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

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

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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, E_2; 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 E_2, 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

48   environment governed by geophysical cycles such as the Earth's rotation around its axis (e.g.

49   day/night changes in solar radiation), around the sun (seasons) and the moon's rotation around

50   the Earth (lunar and tidal cycles).To match this array of cyclic habitats all living organisms

51   have evolved a great variety of adaptive responses, such as biological clocks to keep time and

52   to synchronise with environmental cycles, and flexible mechanisms by means of which

53   individuals can become male or female. Various extrinsic factors have been observed to

54   influence the sex ratio, at least under controlled conditions: for example photoperiod

55   (Bromage, '87; Aida and Amano '95; Taranger et al., '95, '98) and temperature (Colombo et

56   al., '98; Blazquez et al., '98; Pavlidis et al., 2000; Blazquez et al., 2009). The latter factor

57   deserves further consideration since the cyclic infrared radiation from the Sun generates a

58   daily thermocycle: during the day the temperature rises (thermophase, or phase of higher

59   temperature), while during the night the temperature drops (cryophase, or phase of lower

60   temperature). Thus, transitions from cold to warm temperature are roughly associated with

61   dawn, and transitions from warm to cold temperature with dusk (Johnson et al., 2004).

62   However, under artificial rearing conditions, the environmental conditions are set by fish

63   farmers to optimise fish survival and growth, as reported for many aquaculture species

64   (Barahona-Fernandes, '79; Tandler et al., '85; Batty et al., '87; Downing and Litvak, '99), and

65   little attention has been paid to the influence of these daily temperature oscillations in cultured

66   fish.

67   Environmental factors can trigger or determine the process of gonad development in

68   some fish (environmental sex determination, ESD) and can lead to skewed sex ratios in wild

69   or farmed fish (Siegfried, 2010). Moreover, in later stages, these factors can have an impact

70   on the undifferentiated gonads, which are highly susceptible to external stimuli, overriding the

71   genetic sex determination (GSD) and thus switching the fate of the gonad towards the

72   opposite sex. However, the distinction between both mechanisms is not always clear

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73   (Baroiller et al., 2009). In Nile tilapia, the existence of a thermo-sensitive period has been

74   reported in female larvae from 12 to 52 DPH, during which the mortality rate is high and the

75   male proportion increases (Rougeot et al., 2008). This thermo-sensitive window takes place a

76   few hours after fertilization and covers the development of the brain (31 DPH) and the

77   segregation of the primordial germ cells (46 DPH) (Morrison et al., 2001), long before any

78   development of the presumptive gonads.

79   In all vertebrates, sex steroids affect the development of germ cells and other cell-

80   types, as well as the organs involved in sexual differentiation (Devlin and Nagahama, 2002).

81   Estradiol (E_2) is considered to be responsible for inducing and maintaining ovarian

82   development, and its levels are considerably higher in females than in males. Testicular

83   development is mainly regulated by the androgen 11-ketotestosterone (11-KT). In fish in

84   general, testosterone (T) is not directly involved in the mechanisms of sexual differentiation,

85   but participates as precursor of 11-KT and E_2 (Nakamura et al., '84; Baroiller et al., '99).

86   The Senegal sole *Solea senegalensis* Kaup (1858) is a flatfish adapted to temperate

87   waters of around 16-23ºC (Drake et al., '84). This species is extensively exploited in

88   aquaculture, mostly in Spain and Portugal (Dinis et al., '99), although reproduction and

89   culture techniques still need to be optimized (Porta et al., 2007) due to the difficulty of

90   obtaining fertilized eggs from captive broodstock and the commercial interest of this species

91   and the need to get potentially reproductive females and males. In this work, Senegal sole was

92   chosen to investigate the effect of temperature on juvenile sex differentiation. To this end,

93   sole larvae and juveniles were subjected to different temperature cycles (natural or reverse

94   thermocycles vs. constant temperature) to investigate their effect on gonad development and

95   degree of differentiation, sex ratio, and levels of steroid hormones.

