



## Utilising spatial distribution in two-tank systems to investigate the level of aversiveness to crowding in farmed rainbow trout *Oncorhynchus mykiss*

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*Published in:*  
Applied Animal Behaviour Science

*Link to article, DOI:*  
[10.1016/j.applanim.2013.01.010](https://doi.org/10.1016/j.applanim.2013.01.010)

*Publication date:*  
2013

[Link back to DTU Orbit](#)

### *Citation (APA):*

Laursen, D. C., Andersson, M. Å., Silva, P. I. M., Petersson, E., & Höglund, E. (2013). Utilising spatial distribution in two-tank systems to investigate the level of aversiveness to crowding in farmed rainbow trout *Oncorhynchus mykiss*. *Applied Animal Behaviour Science*, 144(3-4), 163-170. <https://doi.org/10.1016/j.applanim.2013.01.010>

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1 Utilising spatial distribution in two-tank systems to investigate the level of aversiveness to crowding in  
2 farmed rainbow trout *Oncorhynchus mykiss*

3

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28 Abstract

29                   In aquaculture, fish are exposed to a range of unfavourable environmental conditions.  
30 Amongst these, stocking density has attracted considerable attention as inappropriate densities may  
31 compromise welfare and negatively impact production. However, the recommendations for stocking remain  
32 elusive. The aim of the present study was to apply a novel method to investigate a level of crowding that  
33 indicated aversiveness in rainbow trout (*Oncorhynchus mykiss*). In a two-tank system, where two identical  
34 tanks were connected via a doorway, it was observed that social behaviour controlled the distribution of the  
35 fish between the tanks. Fish were stocked at equal quantities in each tank of the system. The doorway was  
36 opened and the fish moved between the two tanks. Typically, this resulted in one tank being occupied by a  
37 few highly aggressive dominant individuals (“dominant” tank) and the majority of the fish occupying the  
38 second tank (“crowded” tank). Here, the potential of this unequal spatial distribution for quantifying aversion  
39 to crowding was explored. Fish were stocked in three two-choice systems at a total density of 20, 40 and 80  
40  $\text{kg m}^{-3}$  respectively. The number of fish in each tank was determined every three days throughout the  
41 duration of the experiment and the percentage of fish in the “crowded” tank was used as an indicator of the  
42 distribution pattern in the two-tank systems. The results indicated a negative relationship between the total  
43 density stocked (20, 40 & 80  $\text{kg m}^{-3}$ ) and the percentage of fish in the “crowded” tank. A subsample of  
44 individuals was sacrificed for blood and brain samples every three days from the “crowded” tank, prior to the  
45 fish count. The neuroendocrine indicators of stress, elevated serotonergic activity levels which were not  
46 associated with high plasma levels of cortisol, suggested chronic stress in the fish at the highest total density  
47 stocked (80  $\text{kg m}^{-3}$ ). Taken together, these results indicated that a level of aversiveness to crowding had  
48 been reached at the highest density stocked, where the mean absolute density, irrespective of time of day,  
49 observed in the “crowded” tank was  $126.5 \pm 3.7 \text{ kg m}^{-3}$ .

50

51 Keywords

52 Stocking density, aquaculture, behaviour, spatial distribution, aversiveness, stress

53

54 1. Introduction

55                   Stocking density in aquaculture has received considerable attention in recent years. This has  
56 been the consequence of an increasing public concern for the welfare of fish from aquaculture (Huntingford  
57 et al., 2006) and recognition in the commercial and scientific communities that inappropriate densities  
58 contribute to a reduced welfare status in fish (Ellis et al., 2002).

59                   The general perception is that welfare decreases with increasing density, though there are no  
60 unanimous results of the effect of increasing stocking densities on indicators of welfare, such as general  
61 performance and stress hormone levels (Ellis et al., 2002). Naturally, this may in part be due to species  
62 differences, where welfare may be optimal for some species at higher densities and for others at lower  
63 densities. However, contradictory results have been found even within a species (Ellis et al., 2002; Brännäs  
64 and Johnsson, 2008). This has been attributed to differences between studies in experimental design and  
65 methodology (Ellis et al., 2002). However, it has also highlighted the fact that stocking density is a complex  
66 issue and the negative effects on welfare are likely to be the cause of a combination of factors as a  
67 consequence of stocking density (Bagley et al., 1994; Person-Le Ruyet et al., 2008), such as water quality  
68 and social interactions (Ellis et al., 2002).

69                   The method that has most commonly been used to study the relationship between stocking  
70 density and welfare has been by investigating the effects of varying density levels on indicators of welfare;  
71 such as performance, condition, health and stress levels (Boujard et al., 2002; Ellis et al., 2002; Larsen et al.,  
72 2012; McKenzie et al., 2012; North et al., 2006; Person-Le Ruyet et al., 2008; Skøtt Rasmussen et al., 2007).  
73 Through such studies it has been possible to make general conclusions about the influence of stocking  
74 density on welfare. Ellis et al. (2002) reviewed all the studies to date that had investigated the relationship  
75 between stocking density and welfare for rainbow trout, *Oncorhynchus mykiss*. They concluded that despite  
76 the lack of clear evidence, high stocking density had the potential to reduce welfare. Since then, additional  
77 studies have been carried out, which concluded that low as well as high stocking densities had the potential  
78 to compromise indicators of welfare (Boujard et al., 2002; Ellis et al., 2002; Larsen et al., 2012; McKenzie et  
79 al., 2012; North et al., 2006; Person-Le Ruyet et al., 2008; Skøtt Rasmussen et al., 2007).

80 A number of the published studies on this issue have attempted to make specific  
81 recommendations for maximum stocking densities for rainbow trout based on their experimental results  
82 (Ellis et al., 2002). Depending on the type of rearing system, the recommendations for appropriate stocking  
83 densities made by the studies reviewed ranged from 4 to more than 267 kg m<sup>-3</sup> (Ellis et al., 2002).  
84 Evidently, concrete conclusions regarding the density limits at which welfare and production in rainbow  
85 trout are optimised continue to be ambiguous. Therefore, developing alternative methods to investigate the  
86 density levels that fish experience as critically crowded may provide insight into optimal density limits for  
87 rainbow trout.

