1 # Optimizing rearing and welfare in Senegalese sole (*Solea senegalensis*) broodstock: effect of ambient light intensity and handling time on stress response

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5 Filipe Figueiredo[1,2], Cláudia Aragão[1,3], Wilson Pinto[1,4], Maria Teresa Dinis[1], Catarina
6 Oliveira[1,3]*

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8 [1] CCMAR, Centro de Ciências do Mar, Campus de Gambelas, edf. 7, 8005-139 Faro, Portugal

9 [2] Norwegian College of Fishery Science, University of Tromsø, Tromsø N-9037, Norway

10 [3] Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

11 [4] SPAROS, Lda., Área Empresarial de Marim, Lote C, 8700-221 Olhão, Portugal

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13 * corresponding author's email: ccoliveira@ualg.pt

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Abstract

Broodstock rearing conditions and handling procedures should be optimized in aquaculture species in order to benefit fish welfare and guarantee optimal conditions for spawning. In teleosts, basal cortisol levels display daily rhythms, oscillating along the 24h of the day. In this sense, handling fish at different moments of the day may lead to different stress responses. The present study aimed at investigating the optimal rearing conditions for Senegalese sole broodstock, considering ambient light intensity and handling time. The optimal light intensity (50, 100 or 200 lx) was investigated by measuring fish cortisol levels and monitoring locomotor activity rhythms under each intensity tested. Results showed a significant increase in cortisol levels of fish exposed to 200 lx, when compared to values obtained under 100 lx, accompanied by changes in locomotor activity rhythms in both tanks under study. These results suggested that 200 lx may be too high as light intensity for this species, whereas 100 lx seems to be more adequate. Also, daily rhythms of stress response were investigated in breeders from different origins (Wild and first generation, G1). Basal cortisol levels and cortisol stress response after an acute stressor (air exposure) were monitored at two distinct moments of the day (Mid-Light and Mid-Dark). Basal levels were higher during the day in the wild group, while G1 fish seemed to have lost the daily fluctuations in basal cortisol plasma levels, as well as their daily rhythms of locomotor activity. Both groups showed lower stress responses during night-time, an indication that this is an adequate period of the day to handle this species. Senegalese sole breeders born in captivity presented more pronounced stress responses when compared to wild fish, reflecting their different life history in terms of stress challenges.

1. Introduction

Senegalese sole, *Solea senegalensis*, is among the most interesting and promising species for European marine aquaculture diversification (**Imsland et al., 2003**; **Morais et al., 2016**). While its culture potential has been periodically affirmed by different authors (**Howell, 1997; Dinis et al., 1999**; **Imsland et al., 2003**), intensive production has been slow to take off (**Morais et al., 2016**). Several studies have contributed to the knowledge of the species biology and requirements for production, namely in larval rearing, nutritional requirements and metabolism (**Aragão et al., 2004; Morais et al., 2005, Martins et al., 2011; Bonacic et al., 2016**), genetics, pathologies and skeletal malformations (**Gavaia et al., 2002; Soares et al., 2002; Porta et al., 2006, Fatsini et al., 2016**). Despite this large body of information available on Senegalese sole rearing, broodstock reproduction problems persist, with low fertilization rates (**Chauvigné et al., 2016**), irregular or incomplete spawning (**Agulleiro et al., 2006**) and complete reproductive failure (**Carazo et al., 2011**) being reported in first generation (G1) individuals. The inability of G1 individuals to naturally reproduce in captivity (**García-López et al., 2006; Guzmán et al., 2008**) forces the aquaculture industry to rely only on wild broodstock. This situation is unsustainable, as wild Senegalese sole are already endangered due to fishing pressure (**Morais et al., 2016**) and preservation of its spawning stock biomass is paramount for the recovery of the species.

Photoperiod and temperature cycles play key roles in determining the natural spawning behaviour of *Solea senegalensis'* broodstock (**Oliveira et al., 2009a; Oliveira et al., 2009b; Oliveira et al., 2011**), but the definition of adequate spectral composition and light intensity has been overlooked in the research involving its rearing environment (**Imsland et al., 2003; Cañavate et al., 2006**). The specificity of light perception in terms of spectrum and intensity (**Migaud et al., 2006; Oliveira et al., 2007**) means that lighting systems should be tailored to suit species requirements and preferences.

In an aquaculture environment, altered environmental conditions and routine practices such as handling, sorting, grading or transport, can induce stress in fish that has detrimental effects to health and development (**Barton and Iwama, 1991; Bonga, 1997; Guerreiro and Ciarcia, 2006; Mosconi et al., 2006; Brijs et al., 2018**). Exposure to stressful factors triggers a coping mechanism as a cascading physiological response. A steep rise in cortisol, the main corticosteroid in fish and the most commonly measured indicator of stress (**Small et al., 2008**), aims to increase circulating glucose levels to compensate for higher energy demand (**Mommsen et al., 1999; Mosconi et al., 2006**). Ultimately, stressors result in changes in

performance and fitness, causing reduced resistance to pathogens, inhibition of growth and, especially, reproductive failure (**Barton and Iwama, 1991; Bonga, 1997; Barton, 2002; Tort, 2011**). According to **Schreck (2010)**, cortisol negatively affects egg quality, ovulation/spermiation, mating behaviour and mate selection. Broodstock welfare is crucial in solving issues preventing natural reproduction, so, providing animals with favourable husbandry conditions can positively influence the success of a production cycle. This can be achieved by minimizing the impact of stress factors such as inadequate lighting or handling.

