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High oxygen consumption rates and scale loss indicate elevated aggressive behaviour at low rearing density, while elevated brain serotonergic activity suggest chronic stress at high rearing densities in farmed rainbow trout Oncorhynchus mykiss

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1   Oxygen consumption, scale loss and serotonergic activity indicate aggressive behaviour at low

2   rearing density and chronic stress at high rearing density in farmed rainbow trout

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25 Abstract

26   The effect of stocking density on indicators of welfare has been investigated by

27 several studies on farmed rainbow trout *Oncorhynchus mykiss*. However, the densities at which

28 welfare compromised remain ambiguous. Here three different stocking density treatments were

29 selected based the results of a previous study, where levels of crowding where determined using the

30 spatial distribution of fish in two-tank systems. An un-crowded low density of 25 kg m^{-3}, the

31 highest density accepted by the fish without showing indications of crowding stress of 80 kg m^{-3} as

32 the intermediate density, and the highest density accepted by the fish showing indications of

33 crowding stress of 140 kg m^{-3} as the high density were investigated. The aim of the present study

34 was to examine the effect of being held at these densities on indicators of welfare. This was

35 achieved oxygen through; oxygen consumption measurements using automated respirometry,

36 recording fin erosion, determining scale loss and analysing plasma cortisol and brain serotonergic

37 activity levels. The results obtained in the present study indicated that at the lowest density the fish

38 had the space and opportunity to display their natural aggressive behaviour and that the fish held at

39 the highest density were exposed to a situation of confinement.

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44 Key words:

45 Rearing density, metabolic rate, physical injury, cortisol, brain serotonergic activity

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1. Introduction

A number of studies have been examined the relationship between stocking density and indicators of welfare in rainbow trout *Oncorhynchus mykiss* [1-5]. Being held at low or high stocking density has been demonstrated to potentially impact indicators of welfare in a negative manner [1,3]. Yet, concrete minimum and maximum density levels where welfare indicators are affected continue to be undefined.

Of all the studies to date, a wide range of stocking densities have been investigated [1-5]. However, the majority of these studies lack details about how the densities that were investigated were selected. It may be speculated that previous research and the densities observed in practice influenced these decisions. The present study is unique in this regard, as the densities examined here were reflective of levels of crowding established in a study by Laursen et al. [6]. In the aforementioned study, the spatial distribution of fish held at different densities in two-tank systems was used to determine a level of aversion to crowding [6]. The aim of the present study was to determine if being held at the chosen densities had an influence on indicators of welfare.

Previous research has found that oxygen consumption rates are elevated in fish held at higher stocking density [7,8]. Stress has been attributed as the cause of this increase [8]. Elevated oxygen consumption rates may also reflect high levels of behavioural activity, such as aggressive behaviour and social hierarchy formations [9-12], which is often observed in fish held at low stocking density [1,12]. Therefore, oxygen consumption rates could provide information regarding stress levels at different densities. Furthermore, it could give considerable insight into the behavioural activity levels and social dynamics at different densities. This is especially relevant as aggressive behaviour has been highlighted as a welfare issue in aquaculture [1].

Physical injuries may be considered a welfare concern, based on the five freedoms framework [1,13]. Injury, in the form of fin erosion, is a commonly observed condition in farmed

73 rainbow trout and has been linked to high stocking density [1,3,14]. The condition is thought to be

74 caused by several factors, such as; aggressive encounters with conspecifics [15], abrasion and

75 collision with the rearing environment, water quality, infection and stress [1,16]. As higher stocking

76 density can enhance the occurrence of these factors, it is thought to contribute to this condition [1].

77 Furthermore, injury caused by excessive scale loss could have consequences for welfare. Injury,

78 quantified as scale loss, due to fighting has previously been investigated in cichlids (*Tilapia zillii*)

79 [17]. During aggressive encounters in rainbow trout, individuals are observed to lose a large

80 quantity of scales. Furthermore, abrasion with the environment has been proposed to contribute to

81 this loss [1,16]. To our knowledge, scale loss has not been investigated before as an indicator of

82 welfare, especially in relation to stocking density. In the present study, physical injury, as indicated

83 by fin erosion and scale loss, could give further insight into the level of social interaction within the

84 tank in relation to stocking density.

85   Measures of the physiological stress response have been a focus in previous studies.

86 Chronic activation of the stress response, specifically cortisol, can have deleterious consequences

87 for growth, disease resistance, and reproduction, thereby providing indications for compromised

88 welfare in individuals [18]. Although the influence of stocking density on cortisol levels has given

89 contrasting results [1], it continues to be a valuable indicator of stress in fish. Furthermore, brain

90 serotonergic activity, the ratio between the brain tissue concentration of serotonin (5-HT,

91 monoamine) and 5-hydroxyindoleacetic acid (5-HIAA, metabolite), has previously been used as an

92 indicator of chronic social stress in salmonid fish held in pairs and small groups [19-22]. Recently,

93 it has been used as an indicator of stress in relation to stocking density in rainbow trout [2].

94 Furthermore, in the study by Laursen et al. [6], elevated serotonergic activity levels were found in

95 individuals held in crowded conditions.

96   In summary, here three densities were selected based on levels of crowding

97   determined using the spatial distribution of fish using two-tank systems [6]; an un-crowded low

98   density of 25 kg m^{-3}, the highest density accepted by the fish without showing indications of

99   crowding stress of 80 kg m^{-3}, and the highest density accepted by the fish showing indications of

100   crowding stress of 140 kg m^{-3}. The aim of the present study was to investigate the influence of

101   being held at these densities on indicators of welfare; oxygen consumption rates, physical condition

102   and neuroendocrine indicators of stress.

103

104   2. Materials and Methods

105   2.1 Experimental fish

106   Rainbow trout from Store Restrup fish farm, Denmark were used for the present

107   study. The fish were transported by truck to the Danish Technical University, Institute of Aquatic

108   Resources (DTU Aqua) in Hirtshals and upon arrival stocked directly into quarantine tanks, as a

109   preventative measure against disease and parasites. While in quarantine, the fish were fed at 0.75 %

110   of the total biomass in the tank per day. Additionally, the salt content in the water was slowly

111   increased to 15 ‰ while the fish were held in quarantine, and decreased again before the fish were

112   moved to the experimental facility. The fish were held in quarantine conditions for a period of 15

113   days, after which they were available to be used for experiments. At the time of delivery, the fish

114   had an average individual weight of 130 grams. At the time the fish were used for the experiment,

115   they had an average weight of 170 ± 2.3 grams.

116

117   2.2 Experimental facilities

118   The experiment was carried out using the twelve tank experimental facility previously

119   detailed by Larsen et al. [8] and McKenzie et al. [2] and is briefly outlined here. The fish were held

120   in nine of the white circular tanks. Each tank was one meter in height and diameter, and held a

121   volume of 600 liters.

122   All the tanks were supplied with water from the same recirculating biofilter system.

123   Water from the system was pumped to the inflow of each tank and entered the tank through a

124   vertical inlet pipe (20 mm diameter, 70 cm length), fixed to the wall of the tank. Small holes (4 mm

125   diameter) along the length of the inlet pipe created pressure to the water flowing into the tank,

126   thereby circulating the water around the tank. A circular column (35 cm diameter) standing at the

127   center of the tank aided the circular flow of the water in the tank. The speed of the water current

128   could be adjusted by increasing or decreasing the amount of water entering the inlet pipe. The water

129   left the tank through a drain at the center bottom of the tank and passed through a whirl separator,

130   next to the tank, before returning to the biofilter. Solid waste; faeces, uneaten pellets and fish scales

131   were collected in the whirl separator. A valve at the bottom of the whirl separator could be opened

132   to allow for removal of the collected solid waste.

