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The Effects of Acute and Chronic Hypoxia on Cortisol, Glucose and Lactate Concentrations in Different Populations of Three-Spined Stickleback

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ABSTRACT

The response of individuals from three different populations of three-spined sticklebacks to acute and chronic periods of hypoxia (4.4 kPa DO, 2.2 mg l⁻¹) were tested using measures of whole-body (WB) cortisol, glucose and lactate. Although there was no evidence of a neuroendocrine stress response to acute hypoxia, fish from the population least likely to experience hypoxia in their native habitat had the largest response to low oxygen, with significant evidence of anaerobic glycolysis after two hours of hypoxia. However, there was no measurable effect of a more prolonged period (seven days) of hypoxia on any of the fish in this study, suggesting that they acclimated to this low level of oxygen over time. Between-population differences in the analytes tested were observed in the control fish of the acute hypoxia trial, which had been in the laboratory for 16 days. However, these differences were not apparent among the control fish in the chronic exposure groups that had been held in the laboratory for 23 days suggesting that these site-specific trends in physiological status were acclimatory. Overall, the results of this study suggest that local environmental conditions may shape sticklebacks' general physiological profile as well as influencing their response to hypoxia.

Key words: Three-spined stickleback; hypoxia; cortisol; glucose; lactate.

49 Reduced dissolved oxygen (DO) in the water is an important environmental stressor for fish. Periods of low DO, or
50 hypoxia, can occur as a result of many factors such as eutrophication, elevated ambient temperature and algal
51 blooms. With climate-related warming of freshwater bodies predicted to continue (Bates et al. 2008; Johnson et al.
52 2009), a greater understanding of the response of fish species to consequent environmental factors such as hypoxia
53 could prove beneficial (Gitay et al. 2002). The three-spined stickleback, *Gasterosteus aculeatus*, is a highly
54 adaptable teleost fish, ubiquitous throughout temperate regions of the Northern hemisphere. Their small size, wide-
55 distribution, short generation time and ease of care in captivity have made them a popular study species across a
56 number of different disciplines including ecology, toxicology and molecular biology (Barber and Nettleship 2010;
57 Katsiadaki et al. 2007; Colosimo et al. 2005). However, relatively little is known about how these fish respond to
58 hypoxic stress.

59 Previous studies indicate that the three-spined stickleback is tolerant of DO concentrations approaching 2.0
60 mg l⁻¹, below which point the fish display signs of distress (Feldmeth and Baskin 1976) and may engage in aquatic
61 surface respiration (Walton et al. 2007; Giles 1987). The precise effects of low oxygen tension on fishes depends in
62 part on the ambient temperature because of the interaction between activity and metabolic demand. A small-scale
63 study carried out at water temperatures lower than those employed by Giles (1987) compared the response of
64 sticklebacks from a pond and river population to seven days of 20% of full oxygen saturation (~ 2.2 mg l⁻¹
65 dissolved oxygen at 12°C, 4.4 kPa) and found that while no overt signs of distress were evident among the fish,
66 dominance hierarchies were more disrupted among the river fish than among the pond fish, and after seven days of
67 hypoxic conditions, fish from both populations had significantly elevated whole-body lactate levels (Sneddon and
68 Yerbury 2004). These findings suggested that the type of environment sticklebacks inhabit may influence aspects of
69 their response to hypoxia. Furthermore, there is evidence from other fish species that the availability of oxygen in
70 local habitats can shape hypoxia coping abilities. For example, sailfin mollies, *Poecilia latipinna*, inhabiting a
71 periodically hypoxic salt marsh have been shown to be more tolerant of low oxygen than those from a nearby river
72 with higher oxygen availability (Timmerman and Chapman 2004). When exposed to hypoxia the salt marsh mollies
73 spent less time conducting aquatic surface respiration and had lower gill ventilation rates than the river mollies. This
74 may be mediated in part by the fact that the salt marsh mollies were shown to have 14% larger gill surface area than
75 the river mollies and significantly lower critical oxygen tensions (the oxygen tension required to maintain an
76 individual's metabolic rate).

77 Fishes are known to exhibit a generalised stress response to acute hypoxia involving the activation of the
78 sympathetic response and hypothalamo-pituitary-interrenal (HPI) axis which results in the release of the hormones
79 adrenaline and cortisol respectively (Van Raaij et al. 1996b; Rostrup 1998), as well as downstream changes in the
80 circulating concentrations of metabolites such as glucose (Barton 2002). If insufficient oxygen is available to
81 support aerobic ATP production, fish may resort to anaerobic metabolism resulting in the accumulation of lactate
82 (Van Raaij et al. 1996b; Zhou et al. 2000; Dunn and Hochachka 1986). Changes in the concentrations of these
83 hormones and metabolites provide a means by which the severity and duration of the response of fishes to hypoxia
84 can be quantitatively measured. However, in the case of small fish, such as three-spined sticklebacks, this approach
85 is hampered by the restricted volumes of blood which can be collected. An alternative approach utilises extracts of

86 whole-body (WB) homogenates for measuring cortisol, glucose and lactate and this has successfully been employed
87 with a variety of small teleosts (Pottinger et al. 2002; Reubush and Heath 1996; Scarabello et al. 1992; King and
88 Berlinsky 2006).