Fish display rhythms of varying frequencies in almost every physiological activity (**Singh et al., 2009**). Locomotion, growth, reproduction and immune physiology rhythms are synchronized by extrinsic stimuli that change or reset their phase (**Cermakian & Sassone-Corsi, 2002; Volkoff et al., 2009;**). As cortisol secretion also follows a daily rhythm in fish (**López-Olmeda et al., 2009; Oliveira et al., 2013**), it can be hypothesized that the intensity of stress response is also time-dependent. This leads to the assumption that acute stress response can have a lower impact on an individual when handled at a specific time of day. Indeed, the modulation of stress response in relation to the time of day has been observed in green sturgeon (*Acipenser medirostris*) (**Lankford et al., 2003**), sea bream (*Sparus aurata*) (**Vera et al., 2014**) and Senegalese sole (**López-Olmeda et al., 2013**). The latter authors described different cortisol responses according to the time of day in juvenile G1 soles, however there is no available information for adult breeders, nor on the existence of differences between captivity bred (G1) and wild individuals. It is known that inappropriate environmental conditions seriously compromise fish larvae welfare and early development (**Villamizar et al., 2011**). In Senegalese sole, light characteristics (both photoperiod and spectrum) strongly influenced the development of the biological clock, modifying the onset of daily activity rhythms and bringing out different behavioural responses in larvae (**Blanco-Vives et al., 2012**). Such fact may have implications in the circadian system of the adult animal, thus differences between G1 and Wild soles in terms of cortisol response rhythms deserve to be pursued. Taking into consideration that the use of individuals bred in captivity as broodstock is important towards the development of a species' aquaculture, it is essential that good welfare conditions are attained.

With all this in mind, the present research aims to investigate the optimal rearing conditions for Senegalese sole broodstock, considering light intensity and handling time. Optimal light intensity (50, 100 or 200 lx) has been investigated for wild breeders, by measuring fish cortisol basal levels and monitoring locomotor activity rhythms, while daily rhythms of stress response were characterized for G1 and wild breeders, through the evaluation of stress response at different times of day.

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2. Material and Methods

Experimental procedures were conducted in accordance with the guidelines of the European Directive (2010/63/EU) and Portuguese legislation for the use of laboratory animals. The Centre of Marine Science (CCMAR) facilities and their staff are certified to house and conduct experiments with live animals (Group-C licenses by the Direção Geral de Alimentação e Veterinária, Ministério da Agricultura, Florestas e Desenvolvimento Rural, Portugal). Whenever necessary, experimental procedures were performed under anaesthesia with 2-phenoxyethanol and every effort was made to minimize suffering at all stages.

2.1. Animals and housing

All experiments took place at the CCMAR Research Station *Ramalhete* (Faro, Portugal), using the existing Senegalese sole broodstock, previously acclimated to captivity conditions. Fish were kept indoors in four circular, 3 m^3 tanks, with 1500 L of seawater. Tanks were supplied with flow-through gravel-filtered seawater at a constant flow (± 4 L/min). A total of 40 *Solea senegalensis* (10 per tank) individually identified with a PIT-tag system (ID100 Implantable Transponder, *Trovan*, The Netherlands) were used for all experiments. For the first experiment, two out of four tanks were used (A1 and A2), containing wild origin individuals long adapted to captivity conditions, with similar fish sizes (1167.8 ± 737.8 g) and sex ratios (1:1). In the second experiment, the other two Senegalese sole stocks were used: tank B1, containing wild individuals (530 ± 52.9 g) and tank B2, containing first generation breeders – G1 (1308 ± 776.5 g).

Prior to the start of Experiment 1, all four tanks were covered with a light proof black polyethylene tarpaulin, supported by a polyvinyl chloride structure, and equipped with a lighting system (*AquaBeam 600 Ultra, Tropical Marine Centre^{TM}*, Portugal) on the inside. Each tarpaulin had a flap that could be opened and fully closed, providing access to the interior of the tanks for cleaning and feeding. The lighting system was connected to a controller (*AquaRay Control, Tropical Marine Centre^{TM}*, Portugal), that switched lights on/off at a pre-set time. The system was confirmed to be lightproof by measuring light levels inside each tank with a lux meter. All tanks were exposed to natural conditions of photoperiod and temperature before the start of the trials.

Hydrographic parameters such as dissolved oxygen (mg/L), temperature (˚C) and salinity (ppt) were measured daily to monitor the quality of the rearing water. Individuals were

141 fed 6 out of 7 days of the week during the morning, with food alternating between semi-wet

142 feed (*Sparos Lda.*, Portugal*),* polychaete *Nereis virens* (*Inovsea Lda.*, Portugal) or frozen

143 mussel (*Mytilus spp.* kernel, assorted suppliers) injected with a vitamin and mineral premix

144 (formulation developed by *Sparos Lda.*).

145

146 2.2. Experiment 1. Optimization of rearing conditions – light intensity

147   The objective of the first experiment was to determine the optimal light intensity for

148 Senegalese sole broodstock rearing. Fish in the two tanks (A1 and A2) were exposed to a

149 simulated natural autumn photoperiod (SNP) for the *Faro* region (Portugal) (oscillating

150 between 13:11 to 10:14 L:D) throughout the whole experimental period. Photoperiod was

151 adjusted every 2nd or 3rd day. Average water temperature during the experimental period was

152 19.58 ± 1.90 °C.

153   During the first month (September), light intensity (white light) was set to 50 lx. After

154 this period, blood samples were collected as described below. Light intensity was increased to

155 100 lx for the second month (October), after which blood samples were collected again. In the

156 third part of this experiment, light intensity was increased to 200 lx for one more month

157 (November) and blood sampling took place at the end. Experiments were performed without

158 coinciding with *Solea senegalensis'* spawning season to avoid possible confounding factors

159 from reproductive behaviour or hormones (**Anguis and Cañavate, 2005**).

160

161 2.2.1. Sampling

162   All sampling procedures were performed in the morning, at the same time in relation to

163 dawn, avoiding a masking effect from the daily fluctuation of cortisol in blood (**Oliveira et al.,**

164 **2013**). Before sampling, all individuals of each tank were anesthetized in seawater containing

165 800 ppm of 2-phenoxyethanol (77699 Fluka, Sigma-Aldrich). When unresponsive to touch,

166 approximately 1 ml of blood was extracted by caudal puncture using heparinized syringes and

167 transferred to heparinized microtubes on ice. All samples were collected within 5 min, counting

168 from the moment the fish were removed from the tank, to prevent a rise in cortisol levels due

169 to sampling stress (**Costas et al., 2011**). Blood was then centrifuged at 3000 x *g* for 15 min at

170 room temperature and plasma frozen at -80 ˚C until posterior analysis.

171

172   2.2.2. Locomotor activity assessment

173   In order to evaluate how increasing light intensities would affect fish behaviour,

174   locomotor activity was continuously monitored and recorded in both tanks, enabling the

175   quantification of the daily rhythms of locomotor activity.