88 The aim of the current study was to apply a novel method to investigate a level of  
89 aversiveness to crowding of farmed rainbow trout (*Oncorhynchus mykiss*). This was achieved by studying  
90 the spatial distribution in two-tank systems stocked fish at different densities to establish a level of aversion  
91 to crowding. Here, a two-tank system consisted of two identical tanks which were attached to each other with  
92 a doorway, allowing individuals to move freely between the two tanks. Groups of fish held in this system  
93 were observed to distribute themselves unequally between the two tanks, despite equal initial stocking and  
94 equal feed rations in the two tanks. Social behaviour was established as the controlling factor for this  
95 distribution pattern, as aggression and dominance related behaviours by a few individuals in one tank,  
96 referred to as the “dominant” tank, drove the majority of the group into the second tank, referred to as the  
97 “crowded” tank. The percentage of fish, of the total quantity of fish in the system, occupying the “crowded”  
98 tank was used as an indicator of the distribution pattern between the two tanks at three stocking densities; 20,  
99 40 and 80 kg m<sup>-3</sup>. To support these observations, neuroendocrine indicators of stress, plasma cortisol and  
100 brain serotonergic activity, of individuals from the “crowded” tank were examined to determine crowding  
101 stress.

102

## 103 2. Materials and Methods

### 104 2.1 *Experimental fish*

105 Rainbow trout from Mark Mølle fish farm, Nykøbing Mors in Denmark were used in the  
106 present study. The fish were transported by truck to the Danish Technical University, Institute of Aquatic

107 Resources (DTU Aqua) in Hirtshals and upon arrival unloaded directly into quarantine tanks. While in  
108 quarantine, the fish were put on a feeding regime at 0.75 % of their total body mass per day. Additionally,  
109 the salt content in the water was slowly increased to 15 ‰. The fish were held in quarantine conditions for a  
110 period of 15 days, after which they were available to be used for experiments.

111 Fish were ordered and delivered on two occasions to provide adequate quantities of  
112 individuals for all three trials of the experiment. Fish from the first delivery were used in trial 1 and 2 and  
113 fish from the second delivery were used in trial 3. The fish originated from the same family.  
114 At the time of arrival, the fish from the first delivery had an average individual weight of 150 g. At the time  
115 the fish were used during trial 1 and trial 2, the fish had an average individual weight of 279 g and 390 g  
116 respectively. At the time of arrival, the fish from the second delivery had an average individual weight of  
117 300 g. At the time the fish were used during trial 3, the fish had an average individual weight of 430 g.

118

## 119 *2.2 Experimental facilities*

120 The three trials of the experiment were carried out using two-tank systems. Each system  
121 consisted of two identical 700 liter circular tanks attached to one another via a doorway. The doorway could  
122 be opened by the researcher by removing the sliding door. Each tank was 100 cm in height and had a  
123 diameter of 100 cm. The doorway had a width of 15 cm and ran the height of the tank. Each tank was  
124 individually equipped with a water inflow and outflow, as well as an oxygen and air supply. A water current  
125 of approximately  $0.5 \text{ BL s}^{-1}$  (body lengths per second) was achieved through small holes in inflow pipe  
126 creating pressure, thereby circulating the water around the tank.

127 Three two-tank systems, standing parallel to each other, were used simultaneously during each  
128 trial and were supplied with water from the same recirculating system. The water quality parameters in the  
129 system; temperature, ammonia, nitrate, nitrite and pH were checked daily to ascertain that they were within  
130 optimal levels for the fish. The temperature of the water in the system was  $16 \pm 0.01 \text{ }^\circ\text{C}$ , ammonia  
131 ( $\text{NH}_3/\text{NH}_4^+$ ) levels were  $0 \text{ mg l}^{-1}$ , nitrite ( $\text{NO}_2^-$ ) and nitrate ( $\text{NO}_3^-$ ) were  $37.6 \pm 1.9$  and  $0.4 \pm 0.05 \text{ mg l}^{-1}$   
132 respectively and pH was  $7.6 \pm 0.01$ . Oxygen levels were adjusted manually as the fish moved between the

133 tanks and kept at levels between 90- 100% saturation in both tanks of each system. The fish were on a 12/12  
134 hour light dark regime, with the lights switching on automatically at 08:00 and switching off at 20:00.

135

### 136 *2.3 Experimental design*

137           During pilot studies it was observed that when groups of fish were placed in a two-tank  
138 system, the result was an unequal distribution of individuals between the two tanks. At the start, an equal  
139 quantity of fish was stocked in each tank of the system. Each tank was given the same amount of feed,  
140 throughout the study. The doorway was opened allowing individuals to move freely between the two tanks.  
141 The resulting distribution pattern typically observed was one tank becoming occupied by a few dominant  
142 aggressive individuals and the majority of the fish occupying the second tank. The few dominant individuals  
143 occupying one tank drove out the majority of the group into the second tank, thereby controlling the  
144 distribution of the group in the two-tank system. Although quantifications of their behaviour were not made,  
145 observation of the fish confirmed that they exhibited behaviour that was characteristic for a dominant  
146 individual. They displayed territorial behaviour, monopolising the food resource with chasing out individuals  
147 entering the tank. Furthermore, if more than one individual was present they displayed agonistic behaviour  
148 towards each other. The tank occupied by the dominant individuals will be referred to as the “dominant” tank  
149 and the tank holding the majority of the fish as the “crowded” tank. For the present study, we utilised this  
150 inequality in the distribution pattern of groups of fish in the two-tank system to investigate a level of  
151 aversiveness to crowding.