133   A constant flow of oxygen was supplied to each tank by a diffuser at the inflow of

134   each tank, providing the baseline oxygen level desired for the tank. Each tank was provided with an

135   electrode (Oxyguard standard probe), which measured the oxygen concentrations continuously. The

136   electrode in each tank was connected to a transmitter, where the desired oxygen concentration could

137   be set for the tank. Whenever the oxygen concentration in the tank fell below the desired level, a

138   boost of oxygen was released into the tank until the concentration in the tank again reached the

139   desired level. The lowest oxygen concentration acceptable could be set for each tank at the

140   transmitter. In the present study, the transmitter was programmed so that if the oxygen

141   concentration in a tank fell below 60 % saturation (5.5 mg L^{-1}), an emergency supply of oxygen

142   from an alternative oxygen source was started. The data from the transmitters was saved onto a data

143   logger, for later analysis (see section 2.4.1.).

144   The experimental tanks were modified to function as respirometers. Each tank was

145   fitted with a three-way valve at the inflow to the tank. This valve was open under normal

146   circumstances, allowing oxygen and aerated water from the system to be pumped into the tank. The

147   valve could be closed, cutting off the oxygen and fresh water supply from the system, thereby

148   circulating the existing water in the tank. During this period, the decline in the oxygen

149   concentration in the tank was measured automatically and registered on the data logger. The three-

150   way valve was connected to a digital timer, which could be programmed to close the three-valve for

151   a pre-determined interval.

152

153   2.3 Experimental protocol

154   Three stocking densities were investigated during the experiment. The densities

155   selected reflected the results of a study by Laursen et al. [6], where two-tank systems were used as a

156   method to determine a level of crowding experienced as aversive by the fish. In that study,

157   behavioural and neuroendocrine measures were used as indicators of crowding stress. Here, a

158   density of 25 kg m^{-3} served as an un-crowded low density (LD), the highest density accepted by the

159   fish without showing indications of crowding stress of 80 kg m^{-3} as the intermediate density (ID),

160   and the highest density accepted by the fish showing indications of crowding stress of 140 kg m^{-3} as

161   the high density (HD).

162   The fish were transported from the quarantine facilities to the experimental facilities

163   to be stocked randomly into the experimental tanks; at 25 kg m^{-3}, 80 kg m^{-3} and 140 kg m^{-3} in

164   triplicate. A subsample of 30 individuals for each tank were lightly anaesthetized (Ethylene glycol

165   monophenyl ether) and pit tagged and adipose fin clipped for individual identification throughout

166   the experiment. This was done at initial stocking, to allow for a period of recovery of the fish after

167   the procedure. Subsequently, the sub-sample of 30 individuals was added to each tank and the

7

168 remaining biomass was added to each tank to achieve the desired density. The sub samples of 30

169 fish from each tank were individually weighed, measured for fork length and checked for fin

170 damage at each subsequent weighing session.

171   After stocking, the fish were acclimated in the experimental tanks for a period of

172 approximately two weeks where the feeding level was gradually increased to 1.5% of the total tank

173 biomass per day. This was done to allow the biofilter of the recirculation system to cope with the

174 biomass of fish and to ensure that the water quality parameters were adequate for the fish.

175 Furthermore, it allowed time for making adjustments to the oxygen levels according to the densities

176 in each tank for oxygen consumption measurements. At the end of the acclimation period, the

177 biomass in each tank was determined and re-adjusted to the desired density by removing excess

178 kilograms. The number of fish in the tank was counted.

179   The experimental duration was 28 days, consisting of two growth periods of 12 days.

180 During each growth period, the fish were fed for 12 days, whereafter the biomass in each tank was

181 weighed. Prior to weighing, the fish were given a period of fasting for a day to minimize the risk for

182 infection after weighing.

183   Each tank was fed at 1.5 % of the estimated tank biomass per day, with 3 mm pellets

184 (EFICO Enviro 920, BioMar A/S). The fish were fed in the morning at 09:00 with automatic belt

185 feeders for a period of six hours. In the afternoon after feeding, the solid waste was collected from

186 the whirl separator and the numbers of uneaten pellets were counted to determine feed waste.

187 Additionally, the scales lost by the fish were also separated out from the solid waste and collected

188 for later weighing (see section 2.4.2.).

189   On the day of fasting, to determine basal stress levels, a subsample of four individuals

190 from each tank (un-pit tagged) were sacrificed during daylight hours and blood and brain samples

191 were collected from these fish. The blood samples were for later analysis of plasma cortisol

192 concentration and the brain samples for later analysis of brain monoamine and metabolites (see

193 section 2.4.3.).

194   On the day of weighing, the total biomass in each tank was recorded. The 30 pit

195 tagged individuals were separated from the biomass, and individually weighed, measured for fork

196 length and checked for fin erosion. They were then re-stocked into the tank, and the remaining

197 biomass was added to the tank to achieve the desired density. The excess kilograms were discarded.

198 This process was repeated for the second experimental period.

199   Oxygen consumption was measured continuously throughout the growth period.

200 Measurements were started on the first day of feeding and stopped on the day of weighing. Oxygen

201 levels were set at 80% (8.5 mg L^{-1}) in the tanks held at the low density, 110% (11.5 mg L^{-1}) at the

202 intermediate density, and 120% (12 mg L^{-1}) at the high density. Oxygen concentrations were set at

203 these levels for practical reasons, to obtain a long enough closing period to be able to measure

204 oxygen consumption and to ensure that oxygen levels did not fall below the critical level (60%, 5.5

205 mg L^{-1}) at the intermediate and high densities during the oxygen consumption measurement period.

206   Water quality parameters; nitrite (NO_2^-), nitrate (NO_3^-), ammonia (NH_3/NH_4^+), pH and

207 temperature, were measured daily at the system level to ensure that they were within optimal levels

208 for the fish. The temperature of the water in the system was controlled at 16 °C. A slow water

209 current of approximately 0.5 body lengths per second was provided to each tank to even out the

210 distribution of the fish and the pellets in the tank. Light conditions were at 14.5 light and 9.5 dark

211 hours, with the lights automatically switching on at 07:30 and switching off at 22:00.

212

213 2.4 Measurements

214 *2.4.1. Oxygen consumption*

215   Oxygen consumption measurements were taken continuously using the automated

216   respirometry system. Ever hour, the three-way valve at the inflow to the tank would close and

217   thereby shut off the oxygen supply to each tank. The valve remained closed for a period of 8

218   minutes during the day time hours (09:00 – 17:00) and 6 minutes during the night times hours

219   (18:00 – 08:00). The oxygen concentrations in the tanks were registered from the transmitters to the

220   data logger every 20 seconds during the period when the valve was closed.

221   Oxygen consumption was calculated as previously described by Larsen et al. [8]. For

222   each hourly measurement period on each experimental day, the decline in the oxygen concentration

223   in each tank was used to perform a linear regression. In the present study, the data from the last 5

224   minutes of the measurement period and the last 3 minutes of the measurement period were used

225   from the day time hours and night time hours respectively. The biomass in each tank was estimated

226   for each day using the specific growth rate (SGR). The absolute volume of water in the tank was

227   obtained by subtracting the estimated biomass on the day from the known volume of water the tank

228   could hold. The slope value obtained from the linear regression, the estimated total biomass of fish

229   in the tank on the day and the total volume of water in the tank on the day were used to calculate the

230   oxygen consumed by the fish, as milligrams of oxygen consumed per kilogram of fish per hour (mg

231    $O_2 \, kg^{-1} \, hr^{-1}$).

