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1 **Personality, plasticity, and predation: linking endocrine and behavioural**
2 **reaction norms in stickleback fish**

3
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10
11
12 **Summary**

- 13
14 **1.** Predation plays a fundamental role in evolutionary processes, driving changes in prey
15 morphology, physiology, and behaviour. With organisms being increasingly exposed
16 to rapid environmental changes, there is growing interest in understanding individual
17 phenotypic plasticity in responses to changes in predation pressure.
- 18 **2.** Behavioural and physiological responses to predator exposure are of particular
19 interest as differences in predation pressure are often reflected in correlated suites
20 of behavioural and hormonal profiles across populations. Within populations, the
21 association between endocrine profiles and behaviour is less understood, and often
22 lacking.
- 23 **3.** Adopting a reaction norm approach and a repeated measures design, we assessed
24 within-population effects of changes in perceived predation risk on endocrinology

25 and behaviour in three-spined sticklebacks (*Gasterosteus aculeatus*). We repeatedly
26 exposed subjects to a robotic model predator, and assessed their behavioural
27 response. The fish showed consistent behavioural profiles and were less active and
28 shy when predation risk was higher.

29 **4.** Using non-invasive waterborne hormone analysis, we assessed basal cortisol as well
30 as the cortisol response to changes in predation risk. Individuals showed significantly
31 higher cortisol levels following exposure to the model predator. Individual post-
32 predator exposure cortisol was repeatable but unrelated to behavioural responses.
33 Accounting for between versus within subject effects, we found that basal cortisol
34 and shyness were positively related within individuals, i.e. individuals overall were
35 shy on days they had higher cortisol levels. We also tested if basal testosterone
36 predicted risky behaviour and found no evidence for this hypothesis.

37 **5.** No individual differences in hormonal or behavioural responses to changes in
38 predation risk were found, suggesting that individuals are not constrained by their
39 personalities in their ability to cope with a potentially harmful threat.

40 **6.** Overall, we show that individuals of different personalities are equally “flexible” in
41 their response to changes in predation pressure. Our study offers novel insight into
42 consistent individual differences and plasticity in hormones and behaviour as well as
43 their interplay within populations. Future studies should assess the applicability of
44 these findings to other changes in the environment, as well as the effects of social
45 context on endocrine and behavioural reaction norms.

46

47 **Keywords:** Anti-predator response, Coping styles, Cortisol, Individual differences, Shyness,

48 Stress, Testosterone, Waterborne hormone analysis

49

50 **Introduction**

51

52 Predation plays a fundamental role in animal evolutionary processes (Abrams 2000), driving
53 changes in phenotypic traits of prey (and predators). For example, predation risk can induce
54 changes in nest-site selection and clutch size in the Siberian jay (*Perisoreus infaustus*, Eggers
55 et al. 2006), and can cause lizards (*Anolis sagrei*) to alter their habitat use (Losos, Schoener &
56 Spiller 2004). Such adaptive changes can occur through natural selection across generations
57 (Cousyn et al. 2001), or within the lifetime of an individual, with predation risk influencing
58 the expression of heritable variation during development (Dingemanse et al. 2009), or in
59 direct phenotypic responses to recent experience (Magnhagen et al. 2012). With organisms
60 being increasingly exposed to rapid environmental changes such as habitat disruption or
61 ecological invasions that can increase exposure to novel threats (Sih 2013), there is a
62 growing interest in understanding individual phenotypic plasticity in response to change
63 (Nussey, Wilson & Brommer 2007; Dingemanse et al. 2010; Sih et al. 2012; Dingemanse &
64 Wolf 2013; Wingfield 2013).

65 There is vast evidence documenting individual differences in behaviour, which are
66 consistent across time and contexts, i.e. ‘animal personalities’ (or ‘coping styles’ ,
67 ‘behavioural types’, ‘temperament’, ‘behavioural syndromes’ ; e.g. Koolhaas et al. 1999; Sih,
68 Bell & Johnson 2004; Reale et al. 2007; Réale et al. 2010). However, only recently have these
69 consistent individual differences in behaviour been linked to differences in the
70 responsiveness to environmental change, by adopting the concept of “reaction norms”
71 (Nussey, Wilson & Brommer 2007; Dingemanse et al. 2010; Dingemanse & Wolf 2013).
72 Behavioural reaction norms include information on how an individual behaves on average

73 (i.e. its personality or statistically its intercept) and how its behaviour changes over a specific
74 environmental gradient (i.e. its plasticity or statistically its slope) (Nussey, Wilson &
75 Brommer 2007; Dingemanse et al. 2010; Dingemanse & Wolf 2013). Importantly, reaction
76 norms allow one to assess the effects or constraints of personality upon individual responses
77 to changing environments. In tree swallows (*Tachycineta bicolor*), for instance, more
78 aggressive individuals are better able to adjust to variation in temperature (Betini & Norris
79 2012), and in great tits (*Parus major*), personality predicts responsiveness to predation risk
80 (Quinn et al. 2012).

81 On a proximate level, shifts in behavioural traits are often mirrored or mediated by
82 changes in hormone profiles and there is increasing interest in understanding the
83 relationship between physiology and behaviour (McGlothlin & Ketterson 2008; Ketterson,
84 Atwell & McGlothlin 2009; Killen et al. 2013). In general, the endocrine system responds to
85 environmental changes to help the body cope with challenges, i.e. “stressors”, with the
86 release of glucocorticoids (cortisol in most mammals and fish; corticosterone in birds,
87 amphibians, reptiles, and rodents) from the hypothalamic-pituitary-adrenal (HPA) axis (e.g.
88 Wingfield 2013).

89 Stress coping styles, i.e. “a coherent set of behavioral and physiological stress
90 responses which is consistent over time and which is characteristic to a certain group of
91 individuals” (Koolhaas et al. 1999) are often found on a population-level. For instance,
92 populations of the Panamanian bishop (*Brachirhaphis episcopus*) exposed to high predation
93 pressure are more exploratory and active and exhibit lower cortisol release rates in response
94 to a stressor compared to populations exposed to low predation pressure (Archard &
95 Braithwaite 2011; Archard et al. 2012; see Figure 1A). Likewise, guppies (*Poecilia reticulata*)
96 from high-predation sites have lower basal cortisol levels than conspecifics from low-

97 predation sites (Fischer et al. 2014), and rainbow trout (*Oncorhynchus mykiss*) selected for
98 high cortisol responses are less bold and aggressive than those selected for low cortisol
99 responses (Pottinger et al., 1992). Within populations, however, the link between hormones
100 and behaviour is often lacking (Bell et al. 2007; Archard et al. 2012). A potential reason for
101 this is that researchers tend to adopt a single, rather than repeated measures, sampling
102 design (see Figure 1AB). Single measure designs, by definition, do not allow estimation of
103 within (versus between) subject effects. Given the extensive individual variation in the
104 glucocorticoid stress response across vertebrates (Cockrem 2013), repeated measures
105 designs have potentially important implications for understanding the evolution and
106 maintenance of coping styles (Figure 1C).

