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

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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
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 (E$_2$) 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 E$_2$ (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**
Animals and housing

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

To feed the larvae, *Brachionus picatilis* rotifers were enriched with *Tetraselmis suecica*, *Isochrysis galgana* and commercially available freeze-dried green algae *Nannochloropsis* sp. (Phytobloom Prof® Necton, Portugal) in a proportion of 300,000 cells/ml/day from day 3 to day 7. These enriched rotifers were added to tanks daily as an early live food at an increasing density of 10-20 individuals ml⁻¹ from 3 to 7 DPH. *Artemia* sp. nauplii at a density of 2–3 nauplii ml⁻¹ day⁻¹ were introduced from 8 to 30 DPH. Three to five metanauplii ml⁻¹ day⁻¹ were added from 27 to 30 DPH. Before being provided to the larvae, the metanauplii were enriched with a mixture (ORI-GO, ORI-PRO®, Skretting AS, Spain) of phytoproteins and highly unsaturated fatty acids (HUFA) for 24 hours. From 30 DPH onwards, larvae were fed with dry food (Gemma micro Diamond®, Skretting AS, 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 cylindroconical 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
extraction procedure was based on that described by Feist et al. ('90). Prior to extraction, muscle samples were thawed, finely chopped with a razorblade and homogenized for 20-30 s with 1.5 ml of methanol in a 12 × 75 mm glass culture tube using the Tissue TearorTM motorized homogenizer. The homogenates were centrifuged (1000 g for 10 min) and the aqueous lower phase and pelleted insoluble material were snap frozen in liquid nitrogen. The methanol extract was decanted into a new tube and the lower phase and pellet thawed, mixed with 1.5 ml of methanol, and re-centrifuged. The sample was snap frozen again and the second methanol extract was combined with the first and dried at 37°C under a stream of nitrogen gas. The remaining fraction was double extracted with two 1.5 ml volumes of ethyl ether to remove any remaining particulates and the combined ether extracts were dried at 37°C under nitrogen and finally resuspended in 400 µL of assay buffer before ELISA analysis.

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

**Statistical analysis**

To establish statistical differences in growth and steroid levels between treatments, a one-way ANOVA and Duncan's test were performed, with P<0.05 taken as the statistically significant threshold. Regarding the sex ratio, two one-way ANOVA tests were carried out, 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 analyse were carried out with SPSS 15.0 for Windows. Data are expressed as mean±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 ± 2.6%) than males (21.2 ± 3.4%), whereas sole exposed to CT showed a proportion of males (82.5 ± 5.8%), which was significantly higher than the percentage of females at 152 DPH (17.5 ± 7.6%) (ANOVA, Student’s t-test, p=0.032). Finally, fish under constant temperature showed a greater proportion of males (61.6 ± 6.5%) than of females (38.3 ± 4.3%) (ANOVA, Student’s t-test, p=0.041). (Fig. 5).

**Sex steroids**

Levels of E2 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 E$_2$ 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 E$_2$ (Miura et al., ‘99). The biological significance of such abnormal concentrations remains unknown. In our experiment, the considerable difference in the 11-KT to E$_2$ 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 E$_2$ 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.

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LITERATURE CITED


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content of Solea senegalensis (Pisces: Soleidae) from egg fertilization to

FOOTNOTES

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

FIGURE LEGENDS

Fig. 1. Validation of the ELISA method for analysis of steroids in Senegal sole muscle
samples. Graphs show the parallelism between the standard curves (black
triangles) of testosterone, T (A), 11-ketotestosterone, 11-KT (B) and estradiol,
E_2 (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 significantly 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 (E₂) 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.
Total steroid (pg/g)

Steroid

- T
- 11-KT
- E2

TC
CT
Constant