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Physiological and Genetic Correlates of Boldness: Characterising the Mechanisms of Behavioural Variation in Rainbow Trout, *Oncorhynchus mykiss*

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23 **Abstract**

24 Bold, risk-taking animals have previously been putatively linked with a proactive stress
25 coping style whereas it is suggested shy, risk-averse animals exhibit a reactive coping style.
26 The aim of this study was to investigate whether differences in the expression of bold-type
27 behaviour were evident within and between two lines of rainbow trout, *Oncorhynchus mykiss*,
28 selectively bred for a low (LR) or high (HR) endocrine response to stress, and to link
29 boldness and stress responsiveness with the expression of related candidate genes. Boldness
30 was determined in individual fish over two trials by measuring the latency to approach a
31 novel object. Differences in plasma cortisol concentrations and the expression of eight novel
32 candidate genes previously identified as being linked with divergent behaviours or stress
33 were determined. Bold and shy individuals, approaching the object within 180 s or not
34 approaching within 300 s respectively, were evident within each line, and this was linked
35 with activity levels in the HR line. Post-stress plasma cortisol concentrations were
36 significantly greater in the HR line compared with the LR line, and six of the eight tested
37 genes were upregulated in the brains of LR fish compared with HR fish. However, no direct
38 relationship between boldness and either stress responsiveness or gene expression was found,
39 although clear differences in stress physiology and, for the first time, gene expression could
40 be identified between the lines. This lack of correlation between physiological and molecular
41 responses and behavioural variation within both lines highlights the complexity of the
42 behavioural-physiological complex.

43

44 **Keywords:** boldness; cortisol; HPI axis; novel object; *Oncorhynchus mykiss*; qRT-PCR;
45 stress coping styles.

46

47 **Introduction**

48 Behavioural polymorphisms are a common feature of natural populations (Sih et al., 2004).
49 In some cases intraspecific variation in behaviour may be inherently necessary due to
50 environmental changes, often corresponding with ontogenetic shifts (Slater, 1981). However,
51 for many complex behaviours the full adaptive significance of such variation is not fully
52 understood. Despite this, recent studies have highlighted the underlying role of physiological
53 and genetic factors in driving divergent behaviour, particularly differences in animal
54 personality (Bell, 2007; Koolhaas et al., 1999; Korsten et al. 2010; Øverli et al., 2005). One
55 fundamental personality trait is boldness. An individual's boldness is defined by its response
56 to a novel challenge, with these responses regarded as an indicator of the amount of risk an
57 animal is prepared to take in new circumstances (Koolhaas et al., 1999; Sih et al., 2004;
58 Sneddon, 2003, van Oers et al., 2005). As such, boldness can directly influence an
59 organism's fitness, with costs or benefits dependent upon the environmental context (Brown
60 et al., 2007).

61

62 Boldness is not a discrete trait, but rather represents a continuous range of behavioural
63 profiles from bold to shy (Cockrem, 2007). This bold/shy continuum describes a suite of
64 correlated behaviours which are often considered consistent between contexts. In general, shy
65 animals are more reclusive or unresponsive when faced with an unfamiliar situation, whilst
66 bold organisms will act normally or even actively investigate novel environments or objects
67 more readily under the same conditions (Beausoleil et al., 2008; Carere and van Oers, 2004;
68 Frost et al., 2007; Verbeek et al., 1994; Wilson et al., 1993; Yoshida et al., 2005). Bold
69 animals are also relatively more aggressive, spend more time in the open, recover more
70 quickly (e.g. from fear stimulation) and are able to learn more quickly than shy animals

71 (Carere et al., 2005; Magnhagen, 2007; Sneddon, 2003; van Oers et al., 2005; Verbeek et al.,
72 1996).

73

74 Behavioural profiles within a species have also been linked with the physiological response
75 to a stressor, collectively comprising the individual's 'coping style' (Koolhaas et al., 1999).

76 Stressors are defined as challenges to an individual's homeostasis that result in a stress
77 response: behavioural and neuroendocrine reactions that address the negative effects of that
78 challenge (Wendelaar Bonga, 1997). Intraspecific differences in stress responsiveness reflect
79 variation in the control of hormone release within the neuroendocrine stress axis.

80 Consequently, the proactive (active) coping style, typified by aggression and territoriality, is
81 characterised by high adrenergic (noradrenaline) axis activity and low hypothalamo-pituitary-
82 adrenal/interrenal (HPA/HPI) axis activity. In contrast, reactive (passive) behaviour,
83 characterised by withdrawal and immobility, is linked with a higher HPI response (De Boer
84 et al., 1990). These dichotomous behavioural strategies associated with coping style are often,
85 though not always, correlated with boldness (e.g. Koolhaas et al., 1999; Øverli et al., 2007).

86

87 Behavioural characteristics have a significant genetic component in many natural populations
88 in several taxa (e.g. Álvarez and Bell, 2007; Benus et al., 1991; Fidler et al., 2007; Giles and
89 Huntingford, 1984; Korsten et al., 2010; van Oers et al., 2004). Similarly, the physiological
90 response to stress also appears to have a substantial underlying genetic basis. For example, it
91 was possible to select two lines of rainbow trout, *Oncorhynchus mykiss*, for divergent
92 endocrine response to a confinement stressor; across four generations, post-stress plasma
93 cortisol concentrations remained significantly greater in high (HR) compared with low (LR)
94 stress responding lines, with a moderate to high heritability ($h^2=0.41-0.73$) for HPI-reactivity
95 to stress (Pottinger and Carrick, 1999; Pottinger and Carrick, 2001a). Interestingly, these lines

96 also exhibit divergent behavioural traits which are linked with boldness: LR fish, whose
97 behaviour shares characteristics with a bold phenotype, display longer retention of a
98 classically conditioned response than HR fish which are considered to be relatively shy. LR
99 fish also exhibit proactive behaviours such as enhanced aggression, social dominance, and
100 rapid resumption of feed intake after exposure to a stressor (Øverli et al., 2007). These trout
101 lines thus provide an excellent model to study coping style and the concomitant relationship
102 between heritable stress responses and behavioural phenotype which is, furthermore,
103 reflected in natural populations (Cockrem, 2007; Koolhaas et al., 1999).

104

105 Ultimately, many of these heritable differences in behaviour are manifest as differences in
106 gene expression: a microarray analysis comparing the expression of 20,000 genes in an
107 outbred population of *O. mykiss* highlighted ~1,000 genes which were differentially
108 expressed in the brains of fish showing either consistently bold or shy responses to novelty
109 (Sneddon et al., MS under review). Therefore differential gene regulation between bold and
110 shy fish indicate that bold fish have either a different transcriptomic profile or more
111 profoundly regulate relevant genes, and may also account for divergence of behaviour or
112 stress physiology in these animals. If the genes identified by Sneddon and co-workers
113 (Sneddon et al. 2005; Sneddon et al., MS under review) play a role in defining bold and shy
114 phenotypes, they might be expected to show a different pattern of expression between HR
115 and LR fish. With the exception of a study by Schjolden et al. (2005) there has been little
116 examination of bold/shy behaviour within these lines of rainbow trout, nor has the possibility
117 that behavioural variation between these lines of selected fish may be linked to discrete
118 individual differences in brain gene expression been explored. These lines thus offer a unique
119 opportunity to investigate the putative link between behavioural polymorphism and
120 physiological stress responsiveness. Furthermore these aspects of animal personality and

121 coping style can, for the first time, be correlated by quantification of the expression of a suite
122 of candidate genes.