176   Prior to the experiment, each tank had been equipped with an infrared motion-sensor

177   photocell, installed on the inside wall, 5 cm from the bottom, and pointing towards the centre

178   of the tank. Photocells were individually connected to a relay in a small electrical circuit, and

179   from there to a motherboard (*USB-1024HLS*, *Measurement Computing*^{TM}, Norton,

180   Massachusetts, USA) connected to a computer. Every time an individual interrupted the

181   infrared beam emitted by the photocell, an electrical output signal was produced, which was

182   then converted into a digital signal by the motherboard, and stored in 10 min bins using software

183   specially designed for that purpose (*DIO98USB*, University of Murcia, Spain) **(Oliveira et al.,**

184   **2017; Vera et al., 2009**).

185

186   2.3. Experiment 2. Daily rhythms of stress response in *Solea senegalensis*

187   The second experiment aimed to describe stress response rhythms of *Senegalese sole*

188   breeders in relation to their origin (wild *vs* G1), using animals held in tanks B1 and B2.

189   Considering that cortisol daily rhythms may be affected by the daily patterns of behaviour

190   (**López-Olmeda et al., 2009**), a pre-trial was performed to characterize the circadian system of

191   these stocks in the first place. Daily activity rhythms were attained by monitoring locomotor

192   activity continuously in both tanks during one month prior to the beginning of Experiment 2.

193   As described for Experiment 1, activity was monitored using the previously settled photo-

194   cellular system.

195

196   2.3.1. Sampling

197   Basal and post-stress cortisol levels were assessed for both groups at Mid-Light (ML),

198   the mid-point between dawn and dusk, and Mid-Dark (MD), the equivalent point, but between

199   dusk and dawn. At least a three weeks interval was taken between two sampling points to allow

200   total fish recovery. As in the previous experiment, trials took place between September and

201   January, as to not coincide with *Solea senegalensis'* spawning season, thus avoiding possible

202   confounding factors. Water temperature during the experimental period was 17.16 ± 3.93 °C.

203

204   Sampling procedure for both ML and MD basal levels was similar to that described for
205   Experiment 1: blood was withdrawn from the caudal vein of anaesthetised fish within 5 min
206   from the moment the fish were collected from the tank. For the post-stress ML and MD
207   sampling points, a stressor (air exposure) was applied before blood sampling: individuals were
208   netted from the rearing tank and left out of the water for 3 min and then were returned to the
209   experimental tanks. Blood samples were collected one hour after the applied stressor (**Costas**
210   **et al., 2011**), following the same procedure described above. Sampling was performed under
211   white light during the day and under a dim red light during the night, when Senegalese sole's
212   heads were also covered with aluminium foil (**Oliveira et al., 2007**).

213

214   2.4. Cortisol analysis

215   All plasma cortisol levels were measured using a commercial cortisol Enzyme-Linked
216   Immunosorbent Assay (ELISA) kit (IBL International GmbH, Germany), previously validated
217   for Senegalese sole elsewhere (**Oliveira et al., 2013**). For basal cortisol levels (experiments 1
218   and 2), a kit with a sensitivity of 0.5 ng/mL, and intra- and inter-assay coefficient variation
219   (CV) of 4.7 and 8.2%, respectively, was used, while post-stress cortisol samples (experiment
220   2) were analysed with a similar kit but with a sensitivity of 2.5 ng/mL, and intra- and inter-
221   assay CV of 7.5 and 17%, respectively.

222

223   2.5 Statistical analysis

224   Statistical analysis and data plotting were performed using Excel®, Sigmaplot® and
225   chronobiology specific software *El Temps* (Prof. A. Díez Noguera, University of Barcelona,
226   Spain). Results were expressed as mean ± standard error of the mean (SEM). Cortisol basal
227   plasma levels under different light intensities (Experiment 1) were tested by means of a one-
228   way ANOVA, followed by Tukey's post-hoc test ($p<0.05$). Basal and post-stress cortisol values
229   observed in fish of different origin and at different moments of the day (Experiment 2) were
230   tested for significant differences by means of a two-way ANOVA, with *origin* (wild x G1) and
231   *time of day* (ML x MD) as factors, followed by Tukey's post-hoc test ($p<0.05$).
232   In order to identify behaviour rhythmicity patterns for each group (Experiments 1 and
233   2), locomotor activity data was plotted in the form of actograms and mean waveforms using the
234   software *El Temps* and Sigmaplot®. An actogram is a graphical representation of the locomotor
235   activity along successive day cycles double-plotted on successive horizontal lines, while a mean
236   waveform represents the mean activity along the 24h cycle. Senegalese sole were either

237 categorized as diurnal or nocturnal, depending on when (i.e., day or night, respectively)
238 locomotor activity occurred the most. Significant differences between mean diurnal and
239 nocturnal activity counts were evaluated using a Mann-Whitney rank sum test (significant
240 threshold of $p < 0.05$). When no statistical differences between diurnal and nocturnal activity
241 means were observed for a certain period, the animals were considered arrhythmic (**Vera et al.,**
242 **2009**). Statistical significance of activity daily rhythms was evaluated by COSINOR analysis
243 using the same software: "Amplitude" (one-half the peak-to-trough variation), "mesor" (time
244 series mean), and "acrophase" (peak time relative to the time scale) were determined by least-
245 squares approximation of cosine function (significant threshold of $p < 0.05$) (**Cornelissen,**
246 **2011; Díez, 2007**).

247

248 ## 3. Results

249 ### 3.1. Experiment 1. Optimization of rearing conditions – light intensity

250   Basal cortisol levels varied between the three sampling points of the experiment,
251 coinciding with increasing light intensities during the day period of a SNP (**Figure 1**). After
252 one month of exposure to 50 lx, cortisol plasma levels were 29.09 ± 1.93 ng/mL, decreasing
253 slightly to 16.22 ± 0.99 ng/mL under 100 lx light intensity. The increase of light intensity to
254 200 lx lead to a significant rise ($p<0.05$) in cortisol production (44.47 ± 2.18 ng/mL) (One-way
255 ANOVA, Tukey's post-hoc test, $p<0.05$).