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97   **MATERIAL AND METHODS**

Animals and housing

99   Sole eggs were provided by the Spanish Oceanographic Institute (IEO), Santander

100   (Cantabria, Spain). Wild broodstock was maintained in 14 m^3 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   19ºC (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 picatilis* 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

123   The experiments performed in the present research followed Spanish legislation on

124   Animal Welfare and Laboratory Practices. The experimental protocol was approved by both

125   the National Committee on Animal Welfare and the Bioethics Committee of the University of

126   Murcia.

127   At arrival to our facilities, 1 DPH sole larvae were distributed into six 500 L cylindro-

128   conical tanks, at a density of 50 larvae L^{-1}. The water system was semi-closed with an

129   exchange rate of 10-30% seawater and a flow of 50 L/h in each tank per day . Thermal cycles

130   had duration of 24 hours, the photoperiod was 12 h L:12 h D and light intensity was 0.84 W

131   m^{-2} (200 lux), which was supplied by a mercury vapor lamp (PHILIPS, HPL N 250W). The

132   spectral analysis of lights was performed as indicated above, using a spectroradiometer

133   (FieldSpec® Hand Held spectroradiometer, Colorado, USA) with a wavelength range of 325

134   to 1075 nm, an interval of 1.6 nm and a viewing angle of 25 degrees and a lux meter (MX

135   Elektronik Minilux, Germany) with 6 measuring ranges from 2 lux to 200 klux.

136   From 1 to 97 DPH, three temperature regimes were applied per duplicate: TC cycle

137   (22.1±0.6ºC during the day and 19.0±0.4ºC at night, mean±S.E.M, here and throughout); CT

138   cycle (19.2±0.5ºC during the day and 22.0±0.3ºC at night) and constant temperature

139   (20.7±0.4ºC). After 97 DPH temperature was constant (20.5±0.6ºC). Water temperature was

140   controlled by means of two water coolers (Teco-TR-20, Italy and Astralpool alaska-4) and

141   solenoid. Temperature was continuously recorded by an underwater sensor and a data logger

142   (HOBO PENDANT® Onset Computer Corporation, Massachusetts, USA) placed in the tanks.

143   The sex of the larvae was determined was assessed in 20 specimens per treatment

144   every week from 110 DPH to 173 DPH. This time was chosen a previous study of another

145   flatfish, *Hippoglossus hippoglossus*, showed that sexual differentiation occurred on 140 DPH

146   (Hughes et al., 2008).

147   On 247 DPH, sex steroids (T, 11-KT and E_2) were measured in fish muscle by ELISA.

148   The hormone analysis was performed at this time to ensure that steroid levels in the

149   experimental fish were within the limits of the assay sensitivity.

150   *Data collection and histology analysis*

151   Fragments of sole body were fixed in 1 % gluteraldehyde in distilled water for 24 h at

152   room temperature. After dehydration in increasing concentrations of ethanol, tissue fragments

153   were embedded in glycol methacrylate resin (Technovit 7100, Heraeus Kulzer, Germany).

154   Sections of 2 µm thickness were cut with a Supercut 2065 microtome (Reichert-Jung,

155   Germany) and stained with methylene blue / azure II / basic fucsin (Bennet et al., '76). To

156   ascertain fish sex, pictures were taken with a microscope (Leika) (x 10 magnification), and

157   the sex was verified by histological analysis. Histological procedures were carried out

158   following conventional techniques using the Cleveland Wolfe's methodology for staining

159   (Herlant, '60). Ovary and testis were classified according to previous morphological studies

160   on gametogenesis in females (Mayer et al., '88) and males (Rodríguez et al., 2001) fish.

161   *Steroid analysis in muscle by ELISA*

162   Sex steroids, estradiol (E_2), testosterone (T) and 11-ketotestosterone (11-KT), were

163   analyzed by ELISA, according to the method described and validated by Guzmán et al.,

164   (2009a,b). The ELISA method was previously validated for Senegal sole plasma samples and,

165   for this study, the extraction protocol was modified and the assay further validated for muscle

166   samples.