107 Another endocrine axis, closely linked to the HPA axis, which has been associated
108 with coping styles but has received far less attention is the hypothalamic-pituitary-gonadal
109 (HPG) axis which controls the secretion of sex steroids (androgens, oestrogens, and
110 progestogens). Most research on sex steroids and personality has been on humans,
111 focussing on the positive effect of testosterone on risk-taking and sensation-seeking
112 behaviours in men (reviewed by Caramaschi et al. 2013). In non-human animals, e.g. birds
113 and rodents, testosterone levels have been linked to aggression, coping, and exploration
114 (reviewed by Caramaschi et al., 2013). Although, to date, relatively little is known about HPG
115 functioning and personality traits, the (potential) effect of testosterone on risk-taking may,
116 in addition to glucocorticoids, be an important factor in determining antipredator behaviour.

117 Here, we adopt a reaction norm approach using wild-caught three-spined
118 sticklebacks (*Gasterosteus aculeatus*; Linnaeus, 1758; Figure 2) from the same population,
119 allowing us to investigate potential effects of coping styles on responses to changes in
120 predation risk. We manipulate 'perceived predation risk' (sensu Dingemanse et al. 2010),

121 and repeatedly collect phenotypic data on waterborne hormone levels and behavioural
122 measures from the same individuals. Crucially, the use of a non-invasive sampling method
123 (see methods) allows the same subjects to be sampled repeatedly, which is necessary for
124 assessing individual variation in the glucocorticoid stress response (see Cockrem 2013), and
125 within versus between subjects effects of hormones on behaviour (see Figure 1B,C).
126 Specifically, we aimed: (1) to assess individual consistency and repeatability in behavioural
127 and hormonal responses (i.e. personality), (2) to assess the plasticity, and potential
128 constraints of personality, on the behavioural and glucocorticoid stress response to changes
129 in perceived predation risk, and (3) to test whether hormonal and behavioural responses are
130 linked (across and/or within subjects), i.e. whether cortisol and/or testosterone levels
131 predict phenotypic variation in risky behaviour.

132 Sticklebacks are a major model in behavioural ecology (Huntingford & Ruiz-Gomez
133 2009) and are ideally suited for studying endocrine and behavioural responses to predators
134 in the context described above. They exhibit consistent inter-individual variation in
135 behavioural responses to predation (reviewed by Huntingford & Coyle 2007), and predation
136 pressure generates different behavioural (Dingemanse et al. 2007), morphological
137 (Reimchen 1994), and physiological (Bell, Henderson & Huntingford 2010) profiles. Also,
138 repeated exposure to predator cues leads to significant transcriptomic changes in the brain
139 (Sanogo et al. 2011). Furthermore, sticklebacks show increased glucocorticoid
140 concentrations in response to both acute and chronic stressors (Pottinger, Carrick &
141 Yeomans 2002; Bell et al. 2007; Fürtbauer, King & Heistermann, revision under review).

142

143

144

145 **Materials and Methods**

146

147 STUDY ANIMALS

148

149 Three-spined sticklebacks are known to display both sex-differences in personality (King et
150 al. 2013) and stress-induced cortisol responses (Pottinger et al. 2013). Therefore, this study
151 focussed on non-gravid females ($n=20$; mean \pm standard deviation (sd), body mass and length
152 1.5 ± 0.3 g and 5.3 ± 0.3 cm), wild-caught on Swansea University Campus (Figure 2). Subjects
153 were initially housed in a large holding tank (30x39x122 cm), containing gravel substrate,
154 plants, and drift wood. Fish were kept at a constant temperature/photoperiod regime
155 ($17^{\circ}\text{C}/8\text{L}:16\text{D}$). Two weeks prior to behavioural tests, the subjects were transferred to
156 individual 2.8l gravel-lined aerated tanks in which they were housed throughout the entire
157 test period. Fish were fed once daily between 0830 and 0900 h with defrosted bloodworms.
158 All procedures described were approved by Swansea University's Ethics Committee (IP-1213-
159 3).

160

161 HORMONE SAMPLE COLLECTION, EXTRACTION, AND ANALYSIS

162

163 In fish, exposure to a stressor leads to the activation of the hypothalamic-pituitary-interrenal
164 (HPI) axis, resulting in increased cortisol secretion within minutes (reviewed by Pankhurst
165 2011; Cockrem 2013). Free cortisol (and other steroid hormones) in fish diffuses from the
166 bloodstream into the water through the gills (Vermeirssen & Scott 1996; Ellis, James & Scott
167 2005). Confining a fish to a known volume of water for a known period of time allows to
168 extract these steroids and quantify their concentrations (Hirschenhauser et al. 2004; Ellis,

169 James & Scott 2005; Scott & Ellis 2007; Sebire, Katsiadaki & Scott 2007; Wong et al. 2008;
170 Sebire, Katsiadaki & Scott 2009; Kidd, Kidd & Hofmann 2010; Archard et al. 2012; Fischer et
171 al. 2014; Fürtbauer, King & Heistermann, revision under review) which correlate with
172 concentrations of the free fraction of hormones in the blood (Scott & Ellis 2007; Sebire,
173 Katsiadaki & Scott 2007). This method of measuring waterborne hormones in teleost fish is
174 extremely practical with smaller fish since it negates the need for sacrificing the fish to
175 obtain blood samples for hormone measurements (reviewed by Scott & Ellis 2007).

176 A total of $n=286$ (mean \pm sd: 14.3 ± 2.2 per female, $n=20$ females; two individuals died
177 during the study) waterborne hormone samples were collected in order to assess (i)
178 habituation effects across five consecutive days (Wong et al. 2008; Fischer et al. 2014) prior
179 to behavioural and hormone data collection, (ii) basal cortisol and testosterone levels (i.e.
180 before the predation risk phase) and (iii) post-predator exposure cortisol concentrations
181 (post-predator exposure testosterone was not measured as individuals were tested in a non-
182 social context, see e.g. Oliveira et al. 2002). Subjects were weighed prior to each hormone
183 sample collection. Following previously described procedures for sticklebacks (Sebire,
184 Katsiadaki & Scott 2007; Sebire, Katsiadaki & Scott 2009, Fürtbauer, King & Heistermann,
185 revision under review), fish were confined individually for 1 h in a 150 ml glass beaker
186 (rinsed with 99.9% methanol and distilled water prior to use), filled with 50 ml water (same
187 source as used for tanks). To remove particulate matter, water samples were filtered
188 through a net rinsed with distilled water, and transferred into 60 ml polypropylene tubes,
189 and stored at -18°C until further processing.