89 The aims of this study were (i) to investigate the physiological response of three-spined sticklebacks to
90 hypoxia by comparing the response of individuals from three populations to a short period (two hours) of oxygen
91 depletion and to a more prolonged (seven days) period of hypoxia, and (ii) to assess whether the type of
92 environment these populations originated from affected their response to hypoxia. Cortisol, glucose and lactate
93 concentrations were determined in WB extracts of sticklebacks exposed to two hours or seven days of hypoxia to
94 provide an assessment of the endocrine and metabolic adjustments caused by the treatments (Sneddon and Yerbury
95 2004; Johansen et al. 2006; Van Raaij et al. 1996a). Sticklebacks from three separate populations, whose habitats
96 were characterised by differences in the occurrence of hypoxia, were used to test whether hypoxia tolerance varied
97 between populations. We predicted that sticklebacks from environments that regularly experienced episodes of
98 hypoxia would exhibit a greater tolerance of hypoxic conditions than fish from more oxygenated environments, and
99 that such differences would be reflected more markedly in their initial response to low DO rather than their response
100 to a prolonged period of hypoxia.

101

102 **Materials and Methods**

103 *Experimental subjects*

104 Fish were collected from three different sites on the Wirral in the North-West of England: Ince Marsh (INM, NW
105 53.3 2.8), Peckmill Brook (PMB, NW 53.3 2.7) and Ness Garden's Dipping Pond (NDP, NW 53.2 3.0). Fish from a
106 marsh, small river and pond were used as these environments differed in their general oxygen availability as well as
107 the frequency of hypoxic episodes. DO measurements were taken once on the day the fish were caught using a hand-
108 held oxygen-meter (YSI Pro 20, Fleet, UK) and confirmed that the three sites did differ considerably in DO: INM
109 51.5% (6.2 mg l⁻¹ at 7.1°C), PMB 89.2% (10.9 mg l⁻¹ at 6.4°C) and NDP 19.2% (2.3 mg l⁻¹ at 6.0°C). However as
110 the oxygen profile of any water body can be highly dynamic, single-point measurements may not provide a reliable
111 indication of 'normal' oxygen availability. Logistical limitations prevented repeated measures of DO at the three
112 sites, but the choice of substantially different habitats increased the likelihood that the sites had differential hypoxia
113 tendencies. Monthly water quality data provided by the Environment Agency (EA) showed that between 1994 and
114 2004 INM had a mean DO level of 56.9 ± 1.9% and PMB had a mean DO concentration of 93.8 ± 0.8%. As NDP is
115 both a pond and situated on private land, comparable water quality data were not available for this site. However, as
116 a small lentic water body (~20m x 15m at its widest point), NDP was considered to be a site that was more likely
117 than INM or PMB to become hypoxic. This assumption was supported by the low DO reading taken on the day of
118 fish collection.

119 All fish were collected during the period October to March 2005 and transferred in groups of
120 approximately 30 fish to glass aquaria (38 litres) containing aerated freshwater (12 ± 1°C) in the University of
121 Liverpool's aquarium facility. The water temperature was maintained via the stable ambient environment of a
122 temperature-controlled room. They were held under a continuous light-dark cycle of 10:14 hrs respectively and left

123 undisturbed for 48 hours to recover from the stress of transport. Each tank contained an internal filter (Series 1,
124 Interpet, UK) and a 15cm air-stone. Each tank was a closed system and one third of the water was changed each
125 week. Water quality was routinely monitored to ensure ammonia, nitrite, nitrate and pH were maintained within
126 acceptable limits. No significant fluctuations in these parameters were observed. The fish were fed to satiation daily
127 on defrosted red mosquito larvae.