152           Three stocking densities were used during the experiment; the first two-choice system was  
153 stocked at 20 kg m<sup>-3</sup>, the second at 40 kg m<sup>-3</sup> and the third at 80 kg m<sup>-3</sup>. The experiment was completed in  
154 triplicates as trial 1, trial 2 and trial 3. Between each trial, the stocking density in each two-choice system  
155 was changed. The number of fish in each tank was determined every three days during the experiment for a  
156 period of two weeks. As the distribution of the fish changed between the night time and the day time, the fish  
157 count and sampling of individuals in the “crowded” tank was determined at two time points during the daily  
158 cycle. One time point was chosen at the end of the night time hours, which was in the morning at 07:30 when  
159 it was still dark. The second time point was chosen at the end of the day time hours, which was in the

160 evening at 19:30 when it was still light. These time points will be referred to as in “dark” and “light”,  
161 respectively. During the experiment, sampling was alternated between the morning (“dark”) and the evening  
162 (“light”). Between each trial, the order of the sampling time points was changed. If in trial 1, the first  
163 sampling was done in the “dark”, then during trial 2, the first sampling was done in the “light” and so on. For  
164 each trial, there were a total of four sampling sessions; two at “dark” (session 1 and session 2) and two at  
165 “light” (session 1 and session 2).

166           Additionally, a subsample consisting of six individuals from the “crowded” tank was sampled  
167 for blood and brain parts. The individuals were taken before the number of fish in the tank was determined.  
168 Plasma cortisol concentrations and brain serotonergic activity were analysed to assess the stress levels in this  
169 tank. Cortisol is a commonly used physiological indicator of stress in fish when studying the effects of  
170 stocking density (Ellis et al., 2002; North et al., 2006). Additionally, serotonergic activity, the ratio between  
171 the brain tissue concentration of serotonin (5-HT, monoamine) and 5-hydroxyindoleacetic acid (5-HIAA,  
172 metabolite), has previously been used as an indicator of stress in relation to stocking density in rainbow trout  
173 (McKenzie et al., 2012) and has also been used as an indicator of chronic social stress in salmonid fish in  
174 pairs and small groups (Øverli et al., 1999; Winberg et al., 1991; Winberg et al., 1992; Winberg and Nilsson,  
175 1993).

176

#### 177 *2.4 Experimental procedure*

178           The fish were transported to the experimental facility and stocked into the three two-tank  
179 systems using 20, 40 and 80 kg m<sup>-3</sup>. The two tanks of each system were stocked with equal densities.  
180 During this initial stocking process, the number of fish going into each tank was counted to allow for future  
181 determination of the percentage of fish occupying each tank. After initial stocking, the fish were given an  
182 acclimation period of a week and the doorway separating the two tanks was left closed to hinder any re-  
183 distribution before the start of the experiment. The fish in each tank of the systems were fed at 1% of their  
184 total body weight (grams) from 08:00 to 20:00 using 12 hour automated belt feeders. After an acclimation  
185 period of a week, the doorway between the two tanks in each system was opened, allowing the fish to swim



186 freely between the two environments. The amount of feed given to each tank of the two-tank systems was  
187 kept at the same level as during the acclimation period.

188           The number of fish in each tank was determined every three days. For practical reasons, this  
189 was done by counting the number of fish in the “dominant” tank and subtracting this count from the total  
190 number of known fish in the system. Before determining the number of fish in each tank, a subsample of six  
191 individuals from the “crowded” tank of each system were sacrificed by an overdose of anaesthetic (Ethylene  
192 glycol monophenyl ether). Blood samples were collected from the caudal vein using 1 ml syringes filled with  
193 EDTA (Ethylenedinitrilotetraacetic acid disodium salt dihydrate) powder. The blood samples were  
194 centrifuged and the plasma was separated into 1 ml eppendorf tubes and frozen at -80 °C for later analysis.  
195 Whole brains were dissected out from each fish and separated into four parts; brain stem, hypothalamus,  
196 telencephalon and optic lobes, frozen directly using liquid nitrogen and then stored in the -80 °C freezer for  
197 later analysis.

198

#### 199 *2.5. Analysis of plasma cortisol and serotonin*

200           Cortisol was extracted from the plasma using ethyl ether, evaporated using a vacuum  
201 centrifuge and re-suspended in an extraction buffer (ELISA kit extraction buffer). Concentrations (ng ml<sup>-1</sup>)  
202 were quantified using the ELISA kit standard method (Neogen, Product #402710).

203           Frozen brain parts were homogenised in a homogenising reagent (4% perchloric acid, 0.2%  
204 Ethylenediaminetetraacetic acid, 40 ng ml<sup>-1</sup> dihydroxi benzylamine hydroxide solution) and centrifuged at  
205 10,000 rpm at 4 °C for 10 minutes to separate the supernatant. The supernatant was assayed using High  
206 Performance Liquid Chromatography (HPLC) with electrochemical detection, described in Andersson and  
207 Höglund (2012), to quantify 5-HIAA (metabolite) and 5-HT (monoamine). The supernatant (sample) was  
208 transported through the HPLC system by a mobile phase, which consisted of a buffer solution containing  
209 10.35 g l<sup>-1</sup> sodium phosphate, 0.3252 g l<sup>-1</sup> sodium octyl sulphate, 0.0037 g l<sup>-1</sup> ethylenediaminetetraacetic  
210 acid disodium salt dehydrate, 7% acetonitril in deionised water. The compounds in the sample were analysed  
211 using a computer program (software; Clarity, DataApex Ltd.). The sample 5-HIAA and 5-HT quantities were

212 compared with quantities from solutions of known concentration (standards) to determine the actual  
213 concentrations.

214

## 215 *2.6 Statistical analyses*

216 The percentage of the total number of fish in one tank was used as a measure of crowding.

217 The difference in the proportions of fish occupying the “crowded” tank between density treatments (20, 40 &  
218 80 kg m<sup>-3</sup>), sampling time (“dark” and “light”), trial (1, 2 & 3), two-choice system (1, 2 & 3) and session (1  
219 & 2) was analysed with a generalised linear model (GENMOD). In addition to the mentioned variables (class  
220 variables) initial weight of the fish was used as a covariate. The response variable was number of fish in the  
221 crowded tank/total number of fish (binomial distribution).