190 For hormone extraction, samples were thawed and loaded onto Waters Sep-Pak Plus
191 C18 solid phase extraction cartridges placed onto a 12-port vacuum manifold connected to a
192 vacuum pump. Prior to sample loading, cartridges were primed with 5 ml methanol followed

193 by 5 ml distilled water. After the samples had been passed through, the cartridges were
194 washed with 5 ml distilled water, followed by 20 ml air to remove water. Steroids were
195 eluted with 5 ml absolute methanol, collected in a glass tube and evaporated under nitrogen
196 at 45°C. The dried extracts were sent to the Endocrinology Laboratory of the German
197 Primate Center, Göttingen, Germany.

198 Steroids were redissolved in 350 µl assay buffer, and analysed for immunoreactive
199 cortisol and testosterone using enzyme immunoassays (Palme & Möstl 1994, 1997). All
200 samples were run in duplicate, and samples with a CV above 7% between duplicates were
201 re-measured. Sensitivity of the both assays at 90% binding was 0.5 pg. Intra- and inter-assay
202 coefficients of variation, calculated from replicate determinations of high- and low- value
203 quality controls were 7.4% (n=16) and 11.0% (n=20) (high) and 8.9% (n=16) and 15.2% (n=20)
204 (low) for cortisol, and 6.6% (n=16) and 7.4% (n=16) (high) and 8.9% (n=16) and 14.3% (n=16)
205 (low) for testosterone. Hormone data are expressed as ng/g/h.

206

207 BEHAVIOURAL TESTS AND TRACKING

208

209 Throughout five consecutive weeks, behavioural tests were conducted Mon-Thu. Five fish
210 were tested per day, and each individual was tested each week on the same weekday but at
211 a different time. Behavioural tests were conducted in transparent plastic “test tanks”
212 (WxLxH: 15x54x24), filled up with water to 12.5 cm. A green plastic plant was positioned on
213 one end to provide cover (Figure 3). In order to prevent potential cues from affecting the
214 response of subsequently tested fish, five separate test tanks were used. The test tank was
215 positioned in a rectangular test arena, covered with white sheets on all sides. Behaviours
216 were filmed using a Panasonic HDC-SD60 high definition video camera, mounted above the

217 test tank (Figure 3). Following hormone sample collection to assess cortisol and
218 testosterone baseline concentrations, the subject was placed in the test tank for 20 minutes
219 (“low predation risk phase”). Subsequently, we simulated an aerial predator attack using a
220 polymer clay model heron head which was attached to a Robot Arm with USB PC Interface
221 (Figure 3). The ‘robotic heron’ was hidden behind a curtain before the simulated attack.
222 After exposure to the model predator, the behaviour of the fish was recorded for 20 minutes
223 (“high predation risk phase”). After completion of the tests, water hormone samples were
224 collected as described above, and subjects were returned to their individual tanks.

225 Automated video tracking was performed using EthoVision XT 9 software (Noldus
226 Information Technology Inc.; Noldus, Spink & Tegelenbosch 2001). The detection settings for
227 each video were set to differencing and a sample rate of 5 frames per second. Six
228 behavioural variables were extracted for both low and high predation risk phases: total
229 distance moved [cm], mean speed [cm/s], time spent in cover [s], and transitions out of
230 cover (i.e. the frequency of movements out of cover into the open area of the tank), and two
231 measures of mobility: “immobility” and “high mobility”. These were calculated based on
232 comparisons of the locations of the tracked fish between consecutive images: where there is
233 a perfect overlap of two consecutive images (i.e. the fish is still), there is a 0% change; where
234 there is no overlap (i.e. fish has moved to a new location) there is a 100% change. Immobility
235 (or “freezing”) was the duration of time [s] for which the tracked fish image change was
236 <10%, and high mobility was the duration of time [s] when the velocity of fish movement
237 was >80%.

238

239 DATA ANALYSIS

240

241 In order to summarise the six behavioural variables (see above), we used Principal
242 component analysis (PCA) in SPSS Statistics 17.0. We used Linear Mixed Models (LMMs) in R
243 (R Development Core Team; package lme4, Bates & Maechler, 2010) to analyse behavioural
244 and hormonal patterns across low- and high predation risk phases. Hormone data were log-
245 transformed to achieve normality. To assess whether environmental change (E; i.e.
246 differences in perceived predation risk; fixed effect), the individual (I; random intercept) or
247 the interaction between individual and changes in predation risk (I x E; ID as random
248 intercept; predation risk as random slope) affected cortisol levels and behaviours, we used a
249 reaction norm approach (Nussey, Wilson & Brommer 2007; Dingemanse et al. 2010) and
250 performed three sets of simple model comparisons (for cortisol, activity, and shyness) using
251 log-likelihood ratio tests following Carter, Goldizen & Heinsohn (2012). In each model, we
252 included “day” (i.e. the date) as a random effect to control for temporal effects. To
253 investigate whether cortisol and testosterone concentrations predicted behaviour, and to
254 account for between versus within subject effects, we included daily hormone levels per
255 subject (centred to a mean of zero per subject) as well as the mean hormone levels per
256 subject as fixed effects (van de Pol & Wright 2009). Repeatability of hormones and
257 behaviour was assessed by calculating intraclass correlation coefficients (ICCs) and 95%
258 confidence intervals (CIs) (Lessels & Boag 1987) in SPSS Statistics 17.0.

259

260 **Results**

261

262 INDIVIDUAL CONSISTENCY AND REPEATABILITY IN BEHAVIOURAL AND HORMONAL
263 MEASURES

264

265 Principal Component analysis of the six behavioural variables revealed two Principal
266 Components (PCs), explaining 75% of the total variance (Table 1). Distance, high mobility,
267 speed, and transitions out of cover positively loaded on PC1 (hereafter referred to as
268 “activity”), and immobility and time spent in cover positively loaded on PC2 (hereafter
269 referred to as “shyness”). Activity was repeatable during both low and high perceived
270 predation risk across weeks (low risk: ICC=0.26, p=0.001; high risk: ICC=0.36, p<0.001, Table
271 2). Average activity during low and high predation risk phases were significantly positively
272 correlated (Pearson’s rho=0.641, p=0.002, n=20; Figure 4A). Shyness was not repeatable
273 during low predation risk phase, and a non-significant trend was found for the high
274 predation risk phase (low: ICC=0.08, p=0.157; high: ICC=0.11, p=0.076, Table 2). Average
275 shyness during low and high predation risk phases were significantly positively correlated
276 (Pearson’s rho=0.451, p=0.046, n=20; Figure 4B).