256   Concerning locomotor activity patterns, when experimental groups were held under 50
257 lx during daytime, the actograms (**Figures 2a, d**) and mean waveforms (**Figures 2c, f**) showed
258 higher activity during daylight hours in both tanks (82 and 86% respectively for A1 and A2),
259 starting at 7h10, decreasing from 20h00 onwards and staying at a minimum during the night
260 hours. Counts per 10 min averaged at 2.81 and 4.93 during the day for A1 and A2 individuals,
261 respectively, while during the night that average decreased to 0.70 and 0.95. Such difference in
262 activity levels between day and night periods was confirmed to be statistically significant for
263 both tanks ($p<0.001$; Mann-Whitney rank sum). The COSINOR analyses (**Figure 2b, e**)
264 corroborated the rhythmicity in locomotor activity in both tanks ($p<0.001$): peak acrophase
265 times were at 12h13 and 14h30, respectively for A1 and A2, mesor was 1.86 and 3.01 counts/10
266 min and amplitudes were 1.70 and 2.73 counts/10 min.

267   Under 100 lx, locomotor activity (**Figure 3**) followed a similar pattern to that observed
268 under 50 lx, with higher activity registered during the day (70 and 74%) and a clear decrease
269 after sunset, as evidenced by the significant statistical differences in activity levels between day

270 (2.40 and 5.63 counts/10 min) and night hours (1.00 and 1.80 counts/10 min) ($p<0.001$; Mann-
271 Whitney rank sum). COSINOR analysis (**Figure 3c, f**) proved once more the significance of
272 the daily rhythm in both tanks ($p<0.001$) and, as in the previous phase of the trial, acrophase
273 occurred earlier in A1 (10h36) individuals than in A2 (14h00). Mesor was 1.65 and 3.58
274 counts/10 min and amplitude 1.17 and 2.94 counts/10 min, respectively for A1 and A2.

275 In the last phase of the trial, when animals were reared under 200 lx during daytime,
276 locomotor activity daily pattern changed in tank A1: activity increased during night time (68%)
277 and exhibited its peak before sunrise (**Figure 4a, b, c**). Significant differences between day
278 (2.03 counts/10 min) and night (3.13 counts/10 min) mean locomotor activity were seen
279 ($p<0.05$; Mann-Whitney rank sum) and the COSINOR analysis (**Figure 4b**) indicated
280 rhythmicity, with acrophase occurring at 5h40, a mesor of 2.95 counts/10 min and an amplitude
281 of 2.17 counts/10 min. As for the A2 individuals, daily pattern was not so clear, as suggested
282 by the actogram (**Figure 4d**) and mean waveform (**Figure 4f**). No statistically significant
283 differences were detected between day (1.50 counts/10 min, 52%) and night activity levels (1.00
284 counts/10 min, 48%), thus this tank was considered arrhythmic. However, COSINOR analysis
285 (**Figure 4e**) revealed significant rhythmicity ($p<0.001$), with acrophase taking place at 8h36, a
286 mesor of 1.26 counts/10 min and an amplitude of 0.69 counts/10 min.

287

288 3.2. Experiment 2. Daily rhythms of stress response in *Solea senegalensis*
289 3.2.1. Activity patterns characterization
290 Wild Senegalese sole (**Figure 5 a, b, c**) demonstrated a clear daily rhythm in locomotor
291 activity, with higher activity levels observed during the day (95%), averaging at 16.36 counts/10
292 min. After sunset, that activity decreased to a minimum of 1.15 counts/ 10 min, making this
293 discrepancy between day and night statistically significant ($p<0.001$; Mann-Whitney rank sum).
294 Regarding the G1 individuals, locomotor activity occurred either during day (3.00 counts/10
295 min) or night (2.60 counts/10 min) periods, as illustrated by the actogram (**Figure 5d**) and mean
296 waveform (**Figure 5f**). Since no statistical differences were observed between day and night
297 activity levels ($p<0.05$; Mann-Whitney rank sum), these fish were considered arrhythmic. The
298 different shapes of the mean waveforms of both groups is reflected on the COSINOR analysis
299 results (**Figure 5b, e**): whilst in Wild individuals a significant daily rhythm was described

300 (p<0.001, acrophase located at 11h27, mesor and amplitude 9.86 and 11.57 counts/10 min,
301 respectively), the same did not happen in the G1 group.

302

303 3.2.2. Cortisol stress response rhythms

304   The determination of basal and post-stress cortisol levels at ML and MD revealed a clear
305 influence of time of day in both cases (**Figure 6**). Basal levels exhibited opposite rhythms in
306 relation to fish origin: in wild individuals, levels were higher during the day (60.22 ± 18.31 *vs*
307 13.22 ± 3.89 ng/mL), while for G1 there was a tendency for higher plasma concentrations
308 during night time (19.63 ± 4.45 *vs* 7.07 ± 3.28 ng/mL) (**Figure 6a**). Cortisol levels were seen
309 to be influenced both by fish *origin* ($F_{(1, 38)} = 7.319$, $p<0.05$) and by the interaction of both
310 factors (*origin* x *time of day*: $F_{(1, 38)} = 11.89$, $p<0.01$) and significant differences were detected
311 among sampling points (ML *vs* MD) in the wild group, and between ML samplings of groups
312 with different origins (Wild ML *vs* G1 ML) (Two-Way ANOVA, Tukey's post-hoc test,
313 $p<0.05$).

314   Post-stress cortisol levels (**Figure 6b**) were shown to be influenced by both factors
315 tested (*origin*: $F_{(1, 38)} = 44.37$, $p<0.001$; *time of day*: $F_{(1, 38)} = 57.25$, $p<0.001$), but not by their
316 interaction. First generation individuals had a significantly higher cortisol response in both
317 samplings (597.65 ± 20.19 and 256.27 ± 47.40 ng/mL, respectively for ML and MD) when
318 compared to wild individuals (289.87 ± 50.76 and 70.00 ± 23.47 ng/mL. respectively for ML
319 and MD samplings), and stress response was always greater during daytime within each origin
320 group (Two-Way ANOVA, Tukey's post-hoc test, $p<0.05$). The largest increase in cortisol
321 occurred in G1 individuals sampled during the day, with post-stress levels being 84 times higher
322 than basal concentrations.

323

324 4. Discussion

325   The importance of defining the optimal rearing conditions for Senegalese sole
326 broodstock is well illustrated in the present paper by the impact that ambient light intensity has
327 on the perceived welfare of this species. This research also highlights the necessity of taking
328 the time of day into consideration when handling Senegalese sole broodstock in order to
329 minimize the stress response.