222 To determine if there was a difference in the concentrations of plasma cortisol, concentrations  
223 of 5-HIAA and 5-HT, and ratios of 5-HIAA/5-HT between density treatments (20, 40 & 80 kg m<sup>-3</sup>),  
224 sampling time (“dark” and “light”), trial (1, 2 & 3), and session (1 & 2), was determined using an ANCOVA,  
225 with fish weight (at the time of sampling) as the covariate. The log concentrations of plasma cortisol, log  
226 concentrations of 5-HIAA and 5-HT, or arcsin ratios of 5-HIAA/5-HT were used as the dependent variables.  
227 A Tukey’s post hoc test was used to determine between which treatments the significances occurred.

228

## 229 3. Results

### 230 3.1 Spatial distribution of fish

#### 231 *3.1.1 Percentage of fish in the “crowded” tank*

232 The GENMOD did not indicate any differences between trials (p=0.986), two choice system  
233 (p=0.343), sampling time (p=0.143) or session (p=0.875). The percentage of the fish choosing to be in the  
234 crowded environment decreased with increasing total stocking densities (p<0.001, Fig. 1), with a significant  
235 difference between stocking densities 20 and 40 kg m<sup>-3</sup> (p= 0.007), between 20 and 80 kg m<sup>-3</sup> (p<0.001)  
236 and between 40 and 80 kg m<sup>-3</sup> (p<0.001). At 80 kg m<sup>-3</sup>, of a total of 314 ± 23 individuals in the system,  
237 251 ± 27 occupied the “crowded” tank. At 40 kg m<sup>-3</sup>, 125 ± 11 out of a total of 144 ± 9 individuals  
238 occupied the “crowded” tank. At 20 kg m<sup>-3</sup>, 64 ± 6 out of a total of 77 ± 7 individuals occupied the crowded

239 tank. Furthermore, there was a positive relationship between initial fish weight and density in the crowded  
240 tank ( $p < 0.001$ ).

241

### 242 3.1.2. Absolute density in the “crowded” tank

243 The absolute density ( $\text{kg m}^{-3}$ ) in the “crowded” tank of the two-tank systems was determined  
244 from the percentage of the fish occupying this tank. At stocking density  $20 \text{ kg m}^{-3}$  the mean absolute  
245 density in the “crowded” tank irrespective of sampling time was  $32.5 \pm 1.5 \text{ kg m}^{-3}$  (Fig. 2). At “dark” and  
246 “light” the absolute density was  $30.7 \pm 2.3 \text{ kg m}^{-3}$  and  $34.3 \pm 2.1 \text{ kg m}^{-3}$  respectively. At  $40 \text{ kg m}^{-3}$  the  
247 mean absolute density was  $63.7 \pm 2.4 \text{ kg m}^{-3}$  (Fig. 2), and  $57.4 \pm 3.5 \text{ kg m}^{-3}$  and  $69.9 \pm 3.3 \text{ kg m}^{-3}$  in the  
248 “dark” and “light” respectively. At  $80 \text{ kg m}^{-3}$  the mean absolute density was  $126.5 \pm 3.7 \text{ kg m}^{-3}$  (Fig. 2),  
249 and in the “dark” and “light” was  $115.7 \pm 5.5 \text{ kg m}^{-3}$  and  $137.4 \pm 10.0 \text{ kg m}^{-3}$  respectively.

250

## 251 3.2 Neuroendocrine indicators of stress

### 252 3.2.1 Plasma cortisol

253 Despite a tendency for slight elevation in the plasma cortisol concentrations of individuals in  
254 the “crowded” tank at the highest total density stocked ( $\text{kg m}^{-3}$ ), there was no difference in the levels  
255 between the three densities stocked ( $20, 40 \text{ \& } 80 \text{ kg m}^{-3}$ ;  $p = 0.314$ ; Fig. 3). There was also no significant  
256 difference between the “dark” and “light” (sampling time;  $p = 0.140$ ), between the first and second sampling  
257 session (session;  $p = 0.077$ ), between trials ( $p = 0.948$ ), two-choice system ( $p = 0.128$ ) or fish weight ( $p = 0.217$ ).

258

### 259 3.2.2 Brain ratios (5-HIAA/5-HT)

260 Generally, the serotonergic activity in the brain stem of the individuals in the “crowded” tank  
261 was higher in the “light” compared to the “dark” irrespective of stocking density ( $p = 0.013$ ) and higher in the  
262 first sampling session compared to the second sampling session irrespective of density (session;  $p = 0.001$ ).  
263 Moreover, there was a higher activity level in the individuals in the “crowded” tank of the system stocked at  
264  $80 \text{ kg m}^{-3}$ , compared to the individuals in the two systems stocked at  $20$  and  $40 \text{ kg m}^{-3}$  ( $p < 0.001$ ; Fig. 4A).  
265 Specifically, there were no differences in activity levels between  $20$  and  $40 \text{ kg m}^{-3}$  ( $p = 0.953$ ), but

266 differences between 20 and 80 kg m<sup>-3</sup> (p<0.001) and between 40 and 80 kg m<sup>-3</sup> (p<0.001; Fig. 4A).  
267 Furthermore, there was an effect of trial (p=0.028). Fish weight showed a negative relationship with  
268 serotonergic activity (p=0.004).

269 The serotonergic activity in the telencephalon of the individuals in the “crowded” tank  
270 followed a similar pattern. Activity levels were higher in the individuals in the “light” compared to the  
271 “dark” irrespective of density (sampling time; p≤0.001). In contrast to the brain stem, serotonergic activity  
272 was higher in the second sampling session compared to the first (session; p≤0.001). Furthermore, in the  
273 telencephalon there was only a trend towards higher serotonergic activity in the individuals in the “crowded”  
274 tank of the system stocked at 80 kg m<sup>-3</sup>, compared to 20 and 40 kg m<sup>-3</sup> (p=0.064; Fig. 4B). There was no  
275 effect of trials (p=0.919) or fish weight (0.518).

