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1 **Plasticity of boldness in rainbow trout, *Oncorhynchus mykiss*: do hunger and predation**
2 **influence risk-taking behaviour?**

3

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23 **Abstract**

24 Boldness, a measure of an individual's propensity for taking risks, is an important
25 determinant of fitness but is not necessarily a fixed trait. Dependent upon an individual's
26 state, and given certain contexts or challenges, individuals may be able to alter their
27 inclination to be bold or shy in response. Furthermore, the degree to which individuals can
28 modulate their behaviour has been linked with physiological responses to stress. Here we
29 attempted to determine whether bold and shy rainbow trout, *Oncorhynchus mykiss*, can
30 exhibit behavioural plasticity in response to changes in state (nutritional availability) and
31 context (predation threat). Individual trout were initially assessed for boldness using a
32 standard novel object paradigm; subsequently, each day for one week fish experienced either
33 predictable, unpredictable, or no simulated predator threat in combination with a high (2%
34 body weight) or low (0.15%) food ration, before being reassessed for boldness. Bold trout
35 were generally more plastic, altering levels of neophobia and activity relevant to the
36 challenge, whereas shy trout were more fixed and remained shy. Increased predation risk
37 generally resulted in an increase in the expression of three candidate genes linked to
38 boldness, appetite regulation and physiological stress responses – ependymin, corticotrophin
39 releasing factor and GABA_A – but did not produce a significant increase in plasma cortisol.
40 The results suggest a divergence in the ability of bold and shy trout to alter their behavioural
41 profiles in response to internal and exogenous factors, and have important implications for
42 our understanding of the maintenance of different behavioural phenotypes in natural
43 populations.

44

45 **Introduction**

46 Boldness defines how individuals respond to risk and novelty: bold animals are generally
47 more active, more likely to explore novel objects or environments and spend more time in the

48 open compared with shy conspecifics (Sih et al., 2004; Sneddon, 2003), and this variation
49 exists along a continuum from bold to shy. Whilst many behaviours are, at least partly,
50 heritable (Giles and Huntingford, 1984; van Oers et al., 2004), they can also be shaped by
51 experience and animals may vary their degree of boldness according to extrinsic
52 (environmental; e.g. Chapman et al., 2010; Frost et al., 2007) or intrinsic (age, size, etc.; Bell
53 and Stamps, 2004; Brown and Braithwaite, 2004) factors. Since boldness influences decision
54 making, the fitness consequences of bold or shy behaviour may be determined by the
55 immediate environment (for example, where territory or food is limited, bolder animals may
56 be more successful due to higher aggression and exploration tendency; Dingemanse et al.,
57 2004); the ability to alter behaviour therefore has important implications for fitness,
58 particularly in a fluctuating environment, since an individual may be able to adapt to the
59 environmental conditions. The present study tested this behavioural plasticity by determining
60 the degree to which bold or shy behaviour changes in the context of extrinsic (risk, measured
61 as predation threat) and intrinsic (nutritional status) factors.

62

63 The wrong behavioural choices when exposed to predation threat can lead to mortality, but
64 the optimal behavioural strategy to deal with this threat remains unclear: whilst high threat
65 may encourage risk-taking behaviour to forage (Brown et al., 2005b), a shy strategy limits
66 exposure through reduced activity and exploration (Archard and Braithwaite, 2011; Brydges
67 et al., 2008). The most appropriate behavioural response may depend on additional factors
68 such as habitat stability (Brydges et al., 2008), age (Magnhagen and Borcharding, 2008), size
69 (Werner et al., 1983) and food availability (Borcharding and Magnhagen, 2008). Exposure to
70 predation threat can drive the expression of boldness (Bell and Sih, 2007), but little is known
71 as to how this process may be modulated by individual hunger levels in prey. Nutritional
72 status is an important determinant of activity levels since animals with low energy reserves

73 need to increase activity to forage (Borcherding and Magnhagen, 2008; Vehanen, 2003). Like
74 predator threat, foraging profitability varies spatiotemporally; animals therefore need to
75 adjust foraging rates and activity dependent on both profitability (Croy and Hughes, 1991)
76 and prevailing predation risk (Lima and Bednekoff, 1999; Metcalfe et al., 1987; Vehanen,
77 2003; Werner et al., 1983), and it is likely these decisions may be modulated by an
78 individual's propensity for taking risks.

79

80 The stress response in fish is controlled through activation of the hypothalamo-pituitary-
81 interrenal (HPI) axis, resulting in the release of cortisol (Wendelaar Bonga, 1997). Likewise,
82 much of the control of food intake takes place in the hypothalamus (Kalra et al., 1999), and
83 utilises some of the same biochemistry. Predation threat necessarily invokes a stress response
84 and thus may evoke a reduction in feed intake both through physiological (Scheuerlein et al.,
85 2001) and behavioural (Metcalfe, 1987) changes to reduce feeding rates in the presence of a
86 predator. Coping style theory predicts that bold (proactive) and shy (reactive) animals
87 respond to stress with low or high HPI activity, respectively (Koolhaas et al., 1999; Øverli et
88 al., 2002; Pottinger and Carrick, 2001), and previous studies using lines of rainbow trout,
89 *Oncorhynchus mykiss*, bred for divergent stress responses revealed significantly different
90 patterns of gene expression between low and high stress responsive fish (Backström et al.,
91 2011; Johansen et al., 2011; Thomson et al., 2011). However, how individual differences in
92 HPI activity and coping style reflect antipredator and foraging strategies remains relatively
93 under-studied.