232   To ensure direct comparability of the concentrations of oxygen consumed between the

233   tanks, the data used was selected from the days where the body mass increase of the fish was

234   similar in each of the tanks. In the present study, a period of days from when the fish grew from 190

235   to 220 grams (mean body weight 205 grams) was chosen. The data on the amount of oxygen

236   consumed taken from those days was corrected to a 205 gram body weight fish, using the method

237   detailed by Larsen et al. [7].

238   The hourly oxygen consumption rates during a daily cycle were determined for the

239   three density treatments. The minimum, median and maximum oxygen consumption rates were

240   determined from the daily cycle for comparison between the three density treatments. Generally, the

241   minimum amount of oxygen was consumed between the hours of 06:00 and 08:00 and the

242   maximum between the hours of 11:00 and 13:00. The median rates of oxygen consumption were

243   between the time of 21:00 and 23:00. Furthermore, the total amount of oxygen consumed during the

244   selected days (outlined above) was determined for the three density treatments.

245

246   *2.4.2. Physical indicators*

247   Fish scales were collected from each of the tanks daily. After feeding was finished in

248   the afternoon, the solid waste from the tank that had collected in the whirl separator was flushed out

249   and collected in a bucket. The contents of the bucket were emptied into a sieve where the scales

250   were separated from the faeces and uneaten pellets. The scales were collected in plastic containers

251   for later weighing.

252   The scales were dried and weighed on a pre-weighed filter paper (Qualitative filter

253   paper, 413; WWR). Before weighing, the filter paper with the scales was put in the dryer at 60 °C

254   for a period of one hour. To determine the grams of scales lost per kilogram fish (g kg^{-1}), the weight

255   of the scales was divided by the estimated biomass in the tank (using the SGR). The total amount of

256   scale loss was determined for each density treatments for the same period as for the total oxygen

257   consumed, the selected days from when the fish grew from 190 grams to 220 grams.

258   Fin erosion was determined from the subsample of 30 pit tagged individuals from

259   each tank, using the photographic key developed by Hoyle et al. [12]. During sampling, the

260   individuals were lightly anaesthetized and examined. Each fin type per individual was compared to

261   the pre-developed photographic key and given a score from one to five. A score of one was

262   considered to be a fin in good condition and five a fin showing considerable damage.

263

264   *2.4.3. Neuroendrocrine indicators of stress*

265   A sub sample of four individuals per tank were sacrificed by an overdose of

266   anaesthetic (Ethylene glycol monophenyl ether). Blood samples were collected from the caudal vein

267   using 1 ml syringes. The blood samples were centrifuged at 15,000 rpm for 5 minutes and the

268   plasma was separated into 1 ml eppendorf tubes and frozen at -80 °C for later analysis. During

269   analysis, cortisol was extracted from the plasma by mixing with ethyl ether. The solvent was

270   evaporated using a vacuum centrifuge and the remaining residue was re-suspended in an extraction

271   buffer (ELISA kit extraction buffer). Cortisol concentrations ($ng\ ml^{-1}$) were quantified using the

272   ELISA kit standard method (Neogen, Product #402710).

273   Whole brains were dissected out from each fish and separated into four parts; the brain

274   stem, hypothalamus, telencephalon and optic lobes. Each brain part was frozen separately at -80 °C

275   for later analysis. Before analysis, each frozen brain part was individually weighed. After weighing,

276   the brain part was homogenised in a homogenising reagent (4% perchloric acid, 0.2%

277   Ethylenediaminetetraacetic acid, $40\ ng\ ml^{-1}$ dihydroxi benzylamine hydroxide solution). The

278   solvent was then centrifuged at 10,000 rpm at 4 °C for 10 minutes. The supernatant was assayed by

279   High Performance Liquid Chromatography (HPLC) with electrochemical detection to quantify the

280   concentration of 5-HT (serotonin) and its catabolite 5-Hydroxyindoleacetic acid (5-HIAA). The

281   HPLC system consisted of a mobile phase (buffer solution; $10.35\ g\ l^{-1}$ sodium phosphate, 0.3252 g

282   l^{-1} sodium octyl sulphate, $0.0037\ g\ l^{-1}$ EDTA, 7% acetonitril in deionised water), a solvent delivery

283   system (Shimadzu, LC-10AD), an auto injector (Famos, Spark), a reverse phase column (4.6 mm

284   100 mm, Hichrom, C18, 3.5 mm) and an ESA Coulochem II detector (ESA, Bedford, MA, USA)

285 with two electrodes at -40 mV and +320 mV. A conditioning electrode with a potential of +40 mV

286 is used to oxidize possible contaminants before analysis. Brain 5-HT and 5-HIAA were quantified

287 by comparing them with standard solutions of known concentrations and corrected for recovery of

288 the internal standard using HPLC software (CSW, DataApex Ltd, Czech Republic).

289

290 2.5 Statistical analyses

291   Oxygen consumption rates were analysed using one-way ANOVA's, where density

292 was the independent variable and the dependent variable either the minimum, median, maximum or

293 total oxygen consumption concentration. A tukey's post hoc test was carried to determine where the

294 significances were present. A one-way ANOVA was used to analyse the differences between

295 density treatment on the total amount of scale loss. A Spearman rank test was done to analyse the

296 relationship between the total scale loss and total oxygen consumption. Fin scores were analysed

297 using a Kruskal-Wallis by ranks (comparing multiple independent variables) test, where density

298 was the independent variable and each fin type the dependent variable. A two-way ANOVA was

299 performed to determine if there was a difference in plasma cortisol concentrations between density

300 treatments (LD, ID & HD) and experimental period (1 &2). Density treatment and experimental

301 period were the independent variable and log concentrations of plasma cortisol the dependent

302 variable. Furthermore, a two-way ANOVA was performed to determine if there was a difference in

303 the arcsin ratio of 5-HIAA/5-HT between density treatment and experimental period. The difference

304 between density treatment in the log concentrations of 5-HIAA and 5-HT were determined using a

305 one-way ANOVA. A Tukey post hoc test was done to determine where the significances occurred.

306 All statistical analyses were carried out using the computer program Statistica (version 11). The

307 values presented in the figures are mean ± standard error.

308

309 3. Results

310 3.1. Oxygen consumption

311   The mean daily pattern of mass specific oxygen consumption rates for the three

312 density treatments is shown in Figure 1. The general pattern at all densities is described as follows:

313 consumption rates were lowest from midnight (00:00) until 09:00, the minimum rates being

314 between the hours of 06:00 to 08:00. At 08:00, after the lights automatically turned on at 07:30 and

315 the fish anticipated being fed, there was a slight increase in consumption until 09:00, where after

316 there was a sharp increase. After reaching maximum rates at around mid day (11:00 to 13:00),

317 consumption rates started to decrease rapidly until the hour of 17:00. Thereafter, rates decreased

318 slowly throughout the night. It was observed that in the low density tanks, the pattern was erratic

319 compared to the smooth pattern observed in the tanks at the higher densities.