330   The relation between metabolic and behavioural results in the first experiment seems to
331 suggest that 200 lx is above the optimum light threshold for *Solea senegalensis* broodstock, by
332 comparison with intensities of 50 and 100 lx. When fish were exposed to this high light intensity

a significant increase in basal levels of plasma cortisol was observed, accompanied by an alteration of behavioural rhythms (one tank became arrhythmic, and the other switched from diurnal to nocturnal behaviour), indicating adjustments at the physiological level. Basal plasma cortisol concentrations in Senegalese sole were previously observed to follow a daily rhythm (**Oliveira et al., 2013**) and values obtained under 100 lx (16.22 ±3.56 ng/mL) are coincident with morning values observed by those authors. After the 200 lx period, cortisol concentration increased to 44.46 ± 7.86 ng/mL, which could be associated with a condition of chronic stress. Previous research revealed that cortisol levels around 30 ng/mL or above in Senegalese sole held at high stocking density (**Costas et al., 2008**), or under an osmotic challenge (**Arjona et al., 2009, Aragão et al., 2008**), are related with chronic stress. Comparisons should, however, be cautious due to the diversity in fish ages and sizes among studies. Under 50 lx, plasma cortisol averaged 29.09 ± 6.97 ng/mL, an intermediate value that did not present significant differences with either sampling points, though it seems high for the species when compared with the same studies cited above for chronic stress. In line with our results, very high or very low light intensities were seen to cause elevated plasma cortisol or glucose levels in orange spotted grouper (*Epinephelus coioides*) (**Wang et al., 2013**), blunt snout seabream (*Megalobrama amblycephala*) (**Tian et al., 2015**) and starry flounder (*Platichthys stellatus*) (**Bögner et al., 2018**), reinforcing the idea that inadequate light intensity can impair fish welfare in aquaculture.

As to locomotor activity, both tanks showed diurnal behaviour under 50 or 100 lx, in contrast to what has been described in the species (**Bayarri et al., 2004; Oliveira et al., 2013**). Such a behavioural profile has been previously monitored and associated with this group of fish, due to long term adaptation to the morning feeding schedule (**Oliveira et al., personal communication**), which is a very potent synchronizer of fish daily rhythms (**López-Olmeda, 2017**). In a captive environment, Senegalese sole broodstock is known to habituate to husbandry routines, synchronizing its peak of activity with feeding time during the day (**Carazo et al., 2013; Morais et al., 2016**). During the periods fish were exposed to 50 and 100 lx intensities, the COSINOR parameters (acrophase, mesor and amplitude) did not show major alterations. The change in diurnal daily rhythms occurred under 200 lx, indicating an instability of the circadian system under such light intensity. Other species also showed modifications in their behaviour after a change in ambient light intensity; e.g. in Nile tilapia (*Oreochromis niloticus*) individual aggression markedly decreased at lower light intensities compared to stronger

365 illumination (**Carvalho et al., 2013**), reinforcing the importance of using a species specific
366 suitable light intensity.
367   The influence of light on fish reproduction has been deeply studied solely in terms of
368 photoperiod (**Bromage et al., 2001; Falcón & Zohar 2018**), with only one study focusing on
369 light intensity (**Konkal & Ganesh 2018**). These authors found an effect on tilapia (*O.*
370 *mossambicus*), for which exposure of breeders to low or high light intensity conditions affected
371 the spermatogenetic process, possibly through the suppression of LH secretory activity in the
372 pituitary gland and testicular steroidogenesis (**Konkal & Ganesh 2018**). In a wide range of
373 teleost species, including flatfishes, light intensity has been seen to influence juvenile growth
374 in association with several other physiological parameters, such as feed intake, survival rate,
375 immune response or dietary nutrient composition, according to species preferences (**Kozłowski**
376 **et al., 2010; Wang et al. 2013; Tian et al., 2015; Bögner et al., 2018**). In all cases, better
377 growth was observed at lower light intensities, sometimes associated with differences in stress
378 markers. Also, in fish larvae, evidences were found on the influence of light intensity on growth
379 (common sole, *Solea solea* L., **Bonvini et al., 2016**) or aggression (matrinxã, *Brycon*
380 *amazonicus*, **Lopes et al., 2018**). All the above strengthens the notion that light environment in
381 aquaculture should be carefully considered for each life stage and according to species
382 preferences, assuring optimal welfare conditions and, ultimately, product quality and
383 productivity. Species specific environmental preferences are believed to be an adaptation to
384 each ecological niche and therefore fish are predisposed to perform better under specific light
385 environments. In the wild, Senegalese sole is a benthic species which inhabits areas with
386 minimum light penetration as estuaries and sandy or muddy bottoms. It is very sensitive to dim
387 levels of illumination during the night (**Oliveira et al., 2007, 2009a**), all these in agreement
388 with this preference for lower ambient light intensities during the day. In common sole, light
389 appeared to be more necessary to aid feed intake during the pelagic phase of larvae, but once
390 sensory capacities improve along with metamorphosis, less light is needed during the benthic
391 phase (**Bonvini et al., 2016**). Based on the present results, we may suggest that the optimal light
392 intensity for Senegalese sole broodstock rearing is 100 lx.
393   The second experiment revealed pronounced differences in the circadian system of
394 Senegalese sole breeders born in captivity when compared to wild specimens, both at
395 behavioural and physiological levels. Wild sole presented a very clear daily rhythm in
396 locomotor activity, being predominantly active during the day, in synchronization with feeding
397 time as discussed above. In terms of cortisol concentration, both basal and post-stress values
398 were greater during the day, in agreement with previous findings in the species (**López-Olmeda**

399 **et al. 2013; Oliveira et al. 2013**), regardless of the locomotor activity rhythms. On the other

400 hand, first generation animals did not show clear daily rhythms in locomotor activity nor in

401 basal cortisol concentrations, indicating a disruption of the circadian system development,

402 possibly caused by husbandry during early larval development, as previously suggested for

403 reproduction dysfunction problems (**Howell et al. 2011; Morais et al., 2016**). Production

404 conditions used at early stages to promote feeding intake (high constant temperatures and long

405 darkness periods), might be compromising the normal development of both the brain-pituitary-

406 gonad (BPG) and the hypothalamus-pituitary-interrenal (HPI) axes. The circadian system is

407 responsible for transducing environmental cues, passing timely information to the organism and

408 entraining animals' rhythms at different levels (daily, lunar, seasonal) (**Falcón et al., 2010**).