123

124 The broad aim of this study was to determine the extent to which neuroendocrine responses
125 to stress, within trout selectively bred for divergent responses, correlated with bold or shy
126 behavioural traits; we quantified this not only between the HR and LR lines but also
127 characterised whether individual variation occurred within these lines. Further to this, the
128 expression of a range of novel candidate genes in the brain was determined. We hypothesised
129 (1) that LR individuals would exhibit behaviour typical of a bold phenotype and would
130 approach a novel object more quickly and exhibit a lower stress response than HR individuals
131 whose behaviour would resemble that of a shy phenotype, and (2) that this divergence in
132 behavioural and endocrine responses would be associated with clear differences in the
133 expression of genes associated with boldness (within lines) and/or the stress response
134 (between lines).

135

136 **Materials and Methods**

137 *Experimental fish*

138 The following experiment was conducted humanely under Home Office, UK, guidelines
139 according to the Animal (Scientific Procedures) Act 1986, and following local ethical
140 approval. Rainbow trout, *Oncorhynchus mykiss* Walbaum, from inbred lines selected for high
141 (HR) or low (LR) cortisol responsiveness to a standardised stressor (Pottinger and Carrick,
142 1999) were transferred from CEH Windermere to Liverpool where each line was held
143 separately (~140 fish per tank) in two stock tanks (2x2x0.5 m) in a semi-recirculating system.
144 Tanks were supplied with filtered aerated freshwater and maintained at 13±2°C on an
145 ambient 14:10 h light:dark regime. Half of the tank had an opaque overhead cover for shelter.

146 Fish were inspected twice daily and fed commercial pellets (Skretting, UK) at 1 % body
147 weight per day. After a period of at least 4 months to allow fish to acclimate, trout (HR:
148 $n=44$, 343.0 ± 14.7 g; LR: $n=33$, 356.5 ± 11.0 g) were selected at random from the stock tanks
149 and placed into individual glass tanks (90x50x45 cm) which were screened from visual
150 disturbance. All tanks were supplied with a constant flow of filtered freshwater in a semi-
151 open system maintained at $10\pm 1^{\circ}\text{C}$ with aeration. The trout were left to acclimate for a
152 minimum of one week and fed daily. Experiments were conducted on fish that had resumed
153 feeding after this period.

154

155 *Behaviour*

156 A custom-built low-light video camera was situated in front of the tank and a second camera
157 placed to the side of the tank. Measuring rulers (0.5 cm intervals) were arranged horizontally
158 and vertically along the front of the tank to measure proximity of the fish to the novel object.
159 The fish were allowed 10 minutes to acclimatise to the potential disturbance arising from
160 setting up the cameras. Behaviour of the fish without disturbance was then recorded for 10
161 minutes, before a novel object was added. The novel object test is a standard paradigm to
162 differentiate between bold and shy individuals (Wilson et al., 1993). The novel object was
163 placed as near to the centre of the tank as possible, and the behaviour of the fish was recorded
164 for a further 10 minutes after which the object was carefully removed. This test was repeated
165 a week later to assess the level of consistency of behaviour displayed by the experimental
166 individuals. Novel objects were varied between trials to ensure the fish did not become
167 habituated to a familiar shape, and included an orange frustum-shaped bung (7.05 cm mean
168 diameter, 4.9 cm height) and a bipyramidal Duplo^(R) construct (height 13.5 cm, and
169 maximum widths 7.6x6.3 cm) of black, red and blue bricks.

170

171 Scoring of the behaviour was accomplished using custom designed behavioural analysis
172 software. Three measurements each of three separate behaviours were initially scored based
173 on the activity levels of the subject and its proximity to the novel object (Table 1; see Frost et
174 al., 2007). Principal components analysis (Minitab ver.15.1) was subsequently used to
175 identify the key behaviours that differentiated bold fish from shy. Latency to approach within
176 5 cm (s) of the object was strongly represented in the first principle component
177 (eigenvalue=3.53, loading for 5 cm latency=-0.41) and could be solely used to differentiate
178 between bold and shy groups. This measure has previously been used to identify boldness in
179 fish (Coleman and Wilson, 1998; Frost et al., 2007). Loadings for six of the measurements
180 were well represented in the first principal component, and two of these, frequency of
181 entering a 10 cm zone (min^{-1}) centred on the object (loading=0.459) and duration (s) spent
182 passive (loading=-0.381), were selected for further analysis. Passive behaviour was defined to
183 exclude swimming (movement of the fish generated by propulsion using the fins, of no less
184 than approximately one body length) but include drifting, fish pivoting on their own axis, any
185 minor movements made to maintain position, and resting on the bottom of the tank.

186

187 *Hormone analysis and quantification of gene expression*

188 Subsequent to, and on the same day as, the final behavioural trial, approximately half of the
189 fish ($n=34$) were netted and exposed to air for 60 s to induce an acute physiological stress
190 response before being placed back into their tank (Pickering and Pottinger, 1989). Fifteen
191 minutes after emersion, the trout were netted again before being killed humanely by
192 concussion. To obtain unstressed plasma cortisol concentrations, fish were killed by
193 concussion without this treatment. Individuals were killed at the same time each day to
194 ensure that interpretation of differences in hormone levels was not compromised by diel
195 fluctuations in plasma cortisol (Pickering and Pottinger, 1983). Immediately after euthanasia,

196 a 2 ml blood sample was taken from the caudal vessels using sterile 25 g needles and
197 heparinised 2ml syringes. The supernatant plasma was aspirated, divided into aliquots and
198 frozen at -20°C. Plasma cortisol levels were determined by radioimmunoassay (Pottinger and
199 Carrick, 2001a).

200

201 Immediately following blood sampling, the whole brain was removed and stored at -80°C
202 until RNA extraction, and fish were sexed. Total RNA was extracted from trout brain using
203 TRIzol® (Invitrogen Life Science, UK), with RNA eluted into 50 µl RNase-free water. RNA
204 concentrations were determined by optical density at 260 nm using a NanoDrop ND-1000
205 spectrophotometer (LabTech International, UK) system and the quality of the samples
206 assessed by 2 % agarose gel electrophoresis. For each sample, approximately 1 µg of mRNA
207 was reverse-transcribed into first-strand cDNA using random hexamers and SuperScript™ III
208 reverse transcriptase (Invitrogen Life Science, UK), following the manufacturer's protocol.

209

210 The candidate genes selected for this study were chosen for their roles in behaviours
211 associated with boldness, such as aggression, anxiety and memory, or for their association or
212 direct involvement with the stress response (Table 2). Furthermore, six of these genes,
213 ependymin, GABA_A, calmodulin, MHCI, Hbα4, and a lipocalin, retinol binding protein, were
214 differentially regulated between bold and shy rainbow trout in a previous study (Sneddon et
215 al., 2005; Sneddon et al., MS under review). Eight pairs of primers for these genes were
216 developed using Primer Express® 3.0 software against *O. mykiss* sequences (Table 3). For
217 RT-PCR, ~0.05 µg of the cDNA was amplified in a 10µl PCR (using 5 µl Fast SYBR Green,
218 Invitrogen Life Science, UK) primed with 2pmol each primer. Thermal cycling conditions,
219 using a 7500 Fast Real-Time PCR System (Applied Biosystems), were: 10 min at 95°C,
220 followed by 40X [95°C 3 s, 60°C 30 s] and then [95°C for 15 s, 60°C for 60 s, 95°C for 15 s

221 and 60°C for 15 s], which allowed the construction of a melting curve to assess the specificity
222 of the product.