320   The average minimum (time 06:00 – 08:00; Fig. 2), median (time 21:00 – 23:00; Fig.

321 2), and maximum (time 11:00 – 13:00) oxygen consumption rates were significantly different

322 between the density treatments ($p<0.001$, $p<0.001$, $p<0.001$ respectively; Fig. 2), with the amount

323 of oxygen consumed decreasing with increasing density (Fig. 2). Specifically, at minimum rates,

324 there was a difference between the LD and ID ($p<0.001$), between LD and HD ($p<0.001$), and

325 between ID and HD ($p<0.001$). At median and maximum rates, a similar pattern was observed, with

326 a difference between the densities of LD and ID ($p=0.003$, $p<0.001$ respectively), between LD and

327 HD ($p<0.001$, $p<0.001$ respectively), and between ID and HD ($p=0.017$, $p<0.001$ respectively).

328 This pattern was also reflected in the total oxygen consumed during the selected days, with the rates

329 decreasing with increasing density ($p=0.010$, Fig. 3). Specifically, there was a difference between

330 the LD and HD ($p=0.009$), but not between the LD and ID ($p=0.052$) or between the ID and HD

331 ($p=0.325$).

332

333 3.2. Scale loss

334   The total scale loss differed significantly between the density treatments (p=0.006;

335 Fig. 4), where the amount of scales lost per kg of fish decreased with increasing density.

336 Specifically, there was a difference between the LD and ID (p=0.025), between LD and HD

337 (p=0.006), but not between the ID and HD (p=0.432). Furthermore, there was a positive correlation

338 between total scale loss (g kg^{-1}) and total oxygen consumption (mg O$_2$ kg^{-1}; R^2=0.917, p<0.001;

339 Fig. 5).

340

341 3.3. Fin scores

342   Of the different fin types, there was a significant difference between the density

343 treatments in the damage to the caudal fin (p=0.012; Fig. 6). Specifically, there was a difference

344 between the LD and ID (p=0.016), but not between LD and HD (p=0.450) or the ID and HD

345 (0.553). There was also a significant difference between density treatments in the damage to the left

346 pelvic fin (0.030; Fig. 6). However, no difference was found when looking specifically between

347 densities; LD and ID (p=0.481), LD and HD (p=0.073) or ID and HD (p=1.000).

348

349 3.4. Neuroendocrine indicators

350 *3.4.1. Plasma cortisol*

351   The plasma cortisol concentrations showed no significant differences between the

352 three density treatments (LD, ID & HD), despite a slight elevation in the levels at the highest

353 density stocked (p=0.284; Fig. 7). There was no difference between experimental period (1 & 2;

354 p=0.265), or interaction between density treatment and experimental period (p=0.594).

355

356 *3.4.2. Brain serotonergic activity (5-HIAA/5-HT)*

357   The serotonergic activity levels in the brain stem of the individuals held at LD were

358   lower compared to in the individuals held at the higher densities ($p<0.001$; Fig. 8A). Specifically,

359   there was a difference between the LD and ID ($p<0.001$), between LD and HD ($p<0.001$), and

360   between the ID and HD ($p=0.045$). Significantly higher levels of serotonergic activity was found

361   during the first experimental period compared to the second ($p<0.001$). There was also an

362   interaction between density treatment and experimental period ($p=0.016$). Furthermore, the

363   concentration of 5-HIAA in the brain stem increased with increasing density ($p<0.001$; Table 1).

364   There were no differences in serotonergic activity levels in the telencephalon or hypothalamus

365   between the density treatments ($p=0.594$, $p=0.495$ respectively; Fig. 8B & 8C). However, the

366   concentration of 5-HIAA in the telencephalon was elevated at the HD compared to the ID and LD

367   ($p=0.006$; Table 1). Furthermore, significantly higher levels of serotonergic activity was found

368   during the first experimental period compared to the second in both the telencephalon ($p=0.002$)

369   and hypothalamus ($p=0.006$).

370

371   4. Discussion

372   A negative relationship between oxygen consumption and stocking density was

373   observed in the present study. This is in contrast to previous results in rainbow trout, where high

374   stocking density was associated with elevated metabolic rates compared to low stocking density

375   [2,8]. At higher densities, fish are exposed to crowding and the physiological disturbance caused by

376   chronic stress can result in increased oxygen uptake and usage [7,23]. Behavioural aspects may also

377   explain increased metabolic rates in fish. Bursts of spontaneous activity, such as agonistic

378   interactions, may result in elevated metabolic rates [9,10]. Indeed, high levels of spontaneous

379   activity associated with agonistic behavior have been shown to cause elevated metabolic rates in

380   juvenile sockeye salmon and rainbow trout [9,12]. The elevated oxygen consumption rates found in

381   the tanks held at the lowest density compared to the highest density in the current study may be

382   reflective of high levels of spontaneous aggressive behaviour. The hourly oxygen consumption rates

383   at the lowest density showed a more erratic pattern compared to in the tanks stocked at the higher

384   densities, where a smoother hourly pattern was observed.

385   Additionally to oxygen consumption, a negative relationship between scale loss and

386   stocking density was observed, with a decrease in total scale loss with increasing density. Scale loss

387   has been associated with abrasion with the environment [16]. It could also be the result of

388   aggressive interactions between conspecifics, as has been observed during fights in cichlids [17]

389   and rainbow trout. During a fight between a pair of fish, individuals will violently attack, nip and

390   bite each other [15,22], which results in visible scaring and a large quantity of scale loss. As scale

391   loss was highest at the lowest density in the present study where the fish had abundant space and

392   lowest at the highest density where the fish were crowded together, it is unlikely that scale loss was

393   due to passive abrasion with the environment but rather due to aggressive interactions between

394   individuals. Indeed, aggressive interactions have been observed to increase in rainbow trout with

395   decreasing stocking density [1,12]. Interestingly, the positive correlation between scale loss and

396   oxygen consumption rates further strengthens the implications for the occurrence of natural

397   aggressive behaviour of the fish held in the tanks at the lowest density and that this behaviour was

398   diminished with increasing density.

399   Furthermore, a degree of the erosion observed in certain fin types in rainbow trout has

400   been attributed to aggressive behaviour [1,15] and could therefore be used as an indicator of such

401   behaviour in the present study. As aggressive behaviour was concluded to occur at the lowest

402   density, the same pattern could have been expected in fin erosion. However, in the present study

403   this pattern was difficult to interpret; as of the different fin types, fin erosion was least visible in the

404   caudal and left pelvic fin at the lowest density. Damage to the fins was more evident in the

405 individuals held at the intermediate and high density. However, this may not be surprising as

406 several studies have documented an increase in fin erosion with increasing density [1,3,14]. Poor

407 water quality, abrasion with the environment and elevated stress levels associated with high density

408 situations have been found to increase the severity of fin erosion [1].

409   The plasma cortisol concentrations did not differ between the densities. Furthermore,

410 the values were generally low, suggesting that stress levels were reduced in the individuals held at

411 all of the densities. However, low levels of cortisol have been observed in chronically stressed

412 individuals in some cases [24,25]. For example, it has previously been found that cortisol

413 concentrations returned to basal levels within a week when exposed to a chronic stressor [23]. One

414 explanation given for this observation is that a negative feedback mechanism acts on the

415 hypothalamus causing a down regulation of cortisol [26].

