Histological evaluation of sex differentiation and early sex identification in hatchery produced greater amberjack (*Seriola dumerili*) reared in sea cages

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Abstract

The histological process of gonadal differentiation, together with the endocrine changes of sex steroid hormones and some of their precursors was studied in hatchery-produced greater amberjack (*Seriola dumerili*) from 101 until 408 days post-hatching (dph), with samplings conducted every 50 days. Histological processing showed that sex differentiation began at 101 dph with the formation of the ovarian cavity in females, while the presumptive males did not yet contain any germ cells in their gonad. At 150 dph we observed the first germ cells in the developing testes. Sex differentiation in almost all sampled individuals was complete at 408 dph. No size dimorphism was observed between the sexes, and the sex ratio was 1:1, suggesting that there was no influence of early rearing in captivity on sex differentiation. Plasma concentrations of adrenosterone (Ad), androstenedione (Δ4), 11-ketotestosterone (11KT), testosterone (T), estradiol (E_2), progesterone (P4) and 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) 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 seven hormones, the only one that exhibited differences between the sexes was 11-KT and the plasma 11-KT concentration was found to be a useful indication of greater amberjack sex. Variations were observed in the mean values of Ad, Δ4, 11-KT, T, P4 and 17,20βP over time in one or both sexes, indicating their involvement in the sex differentiation process.

Keywords: greater amberjack, *Seriola dumerili*, sex differentiation, LC-MS/MS, adrenosterone, androstenedione, 11-ketotestosterone, testosterone, estradiol, progesterone, 17,20β-dihydroxy-4-pregnen-3-one.

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°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 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, 17β-estradiol (E_2) is the female-specific estrogen (Yamamoto 1969) and 11-ketotestosterone (11-KT) is the male-specific androgen (Borg 1994), with its role in females having been recently recognized (Akhavan et al. 2019) . 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 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) as gametogenesis progresses (Nagahama 1994). The involvement of progestogens in the sex differentiation process has been shown recently (Jiang et al. 2019; Xia et al. 2019). Moreover, the study of gene expression (Banh et al. 2017; Bertho et al. 2018; Gonzalez et al. 2015) and of epigenetic mechanisms involved in fish sex differentiation (Anastasiadi et al. 2018; Piferrer et al. 2019) have shed more light in the mechanisms controlling the process. 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 concentrations or 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_2 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, knowing the sex of the fish during the reproductively inactive period is of great importance in sequentially hermaphroditic fishes, that change sex between reproductive seasons. In these fishes, 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 a plasma hormone concentration or an androgen/estrogen ratio for sex prediction 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 hatched larvae (two days after spawning) were reared until 50 days post-hatching (dph). Then, fingerling fish were moved to the pilot sea cages of HCMR at Souda Bay, Chania, Crete, Greece (GR94FISH0001), where they were maintained in grow out cages (6x6 m) throughout the experiment.

A total of seven samplings were conducted from October until August at intervals of about 50 days, between 101 and 408 dph (**Fig. 1**). At each sampling, a small number of fish (approximately 200) from the rearing cage was randomly culled with the use of a seine leading to a tarpaulin sack (20 m length x 8 m depth). Specifically, one side of the sack was tied to one side of the cage, while the other part was thrown in the water and pulled in order

to create a cavity where the fish were restricted. Once this was accomplished fish (n=17-23 individuals per sampling) were immediately collected, transferred to another tank where they were anaesthetized with phenoxy-ethanol and blood was collected into heparinized syringes. Blood samples were kept on ice until transferred to the lab, where they were centrifuged at 6000 rpm and the collected plasma was stored individually at -80°C until analysis. After bleeding, the fish were sacrificed in an overdose of anesthetic and total length (TL, mm) and wet weight (WW, g) were measured. The gonads were then extracted and fixed in 4% formaldehyde:1% glutaraldehyde (McDowell and Trump 1976) for histological processing.

2.2 Histological analysis

The excised gonads of all the sampled individuals (n=17-23 per sampling, total number of samples 139) 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 seven steroid hormones under investigation, *i.e.* 11-KT, adrenosterone (Ad), androstenedione (Δ4), E_2, T, P4, 17,20βP; ≥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^{-1}), the working solution of internal standard (2 ng μL^{-1}), as well as the calibration standard mixtures of hormones (0.2 to 5000 pg μL^{-1}) 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 33μm 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 μL of methanol and 500 μL of water. Subsequently, a 200-μL 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 μL of water and 350 μL of methanol 40% v/v, the hormones were selectively eluted using 450 μL 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 μL of internal standard solution (2 ng μL^{-1}), evaporated to dryness using a Centrivac VR-1 vacuum concentrator (Heraeus, Germany) and finally redissolved in 200 μL of methanol.

2.3.3 LC-MS/MS analysis

All hormonal analyses (n=17-23 individuals per sampling, total number of samples 139) 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 μm 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% to 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^{-1}.

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^{-1}; sheath gas temperature 380°C; sheath gas flow rate 12 L min^{-1}; 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, and their d-MRM parameters were optimized (**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).

2.3.4. Matrix effects and recovery evaluation

The performance of the final method was investigated by assessing recovery and matrix effect. The recovery (%RE) of each hormone was assessed at two concentration levels (25, 150 pg μL^{-1}) in triplicate by calculating the ratio of peak areas (i.e. LC-MS/MS response) of each analyte spiked in a blank serum sample before (pre-spiked) and after SPE procedure (post-spiked). Matrix effect (%ME) was also assessed at the same concentration levels by measuring the peak area of each analyte in a post-spiked blank serum sample against the respective peak area of hormone standard solutions prepared in pure solvent (i.e. methanol).

In the case of %ME calculation the peak areas of hormones were normalized against the peak area of the internal standard.

The analytical procedure provided acceptable recoveries in the 70–100% range for all hormones. Acceptable levels of matrix effect were also observed for the different hormones. For the majority of hormones %ME ranged from 92% to 107% (**Table 2**).

2.4 Statistical analysis

Although we examined the gonads and measured the hormone levels in all sampled individuals (total sample number 139), we are only reporting the means of the fish that were clearly identified as males or females (n=93, supplemental table). Undifferentiated individuals were excluded from the 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/E_2 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 for samplings 260, 305, 357 and 408 dph, when the number of the individuals belonging to each sex was greater than 5, with $\alpha=0.05$ as criterion for significance.

