



**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

3

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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<sup>-3</sup>, the  
31 highest density accepted by the fish without showing indications of crowding stress of 80 kg m<sup>-3</sup> as  
32 the intermediate density, and the highest density accepted by the fish showing indications of  
33 crowding stress of 140 kg m<sup>-3</sup> 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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49 1. Introduction

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

55 Of all the studies to date, a wide range of stocking densities have been investigated [1-  
56 5]. However, the majority of these studies lack details about how the densities that were  
57 investigated were selected. It may be speculated that previous research and the densities observed in  
58 practice influenced these decisions. The present study is unique in this regard, as the densities  
59 examined here were reflective of levels of crowding established in a study by Laursen et al. [6]. In  
60 the aforementioned study, the spatial distribution of fish held at different densities in two-tank  
61 systems was used to determine a level of aversion to crowding [6]. The aim of the present study was  
62 to determine if being held at the chosen densities had an influence on indicators of welfare.

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

71 Physical injuries may be considered a welfare concern, based on the five freedoms  
72 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<sup>-3</sup>, the highest density accepted by the fish without showing indications of  
99 crowding stress of 80 kg m<sup>-3</sup>, and the highest density accepted by the fish showing indications of  
100 crowding stress of 140 kg m<sup>-3</sup>. 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 ( $5.5 \text{ mg L}^{-1}$ ), 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<sup>-3</sup> 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<sup>-3</sup> as the intermediate density (ID),  
160 and the highest density accepted by the fish showing indications of crowding stress of 140 kg m<sup>-3</sup> 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<sup>-3</sup>, 80 kg m<sup>-3</sup> and 140 kg m<sup>-3</sup> 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



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% ( $8.5 \text{ mg L}^{-1}$ ) in the tanks held at the low density, 110% ( $11.5 \text{ mg L}^{-1}$ ) at the  
202 intermediate density, and 120% ( $12 \text{ mg L}^{-1}$ ) 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  $\text{mg L}^{-1}$ ) at the intermediate and high densities during the oxygen consumption measurement period.

206 Water quality parameters; nitrite ( $\text{NO}_2^-$ ), nitrate ( $\text{NO}_3^-$ ), ammonia ( $\text{NH}_3/\text{NH}_4^+$ ), 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  $16 \text{ }^\circ\text{C}$ . 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. Every 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 time 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  $\text{O}_2 \text{ kg}^{-1} \text{ hr}^{-1}$ ).

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 ( $\text{g kg}^{-1}$ ), 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. *Neuroendocrine 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 ( $\text{ng ml}^{-1}$ ) 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  $\text{ng ml}^{-1}$  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  $\text{g l}^{-1}$  sodium phosphate, 0.3252  $\text{g l}^{-1}$   
282 sodium octyl sulphate, 0.0037  $\text{g l}^{-1}$  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  $\pm$  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 ( $\text{g kg}^{-1}$ ) and total oxygen consumption ( $\text{mg O}_2 \text{ kg}^{-1}$ ;  $R^2=0.917$ ,  $p<0.001$ ;  
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