94

95 Divergent bold/shy phenotypes reflect differences in how animals respond to threat, but
96 whilst the strategies of bold and shy fish are established under risk or food-deprived regimes
97 it remains unclear how animals exhibiting bold or shy strategies, and consequently

98 differential physiological activity, respond to an interaction between risk and hunger.
99 Individual genes are important in shaping behaviour (e.g. Greenwood et al., 2008; Sneddon et
100 al., 2011), and to understand this relationship it is thus imperative to identify correlations
101 between gene expression and behavioural or physiological responses to such challenges. The
102 aim of this study was therefore to determine how behavioural decisions in bold and shy
103 rainbow trout may be influenced by exposure to varying levels of predator threat and
104 metabolic demand. Circulating plasma cortisol levels were assessed to determine variation in
105 stress levels in these fish, and behavioural and physiological differences were related to the
106 expression of three specific genes known to be involved in processes of behaviour, the
107 physiological stress response and appetite regulation: ependymin, CRF and the GABA_A
108 receptor (Table 1). Ependymin is involved in behaviours strongly linked with boldness, such
109 as aggression (Sneddon et al., 2011) and behavioural plasticity and learning (Shashoua,
110 1985), but is also involved in responses to environmental stress (e.g. Tang et al., 1999). CRF
111 plays an integral role in the corticosteroid response to stress, initiating the HPI axis through
112 binding to CRF Type I receptors in the pituitary to stimulate the secretion of ACTH
113 (Chrousos and Gold, 1992). CRF is also a critical hormone for the integration of sensory cues
114 and dietary (or energetic) information with stress status, and translating this into orexigenic or
115 anorexigenic signals (see Bernier, 2006, and references therein). Finally, the GABA_A
116 receptor, and the GABA system in general, has broad functionality and has been linked with
117 fearfulness (Caldji et al., 2000) and aggression (Miczek et al., 2003), both indicators of
118 boldness and stress responsiveness or coping style (Koolhaas et al., 1999). Furthermore,
119 evidence suggests GABA_A receptors may be linked with the control of appetite and feed
120 intake (Wu et al., 2009). Divergent mRNA expression for each of these genes has previously
121 been demonstrated in lines of rainbow trout bred for divergent stress responses (Backström et
122 al., 2011; Thomson et al., 2011), and they are therefore excellent candidate genes to

123 investigate links between boldness and stress responsiveness in unselected trout in the
124 contexts presented.

125

126 Specifically, we hypothesised that (1) individuals would vary their behaviour according to
127 prevailing risk, with the prediction that satiated fish would reduce risk-taking activity whilst
128 food-deprived animals would take more chances; (2) fish under higher predation and/or
129 restricted dietary regimes would experience elevated activation of the stress response, and
130 alterations in the expression of three candidate genes implicated in feeding- and boldness-
131 related behavioural processes.

132

133 **Methodology**

134 *Test Animals*

135 The following experiment was conducted under Home Office, UK, guidelines according to
136 the Animal (Scientific Procedures) Act 1986, and following local ethics approval. Rainbow
137 trout, *Oncorhynchus mykiss*, were maintained in stock tanks (2 x 2 x 0.5 m) with a semi-
138 recirculating system on a 14:10 h light:dark cycle at $13 \pm 1^\circ\text{C}$, and fed 1% body weight per
139 day on commercial trout feed (Skretting, UK). Experimental fish ($n = 75$, 93.48 ± 3.94 g)
140 were caught at random and transferred to individual glass aquaria (90 x 50 x 45 cm) which
141 were screened from visual disturbance, and maintained at $10 \pm 1^\circ\text{C}$ with constant aeration.
142 Feed were provided 1% body weight feed per day at the same time each day. The next day
143 trout were netted, anaesthetised in 0.033 g l^{-1} , benzocaine (Sigma-Aldrich Co., UK) and
144 weighed, and then returned to their individual aquaria and allowed to acclimate for at least
145 one week or until the resumption of feeding. Fish that did not resume feeding after 14 days
146 were not used in the study.

147

148 *Novel Object Tests*

149 Boldness was assessed using a standard novel object paradigm (Frost et al., 2007; Thomson
150 et al., 2011) whereby a novel object was placed centrally into the tank and the behaviour was
151 recorded for ten minutes (after which the object was removed). Novel objects comprised an
152 orange rubber stopper (7.1 cm mean diameter, 4.9 cm height) and a blue transparent box
153 weighted with gravel (7.5 x 5.3 x 3.8 cm). We focussed on two key behavioural responses
154 (see Thomson et al., 2011 for details): 1) latency to approach to within 5cm of the novel
155 object (s); and, 2) The duration of passive behaviour (s), which included the subject resting at
156 the base of the tank, pivoting on its own axis, and drifting across the tank, but excluded
157 swimming greater than one body length. Bold fish were defined as those approaching the
158 novel object within 180 s ($n = 35$) and shy fish as those which did not approach within 300 s
159 ($n = 36$); the remainder were classed as intermediate ($n = 4$) and discarded from further
160 analysis. These were therefore clearly distinct behavioural groups, and trout exhibiting
161 discrete suites of behavioural differences in response to a novel object have previously been
162 successfully selected on this basis (Thomson et al., 2011).

163

164 *Predation Risk and Diet Manipulations*

165 Once each day, subsequent to the first behavioural test, fish were subjected to one of three
166 treatments varying the level of perceived predation risk, and imposing one of two levels of
167 feed provision (Table 2). Predation risk was simulated by using a plastic heron head (*Ardea*
168 *cinerea*) mounted on a pole to simulate a predator attack (see Johnsson et al., 2001b; Jönsson
169 et al., 1996). Attacks were made from behind a screen to prevent association with the
170 presence of a human, and consisted of three swift strikes into the water followed by
171 immediate removal of the model. Simulated attacks coincided with the injection of 20 ml
172 trout alarm substance into the water to provide a chemical stimulus of risk. Alarm substance

173 extracted from conspecific skin has been demonstrated to increase antipredator behaviour in
174 fish (Smith, 1992) with rainbow trout increasing cover use and decreasing activity and
175 feeding (Ashley et al., 2009; Brown and Smith, 1998). Alarm substance was prepared from
176 dissected skin from non-experimental trout that was then washed with sterile distilled water
177 (SDW) and homogenised in 50 ml Falcon tubes containing 6.25 ml SDW per 1 g skin. After
178 centrifugation at 4°C, the supernatant was aliquoted and frozen at -20°C. For experiments,
179 aliquots were further diluted (1 ml pheromone per 7 ml SDW) and a final volume of 20 ml
180 solution used for each tank.

181

182 Fish experiencing no risk did not experience a simulated predator attack. Low risk fish were
183 subjected to a simulated attack each day at the same time, one hour after feeding, to create a
184 predictable threat. High risk trout were exposed to an unpredictable environment where the
185 timing of the attack varied each day at random.