416   Similarly to the plasma cortisol concentrations, rearing density did not affect

417 serotonergic activity in the hypothalamus and telencephalon. On the other hand, serotonergic

418 activity was found to be significantly higher in the brain stem. This region specificity may be

419 related to that serotonin (5-HT) is mainly synthesised in the raphe nuclei, a structure found in this

420 brain part [27]. Previous studies have shown a strong positive relationship between 5-HTergic

421 activity in this brain part and cortisol during moderate to short term stress [19-22]. However, during

422 periods of chronic stress this relationship may weaken, where serotonergic activity remains high

423 while cortisol concentration declines [28]. Hence, in the present study, chronic stress was indicated

424 by low concentrations of plasma cortisol and elevated serotonergic activity (5HIAA/5HT) levels.

425 Indeed, low levels of cortisol and elevated brain stem 5-HTergic activity levels have previously

426 been associated with chronic stress in fish held at higher densities [6]. As a result, the

427 neuroendocrine indicators in the present study showed that stress levels were most elevated at the

428 highest density stocked, followed by the intermediate density and lowest at the low density.

429   Taken together, the results discussed here give insight into to the suggestion that at the

430   lowest density the fish had the space and opportunity to display their natural behaviour and that the

431   fish held at the highest density were exposed to a situation of confinement. At the lowest density,

432   the high oxygen consumption rates and high scale loss indicated increased natural behaviour levels,

433   in the form of aggressive encounters. Additionally, the low stress levels could indicate that the

434   behaviour displayed had a stress reducing effect. Displaced aggression is a stress reducing

435   behavioural outlet, where aggression towards others functions as a coping strategy to reduce stress

436   [29]. This type of behaviour has been observed in rainbow trout held in small groups [29]. At the

437   highest density, the lower levels of oxygen consumption and scale loss suggest that due to the

438   confined conditions the fish were unable to display natural behaviour. Furthermore, the individuals

439   held at this density showed signs of elevated stress levels, which are indicative of a crowded

440   environment.

441

442   5. Conclusion

443   The aim of the present study was to assess indicators of behaviour and welfare in fish

444   held at three different densities; a low, intermediate and high density. The densities investigated in

445   the present study reflect levels of crowding that were identified by the spatial distribution of the fish

446   in two-tank systems. The results discussed here indicated that at the lowest density the fish had the

447   space and opportunity to display their natural behaviour and that the fish held at the highest density

448   were exposed to a situation of confinement.

449

450   6. Acknowledgements

454

455   7. References

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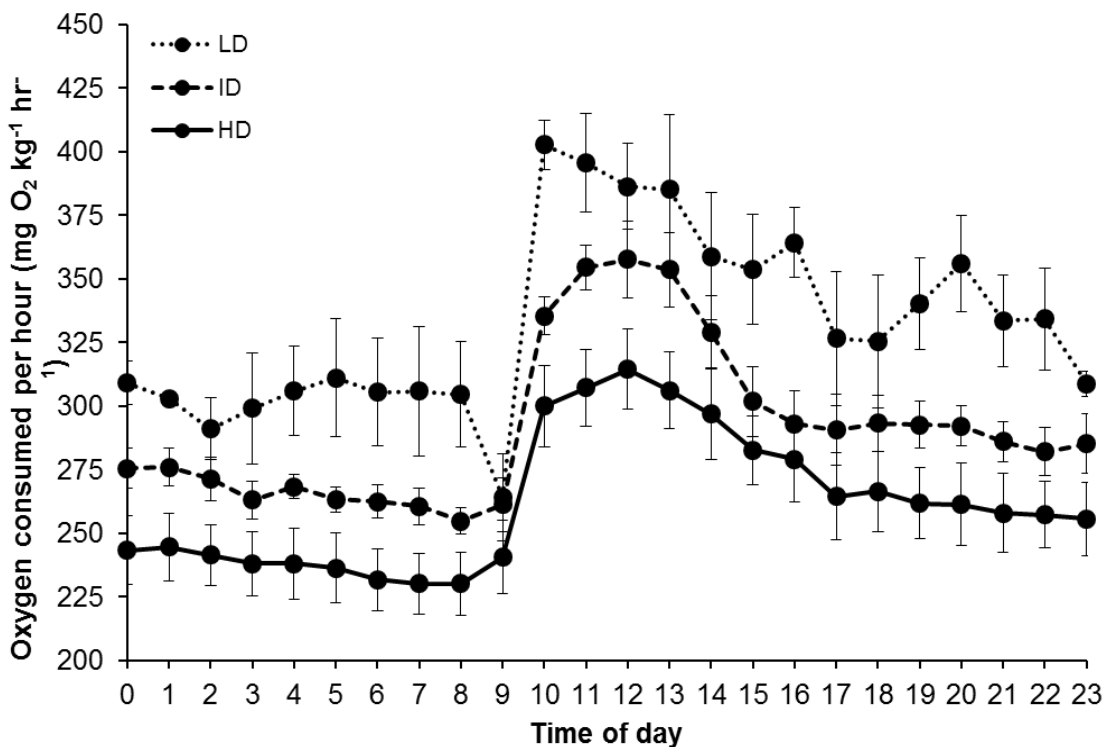
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555

556   8. Figure captions

557

558

559   Figure 1. The hourly oxygen consumption rates (mg O_2 kg^{-1} hr^{-1}) between the three density

560   treatments (n=3 tanks per treatment); 25 kg m^{-3} (LD), 80 kg m^{-3} (ID) and 140 kg m^{-3} (HD),

561 normalised for a 205 gram fish. The days during which the fish grew from 190 grams to 220 grams

562 are used.

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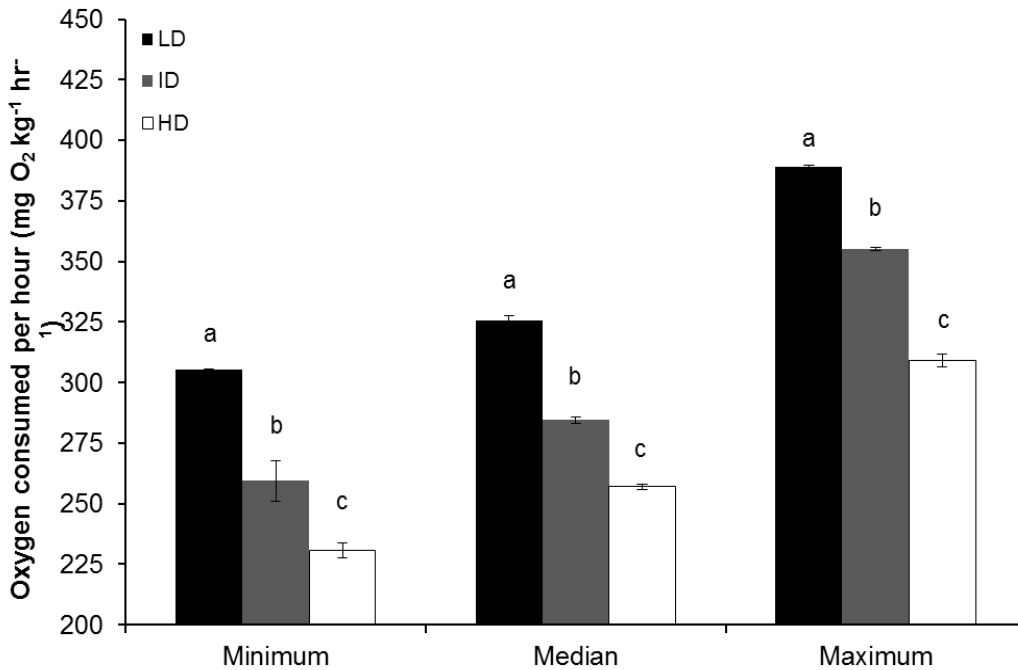
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565 Figure 2. The minimum, median and maximum oxygen consumption rates (mg O_2 kg^{-1} hr^{-1}) during

566 the daily cycle (Fig. 1) between the density treatments (n=3 tanks per treatment); 25 kg m^{-3} (LD),

567 80 kg m^{-3} (ID) and 140 kg m^{-3} (HD). The minimum rates were between time of day 6 to 8, the

568 median rates were from time of day 21 to 23, and maximum rates from time of day 11 to 13 of the

569 daily cycle from Figure 1. The letters denote where the significances lie.