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454

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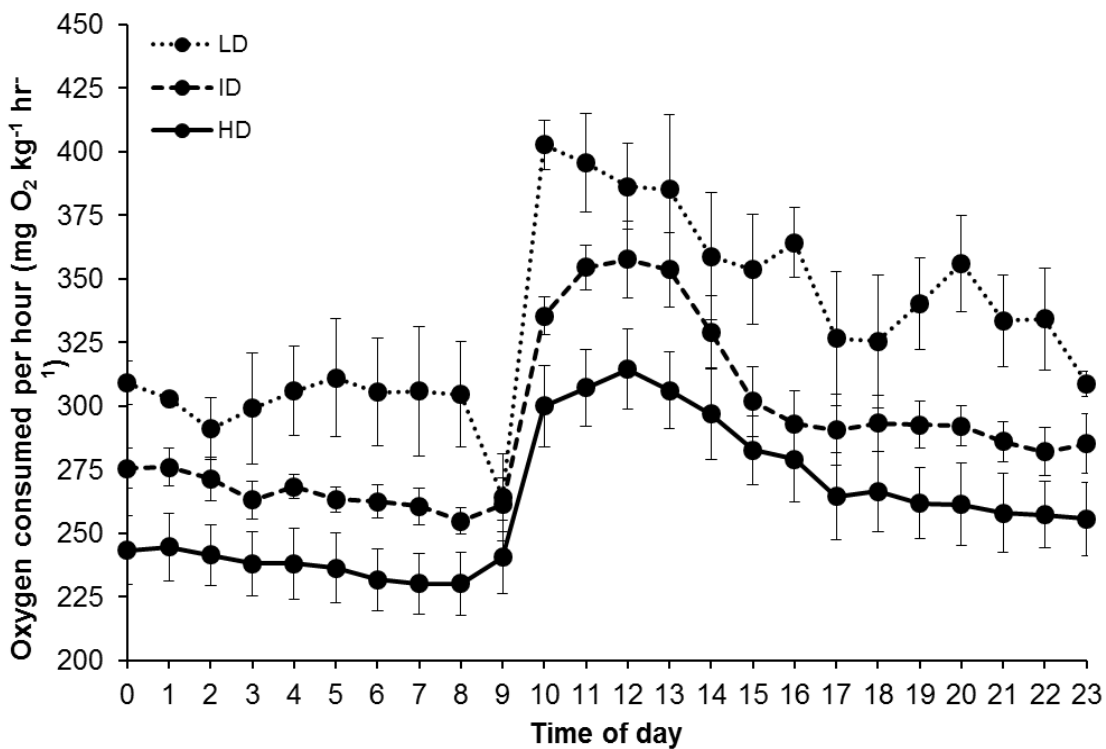
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556 8. Figure captions

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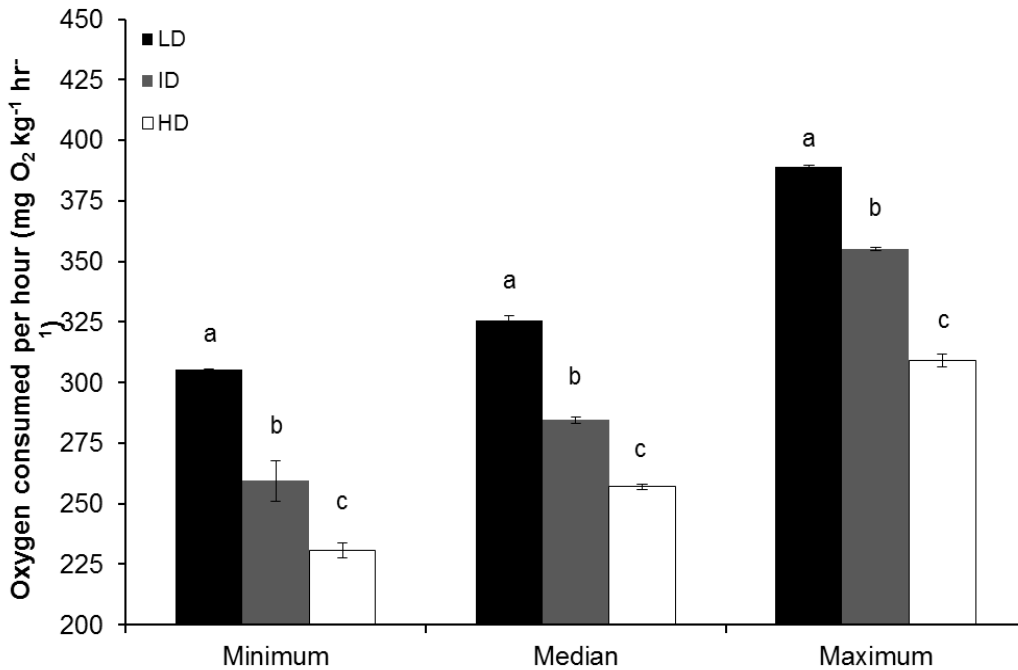
558

559 Figure 1. The hourly oxygen consumption rates (mg O<sub>2</sub> kg<sup>-1</sup> hr<sup>-1</sup>) between the three density

560 treatments (n=3 tanks per treatment); 25 kg m<sup>-3</sup> (LD), 80 kg m<sup>-3</sup> (ID) and 140 kg m<sup>-3</sup> (HD),