186

187 To generate different levels of hunger in the subjects, fish were fed one of two quantities of
188 feed. Those on a high diet were fed $2\% \pm 0.01\text{g}$ body weight per day, whilst those fed on a
189 low feed routine were only provided with $0.15\% \pm 0.01\text{g}$ body weight per day to induce
190 fasting. Short-term food deprivation occurs in the wild due to limited food availability or low
191 temperatures, and therefore fasting is a non-stressful natural process in rainbow trout causing
192 a reduction in weight and condition factor (Pottinger et al., 2003); upon resumption of
193 feeding, individuals quickly return to their former health status with no further ill-effects.
194 Indeed, the subjects in this study did not lose weight during the trial period (Fig. 3). Fish were
195 fed at the same time each day regardless of threat or diet regime. These conditions were
196 applied each day for seven days, at which point a second novel-object test was used to
197 reassess behavioural phenotype.

198

199 *Hormone Analysis and Quantification of Gene Expression*

200 After reassessing behaviour, fish were killed by concussion at the same time each day (15:00
201 \pm 1 hr) to account for diel fluctuations in levels of plasma cortisol (Pickering and Pottinger,
202 1983). The fish were reweighed and a 2 ml blood sample taken from the caudal vessels into a
203 heparinised syringe. After centrifugation, the plasma was divided into aliquots and frozen
204 at -20°C . Plasma cortisol levels were determined by radioimmunoassay (see Pottinger and
205 Carrick 2001 for details of protocol). The antibody employed was rabbit anti-cortisol
206 antibody IgGF2 (IgG Corporation, Nashville). Sensitivity (minimal detection limit) of the
207 assay was 0.3 ng ml^{-1} . The inter-assay coefficients of variation for a low (5 ng ml^{-1}) and high
208 (53 ng ml^{-1}) plasma sample were 8.4 and 8.1% ($n = 8$) and the corresponding intra-assay
209 coefficients of variation were 5.5 and 5.1% ($n = 8$). Cross-reactivity of the antibody with
210 cortisone, the most significant potential competitor in rainbow trout plasma, was 2.6%.

211

212 Whole brains were removed and stored at -80°C until RNA extraction. Total RNA was
213 extracted using TRIzol® (Invitrogen Life Science, UK). For each sample, $\sim 1\text{ }\mu\text{g}$ of mRNA
214 was reverse-transcribed into first strand cDNA using random hexamers and SuperScript™ III
215 reverse transcriptase (Invitrogen Life Science, UK). For RT-PCR, $\sim 0.15\text{ }\mu\text{g}$ cDNA was
216 amplified in a $10\text{ }\mu\text{l}$ PCR (using $5\text{ }\mu\text{l}$ Fast SYBR Green, Invitrogen Life Science, UK) primed
217 with 2 pmol of each primer. Four primer pairs were developed using Primer Express® 3.0
218 (Applied Biosystems, USA) software against *O. mykiss* sequences, which were: ependymin,
219 5-CTCATGCTCACGCTCTGGAA-3 and 5-CCAAAACAGCTCAACCTGATG-3; CRF:
220 5-GTGGTTCTGCTCATTGCTTTCTT-3 and 5-CGCCAGGGCTCTCGATAG-3; GABA_A
221 Receptor: 5-CTCATCCGAAAGCGAATCCA-3 and 5-CACACTCTCGTCACTGTAGG-3;
222 GAPDH: 5-TGTTGTGTCTTCTGACTTCATTGG-3 and 5-CCAGCGCCAGCATCAAA-3.

223 Thermal cycling conditions, using a 7500 Fast Real-Time PCR System (Applied
224 Biosystems), were: 10 min at 95°C, followed by 40X [95°C 3 s, 60°C 30 s] and then [95°C
225 for 15 s, 60°C for 6 s, 95°C for 15 s and 60°C for 15 s], and the relative quantitation of PCR
226 product was determined using comparisons of ΔCt (Ct of target – Ct of reference [GAPDH]).

227

228 *Data Analysis*

229 All analyses were performed in R (ver. 2.7.0; R Development Core Team, 2009). Response
230 variables (change in behaviour between the trials; change in weight (%); plasma cortisol (ng
231 μl^{-1}); ΔCt of target genes) were analysed with a factorial analysis using a generalized linear
232 model; non-significant terms were systematically removed, and degrees of freedom and AIC
233 values compared using the stepAIC function (MASS package; Venables and Ripley, 2002) to
234 obtain the minimum adequate model (Table 3). With the exception of cortisol, all response
235 variables fit the assumptions of GLM; cortisol data were \log_{10} -transformed for analysis. Since
236 bold and shy fish exhibited a difference in the degree to which they changed their latency to
237 approach to within 5 cm of the object, a Wilcoxon signed rank test was used to determine if
238 these changes between trials was significant. Mann-Whitney U Tests were subsequently
239 utilised to determine whether either 5 cm latency or the duration of passive behaviour were
240 equivalent for bold and shy fish after the second trial.

241

242 Plasma cortisol concentration data included three points with large residuals: one shy
243 individual (high risk) had a cortisol concentration of 110.4 ng ml^{-1} , typical of the response to
244 a moderate stressor in this species (Øverli et al., 2002; Pottinger and Carrick, 1999) and far in
245 excess of those exhibited by the remainder of the group. Two bold fish, one each in the no
246 threat and low threat group exhibited cortisol concentrations (20.5 and 19.9 ng ml^{-1}
247 respectively) which, whilst high, are of a magnitude previously observed in unstressed trout

248 (Øverli et al., 2002). However, it is unlikely these fish exhibited the same physiological
249 profile as the remainder of their groups, which exhibited cortisol concentrations $<4\text{ng ml}^{-1}$
250 which are more typical of an unstressed state (Balm and Pottinger, 1995; Thomson et al.,
251 2011). On these bases, and verified through Grubbs' Tests, these data were assumed to be
252 outliers and all analyses are thus presented with these data excluded.