277 Cortisol concentrations varied significantly across the five habituation days (Friedman
278 test: $\chi^2=29.32$, df=4, p<0.001, n=20) and decreased significantly from day 1 to day 3 after
279 which they remained stable (Wilcoxon signed ranks test: day 1 to day 3: Z= -2.389, p=0.017,
280 n=20; Figure 5). Post-predator exposure cortisol concentrations were repeatable (ICC=0.34,
281 p<0.001; Table 3) and a non-significant trend was found for basal cortisol (ICC=0.11,
282 p=0.087; Table 3). Cortisol levels before and after exposure to the model predator were not
283 correlated (Spearman’s rho=0.179 p=0.450, n=20). No significant difference was found in
284 testosterone concentrations across the five habituation days (Friedman test: $\chi^2=2.08$, df=4,
285 p=0.721, n=20; Figure 4), and testosterone was not repeatable between weeks (ICC=-0.01,
286 p=0.515; Table 2).

287

288 BEHAVIOURAL AND GLUCOCORTISCOID STRESS RESPONSES TO PERCEIVED PREDATION RISK

289

290 Both activity and shyness altered in response to the change in perceived predation risk (E;
291 Table 3). Individuals showed significantly lower activity during the high- compared to low
292 predation risk phase (estimate±standard error (se)=-0.43±0.12, t=-3.62, p=0.003; Figure 6A).
293 Shyness was higher during the high- compared to the low predation risk phase
294 (estimate±se=0.40±0.12, t=2.91, p=0.004; Figure 6B). Individual had a significant effect on
295 activity but not shyness (I, Table 3). Neither activity nor shyness showed a significant E x I
296 interaction (Table 3), indicating no individual differences in the behavioural response to
297 changes in predation risk.

298 Comparing basal and post-predator exposure cortisol revealed that cortisol was
299 significantly higher after exposure to the simulated aerial predator (estimate±se=0.62±0.10,
300 t=6.26, p<0.001; Figure 6C, Table 3). Individual had a significant effect on cortisol levels but
301 no E x I interaction was found (Table 3), indicating no individual differences in HPI axis
302 sensitivity to perceived predation.

303

304 THE LINK BETWEEN HORMONAL AND BEHAVIOURAL RESPONSES TO PERCEIVED PREDATION 305 RISK

306

307 Neither basal nor post-predator exposure cortisol predicted activity (p>0.05; Table 4). Post-
308 predator exposure cortisol did not predict shyness (p>0.05; Table 4). No relationship of basal
309 cortisol on shyness was found across subjects (p=0.816; Table 4) but there was a significant
310 within-subjects effect (p=0.038; Table 4). A post-hoc investigation into immobility and time
311 spent in cover (see Table 1), revealed a significant within-subject effect of basal cortisol on
312 the time spent in cover (estimate±se=50.35±23.01, t=2.19, p=0.035) but not immobility

313 (estimate±se=38.98±26.96, t=1.45, p=0.160), i.e. individuals, across low- and high-predation
314 risk phases, spent more time in cover on days where they had higher basal cortisol levels.
315 Testosterone neither predicted activity or shyness (p>0.05; Table 4).

316

317 **Discussion**

318

319 How animals respond to changes in their environment and whether certain individuals are
320 better able to cope with challenges are crucial questions given the rapid environmental
321 changes caused by human activity. Our repeated measures sampling design allowed us to (1)
322 investigate behavioural and endocrine personalities and (2) their potential impact on
323 phenotypic plasticity in response to changes in perceived predation risk, and (3) to account
324 for within- versus between-subject effects of hormones on behaviour (see Figure 1B,C).

325 First, we found individual behaviours and hormone levels to be consistent and
326 repeatable, indicative of personality. In the case of individual differences in behaviour, we
327 found that both “activity” and “shyness” were significantly correlated across low and high
328 predation risk phases, demonstrating behavioural consistency within our test treatments.
329 Interestingly, we only found significant individual differences and repeatability in activity and
330 not shyness. This suggests that our measure of shyness does not represent an individual trait
331 (see e.g. Carter, Goldizen & Heinsohn 2012). However, the two variables that loaded onto
332 PCA axis of shyness ‘immobility’ and ‘time spent in cover’ were affected by ‘individual’, and
333 thus, can be considered personality traits (data not shown). Measuring multiple behaviours
334 can be important, since some may be representative of individual traits whilst others may
335 not (Carter, Goldizen & Heinsohn 2012; Carter et al. 2013; Weiss & Adams 2013). Our study,

336 further suggests that caution is required when applying Principal Component Analysis to
337 combine correlated behavioural traits.

338 Regarding individual differences in HPI axis activity, we found that post-predator
339 exposure cortisol concentrations were repeatable, indicating a strong individual consistency
340 in the physiological stress response, i.e. the sensitivity of the HPI axis, to perceived predation
341 risk ('endocrine personalities'). In contrast, we only found a trend for repeatability in basal
342 cortisol. Across vertebrates, most of our knowledge on repeatabilities of glucocorticoid
343 responses comes from studies on birds which generally show high repeatabilities (reviewed
344 by Cockrem 2013). Comparable data on individual differences in the glucocorticoid stress
345 response for fish are scarce (reviewed by Cockrem 2013); nevertheless, similar
346 repeatabilities have been reported for bluegill sunfish (*Lepomis macrochirus*: Cook et al.
347 2012) and, on a population level, for rainbow trout and the Panamanian bishop (Pottinger,
348 Pickering & Hurley 1992; Archard et al. 2012).

349 Second, we were interested if and how subjects respond to changes in perceived
350 predation risk, both behaviourally and hormonally, and whether different personalities (see
351 above) respond differently. Both activity and shyness were affected by changes in predation
352 risk, i.e. individuals were significantly less active and shyer, and also had significantly higher
353 cortisol levels after, compared to before, exposure to the model predator. Since individuals
354 habituated to the hormone sampling method within three days (for similar findings see
355 Wong et al. 2008; Fischer et al. 2014), we are confident that confinement, which itself is a
356 stressor (e.g. Cockrem 2013, this study), did not - or only marginally - affected the 'true'
357 physiological state/response of our subjects. Despite the individual differences in activity
358 and cortisol levels (see above), we found no evidence for individuals varying in the level of
359 endocrine and behavioural plasticity with respect to predation risk (E x I interaction),

360 suggesting that individuals are not constrained by their personality in their response to
361 changes in their environment. Having said this, sticklebacks, as numerous other animals, are
362 not solitary – they are social and respond to the movements of others (e.g. Harcourt et al.,
363 2009). Sociality, in fact, can have moderating effects upon personality (e.g. King et al. in
364 press; for review see Webster & Ward 2011) as well as the glucocorticoid stress response
365 (e.g. Fürtbauer et al. 2014). Also, phenotypic plasticity could be context-dependant, making
366 it entirely possible that individuals differ in their response to other (non-predator) stimuli, or
367 when trade-offs are involved (see e.g. Quinn et al., 2012).

