisk (one-way ANOVA, Tukey HSD,  $P < 0.05$ )