In order to check for a sex prediction model, logistic regression was applied, using all the sexed samples of the study (n=93). Two different models for sex prediction were estimated, using 11-KT and 11-KT/E_2 ratio as predictor variables.

Unless otherwise mentioned, results are presented as means ± 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 ± 3.83 cm and 41.7 ± 1.99 cm TL and 809.56 ± 193.82 g and 827.3 ± 104.22 g WW in females and males, respectively, at the last sampling in August **(Figs. 1b and c)**.

3.2 Sex differentiation

The first morphological indication of female differentiation was seen at 101 dph (**Fig. 2,** 14.98 ± 6.20 cm TL), when the gonad that was attached to the swim bladder wall had formed the ovarian cavity and scattered germ cells were visible around the cavity in histological sections (**Fig. 2**). A more developed ovarian cavity with more proliferating germ cells was found at 150 dph (**Fig. 2b**, 25.5 ± 1.29 cm TL), while at 198 dph (**Fig. 2c,** insert, 25.8 ± 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 ± 1.89 cm TL). At 305 dph (**Fig. 2e**, 28.41 ± 1.29 cm TL) and at 357 dph (**Fig. 2f**, 34.86 ± 2.15 cm TL) the number of primary oocytes kept increasing, to reach complete ovary differentiation at 408 dph (**Fig. 2g**, 41.22 ± 3.83 cm TL), when the ovarian lamellae were filled with primary oocytes.

In presumptive males, at 101 dph the gonads were attached along the length of the swim bladder and were identified by the absence of a cavity, and contained exclusively somatic cells and connective tissue, and still no germ cells (**Fig. 3a**, 14.47 ± 6.60 cm TL), while occasionally the blood vessels of the testis were also visible (not shown). The first germ cells in the testes appeared at 150 dph, when spermatocytes could be found (**Fig. 3b**, insert, 24.71 ± 3.09 cm TL). The number of proliferating and differentiating germ cells increased at 198 and 260 dph (**Figs. 3c and d**, 28 ± 2.39 cm and 29.75 ± 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 ± 2.85 cm TL). This structure was obviously maintained in the following samplings (357 and 408 dph, **Figs. 3f and g**, 34.25 ± 2.98 cm and 41.7 ± 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**). Sex ratio was not different from the expected 1:1 sex ratio in the samplings tested (260, 305, 357, 408 dph, $\chi^2(1, N=67) = 0.809$, $P > 0.05$). Undifferentiated gonads were encountered in all the samplings. Their percentage was high at the first three samplings, until 198 dph, and decreased thereafter, with a very small number of undifferentiated gonads found in the last samplings (**Fig. 4**).

3.3 Hormonal profile of males and females during sex differentiation and 11-KT/E_2 ratio

Of the seven measured hormones in greater amberjack females and males during the sex differentiation process, the ones that exhibited statistically significant changes in time were Ad, Δ4, 11-KT, T and P4 in females and Δ4, 11-KT, T, P4 and 17,20βP in males (**Fig. 5**). Ad exhibited variations in its values as the sex differentiation period was progressing in

females; on the other hand, Δ4, 11-KT, T and P4 in both sexes and 17,20βP 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 when only two females were found **(Fig. 6A)**. The 11-KT/E_2 ratio was also significantly different between the sexes, beginning from the second sampling at 150 dph until the last sampling at 408 dph (**Fig. 6B**). According to the logistic regression analysis, both 11-KT concentration and 11-KT/E_2 ratio can be used to predict the sex of young greater amberjack ($P<0.0001$). The threshold value for 11-KT concentration was 0.13 ng ml^{-1}, with 98% probability of an individual being a female when exhibiting a lower value. For the 11-KT/E_2 ratio, the threshold value was 1.942, with 80% possibility of an individual being a female when exhibiting a lower value. Therefore, 11-KT plasma concentration could be used as a more accurate predictor of sex in greater amberjack from 101-408 dph, similar to another study in fish older than 412 dph, which had completed sex differentiation (Aoki et al. 2019). However, since more differentiated fish were encountered after the sampling of 260 dph, we propose that the sex prediction model should be used in fish older than 260 dph, when the number of undifferentiated individuals is lower.

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 in the samplings tested 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). On the other hand, in the Gulf of Mexico, more females were found in larger size and age classes than males (Thompson et al. 1999), but this was attributed to females living longer and not

growing faster 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 (June) reached around 1000-1200 g (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, compared to 17 or 22°C (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 almost all fish sampled 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). On the contrary, in another study on tank-reared wild-caught fish, 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. Therefore, although more than 95% of the population is differentiated during the first year, a small percentage of individuals may take longer to undergo sex differentiation.

Sex steroid hormones have been linked to the sex differentiation process and different studies have correlated their concentrations with sex differentiation. Testosterone, 11-KT and Δ4, but not E_2 or 17,20βP concentrations were found to be different between sexes in the coho salmon *Oncorhynchus kisutsch* (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). Plasma testosterone concentration and gonadal aromatase activity were sexually dimorphic in grey mullet *Mugil cephalus*, whereas plasma 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 thus can be considered related to the sex differentiation procedure. More specifically, Ad exhibited variations and Δ4, 11-KT, T and P4 increased in females and Δ4, 11-KT, T, P4 and 17,20βP increased in males during the sex differentiation 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 progestins (P4) produced more females and treatment with synthetic progestins (norgestrel) produced more males (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, Δ4, Ad, T, and 17,20βP are produced from P4 (Depeche and Sire 1982).

The latter progestin, 17,20βP, was shown in the present study to be linked to the sex differentiation procedure of male greater amberjack. Best known to be controlling oocyte maturation in fully vitellogenic oocytes (Nagahama and Yamashita 2008), 17,20βP was also 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 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_2 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 female differentiation in fish, whereas male differentiation results from down-regulation of female differentiating genes and hormones (Kobayashi et al. 2013; Li et al. 2019). However, the simultaneous rise of androgens in both sexes of the present study pinpoints to an important role of androgens in the gonadal differentiation of both males and females in greater amberjack. In the rainbow trout, it was shown that the enzyme 11β-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 (*cyp17α1*) 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 during early oogenesis, stimulating oocyte growth and lipid accumulation in previtellogenic ovaries, suggesting that this hormone plays a significant role in female reproductive physiology as well (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_2 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 sexually differentiated fish older than 412 dph and larger than 39 cm FL. In the present study, plasma 11-KT concentration was shown to be a potential predictor of sex also in younger aged greater amberjack during the process of sex differentiation, with a threshold value of 0.13 ng ml^{-1}. Above this value, individuals would be predicted to be males.