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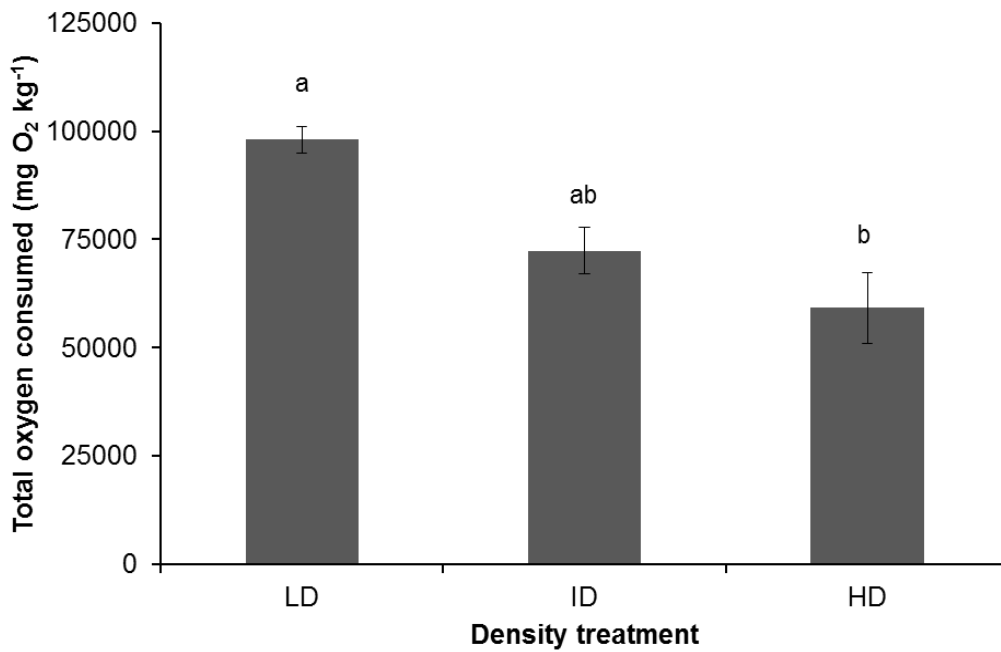
Figure 3. The total oxygen consumption rates (mg O_2 kg^{-1}) between the density treatments (n=3

tanks per treatment); 25 kg m^{-3} (LD), 80 kg m^{-3} (ID) and 140 kg m^{-3} (HD) during the period

(selected days) when the fish grew from from 190 grams to 220 grams. The letters denote where the
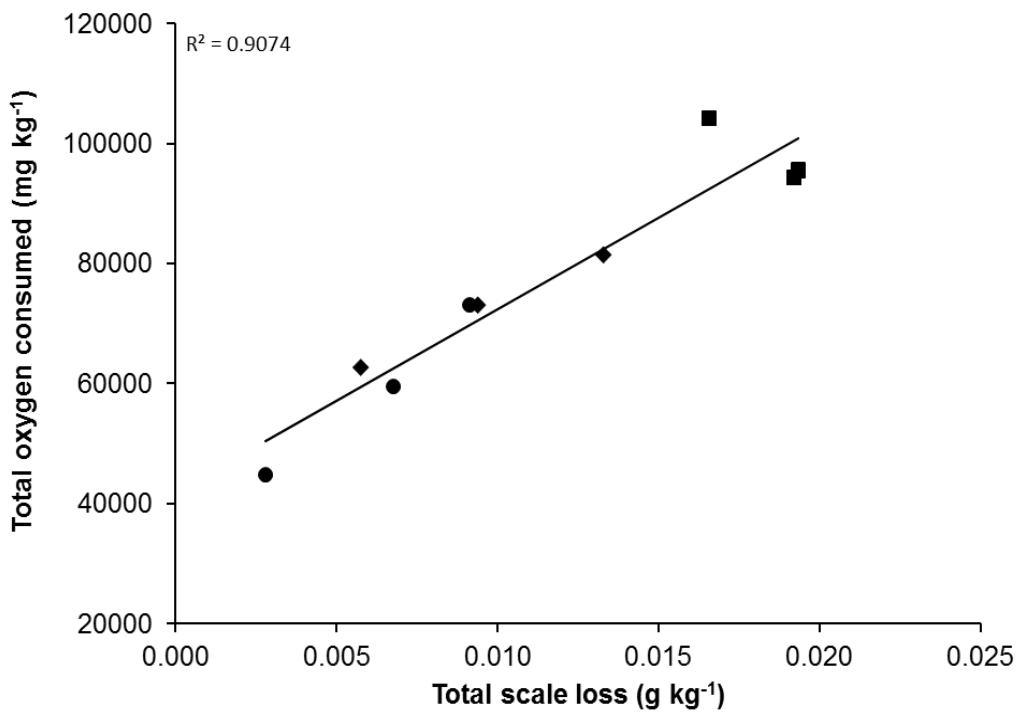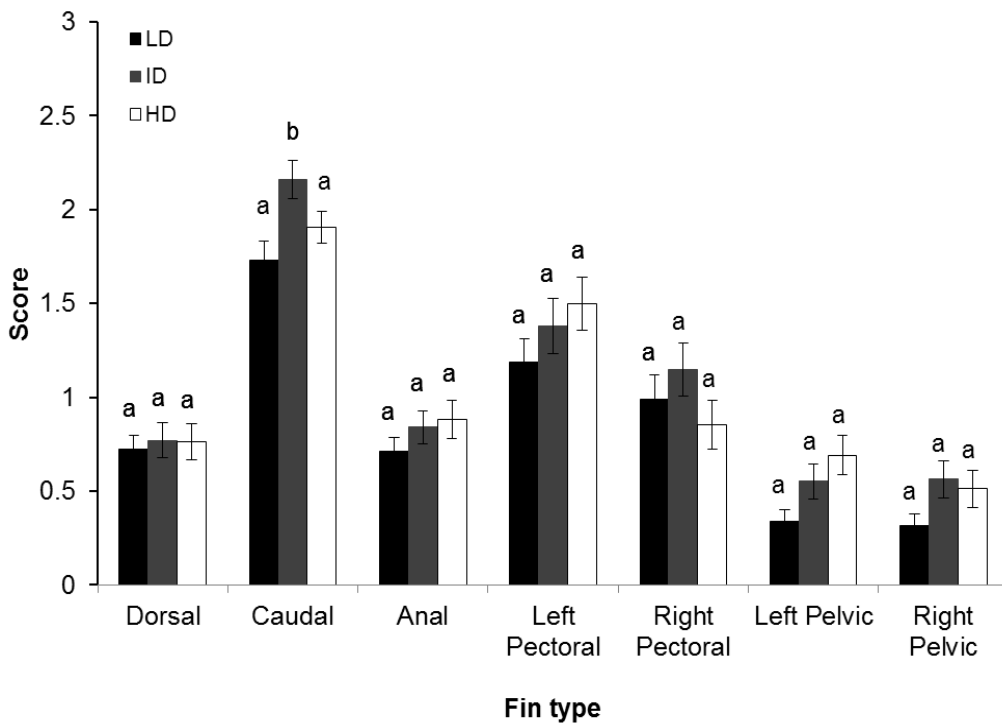
significances lie.

