

# 1 Genetic integration of the stress response

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21

## 22 **Abstract**

23 The stress response is a product of selection for an integrated suite of behavioural and  
24 physiological traits that facilitate coping with acute stressors. As such, genetic variation  
25 in the stress response is expected to reflect genetic variation in, and genetic covariation  
26 among, its behavioural and physiological components. Such genetic integration among  
27 stress response components has yet to be formally demonstrated using multivariate  
28 quantitative genetics, despite its profound implications for optimising human and  
29 animal health and understanding the responses of wild populations to natural and  
30 anthropogenic stressors. Here we use a laboratory population of wild-derived  
31 Trinidadian guppies (*Poecilia reticulata*) to determine levels of genetic variation in  
32 behavioural and physiological components of the acute stress response, and to establish  
33 whether such variation is integrated into a single major axis of genetic (co)variation.  
34 First, using a novel method to characterise behavioural components of the stress  
35 response from a widely used Open Field Trial paradigm, we find genetic variation in,  
36 and genetic covariation among, behavioural parameters that characterise movement  
37 patterns under stress. Second, we find a strong genetic component to variation in both  
38 the endocrine response to a confinement stressor and the rate at which this response  
39 attenuates following repeated exposures to the stressor. Finally, we show that these  
40 behavioural and physiological components of the stress response align on a major axis  
41 of genetic (co)variation as predicted, suggesting correlational selection in the past has  
42 led to genetic integration. This genetic integration could either facilitate or constrain  
43 future responses to selection, depending upon the extent to which the direction of  
44 selection aligns with this major axis of genetic covariation among stress response traits.  
45 This genetic integration also suggests that, while stress-related disease typically arises  
46 from physiological stress responses, selection on the genetically correlated behavioural  
47 responses could offer a viable non-invasive route to the genetic improvement of health  
48 and welfare in captive animal populations.

## 49 **Introduction**

50           Stress responses are comprised of physiological and behavioural traits that  
51 enable individuals to cope with adverse environmental conditions (Romero 2004; Øverli  
52 *et al.* 2007; McEwen & Wingfield 2010). Although exposure to such stressors tends to  
53 negatively impact organismal performance, populations harbour considerable variation  
54 in stress response traits (Korte *et al.* 2005; Koolhaas *et al.* 2007, 2010), such that some  
55 individuals may be better at coping with adversity than others. This variation has  
56 immediate implications for understanding and predicting susceptibility to stress-related  
57 disease in humans and animals (Barton & Iwama 1991; Koolhaas *et al.* 1999; McEwen &  
58 Wingfield 2003; Romero 2004; Koolhaas 2008), and for improving animal welfare (e.g.,  
59 in livestock production; Broom & Johnson 1993; von Borell 1995; Möstl & Palme 2002).  
60 Among-individual differences in stress responses are likely underpinned by genetic  
61 variation (Koolhaas *et al.* 1999, 2007), enabling a response to selection. This is vital for  
62 artificial selection regimes in non-wild populations (Mignon-Grasteau *et al.* 2005). In  
63 addition, differences in stress response are expected to be linked directly to fitness  
64 variation (Wingfield 2003; Koolhaas 2008), and should therefore be an important target  
65 of natural selection. The evolutionary dynamics of the stress response will therefore  
66 impact the resilience of wild populations to environmental stressors, whether natural (  
67 e.g. predation risk; Clinchy, Sheriff & Zanette 2013) or anthropogenic (Tarlow &  
68 Blumstein 2007; Busch & Hayward 2009; Angelier & Wingfield 2012).

69           Selection – whether natural or artificial – does not act on single traits in  
70 isolation, but rather on multivariate phenotypes (Lande & Arnold 1983; Blows 2007).  
71 Glucocorticoid (GC) levels are often used as univariate proxies for the physiological  
72 stress response (McEwen & Wingfield 2003; Korte *et al.* 2005). However, when coping  
73 with acute stressors, an individual’s first line of defence against a sudden threat is  
74 typically a behavioural one (Moberg 2000). For instance, the well-known ‘fight-or-flight’  
75 response occurs rapidly, with subsequent GC release then helping to mediate

76 physiological (and further behavioural) responses to acute stressors (Wingfield *et al.*  
77 1998; Wingfield & Kitaysky 2002). The mechanisms that link GCs with the expression of  
78 numerous traits are well established (Sopinka *et al.* 2015; Crossin *et al.* 2016), but if past  
79 selection has favoured particular combinations of trait values, then genetic integration  
80 of behavioural and physiological stress response traits should also be apparent  
81 (McGlothlin & Ketterson 2008; Ketterson, Atwell & McGlothlin 2009; Cox, McGlothlin &  
82 Bonier 2016). The premise that the physiological and behavioural components of the  
83 stress response will be tightly integrated is pervasive in the literature, as encapsulated  
84 in the 'stress coping style' model that predicts an axis of variation from 'reactive'  
85 (behavioural immobility coupled with low endocrine reactivity) to 'proactive'  
86 (behavioural avoidance and high endocrine reactivity) styles of stress response  
87 (Koolhaas *et al.* 1999; Øverli *et al.* 2007). However, with respect to genetic integration,  
88 there is a relative paucity of supporting empirical work.

89         To date, the best evidence for genetic integration of behavioural and  
90 physiological stress response traits comes from artificial selection studies on  
91 domesticated animal populations. In rainbow trout (*Oncorhynchus mykiss*), successful  
92 selection on stress-induced plasma cortisol levels revealed heritable variation in stress  
93 physiology (Pottinger and Carrick, 1999), while subsequent comparison of high-  
94 responding and low-responding selection lines identified correlated changes in stress-  
95 related behaviour (Øverli *et al.* 2001, 2002; Øverli, Winberg & Pottinger 2005; Pottinger  
96 & Carrick 2001; Schjolden *et al.* 2005; Ruiz-Gomez *et al.* 2011). However, some results  
97 lack consistency and/or appear highly context-dependent: low-responding lines showed  
98 more pronounced metabolic stress response under confinement relative to high-  
99 responding (Trenzado, Carrick & Pottinger 2003); boldness was not linked to stress-  
100 responsiveness under standardised testing procedures (Thomson *et al.* 2011); and  
101 behavioural components only of stress coping style were modified by an environmental  
102 change (Ruiz-Gomez *et al.* 2008). Artificial selection on stress-related GC levels has also

103 produced correlated stress-related behavioural responses (and/or vice versa) in  
104 Japanese quail (*Coturnix japonica*; Hazard et al., 2008b; Hazard et al., 2008a; Jones et al.,  
105 1994) and house mice (*Mus musculus domesticus*; Veenema et al., 2003a; Veenema et al.,  
106 2003b). In the great tit (*Parus major*), there is evidence for cortisol levels evolving in  
107 response to selection on behavioural ‘personality’ traits in the great tit (Carere *et al.*  
108 2003; Stöwe *et al.* 2010; Baugh *et al.* 2012), and this association has also been shown at  
109 the phenotypic level in the wild (Baugh *et al.* 2013).