A major advantage of using LC-MS/MS in the present study 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 enzyme-linked immunosorbent assay (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). Using LCMS/MS, with just 200 μL 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. This method could be adapted to measuring a number of other molecules in a small plasma sample from small fish.

In conclusion, the present study showed that hatchery produced greater amberjack exhibits no sexual size dimorphism and the sex ratio in cultured population is 1:1, underlining that the early life rearing method did not have any influence on the process of sex differentiation. Sex could be predicted in under yearling fish before the completion of sex differentiation, with the use of the 11-KT plasma concentration. More studies are needed in order to decipher the exact role of each measured hormone in sex differentiation, both in greater amberjack and other species.

5. Author declarations

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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 (Metcalfe 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.

Consent for publication: All authors have agreed to submit the manuscript for publication.

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Figure captions

Fig. 1 a. Water temperature (ºC) in the sea cages where the hatchery produced greater amberjack were kept, from 101 until 408 days post-hatching. b. Mean (± S.E.M) growth in total length (cm) of greater amberjack from 101 until 408 days post-hatching, in females and males. c. Mean (± 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, $P < 0.05$)

Fig. 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 µm (insert), 200 µm (c, d, e, f and g) and 500 µm (a and b)

Fig. 3 Histological sections of testes from hatchery produced greater amberjack during the process of sex differentiation. a. Presumptive undifferentiated testis at 101 dph, lacking a cavity, but also any germ cells. 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 different germ cell stages, from sg to sp. The scale bars indicate 50 μm (insert), 100 μm (d, e, f and g), 200 μm (c) and 500 μm (a and b)

Fig. 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 sampling time is shown above the bar. There was no difference from the 1:1 sex ratio in the samplings of 260, 305, 357 and 408 days post-hatching (chi-square test, $P>0.05$). The test was not performed in the other sampling times, due to a small sample size for males and females (<5 individuals).

Fig. 5 Hormone plasma levels of adrenosterone (Ad), androstenedione (Δ4), 11-ketotestosterone (11-KT), testosterone (T), estradiol (E2), progesterone (P4) and 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) in hatchery produced greater amberjack in relation to time. Different letter superscripts indicate differences between steroid hormones in time (one-

748 way ANOVA, Tukey HSD, $P < 0.05$). Between the sexes, only 11-KT exhibited differences (one-way ANOVA, Tukey HSD, $P < 0.05$)

750

Fig. 6 Evolution of 11-ketotestosterone (11-KT) plasma concentration (A), and 11-KT/estradiol (E_2) ratio (B) in hatchery produced greater amberjack males and females in relation to time. Differences in the plasma 11-KT levels and in the 11-KT/E_2 ratio between males and females are indicated with the P values over each sample time (one-way ANOVA, Tukey HSD, $P < 0.05$).

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Figure 1

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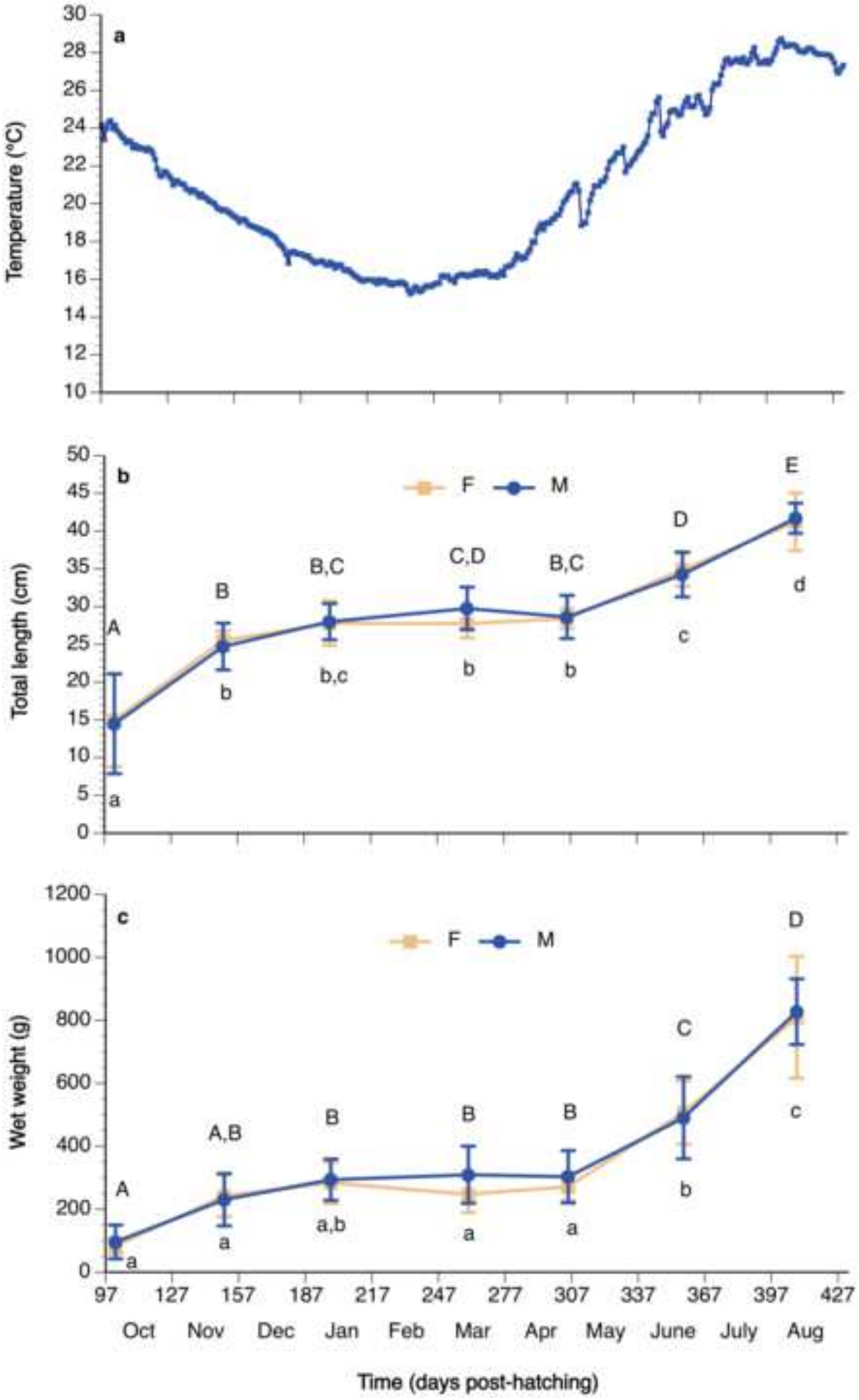