409 When the early development of such system is disrupted, all rhythms may be compromised,

410 namely behavioural, endocrinal and reproductive. The G1 group, however, did show daily

411 fluctuations in terms of cortisol response to an acute stress challenge, which was significantly

412 higher during the day, as seen elsewhere in juveniles of this species (**López-Olmeda et al.,**

413 **2013**), highlighting the importance of the time of day when handling Senegalese sole. To the

414 best of our knowledge, the existence of daily rhythms in stress response is barely explored in

415 fish, with only a few studies available in other species (**Lankford et al., 2003; Vera et al.,**

416 **2014**). However, this could be an interesting tool to minimize the effect of unavoidable stressors

417 under captive conditions (sampling, sorting, fishing, etc.), both for farming and research

418 purposes, thus increasing fish welfare. Handling Senegalese sole during the night period will

419 always induce a minor stress response.

420   The group of Senegalese sole born and raised under aquaculture conditions showed

421 more pronounced stress response than their wild counterparts, either when exposed to the acute

422 stressor during the day or during the night. As to basal levels, the opposite happened during the

423 day, when groups were compared. Such differences likely reflect their different origin and life

424 history. Wild fish were captured as adults, thus experienced the dangers and challenges of a

425 natural environment (escaping from predators, searching for food, hiding from a storm, etc),

426 which are not present in a protected and predictable environment as an aquaculture system, and

427 this history can be determinant of subsequent corticosteroid status (**Pankhurst, 2011**). In

428 rainbow trout (*Oncorhynchus mykiss*) it was seen that brief stress episodes, or cortisol applied

429 very early in ontogeny, resulted in a reduced cortisol stress response at 5 months of age,

430 suggesting that different stress responses among adults might reflect their experience during

431 ontogeny, as well as heritable traits (**Auperin & Geslin, 2008**). Even cortisol treatment of

432 females prior to spawning induced an effect on the offspring of wild largemouth bass

433 (*Micropterus salmoides*), which resulted in attenuated cortisol response to an acute stressor,
434 together with less anxiety, exploratory behaviour, boldness and aggression of juveniles
435 (**Redfern et al., 2017**). Both these evidences support the hypothesis that non-genetic factors
436 acting early in life will organize or imprint physiological systems in fish (**Auperin & Geslin,
437 2008**), in line with our results, which reflected different stress responses according to fish life
438 history. All this suggests that early exposure of fish to mild stress can be beneficial to juvenile
439 and adult stages, as it can reduce their sensitivity to stressful events, being more adapted to the
440 ongoing environmental demand and perturbations (**Auperin & Geslin, 2008**).

441   The present study represents the first description of the influence of light intensity on
442 broodstock welfare in aquaculture of a flatfish species, suggesting that either too high or too
443 low ambient intensities are not the most adequate. For Senegalese sole, 100 lx seems to be the
444 most suitable light intensity in terms of basal cortisol levels and daily rhythms of locomotor
445 activity. Under captivity, such assessment is an extremely important issue in fish welfare
446 management, as according to each species' habitat preferences, optimal environmental
447 conditions may vary. Our results also demonstrated that Senegalese sole breeders from different
448 origins (wild *vs* G1) presented different stress response rhythms, reflecting their life history in
449 terms of exposure to stress challenges. However, both groups had lower stress responses during
450 nighttime, thus this is the period of the day more suited to handle this species. Senegalese sole
451 breeders born in captivity seemed to have lost the daily fluctuations in basal cortisol plasma
452 levels, as well as their daily rhythms of locomotor activity, revealing that also the circadian
453 system and the HPI axis could be impaired due to rearing conditions, along with the known
454 disruption in the BPG axis of these fish. More research is needed into the ontogeny of such
455 systems under different conditions, to develop better selection programs to obtain breeders
456 more fitted to cope with stress challenges in an aquaculture environment.

457

458 5. Acknowledgements

469

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681

682

683   Figure Captions

684   **Figure 1** – Cortisol basal levels (Mean ± SEM) of Senegalese sole breeders after one month

685   exposure to each of three different ambient light intensities (50, 100 and 200 lx) during daytime.

686   Different letters represent significant statistical differences between light intensities (One-way

687   ANOVA, Tukey's post-hoc multiple comparisons test, $p < 0.05$).

688   **Figure 2** – Actograms of daily locomotor activity of Senegalese sole exposed to 50 lux daytime

689   intensity on a simulated natural photoperiod (**a** - tank A1 and **d** – tank A2); respective polar

690   representation of the COSINOR analysis, depicting clockwise the daily cycle of activity (**b** -

691   tank A1 and **e** – tank A2); and mean (area chart) + SEM (dashed line) daily waveform (**c** - tank

692   A1 and **f** – tank A2). The actogram is double-plotted for better visualization. White and black

693   bars at each graph represents the light (day) and dark (night) periods, respectively. Triangle in

694   COSINOR graphs delimits the acrophase confidence interval.

695   **Figure 3** – Actograms of daily locomotor activity of Senegalese sole exposed to 100 lux

696   daytime intensity on a simulated natural photoperiod (**a** - tank A1 and **d** – tank A2); respective

697   polar representation of the COSINOR analysis, depicting clockwise the daily cycle of activity

698   (**b** - tank A1 and **e** – tank A2); and mean (area chart) + SEM (dashed line) daily waveform (**c** -

699   tank A1 and **f** – tank A2). The actogram is double-plotted for better visualization. White and

700   black bars at each graph represents the light (day) and dark (night) periods, respectively.

701   Triangle in COSINOR graphs delimits the acrophase confidence interval.

702   **Figure 4** – Actograms of daily locomotor activity of Senegalese sole exposed to 200 lux

703   daytime intensity on a simulated natural photoperiod (**a** - tank A1 and **d** – tank A2); respective

704   polar representation of the COSINOR analysis, depicting clockwise the daily cycle of activity

705   (**b** - tank A1 and **e** – tank A2); and mean (area chart) + SEM (dashed line) daily waveform (**c** -

706   tank A1 and **f** – tank A2). The actogram is double-plotted for better visualization. White and

707   black bars at each graph represents the light (day) and dark (night) periods, respectively.