253

254 **Results**

255 *Behaviour*

256 Median (min - max) initial latency to approach to within 5 cm of the object were 19.49 s
257 (0.00 – 135.12 s) for bold fish and 600.00 (403.04 – 600.00) for shy fish. The change in
258 latency to approach to within 5 cm of the novel object differed between bold and shy trout
259 ($F_{1,66} = 26.04, p < 0.001$; Fig. 1). This was reflected by a significant increase in latency in
260 bold fish between trials ($W_{33} = 462.0, p < 0.001$), but shy fish exhibited a non-significant
261 decrease in latency ($W_9 = 8.0, p = 0.097$). Despite these changes, after the second trial bold
262 trout did not become as neophobic as shy trout (median [min - max] latency after treatments
263 = 137.86 s [0.00 – 600.00 s] for bold fish and 600.00 s [4.01 – 600.00 s] for shy fish; $W =$
264 293.0, $p < 0.001, n_1n_2 = 33, 35$).

265

266 Bold fish were also less passive than shy trout during the first trial (median [min - max]
267 passive duration = 476.84 s [163.79 – 582.75 s] for bold fish and 572.95 s [442.42 – 600.00
268 s] for shy fish; $W = 151.0, p < 0.001, n_1n_2 = 33, 35$). The change in the duration of passive
269 behaviour between the trials varied according to a three-way interaction of all parameters
270 ($F_{2,56} = 4.95, p = 0.010$; Fig. 2), and varied more in bold fish than shy. Median passive
271 duration generally increased for bold fish except for some individuals showing a decrease in
272 activity when on a low diet and confronted with low or high predation risk. In contrast, shy

273 fish generally did not alter their level of passive behaviour, with the exception of an increase
274 in activity for animals under high threat and high diet.

275

276 Unsurprisingly, those fish on a higher diet gained proportionally more weight than those on a
277 low diet ($F_{1,62} = 72.82, p < 0.001$; Fig. 3), but no other treatment had a significant effect.

278

279 *Cortisol Measurements*

280 After removal of outliers, no significant trend was observed between \log_{10} -transformed
281 plasma cortisol and any treatment (risk, diet or boldness, or any interaction thereof: $p > 0.05$),
282 although a possible interaction between risk and initial boldness cannot be discounted ($F_{2,54} =$
283 $2.82, p = 0.068$). The greatest plasma cortisol was recorded from shy fish under a low-threat
284 regime, whilst all fish in the high risk group generally showed higher concentrations than
285 those under low risk (Fig. 4).

286

287 *Gene Expression*

288 Diet did not influence gene expression and all effects of diet were removed from the model.
289 Relative expression of all three genes varied according to the levels of threat experienced by
290 individual trout. Expression of CRF was significantly greater in fish under high threat than
291 those under no threat ($F_{2,51} = 9.20, p < 0.001$; Fig. 5). In contrast, expression of both
292 ependymin ($F_{2,51} = 3.65, p = 0.033$) and GABA_A receptor ($F_{2,50} = 4.46, p = 0.016$) varied
293 according to an interaction between threat and boldness: in each case, expression generally
294 increased with increasing threat but was highest in shy fish under unpredictable predation
295 threat (Fig. 5).

296

297 **Discussion**

298 Being able to alter behaviour may provide an adaptive advantage, particularly in fluctuating
299 environments, but not all individuals exhibit the same degree of behavioural plasticity. Here
300 we demonstrate that, at least in some contexts, personality (in this case boldness) may be one
301 determinant of the extent to which individuals can alter their behavioural profile. Bold fish
302 were generally labile, and altered their strategy according to context, but in contrast the
303 behaviour of shy fish was relatively inflexible in these contexts. Risk itself appeared to
304 induce physiological and gene expression changes in these fish regardless of food availability
305 or, to some extent, the level of boldness; thus, bold and shy behavioural profiles may arise
306 from individual differences in the ability to respond to both external and internal cues.

307

308 *Behaviour*

309 In response to high predation threat either bold (e.g. Brown et al., 2005b) or shy (e.g. Brydges
310 et al., 2008) strategies can be preferred, suggesting both can be adaptive antipredator
311 strategies but likely dependent upon additional environmental contexts (Coleman and Wilson,
312 1998). Bold fish in this study may have behaved according to the risk allocation hypothesis
313 and allocated food acquisition behaviour to periods when risk was low (Lima and Bednekoff,
314 1999); these fish altered levels of activity dependent upon predation risk and dietary regime.
315 In general, bold trout decreased activity levels which may be an attempt to lower the
316 encounter rate with any potential predator given the unpredictability of attacks (Anholt and
317 Werner, 1995; Ferrari et al., 2008). Inactivity would, furthermore, conserve energy if fish
318 were habituated to a particular feeding time (Chen and Tabata, 2002). When threat was
319 unpredictable and food availability low, however, some bold fish increased activity, possibly
320 to maximise food acquisition (Vehanen, 2003); greater risk-taking behaviour in bold animals
321 may therefore be adaptive in these conditions (Brown et al., 2005b).

322

323 In comparison, shy fish exhibited an ostensibly reduced behavioural plasticity in response to
324 a novel object compared to bold trout. Whilst potentially adaptive, phenotypic plasticity is
325 expensive (DeWitt et al., 1998), and shy fish may be able to place less investment in
326 plasticity as some costs (e.g. the acquisition of environmental information) may favour risk-
327 taking behaviour. One argument may be that shy behaviour was simply the most suitable
328 strategy in response to the presented treatments. However, changes to internal state can lead
329 to a need to change behaviour: for instance, the potential cost of exposure to threat may be
330 mitigated by a need to forage driven by low nutritional state, and thus below a certain
331 nutritional threshold shy animals may begin to forage even in risky environments (Dall et al.,
332 2004; Höjesjö et al., 1999). However, even animals in the low dietary groups in this study
333 gained weight, and thus this threshold may not have been reached and the need to forage
334 regardless of danger not achieved. Since shy animals are less prone to taking risks than bold
335 conspecifics, the threshold nutritional level before which shy animals are prepared to forage
336 under threat may be lower. Reduction in feeding (such as that which could be caused by the
337 threat of predation; Metcalfe et al., 1987) can result in a reduced body condition (e.g. Höjesjö
338 et al., 1999) which was not observed in the present study despite similar quantities of feed
339 being provided, possibly suggesting a difference in the quality of feed. Nutrient profiles of
340 feed may thus be a more useful measure in future studies than supply by weight (e.g. in
341 Borcharding and Magnhagen, 2008). However, whilst growth rates were positive in the
342 present study, the high threat group presented a possible lower rate of growth compared to
343 low and no threat, and a sustained period of high threat may result in a more conspicuous
344 deterioration in weight gain relative to exposure to lower threat levels.