223

224 *Data Analysis*

225 None of the data were normally distributed (Anderson-Darling; Minitab, ver.15.1) and thus
226 non-parametric tests were applied. These tests also reduce Type 1 errors since there were
227 unbalanced sample sizes due to unequal numbers of bold and shy fish in each line. A
228 Wilcoxon Signed Rank Test was used to analyse the difference between behavioural scores
229 of the first and second trial to test for consistency in latency to approach within 5cm of the
230 novel object (Minitab, ver.15.1). Subsequently, data were separated for trout showing
231 consistently bold (approach to 5 cm of the object within 180 s in both trials; $n=28$) or shy (do
232 not approach to 5 cm within 300 s in both trials; $n=13$) behaviour. Scores for each of the
233 behaviours were then averaged over the two trials and compared between bold and shy
234 groups within both the HR and LR line using Mann-Whitney U-tests (R, ver.2.7.0), including
235 sequential Bonferroni treatment (Rice, 1989) for multiple tests.

236

237 Plasma cortisol concentrations for stressed and unstressed trout were compared between the
238 two stress lines (unstressed: HR $n=13$, LR $n=23$; stressed: HR $n=27$, LR=7), between
239 consistently bold and shy trout (unstressed: bold $n=12$, shy $n=5$; stressed: bold $n=14$, shy
240 $n=7$) and between sexes (female $n=17$, male $n=15$) using Mann-Whitney U Tests (R,
241 ver.2.7.0). For RT-PCR, cycle threshold (Ct; the first cycle number at which fluorescence is
242 significantly greater than background levels) and efficiency values for each gene were
243 exported into REST (ver.2.0.7; Pfaffl et al., 2002) whereby the relative expression of each
244 gene between bold and shy fish or between fish from each of the two stress lines, normalised

245 to a reference gene (GAPDH), was calculated. Statistical analysis was subsequently
246 accomplished through REST's bootstrap randomisation procedure.

247

248 **Results**

249 In unstressed rainbow trout, (Fig. 1A) plasma cortisol concentrations were significantly
250 greater in LR fish compared with the HR line (3.16 and 1.34 ng ml⁻¹ respectively; $W=47.0$,
251 $p<0.01$, $n_1n_2=23,13$), with no significant difference between sexes ($W=89.0$, $p=0.15$,
252 $n_1n_2=17,15$). By contrast, after exposure to a stressor, HR trout had a greater plasma cortisol
253 response than did LR fish (67.42 ng ml⁻¹ and 27.14 ng ml⁻¹ respectively; $W=158.0$, $p<0.01$,
254 $n_1n_2=27,7$; Fig. 1B), and while blood-cortisol concentrations were higher in female trout
255 (73.53 ng ml⁻¹) than in males (46.36 ng ml⁻¹), the response was highly variable so
256 insignificant ($W=177.0$, $p=0.06$, $n_1n_2=17,15$).

257

258 Consistent with other studies, boldness showed a bimodal (i.e. u-shaped) distribution and
259 tended towards extremes in individual trials both as a group (Fig. 2A) and separated by line
260 (Fig. 2B, 2C), with fish exhibiting clear bold (approaching 5 cm of the object within 60 s;
261 $n=63$) or shy (not approach within 5 cm during the trial; $n=42$) behaviour. Individual trout
262 were consistent in their latency to approach within 5 cm of a novel object over two trials
263 ($W=913.0$, $p=0.113$, $n=77$), thus confirming the utility of this measure. Rather than being
264 associated predominantly with one or other line, both bold and shy fish were identified within
265 each line. Moreover, there was a tendency for fish to be bold rather than shy in both lines
266 (Fig. 3); although there were proportionately more shy fish in the HR line compared to the
267 LR line (15:9 bold and shy compared to 13:4 bold and shy respectively), this difference was
268 not significant ($\chi^2_1=0.891$, $p=0.344$). Furthermore, although plasma cortisol concentrations
269 profoundly differed between the two lines, there was no significant difference observed in

270 cortisol concentration between bold and shy fish, regardless of whether they were unstressed
271 ($W=37.0$, $p=0.51$, $n_1n_2=12,5$; Fig. 1A) or stressed ($W=89.0$, $p=0.15$, $n_1n_2=17,15$; Fig. 1B).

272

273 Although bold and shy fish could be distinguished within each line by their approach latency
274 to within 5 cm of a novel object, trends in other behaviours were apparent in HR trout but not
275 in LR fish. Within the HR line, consistently bold fish spent less time overall being passive
276 ($W=244.5$, $p<0.01$, $n_1n_2=15,9$; Fig. 4A) than shy trout but this was not true of trout from the
277 LR line ($W=103.0$, $p=0.126$, $n_1n_2=13,4$). Similarly, bold HR trout also entered the 10 cm
278 zone about the object more frequently ($W=138.0$, $p<0.01$, $n_1n_2=15,9$; Fig. 4B) than shy fish,
279 but no significant difference was detected between bold and shy fish in the LR line after
280 Bonferroni treatment for multiple tests ($W=135.0$, $p=0.048$, $n_1n_2=13,4$).

281

282 Differences between the stress lines were evident in the relative expression levels of six
283 candidate genes: endymin, calmodulin, MHCI, GABA_A, vasotocin and RBP were
284 significantly upregulated in the brains of LR fish compared with HR fish (Table 4; Fig. 5B).
285 Average fold change varied from an upregulation factor of 1.89 for AVT up to 5.92 for
286 MHCI. In contrast, expression of both POMC and Hb α 4 were almost identical between the
287 lines. However, bold and shy fish, independent of selection line, did not significantly differ in
288 the expression levels of any of these genes, with the expression of most genes marked by
289 large variance due to pooling of samples within the stress lines (Fig. 5A).

290

291 **Discussion**

292 Boldness is a complex behavioural trait that has previously been associated with coping style
293 (Koolhaas et al., 2007), and may thus be assumed to correlate with the magnitude of the
294 physiological stress response. In this study, bold and shy rainbow trout were identified within

295 distinct stress-response lines of rainbow trout by measuring their behavioural response to
296 novelty: this is the first characterisation of both bold and shy phenotypes within these lines.
297 Whilst divergent plasma cortisol responses to a stressor were evident between the HR and LR
298 lines, consistent with earlier findings (summarised in Øverli et al., 2005), no significant
299 relationship between boldness and stress responsiveness was found either between or within
300 lines. Although a slightly larger proportion of LR trout exhibited a bold phenotype than HR
301 trout this was not significant and no associated differences were observed in post-stress
302 plasma cortisol levels between bold and shy individuals independent of selection line.
303 Similarly, physiological divergence between the HR and LR lines was correlated with
304 differences in regulation of six candidate genes in the brain, but bold and shy fish did not
305 exhibit any dissimilarity in the regulation of these candidate genes.