368 Our third and final aim was to investigate whether cortisol predicted behaviour
369 across low and high predation phases. Neither basal cortisol nor post-predator exposure
370 cortisol predicted activity and we found no link between post-predator exposure cortisol and
371 shyness. Conversely, basal cortisol levels predicted shyness; however only within, but not
372 across, individuals (see Figure 1B,C). Together, these findings have several potentially
373 important implications for past and future studies: (1) it is possible that a relationship
374 between hormones and behaviour (here, cortisol and shyness) is present within, but not
375 across, individuals (Figure 1). Such a relationship is only quantifiable by measuring cortisol
376 and behaviour repeatedly in the same individuals (Figure 1C), rather than just once, or taking
377 the average of multiple measures (Figure 1B). Notably, the existence of a within-subject
378 effect of cortisol on shyness within a population may shed new light on coping styles found
379 across populations (see e.g. Archard & Braithwaite 2011; Archard et al. 2012; Figure 1A). (2)
380 Some behavioural variables may be linked to cortisol levels whereas others are not, e.g. in
381 our study, basal cortisol was linked to the time spent in cover which essentially is the
382 amount of time the subjects were ‘hiding’ and, in our opinion, probably the most meaningful
383 measure of ‘boldness’ (c.f. Carter et al. 2013). (3) There may be an important distinction in

384 the relationship between basal versus post- predator exposure cortisol and behaviour, i.e.
385 basal versus reactive cortisol levels. Single measures studies usually measure post-predator
386 exposure cortisol (e.g. Pottinger, Carrick & Yeomans 2002; Bell et al. 2007) which, in our
387 study, was unrelated to behaviour. Clearly, more careful consideration of study and sampling
388 designs is needed when linking endocrine and behavioural profiles in an animal personality
389 framework.

390 In addition, we tested whether testosterone, a sex steroid hormone which has been
391 linked to risk-taking and sensation-seeking behaviours (reviewed by Caramaschi et al. 2013),
392 affects antipredator behaviour in our female sticklebacks. We found no evidence in support
393 of this hypothesis; however, it remains to be investigated whether testosterone is a marker
394 of male personalities. Generally, sex differences in testosterone (a “male hormone”)
395 concentrations may, at least partly, be the reason why testosterone has been devoted more
396 attention with respect to phenotypic variation in males, compared to females (Ketterson &
397 Nolan 2005; Ketterson, Atwell & McGlothlin 2009). Testosterone is an interesting hormone
398 regarding its presence/function in fish. Usually, in teleost fish, 11-keto testosterone is the
399 most important androgen in males in terms of secondary sexual characters,
400 spermatogenesis, and reproductive behaviour (Borg 1994), and can only be found in very
401 low concentrations or is absent in females. Testosterone, in contrast, is ubiquitous in males
402 and females (Borg 1994; Sebire, Katsiadaki & Scott 2007), and when breeding, females show
403 increased plasma testosterone levels compared to males (Borg & Mayer 1995), and are
404 bolder towards a predator than non-breeding females (Frommen, Mehlis, & Bakker, 2009).
405 These findings, despite the non-significant result in our study, provide an interesting basis
406 for future research into the link between testosterone and risky behaviour in different, e.g.
407 reproductive, contexts.

408 In summary, our study has revealed consistent individual differences in behaviour
409 and cortisol levels (i.e. behavioural and physiological personalities), plasticity in hormonal
410 and behavioural responses to changes in predation risk, and a relationship between
411 behaviour and basal cortisol within, but not across, individuals. However, we found no
412 individual differences in behavioural and endocrine plasticity, indicating that individuals are
413 not constrained by their personalities, and thus, are equally capable to respond to a
414 potentially harmful threat. Future research should focus on assessing the effects and
415 constraints that conspecifics may impose on personality (see Webster & Ward 2011) but also
416 phenotypic plasticity in the response to environmental change.

417

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419

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425

426 **Data accessibility**

427 All data analysed in this study are available online in the supporting information.

428

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614 **Supporting Information**

615

616 Additional supporting information may be found in the online version of this article.

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618 Table S1 Data analysed in this study

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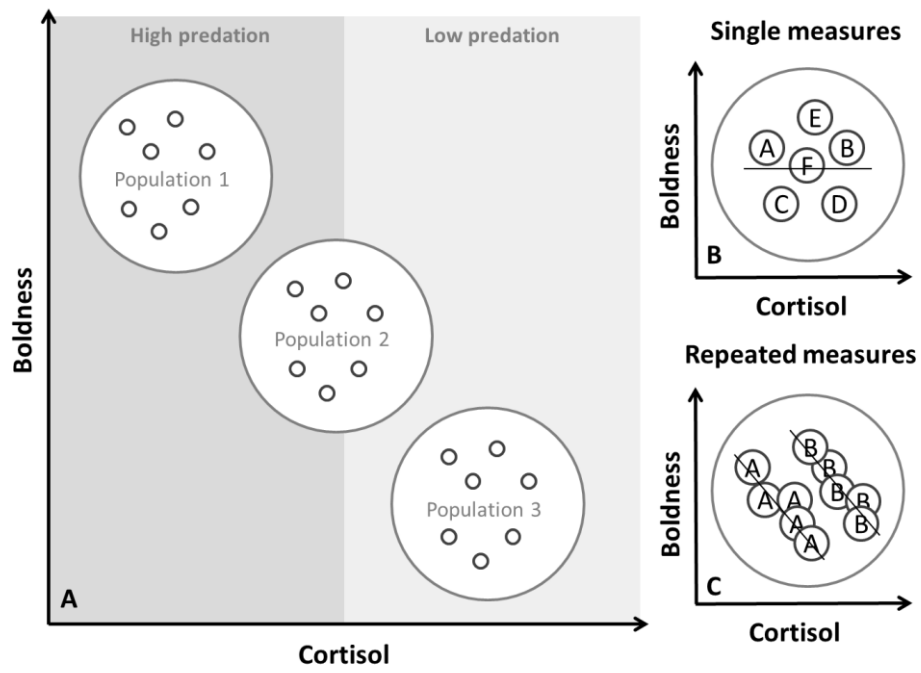
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640 **Figures**