345

346 The antipredator response often derives from experience (Kelley and Magurran, 2003),
347 without which animals may have impaired behavioural and physiological reactions to a threat

348 (Brown et al., 2005a; Johnsson et al., 2001a) which may be evident in cultured fish such as
349 those used here (Álvarez and Nicieza, 2003). Whilst this should be considered when
350 interpreting behaviour, alarm pheromone does elicit antipredator responses even in farmed
351 trout (Ashley et al., 2009), though comparisons between wild and farmed individuals could
352 be explored in future studies.

353

354 *Cortisol Measurements*

355 In contrast to the behavioural responses of these fish, the endocrine and gene expression data
356 suggest that both bold and shy fish were experiencing similar physiological and genetic
357 responses to the stimuli. Cortisol levels were marginally higher, although not significantly so,
358 in both bold and shy fish under high predation threat compared to none: in general, fish in or
359 from a riskier environment tend to show heightened stress indicators (Brown et al., 2005a),
360 though in this case the cortisol increase was not as profound as what would be expected after
361 an acute and substantial stress (cf. confinement: Øverli et al., 2002; Pottinger and Carrick,
362 1999; emersion: Sloman et al., 2001; Thomson et al., 2011). Rather than being an acute
363 response to the behavioural test, these values may therefore reflect baseline HPI activity
364 which has been modified by exposure to the treatment regimes. The functional significance of
365 variation in blood cortisol levels of unstressed fish remains relatively unknown. Here, no
366 significant differentiation in plasma cortisol concentrations was observed, yet the data
367 suggest a possible divergence in HPI activity between bold and shy fish exposed to a
368 predictable threat. Rainbow trout are able to precisely anticipate daily feeding times (Chen
369 and Tabata, 2002), and it seems likely that other types of routine event can likewise be
370 predicted. Animals of different personality or coping style may have divergent abilities to
371 predict such events, and certainly there are marked differences in cognitive performance
372 between trout exhibiting divergent coping styles (Moreira et al., 2004). Shy (reactive)

373 animals may be better at dealing with unpredictable events than bold (proactive) animals,
374 which are better at learning and retaining conditioned stimuli and dealing with routines
375 (Koolhaas et al., 1999; Sneddon, 2003, Ruiz-Gomez et al., 2010). Therefore, elevated cortisol
376 levels in shy fish may represent an anticipatory response to the stimulus which the bold fish
377 have already learned is not a threat. On the other hand, unpredictability may drive the
378 development of bold behaviour as risk-taking, not risk-averse, strategies will be favoured
379 when environmental variables cannot be accurately predicted (Chapman et al., 2010). Current
380 literature, therefore, provides inconsistent evidence in behavioural and physiological
381 comparisons of animal responses to predictable and unpredictable events, aversive or positive
382 (Bassett and Buchanan-Smith, 2007). Additionally, the influence of personality on the
383 physiological responses to predictability of an aversive stimulus remains unclear, and future
384 work should address this.

385

386 *Gene Expression*

387 Two of the genes utilised in this study, CRF and GABA_A receptor, have been implicated as
388 having roles in neural pathways controlling appetite and feed intake (Bernier and Craig,
389 2005; Pu et al., 1999), yet expression of these genes in this study was not correlated with
390 dietary regime. Instead, since these genes are also linked with stress reactivity (CRF:
391 Chrousos and Gold, 1992; GABA, Makara and Stark, 1974), and GABA_A receptor in the
392 expression of fear (Caldji et al., 2000), it is likely that increased expression of these genes
393 with higher risk represents a molecular response to predation risk. Risk can, however, reduce
394 foraging without affecting appetite (Metcalf, 1987), and since exposure to a predator induces
395 a stress response it seems likely that upregulation of CRF and GABA_A receptor in high threat
396 conditions may represent simultaneous activation of both physiological stress response and
397 appetitive pathways.

398

399 Ependymin plays an important role in behavioural responses to the environment (Shashoua,
400 1991). One such role is that of memory formation (Shashoua, 1991), and variation in its
401 expression may therefore be linked with the anticipation of events, such as predator attacks as
402 observed in this study. This effect was strongest in shy fish, which are known for reduced
403 competitive ability compared to bold animals, an effect also linked with upregulation of
404 ependymin (Aubin-Horth et al., 2005; Sneddon et al., 2011). These data may therefore
405 suggest an important link, at the level of gene expression, between individual boldness and
406 how individuals react to the regularity of predator threat.

407

408 *Conclusions and Implications*

409 In the present study we demonstrated clear differences in the behavioural responses of bold
410 and shy rainbow trout to variations in nutritional state and predation threat, where bold fish
411 exhibited greater behavioural plasticity in their response to novelty than shy fish. In contrast,
412 only slight differences between bold and shy fish in plasma cortisol concentrations were
413 observed and trends in gene expression were dominated by responses to predation threat
414 rather than initial boldness. These data therefore highlight the divergence of phenotypic
415 plasticity in response to a particular stimulus within a species whilst providing information on
416 the roles of physiology and gene expression in response to these contexts. State and context-
417 dependent effects, such as prevailing predation pressure, may generate evolutionary
418 constraints particular to one species or population. Future work therefore needs to focus on
419 how personality affects responses to multiple challenges, and apply this to complex
420 environments to elucidate the functional significance of variation in both behaviour and its
421 plasticity in nature. Furthermore, studies should continue to attempt to determine how

422 behaviour and plasticity are related to key physiological and genetic mechanisms which may
423 drive these differences in personality.

424

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431

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595 **Table 1:** Genes used in this study, their abbreviations and relevant roles.