110 While selection studies provide one useful strategy to test for genetic  
111 integration, an alternative approach is to directly estimate the genetic covariance matrix  
112 (**G**) among stress-related traits in a population. Selection over long time periods for  
113 particular trait combinations is expected to result in tight correlation structure among  
114 those traits being evident in **G** (Ketterson *et al.* 2009). **G** has been widely used to  
115 scrutinise genetic integration among sets of traits describing behaviour (Oswald, Singer  
116 & Robison 2013), male attractiveness to females (Blows, Chenoweth & Hine 2004; Hine,  
117 Chenoweth & Blows 2004; Hunt *et al.* 2007), and (most commonly) morphology  
118 (following, e.g., Cheverud 1982). Here, we use a lab-based pedigreed population of wild-  
119 derived Trinidadian guppies (*Poecilia reticulata*) to estimate the genetic covariance  
120 matrix **G** for behavioural and physiological components of the acute stress response, in  
121 order to determine whether (i) these components are genetically integrated into a single  
122 major axis of genetic (co)variation, and (ii) the make-up and orientation of this axis  
123 conforms with the expectations of the stress coping style model (Koolhaas *et al.* 2010;  
124 Boulton *et al.* 2015).

125 The population of guppies studied here is derived from wild individuals sampled  
126 from the lower Aripo River, Trinidad in 2008. The colony has subsequently been  
127 maintained at high population size with no deliberate inbreeding or selection.  
128 Introducing a lone fish to a novel ‘open field trial’ (OFT) environment is considered a  
129 mild stressor for this shoaling species, offering an opportunity to utilise behavioural

130 responses to this treatment as a proxy for their behavioural stress response (Houslay *et*  
131 *al.* 2018). Previous work has demonstrated significant additive genetic (co)variance in  
132 risk-taking and exploratory behavioural responses in this context (White & Wilson  
133 2018; White, Houslay & Wilson 2018), behaviours that also exhibit strong plastic  
134 responses to other acute stressors, including simulated predation events by birds and  
135 predatory fish (Houslay *et al.* 2018). Using a non-invasive waterborne hormone  
136 sampling method, we have also shown that individuals differ in their GC (specifically  
137 free circulating cortisol) response to an acute stressor (handling and short term  
138 confinement associated with the sampling itself; Houslay *et al.* 2019) and that, on  
139 average, this GC response decreases with repeated exposure to the sampling event  
140 stressor. However, nothing is known about the genetic basis of variation in these  
141 physiological components of the stress response, or of their integration with genetic  
142 variation in stress-related behaviours.

143         Here we test for evidence of genetic integration of the behavioural and  
144 endocrine components of the stress response. First, we characterise multiple  
145 behavioural components of the stress response, by developing a novel phenotyping  
146 approach for OFTs, to better distinguish ‘flight’ behaviour from potentially confounding  
147 variation in activity and exploration traits, and by complementing OFTs with additional  
148 ‘emergence trials’ (ET) and ‘shoaling trials’ (ST). Second, we characterise two key  
149 physiological components of the stress response by assaying GC levels following  
150 exposure to the first and third handling stress treatments in a stress habituation  
151 paradigm (see methods). All behavioural and physiological traits were assayed multiple  
152 times per individual using fish within a known genetic pedigree structure, enabling us to  
153 partition phenotypic (co)variation in the suite of focal traits into its additive genetic,  
154 permanent environment (here, among-individual), housing group, and residual  
155 components. We predict that there will be a heritable (genetic) component to each trait  
156 measured and that the **G** matrix (the pattern of genetic covariation among traits) will

157 contain significant among-trait correlation structure. More specifically, based on the  
158 ‘stress coping style’ model (Koolhaas *et al.* 1999) we predict genetic integration of  
159 behavioural and physiological traits in **G** such that a major axis of genetic variation is  
160 present: at one end, genotypes will be predisposed to a low-activity, exploratory  
161 phenotype with lower GC levels, and at the other a ‘flighty’ phenotype with high GC  
162 levels.

163

## 164 **Results**

165 Our data set includes behavioural observations from 7,637 trials (3,812 OFTs, 1,548 ETs  
166 and 1,039 STs) and physiological measures from 1,238 waterborne assays of cortisol  
167 (CORT hereafter). These phenotypic data were collected from 1,518 individual fish  
168 within a genetic pedigree structure comprised of maternal full-sibships nested within  
169 paternal half-sibships (with some connectedness across 5 ‘generations’, as offspring  
170 from one round of breeding were used, where available, as parents in the next). The  
171 number of individuals phenotyped (OFTs = 1,487, ETs = 806, STs = 532) and the mean  
172 number of observations per fish (OFTs = 2.6, ETs = 1.9, STs = 2.0) varied across the  
173 behavioural data types. For the 629 fish that were assayed for cortisol (almost all from  
174 the final generation), the handling and confinement stressor applied in this assay was  
175 performed 3 times (at 48h intervals) for all fish, with the magnitude of the GC response  
176 to the stressor being assessed only for the first and last stressor exposures  
177 (subsequently Cortisol<sub>1</sub> and Cortisol<sub>3</sub>). Full details of husbandry, phenotyping and  
178 analysis are provided in Materials and Methods.

179

## 180 **Genetic variance in behavioural components of the stress response**

181 We extracted behavioural data from videos of each trial type using video tracking of fish  
182 (as described in White, Kells & Wilson 2016; Houslay *et al.* 2018). Time to emerge from  
183 the shelter (*‘emergence time’*) was extracted from ETs and log<sub>n</sub> transformed for analysis,

184 while *shoaling tendency* was calculated from STs as the time spent in the third of the  
185 tank closest to a same-sex shoal (which was visible but physically separated) minus the  
186 time spent in the third of the tank farthest from the shoal. Note that both trials are  
187 considered to assay the behavioural stress response because the fish are subject to  
188 capture and solitary transfer into novel, brightly lit, arenas away from their home tanks  
189 and familiar tank mates.