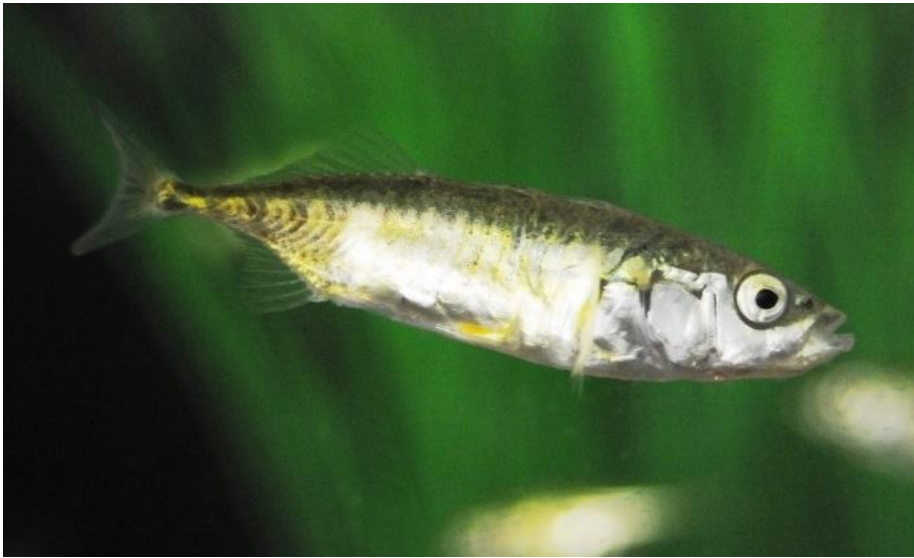
641 **Figure 1:**



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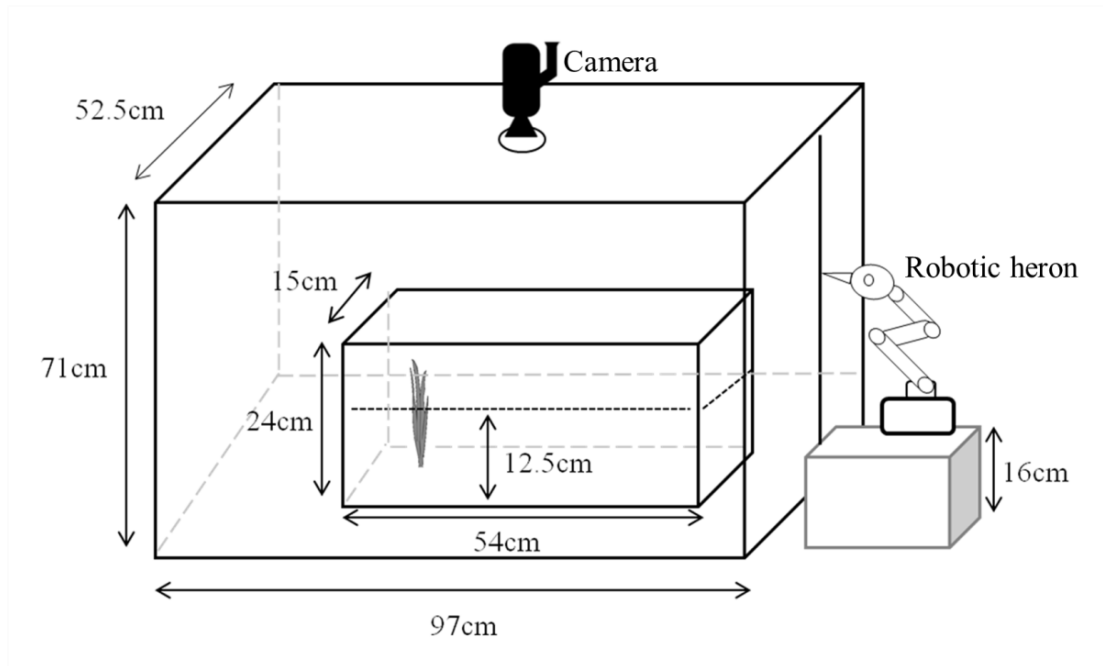
644 Figure 2:



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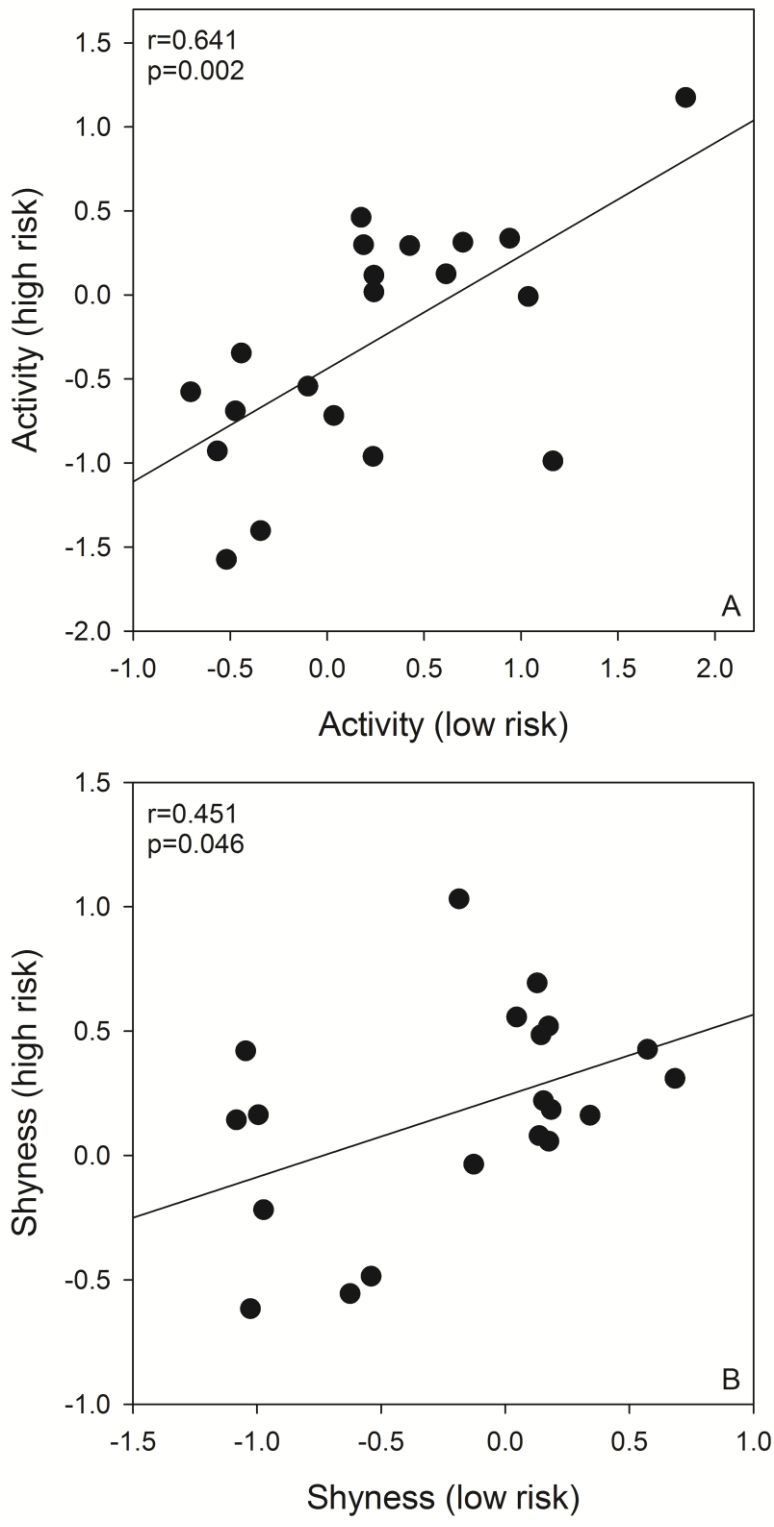
647 Figure 3:



