Exposure of larvae to daily thermocycles affects gonad development, sex ratio and sexual steroids in *Solea senegalensis*, Kaup.

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Running title: Early exposure to daily thermocycles in *Solea senegalensis*.

Grant sponsor: Spanish Ministry of Science and Innovation (MICINN); Grant number: AGL2006-13777-C03.

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ABSTRACT

The effect of water temperature during the development of fish larvae on sex differentiation is well known, but not so well known is the impact of the daily thermocycles. Our aim was to investigate the effect of early exposure of Senegal sole larvae to different temperature cycles on gonad development, sex ratio and sex steroid (11-ketotestosterone, 11-KT; estradiol, E₂; and testosterone, T) content in muscle extracts of juveniles. From 1 to 97 DPH (days post-hatching) fish larvae and post larvae were subjected to three temperature regimes: TC (Thermophase-Cryophase); CT (Cryophase-Thermophase); and constant temperature. In fish exposed to TC, sex determination occurred earlier, since 90% of soles were males/females at 110 DPH, whereas 45% of fish under CT were undifferentiated at that time. Fish under TC showed the highest growth rates, followed by fish under constant temperature and by fish under CT, the differences being statistically significant between the TC and CT groups. Regarding sex ratio, juveniles exposed to TC showed a higher proportion of females than fish under CT or constant temperature. Under TC, fish showed the highest concentration of E₂, while 11-KT concentration was highest in fish under CT and constant temperature. Fish under constant temperature and CT showed higher T levels than those under TC. These results provide the first insights into the effect of daily thermocycles on sex differentiation in fish, and underline the key role of natural environmental cycles on the control of sex ratios during larval development, which may be applied to the manipulation of sex ratio in aquaculture.

INTRODUCTION

Fish, with almost 30,000 species, represent half of all vertebrates (Helfman et al., '09). They colonize nearly all aquatic habitats, which are subjected to cyclic changes in the

environment governed by geophysical cycles such as the Earth's rotation around its axis (e.g. day/night changes in solar radiation), around the sun (seasons) and the moon's rotation around the Earth (lunar and tidal cycles). To match this array of cyclic habitats all living organisms have evolved a great variety of adaptive responses, such as biological clocks to keep time and to synchronise with environmental cycles, and flexible mechanisms by means of which individuals can become male or female. Various extrinsic factors have been observed to influence the sex ratio, at least under controlled conditions: for example photoperiod (Bromage, '87; Aida and Amano '95; Taranger et al., '95, '98) and temperature (Colombo et al., '98; Blazquez et al., '98; Pavlidis et al., 2000; Blazquez et al., 2009). The latter factor deserves further consideration since the cyclic infrared radiation from the Sun generates a daily thermocycle: during the day the temperature rises (thermophase, or phase of higher temperature), while during the night the temperature drops (cryophase, or phase of lower temperature). Thus, transitions from cold to warm temperature are roughly associated with dawn, and transitions from warm to cold temperature with dusk (Johnson et al., 2004). However, under artificial rearing conditions, the environmental conditions are set by fish farmers to optimise fish survival and growth, as reported for many aquaculture species (Barahona-Fernandes, '79; Tandler et al., '85; Batty et al., '87; Downing and Litvak, '99), and little attention has been paid to the influence of these daily temperature oscillations in cultured fish.

Environmental factors can trigger or determine the process of gonad development in some fish (environmental sex determination, ESD) and can lead to skewed sex ratios in wild or farmed fish (Siegfried, 2010). Moreover, in later stages, these factors can have an impact on the undifferentiated gonads, which are highly susceptible to external stimuli, overriding the genetic sex determination (GSD) and thus switching the fate of the gonad towards the opposite sex. However, the distinction between both mechanisms is not always clear

(Baroiller et al., 2009). In Nile tilapia, the existence of a thermo-sensitive period has been reported in female larvae from 12 to 52 DPH, during which the mortality rate is high and the male proportion increases (Rougeot et al., 2008). This thermo-sensitive window takes place a few hours after fertilization and covers the development of the brain (31 DPH) and the segregation of the primordial germ cells (46 DPH) (Morrison et al., 2001), long before any development of the presumptive gonads.

In all vertebrates, sex steroids affect the development of germ cells and other cell-types, as well as the organs involved in sexual differentiation (Devlin and Nagahama, 2002). Estradiol (E₂) is considered to be responsible for inducing and maintaining ovarian development, and its levels are considerably higher in females than in males. Testicular development is mainly regulated by the androgen 11-ketotestosterone (11-KT). In fish in general, testosterone (T) is not directly involved in the mechanisms of sexual differentiation, but participates as precursor of 11-KT and E₂ (Nakamura et al., '84; Baroiller et al., '99).