Figure 2

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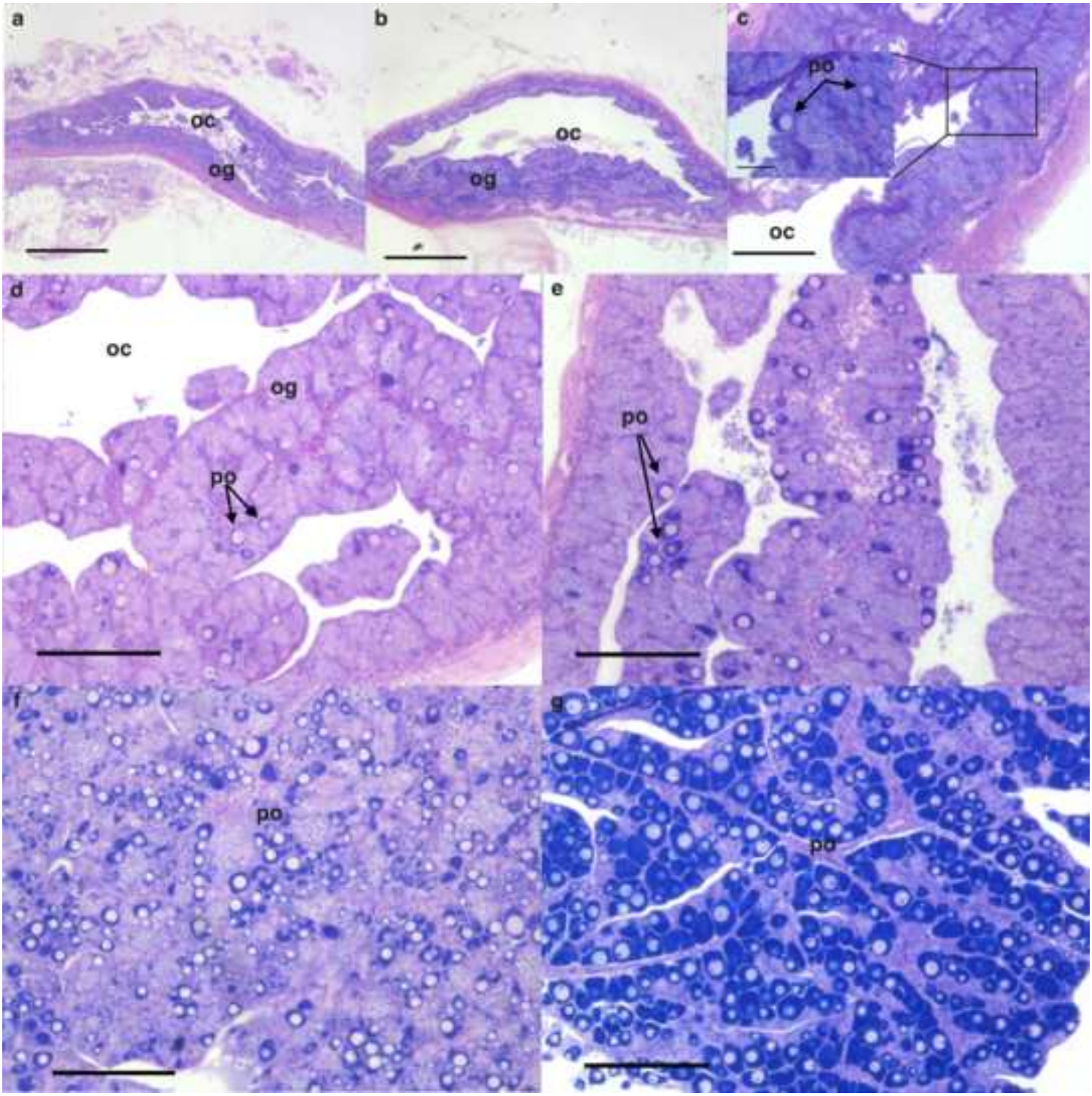