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650 Figure 4:

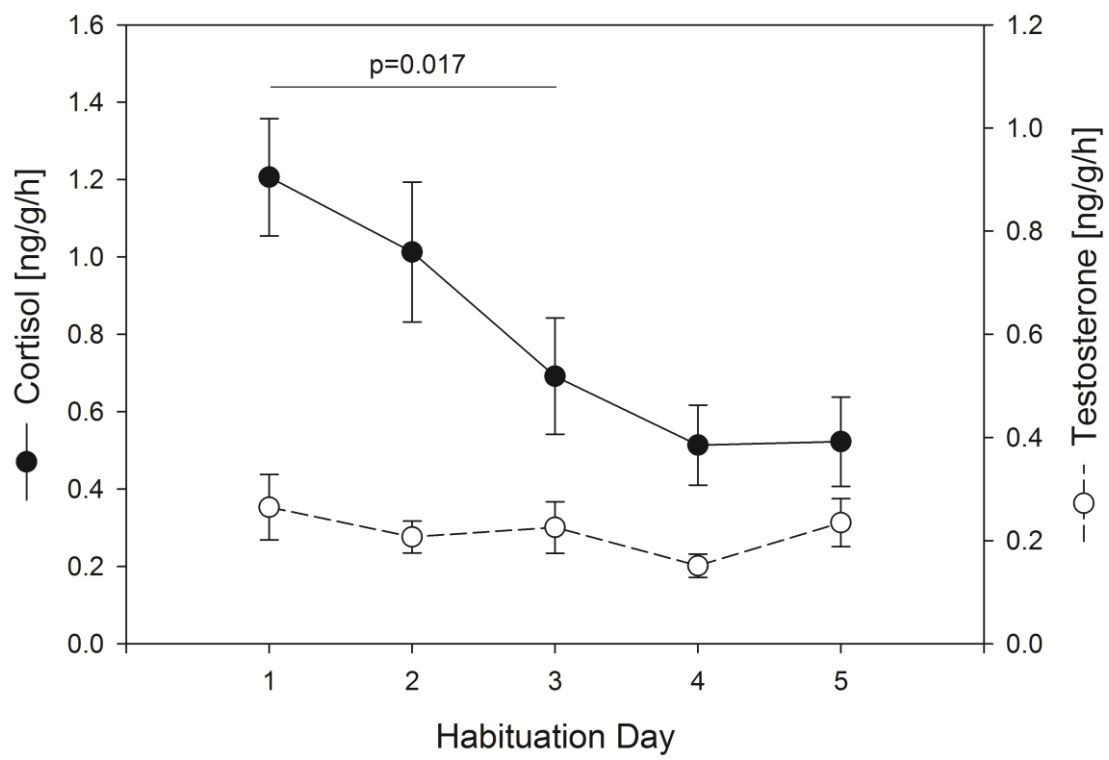


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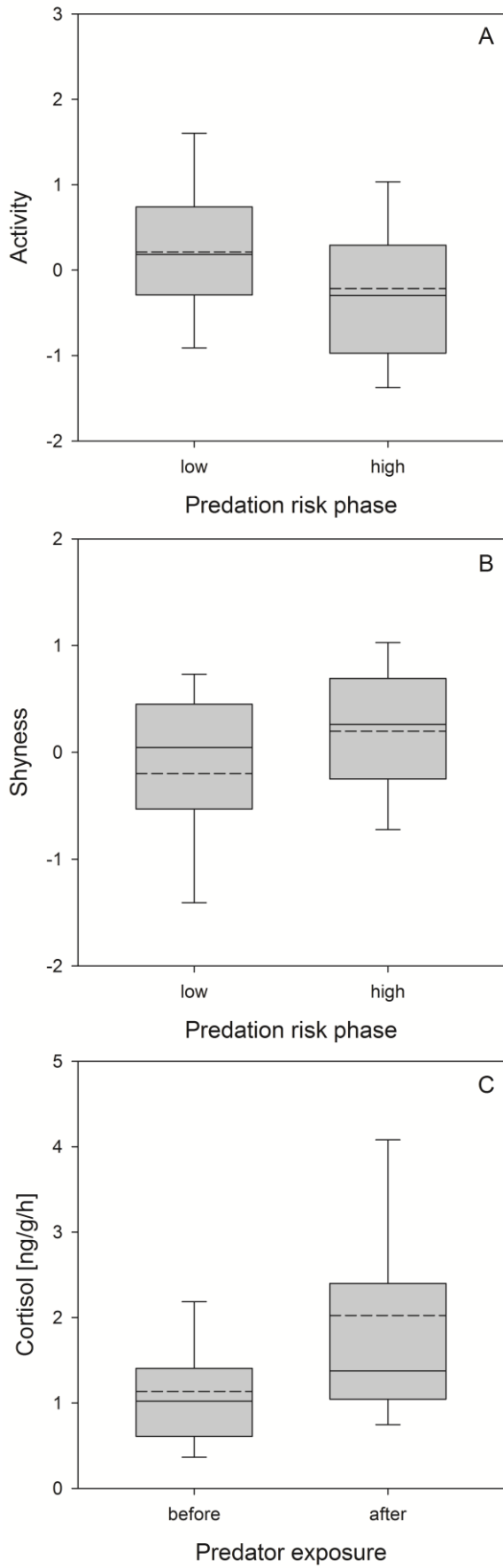
654 Figure 5:



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657 Figure 6:



659 **Figure Legends**

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661 Figure 1: Schematic representations of potential relationships between cortisol levels and
662 boldness. (A) Across populations, those populations exposed to high predation pressure are
663 often bolder and exhibit lower cortisol levels (Population 1) compared to populations
664 exposed to low predation pressure (Population 3), representative of 'stress coping styles',
665 i.e. consistent sets of behavioural and physiological stress responses. (B) Within populations,
666 studies are usually based on single measurements per subject (A-F), and a relationship
667 between cortisol and boldness is often lacking. (C) A repeated measures design, i.e. multiple
668 measurements per subject (A, B) may reveal a within-subject correlation between cortisol
669 and boldness.