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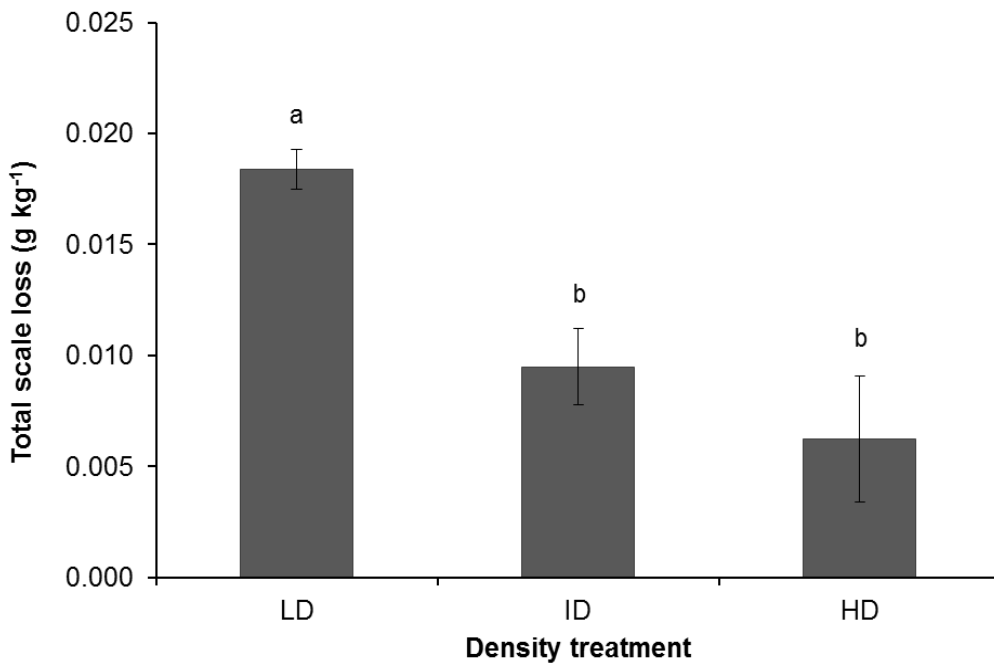
Figure 4. The total scale loss (g kg^{-1}) between the density treatments (n=3 tanks per treatment); 25

kg m^{-3} (LD), 80 kg m^{-3} (ID) and 140 kg m^{-3} (HD) during the period (selected days) when the fish

grew from from 190 grams to 220 grams. The letters denote where the significances lie.

581

582

583   Figure 5. The relationship between the total scale loss (g kg^{-1}) and the total oxygen consumption

584   (mg O$_2$ kg^{-1}) between the density treatments (n=3 tanks per treatment); 25 kg m^{-3} (LD), 80 kg m^{-3}

585   (ID) and 140 kg m^{-3} (HD) during the period (selected days) when the fish grew from from 190

586   grams to 220 grams. Cirlces represent the LD tanks, diamonds represent the ID tanks and squares
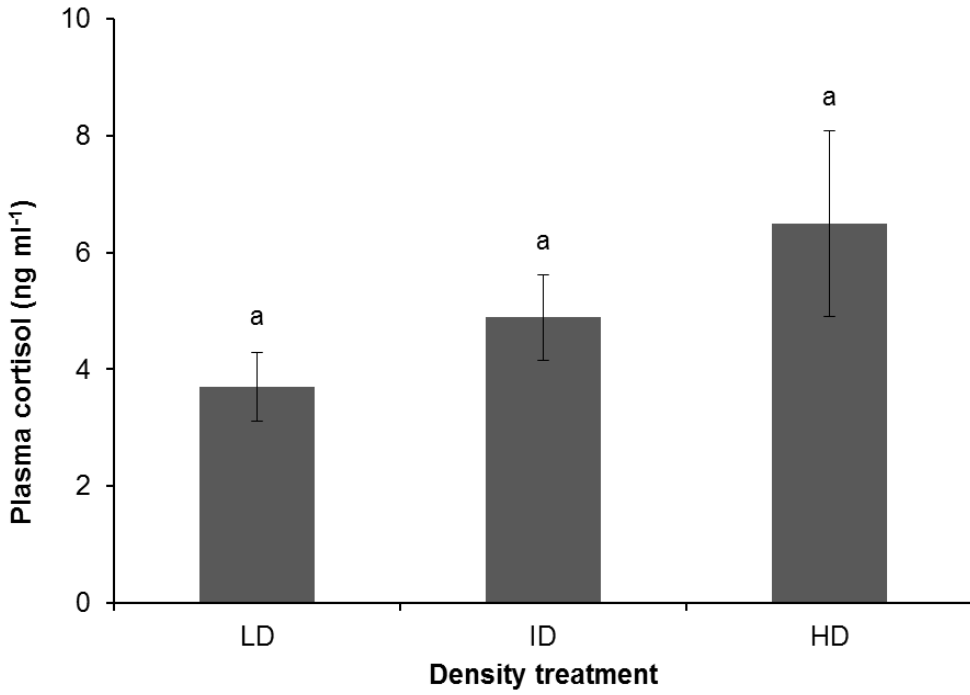
587   represent HD tanks.

588

589

590   Figure 6. The fin score for each fin type of individuals (n=30 individuals per tank in triplicate)

591   between the density treatments; 25 kg m^{-3} (LD), 80 kg m^{-3} (ID) and 140 kg m^{-3} (HD) at termination