306

307 *Differences between HR and LR lines*

308 The clear bimodal response to novel objects and the frequency of bold and shy fish within
309 line and as a whole were similar to those observed in outbred rainbow trout (Frost et al.,
310 2007). Boldness thus appears to be bimodally distributed in this species, a response seemingly
311 maintained even in lines selected for divergent responsesiveness to a stressor. Other species
312 may exhibit different distributions, such as a normal distribution with relatively fewer bold
313 and shy compared to intermediate fish in pumpkinseed sunfish (Wilson et al., 1993). Thus
314 bold/shy distributions may reflect interspecific or between-population differences in intrinsic
315 factors or extrinsic pressures that may drive variation in personality. Even rearing conditions
316 can cause a prevalence of certain behavioural types within a population of salmonid fish
317 (Sundström et al., 2004).

318

319 The consistent divergence in the HPI reactivity to stress between the two stress lines is in
320 accordance with earlier studies on these selected lines using confinement to induce a stress
321 response (Pottinger and Carrick, 1999; Schjolden et al. 2005). However, the equally strong
322 divergence among some genes involved in the stress response has not previously been
323 demonstrated and emphasises the strong genetic basis that underpins stress physiology in
324 rainbow trout (e.g. Pottinger and Carrick, 1999; Pottinger and Carrick, 2001a) and possibly
325 other vertebrates (Yao and Denver, 2007). Further work should focus on determining whether
326 these responses are consistent throughout the entire pathway or whether genetic regulation
327 occurs only at key loci within the response. In unstressed fish plasma cortisol concentrations
328 were higher in LR fish than in HR fish, the reverse of an earlier observation in these lines
329 (Pottinger and Carrick, 2001b), and may reflect factors responsible for modulation of the
330 unstimulated HPI axis that have yet to be identified in fish.

331

332 Differences in whole-brain gene expression between the stress lines represent the first
333 evidence that the key phenotypic difference between the lines, divergence in stress
334 responsiveness, is reflected in a broader suite of correlated molecular responses linked with
335 boldness or stress physiology. Immune function can be compromised by chronic stress
336 possibly explaining why MHC, CaM and RBP were each upregulated in LR fish relative to
337 HR fish, since the corresponding proteins are associated with the immune system or response.
338 The Ca^{2+} /CaM complex directly or indirectly controls a number of mechanisms and enzymes
339 involved in the immune response, including aspects of the MHC and the serine-threonine
340 kinases CaMK I, II and IV (Racioppi and Means, 2008). RBP meanwhile has been implicated
341 in inflammatory processes associated with immune responses (Flower, 1996). Low stress-
342 responding animals are often characterised as having improved health over those with a high
343 response, and a major issue associated with sustained elevation of cortisol is a reduction in

344 immunocompetence and increased susceptibility to pathogens (Wendelaar Bonga, 1997).
345 Some aspect of divergent immunological parameters between low and high stress responders
346 thus appears to be controlled at the molecular level; divergence in gene expression,
347 particularly that of proinflammatory genes, has been identified between stress coping styles
348 (MacKenzie et al., 2009) and may reflect differences in circulating steroid concentrations.

349

350 Both GABA_A and AVT genes were upregulated in LR fish, and changes in expression of both
351 genes have been related to aggressive behaviour (Backström and Winberg, 2009; Miczek et
352 al., 2003), a defining characteristic of stress coping styles and also of these stress lines, where
353 LR trout are more aggressive (Pottinger and Carrick, 2001a). However, high levels of AVT
354 tend to inhibit aggression in territorial teleosts such as rainbow trout (Backström and
355 Winberg, 2009), so higher expression of AVT in LR trout is seemingly paradoxical and
356 merits further investigation. Backström and Winberg (2009) suggest that the aggressive
357 output influenced by AVT could be mediated by other systems, in particular the brain
358 serotonergic system, and thus studies that evaluate serotonergic activity together with AVT
359 concentration or expression may throw light on these observations.

360

361 Expression of POMC may not differ between subjects with different stress-coping abilities
362 (Centeno et al., 2007), but rather physiological variation in the HPI axis may occur
363 downstream during post-translational modification, or via differences in target tissue
364 sensitivity, and this may indeed be the case for the HR and LR trout lines. Concentrations of
365 adrenocorticotrophic hormone (ACTH) in the blood of HR and LR fish did not differ
366 significantly during stress; instead, the responsiveness of the interrenal to ACTH differed
367 between the lines (Pottinger and Carrick, 2001b), and a similar process may operate here.

368

369 *Physiology and boldness within the lines*

370 The results suggest that the distribution of bold and shy individuals within each line was not
371 consistently influenced by the selection process despite evidence from earlier studies that the
372 two lines differ consistently in certain key behavioural traits (Pottinger & Carrick, 2001;
373 Øverli et al., 2005, 2007). Furthermore, within the HR line the existence of a behavioural
374 syndrome was evident where boldness was significantly linked with activity levels,
375 suggestive of risk-taking and risk-averse strategies in bold and shy fish respectively
376 (Sneddon, 2003). Indeed the bold fish in this study were characterised by making more use of
377 the available tank space and making less effort to avoid the object. In contrast, a clear
378 behavioural syndrome was not apparent in the LR line. Behaviour of shyer fish within the LR
379 line perhaps was not as well defined compared to natural populations (e.g. Wilson et al.,
380 1993), which may reflect the generally more bold or proactive coping style exhibited by low
381 stress-responding animals (Koolhaas et al., 1999). Alternatively, coping style theory predicts
382 that proactive animals are more rigid in behaviour whereas reactive animals are flexible
383 (Koolhaas et al., 1999), which could suggest they are able to draw on a greater pool of
384 behaviours when reacting to environmental stimuli. These LR and HR trout may be
385 exhibiting these same trends, where LR animals may simply have a less diverse or more
386 limited behavioural repertoire. However, a particularly low sample size for consistently shy
387 fish in the LR line, although originally expected considering previous theory regarding
388 behaviour in LR animals, may limit the power to draw robust conclusions. Nonetheless
389 previous studies have been unable to conclusively link novelty-induced boldness with stress
390 physiology (e.g. Schjolden et al., 2005); our data indicate that this is due to both bold and shy
391 phenotypes existing amongst low and high stress-responding groups.

392

393 The absence of a well-defined link between cortisol levels and boldness within the lines was
394 surprising given previously observed correlations between the magnitude of the stress
395 response and behaviour (Koolhaas et al., 1999; Øverli et al., 2005). Both boldness and
396 shyness were represented within each selected line, and so the correlations between stress
397 responsiveness and behaviour or boldness that have previously been reported (e.g. Øverli et
398 al., 2007; Schjolden et al., 2005) are not always observed. One reason may be that if boldness
399 is context-specific individual behaviour will vary dependent upon the situation (e.g. in
400 familiar compared to unfamiliar environments; Schjolden et al., 2005). This would potentially
401 confer adaptive advantages particularly in an inconsistent environment (Bell, 2007; Coleman
402 and Wilson, 1998; Wilson and Stevens, 2005). Such variation may be elicited by the type or
403 severity of the stressor or by familiarity with the test environment (Brelvi et al., 2008; Mislin
404 and Ropartz, 1981; Schjolden et al., 2005). Contrasting behavioural responses observed
405 between studies may additionally arise from variation in methodological approach to
406 characterising boldness. Furthermore, Schjolden et al. (2005) could not find consistent
407 differences in behavioural responses between HR and LR rainbow trout across several tests
408 including the response of the subjects to a novel object, which may be a result of comparing
409 average behaviours between the lines rather than characterising boldness within each line as
410 in the present study. Thus whilst aggression, a defining component of coping styles and a
411 putative element of boldness, may strongly and consistently correlate with HPI axis reactivity
412 the same is not necessarily true of responses to novelty. It therefore seems apparent that
413 boldness may not directly correlate with stress coping style, and future studies should explore
414 the extent to which the stress response is linked with behavioural phenotype. However, there
415 is a need for standardisation in protocol to determine the degree of boldness and which
416 features of an individual's behavioural repertoire are dependent on or act congruously with
417 hormonal stimulation under greater homeostatic threat.