167   For hormone analysis, 500 mg samples of skeletal muscle tissue taken from the muscle

168   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 TearorTM

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 (E_2, 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 E_2.

187   The sensitivities of the ELISAs were 5.2, 8.8 and 0.4 pg ml^{-1}, for the E_2, 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

194   Student's t-test was used to assess differences in the percentage of males and females within

195   each group. All percentage data were normalized and arcsin transformed before statistical

196   analysis. All statistical analyse were carried out with SPSS 15.0 for Windows. Data are

197   expressed as mean±S.E.M. values.

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RESULTS

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Growth performance

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201   The growth of soles under the three experimental thermocycles was similar until 145

202   DPH. At 152 DPH, the juveniles under the TC thermal cycle were larger in size (6.6 ± 0.2

203   cm) than those exposed to CT (5.7 ± 0.2 cm) (ANOVA, Duncan's test, p=0.043). Fish

204   exposed to constant temperature were 6.3 ± 0.2 cm long and showed no statistical differences

205   from the TC and CT groups (Fig. 2).

206   As regards body weight, there were no significant differences between groups until

207   138 DPH. From 145 DPH onwards, juveniles subjected to TC showed a significantly higher

208   weight (2.4 ± 0.1 g) than sole under CT (1.9 ± 0.2 g) (ANOVA, Duncan's test, p=0.033). The

209   mass of fish exposed to a constant temperature was similar at 145 DPH (2.3 ± 0.2 g) to that of

210   the TC and CT groups, but at 152 DPH (2.8 ± 0.2 g) it was higher than the mass of CT fish

211   (Fig. 2).

Gonad development and differentiation

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213   In fish exposed to TC sex differentiation occurred earlier, since at 110 DPH only 10%

214   of sole were not distinct, whereas 45% of fish under CT were still undifferentiated (ANOVA,

215   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

237   Although the effect of (constant) water temperature on larvae development and sex

238   determination in fish is well known, the effect of daily thermocycles has never been explored.

239   The results provided by the present research reveal the strong effect of daily cycles of water

240   temperature on gonad differentiation, sex ratio and sex steroids in *Solea senegalensis.*

241   Therefore, this species should be considered a thermosensitive species, as seen in previous

242   studies investigating other fish species, in which temperature was seen to affect the sex ratio

243   (Blazquez et al., '98; Baroiller et al., 2009). Indeed, water temperature seems to be the most

244   prevalent environmental factor influencing sex determination, as documented in at least 61

245   fish species belonging to very divergent orders (Baroiller et al.,, '99; Baroiller and D'Cotta,

246   2001; Devlin and Nagahama, 2002; Conover, 2004; Ospina-Alvarez and Piferrer, 2008). The

247   sex of amphibians and reptiles is also determined by environmental factors, including

248   temperature-dependent sex determination (Nakamura, 2010).

249   Conover ('84) observed that sensitivity to environmental factors was directly related to

250   the change in growth rate induced by these factors, suggesting an adaptive role of

251   environmental determination in fish species. In our study the group of fish showing the

252   greatest growth (size and weight) was that under TC, coinciding with the group that showed a

253   higher proportion of females, while the CT group showed reduced growth and weight and had

254   a higher percentage of males. Fish under constant temperature showed a greater equality of

255   sexes and showed no significant differences in growth and weight with TC or CT, suggesting

256   that temperature cycle (but not average temperature itself) may cause the increased growth

257   and differentiation observed in the females exposed to TC.