Figure 3

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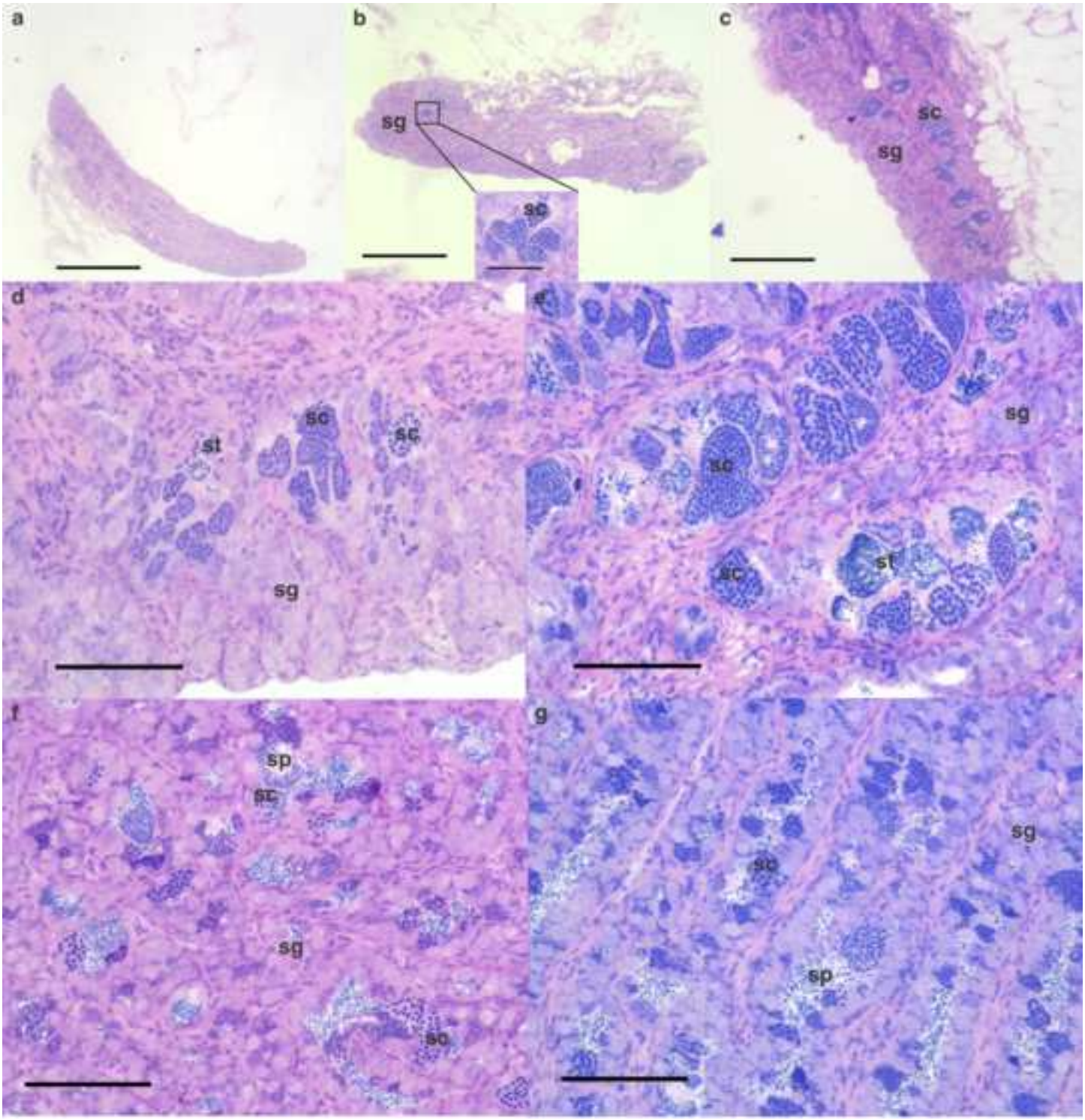

Figure 4

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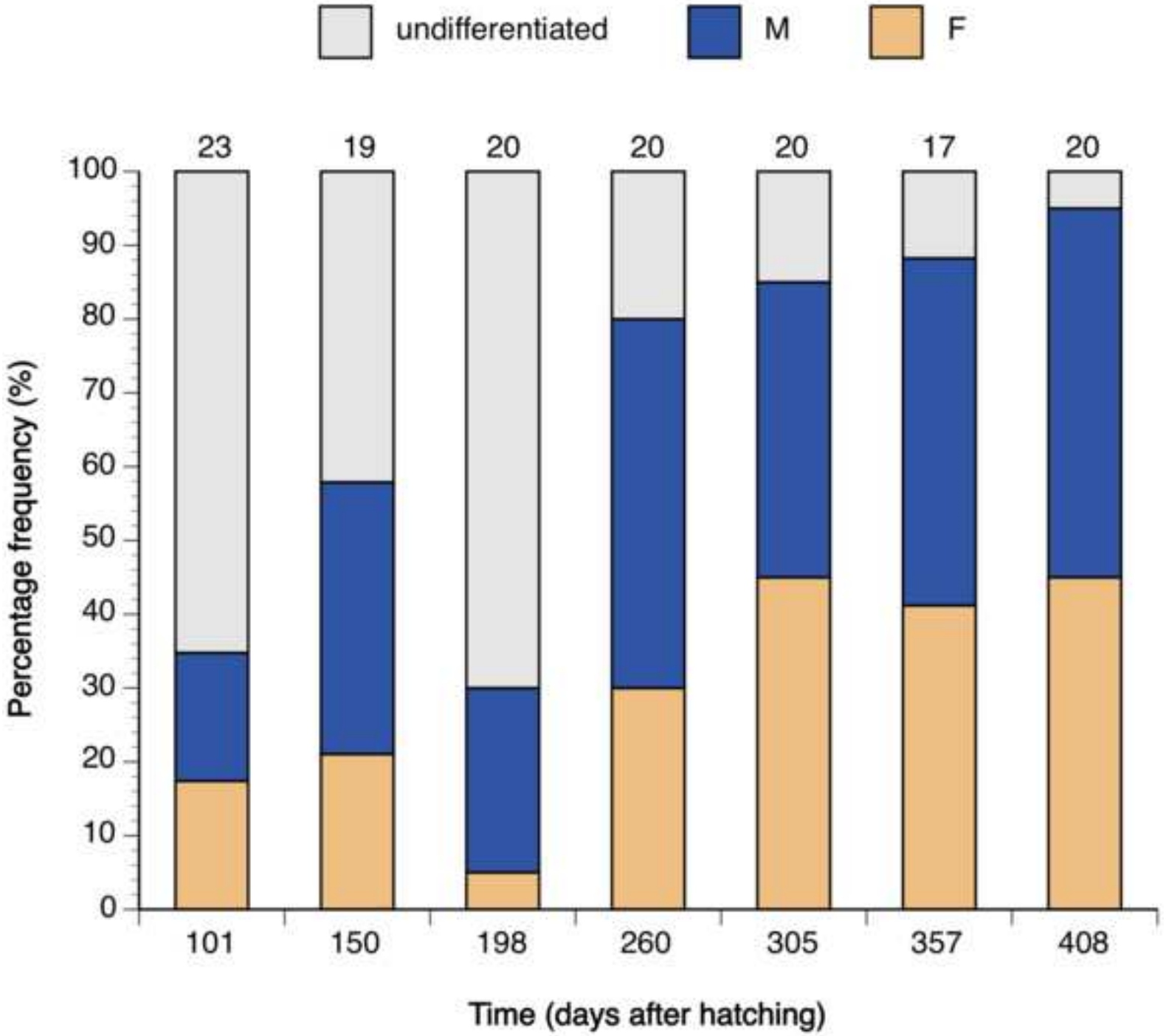