The Senegal sole *Solea senegalensis* Kaup (1858) is a flatfish adapted to temperate waters of around 16-23°C (Drake et al., '84). This species is extensively exploited in aquaculture, mostly in Spain and Portugal (Dinis et al., '99), although reproduction and culture techniques still need to be optimized (Porta et al., 2007) due to the difficulty of obtaining fertilized eggs from captive broodstock and the commercial interest of this species and the need to get potentially reproductive females and males. In this work, Senegal sole was chosen to investigate the effect of temperature on juvenile sex differentiation. To this end, sole larvae and juveniles were subjected to different temperature cycles (natural or reverse thermocycles vs. constant temperature) to investigate their effect on gonad development and degree of differentiation, sex ratio, and levels of steroid hormones.

MATERIAL AND METHODS

99 Sole eggs were provided by the Spanish Oceanographic Institute (IEO), Santander
100 (Cantabria, Spain). Wild broodstock was maintained in 14 m³ tanks, in a female:male
101 proportion of 1:1. The tanks were covered with a shadow net that provided 0.21 W m⁻² (50 lx)
102 on the water surface. The spectral analysis of lights was performed using a spectroradiometer
103 (FieldSpec®, ASD, Colorado, USA). Fish were fed five days a week, three days with mussels
104 and two days with small cuttlefish; one of the days on which they were fed with mussels,
105 frozen polychaetes were added (Sebait Ltd., UK). Water temperature varied between 16 and
106 19°C (simulating natural temperature fluctuations) and the photoperiod was 12 h L:12 h D. An
107 egg collector was placed at the water outflow of the tank. Fertilised eggs were collected and
108 incubated in 70 L incubators under continuous darkness until hatching. At 1 DPH, fish larvae
109 were transported in darkness to the Institute of Aquaculture Torre la Sal (IATS-CSIC,
110 Castellón, Spain), where the thermocycle trials were performed.

111 To feed the larvae, *Brachionus plicatilis* rotifers were enriched with *Tetraselmis*
112 *suecica*, *Isochrysis galgana* and commercially available freeze-dried green algae
113 *Nannochloropsis* sp. (Phytobloom Prof® Necton, Portugal) in a proportion of 300,000
114 cells/ml/day from day 3 to day 7. These enriched rotifers were added to tanks daily as an
115 early live food at an increasing density of 10-20 individuals ml⁻¹ from 3 to 7 DPH. *Artemia*
116 sp. nauplii at a density of 2–3 nauplii ml⁻¹ day⁻¹ were introduced from 8 to 30 DPH. Three to
117 five metanauplii ml⁻¹ day⁻¹ were added from 27 to 30 DPH. Before being provided to the
118 larvae, the metanauplii were enriched with a mixture (ORI-GO, ORI-PRO®, Skretting AS,
119 Spain) of phytoproteins and highly unsaturated fatty acids (HUFA) for 24 hours. From 30
120 DPH onwards, larvae were fed with dry food (Gemma micro Diamond®, Skretting AS,
121 Spain).

Experimental design

The experiments performed in the present research followed Spanish legislation on Animal Welfare and Laboratory Practices. The experimental protocol was approved by both the National Committee on Animal Welfare and the Bioethics Committee of the University of Murcia.

At arrival to our facilities, 1 DPH sole larvae were distributed into six 500 L cylindro-conical tanks, at a density of 50 larvae L⁻¹. The water system was semi-closed with an exchange rate of 10-30% seawater and a flow of 50 L/h in each tank per day. Thermal cycles had duration of 24 hours, the photoperiod was 12 h L:12 h D and light intensity was 0.84 W m⁻² (200 lux), which was supplied by a mercury vapor lamp (PHILIPS, HPL N 250W). The spectral analysis of lights was performed as indicated above, using a spectroradiometer (FieldSpec® Hand Held spectroradiometer, Colorado, USA) with a wavelength range of 325 to 1075 nm, an interval of 1.6 nm and a viewing angle of 25 degrees and a lux meter (MX Elektronik Minilux, Germany) with 6 measuring ranges from 2 lux to 200 klux.

From 1 to 97 DPH, three temperature regimes were applied per duplicate: TC cycle (22.1±0.6°C during the day and 19.0±0.4°C at night, mean±S.E.M, here and throughout); CT cycle (19.2±0.5°C during the day and 22.0±0.3°C at night) and constant temperature (20.7±0.4°C). After 97 DPH temperature was constant (20.5±0.6°C). Water temperature was controlled by means of two water coolers (Teco-TR-20, Italy and Astralpool alaska-4) and solenoid. Temperature was continuously recorded by an underwater sensor and a data logger (HOBO PENDANT® Onset Computer Corporation, Massachusetts, USA) placed in the tanks.