258   Some studies have demonstrated the existence of daily rhythms of temperature

259   selection in fish in wild conditions. In such studies, fish showed daily migrations as they

260   searched for a preferred temperature for physiological activity and growth (Gibson et al., '98;

261   Sims et al., 2006). In Senegalese sole, most studies on biological development and

temperature have used a constant temperature of 20ºC (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 E_2: 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 fluvialitilis* 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),

287   while the concentration of E_2 was significantly higher (Fig. 6). Some studies have reported

288   that in several species females have similar 11-KT blood concentrations to males (Borg, 1994;

289   D'Cotta et al., 2001; Lokman et al., 2002), while males can display high levels of E_2 (Miura

290   et al., '99). The biological significance of such abnormal concentrations remains unknown. In

291   our experiment, the considerable difference in the 11-KT to E_2 ratio (0.72) between mixed-

292   sex progenies strongly suggest that sex differentiation in Senegal sole is closely controlled by

293   this ratio, as in Eurasian perch (Rougeot et al., 2007), where an excess of E_2 induces the

294   female differentiation process while an excess of 11-KT induces the male differentiation

295   process. This hypothesis, suggested by Baroiller et al., ('99) and Bogart ('87), is supported by

296   results obtained in the present study, as sex differentiation of sole appeared to be correlated

297   with the sex steroids ratio.

298   During larval development, temperature and light cycles are required for the circadian

299   clock to work properly. In fish, the circadian clock matures extremely early during larval

300   development (within 24-48 h) and is thought to regulate the temporal co-ordination of many

301   physiological processes (Vallone et al., 2007). In the present study, differences in the

302   development of the circadian system of sole under different thermal cycles may explain

303   differences in development and, thus, in gonad differentiation and the sex ratio. This

304   hypothesis is supported by ongoing research aiming at characterising rhythmic clock gene

305   expression in sole larvae, which appears very early (Dr. Muñoz-Cueto, personal

306   communication) and is very probably influenced by light and temperature conditions during

307   sole ontogeny.

308   In conclusion, the present paper has revealed that daily thermocycles applied during

309   early larval development have a strong impact on gonad development and the sex ratio, as

310   well as on sex steroids concentrations. These findings should be considered when designing

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311 larva rearing protocols to manipulate the sex ratio of Senegalese sole in aquaculture, since

312 such protocols aim to produce more females, which have better growth performance.

313

ACKNOWLEDGEMENTS

325

LITERATURE CITED

327 Aida K, and Amano M. 1995. Salmon GnRH gene expression following photoperiod

328   manipulation in precocious male masu salmon. In: Goetz FW, Thomas P. Eds.,

329   Proceedings of the Fifth International Symposium on the Reproductive

330   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 gycol

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. Adaptative 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 GnRHa and sex steroids in cultured female Senegalese sole (*Solea

395   senegalensis*) treated with different GnRHa-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.

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

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

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

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

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

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

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

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

Porta J, Porta JM, Cañavate P, Martínez-Rodríguez G, Álvarez, MC. 2007. Substantial loss 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, Morrit 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-17β 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

FOOTNOTES

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

486 authors.

487

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   E_2 (C) and serial dilutions of Senegal sole muscle samples (black circles).

493   Fig. 2. Increase of length (A) and mass (B) of Senegal sole juveniles in the experimental

494   groups subjected to different thermocycles. Data are expressed as mean±S.E.M.

495   The sample sizes are N=20 in each group. Letters indicate significant differences

496   (p<0.05) between groups within each sampling point (age DPH).

497   Fig. 3. Percent sexually undifferentiated juveniles Senegal sole under different thermocycles.

498   Data are expressed as mean±S.E.M. The sample sizes are N=20. Different

499   letters indicate means within age significantly different from each other

500   (p<0.05).

501   Fig. 4. Gonads of Senegal sole juveniles in TC (A) and CT (B). Ovary (A1) and testis (A2)

502   from fish sampled at 138 DPH. Ovary (B1) and testis (B2) from fish sampled at

503   152 DPH. Scale bars are 0.5 cm for pictures A1 and B1 and 0.7 cm for pictures

504   A2 and B2.

505   Fig. 5. Sex ratio of the population (%) in the three Senegal sole groups exposed to the

506   different experimental thermocycles. Data are expressed as mean±S.E.M. The

507   sample sizes are N=20. Different letters indicate significantly differences from

508   each other (capital letters refer to females and lower case letters refer to the

509   males). The asterisk refers to significant differences within each group.