Gene	Abbreviation	Role
Ependymin	Epd	A glycoprotein implicated in environmental adaptation, particularly linked to boldness ¹ .
Corticotrophin Releasing Factor	CRF	A hypothalamic neurotransmitter hormone which activates the HPI axis by binding with CRF Type I receptors in the anterior lobe of the pituitary to stimulate the release of adrenocorticotrophic hormone ² . Acts as an anorexigenic factor ³ .
γ -Aminobutyric Acid A Receptor	GABA _A	A receptor protein with diverse functionality, including roles in the control of ACTH release in the stress response ⁴ , the control of appetite ⁵ , and also lined with the expression of boldness (e.g. aggression ⁶).
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	Reference gene

596 ¹Sneddon et al. (2011); ²Chrousos and Gold (1992); ³Bernier and Craig (2005); ⁴Makara and

597 Stark (1974); ⁵Pu et al. (1999); ⁶Miczek et al. (2003).

598 **Table 2:** Treatment combinations and sample size for individual rainbow trout,
 599 *Oncorhynchus mykiss*, used in this study; each trout was exposed to one level of threat
 600 alongside one diet regime throughout the seven-day test period. Asterisks indicate groups
 601 where a subject was removed from analysis due to high cortisol levels (see Methodology).
 602

Threat regime	Diet regime	<i>n</i>	
		<i>Bold</i>	<i>Shy</i>
None	Low (0.15% bw)	7	8
	High (2% bw)	7*	8
Low (Predictable)	Low (0.15% bw)	5	5
	High (2% bw)	4*	5
High (Unpredictable)	Low (0.15% bw)	5	4*
	High (2% bw)	5	5

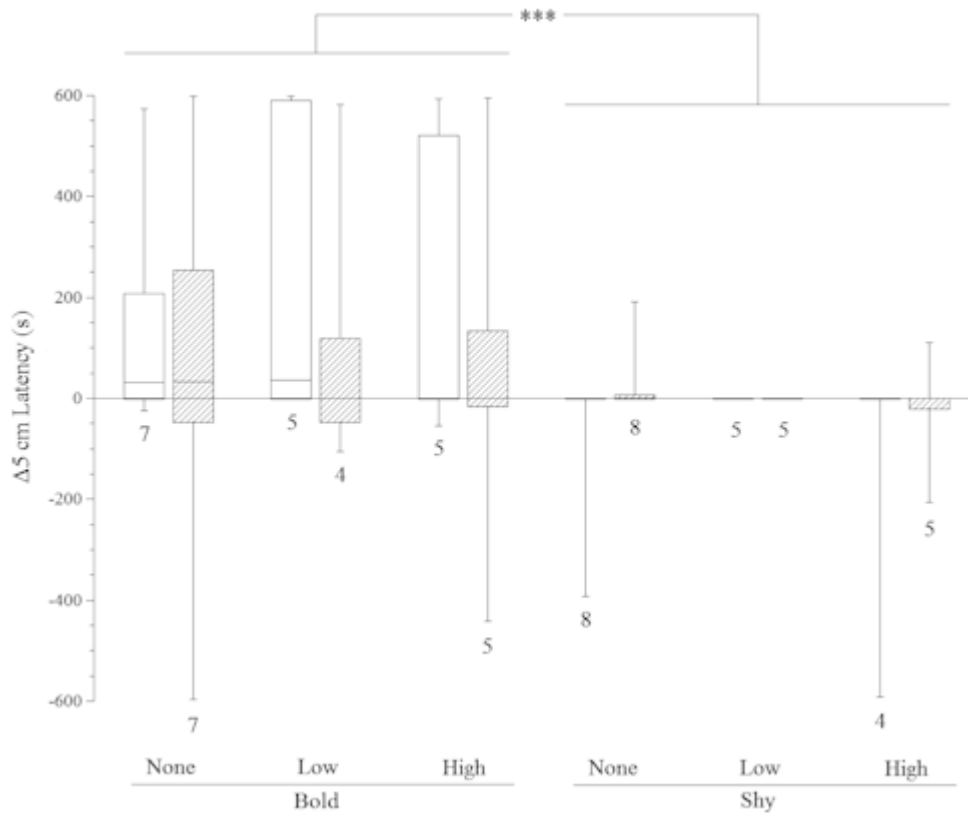
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605 **Table 3:** Akaike Information Criteria (AIC) and residual degrees of freedom (d.f.) for full
 606 models and, where appropriate, minimum adequate models for each response variable. Full
 607 model was defined as $response \sim risk \times diet \times boldness$, and terms retained in the selected
 608 model are also presented.

Response Variable	Full Model		Minimum Adequate Model		
	AIC	d.f.	Retained terms	AIC	d.f.
$\Delta 5\text{cm Latency (s)}$	955.5	56	<i>boldness</i>	940.9	66
$\Delta \text{Passive Duration (s)}$	875.5	56	-	-	-
$\Delta \text{Weight (\%)}$	474.5	50	<i>risk, diet</i>	465.5	58
$\log_{10}\text{-Cortisol (ng ml}^{-1}\text{)}$	120.1	48	[<i>risk</i> \times <i>boldness</i>]	108.6	54
$\Delta \text{Ct(Ependymin)}$	193.5	45	[<i>risk</i> \times <i>boldness</i>]	187.8	51
$\Delta \text{Ct(CRF)}$	206.7	45	[<i>risk</i> \times <i>boldness</i>]	200.5	51
$\Delta \text{Ct(GABA}_A\text{)}$	192.6	44	[<i>risk</i> \times <i>boldness</i>]	185.0	50