276 The 5-HTergic activity in the hypothalamus of the individuals in the “crowded” tank, of all  
277 systems combined, did not differ between the “dark” and “light” (sampling time; p=0.127), between the first  
278 and second sampling session (p=0.064), between trial (p=0.058), fish weight (p=0.109) or the total densities  
279 stocked (p=0.263; Fig. 4C).

280

### 281 3.2.3 Brain 5-HT and 5-HIAA

282 The concentration of the main metabolite (5-HIAA) of serotonin and monoamine serotonin (5-  
283 -HT) in the brain stem, telencephalon and hypothalamus between the three density treatments (20, 40, & 80  
284 kg m<sup>-3</sup>) are given in Table 1.

285 In the brain stem, there was a significant effect on 5-HIAA concentration by sampling time  
286 (p=0.013), session (p=0.001) and density treatment (p=0.012) and trial (p=0.011), but there was no effect of  
287 fish weight (p=0.468). There was a significant difference in 5-HT concentration between session (p<0.001)  
288 and trial (p=0.001), but not sampling time (p=0.301), fish weight (p=0.368) or density treatment (0.703).

289 In the telencephalon, there was a difference in 5-HIAA concentration between sampling time  
290 (p=0.012), but not between trials (p=0.069), session (p=0.975), fish weight (p=0.329) or density treatment  
291 (p=0.345). A similar pattern was observed in 5-HT concentrations, where an effect of sampling time

292 ( $p < 0.001$ ) and trials ( $p < 0.001$ ) was observed. However, no effect of session ( $p = 0.116$ ), fish weight ( $p = 0.846$ )  
293 or density treatment ( $p = 0.146$ ) were detected.

294 In the hypothalamus, there was a difference in 5-HIAA concentration between sampling time  
295 ( $p = 0.008$ ), trials ( $p < 0.001$ ), session ( $p = 0.044$ ), but not fish weight ( $p = 0.173$ ) or density treatment ( $p = 0.321$ ). In  
296 5-HT concentrations there was a difference between session ( $p \leq 0.001$ ), trials ( $p < 0.001$ ), but not sampling  
297 time ( $p = 0.986$ ), fish weight ( $p = 0.643$ ) or density treatment ( $p = 0.798$ ).

298

#### 299 4. Discussion

300 In the present study, the distribution of the fish in the two-tank systems was unequal,  
301 irrespective of total density, with a few highly aggressive dominant individuals controlling one tank  
302 (“dominant” tank) and the majority of the fish preferring to occupy the second tank (“crowded” tank). This  
303 distribution pattern resembled an Ideal Despotic Distribution (IDD), first described in birds, where  
304 movement between patches was controlled by intraspecific competition (Fretwell, 1972). The IDD has  
305 previously been described in laboratory situations in Salmonids, where dominant individuals excluded other  
306 individuals from a favourable patch (Hakoyama and Iguchi, 2001; Maclean et al., 2005). In our study,  
307 although behavioural quantifications of the individuals in the “dominant” tank were not carried out,  
308 observation of the fish confirmed that they displayed agonistic behaviours towards other individuals in the  
309 tank and fish attempting to enter the tank. Furthermore, it was observed that with increasing density, apart  
310 from the few dominant aggressive individuals occupying the “dominant” tank, there was a spillover of  
311 individuals from the “crowded” tank entering the “dominant” tank that did not perform aggressive acts.  
312 These individuals stayed immobile and accumulated in the “dominant” tank close to the doorway between  
313 the two tanks, a behaviour which is typically observed in subordinate fish (Abbott et al., 1985; Øverli et al.,  
314 1999; Winberg and Nilsson, 1993; Øverli et al., 1998). This distribution pattern was especially distinct in the  
315 two-tank system stocked with the highest total density, with the “dominant” tank occupied by a few  
316 dominant aggressive individuals and a gradual accumulation of subordinate individuals. The results indicated  
317 a negative relationship between the percentage of fish in the “crowded” tank and the total density stocked.

318 Specifically, the percentage of fish in the “crowded” tank decreased significantly with increasing total  
319 stocking density (20, 40 and 80 kg m<sup>-3</sup>). As a result of this distribution pattern in the tanks, irrespective of  
320 the time of day (sampling time, “dark” or “light”), the mean absolute density in the “crowded” tank stocked  
321 at a total density of 20 kg m<sup>-3</sup> was 33 kg m<sup>-3</sup>, at 40 kg m<sup>-3</sup> was 64 kg m<sup>-3</sup>, and 80 kg m<sup>-3</sup> was 127 kg m<sup>-3</sup>.  
322 Moreover, although not significantly different, the spatial distribution was observed to be more unequal  
323 during the hours when it was light (evening sampling) than during the hours when it was dark (morning  
324 sampling). During the day the fish were provided with a food resource to compete for, resulting in a few  
325 individuals monopolising this resource in one tank (“dominant” tank) and driving out the majority of the  
326 individuals into the second tank (“crowded” tank).

327           Neuroendocrine indicators of stress were examined to support our behavioural observations.  
328 Interestingly, the significantly higher serotonergic activity found in the brain stem and telencephalon of the  
329 individuals in the “crowded” tank under light conditions, irrespective of density, indicated higher stress  
330 levels in these fish. This suggests that stronger social competition in the “dominant” tank during the day led  
331 to greater inequality in the observed distribution of the fish in the two-choice systems which resulted in  
332 higher stress levels in the “crowded” tank. Furthermore, we observed elevated serotonergic activity, as 5-  
333 HIAA concentrations and 5-HIAA/5-HT ratios, in the brain stem and a tendency for elevated levels in the  
334 telencephalon of the individuals in the “crowded” tank of the system stocked at the highest density (80 kg m<sup>-3</sup>).  
335 Previous studies investigating social behaviour in pairs or small groups of fish found an elevation in  
336 serotonergic activity levels in individuals exposed to prolonged periods of social stress (socially subordinate  
337 individuals) (Øverli et al., 1999; Winberg et al., 1991; Winberg et al., 1992; Winberg and Nilsson, 1993), as  
338 indicated by elevated concentrations of 5-HIAA and 5-HIAA/5-HT ratios (Winberg and Nilsson, 1993;  
339 Winberg and Lepage, 1998). Often in parallel to this is an elevation in plasma cortisol concentration,  
340 suggesting a stimulatory role of 5-HT activity on the HPI axis (Øverli et al., 1999). However, this  
341 relationship tends to weaken during prolonged stress, where HPI axis reactivity decreases while 5-HT  
342 activity remains high (Winberg and Lepage, 1998). Indeed, the plasma cortisol levels found in the  
343 individuals in the “crowded” tank of the two-choice systems in the present study were generally low, and did