The sex of the larvae was determined was assessed in 20 specimens per treatment every week from 110 DPH to 173 DPH. This time was chosen a previous study of another

flatfish, *Hippoglossus hippoglossus*, showed that sexual differentiation occurred on 140 DPH (Hughes et al., 2008).

On 247 DPH, sex steroids (T, 11-KT and E₂) were measured in fish muscle by ELISA. The hormone analysis was performed at this time to ensure that steroid levels in the experimental fish were within the limits of the assay sensitivity.

Data collection and histology analysis

Fragments of sole body were fixed in 1 % gluteraldehyde in distilled water for 24 h at room temperature. After dehydration in increasing concentrations of ethanol, tissue fragments were embedded in glycol methacrylate resin (Technovit 7100, Heraeus Kulzer, Germany). Sections of 2 µm thickness were cut with a Supercut 2065 microtome (Reichert-Jung, Germany) and stained with methylene blue / azure II / basic fucsin (Bennet et al., '76). To ascertain fish sex, pictures were taken with a microscope (Leika) (x 10 magnification), and the sex was verified by histological analysis. Histological procedures were carried out following conventional techniques using the Cleveland Wolfe's methodology for staining (Herlant, '60). Ovary and testis were classified according to previous morphological studies on gametogenesis in females (Mayer et al., '88) and males (Rodríguez et al., 2001) fish.

Steroid analysis in muscle by ELISA

Sex steroids, estradiol (E₂), testosterone (T) and 11-ketotestosterone (11-KT), were analyzed by ELISA, according to the method described and validated by Guzmán et al., (2009a,b). The ELISA method was previously validated for Senegal sole plasma samples and, for this study, the extraction protocol was modified and the assay further validated for muscle samples.

For hormone analysis, 500 mg samples of skeletal muscle tissue taken from the muscle tissue anterior to the urogenital pore were obtained from the abdomen of each fish. The

169 extraction procedure was based on that described by Feist et al. ('90). Prior to extraction,
170 muscle samples were thawed, finely chopped with a razorblade and homogenized for 20-30 s
171 with 1.5 ml of methanol in a 12 × 75 mm glass culture tube using the Tissue Tearor™
172 motorized homogenizer. The homogenates were centrifuged (1000 g for 10 min) and the
173 aqueous lower phase and pelleted insoluble material were snap frozen in liquid nitrogen. The
174 methanol extract was decanted into a new tube and the lower phase and pellet thawed, mixed
175 with 1.5 ml of methanol, and re-centrifuged. The sample was snap frozen again and the
176 second methanol extract was combined with the first and dried at 37°C under a stream of
177 nitrogen gas. The remaining fraction was double extracted with two 1.5 ml volumes of ethyl
178 ether to remove any remaining particulates and the combined ether extracts were dried at
179 37°C under nitrogen and finally resuspended in 400 µL of assay buffer before ELISA
180 analysis.

181 The assay was validated for analysis of Senegal sole muscle by testing the parallelism
182 between the standard curves (E₂, T and 11-KT) and serial dilutions of muscle extracts
183 obtained from juvenile Senegal sole. Validation and accuracy of the assay was further tested
184 by the overloading test, checking the parallelism between the standard curves and serial
185 dilutions of Senegal sole muscle samples with increasing doses of the corresponding steroid
186 (Fig. 1). The calculated recovery rates were 74.9% for 11-KT, 56.1% for T and 38.6% for E₂.
187 The sensitivities of the ELISAs were 5.2, 8.8 and 0.4 pg ml⁻¹, for the E₂, T and 11-KT
188 ELISA, respectively (Guzmán et al., 2009a,b).

189 *Statistical analysis*

190 To establish statistical differences in growth and steroid levels between treatments, a
191 one-way ANOVA and Duncan's test were performed, with P<0.05 taken as the statistically
192 significant threshold. Regarding the sex ratio, two one-way ANOVA tests were carried out,
193 one to test the percentage of males and the other to check the percentage of females. A

Student's t-test was used to assess differences in the percentage of males and females within each group. All percentage data were normalized and arcsin transformed before statistical analysis. All statistical analyses were carried out with SPSS 15.0 for Windows. Data are expressed as mean $\pm$ S.E.M. values.

RESULTS

Growth performance

The growth of soles under the three experimental thermocycles was similar until 145 DPH. At 152 DPH, the juveniles under the TC thermal cycle were larger in size (6.6 ± 0.2 cm) than those exposed to CT (5.7 ± 0.2 cm) (ANOVA, Duncan's test, $p=0.043$). Fish exposed to constant temperature were 6.3 ± 0.2 cm long and showed no statistical differences from the TC and CT groups (Fig. 2).