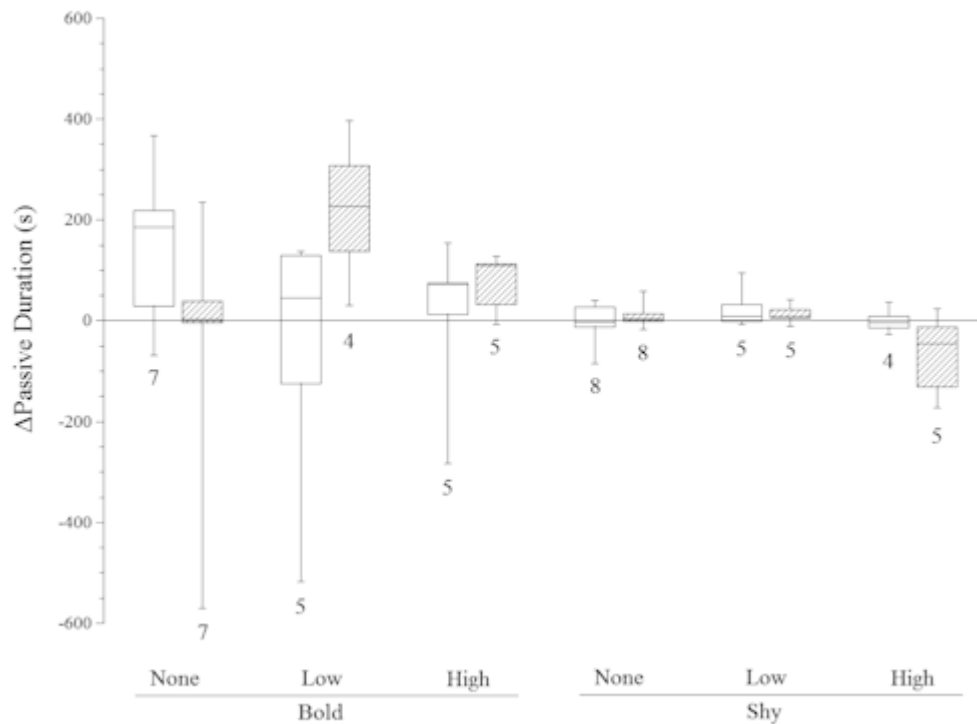
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610

611 **Figure 1:** Median (upper and lower boundaries are 25th and 75th percentiles) change in
 612 latency between trials (s) for bold and shy rainbow trout, *Oncorhynchus mykiss*, to approach
 613 within 5 cm of a novel object. Individual trout experienced no, low or high predator threat
 614 and had either low (white, left box) or high (hatched, right box) feed availability. *n* for each
 615 treatment indicated below boxes. Asterisks represent a significant difference between groups
 616 (***, $p < 0.001$).

617



618

619 **Figure 2:** Median (upper and lower boundaries are 25th and 75th percentiles) change in

620 duration of passive behaviour between trials (s) for bold and shy rainbow trout,

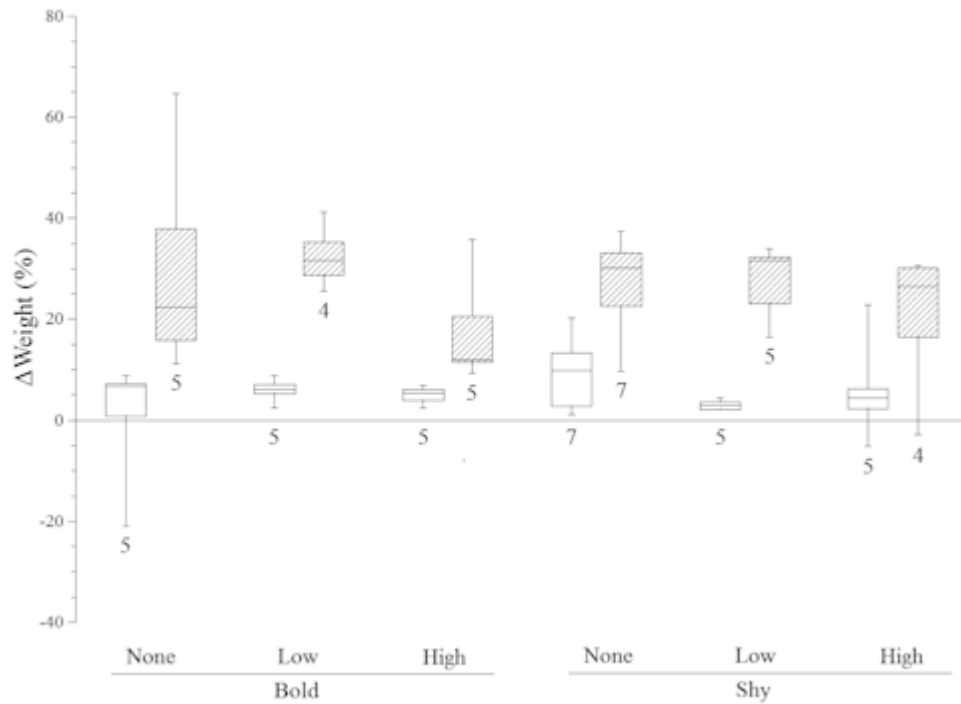
621 *Oncorhynchus mykiss*, under a no, low or high threat regime and low (white) or high

622 (hatched) food availability. *n* for each treatment indicated below the boxes. Overall change in

623 passive duration differed between treatments according to a three-way interaction between

624 level of risk, diet and initial boldness ($p = 0.010$).

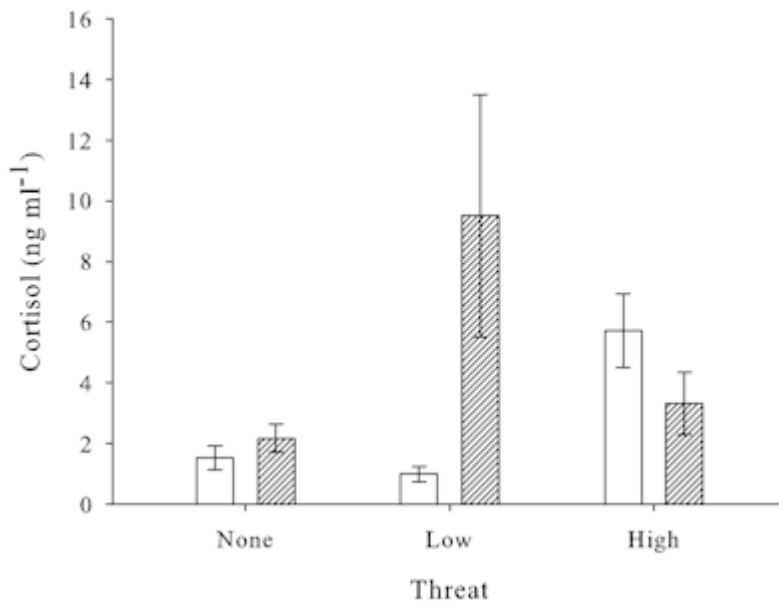
625



626

627 **Figure 3:** Mean (\pm SE) change in weight (%) in rainbow trout, *Oncorhynchus mykiss*, under
 628 no, low or high predation threat and low (white) or high (hatched) feed availability. n for each
 629 treatment indicated below the boxes. Means that do not share a common lowercase letter
 630 were significantly different ($p < 0.05$).