344 not co-vary with serotonergic activity. Nevertheless, these findings are not uncommon in Salmonids. Basal  
345 levels of plasma cortisol in unstressed fish below  $5 \text{ ng ml}^{-1}$ , usually between  $1\text{-}2 \text{ ng ml}^{-1}$ , have been found,  
346 and in chronically stressed individuals, below  $10 \text{ ng ml}^{-1}$  (Pickering and Stewart, 1984; Pickering and  
347 Pottinger, 1989). In some cases, when subjected to chronic stress, plasma cortisol levels ( $10 \text{ ng ml}^{-1}$ )  
348 eventually returned to basal levels ( $\text{ng ml}^{-1}$ ) after a period of time, despite the continued presence of stress  
349 (Barton et al., 1980; Pickering, 1992; Strange and Schreck, 1978). Hence, in the present study, elevated  
350 levels of serotonergic activity and low concentrations of cortisol in the “crowded” tank of the two-choice  
351 system stocked at  $80 \text{ kg m}^{-3}$  should reflect chronic stress in a crowded situation.

352           The positive relationship between density and fish weight suggested that larger fish accepted  
353 to be at a higher density than smaller individuals. The negative relationship between fish weight and  
354 serotonergic activity in the brain stem suggests that of the fish that have accepted to stay in the “crowded”  
355 tank, the smaller fish had higher stress levels compared to larger fish. Additional studies are needed to assess  
356 how fish size influences the distribution of fish, but our results indicate that fish size is an important factor to  
357 consider when investigating critical stocking densities. Furthermore, although water quality parameters were  
358 checked at a system level daily, they were not measured specifically at the tank level. It may be speculated  
359 that as there was such a high number of fish in the “crowded” tank of the system stocked at the highest  
360 density, the water quality may have been influenced. As a result, we cannot exclude the fact that the density  
361 effects observed on the neuroendocrine stress levels could be, in part, influenced by water quality. Therefore,  
362 additional studies are necessary to exclude the influence of this factor.

363

## 364 5. Conclusion

365           Here we have presented a method using two-tank systems to determine a level of crowding  
366 that showed signs of aversiveness in farmed rainbow trout. A negative relationship between stocking density  
367 and the percentage of fish occupying the “crowded” tank was observed. Furthermore, the neuroendocrine  
368 indicators of stress suggested the presence of chronic stress in the fish of the two-tank system stocked at the  
369 highest density ( $80 \text{ kg m}^{-3}$ ), with low concentrations of plasma cortisol but elevated levels of serotonergic  
370 activity found in the brain stem of the individuals in the “crowded” tank of this system. Overall, these results

371 indicated that a level of aversiveness to crowding had been reached at the highest total density stocked,  
372 where the mean absolute density that was observed in the “crowded” tank was  $126.5 \pm 3.7 \text{ kg m}^{-3}$ . A follow  
373 up study is necessary to assess if being held at the densities accepted by the fish in the present study has an  
374 impact on indicators of welfare and performance in farmed rainbow trout.

375

## 376 6. Acknowledgements

377 This study was funded by the Danish Ministry of Food, Agriculture and Fisheries. We thank  
378 Ole Madvig Larsen, Rasmus Freydenlund Jensen, Erik Poulsen and Alfred Jokumsen from the Danish  
379 Technical University, Institute of Aquatic Resources for their practical assistance with the fish.

380

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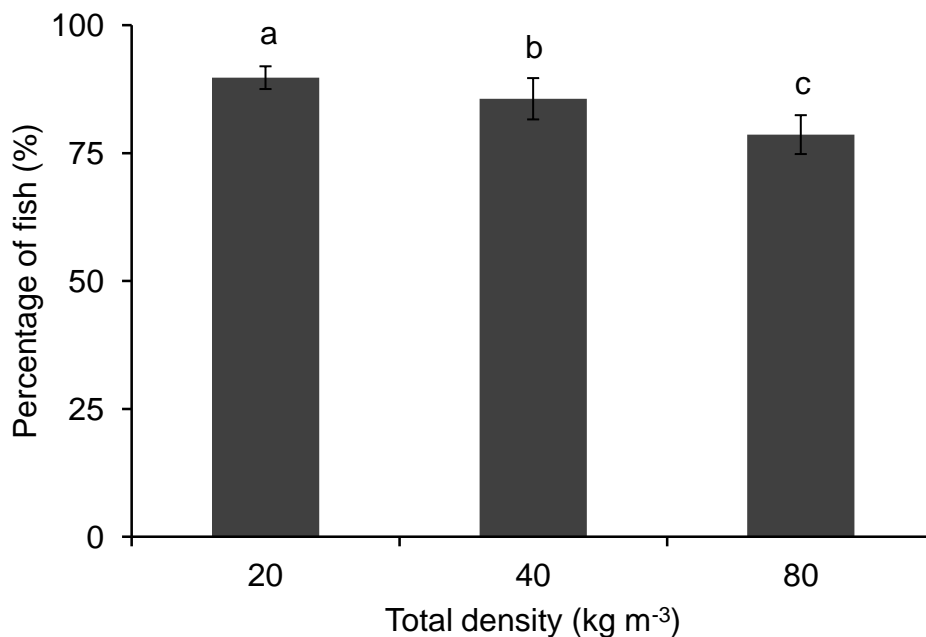
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446 8. Figure captions