190 The OFT was used to quantify three traits over a 4m 30s period following  
191 release into the tank and 30s acclimation: *track length* (distance swum), *area covered* (as  
192 a proportion of the arena floor area), and *time in the middle* (i.e., in the central area of  
193 the open field arena away from the tank walls, which is assumed to be perceived as  
194 riskier). Though these three OFT traits have previously been found to be repeatable and  
195 heritable in this population (White *et al.* 2016, 2018; Houslay *et al.* 2018; White &  
196 Wilson 2018), there is not a strong positive association between *track length* and *area*  
197 *covered* (Fig 1A). This is interesting because, if fish moved randomly in the arena, then  
198 we would expect *area covered* to increase asymptotically to 100% as *track length*  
199 increases. A plausible explanation is that similar *track lengths* can and do arise from two  
200 distinct stress-response scenarios: a (putatively) less stressed fish could be exhibiting  
201 an exploratory response, leading to relatively high *track length* and *area covered* (fish 1  
202 in Fig 1B), while a (putatively) more stressed fish could be exhibiting a flight response,  
203 which typically manifests as rapid swimming around the walls of the arena, leading to a  
204 similarly high *track length* but lower *area covered* (Fish 4 in Fig 1B).

205 To quantitatively discriminate these hypothetical exploratory and flight  
206 responses we derived a new trait, '*relative area covered*', using a simple simulation  
207 procedure (see Methods). This allowed us to predict the expected *area covered* for a  
208 given *track length* under the null model of a 'random swim' within the arena (Fig 1C).  
209 *Relative area covered* is then calculated as *observed area covered* – *expected area covered*  
210 (Fig 1D). Thus, higher values of *track length* may reflect an exploratory response when



211 coupled to high values of *relative area covered* and *time in the middle*, but a ‘flight’  
212 response when coupled to low values of *relative area covered* and *time in the middle*.  
213 We used univariate pedigree-based ‘animal models’ (Kruuk 2004; Kruuk &  
214 Hadfield 2007; Wilson *et al.* 2010) to test each trait for additive genetic variation while  
215 controlling for additional random effects of social housing group and non-genetic  
216 among-individual variance (as well as several fixed effects; see methods for full details).  
217 These models confirm the presence of significant additive genetic variation for the  
218 derived *relative area covered* trait, as well as for *track length* and *time in the middle* (  
219 consistent with previous findings; White & Wilson 2018; White *et al.* 2018) and  
220 *emergence time* (Table 1). The heritabilities of these behavioural traits (adjusted for  
221 fixed effects; see methods) are low to medium (Table 1) and within the range expected  
222 for behavioural traits (Stirling, Réale & Roff 2002). We detected no significant additive  
223 genetic variance for *shoaling tendency* (Table 1), despite there being repeatable  
224 differences among individuals;  $R = 0.19 \pm 0.04$ ;  $\chi^2_{0,1} = 20.01$ ,  $P < 0.001$ ).

225

## 226 **Genetic variation in physiological components of the stress response**

227 Using a series of nested bivariate animal models, we tested for the presence of additive  
228 genetic variation in cortisol levels ( $\log_n$  transformed) following stressor exposure  
229 (handling and confinement), and for genotype-by-environment interaction (GxE) in  
230 cortisol levels across the two samples assayed per fish (i.e. following stressors 1 and 3).  
231 In this context, the environment (E) is the time point at which the fish was exposed to  
232 the stressor. Any GxE present can therefore be interpreted as genetic variance for  
233 habituation to the stressor, given that the average cortisol level was lower following  
234 exposure to the third stressor than the first ( $\log_n$  transformed mean  $\pm$  SE; Cortisol<sub>1</sub> =  
235  $8.50 \pm 0.05$ , Cortisol<sub>3</sub> =  $8.05 \pm 0.06$ , Wald  $F_{1,12.9} = 120.5$ ,  $P < 0.001$ ). We first modelled  
236 Cortisol<sub>1</sub> and Cortisol<sub>3</sub> as distinct response variables in a bivariate framework but  
237 assuming no GxE (such that we assume  $V_{A-Cortisol1} = V_{A-Cortisol3}$  and the cross context

238 additive genetic correlation  $r_{A-Cortisol1, Cortisol3} = 1$ ). This model revealed a significant  
239 additive genetic component to variation among individuals in their cortisol levels  
240 following stressor exposure ( $\chi^2_{0,1} = 6.58, P = 0.005$ ).

241 Expanding the model to allow GxE (by freely estimating separate genetic  
242 variances for Cortisol<sub>1</sub> and Cortisol<sub>3</sub> as well as the cross-context genetic correlation)  
243 provides a significantly better fit to the data ( $\chi^2_2 = 9.65, P = 0.008$ ). This means that  
244 GxE is present, or (equivalently) that the magnitude of change in cortisol levels from the  
245 first to the third sampling (i.e., the degree of habituation to the stressor) varies among  
246 genotypes. The cross-context genetic correlation is strongly positive ( $r_{A-Cortisol1, Cortisol3} \pm SE$   
247  $= 0.74 \pm 0.25$ ), while genetic variance in cortisol levels is greater following exposure to  
248 the first stressor than the third ( $V_{A-Cortisol1} = 0.076 \pm 0.028, V_{A-Cortisol3} = 0.047 \pm 0.029$ ).

249 Both findings are apparent when visualising model predictions (Figure 2): while there is  
250 genetic variation in habituation rates (indicated by variation in the slopes of genotypic  
251 reaction norms; Figure 2), there is still positive cross-context genetic correlation  
252 (indicated by little change in the rank order of individuals across the two contexts;  
253 Figure 2). The reduction in genetic variance for cortisol levels between the first and  
254 third stressor exposure arises because genotypes with higher than average cortisol  
255 levels following the first exposure habituate more rapidly following subsequent  
256 exposures (reflected in the model as GxE). In this model we also find that variance in  
257 cortisol explained by average differences among groups is similar across contexts  
258 ( $V_{Group-Cortisol1} = 0.034 \pm 0.013, V_{Group-Cortisol3} = 0.045 \pm 0.016$ ), but that residual variance is  
259 greater following exposure to the third stressor than the first ( $V_{R-Cortisol1} = 0.166 \pm 0.021,$   
260  $V_{R-Cortisol3} = 0.229 \pm 0.025$ ). This means that there is no change in variance explained  
261 among groups across contexts, but variance within groups is greater after the third  
262 stressor relative to the first. Overall, this pattern creates a higher adjusted heritability  
263 (the ratio of additive genetic variance to the sum of additive genetic, group and residual

264 variances after adjusting for fixed effects) for cortisol levels following the first stressor  
265 exposure than the third ( $h^2_{\text{Cortisol1}} = 0.275 \pm 0.093$ ,  $h^2_{\text{Cortisol3}} = 0.146 \pm 0.088$ ).