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

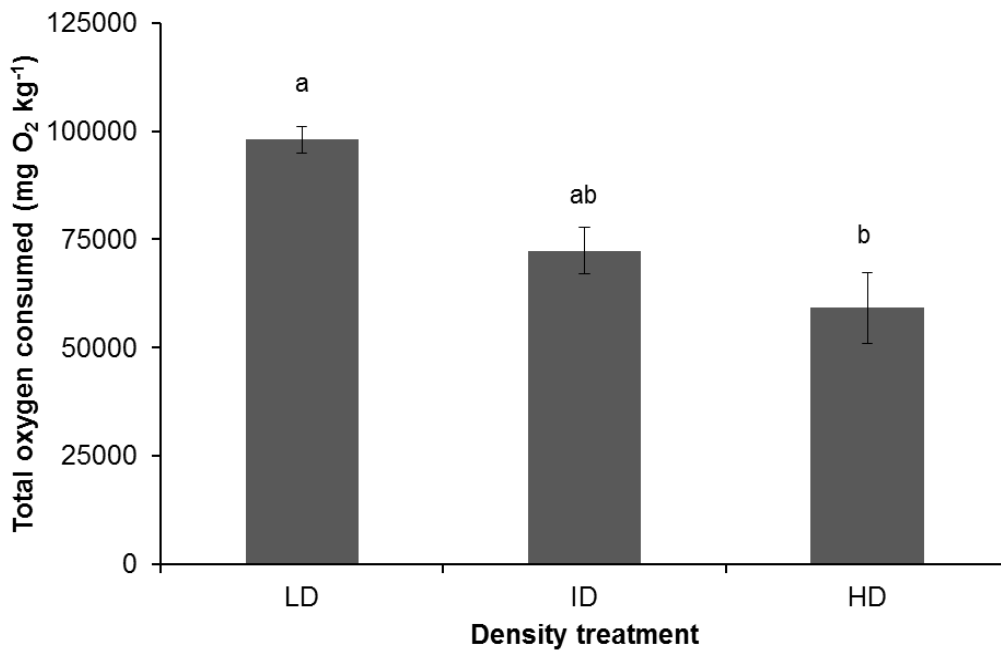
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564

565 Figure 2. The minimum, median and maximum oxygen consumption rates (mg O<sub>2</sub> kg<sup>-1</sup> hr<sup>-1</sup>) during  
566 the daily cycle (Fig. 1) between the density treatments (n=3 tanks per treatment); 25 kg m<sup>-3</sup> (LD),  
567 80 kg m<sup>-3</sup> (ID) and 140 kg m<sup>-3</sup> (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.

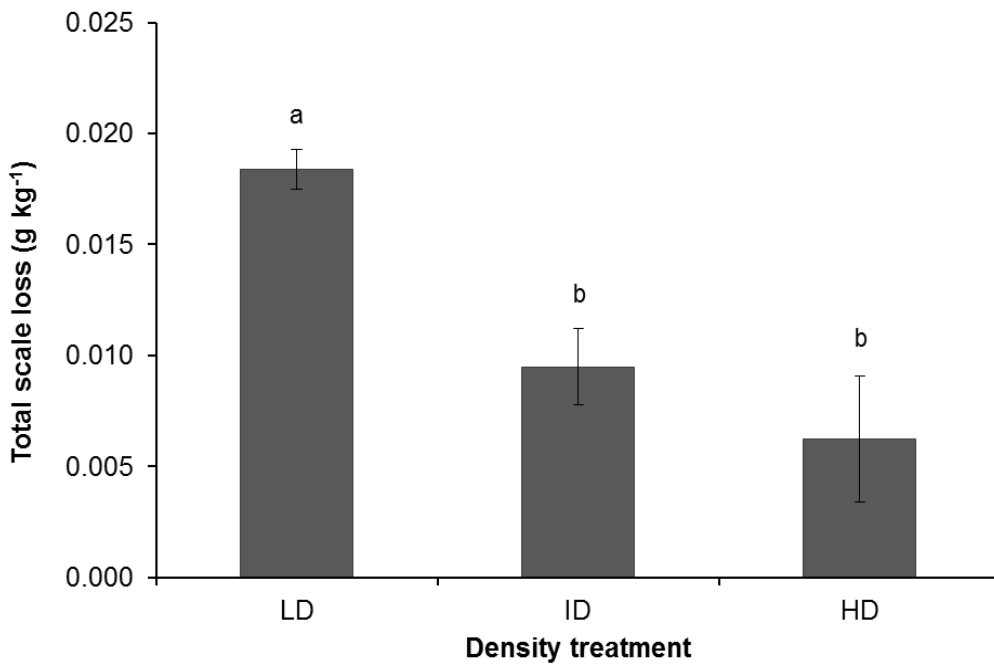
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571