447

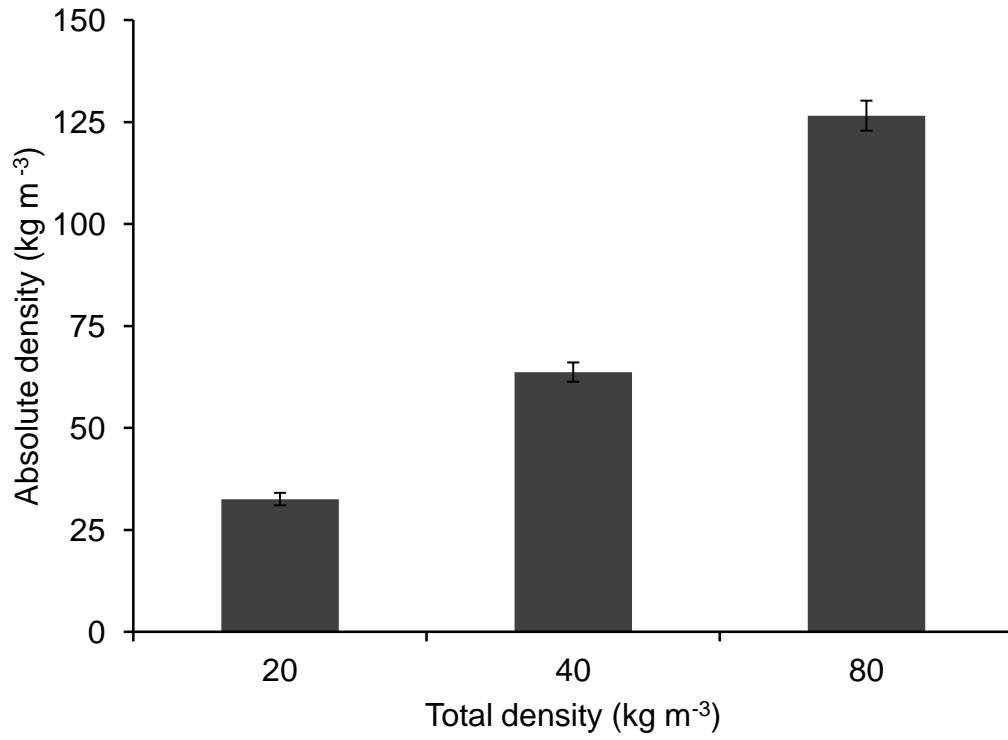


448

449 Figure 1. The percentage of fish in the “crowded” tank of the two-choice system between the three total  
450 densities (n=3). The letters (a, b & c) indicate a significant difference between treatments.

451

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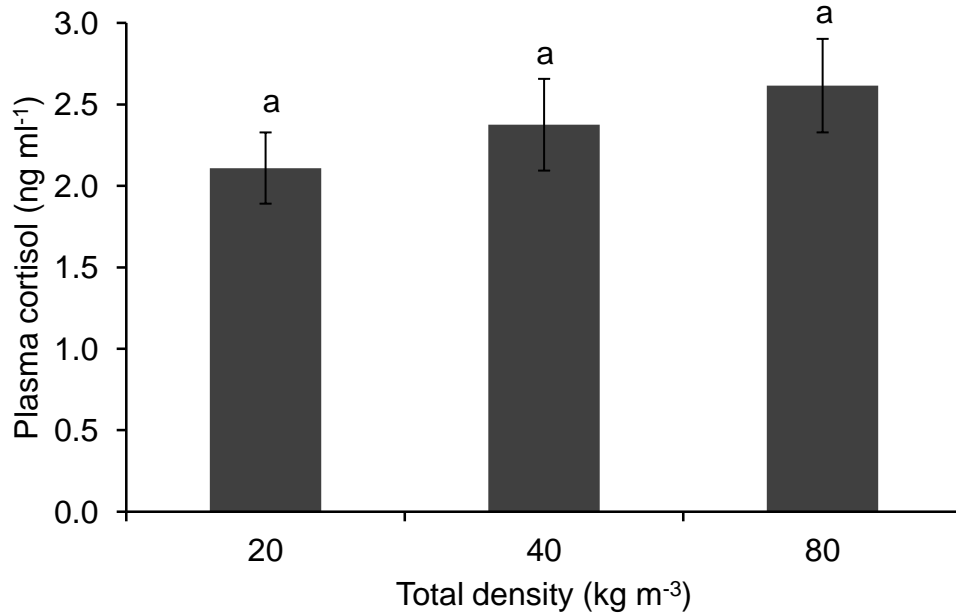