128

129 *Acute hypoxia trial*

130 For the acute hypoxia trial 24 fish from each population (INM $0.86 \pm 0.06g$, PMB = $0.91 \pm 0.06g$ and NDP = $0.98 \pm$
131 $0.08g$) were taken from the holding tanks 48 hours after capture and divided into groups of four visually size-
132 matched fish from the same population before being transferred to six new tanks (38 litres). Half of the tanks were
133 randomly designated control tanks and the other half treatment tanks. There were three control and three treatment
134 tanks of fish from each population (a total of 18 tanks). Sex of the fish was not determined as they were not in
135 breeding condition and, therefore, no major sex differences in stress response were anticipated (Pottinger and
136 Carrick 2000). Each tank contained an internal filter (Series 1, Interpet, UK) and a 15cm air-stone. Dark grey
137 polystyrene batons were placed on the surface of the water to minimise the air-water interface. Fish were maintained
138 under these conditions for 14 days and fed mosquito larvae daily to satiation. The water was maintained close to full
139 oxygen saturation ($12 \pm 1^\circ C$, 22kPa DO) over the experimental period by constant aeration. This was periodically
140 checked using a hand-held oxygen meter (YSI Pro 20, Fleet, UK). On day 14 the fish in the control tanks were killed
141 by concussion and immediately immersed in liquid nitrogen. This process was conducted in less than one minute
142 and the frozen samples were stored at $-20^\circ C$ until required for analysis. In the treatment tanks, the oxygen level was
143 reduced to $20 \pm 1\%$ of full oxygen saturation ($12 \pm 1^\circ C$, 4.4 kPa DO, 2.2 mg l^{-1}) on day 14 for two hours. This level
144 of oxygen depletion was chosen as it has been used in a previous study of the effect of hypoxia on sticklebacks
145 (Sneddon and Yerbury 2004). Dissolved oxygen concentrations were reduced by bubbling nitrogen through the
146 airstone to displace oxygen from solution; the flow of nitrogen was controlled by a solenoid valve connected to an
147 oxygen controller and temperature compensated oxygen probe (Cole Parmer, USA). A permanent oxygen probe was
148 randomly assigned to one of the treatment tanks to monitor DO. After two hours of hypoxic conditions the fish in
149 the treatment tanks were killed and stored in the same manner as the control fish.

150

151 *Chronic hypoxia trial*

152 The chronic hypoxia trial followed an identical protocol to the acute hypoxia trial with the exception that fish were
153 kept under the experimental conditions for 21 days and the hypoxia treatment tanks were maintained at $20 \pm 1\%$ of
154 full oxygen saturation for the final seven days of the trial before being killed as described above. A period of seven
155 days of hypoxia was chosen to make this trial comparable to that of Sneddon and Yerbury (2004). There were six
156 control and six treatment tanks of INM fish ($0.99 \pm 0.04g$), seven control and seven treatment tanks of PMB fish
157 ($0.71 \pm 0.03g$) and four control and four treatment tanks from NDP ($0.95 \pm 0.06g$).

158 All work was carried out with Local Ethical Committee approval and in accordance with Home Office
159 guidelines under project licence PPL 40/2573.

160

161 *Physiological assays*

162 Whole-body homogenates (one part tissue to five parts distilled water) were prepared in polypropylene tubes on ice
163 from each fish carcass using an Ultra-Turrax TP18/10. The homogenate was centrifuged (13,000 RPM) at 5°C for
164 ten minutes and all assays were performed on the resultant supernatant. Cortisol immunoreactivity was determined
165 in ethyl acetate extracts from the supernatant by radioimmunoassay as previously described by Pottinger *et al.*
166 (2002). Recovery of cortisol from the extracts was >95%. Glucose (glucose oxidase method; Diagnostic Chemicals
167 Limited, Oxford Connecticut USA) and lactate assays (lactate oxidase method; Trinity Biotech, Didcot UK) were
168 performed using commercial test kits. All assays were run on single samples, with re-assays carried out for
169 anomalous results.

170

171 *Statistical analyses*

172 All statistical analyses were performed using SAS version 9.2 (Littell et al. 2006). Generalised Linear Mixed Models
173 (GLMM) were used to analyse the hormone and metabolite data from the acute and chronic hypoxia trial separately.
174 The threshold for statistical significance was set at $P < 0.05$. The response variable was cortisol, glucose or lactate.
175 The error distribution for the cortisol data in both trials was log normal, for glucose it was normal and for lactate it
176 was normal and log normal for the acute and chronic hypoxia trials respectively. Experimental group (control or
177 treatment) and population were specified as fixed factors, with tank as a random factor to account for the non-
178 independence of fish within the same tanks. An interaction between treatment and population was also entered into
179 the model to test whether there were population differences in response to hypoxia. Where a significant interaction
180 between treatment and population was found, the difference in the effect of treatment conditions on each population
181 was examined using least squares means (LSM) output from the GLMM.

182 To compare WB cortisol, glucose and lactate of fish from different populations, data from only the control
183 fish were used. This was done separately for fish in the acute and chronic hypoxia trials. Identical GLMMs to those
184 described above were used for these tests but with a single fixed factor of location. Specific differences between
185 populations were examined using LSM output. The error distributions of the data for these tests were as described
186 previously.