572 Figure 3. The total oxygen consumption rates (mg O<sub>2</sub> kg<sup>-1</sup>) between the density treatments (n=3  
573 tanks per treatment); 25 kg m<sup>-3</sup> (LD), 80 kg m<sup>-3</sup> (ID) and 140 kg m<sup>-3</sup> (HD) during the period  
574 (selected days) when the fish grew from from 190 grams to 220 grams. The letters denote where the  
575 significances lie.

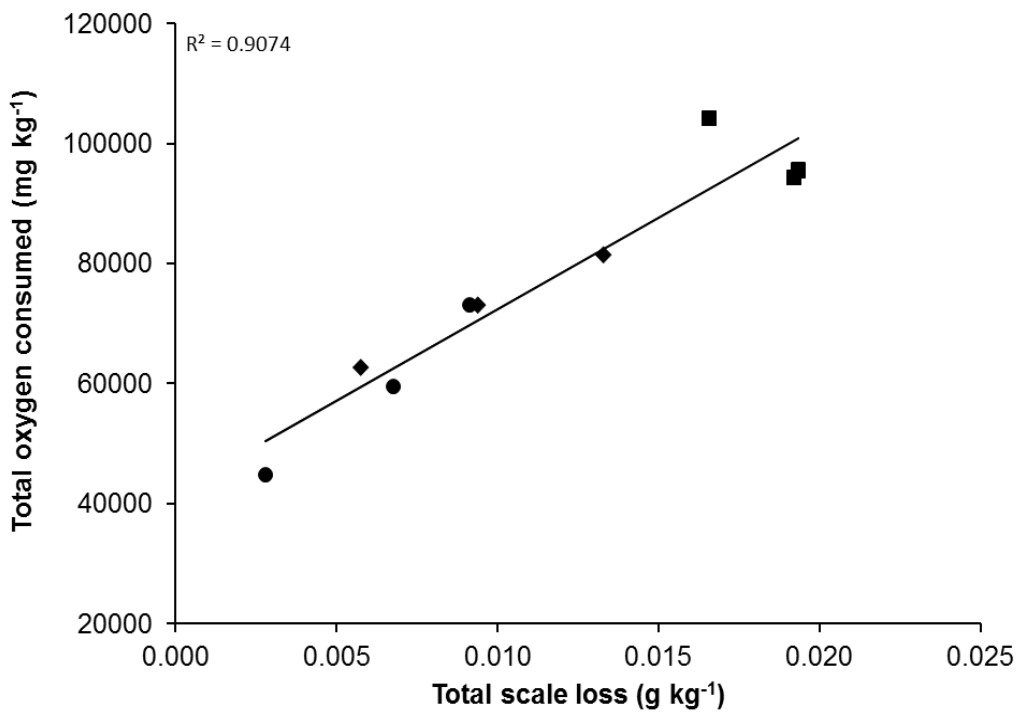
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577

578 Figure 4. The total scale loss (g kg<sup>-1</sup>) between the density treatments (n=3 tanks per treatment); 25  
 579 kg m<sup>-3</sup> (LD), 80 kg m<sup>-3</sup> (ID) and 140 kg m<sup>-3</sup> (HD) during the period (selected days) when the fish  
 580 grew from from 190 grams to 220 grams. The letters denote where the significances lie.