453

454 Figure 2. The absolute density (kg m<sup>-3</sup>) in the “crowded” tank at each total density (n=3).

455

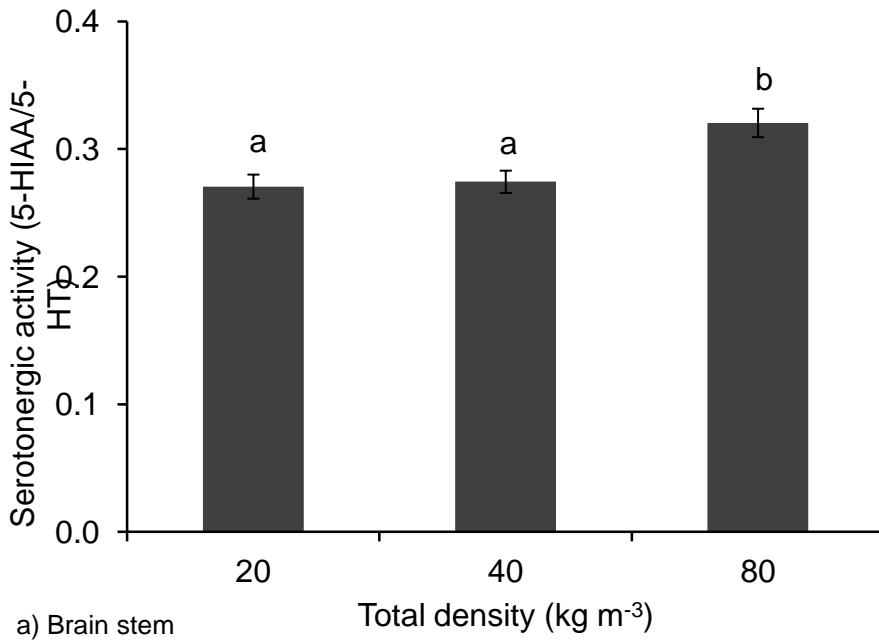
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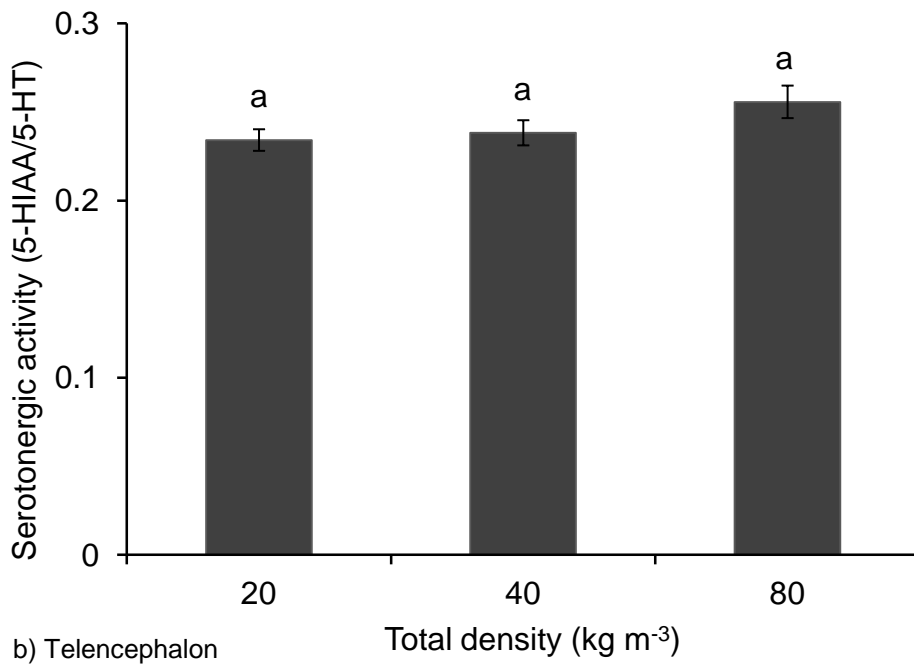
457 Figure 3. Plasma cortisol concentrations of individuals taken from the “crowded” tank at each total density

458 (n=18).

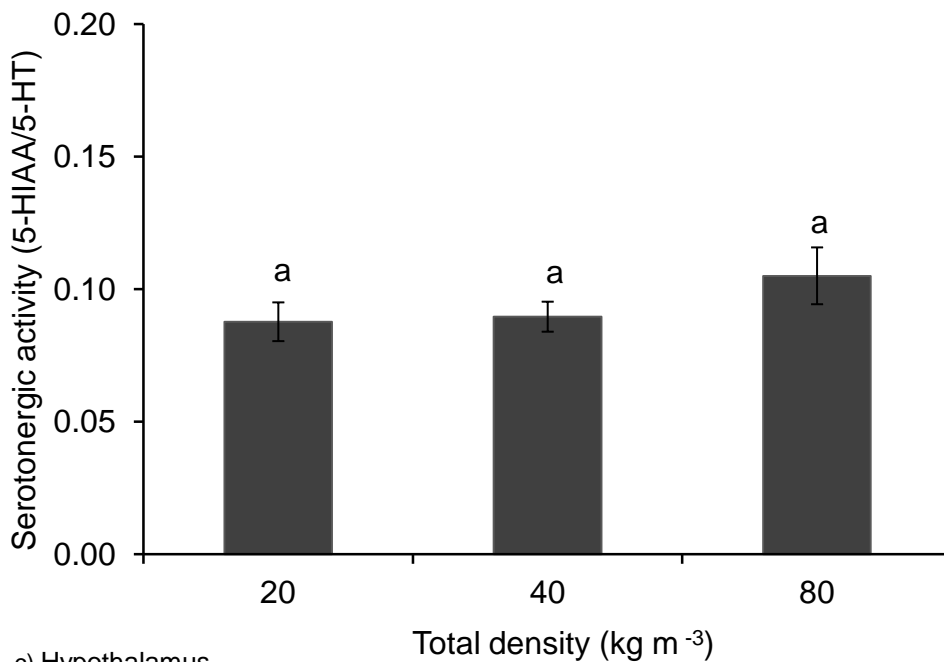
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461 a) Brain stem



462 b) Telencephalon



463 c) Hypothalamus

464 Figure 4. Serotonergic activity (5-HIAA/5-HT) in the a) Brain stem, b) Telencephalon and c) Hypothalamus  
 465 of individuals (n=18) in the “crowded” tank at each total density. The letters (a & b) indicate a significant  
 466 difference between treatments.

467

468 9. Table captions

469 Table 1. The concentrations (mean ± SEM) of monoamine and metabolites in the different brain regions of  
 470 the individuals (n=18) in the “crowded” tank at each total density.

Brain region	Metabolite and metabolite	Density treatment (kg m <sup>3-1</sup> )			p value
		20	40	80	
Brain stem	5-HIAA	363.1 ± 21.0	404.5 ± 23.4	438.9 ± 19.7	0.013
	5-HT	1419.5 ± 89.3	1574.7 ± 113.6	1444.9 ± 67.7	0.653
Telencephalon	5-HIAA	1094.9 ± 55.7	1161.1 ± 51.3	1077.8 ± 47.9	0.398
	5-HT	4954.33 ± 297.4	5201.8 ± 277.9	4550.1 ± 230.2	0.190
Hypothalamus	5-HIAA	390.5 ± 17.3	410.7 ± 25.8	457.8 ± 29.5	0.439
	5-HT	5589.9 ± 373.0	5326.3 ± 372.0	5633.1 ± 420.9	0.850

471