As regards body weight, there were no significant differences between groups until 138 DPH. From 145 DPH onwards, juveniles subjected to TC showed a significantly higher weight (2.4 ± 0.1 g) than sole under CT (1.9 ± 0.2 g) (ANOVA, Duncan's test, $p=0.033$). The mass of fish exposed to a constant temperature was similar at 145 DPH (2.3 ± 0.2 g) to that of the TC and CT groups, but at 152 DPH (2.8 ± 0.2 g) it was higher than the mass of CT fish (Fig. 2).

Gonad development and differentiation

In fish exposed to TC sex differentiation occurred earlier, since at 110 DPH only 10% of sole were not distinct, whereas 45% of fish under CT were still undifferentiated (ANOVA, Duncan's test, $p=0.042$). All fish under TC were differentiated at 117 DPH, whereas complete

sex differentiation of the population in the groups under constant temperature and CT did not occur until 131 and 138 DPH, respectively (Fig. 3).

Figure 4 shows a clearly differentiated ovary in sole subjected to TC (Fig. 4A) and a differentiated teste in sole under CT (Fig. 4B).

Sex ratio

Fish under TC showed a higher proportion of females ($70.8 \pm 2.6\%$) than males ($21.2 \pm 3.4\%$), whereas sole exposed to CT showed a proportion of males ($82.5 \pm 5.8\%$), which was significantly higher than the percentage of females at 152 DPH ($17.5 \pm 7.6\%$) (ANOVA, Student's t-test, $p=0.032$). Finally, fish under constant temperature showed a greater proportion of males ($61.6 \pm 6.5\%$) than of females ($38.3 \pm 4.3\%$) (ANOVA, Student's t-test, $p=0.041$). (Fig. 5).

Sex steroids

Levels of E_2 were higher in fish under TC (15.5 ± 2.4 pg/g) than in fish under CT (9.9 ± 1.7 pg/g) or kept at constant temperature (10.5 ± 2.2 pg/g) (ANOVA, Duncan's test, $p=0.041$). Muscle 11-KT concentration was higher in fish under CT (8.5 ± 1.3 pg/g) or constant temperature (7.6 ± 1.2 pg/g) than in fish under TC (5.4 ± 0.8 pg/g) (ANOVA, Duncan's test, $p=0.039$). As regards T, fish exposed to constant temperature and CT (23.3 ± 2.3 pg/g) showed higher levels than those under TC (13.4 ± 1.0 pg/g) (ANOVA, Duncan's test, $p=0.039$) (Fig. 6).

DISCUSSION

Although the effect of (constant) water temperature on larvae development and sex determination in fish is well known, the effect of daily thermocycles has never been explored. The results provided by the present research reveal the strong effect of daily cycles of water temperature on gonad differentiation, sex ratio and sex steroids in *Solea senegalensis*. Therefore, this species should be considered a thermosensitive species, as seen in previous studies investigating other fish species, in which temperature was seen to affect the sex ratio (Blazquez et al., '98; Baroiller et al., 2009). Indeed, water temperature seems to be the most prevalent environmental factor influencing sex determination, as documented in at least 61 fish species belonging to very divergent orders (Baroiller et al., '99; Baroiller and D'Cotta, 2001; Devlin and Nagahama, 2002; Conover, 2004; Ospina-Alvarez and Piferrer, 2008). The sex of amphibians and reptiles is also determined by environmental factors, including temperature-dependent sex determination (Nakamura, 2010).

Conover ('84) observed that sensitivity to environmental factors was directly related to the change in growth rate induced by these factors, suggesting an adaptive role of environmental determination in fish species. In our study the group of fish showing the greatest growth (size and weight) was that under TC, coinciding with the group that showed a higher proportion of females, while the CT group showed reduced growth and weight and had a higher percentage of males. Fish under constant temperature showed a greater equality of sexes and showed no significant differences in growth and weight with TC or CT, suggesting that temperature cycle (but not average temperature itself) may cause the increased growth and differentiation observed in the females exposed to TC.

Some studies have demonstrated the existence of daily rhythms of temperature selection in fish in wild conditions. In such studies, fish showed daily migrations as they searched for a preferred temperature for physiological activity and growth (Gibson et al., '98; Sims et al., 2006). In Senegalese sole, most studies on biological development and

temperature have used a constant temperature of 20°C (Parra and Yúfera, '99; Yúfera et al., '99; Cañavate et al., 2006), neglecting the effects that temperature fluctuations in the natural environment may cause. Nevertheless, previous investigations carried out in goldfish pointed to the existence of a daily pattern of temperature selection (Reynolds et al., '78), which seemed to be related to body weight gain and gonadal growth (Spieler et al., '77). Such findings support our hypothesis which relates better performance in sole with the existence of a particular daily cycle of temperature (TC).