418

419 Alternatively, the existence of bold and shy phenotypes within line instead of correlating with
420 stress responsiveness suggests that coping style theory (Koolhaas et al., 1999) may simply
421 not be true in all cases. Here, we provide novel data to suggest that divergent personality
422 traits persist within a population or species irrespective of stress coping style. Experience,
423 brought about by environmental or social influences, can shape an individual's behavioural
424 strategy (Brown et al., 2007; Frost et al., 2007). Moreover, behavioural variation can occur
425 within a group regardless of genetic background, and when environmental conditions are
426 identical for each individual (Metcalf et al., 1989). With this in mind, it is not surprising that
427 this study and other recent work have highlighted the complexity inherent in the genetic
428 control of personalities (Korsten et al., 2010). Our data reinforce this, since, despite previous
429 studies that identified different gene expression profiles between outbred rainbow trout with
430 different behaviours (e.g. dominance, Sneddon et al., 2005; boldness, L.U. Sneddon, MS
431 under review), no such divergence between bold and shy fish was uncovered in this study.
432 Gene expression may vary between discrete regions of the brain (Bernier et al., 1999; Feldker
433 et al., 2003; Larson et al., 2006), and can relate directly to behavioural differentiation
434 (Greenwood et al., 2008), and thus a single measurement encompassing all brain regions
435 could obscure more fine-scale differences in expression. Thus, whilst no difference in
436 expression of the studied genes was found across the entire brain, that is not to say that bold
437 and shy individuals express these genes in different localised areas of the brain: whilst
438 differential expression of these genes between the stress lines was profound, variation
439 amongst bold and shy groups may be more subtle. It is of course possible that the lines lack
440 genetic diversity, or that different genes may be involved in the expression of bold/shy
441 behaviour. However, the clear divergence in expression of some of the examined genes in a
442 previous study (LU Sneddon, MS under review) suggests the latter not to be the case, but

443 does emphasise the complexity of bold and shy personalities in rainbow trout. Given that the
444 expression of boldness was independent of selection line, it is likely that the genetic control
445 of boldness may be unrelated to the controlling divergent elements of the selected stress
446 response.

447

448 *Conclusions and Implications*

449 The results of this study indicate a complex relationship between stress responsiveness and
450 behaviour in the HR and LR lines of rainbow trout. Stress responsiveness is a heritable trait
451 in trout (Pottinger and Carrick, 1999; Pottinger and Carrick, 2001a) and the present study
452 demonstrated that divergence in stress responsiveness correlates with differential expression
453 of six novel candidate genes with functions in relevant behaviour and physiology. However,
454 contrary to our hypothesis, the physiological and gene expression responses evident in the
455 selected HR and LR lines did not correlate with boldness or shyness, traits that were
456 identified in substantial numbers within each line. This suggests that the adoption of these
457 contrasting behavioural strategies may not be explained entirely by genetic background or
458 stress coping style and may instead be influenced by external factors that should be
459 considered in theoretical and empirical studies. Experience and environmental influences
460 may cause quite distinct changes in behavioural responses throughout an animal's life history
461 (Frost et al., 2007; Ruiz-Gomez et al., 2008), which may result in behavioural polymorphism
462 even within coping styles. Therefore, it is important for future studies to take into account of
463 how experience and external factors may mould boldness. This may explain why variation in
464 these behavioural phenotypes persists in natural populations to ensure a proportion of
465 individuals can adapt to and survive any perturbations.

466

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470 technical assistance.

471

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635

636 **Table 1:** The definition and measurements recorded of the behaviours assessed during the
 637 novel object tests in rainbow trout, *Oncorhynchus mykiss*.

| Behaviour | Definition and measures |
|------------------|---|
| Within 5cm | The subject was within a delineated zone extending to 5cm around the object. Three measurements were taken: 1) latency , the time (s) taken to enter this zone for the first time; 2) duration , the total time (s) spent within this zone; 3) frequency , how often the subject entered this zone. |
| Within 10cm | The subject was within a delineated zone extending to 10cm around the object. Three measurements were taken: 1) latency , the time (s) taken to enter this zone for the first time; 2) duration , the total time (s) spent within this zone; 3) frequency , how often the subject entered this zone. |
| Passive | Inactivity; includes drifting, minor movements to maintain position within the tank, pivoting on its own axis and resting on the bottom of the tank, but excludes swimming. Three measurements of passive behaviour were recorded: 1) latency , the time taken (s) to begin displaying passive behaviour; 2) duration , the total time the subject spent (s) displaying passive behaviour; 3) frequency , how often the subject displayed passive behaviour. |

638

639 **Table 2:** Genes (including abbreviations and known major functions) used in this study.
 640 Italicised genes showed differential expression between bold and shy rainbow trout,
 641 *Oncorhynchus mykiss*, in a previous microarray study (Sneddon et al. 2005; LU Sneddon, MS
 642 under review).

| Gene | Abbr. | Functions |
|---|-------------------|--|
| <i>Ependymin</i> | Epd | Memory/learning ¹ ; Cold tolerance ² ; Regeneration ³ |
| <i>γ-Aminobutyric acid A</i> | GABA _A | Anxiety ⁴ ; Aggression ⁵ ; Memory ⁴ |
| <i>Calmodulin</i> | CaM | Calcium binding (Memory ⁶ ; Nerve growth ⁶ ; Immune system ⁷) |
| <i>Major histocompatibility complex Class I</i> | MHC I | Immune system ⁸ ; Kin recognition ⁸ |
| <i>Haemoglobin α4 subunit</i> | Hbα4 | Oxygen transport |
| (Arginine) vasotocin | AVT | ACTH secretion ⁹ ; Modulation of social and non-social behaviour ⁹ |
| Proopiomelanocortin | POMC | Stress response ¹⁰ |
| Retinol binding protein | RBP | Vitamin A transport ¹¹ ; Stress/Immune response ¹² |

643
 644 ¹Shashoua (1991), ²Tang *et al.* (1999), ³Suárez-Castillo *et al.* (2004), ⁴Kalueff and Nutt (1997),
 645 ⁵Miczek *et al.* (2003), ⁶Stevens (1983), ⁷Racioppi and Means (2008), ⁸Götze (1977), ⁹Goodson and
 646 Bass (2001), ¹⁰Winberg and LePage (1998), ¹¹Goodman (1980) ¹²Flower (1996).