187

188 **Results**

189 *Acute hypoxia*

190 There was no effect of acute hypoxia on the WB cortisol concentrations of fish from any of the three populations
191 tested (treatment: $F_{1, 15} = 0.06$ $P = 0.81$, population*treatment: $F_{2, 13} = 0.99$ $P = 0.40$, Table 1). However, fish from
192 different populations did have significantly different cortisol levels (population: $F_{2, 7} = 13.69$ $P < 0.01$). NDP fish
193 had higher WB cortisol concentrations (6.81 ± 1.36 ng/g) than both PMB (1.83 ± 0.58 ng/g $P = 0.03$) and INM (0.19
194 ± 0.13 ng/g, $P < 0.001$) and the WB cortisol of PMB was significantly higher than that of INM fish ($P = 0.03$).

195 For WB glucose levels there was a significant population*treatment interaction ($F_{2, 12} = 8.25$ $P < 0.01$, Fig
196 1a) with sticklebacks from INM exhibiting significantly higher WB glucose when exposed to two hours of hypoxia

197 compared to controls ($P = 0.01$), whereas PMB fish had significantly lower WB glucose in the hypoxic tanks
198 compared to controls ($P = 0.03$). For NDP fish there was no significant difference in WB glucose between the
199 groups exposed to acute hypoxia and control fish ($P = 0.24$). There was a significant effect of the population the fish
200 originated from on WB glucose levels ($F_{2,6} = 6.85$ $P = 0.03$). Fish from NDP and PMB both had higher mean
201 concentrations of glucose (NDP 2.20 ± 0.16 mg/g, PMB 1.83 ± 0.17 mg/g) than INM (1.29 ± 0.17 mg/g, NDP vs.
202 INM $P = 0.01$, PMB vs INM, $P = 0.06$). The WB glucose of control fish from PMB and NDP was not significantly
203 different ($P = 0.21$).

204 Acute hypoxia also had a different effect on the WB lactate levels of fish depending on the population they
205 originated from (population*treatment: $F_{2,13} = 5.99$ $P = 0.01$, Fig 1b); only the fish from PMB had significantly
206 higher WB lactate levels in the groups that experienced two hours of hypoxia compared to controls ($P < 0.001$). The
207 WB lactate levels of control fish and those exposed to acute hypoxia were not significantly different for INM ($P =$
208 0.84) or NDP fish ($P = 0.61$). Overall, there were significant population-level differences in WB lactate levels ($F_{2,3}$
209 $= 40.86$ $P < 0.01$). Fish from INM had higher WB lactate (0.32 ± 0.02 mg/g) than either PMB (0.23 ± 0.01 mg/g, P
210 $= 0.04$) or NDP (0.10 ± 0.01 mg/g, $P < 0.01$). The lactate levels of PMB fish were significantly higher than those of
211 NDP fish ($P = 0.01$).

212 213 *Chronic hypoxia*

214 Overall, there was no significant effect of chronic hypoxia on WB cortisol, glucose or lactate levels of the fish
215 irrespective of population (cortisol $F_{1,28} = 0.27$ $P = 0.60$, glucose $F_{1,30} = 1.94$ $P = 0.14$ and lactate $F_{1,30} = 0.68$ $P =$
216 0.41 , Table 2). Nor were there significant treatment-related differences within populations (population*treatment:
217 cortisol $F_{2,27} = 0.80$ $P = 0.46$, glucose $F_{1,28} = 0.07$ $P = 0.93$ and lactate $F_{2,28} = 0.21$ $P = 0.81$). However, fish from
218 the three populations did have significantly different WB lactate levels in general ($F_{2,14} = 12.83$ $P < 0.01$) Fish from
219 PMB had the higher WB lactate concentrations (1.61 ± 0.26 mg/g) than fish from both INM (0.50 ± 0.05 mg/g, $P <$
220 0.01) and NDP (0.27 ± 0.05 mg/g, $P < 0.01$). Although the lactate levels of fish from INM were higher than those of
221 fish from NDP, this difference was not statistically significant ($P = 0.07$). There were no population differences in
222 either cortisol ($F_{2,14} = 0.32$ $P = 0.73$) or glucose ($F_{2,14} = 0.91$ $P = 0.42$) concentrations.