510   Fig. 6. Concentration of testosterone (T), 11-ketotestosterone (11-KT) and estradiol (E_2) in

511   the muscle of Senegal sole juveniles in each of the three experimental groups

512   subjected to different thermocycles. Data are expressed as mean±S.E.M. The

513   sample sizes are N=16. Small letters indicate significant differences (p<0.05)
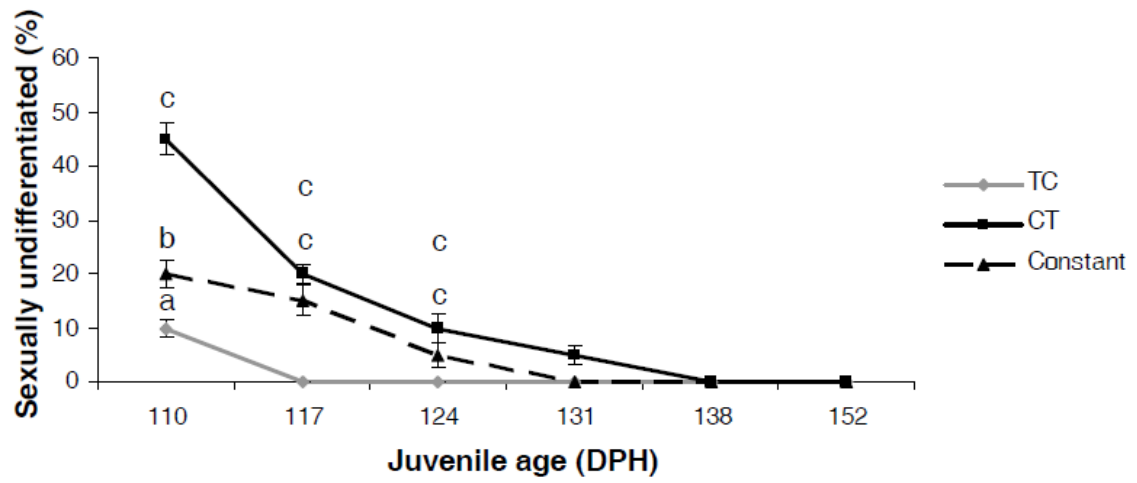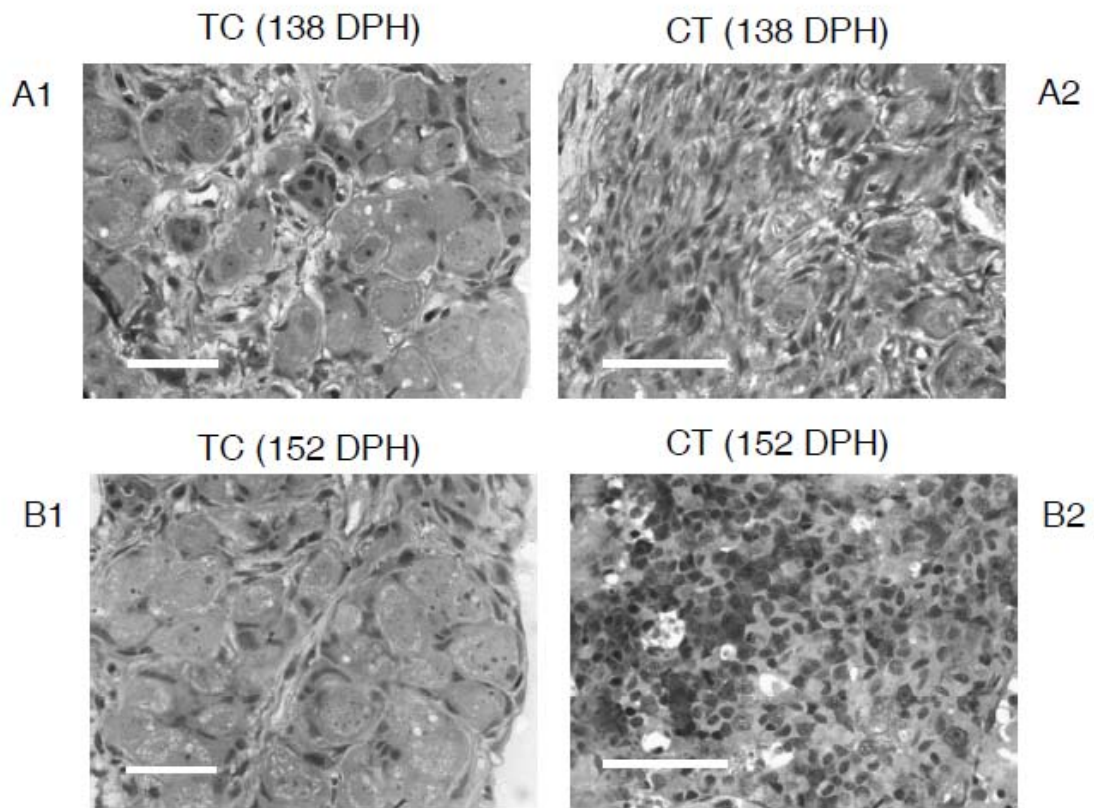
514   between groups for each steroid.