631

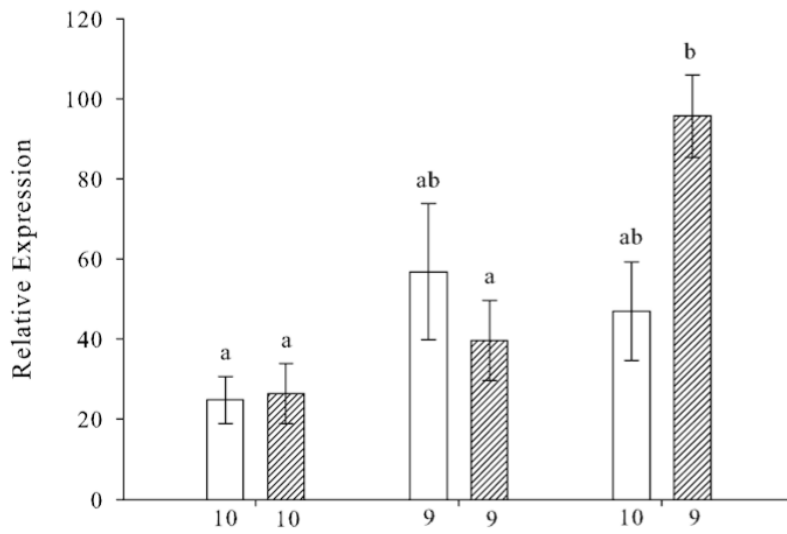


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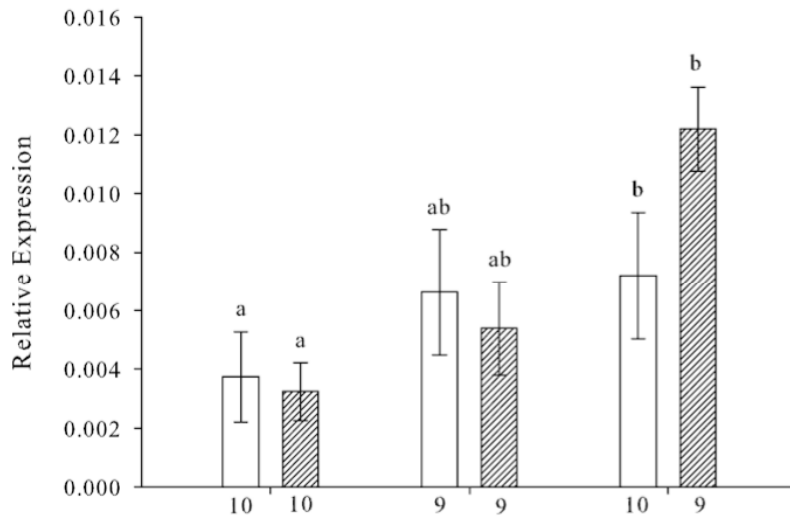
633 **Figure 4:** Mean (\pm SE) plasma cortisol (ng ml^{-1}) in bold (white) and shy (hatched) rainbow
 634 trout, *Oncorhynchus mykiss*, exposed to no, low or high levels of predation threat. n (no, low,
 635 high threat) for bold trout = 10, 9, 10 and for shy trout = 14, 10, 10, with three outlier points
 636 removed.

637

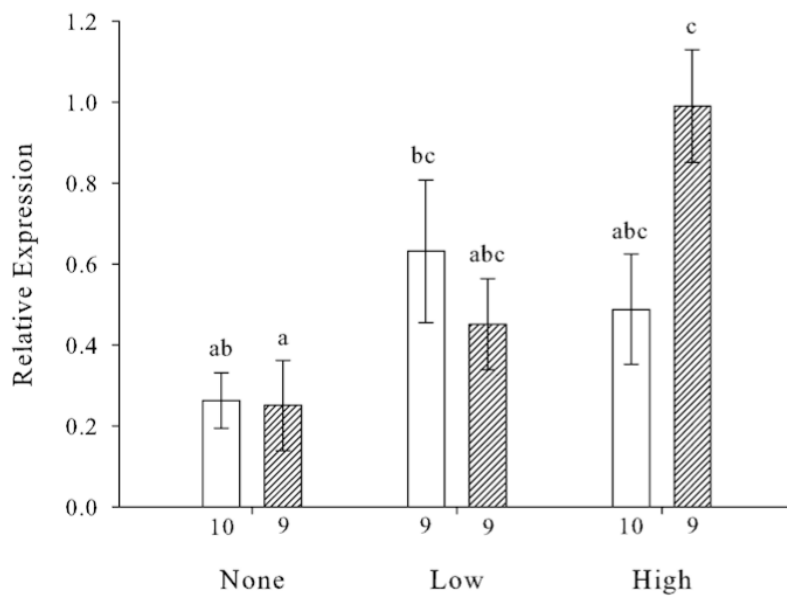
A - Ependymin



B - CRF



C - GABA_A



639 **Figure 5:** Mean (\pm SE) relative expression, $[(E_{\text{Ref}})^{\text{Ct}_{\text{Ref}}}] / [(E_{\text{Target}})^{\text{Ct}_{\text{Target}}}]$ where $E =$
640 efficiency of the reaction, of (A) ependymin, (B) CRF (corticotrophin releasing factor) and
641 (C) GABA_A (γ -aminobutyric acid A) receptor in the brains of bold (white) and shy (hatched)
642 rainbow trout, *Oncorhynchus mykiss* under no, low and high predation threat. Sample sizes
643 are presented beneath each bar. Means that do not share a common lower case letter were
644 significantly different (Tukey HSD, $p < 0.05$).