266

267

## 268 **Testing for genetic integration and identifying the major axis of genetic variance**

269

270 Lastly, to test for the hypothesised genetic integration between behavioural and  
271 physiological components of the stress response, we built a multivariate animal model  
272 to estimate patterns of genetic covariances among these traits (**G**). We excluded *shoaling*  
273 *tendency* given the absence of detectable genetic variance in the univariate model of this  
274 trait. We also elected to treat cortisol as a single trait (allowing for a fixed effect of  
275 stressor exposure number (1 vs 3) on the mean). Although the analysis described above  
276 shows evidence of GxE, the strong positive genetic correlation between Cortisol<sub>1</sub> and  
277 Cortisol<sub>3</sub> justifies combining into a single trait here in order to maximise power to detect  
278 overall genetic integration of behaviour and GC expression.

279 Our final model contained 5 response traits: *relative area covered*, *time in the*  
280 *middle* and *track length*, (log-transformed) *emergence time*, and (log-transformed)  
281 *Cortisol* (treated as two repeats of a single trait; see above). Following transformation  
282 (where applicable), we standardised all traits to standard deviation units, to assist  
283 multivariate model fitting and to prevent eigenvectors (see below) of **G** being dominated  
284 by traits with higher variance in observed units. To simplify interpretation of the  
285 estimated correlation (and covariance) structure in **G** we multiplied log-transformed  
286 *emergence time* by -1, resulting in higher values for all behavioural traits indicating  
287 individuals that mounted 'bolder' behavioural responses to stressors. Our estimate of **G**  
288 (Table 2) contains significant genetic covariance structure overall (Likelihood Ratio Test  
289 of the full model vs. a model in which we did not allow for additive genetic covariance:  $\chi$   
290  $^2_{10} = 36.79$ ,  $P < 0.001$ ). **G** also contains a number of strong pairwise genetic correlations,

291 both negative and positive, that are statistically significant (based on the bootstrapped  
292 95% confidence intervals not crossing zero). Log-transformed *Cortisol* is correlated  
293 positively with *track length* at the genetic level, and negatively with *relative area covered*  
294 and *time in the middle* (though not all pairwise correlations are individually significant;  
295 Figure 3). *Relative area covered* shows significant genetic correlations with both of the  
296 other OFT traits: positive with *time in the middle*, and negative with *track length*.

297 Eigen decomposition of **G** provides a more holistic view of the genetic  
298 covariance structure and the level of integration among traits. Here the major axis (first  
299 principal component, PC1) explains 57.8% of the genetic variance in multivariate  
300 phenotype (PC2 = 25.7%; PC3 = 9.3%; PC4 = 5.6%; PC5 = 1.6%). All of the traits except  
301 *emergence time* load significantly on this axis (Figure 4). *Relative area covered* and *time*  
302 *in the middle* load in one direction, while *track length* and *Cortisol* load in the other  
303 direction. This suggests that genotypes at one end of this major axis of genetic  
304 (co)variation have more ‘exploratory’ behavioural responses to stress (i.e., swim shorter  
305 distances, spend more time in the central area of the tank, and cover a high area relative  
306 to their distance travelled in OFTs) and have weaker physiological responses to stress  
307 (i.e., produce lower cortisol levels in response to the stressor). While genotypes at the  
308 other end of this axis have more of a ‘flight’ type of behavioural response to stress (i.e.,  
309 swim longer distances, stay closer to the edges of arena, and cover a small area relative  
310 to their distance travelled in OFTs) and have stronger physiological responses to stress  
311 (i.e., produce higher cortisol levels in response to the stressor).

312

## 313 Discussion

314

315 In this study we sought to determine whether – and to what extent – there exists  
316 genetic variation for, and integration between, behavioural and physiological  
317 (endocrine) components of stress response. Our results provide three main novel  
318 insights. First, we find that genetic variation underpins individual differences in not only  
319 stress-related behaviours (including but not limited to relative area) but also stress  
320 physiology. Second, we find genetic covariance structure among these stress response  
321 traits – including between behavioural and physiological components. Thirdly, the  
322 major axis of genetic covariation conforms to that hypothesised in the stress coping  
323 styles model. Overall, by estimating genetic correlation structure among traits we find at  
324 least some support for the hypothesis of evolutionary integration between behavioural  
325 and endocrine components of the stress response.

326 The open field trial (OFT) paradigm used here is widely applied to study ‘shy-  
327 bold’ type personality variation in fishes (Toms, Echevarria & Jouandot 2010) and other  
328 vertebrates (Carter *et al.* 2013; Perals *et al.* 2017). Furthermore, behavioural differences  
329 expected to occur along a ‘shy – bold’ personality axis align closely with predictions  
330 from the ‘reactive – proactive’ model of stress coping (see Boulton *et al.* 2015).  
331 However, whether actual behavioural variation matches expectations of these verbal  
332 models is an empirical question. In this guppy population, using the directly observed  
333 OFT traits, it does not (White *et al.* 2016; Houslay *et al.* 2018). By identifying the ‘null’  
334 expected relationship between track length and area covered under a random swim we  
335 greatly increase biological interpretability. Specifically, using ‘*relative area covered*’ we  
336 are able to demonstrate an axis of repeatable and heritable variation that spans from  
337 methodical exploration of the arena by (putatively) unstressed fish through to a ‘flight’  
338 type response to being placed in the OFT arena. Individuals – and genotypes –  
339 predisposed to low relative area also have longer track lengths and spend less time in

340 the middle (i.e., are more thigmotaxic). Given the wide use of OFTs in biomedical  
341 research (e.g., Rex *et al.* 1998; von Horsten, Karl & Pabst 2003) as well as in animal  
342 behaviour, our approach may have broad applicability for discriminating between  
343 exploration and stress/anxiety-related behaviours.