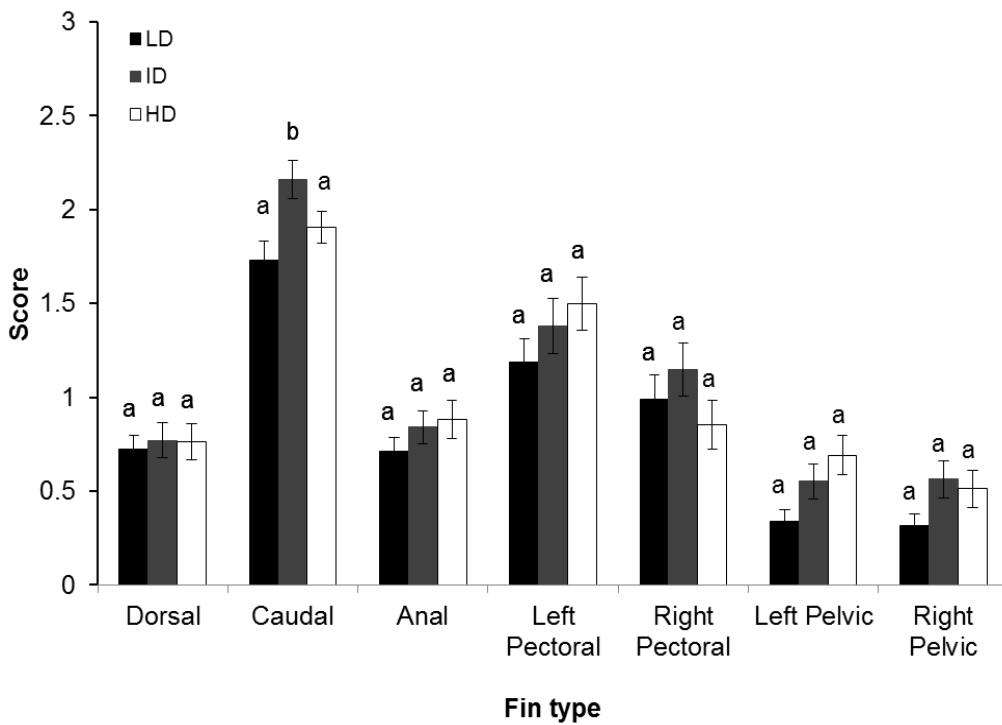
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582

583 Figure 5. The relationship between the total scale loss ( $\text{g kg}^{-1}$ ) and the total oxygen consumption  
 584 ( $\text{mg O}_2 \text{ kg}^{-1}$ ) between the density treatments ( $n=3$  tanks per treatment);  $25 \text{ kg m}^{-3}$  (LD),  $80 \text{ kg m}^{-3}$   
 585 (ID) and  $140 \text{ kg m}^{-3}$  (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.

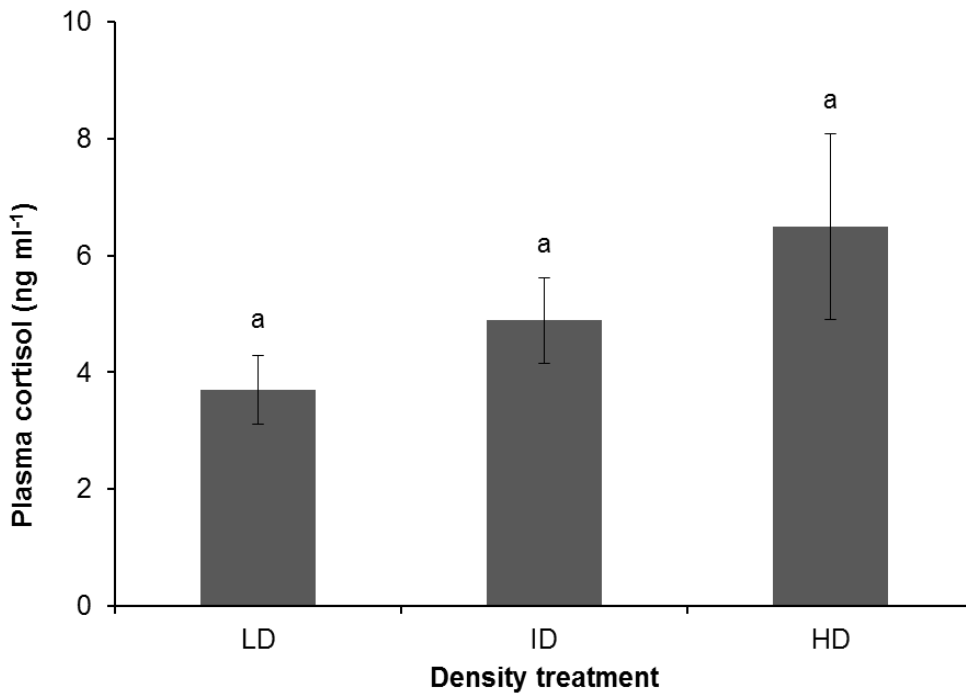
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589

590 Figure 6. The fin score for each fin type of individuals ( $n=30$  individuals per tank in triplicate)  
 591 between the density treatments;  $25 \text{ kg m}^{-3}$  (LD),  $80 \text{ kg m}^{-3}$  (ID) and  $140 \text{ kg m}^{-3}$  (HD) at termination  
 592 of the experiment. The letters denote where the significances lie.