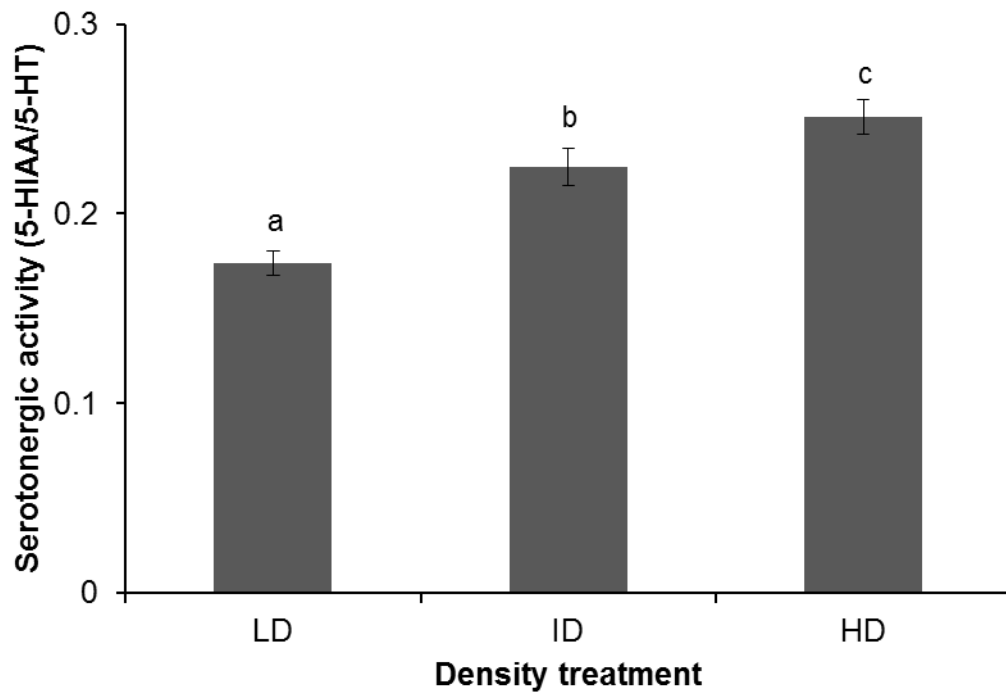592   of the experiment. The letters denote where the significances lie.
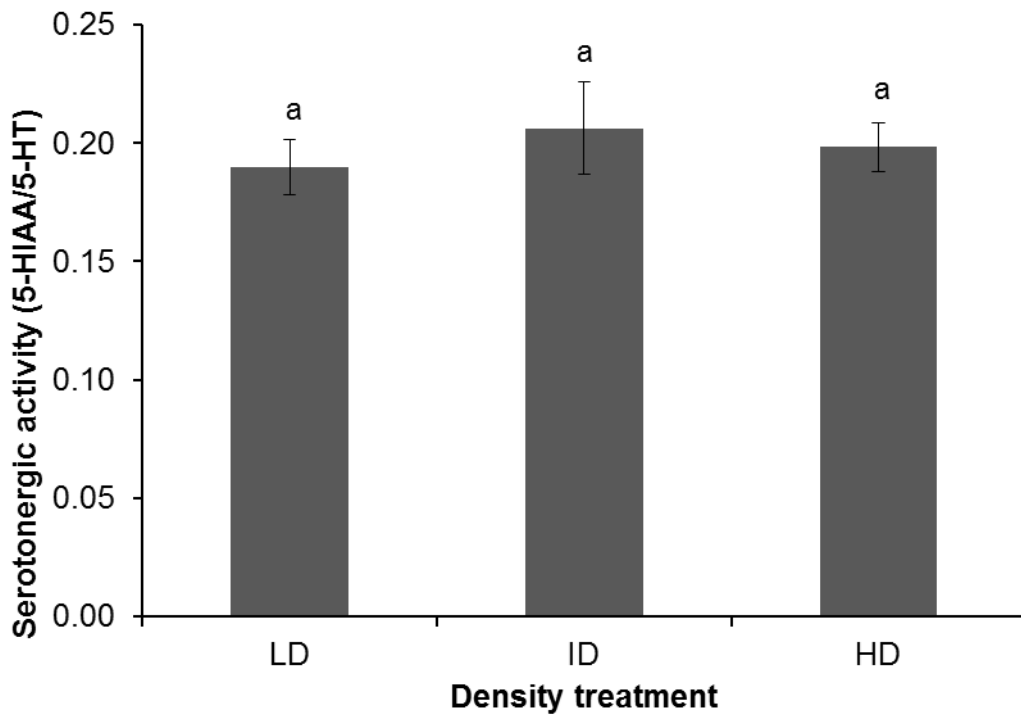
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595   Figure 7. The plasma cortisol concentrations (ng ml^{-1}) of individuals between the density treatments

596   (n=3 tanks per treatment); 25 kg m^{-3} (LD), 80 kg m^{-3} (ID) and 140 kg m^{-3} (HD).
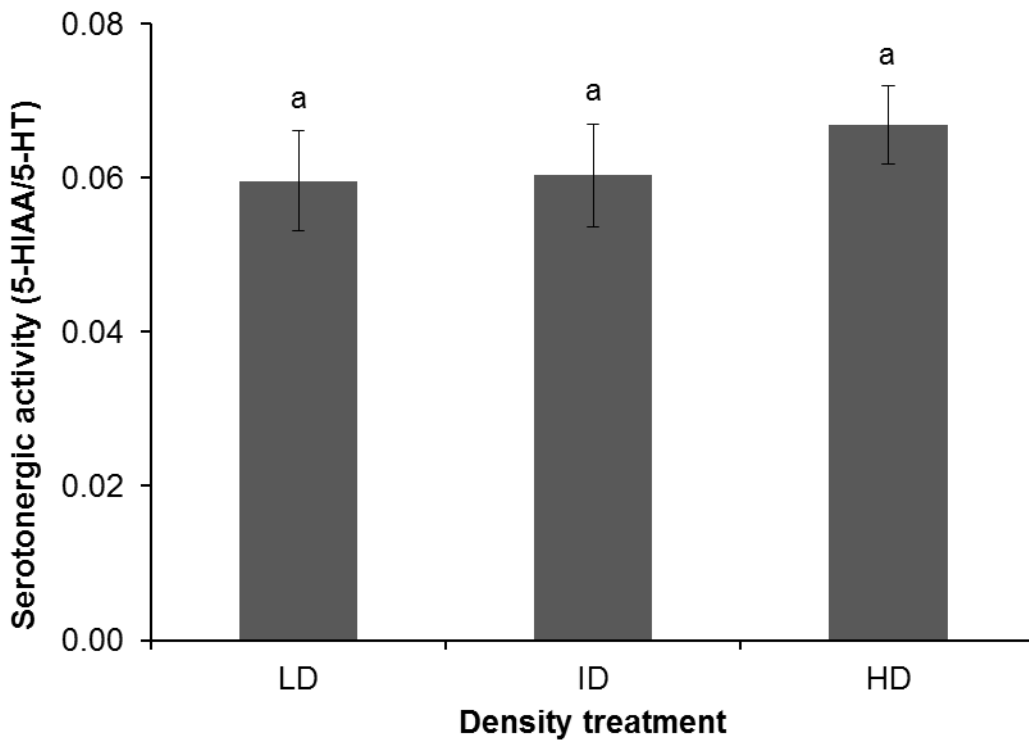
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a)   Brain stem

b) Telencephalon

c) Hypothalamus

598

599   Figure 8. The serotonergic activity level (5-HIAA/5-HT) in the a) Brain stem, b) Telencephalon,

600   and c) Hypothalamus of individuals (n=8 per tank in triplicate) between the density treatments; 25

601   kg m^{-3} (LD), 80 kg m^{-3} (ID) and 140 kg m^{-3} (HD). The letters denote where the significances lie.

602

603   Table 1: The concentration (ng g^{-1}) of the metabolite (5-HIAA) and monoamine (5-HT) in the

604   different brain regions of individuals between the different density treatments (n=3 tanks per

605   treatment); 25 kg m^{-3} (LD), 80 kg m^{-3} (ID) and 140 kg m^{-3} (HD).

		Density treatment			
Brain region	Metabolite and monoamine	LD	ID	HD	p value
Brain stem	5-HIAA	51,90 ± 2,32	63,64 ± 3,05	72,07 ± 4,08	0,001
	5-HT	304,35 ± 12,80	296,42 ± 15,04	287,62 ± 11,86	0,632
Telencephalon	5-HIAA	170,69 ± 8,75	171,70 ± 12,08	218,70 ± 16,18	0,006
	5-HT	964,52 ± 67,67	972,80 ± 122,92	1228,45 ± 146, 95	0,106
Hypothalamus	5-HIAA	96,45 ± 5,39	115,38 ± 9,85	113,26 ± 7,72	0,224
	5-HT	1898,0 ± 138,66	2013,82 ± 129,33	1733,67 ± 138,0	0,299

606