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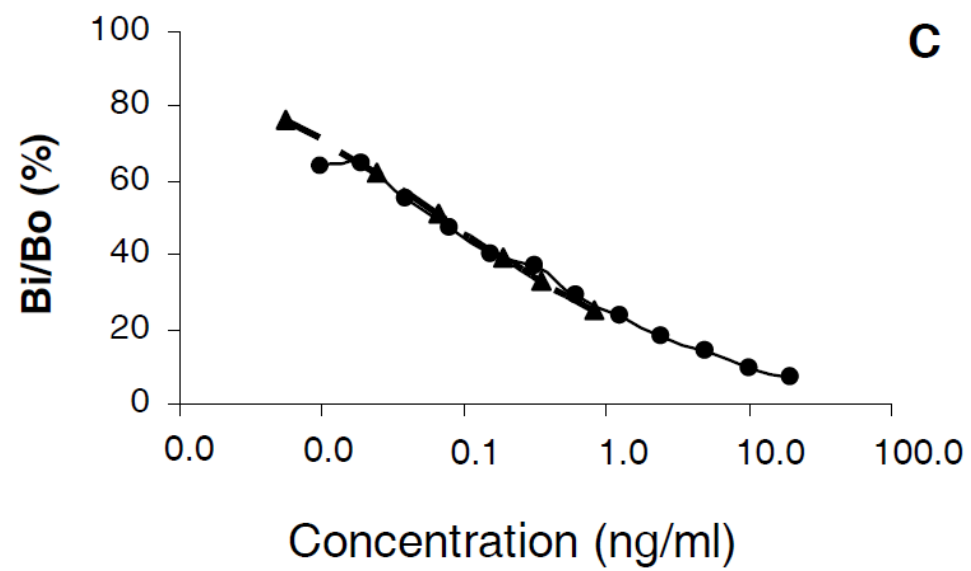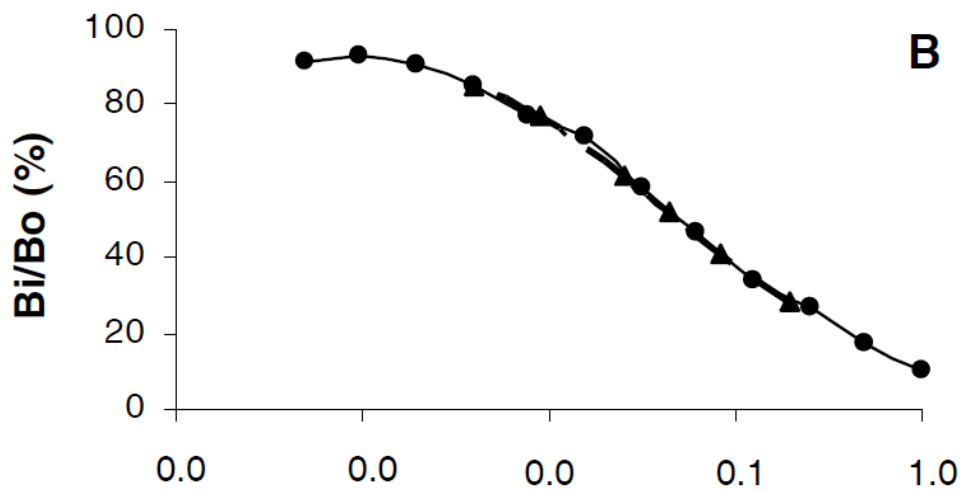