344         Among the non-OFT behaviours assayed, we find less evidence for genetic  
345 variation and/or integration. Tendency to shoal varies among individuals but is not  
346 detectably heritable. Though not generally considered a stress-response trait *per se*,  
347 shoaling is an anti-predator behaviour in guppies (Herbert-Read *et al.* 2017). We had  
348 therefore predicted that heightened perception of risk in the open field might be  
349 associated with increased shoaling tendency. The absence of detectable genetic variance  
350 meant that we could not test this prediction in **G**, though there was no evidence for such  
351 a pattern at the level of individual phenotype.

352         By contrast, emergence time is heritable but not significantly correlated with  
353 OFT traits in **G** (nor among individuals). While longer emergence times are typically  
354 interpreted as reflecting a fear of the open arena (i.e., lack of 'boldness'; see Burns  
355 2008), it is notable that the qualitative pattern in **G** in our study actually ran counter to  
356 this expectation. Genotypes predisposed to longer emergence times are associated  
357 (albeit non-significantly) with higher relative area, lower track length, and more time in  
358 the middle during OFT trials. That is, longer emergence times are associated with the  
359 bolder, exploratory and (putatively) less stressed end of the stress response continuum.  
360 This may indicate that at least some individuals do not perceive the shelter area as safer  
361 than the open arena, a possibility also suggested by the fact that some individual  
362 guppies decrease (rather than increase) shelter use following simulated predation  
363 events (Houslay *et al.* 2018).

364         We also find evidence of significant additive genetic variance in a key  
365 physiological component of the stress response: waterborne cortisol concentrations  
366 following exposure of the fish to a handling stressor. Our findings suggest that

367 previously detected differences among individuals in cortisol response to a stressor  
368 (Houslay *et al.* 2019) are primarily attributable to genetic effects, with the estimated  
369 heritability ( $h^2 = 0.26$ ) being >75% of the individual-level repeatability for log(Cortisol).  
370 Moreover, by adopting a reaction norm approach to modelling stress physiology, as  
371 recently advocated by ourselves and others (e.g., Fürtbauer *et al.* 2015; Hau & Goymann  
372 2015; Taff & Vitousek 2016), we detect GxE reflecting genetic differences in the extent of  
373 habituation to the stressor over repeated exposures. This result is potentially important  
374 since poor habituation of the hypothalamic-pituitary-adrenal/interrenal (HPA/I)  
375 response to repeated or ongoing stressors can lead to well documented health problems  
376 in human and animal populations (Segerstrom & Miller 2004; Koolhaas 2008; Romero,  
377 Dickens & Cyr 2009; Mason 2010). Heritable variation in habituation to stressors raises  
378 the possibility of developing targeted selection strategies to improve welfare in captive  
379 populations (e.g., Frankham *et al.* 1986; Muir & Craig 1995; Oltenacu & Algers 2009).  
380 These findings also highlight the possibility that habituation could be important for  
381 adaptation in the wild, as many agents of natural selection can be viewed as  
382 environmental stressors (Bijlsma & Loeschcke 2005).

383         Our findings also highlight that there is greater additive genetic variance (and  
384 heritability) for cortisol levels following the first exposure to the stressor than following  
385 the third. This pattern, which occurs because genotypes that produce the highest  
386 cortisol response at first exposure also show the most marked habituation, is consistent  
387 with the idea of cryptic genetic variance (Paaby & Rockman 2014) being ‘released’ by  
388 exposure to novel, and so potentially stressful, environments (Ledón-Rettig, Pfennig &  
389 Crespi 2010; Ledón-Rettig *et al.* 2014; Berger *et al.* 2011). All else being equal, it also  
390 means that selection on cortisol levels following stressor exposure should induce a  
391 stronger evolutionary response in naïve relative to habituated fish. However, the strong  
392 positive cross-environment correlation means that the ranking of genotypes is  
393 consistent across repeated stressor exposures. It also means that we would expect a

394 positively correlated response of habituation to any selection on (average) GC response  
395 and *vice versa*.

396 Our estimate of **G** shows genetic integration between behavioural and endocrine  
397 components of the stress response: genotypes with more exploratory behavioural  
398 phenotypes in the OFT also produce lower levels of cortisol following the handling and  
399 confinement stressor. This genetic integration of behaviour and physiology is consistent  
400 with the idea that correlational selection in the past has led to the coevolution of these  
401 stress response components. Covariance structure in **G** will also modify, and potentially  
402 constrain, future evolutionary responses to contemporary selection (whether natural or  
403 artificial). Here we have no direct knowledge of selection acting in the wild but note that  
404 we do expect acute stress responses in natural population to be broadly adaptive. In  
405 contrast, it is clear that prolonged, chronic activation of stress response pathways  
406 (notably the HPA(I) axis) frequently jeopardises health and survival in captive animal  
407 populations (Huether 1996; Boonstra 2013). Thus, it could follow that more stress-  
408 responsive genotypes are disadvantaged in captivity, if responsiveness to acute  
409 stressors positively predicts susceptibility to chronic stress. However, even in this  
410 scenario it does not follow that high stress-responsiveness is disadvantageous in the  
411 wild. Rather, since natural selection should purge alleles that are universally  
412 detrimental, it seems more plausible that genetic variation along the major axis  
413 described here is maintained by some form of selective trade-off (as widely  
414 hypothesised for maintenance of personality variation; e.g., Stamps 2007; Wolf *et al.*  
415 2007; Réale *et al.* 2010).

416

#### 417 **Conclusions and future directions**

418 Here we find evidence for genetic variation in – and integration of – behavioural  
419 and physiological (endocrine) components of the stress response. The structure of **G**  
420 holds with the widely used ‘reactive – proactive’ model of stress coping style variation



421 (Koolhaas *et al.* 2007), but is more consistent with a continuous axis of variation in  
422 acute stress responsiveness. Interestingly, genotypes that are more responsive (in the  
423 sense of displaying flight-type behaviour and higher cortisol levels) also exhibit faster  
424 habituation of GC physiology when subject to repeated stressors.