In the present study, daily thermocycles influenced not only the sex ratio but the timing of gonad differentiation. In TC, sex differentiation in juvenile Senegal sole took place earlier than in fish under CT or constant temperature. These findings are consistent with the findings of a previous study reporting that Senegal sole larvae kept under TC showed faster development and metamorphosis (Blanco-Vives et al., 2010). In that report, fish under CT or constant temperature exhibited delayed metamorphosis, especially in larvae exposed to CT, which also showed the slowest development, as seen in the present investigation.

In an early paper, Hontela and Peter ('83a,b) found that daily thermocycles in goldfish affected gonadotropin hormone (GTH), which showed relatively high levels throughout the day under constant temperature, but fluctuated or decreased when a warm temperature was applied during the day or night, respectively. Sex steroid hormones are crucial in the regulation of sexual differentiation in fish (Baroiller and Guiguen, 2001), although the effect of daily thermocycles has never been reported. According to Bogart ('87) and Baroiller et al. ('99), sexual differentiation depends on the balance between 11-KT and E₂: a higher proportion of 11-KT induces masculine differentiation, while the inverse situation induces feminine differentiation, as observed in several species of teleost, e.g. *Perca fluviatilis* and *Oreochromis niloticus* (Rougeot et al., 2007). In the present study, the concentrations of 11-KT and T were significantly lower in the TC group (with the highest proportion of females),

while the concentration of E₂ was significantly higher (Fig. 6). Some studies have reported that in several species females have similar 11-KT blood concentrations to males (Borg, 1994; D'Cotta et al., 2001; Lokman et al., 2002), while males can display high levels of E₂ (Miura et al., '99). The biological significance of such abnormal concentrations remains unknown. In our experiment, the considerable difference in the 11-KT to E₂ ratio (0.72) between mixed-sex progenies strongly suggest that sex differentiation in Senegal sole is closely controlled by this ratio, as in Eurasian perch (Rougeot et al., 2007), where an excess of E₂ induces the female differentiation process while an excess of 11-KT induces the male differentiation process. This hypothesis, suggested by Baroiller et al., ('99) and Bogart ('87), is supported by results obtained in the present study, as sex differentiation of sole appeared to be correlated with the sex steroids ratio.

During larval development, temperature and light cycles are required for the circadian clock to work properly. In fish, the circadian clock matures extremely early during larval development (within 24-48 h) and is thought to regulate the temporal co-ordination of many physiological processes (Vallone et al., 2007). In the present study, differences in the development of the circadian system of sole under different thermal cycles may explain differences in development and, thus, in gonad differentiation and the sex ratio. This hypothesis is supported by ongoing research aiming at characterising rhythmic clock gene expression in sole larvae, which appears very early (Dr. Muñoz-Cueto, personal communication) and is very probably influenced by light and temperature conditions during sole ontogeny.

In conclusion, the present paper has revealed that daily thermocycles applied during early larval development have a strong impact on gonad development and the sex ratio, as well as on sex steroids concentrations. These findings should be considered when designing

larva rearing protocols to manipulate the sex ratio of Senegalese sole in aquaculture, since such protocols aim to produce more females, which have better growth performance.

ACKNOWLEDGEMENTS

This research was funded by the Spanish Ministry of Science and Innovation (MICINN) by projects “*Circasole*” AGL2007-66507-C02-02 and “*Reprosole*” AGL2006-13777-C03. AQUAGENOMICS 28502 (Consolider-Ingenio Program), and FUNDACIÓN SENECA by project 05690/PI/07 awarded to Francisco Javier Sánchez Vázquez and Generalitat Valenciana project ACOMP/2006/202 to Evaristo Mañanós. Thanks are also due to COST ACTION 867 (Fish Welfare) and FA0801 (Larvanet), where part of these results were presented and discussed. Borja Blanco Vives was supported by an FPI scholarship from the MICINN. Thanks are also due to the staff of IEO at Santander for kindly providing the Senegalese sole eggs. The authors would also like to thank Vanesa Piquer and Juan Antonio Rielo for their technical assistance.

LITERATURE CITED

Aida K, and Amano M. 1995. Salmon GnRH gene expression following photoperiod manipulation in precocious male masu salmon. In: Goetz FW, Thomas P. Eds., Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish. University of Texas, Port Aransas, Austin, TX, p 161–163.

331 Barahona-Fernandes MH. 1979. Some effects of light intensity and photoperiod on the sea
332 bass larvae (*Dicentrarchus labrax*) reared at the Centre Oceanologique de
333 Bretagne. *Aquaculture* 17:311-321.

334 Baroiller JF, Guiguen Y, Fostier A. 1999. Endocrine and environmental aspects of sex
335 differentiation in fish. *Cell Mol Life Sci* 55:910–931.

336 Baroiller JF and D’Cotta H. 2001. Environment and sex determination in farmed fish. *Comp.*
337 *Biochem. Physiol. C Toxicol Pharmacol* 130:399– 409.