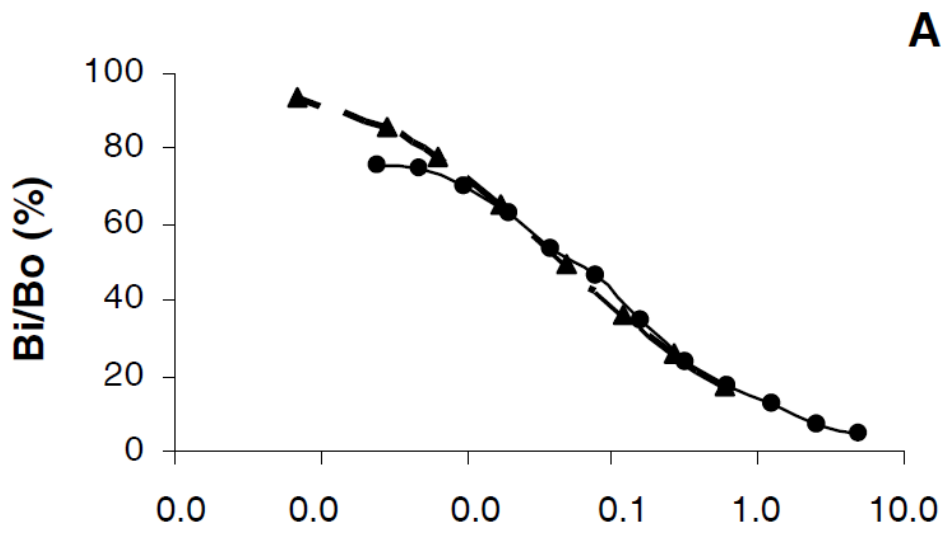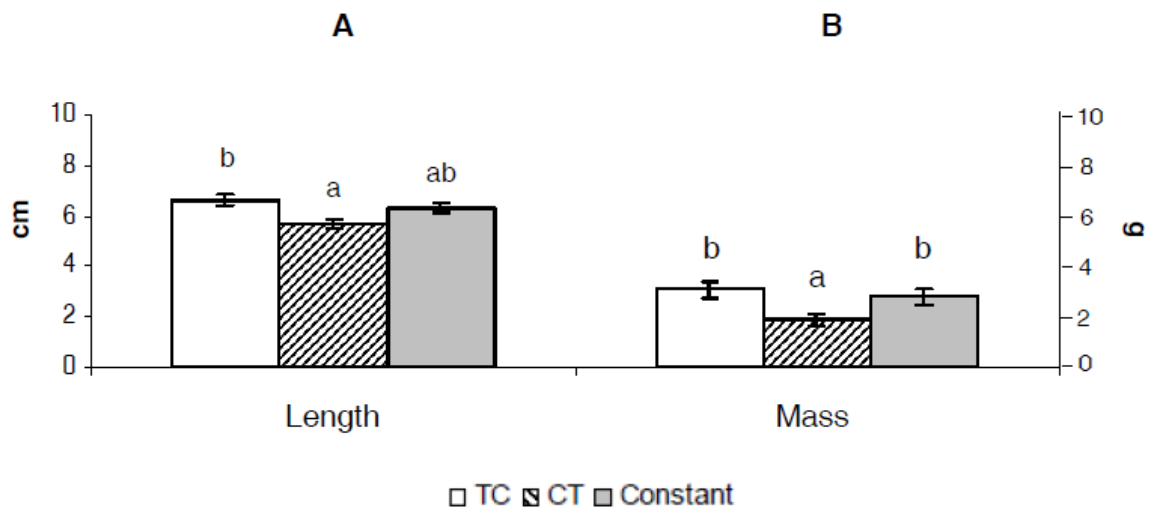
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A1 — TC (138 DPH)   A2 — CT (138 DPH)