223 224 **Discussion**

225 The aim of the present study was to assess selected physiological effects of acute and chronic hypoxia on three-
226 spined sticklebacks at levels of DO known to be tolerated by this species (Giles, 1987) but to have effects on
227 behaviour (Sneddon and Yerbury, 2004), and to identify whether between-population differences were evident in
228 these responses. Two hours of exposure to hypoxia (2.2 mg l^{-1}) had no systematic effect on WB cortisol
229 concentrations, suggesting that the HPI axis was not activated in fish from any of the populations by this treatment.
230 Catecholamines were not measured so the possibility that the sympathetic response was activated cannot be
231 discounted. Chronic stress can alter the responsiveness of the HPI axis to additional stressors, sometimes leading to
232 hyporeactivity of the corticosteroid response (Rotllant et al. 2000). Therefore, it could be suggested that absence of a
233 difference in WB cortisol between control fish and those subjected to acute hypoxia was the result of fish entering

234 the experiment in a state of chronic stress. However, this seems unlikely as the WB cortisol levels of the fish in the
235 current study were similar to those reported for the control fish (<8 ng/g) in a previous study (Pottinger et al., 2002)
236 of the effect of various stressors on WB cortisol in three-spined sticklebacks. In this earlier study chronically
237 stressed fish had WB cortisol levels of up to 50 ng/g after four days of crowding and confinement. We therefore
238 conclude that in the present study there is no evidence for stress-induced elevation of cortisol levels among the
239 controls or in the fish exposed to two hours of hypoxia. However, differences in glucose concentrations in two of
240 the populations (INM and PMB) and in lactate concentrations in one of the populations (PMB) were observed in
241 response to acute hypoxia. Fish from PMB had lower WB glucose and higher WB lactate concentrations after two
242 hours of hypoxia compared to control fish. These results suggest that PMB fish placed greater reliance on anaerobic
243 glycolysis under hypoxia than fish from the other two populations, leading to an accumulation of lactate and
244 depletion of glucose, during exposure to two hours of hypoxia (Dunn and Hochachka 1986; Muusze et al. 1998).
245 Fish from INM had higher WB glucose concentrations when exposed to hypoxia, indicating some degree of
246 metabolic disturbance. However, it is difficult to interpret the functional significance of this response given that
247 there was no observable change in the other analytes tested. In contrast, acute hypoxia did not appear to have any
248 significant effect on the parameters measured in the NDP fish.

249 As the greatest response to acute hypoxia was seen in the population that was considered least likely to
250 experience substantial reductions in oxygen availability (PMB) and the fish on which acute hypoxia had no observed
251 effect were from the site considered most likely to regularly experience hypoxia (NDP), it is reasonable to speculate
252 that these putative population differences in hypoxia response could reflect acclimation to oxygen availability at
253 their sites of origin. However, it is possible that environmental factors other than oxygen availability also influenced
254 the population differences in the response of these fish to acute hypoxia. For example, fish from INM, PMB and
255 NDP may have had different nutritional status and energy reserves prior to capture, not ameliorated by the
256 habituation period, which could have altered the effect of acute hypoxia on the WB indices measured. Just three
257 populations were involved in the current study, but a previous investigation (Sneddon and Yerbury 2004) found that
258 the dominance hierarchies of a population of three-spined sticklebacks from a river were more disrupted by oxygen
259 depletion (4.4 kPa DO, 12°C, 2.2 mg l⁻¹) than those of a pond population of sticklebacks. Both sets of data suggest
260 that local environmental conditions shape the response of sticklebacks to low oxygen, which warrants further
261 investigation. Populations in close geographical proximity to one another were chosen in this study to minimise the
262 likelihood that large genotypic differences determined their response to experimentally-induced hypoxia, making it
263 more likely that the population differences were shaped by the availability of oxygen in their local habitats.
264 However, it is possible that genetic differences between these populations did influence their hypoxia responses.
265 This is particularly the case if selective pressure has altered the prevalence of genes associated with coping with low
266 DO. Therefore, future studies of this subject may benefit from an approach whereby the relative genetic and
267 environmental influences that determine the hypoxia response of these fish can be teased apart.

268 Although a significant effect of acute hypoxia was observed in two of the populations tested, there was no
269 observable effect of seven days of hypoxia on these fish. This suggests that fish from INM and PMB were able to
270 adjust to hypoxia over the longer period of exposure. Acclimation to sustained oxygen depletion is known to occur

271 in other fishes and is often mediated by a reduction in their oxygen requirement or optimisation of their oxygen
272 utilisation (Kramer 1987; Bickler and Buck 2007; Chapman et al. 2002). For example, oxygen supply and demand
273 may be behaviourally adjusted under conditions of low DO by reducing high energy behaviours such as aggression
274 and/or increasing their ventilation rate (Kramer 1987; Sneddon and Yerbury 2004).