338 Baroiller JF, D’Cotta H, Bezault E, Wessels S, Hoerstgen-Schwark G. 2009. Tilapia sex
339 determination: Where temperature and genetics meet. *Comp Biochem Physiol A*
340 *Mol Integr Physiol* 153:30–38.

341 Batty RS. 1987. Effect of light intensity on activity and food-searching of larval herring,
342 *Clupea harengus*: a laboratory study. *Mar Biol* 94:323–327.

343 Bennett HS, Wyrick AD, Lee SW, McNeil JH. 1976. Science and art in preparing tissues
344 embedded in plastic for light microscopy, with special reference to glycol
345 methacrylate, glass knives, and simple stains. *Stain Technol* 51:71-94.

346 Blanco-Vives B, Villamizar N, Ramos J, Bayarri MJ, Chereguini O, Sánchez-Vázquez, F. J.
347 2010. Effect of daily thermo- and photo-cycles of different light spectrum on the
348 development of Senegal sole (*Solea senegalensis*) larvae. *Aquaculture* 306:137-
349 145.

350 Blazquez M, Zanuy S, Carrillo M, Piferrer F. 1998. Effects of rearing temperature on sex
351 differentiation in the European sea bass *Dicentrarchus labrax* L. *J Exp Zool*
352 282:207–216.

353 Blázquez M, Navarro-Martín L Piferrer F. 2009. Expression profiles of sex differentiation-
 354 related genes during ontogenesis in the European sea bass acclimated to two
 355 different temperatures. *J Exp Zool* 312:686–700.

356 Bogart MH. 1987 Sex determination: a hypothesis based on steroid ratios. *J Theor Biol*
 357 128:349–57.

358 Borg B. 1994. Androgens in teleost fishes. *Comp Biochem Physiol* 109:219–45.

359 Bromage NR. 1987. The advancement of puberty or time of first-spawning in female rainbow
 360 trout *Salmo gairdneri* maintained on altered seasonal-light cycles. In: Idler DL,
 361 Crim LW, Walsh JM. Eds., *Proceedings of the Third International Symposium*
 362 *on the Reproductive Physiology of Fish*. Memorial University of Newfoundland,
 363 St. John's, Newfoundland, Canada, p 303.

364 Cañavate JP, Zerolo P, Fernandez-Diaz C. 2006. Feeding and development of Senegal sole
 365 (*Solea senegalensis*) larvae reared in different photoperiods. *Aquaculture*
 366 258:368-377.

367 Colombo L, Barbaro A, Francescon A, Libertini A, Bortolusi M, Argenton F, Dalla Valle L,
 368 Vianello S, Belvedere P. 1998. Towards an integration between chromosome set
 369 manipulations, intergeneric hybridization and gene transfer in marine fish
 370 culture. *Cah Opt Mediterran* 34: 77–122.

371 Conover DO. 1984. Adaptive significance of temperature- dependent sex determination in a
 372 fish. *Am Nat* 123:298–313.

373 Conover DO. 2004. Temperature-dependent sex determination in fishes, In: Valenzuela N,
 374 Lance V. Eds., *Temperature-Dependent Sex Determination in Vertebrates*
 375 (Smithsonian Books, Washington) p 11–20.

376 D'Cotta H, Guiguen Y, Govoroun MS, McMeel O, Baroiller JF. 2001. Aromatase plays a key
377 role during normal and temperature-induced sex differentiation of tilapia
378 *Oreochromis niloticus*. Mol Reprod Dev 59:265–276.

379 Devlin RH, Nagahama Y. 2002. Sex determination and sex differentiation in fish: an
380 overview of genetic, physiological, and environmental influences. Aquaculture
381 208:191–364.

382 Dinis MT, Ribeiro L, Soares F, Sarasquete C. 1999. A review on the cultivation potential of
383 *Solea senegalensis* in Spain and in Portugal. Aquaculture 176:27-38.

384 Downing G and Litvak MK. 1999. The influence of light intensity on growth of larval
385 haddock. N Am J Aquacult 61:135-140.

386 Feist G, Schreck CB, Fitzpatrick MS, Redding JM. 1990. Sex steroid profiles of coho salmon
387 (*Oncorhynchus kisutch*) during early development and sexual differentiation.
388 Gen Comp Endocrinol 80:299–313.

389 Gibson RN, Pihl L, Burrows MT, Modin J, Wennhage H, Nickell LA. 1998. Diel movements
390 of juvenile plaice *Pleuronectes platessa* in relation to predators, competitors,
391 food availability and abiotic factors on a microtidal nursery ground. Mar Ecol
392 Prog Ser 165:145-159.

393 Guzmán JM, Ramos J, Mylonas CC, Mañanós E. 2009a. Spawning performance and plasma
394 levels of GnRH α and sex steroids in cultured female Senegalese sole (*Solea*
395 *senegalensis*) treated with different GnRH α -delivery systems. Aquaculture
396 291:200-209.