B1 — TC (152 DPH)   B2 — CT (152 DPH)

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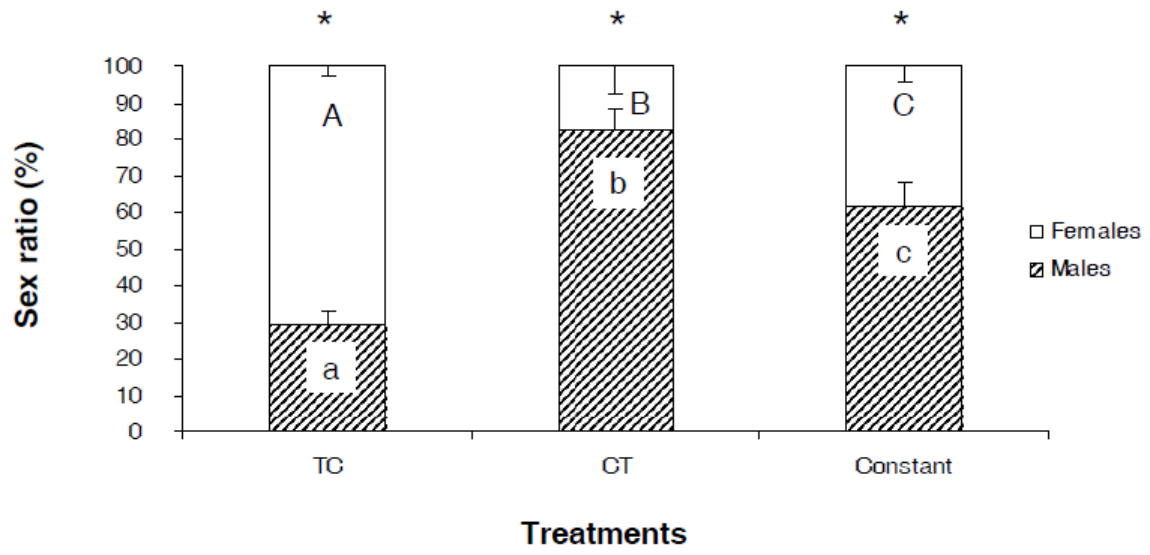559

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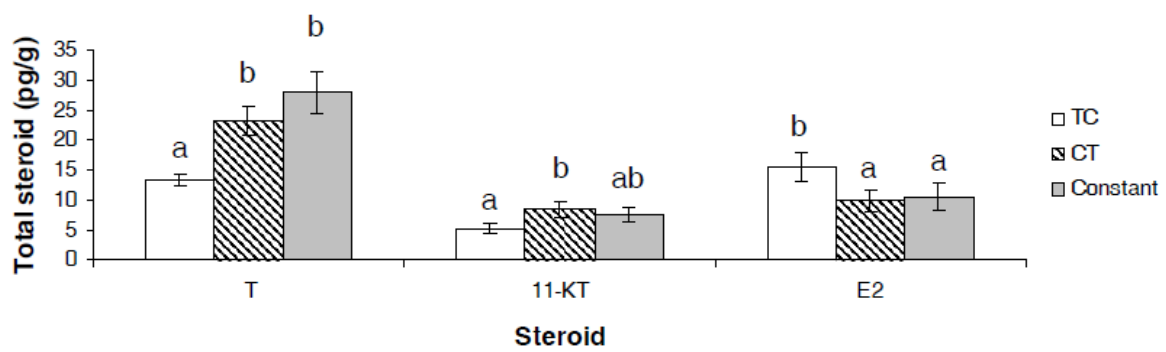585