275 The lack of evidence to suggest that the fish were physiologically challenged by chronic hypoxia is
276 consistent with earlier observations that the three-spined stickleback is a relatively hypoxia tolerant species (Jones
277 1964); the level of hypoxia tested in this study (4.4 kPa DO, 12°C, 2.2 mg l⁻¹) is known to cause significant
278 physiological stress to several other teleost fishes (Johansen et al. 2006; Bernier et al. 1996; Herbert and Steffensen
279 2005). However, this finding is somewhat in contrast to Sneddon and Yerbury's (2004) who reported significantly
280 elevated WB lactate in sticklebacks from both a Scottish pond and river after seven days of exposure to the same
281 level of DO. Even accounting for differences in DO profiles between ponds and rivers, it is possible that the
282 populations of fish used by Sneddon and Yerbury (2004) were from more pristine sites overall than the fish used in
283 the present study. The Wirral is a particularly industrialised region of the UK and this may have implications for the
284 DO regime of local waterbodies.

285 In the acute hypoxia trial, control fish from different populations exhibited substantial differences in the
286 levels of cortisol and metabolites. NDP and PMB fish both had higher WB cortisol and glucose and lower WB
287 lactate than fish from INM and the WB cortisol and lactate of PMB fish was significantly higher than that of NDP
288 fish. As no directly comparable data on other stickleback populations has currently been published, it is difficult to
289 identify the causal factors underlying these apparent between-population differences in the physiology of the fish.
290 However, the fact that these population differences were not evident in the control fish of the chronic hypoxia trial
291 suggests that they are acclimatory (i.e. plastic) rather than adaptive in nature and are likely to be potentially
292 attributable to a wide range of differing environmental influences prevailing in each habitat. As the fish in the acute
293 hypoxia trial had been removed from their native habitat to the aquarium facilities more recently than the fish in the
294 chronic hypoxia trials (16 days and 23 days respectively) it is likely that these fish were at different in their stage of
295 adjustment to the aquarium conditions. It may be that 23 days of being housed in the aquarium was sufficient to
296 ameliorate the population differences observed in the shorter trial. If this was the case, it would suggest that it took
297 these fish somewhere between 16 and 23 days to lose their site-specific physiological profiles. To our knowledge,
298 this is the first evidence of the time course of this adjustment for three-spined sticklebacks and this information may
299 be of value to future laboratory-based research on this species. Although site-specific physiology was no longer
300 evident in these fish at day 23, some population differences are likely to still persist. Genotypic disparity between
301 populations may still shape their behavioural and physiological responses to experimental conditions.

302 PMB fish had the highest WB lactate of all three populations after 23 days in the aquarium, but not at 16
303 days, which suggests that the effect of being housed in this environment may have been cumulative for these fish.
304 The lactate concentrations in the WB extracts of the PMB fish were more than three times higher than any of the
305 other control fish from either the acute or chronic hypoxia trials. Interestingly, Sneddon and Yerbury (2004) also
306 reported higher WB lactate in the river population of sticklebacks utilised in their study compared to the pond
307 population. These fish had been collected from the wild and kept in near-identical conditions to the fish in the

308 current study and for a similar length of time. One possible explanation for this is that lentic sticklebacks develop
309 more muscle than pond sticklebacks as a result having to swim against flowing water, which could increase their
310 overall aerobic demand (Killen et al. 2010). This could lead to them having to resort to anaerobic metabolism more
311 often than lentic fish even in conditions where oxygen availability is relatively high, as it was for control fish in both
312 the present and Sneddon and Yerbury's (2004) study. However, this is just one possible explanation. Another may
313 be that river sticklebacks engage in more high-energy swimming in a laboratory environment increasing their
314 aerobic demand. Further work is required to ascertain why river sticklebacks tend to have higher WB lactate than
315 pond sticklebacks when housed in these conditions.

316 In summary, although the populations of sticklebacks tested all appeared tolerant of chronic hypoxia, there
317 was evidence for population differences in their responses to acute hypoxia, with fish from the population least
318 likely to have regularly experienced hypoxic conditions (PMB) having the most marked response. The ability of
319 three-spined sticklebacks to tolerate prolonged periods of hypoxia will be advantageous in coping with an increase
320 in the frequency of extreme perturbations in the freshwater environment likely to be brought about by anthropogenic
321 habitat change. However, the limitations of this study mean that further work is required to fully elucidate the role of
322 the local environment in the response of these fish to low DO. Future studies should sample fish from a larger
323 number of lentic and lotic stickleback populations and acquire more comprehensive water quality data with which to
324 characterise the habitat of each population. Furthermore, although indices such as WB cortisol and glucose have
325 been shown to be effective in measuring the acute and chronic stress response of a variety of small fishes, including
326 sticklebacks (Reubush and Heath 1996; Scarabello et al. 1992; King and Berlinsky 2006; Pottinger et al. 2002) these
327 are relatively crude measures of a complex biological response. Future investigations may benefit from a more
328 refined approach to the assessment of HPI activity, including repeat measures in individual fish by the collection of
329 water-borne cortisol (Sebire et al., 2007) and quantification of transcription factors such as hypoxia inducible factor-
330 1, that are known to be associated with regulating the expression of physiologically relevant genes (Terova et al.
331 2008). Results from the control fish in this study also highlighted the existence of between-population differences in
332 corticosteroid and metabolite concentrations that appeared to ameliorate over the time fish were in the laboratory.
333 Therefore, researchers should ensure that any population specific responses to captivity do not confound the
334 outcome of experiments when subjects are obtained from several natural sites.