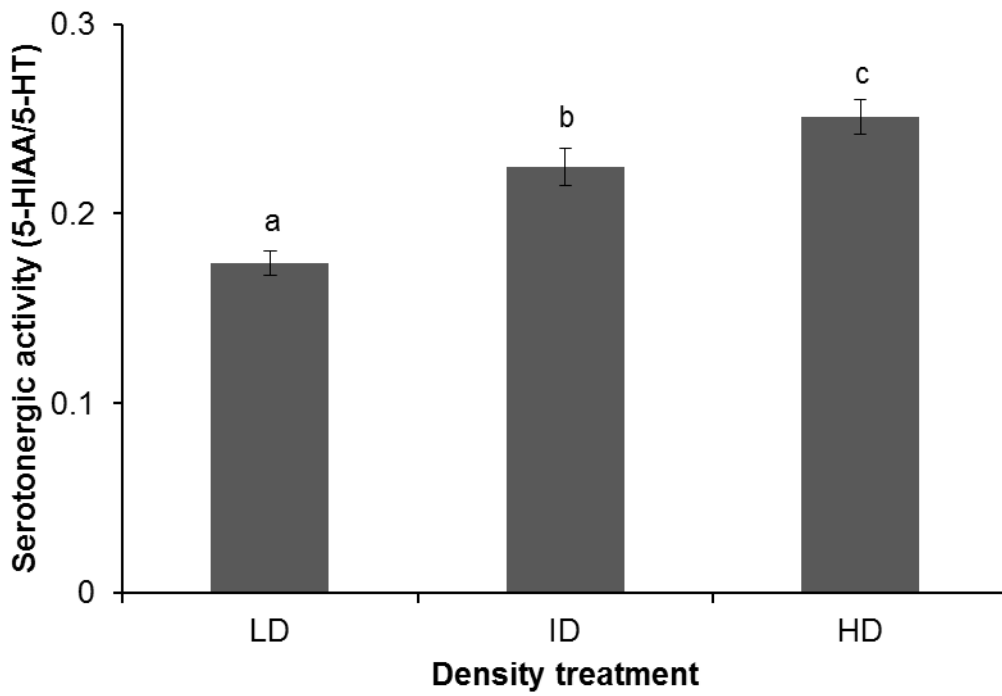
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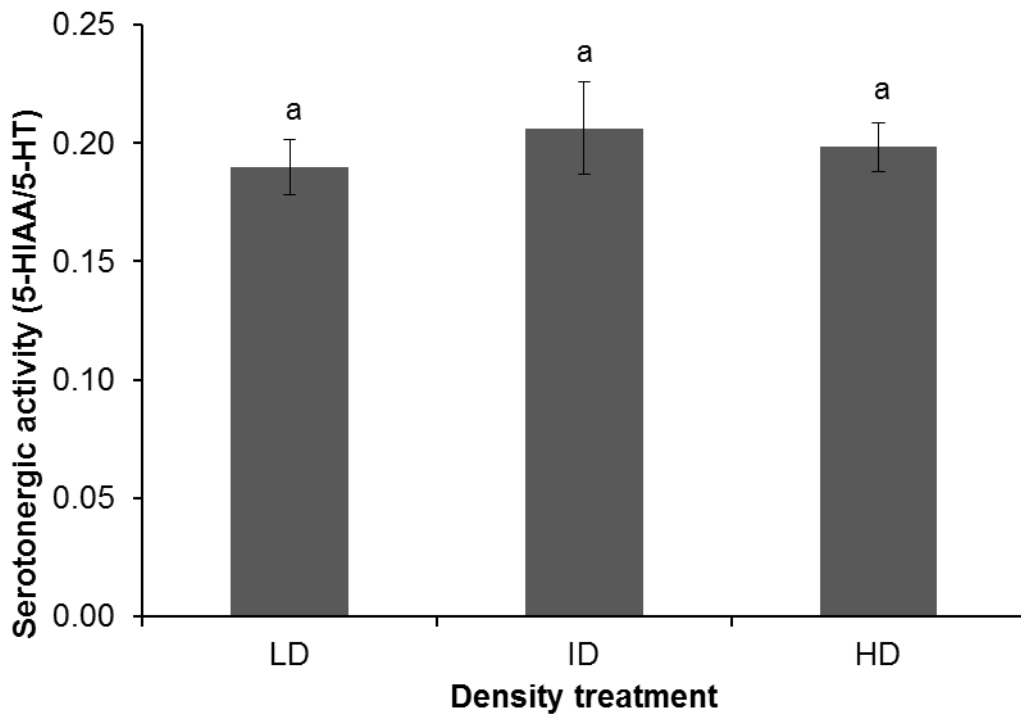
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595 Figure 7. The plasma cortisol concentrations (ng ml<sup>-1</sup>) of individuals between the density treatments  
 596 (n=3 tanks per treatment); 25 kg m<sup>-3</sup> (LD), 80 kg m<sup>-3</sup> (ID) and 140 kg m<sup>-3</sup> (HD).