670

671 Figure 2: Adult female *Gasterosteus aculeatus* in tank © the authors.

672

673 Figure 3: Test setup. Test tank and arena, containing a green plastic plant to provide cover.
674 The 'robotic heron', made of polymer clay and attached to robotic arm with a USB PC
675 interface, was hidden behind a curtain before the simulated attack. The behaviour of the fish
676 during low and high predation risk phases was filmed using a Panasonic HDC-SD60 high
677 definition video camera, mounted above the test tank.

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679 Figure 4. Correlation in behaviour before and after predator exposure. Plots show significant
680 and positive correlation between mean average (A) activity and (B) shyness during low and
681 high predation risk phases In female sticklebacks (n=20). Values were averaged across 5
682 weeks.

683

684 Figure 5: Habituation to beaker confinement. Waterborne cortisol (solid line, filled circles)
685 and testosterone (dashed line, open circles) concentrations (mean±SE) during five
686 consecutive beaker habituation days in female three-spined stickleback fish (n=20). Cortisol
687 concentrations decreased significantly from day 1 to day 3 ($p=0.017$), whereas testosterone
688 concentrations remained constant ($p=0.721$).

689

690 Figure 6: Population-level effect of changes in perceived predation risk. Data are shown for
691 the (A) activity and (B) shyness of n=20 female three-spined sticklebacks during a low and
692 high predation risk phase, and the same fishes' (C) cortisol concentrations before and after
693 exposure to a model predator (total n=186). The boxes indicate means (dashed line),
694 medians (solid line) and upper and lower quartiles. The whiskers indicate the 90th and 10th
695 percentiles.

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708 Table 1: Loadings of the six behavioural variables extracted from video on the Principal
 709 Components (PCs) "activity" and "shyness", Eigenvalue, and percent variance explained.

710

Behavioural parameter	PC 1 "Activity"	PC 2 "Shyness"
Distance	.846	-.247
Time spent in cover	-.028	.896
Immobility	-.594	.682
High mobility	.731	-.356
Speed	.347	-.735
Transitions out of cover	.893	-.055
Eigenvalue	3.5	1
% variance explained	58.2	17.2

711

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713 Table 2: Individual repeatability of hormones and behaviour in female sticklebacks (n=20)
 714 across five weeks.

715

Variable	ICC	95% CI		F_{17,72}	P
Cortisol before	0.11	-0.04	0.36	1.60	0.087
Cortisol after	0.34	0.14	0.60	3.52	<0.001
Testosterone	-0.01	-0.12	0.20	0.96	0.515
Activity low risk	0.26	0.13	0.60	3.50	0.001
Activity high risk	0.36	0.16	0.62	3.87	<0.001
Shyness low risk	0.08	-0.06	0.32	1.41	0.157
Shyness high risk	0.11	-0.04	0.36	1.64	0.076

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723 Table 3: Comparisons of models with different fixed and random effects using log-likelihood
 724 ratio tests (n=186 observations). "Date" was included as random effect in all models.

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Comparison	Model	Models compared	Log-likelihood	df	χ^2	p
Activity						
E	M1: predator as fixed effect	M1 vs M2	-259.05	4	8.75	0.003
	M2: without predator		-263.42	3		
I	M3: ID as random intercept	M3 vs M4	-239.75	5	38.59	<0.001
	M4: without ID		-259.05	4		
I x E	M5: ID as random intercept; predator as random slope	M5 vs M6	-239.74	7	0.03	0.987
	M6: without predator as random slope		-239.75	5		
Shyness						
E	M1: predator as fixed effect	M1 vs M2	-256.90	4	8.27	0.004
	M2: without predator		-261.03	3		
I	M3: ID as random intercept	M3 vs M4	-256.31	5	1.17	0.279
	M4: without ID		-256.90	4		
I x E	M5: ID as random intercept; predator as random slope	M5 vs M6	-255.55	7	1.52	0.468
	M6: without predator as random slope		-256.31	5		
Cortisol						
E	M1: predator as fixed effect	M1 vs M2	-204.91	4	31.32	<0.001
	M2: without predator		-220.57	3		
I	M3: ID as random intercept	M3 vs M4	-201.08	5	7.66	0.006
	M4: without ID		-204.91	4		
I x E	M5: ID as random intercept; predator as random slope	M5 vs M6	-199.47	7	3.21	0.201
	M6: without predator as random slope		-201.08	5		

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735 Table 4: Results from LMMs testing the effects of endocrine variables on activity and shyness
736 in female three-spined sticklebacks (n=20). "Predation risk" (low/high) was included as fixed
737 effect, and "ID" and "Date" were included as random effects in all models. To account for
738 between versus within subject effects of hormones on behaviour, daily hormone levels per
739 subject as well as the mean hormone levels per subject were included (see text for details).
740

Response variable	Predictor variable	estimate±se	t value	p-value
<i>Activity</i>	mean testosterone	-0.17±1.71	-0.10	0.921
	within-subject testosterone	0.13±0.37	0.34	0.735
	mean basal cortisol	-0.14±0.35	-0.40	0.693
	within-subject basal cortisol	-0.03±0.11	-0.24	0.820
	mean post-predator exposure cortisol	0.02±0.12	-0.13	0.901
	within-subject post-predator exposure cortisol	0.02±0.10	0.38	0.711
<i>Shyness</i>	mean testosterone	0.83±1.15	0.72	0.478
	within-subject testosterone	-0.04±0.49	-0.09	0.932
	mean basal cortisol	-0.06±0.25	-0.24	0.816
	within-subject basal cortisol	0.23±0.11	2.10	0.038
	mean post-predator exposure cortisol	0.01±0.09	0.08	0.937
	within-subject post-predator exposure cortisol	0.09±0.10	1.29	0.242

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