425         Our results suggest that correlational selection in the past has likely shaped the  
426 multivariate stress response, and that continued evolution of stress-related behaviour  
427 will have consequences for glucocorticoid physiology and *vice versa*. Determining  
428 contemporary selection on the stress response, and testing the possibility that genetic  
429 variation is maintained by fitness trade-offs, is thus an obvious – if empirically  
430 challenging – next step. In a more applied context, integration of behavioural and  
431 endocrine stress-response components at the genetic level has potential utility for  
432 genetic improvement of captive populations. Specifically, it may be possible to identify  
433 non-invasive, high throughput, behavioural biomarkers and target them in selection  
434 schemes to reduce chronic activation of the HPA/I axis and its attendant deleterious  
435 effects. The efficacy of this approach will necessarily depend on (i) the presence of  
436 standing genetic variance for health and welfare under chronic stress, and (ii) strong  
437 genetic correlation structure between target traits and behavioural markers of acute  
438 stress-response.

439 Table 1: Estimated variance components, along with adjusted heritability, for each trait as estimated in a univariate model ( $\pm$  standard error). Chi-  
 440 square test statistics and p-values are provided for the pedigree term, testing for the presence of significant additive genetic variance ( $V_a$ ).

| Trait                        | $V_a$                  | $V_{pe}$               | $V_{group}$          | $V_{residual}$         | $h^2$           | $\chi^2_{0,1}$ | P      |
|------------------------------|------------------------|------------------------|----------------------|------------------------|-----------------|----------------|--------|
| <i>Relative area covered</i> | 26.35 $\pm$ 9.37       | 72.42 $\pm$ 9.28       | 33.33 $\pm$ 6.86     | 205.22 $\pm$ 5.90      | 0.08 $\pm$ 0.03 | 20.7           | <0.001 |
| <i>Time in the middle</i>    | 588.48 $\pm$ 139.61    | 554.58 $\pm$ 109.57    | 203.29 $\pm$ 53.41   | 2070.06 $\pm$ 60.08    | 0.17 $\pm$ 0.04 | 53.7           | <0.001 |
| <i>Track length</i>          | 26832.64 $\pm$ 5925.25 | 32204.05 $\pm$ 4868.88 | 9956.4 $\pm$ 2626.41 | 93921.54 $\pm$ 2711.28 | 0.16 $\pm$ 0.03 | 86.3           | <0.001 |
| <i>Shoaling tendency</i>     | 0 $\pm$ 0              | 2457.36 $\pm$ 570.96   | 708.87 $\pm$ 316.30  | 9900.95 $\pm$ 622.10   | 0 $\pm$ 0       | 0              | 0.5    |
| <i>log(Emergence time)</i>   | 0.12 $\pm$ 0.05        | 0.06 $\pm$ 0.06        | 0.05 $\pm$ 0.02      | 1.07 $\pm$ 0.05        | 0.09 $\pm$ 0.04 | 23.2           | <0.001 |
| <i>log(Cortisol)</i>         | 0.07 $\pm$ 0.02        | 0.02 $\pm$ 0.02        | 0.01 $\pm$ 0.01      | 0.15 $\pm$ 0.01        | 0.26 $\pm$ 0.08 | 22.0           | <0.001 |

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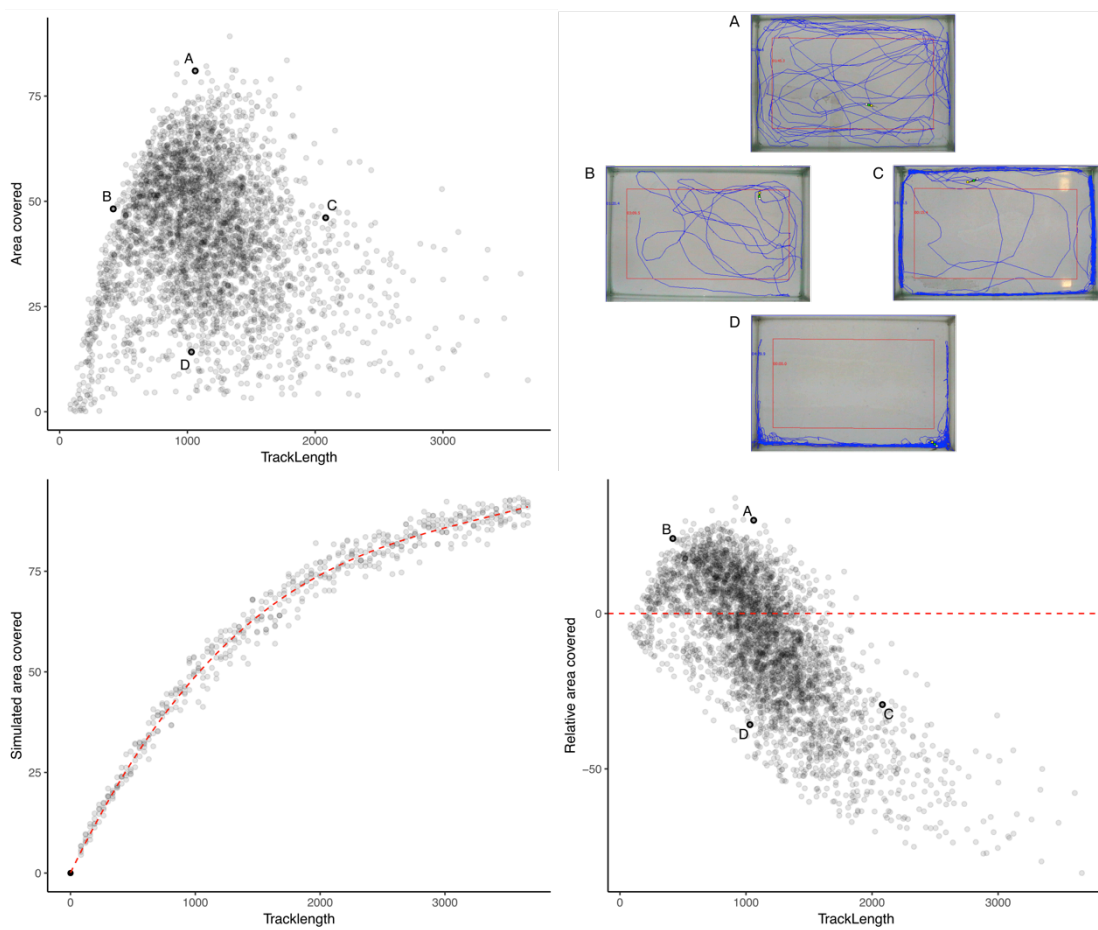
444 Table 2: Additive genetic covariance-correlation matrix (G) from the full multivariate animal model. Genetic variances provided on the shaded  
 445 diagonal, with genetic covariances below and genetic correlations above. 95% confidence intervals in parentheses are estimated from 5000  
 446 bootstrapped replicates. Where the confidence intervals for any estimate do not cross zero the estimate is considered statistically significant (at the  
 447 0.05 alpha level) and are shown in bold.