Figure 6

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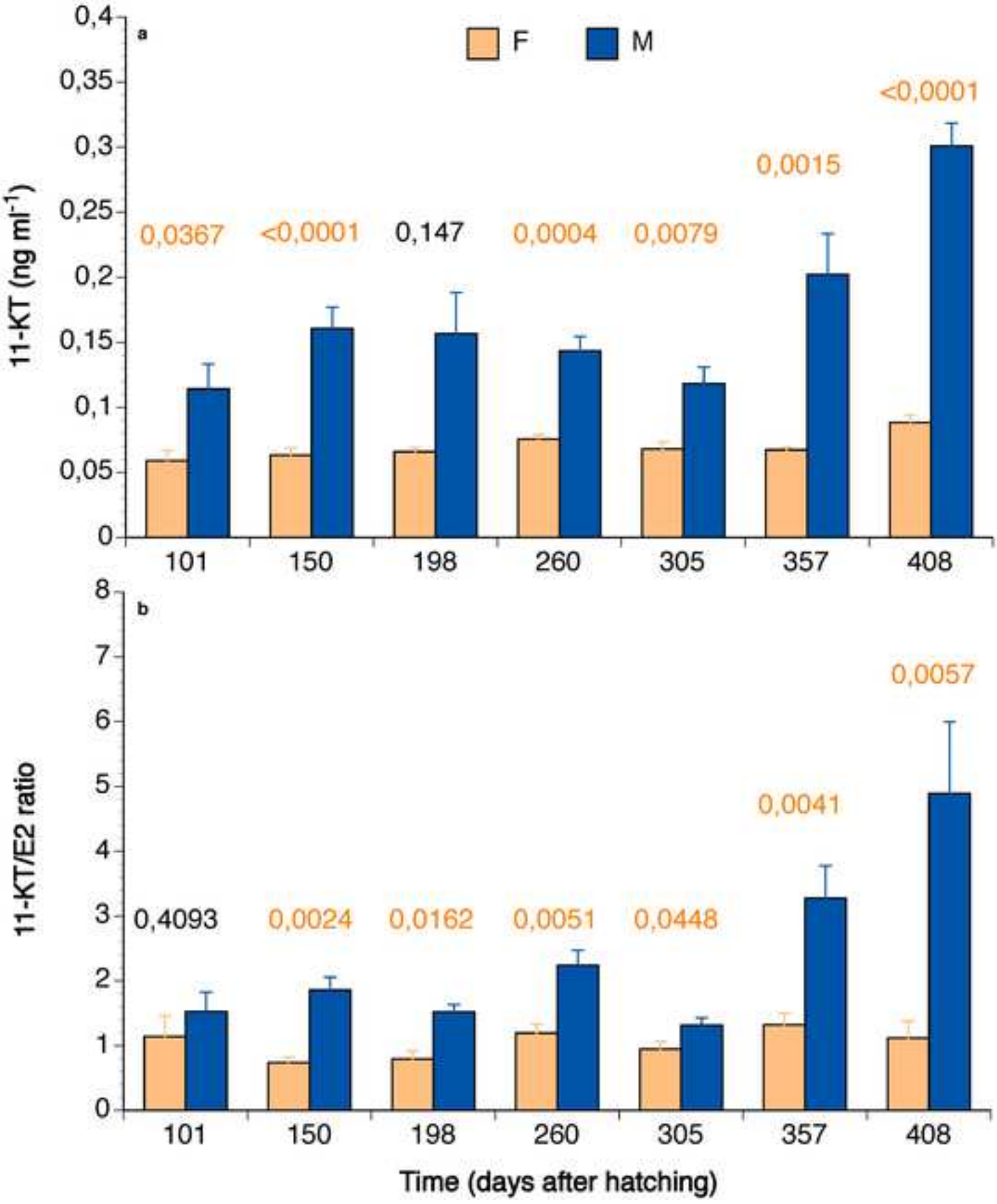

Figure 5

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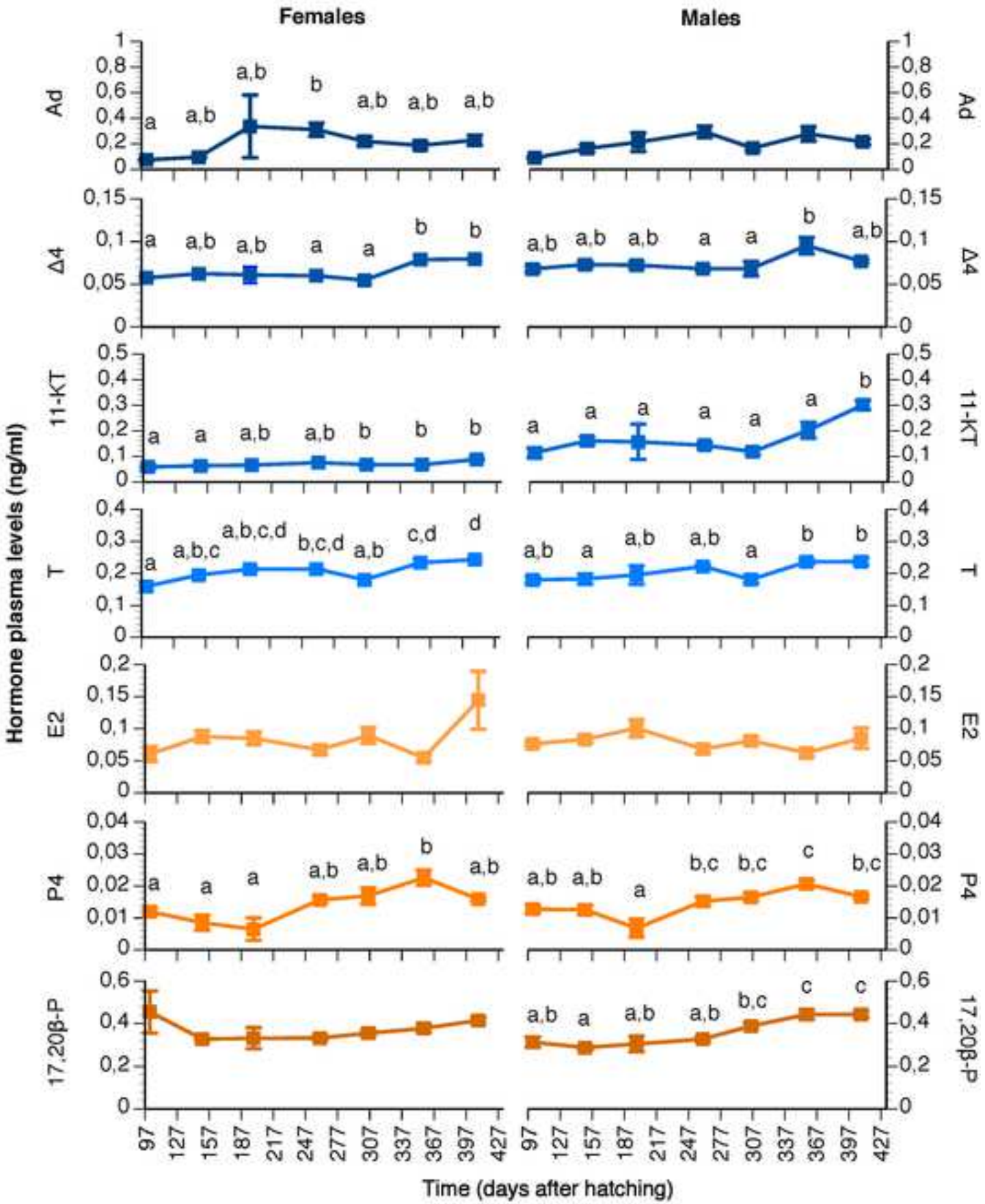