335

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340

341 **References**

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Table 1 Mean (+ s.e.m.) WB cortisol concentrations of control and treatment fish in the acute hypoxia trial.

Letters in superscript indicate where statistically significant ($P < 0.05$) differences between values exist. Values with no letters in common were significantly different to one another whereas those with the same letters were not significantly different. $N_{\text{tanks}} = 3$ per treatment group for each population.

	INM		PMB		NDP	
	Control	Treatment	Control	Treatment	Control	Treatment
Cortisol ng/g	0.19 ^a	0.17 ^a	1.83 ^b	1.29 ^b	6.81 ^c	10.37 ^c
	(0.13)	(0.01)	(0.58)	(0.40)	(6.81)	(1.58)

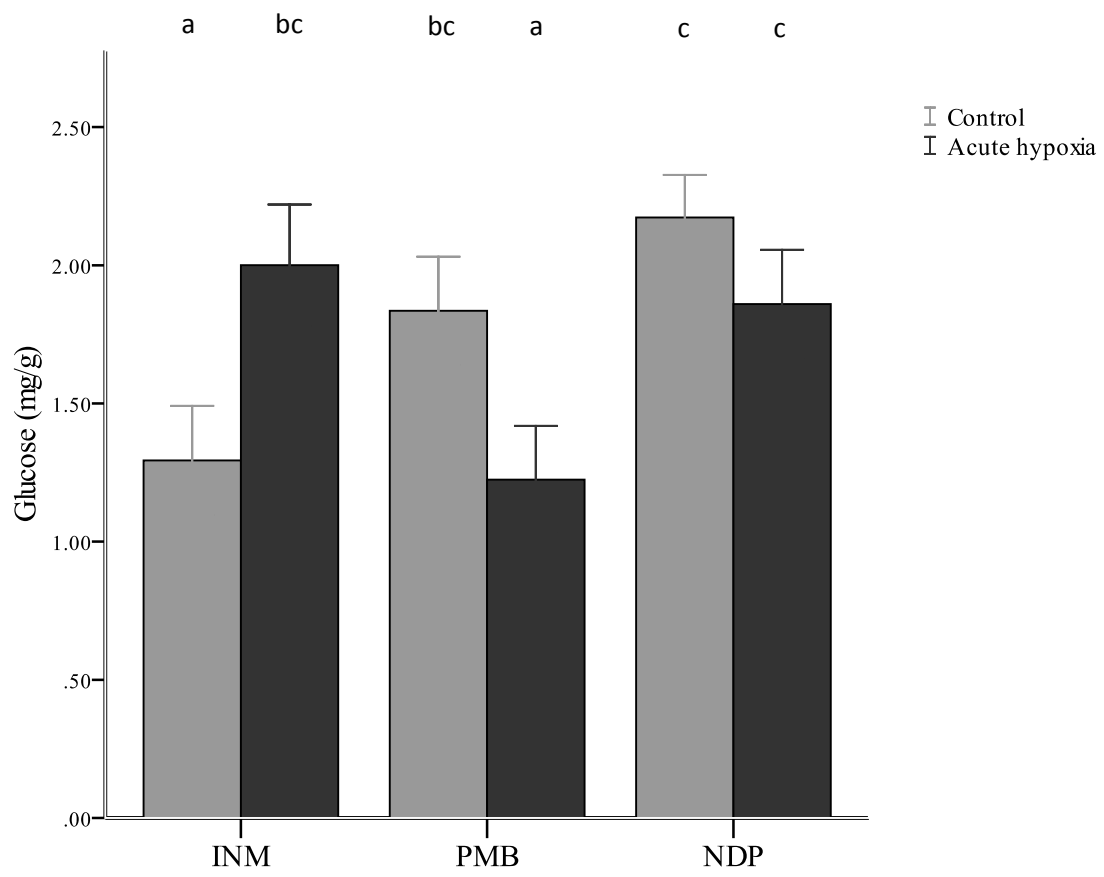
Table 2 Means (+ s.e.m.) of hormone and metabolite data of control and treatment fish in the chronic hypoxia trial.