|                              | <i>Relative area covered</i>  | <i>Time in the middle</i>     | <i>Track length</i>           | <i>-log(Emergence time)</i> | <i>log(Cortisol)</i>          |
|------------------------------|-------------------------------|-------------------------------|-------------------------------|-----------------------------|-------------------------------|
| <i>Relative area covered</i> | <b>0.074 (0.029,0.122)</b>    | <b>0.761 (0.549,0.955)</b>    | <b>-0.506 (-0.758,-0.184)</b> | -0.503 (-1.394,0.256)       | -0.414 (-1.035,0.225)         |
| <i>Time in the middle</i>    | <b>0.075 (0.031,0.124)</b>    | <b>0.130 (0.062,0.191)</b>    | <b>-0.554 (-0.774,-0.295)</b> | -0.117 (-0.791,0.531)       | <b>-0.686 (-1.165,-0.220)</b> |
| <i>Track length</i>          | <b>-0.048 (-0.086,-0.008)</b> | <b>-0.070 (-0.116,-0.022)</b> | <b>0.121 (0.067,0.171)</b>    | 0.559 (-0.026,1.256)        | 0.279 (-0.238,0.823)          |
| <i>-log(Emergence time)</i>  | -0.038 (-0.083,0.014)         | -0.012 (-0.070,0.040)         | <b>0.055 (0.001,0.106)</b>    | <b>0.079 (0.011,0.149)</b>  | -0.177 (-0.910,0.560)         |
| <i>log(Cortisol)</i>         | -0.038 (-0.091,0.011)         | <b>-0.082 (-0.138,-0.021)</b> | 0.032 (-0.026,0.085)          | -0.017 (-0.073,0.038)       | <b>0.111 (0.036,0.191)</b>    |

448

449

450 Figure 1: The lack of a strong positive relationship between observed *tracklength* and *area covered*  
451 (panel 1), is initially puzzling given expected autocorrelation and that both are used as positive  
452 indicators of exploratory (or ‘bold’) behaviour. Inset examples of OFT tracks from 4 individuals (panel  
453 2) shed light on this. Fish A and B appear to be exploring the tank, while C and D are engaging in a  
454 stereotypical ‘flight’ behaviour characterised by strong thigmotaxis (remaining close to tank walls)  
455 and/or rapid movement along tank walls. As a consequence, individuals B and C have similar *area*  
456 *covered* during the OFT as a consequence, but very different *track lengths*. We simulated random  
457 movements to define an expected null relationship between *area covered* and *track length* (panel 3;  
458 dashed red line shows the third order polynomial model fit; see Appendix A). The polynomial  
459 regression was then used to predict the expected area covered under random movement for each  
460 trial’s observed *track length*, and the ‘*relative area covered*’ was calculated as the observation minus  
461 this prediction. Panel 4 shows the resultant *relative area covered* plotted against *track length* for all  
462 trials (dashed red line at *relative area covered* = 0, shows where individuals of any *track length* are  
463 expected to lie if they move randomly with respect to direction). From this it is apparent that fish A  
464 and B have high *relative area covered*, while C and D do not.



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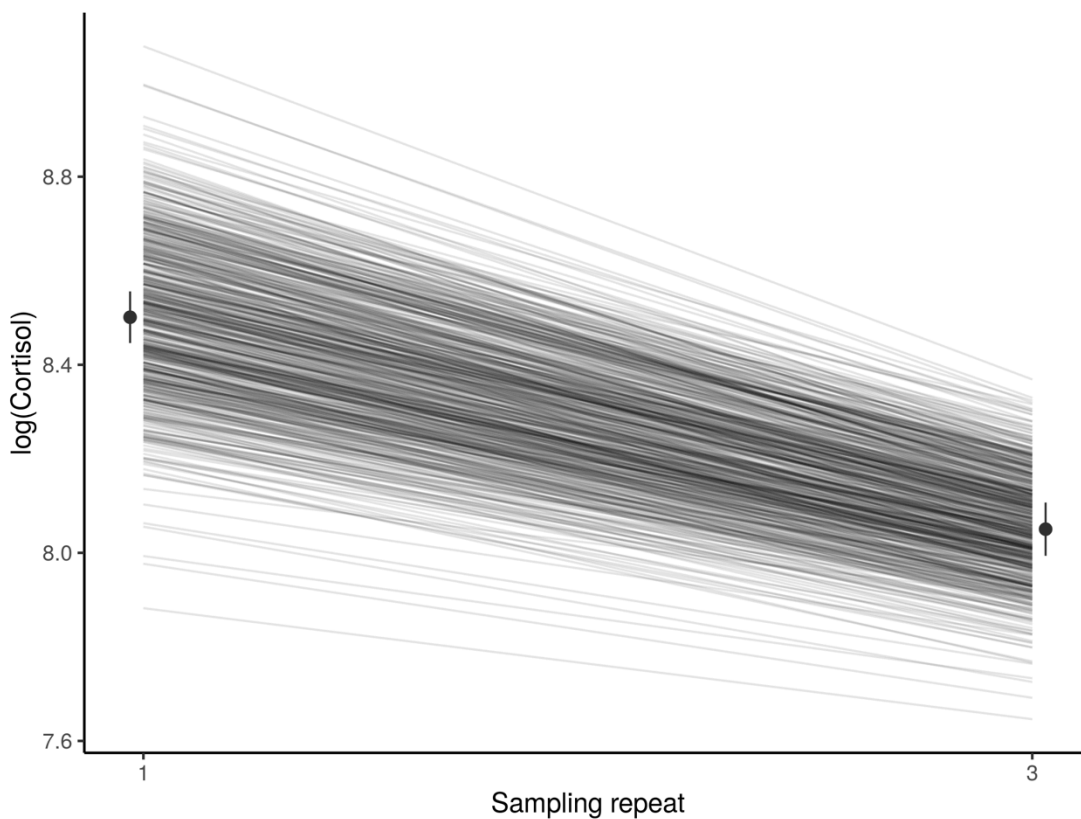
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472 Figure 2: Guppies habituate to the waterborne sampling procedure, as shown by a decline in log-  
473 transformed cortisol levels following stressor exposure declining from their first stressor exposure to  
474 their third. Black circles and associated bars denote predicted population means ( $\pm$  standard error)  
475 from mixed model analysis. Reaction norms (grey lines) depict the predicted genetic deviations for  
476 each individual from the average pattern. GxE is evidenced by the slight reduction in genetic variance  
477 over the repeated sampling events (i.e., that the reaction norms (therefore) are not parallel; see main  
478 text).