708   Triangle in COSINOR graphs delimits the acrophase confidence interval.

709   **Figure 5 -** Actograms of daily locomotor activity of Wild (**a**) and G1 (**d**) Senegalese sole, reared

710   under natural conditions of photoperiod and temperature; respective polar representation of the

711   COSINOR analysis, depicting clockwise the daily cycle of activity (**b** - Wild and **e** – G1); and

712   mean (area chart) + SEM (dashed line) daily waveform (**c** - Wild and **f** – G1). The actogram is

713   double-plotted for better visualization. White and black bars at each graph represents the light

714   (day) and dark (night) periods, respectively. Triangle in COSINOR graphs delimits the

715   acrophase confidence interval.

716   **Figure 6** – Cortisol basal (**a**) and post-stress levels (**b**) (Mean ± SEM) in wild and G1
717   Senegalese sole sampled at different times of day (Mid-Dark, MD and Mid-Light, ML). Two-
718   Way ANOVA with Origin and Time of Day as factors followed by Tukey's post-hoc multiple
719   comparisons test. Different letters represent significant statistical differences between time
720   points, within each fish group ($p<0.005$); * represents significant statistical differences between
721   fish groups, for the same time point ($p<0.05$).
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723 **Figure 1**
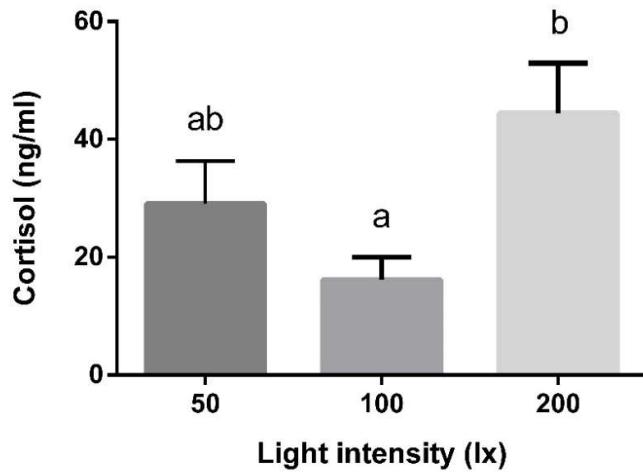
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726 **Figure 2**
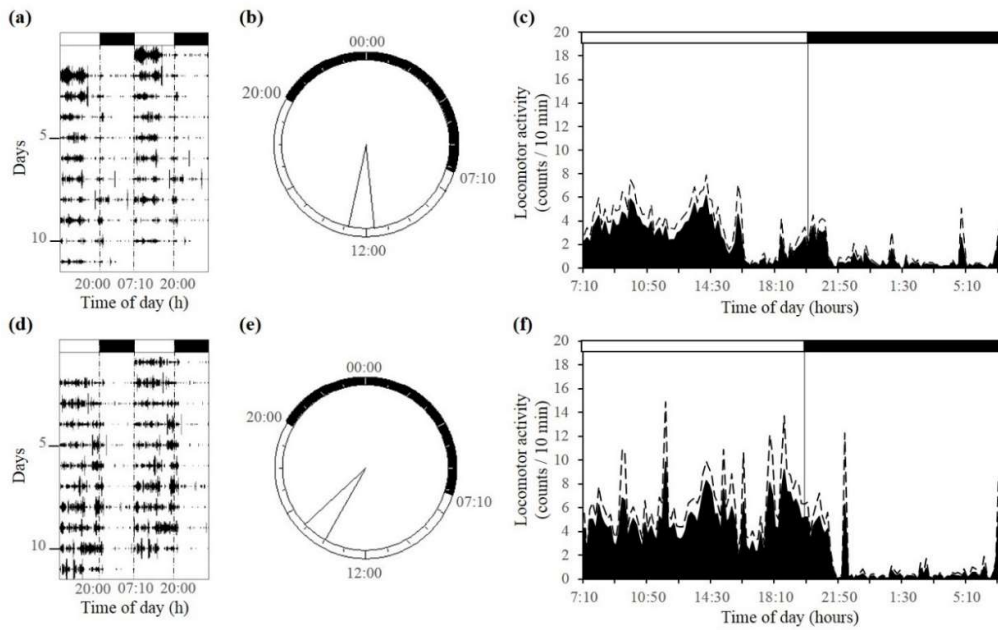
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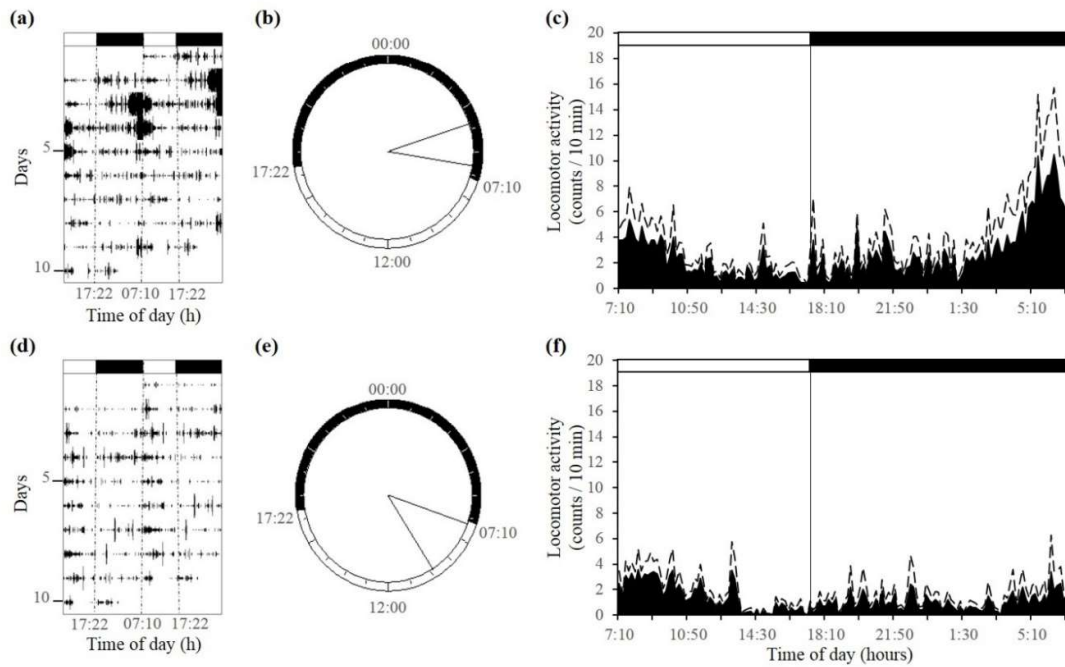
735   **Figure 3**
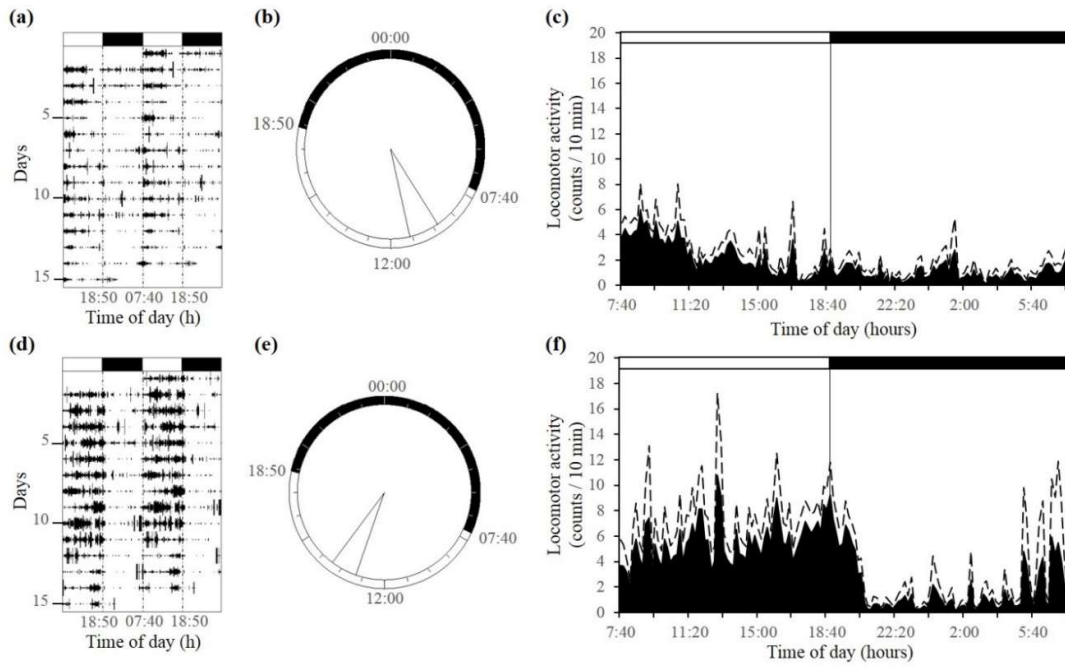
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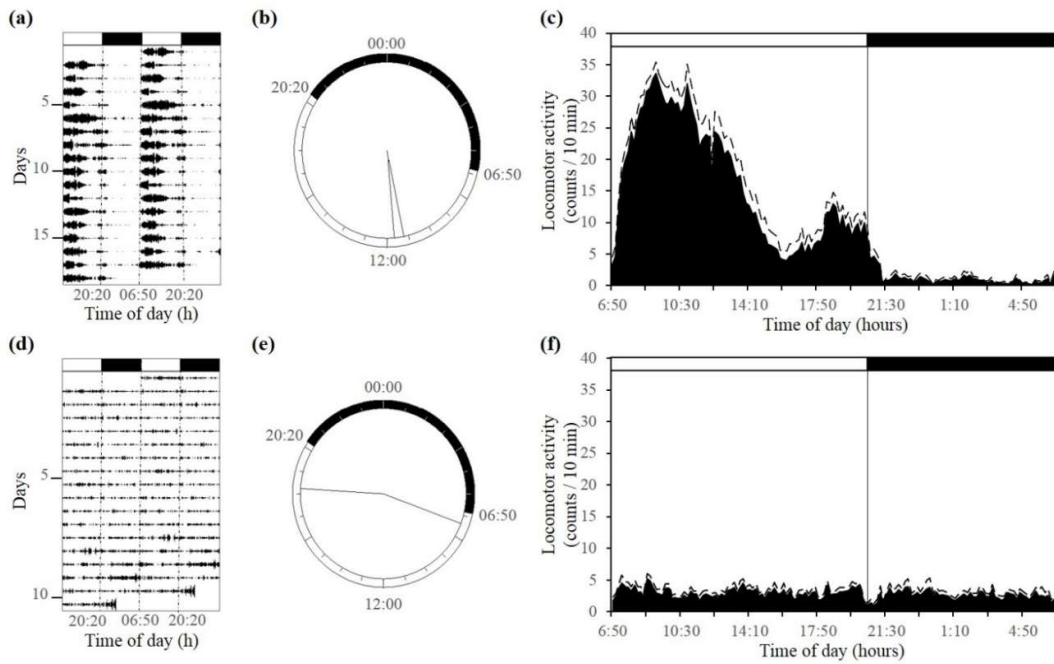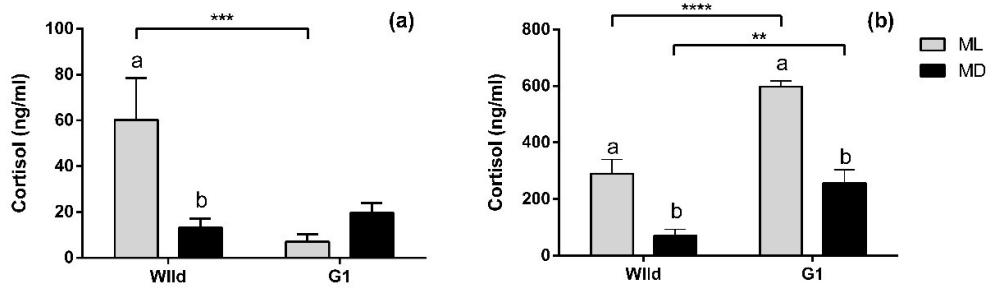737

738   **Figure 4**

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743   **Figure 5**

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746   **Figure 6**

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