647

648 **Table 3:** Primer sequences for RT-PCR for eight genes implicated in behavioural responses,
649 and for a reference gene (*), including accession number (where primers were generated from
650 a single sequence), and amplicon size and melting temperature, T_m . Primers were developed
651 using Primer Express® 3.0 software, and were diluted to a working concentration of 10 pmol
652 μl^{-1} .

| Gene Accession No. | Forward (5' – 3') | Reverse (5' – 3') | Size (bp) | T_m (°C) |
|-------------------------------|------------------------------------|------------------------------------|--------------|---------------|
| Ependymin NM_001124693 | CTC ATG CTC ACG CTC TGG AA | CCA AAA ACA GCT CAA CCT GAT G | 60 | 83 |
| GABA _A BT073523 | CTC ATC CGA AAG CGA ATC CA | CAC ACT CTC GTC ACT GTA GG | 156 | 81 |
| Calmodulin | CCG GGA GGC TGA TAT CGA T | CGT CAT CAT CTG CAC AAA TTC TTC | 64 | 81 |
| MHC1 | AGT CCC TCC CTC TGT GTT TCT G | TCG CGT GGC AGG TCA CT | 62 | 62 |
| POMC NM_001124718 | AGC GCT ATG GAG GGT TCA TG | CAA CGT GAG CAG TGG TTT CTG | 62 | 82 |
| Hb α 4 BT074353 | GAA GAA GCG CGG CAT CAC | TCG TCC ATG TGG CCA ACA | 60 | 81 |
| AVT DQ291141 | ACC CAG CGG TCC TAT ATT ATG ATC | GGC ATG CTG AGG ACC AGA CT | 62 | 81 |
| RBP NM_001124278 | GGA CAA TGT CGT CGC TCA GTT | CGT GGG CAG TTG CAG TCA | 62 | 80 |
| <i>GAPDH</i> * AF027130 | TGT TGT GTC TTC TGA CTT CAT TGG | CCA GCG CCA GCA TCA AA | 60 | 81 |

653

654 **Table 4:** Relative expression (normalised to a control gene, GAPDH; RE) and *p* values for
 655 the comparisons of expression of eight genes, selected for implicated roles in boldness,
 656 between bold and shy or between high (HR) and low (LR) stress responsive rainbow trout,
 657 *Oncorhynchus mykiss*. Asterisks denote significant difference between the groups (REST, in
 658 Pfaffl et al., 2002): *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

659

| | Boldness | | Stress Line | |
|-------------------|-----------------|-----------------|--------------------|-----------------|
| | RE | <i>p</i> | RE | <i>p</i> |
| Epd | 0.82 | 0.52 | 2.63 | *** |
| MHC I | 0.69 | 0.46 | 5.92 | *** |
| CaM | 0.75 | 0.31 | 2.09 | ** |
| GABA _A | 1.02 | 0.96 | 1.93 | ** |
| POMC | 1.03 | 0.98 | 0.76 | 0.70 |
| Hb α 4 | 0.94 | 0.88 | 0.99 | 0.98 |
| AVT | 0.90 | 0.72 | 1.89 | * |
| RBP | 0.80 | 0.42 | 2.01 | ** |

660

661 **Figure 1:** Median plasma cortisol (ng ml^{-1} ; $\pm 90^{\text{th}}$ and 10^{th} percentiles) in unstressed
662 (A; $n=36$) and stressed (B; $n=34$) rainbow trout, *Oncorhynchus mykiss*. In each case,
663 comparisons were made between high (HR) and low (LR) stress responsive lines, between
664 individuals determined bold and shy by a novel object test, and by sex. Asterisks denote
665 significant difference between groups (Mann-Whitney test): **, $p<0.01$; ***, $p<0.001$.

666

667 **Figure 2:** Frequency of individual trials in which individual rainbow trout, *Oncorhynchus*
668 *mykiss* ($n=154$), either (A) as a whole ($n=154$) or separated into (B) the HR ($n=88$) or (C) the
669 LR ($n=66$) stress lines, approached within 5 cm of a novel object within a certain period of
670 time ($n=154$).

671

672 **Figure 3:** Percentage of rainbow trout, *Oncorhynchus mykiss*, showing consistently bold
673 (white) or shy (grey) behaviour in lines bred for high (HR; $n=24$) and low (LR; $n=17$) cortisol
674 response to stress, and in both groups combined.