Table 1. Optimized MRM parameters of the quantifier and qualifier transitions that were used for the LC-MS/MS analysis of hormones.

				Quantifier			Qualifier		
Compound	**Retention Time (min)**	**Precursor ion m/z**	**Fragmentor**	**Product ion m/z**	**Collision Energy**	**Cell Accelerator Voltage**	**Product ion m/z**	**Collision Energy**	**Cell Accelerator Voltage**
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
17β-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
17α,20β-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

Table 2. Percentage recovery and matrix effect of the studied hormones in fish serum.

Compound	% Recovery		% Matrix Effect	
	C=25 pg µl^{-1}	C=150 pg µl^{-1}	C=25 pg µl^{-1}	C=150 pg µl^{-1}
11-Ketotestosterone	80±5	91±12	104±8	98±3
Adrenosterone	95±4	96±12	105±8	102±3
17β-Estradiol	72±4	84±10	92±4	94±5
Testosterone	80±2	83±4	107±6	97±3
17α,20β-Dihydroxy-4-pregnen-3-one	99±3	100±14	106±5	99±6
4-Androstene-3,17-dione	95±5	98±12	106±8	98±3
Progesterone	97±4	99±15	105±6	92±6

Supplemental table

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Name	Age (dph)	Sex	(cm)	(g)	(ng/ml)	(ng/ml)
Name	Age (dph)	Sex	Length	Weight	11-ketotestosterone Results	Adrenosterone Results
Seriola#001	101	F	16	66	0.074	0.070
Seriola#002	101	M	21	132	0.168	0.110
Seriola#003	101	M	15	50	0.094	0.088
Seriola#004	101	M	22	152	0.082	0.087
Seriola#005	101	F	20.5	118	0.037	0.057
Seriola#006	101	F	15.5	64	0.058	0.095
Seriola#007	101	M	15	50	0.113	0.078
Seriola#008	101	F	17.7	85	0.067	0.077
Seriola#009	150	M	25	190	0.146	0.122
Seriola#010	150	M	28	320	0.250	0.183
Seriola#011	150	M	24	210	0.154	0.131
Seriola#012	150	F	26	270	0.072	0.122
Seriola#013	150	F	25	210	0.055	0.086
Seriola#014	150	M	29	360	0.144	0.096
Seriola#015	150	M	25	230	0.169	0.237
Seriola#016	150	M	21	180	0.153	0.288
Seriola#017	150	F	27	320	0.052	0.094
Seriola#018	150	F	24	170	0.074	0.083
Seriola#019	150	M	21	120	0.108	0.085
Seriola#020	198	M	25.5	252	0.276	0.212
Seriola#021	198	F	29.9	330	0.063	0.581
Seriola#022	198	M	25.9	228	0.131	0.205
Seriola#023	198	M	30.7	387	0.164	0.483
Seriola#024	198	M	27.7	267	0.102	0.107
Seriola#025	198	M	30.2	334	0.111	0.058
Seriola#026	198	F	25.7	237	0.069	0.091
Seriola#027	260	M	30	305	0.127	0.314
Seriola#028	260	M	27	257	0.184	0.218
Seriola#029	260	M	26	207	0.189	0.287
Seriola#030	260	M	28	230	0.132	0.509
Seriola#031	260	M	31	343	0.115	0.146
Seriola#032	260	F	29	302	0.075	0.249
Seriola#033	260	M	35	495	0.198	0.256
Seriola#034	260	F	27	216	0.090	0.383
Seriola#035	260	M	29	249	0.150	0.150
Seriola#036	260	M	33	421	0.116	0.581
Seriola#037	260	F	26	193	0.072	0.382
Seriola#038	260	F	31	335	0.067	0.444
Seriola#039	260	M	27.5	259	0.120	0.224
Seriola#040	260	F	27	230	0.079	0.319
Seriola#041	260	M	31	327	0.103	0.252
Seriola#042	260	F	26.5	206	0.071	0.087
Seriola#043	305	F	29.5	230	0.100	0.165
Seriola#044	305	F	29.5	290	0.082	0.248
Seriola#045	305	M	31.5	382	0.129	0.126
Seriola#046	305	F	29.5	337	0.073	0.229
Seriola#047	305	F	27.5	245	0.072	0.283
Seriola#048	305	M	30	340	0.143	0.109