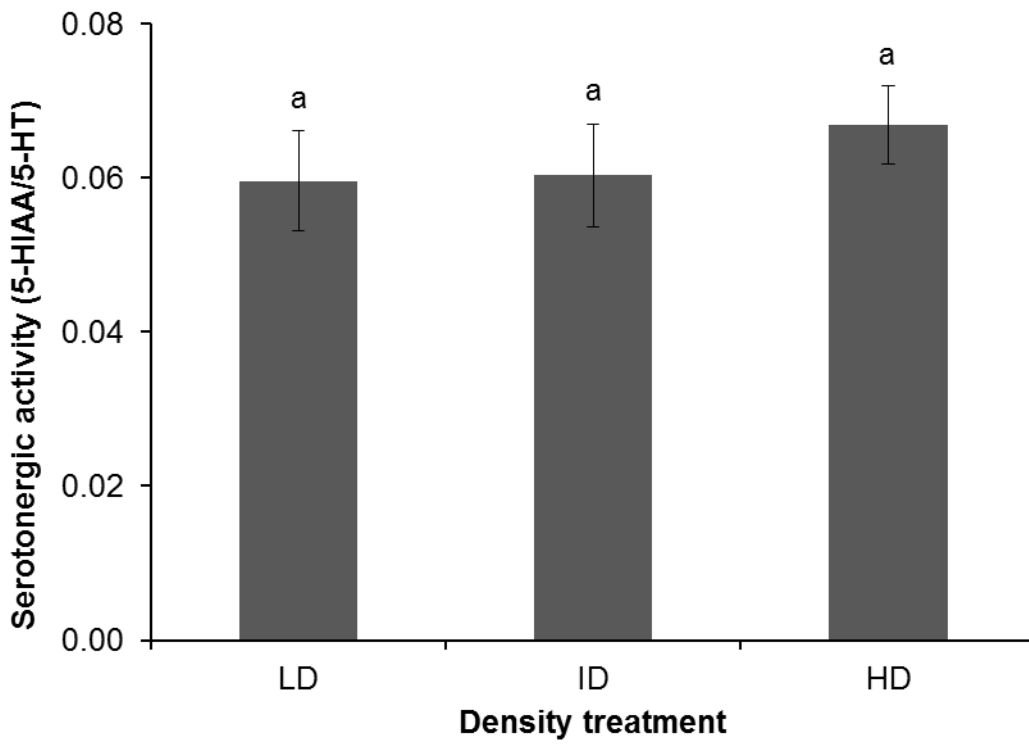
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a) Brain stem



b) Telencephalon



c) Hypothalamus

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<sup>-3</sup> (LD), 80 kg m<sup>-3</sup> (ID) and 140 kg m<sup>-3</sup> (HD). The letters denote where the significances lie.

602

603 Table 1: The concentration (ng g<sup>-1</sup>) 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<sup>-3</sup> (LD), 80 kg m<sup>-3</sup> (ID) and 140 kg m<sup>-3</sup> (HD).

Brain region	Metabolite and monoamine	Density treatment			p value
		LD	ID	HD	
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