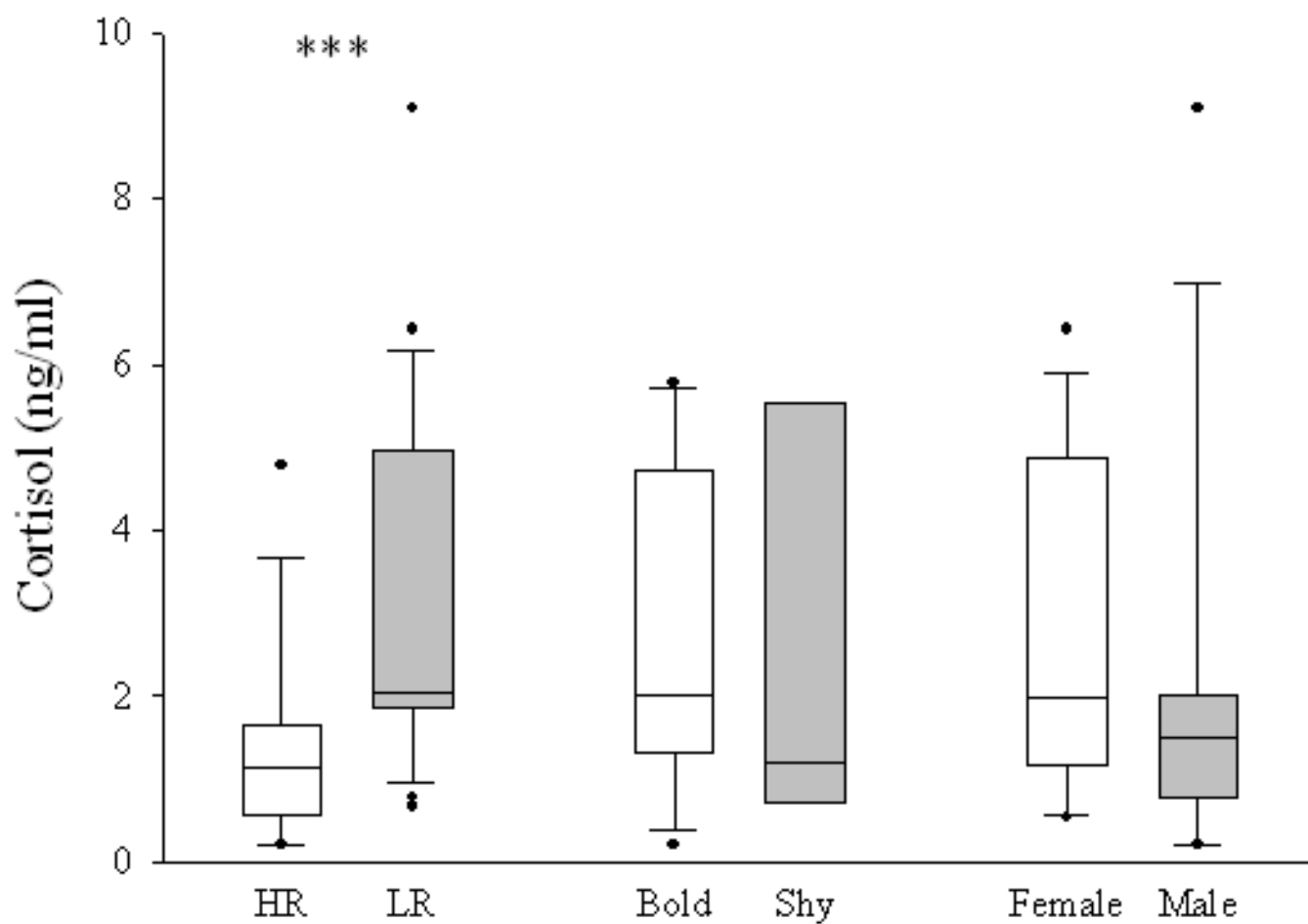
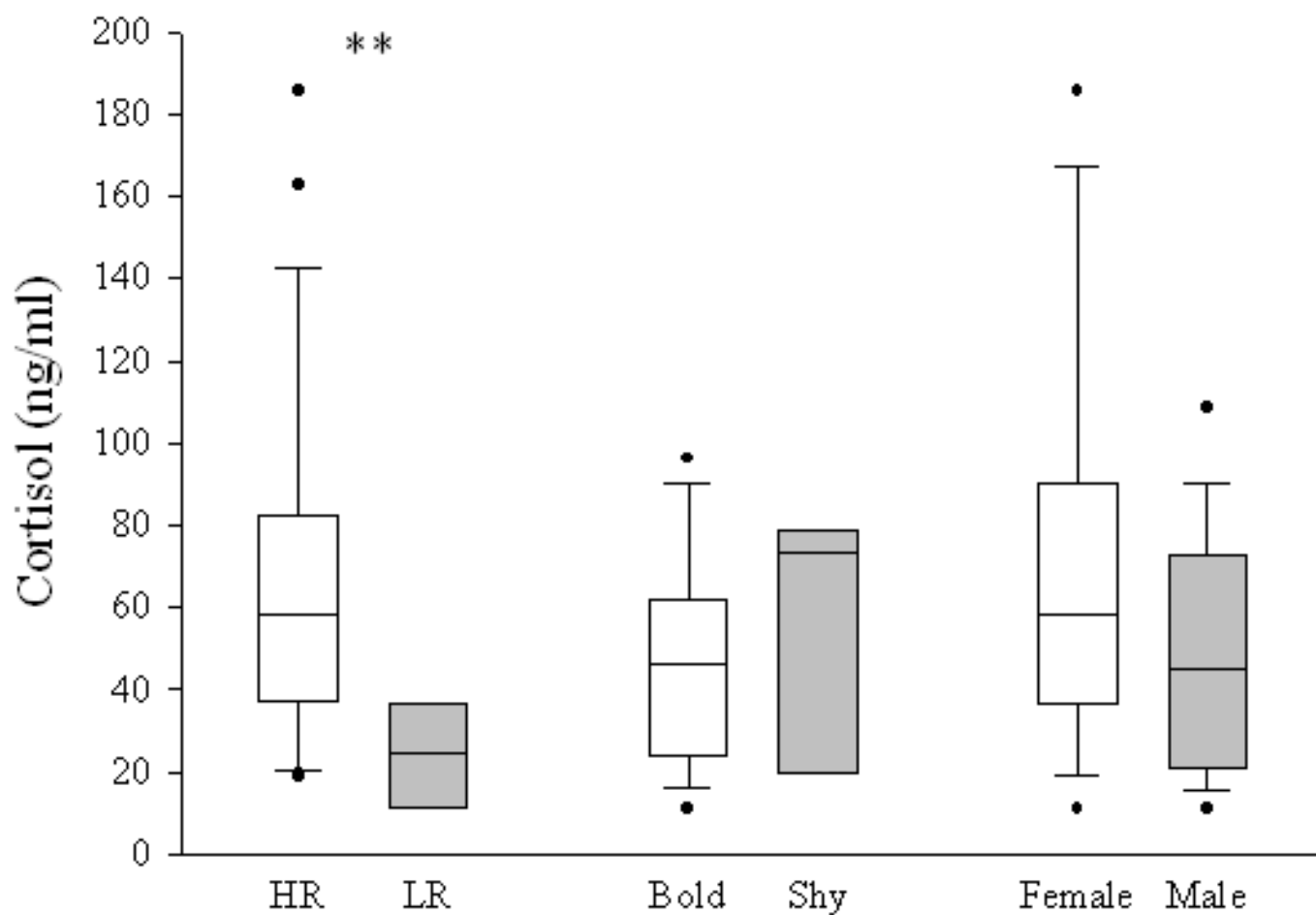
675

676 **Figure 4:** Median ($\pm 90^{\text{th}}$ and 10^{th} percentiles) (A) duration of passive behaviour and (B)
677 frequency of approaching to within 10 cm of a novel object for bold and shy rainbow trout,
678 *Oncorhynchus mykiss*, within the HR (white; n for bold=15, n for shy=9) and LR (grey; n for
679 bold=13, n for shy=4) stress lines. Asterisks denote significant difference between groups
680 (Mann-Whitney test): **, $p<0.01$; ***, $p<0.001$.

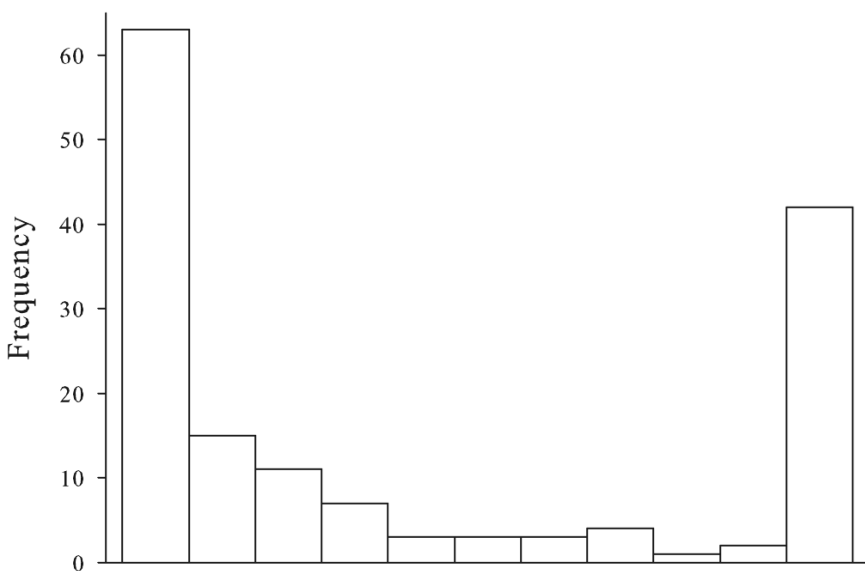
681

682 **Figure 5:** Median relative expression ($\Delta Ct_{\text{reference}} - \Delta Ct_{\text{target}}$; $\pm 90^{\text{th}}$ and 10^{th} percentiles) of
683 eight candidate genes compared between (A) bold ($n=28$; white) and shy ($n=13$; grey), and
684 (B) high (HR; white; $n=22-25$) and low (LR; grey; $n=17$) stress responding rainbow trout,
685 *Oncorhynchus mykiss*. Epd = Ependymin; MHCI = major histocompatibility complex I;