397 Guzmán JM, Rubio M, Ortiz-Delgado J, Klenke U, Kight K, Cross I, Sánchez-Ramos I, Riaza
398 A, Rebordinos L, Sarasquete C, Zohar Y, Mañanós E. 2009b. Comparative gene

399 expression of gonadotropins (FSH and LH) and peptide levels of gonadotropin-
 400 releasing hormones (GnRHs) in the pituitary of wild and cultured Senegalese
 401 sole (*Solea senegalensis*) broodstocks. Comp Biochem Phys A 153:266-277.

402 Helfman GS, Collette BB, Facey DE, Bowen B. 2009. The Diversity of Fishes. Biology,
 403 Evolution, and Ecology, 2nd ed. (Blackwell Science, Malden).

404 Herlant M. 1960. Étude critique de deux techniques nouvelles afin de mettre en évidence les
 405 différentes catégories cellulaires présente dans la glande pituitaire. Bull Microsc
 406 Appl 10:37–44.

407 Hontela A, Peter RE. 1983. Entrainment of daily serum gonadotropin cycles in the goldfish to
 408 photoperiod, feeding, and daily thermocycles. J Exp Zool 228:129-134.

409 Hontela A, Peter RE. 1983. Characteristics and functional significance of daily cycles in
 410 serum gonadotropin hormone levels in the goldfish. J Exp Zool 228: 543-550.

411 Hughes V, Benfey TJ, Martin-Robichaud DJ. 2008. Effect of rearing temperature on sex ratio
 412 in juvenile Atlantic halibut, *Hippoglossus hippoglossus*. Environ Biol Fish
 413 81:415–419

414 Johnson CH, Elliott J, Foster R, Honma K, Kronauer R. 2004. Fundamental properties of
 415 circadian rhythms. In: Dunlap JC, Loros JJ, DeCoursey PJ. Chronobiology.
 416 Biological timekeeping. Sunderland, MA, USA: Sinauer Associates, p 67-105.

417 Lokman PM, Harris B, Kusakabe M, Kime DE, Schulz RW, Adachi S, Young G. 2002. 11
 418 oxygenated androgens in female teleosts: prevalence, abundance, and life history
 419 implications. Gen Comp Endocrinol 129:1–12.

420 Mayer I, Shackley SE, Ryland JS. 1988. As of the reproductive biology of the bass
 421 *Dicentrarchus labrax*. A histological and histochemical study of oocyte
 422 development. J Fish Biol 33:609-622.

423 Miura T, Miura C, Ohta T, Nader MR, Todo T, Yamauchi K. 1999. Estradiol-17 β stimulates
 424 the renewal of spermatogonial stem cells in males. Biochem Biophys Res
 425 Commun 264:230–4.

426 Morrison CM, Miyake T, Wright JR. 2001. Histological study of the development of the
 427 embryo and early larva of *Oreochromis niloticus* (Pisces: Cichlidae). J Morphol
 428 247:172–195.

429 Nakamura M. 1984. Effects of 17 β -estradiol-17 on gonadal sex differentiation in two species
 430 of salmonids: The Masu salmon, *Oncorhynchus masu*, and the chum salmon, *O.*
 431 *keta*. Aquaculture 43:83–90.

432 Nakamura M. 2010. The mechanism of sex determination in vertebrates-are sex steroids the
 433 key-factor? J Exp Zool 313A:381-398.

434 Ospina-Alvarez N, Piferrer F. 2008. Temperature-dependent sex determination in fish
 435 revisited: prevalence, a single sex ratio response pattern, and possible effects of
 436 climate change. PLoS ONE 3:e2837.

437 Parra G, Yúfera M. 1999. Tolerance response to ammonia and nitrite exposure in larvae of
 438 two marine fish species (gilthead seabream *Sparus aurata* L. and Senegal sole
 439 *Solea senegalensis* Kaup). Aquac Res 30:857-863.

440 Pavlidis M, Komoundouros G, Sterioti A, Somarakis S, Divanach P, Kentouri M. 2000.
 441 Evidence of temperature dependent sex determination in the European sea bass
 442 *Dicentrarchus labrax* L. J Exp Zool 287:225–232.

443 Porta J, Porta JM, Cañavate P, Martínez-Rodríguez G, Álvarez, MC. 2007. Substantial loss
 444 of genetic variation in a single generation of Senegalese sole (*Solea*

445 *senegalensis*) culture: implications in the domestication process. J Fish Biol
446 71:223-234.

447 Reynolds WW, Casterlin ME, Matthey JK, Millington ST, Ostrowski AC. 1978. Diel patterns
448 of preferred temperature and locomotor activity in the goldfish *Carassius*
449 *auratus*. Comp Biochem Physiol 59A:225-227.