Seriola#049	305	M	32.5	434	0.139	0.136
Seriola#050	305	F	29	280	0.057	0.259
Seriola#051	305	F	27.2	250	0.057	0.111
Seriola#052	305	M	27	255	0.102	0.262
Seriola#053	305	M	30	315	0.181	0.188
Seriola#054	305	F	28	300	0.052	0.431
Seriola#055	305	M	25.5	200	0.086	0.115
Seriola#056	305	M	24.5	200	0.079	0.184
Seriola#057	305	F	26	200	0.069	0.131
Seriola#058	305	M	28	298	0.088	0.211
Seriola#059	305	F	29.5	315	0.051	0.120
Seriola#060	357	M	29	260	0.081	0.161
Seriola#061	357	F	33.5	454	0.073	0.179
Seriola#062	357	F	32.5	415	0.071	0.256
Seriola#063	357	M	35	563	0.328	0.257
Seriola#064	357	M	31	345	0.141	0.174
Seriola#065	357	F	35	512	0.070	0.124
Seriola#066	357	F	34.5	465	0.061	0.295
Seriola#067	357	M	34	447	0.135	0.095
Seriola#068	357	M	37	546	0.269	0.375
Seriola#069	357	M	34	530	0.186	0.590
Seriola#070	357	F	33	412	0.072	0.129
Seriola#071	357	F	37.5	648	0.065	0.095
Seriola#072	357	M	37.5	646	0.173	0.192
Seriola#073	357	M	36.5	587	0.305	0.370
Seriola#074	357	F	38	645	0.060	0.235
Seriola#075	405	F	41.5	751	0.063	0.215
Seriola#076	405	M	40	722	0.367	0.257
Seriola#077	405	F	45.5	1005	0.105	0.191
Seriola#078	405	M	41	836	0.311	0.180
Seriola#079	405	F	41	742	0.104	0.136
Seriola#080	405	F	41	794	0.103	0.343
Seriola#081	405	F	32	376	0.091	0.202
Seriola#082	405	F	43.5	960	0.074	0.109
Seriola#083	405	M	39	639	0.233	0.107
Seriola#084	405	M	43.5	930	0.341	0.307
Seriola#085	405	F	42	875	0.093	0.208
Seriola#086	405	M	40	790	0.335	0.197
Seriola#087	405	M	43	915	0.317	0.183
Seriola#088	405	M	42	840	0.243	0.356
Seriola#089	405	M	40	734	0.368	0.202
Seriola#090	405	M	43.5	927	0.277	0.180
Seriola#091	405	M	45	940	0.216	0.188
Seriola#092	405	F	44	1005	0.065	0.164
Seriola#093	405	F	40.5	778	0.098	0.486

(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)
b-Estradiol Results	Testosterone Results	17a,20b-dihydroxy-4-pregnen-3-one Results	4-Androstene-3,17-dione Results
0.082	0.162	0.742	0.061
0.071	0.169	0.261	0.072
0.070	0.196	0.294	0.069
0.085	0.157	0.320	0.057
0.073	0.145	0.298	0.052
0.029	0.136	0.413	0.055
0.079	0.197	0.377	0.074
0.058	0.194	0.366	0.062
0.089	0.132	0.242	0.055
0.099	0.152	0.298	0.084
0.078	0.230	0.291	0.073
0.073	0.167	0.322	0.059
0.078	0.183	0.345	0.056
0.085	0.170	0.305	0.086
0.081	0.232	0.326	0.073
0.059	0.211	0.290	0.072
0.085	0.225	0.308	0.076
0.115	0.205	0.334	0.059
0.094	0.153	0.263	0.069
0.141	0.238	0.286	0.071
0.095	0.222	0.381	0.071
0.089	0.213	0.305	0.073
0.124	0.269	0.441	0.088
0.076	0.117	0.225	0.061
0.074	0.139	0.267	0.069
0.075	0.204	0.282	0.051
0.046	0.222	0.288	0.080
0.086	0.224	0.298	0.073
0.070	0.234	0.346	0.068
0.107	0.205	0.324	0.066
0.080	0.215	0.308	0.072
0.086	0.192	0.335	0.055
0.070	0.244	0.356	0.072
0.051	0.230	0.345	0.062
0.044	0.206	0.304	0.059
0.077	0.216	0.313	0.064
0.053	0.221	0.315	0.064
0.053	0.221	0.380	0.066
0.047	0.220	0.401	0.061
0.088	0.214	0.322	0.058
0.056	0.233	0.337	0.067
0.071	0.203	0.298	0.055
0.115	0.219	0.368	0.061
0.084	0.211	0.374	0.051
0.087	0.217	0.384	0.066
0.060	0.210	0.329	0.029
0.046	0.184	0.307	0.047
0.098	0.180	0.337	0.023

0.045	0.209	0.381	0.096
0.085	0.161	0.336	0.050
0.090	0.157	0.302	0.062
0.083	0.166	0.365	0.064
0.097	0.202	0.376	0.101
0.174	0.145	0.346	0.060
0.081	0.180	0.382	0.073
0.074	0.172	0.417	0.065
0.066	0.189	0.452	0.072
0.085	0.121	0.470	0.059
0.086	0.144	0.381	0.061
0.034	0.208	0.540	0.061
0.072	0.236	0.344	0.086
0.061	0.195	0.333	0.052
0.083	0.266	0.463	0.146
0.051	0.226	0.432	0.092
0.045	0.295	0.449	0.082
0.057	0.226	0.425	0.082
0.067	0.234	0.494	0.075
0.072	0.256	0.437	0.101
0.072	0.212	0.426	0.074
0.032	0.229	0.362	0.081
0.050	0.211	0.326	0.077
0.072	0.239	0.413	0.095
0.048	0.246	0.338	0.118
0.066	0.242	0.407	0.092
0.077	0.234	0.354	0.079
0.139	0.254	0.421	0.075
0.041	0.253	0.449	0.078
0.068	0.268	0.424	0.082
0.058	0.255	0.470	0.079
0.111	0.260	0.394	0.078
0.054	0.238	0.438	0.070
0.064	0.241	0.375	0.073
0.053	0.244	0.346	0.079
0.061	0.276	0.441	0.095
0.139	0.245	0.382	0.076
0.178	0.279	0.462	0.080
0.079	0.223	0.498	0.073
0.140	0.218	0.426	0.076
0.056	0.173	0.485	0.072
0.020	0.202	0.500	0.068
0.059	0.233	0.431	0.071
0.379	0.202	0.407	0.087
0.378	0.258	0.465	0.097

(ng/ml)
Progesterone Results
0.012
0.011
0.015
0.010
0.010
0.011
0.015
0.015
0.013
0.019
0.014
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0.012
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0.015
0.017
0.015
0.013
0.025
0.017
0.017
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