Letters in superscript indicate where statistically significant ($P < 0.05$) differences between values exist.

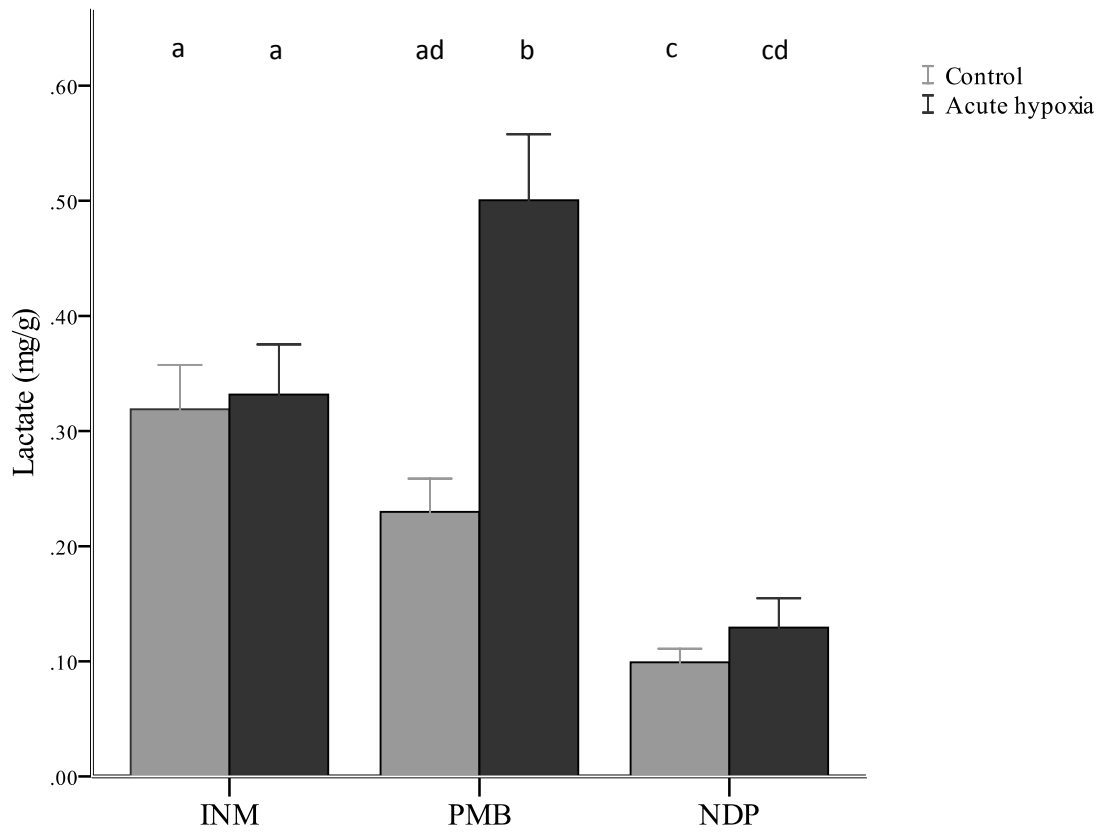
Values with no letters in common were significantly different to one another whereas those with the same letters were not significantly different. Tanks per treatment group: INM = 6, PMB = 7 and NDP = 4.

	INM		PMB		NDP	
	Control	Treatment	Control	Treatment	Control	Treatment
Cortisol ng/g	2.71 ^a	4.80 ^a	2.51 ^a	1.23 ^a	3.02 ^a	3.21 ^a
	(0.71)	(1.68)	(0.41)	(0.27)	(0.59)	(0.95)
Glucose mg/g	2.59 ^a	2.33 ^a	2.04 ^a	1.72 ^a	2.34 ^a	1.82 ^a
	(0.22)	(0.24)	(0.19)	(1.18)	(0.20)	(0.21)
Lactate mg/g	0.50 ^{ac}	0.66 ^{acd}	1.61 ^{bc}	1.79 ^b	0.27 ^e	0.35 ^e
	(0.05)	(0.06)	(0.26)	(0.22)	(0.05)	(0.10)

Figures



1a



1b

Figs 1a & b Mean (+ s.e.m.) WB glucose and lactate levels of fish from each population in control and acute hypoxia tanks. $N_{\text{tanks}} = 3$ per treatment group for each population. Grey bars = control fish, black bars = fish subject to 2h hypoxia. Letters above bars indicate where statistically significant ($P < 0.05$) differences exist; bars with no letters in common were significantly different to one another whereas those with the same letters were not significantly different.