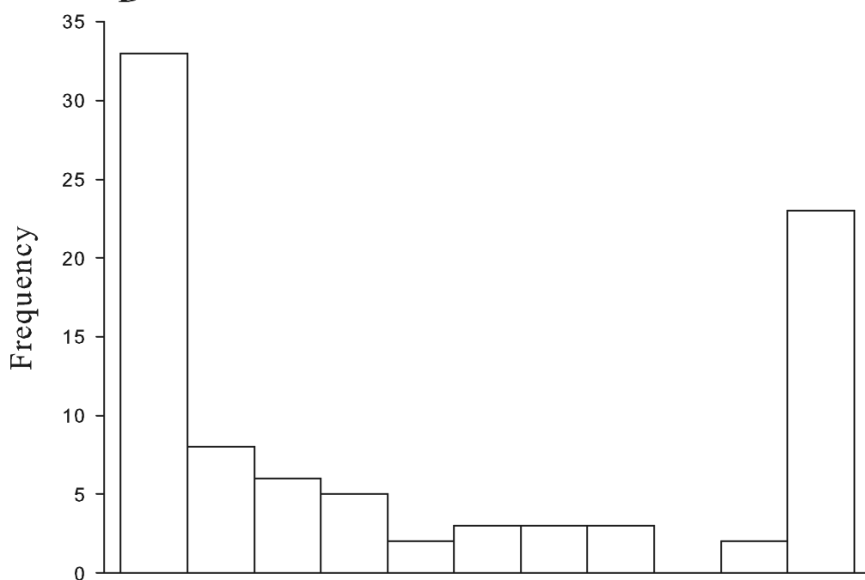
686 CaM = calmodulin; GABA = γ -Aminobutyric acid A; POMC = proopiomelanocortin;
687 Hba4 = haemoglobin α 4 subunit; AVT = vasotocin; RBP = retinol binding protein. Asterisks
688 denote significant difference between the groups (REST, in Pfaffl et al., 2002): *, $p \leq 0.05$;
689 **, $p \leq 0.01$; ***, $p \leq 0.001$.

A**B**

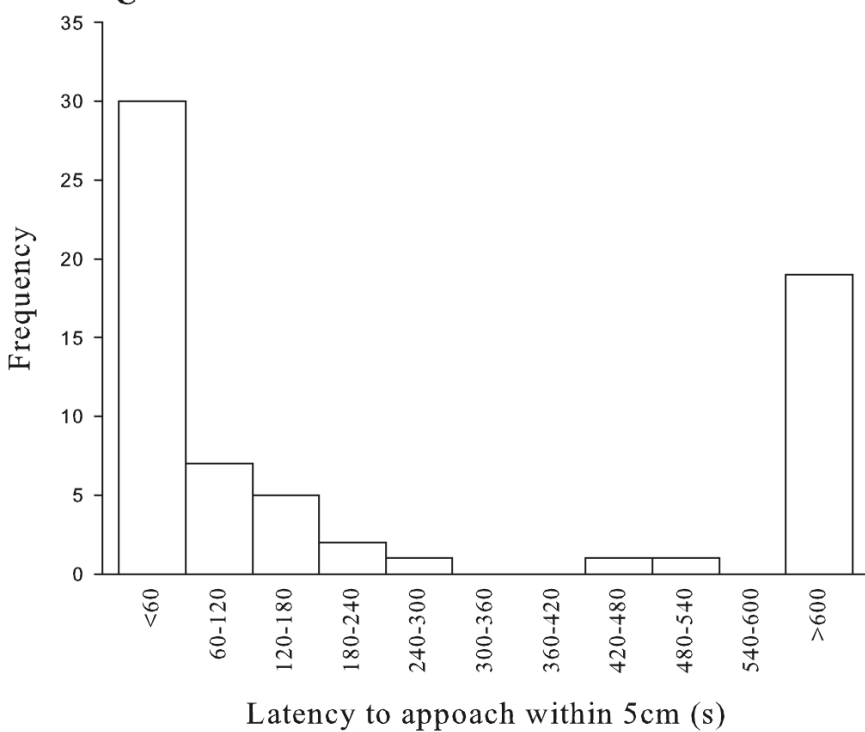
5-Figure 2
A



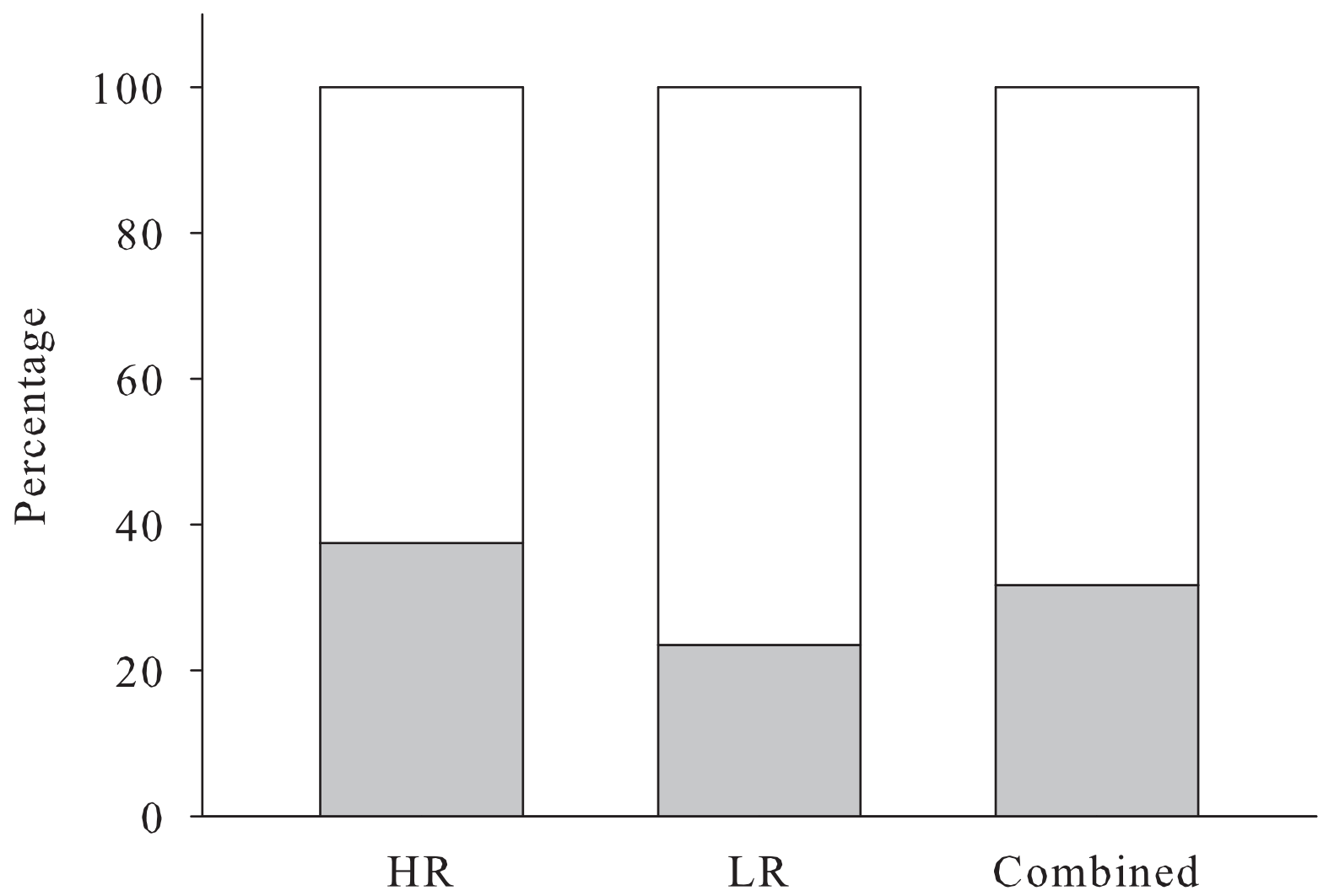
B



C



5-Figure 3



5-Figure 4