450 Rodriguez L, Zanuy S, Carrillo M. 2001. Influence of day length on the age at first maturity
451 and somatic growth in male sea bass (*Dicentrarchus labrax*, L.). Aquaculture
452 196:159-175.

453 Rougeot C, Prignon C, Ngouana Kengne CV, Mélard C. 2008. Effect of high temperature
454 during embryogenesis on the sex differentiation process in the Nile tilapia,
455 *Oreochromis niloticus* . Aquaculture 276:205–208.

456 Siegfried KR. 2010. In search of determinants: gene expression during gonadal sex
457 differentiation. J Fish Biol 76: 1879-1902.

458 Sims DW, Wearmouth VJ, Southall EJ, Hill JM, Moore P, Rawlinson K, Hutchinson N, Budd
459 GC, Righton D, Metcalfe JD, Nash JP, Morritt D. 2006. Hunt warm, rest cool:
460 bioenergetic strategy underlying diel vertical migration of a benthic shark. J
461 Anim Ecol 75:176-190.

462 Spieler RE, Noeske TA, Devlaming V, Meier AH. 1977. Effects of thermocycles on body
463 weight gain and gonadal growth in goldfish, *Carassius auratus*. T Am Fish Soc
464 106:440-444.

465 Tandler A, Helps S. 1985. The effects of photoperiod and water exchange on growth and
466 survival of gilthead sea bream (*Sparus aurata*, Linnaeus; Sparidae) from
467 hatching to metamorphosis in mass rearing systems. Aquaculture 48:71-82.

468 Taranger GL, Daae H, Jørgensen KO, Hansen T. 1995. Effects of continuous light on growth
469 and sexual maturation in sea water reared Atlantic salmon. In: Goetz FW,

470 Thomas P. Eds., Proceedings of the Fifth International Symposium on the
471 Reproductive Physiology of Fish. University of Texas, Port Aransas, Austin, TX,
472 p 200.

473
474 Taranger GL, Haux C, Stefansson SO, Bjorsson BT, Walther BTh, Hansen T. 1998. Abrupt
475 changes in photoperiod affect age at maturity, timing of ovulation and plasma
476 testosterone and estradiol-17 β profiles in Atlantic salmon, *Salmo salar*.
477 Aquaculture 162:85–98.

478 Vallone D, Lahiri K, Dickmeis T, Foulkes N. 2007. Start the clock! Circadian Rhythms and
479 development. Dev Dynam 236:142-155.

480 Yúfera M, Parra G, Santiago R, Carrascosa M. 1999. Growth, carbon, nitrogen and caloric
481 content of *Solea senegalensis* (Pisces: Soleidae) from egg fertilization to
482 metamorphosis. Mar Biol 134:43-49.

483

484 FOOTNOTES

485 All the authors have read the paper and have agreed to have their names listed as
486 authors.

487

488 FIGURE LEGENDS

489 Fig. 1. Validation of the ELISA method for analysis of steroids in Senegal sole muscle
490 samples. Graphs show the parallelism between the standard curves (black
491 triangles) of testosterone, T (A), 11-ketotestosterone, 11-KT (B) and estradiol,
492 E₂ (C) and serial dilutions of Senegal sole muscle samples (black circles).

Fig. 2. Increase of length (A) and mass (B) of Senegal sole juveniles in the experimental groups subjected to different thermocycles. Data are expressed as mean±S.E.M. The sample sizes are N=20 in each group. Letters indicate significant differences ($p<0.05$) between groups within each sampling point (age DPH).

Fig. 3. Percent sexually undifferentiated juveniles Senegal sole under different thermocycles. Data are expressed as mean±S.E.M. The sample sizes are N=20. Different letters indicate means within age significantly different from each other ($p<0.05$).

Fig. 4. Gonads of Senegal sole juveniles in TC (A) and CT (B). Ovary (A1) and testis (A2) from fish sampled at 138 DPH. Ovary (B1) and testis (B2) from fish sampled at 152 DPH. Scale bars are 0.5 cm for pictures A1 and B1 and 0.7 cm for pictures A2 and B2.

Fig. 5. Sex ratio of the population (%) in the three Senegal sole groups exposed to the different experimental thermocycles. Data are expressed as mean±S.E.M. The sample sizes are N=20. Different letters indicate significant differences from each other (capital letters refer to females and lower case letters refer to the males). The asterisk refers to significant differences within each group.

Fig. 6. Concentration of testosterone (T), 11-ketotestosterone (11-KT) and estradiol (E_2) in the muscle of Senegal sole juveniles in each of the three experimental groups subjected to different thermocycles. Data are expressed as mean±S.E.M. The sample sizes are N=16. Small letters indicate significant differences ($p<0.05$) between groups for each steroid.

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