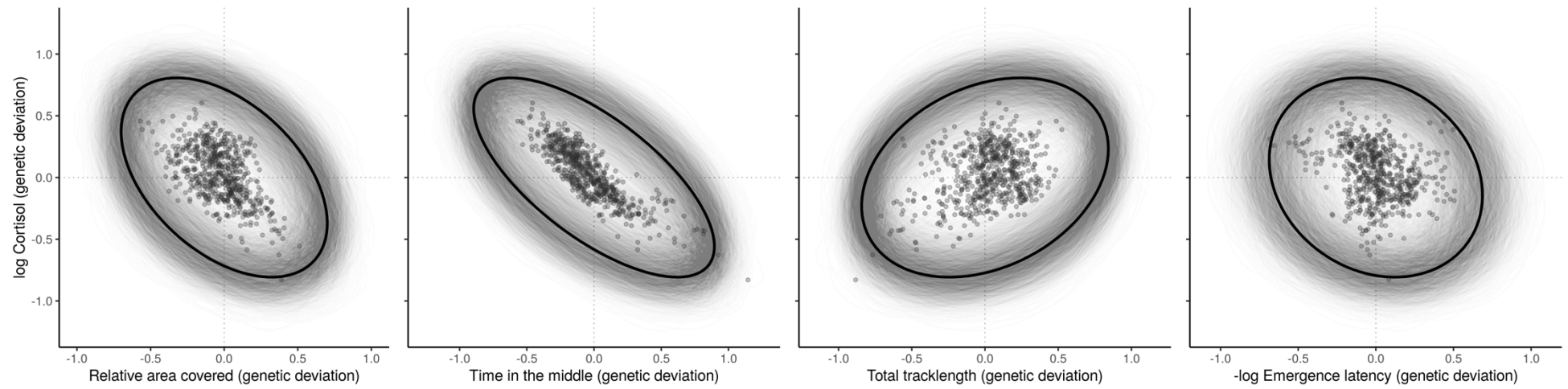


479

480

481 Figure 1: The additive genetic relationship between log-transformed cortisol (y-axis) and four behaviours (a, *relative area covered*; b, *time in the middle*; c,  
482 *track length*; d, *-log emergence time*). Points show (predicted) bivariate genetic deviations from the population means, plotted for those individuals in the  
483 pedigree with cortisol data. In each case the black ellipse depicts the shape of the relationship as estimated from  $\mathbf{G}$ , with 5000 bootstrapped replicates in  
484 grey showing the associated uncertainty.

485

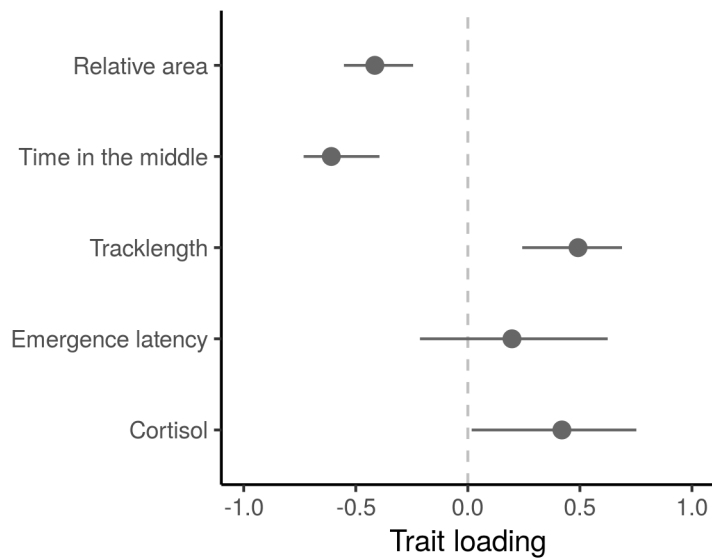


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488

489 Figure 2: Trait loadings from the first eigen vector (principal component) of **G**. This axis explains  
490 57.8% of the genetic (co)variation found in the focal behavioural and physiological components of the  
491 stress response in our guppy population. Points show trait loadings from the first eigen vector of our  
492 estimate of **G**, with bars representing 95% confidence intervals on each loading (calculated from 5000  
493 bootstrapped replicates of the model).



494



## 495 **Materials and methods**

496

### 497 *Husbandry and breeding*

498 We used fish taken from our captive population housed at the University of Exeter's Penryn campus,  
499 which is descended from wild fish collected in 2008 from the lower Aripo River in Trinidad. This  
500 population has been maintained at a population size of several thousand, and has undergone no  
501 deliberate selection or inbreeding. All fish are fed to satiation twice daily (0800 – 1000h and again at  
502 1600 – 1800h) using commercial flake food and live *Artemia nauplii*. Water temperature is maintained  
503 at 23-24°C in well-aerated closed system tank stacks that undergo 25% water changes each week and  
504 with weekly tests for ammonia, nitrate and nitrite levels. Lighting is kept at a 12:12 light/dark cycle.  
505 The experiment described in this study was carried out in accordance with the UK Animals (Scientific  
506 Procedures) Act 1986 under licence from the Home Office (UK), and with local ethical approval from  
507 the University of Exeter.

508 To create our pedigreed sub-population, female fish were sampled haphazardly from the stock  
509 tanks and kept in female-only groups for 3 months. Isolation from male contact minimised the chance  
510 of females carrying viable sperm from previous matings. For the first generation of offspring, we used  
511 a group breeding design (as detailed in White and Wilson, 2018); briefly, females were tagged under  
512 anaesthetic (buffered MS222 solution) using visible implant elastomer (VIE) to allow individual  
513 identification. We then assigned groups of 4 females to 1 male in 15L breeding tanks (18.5cm x 37cm x  
514 22cm), and inspected females daily for high gravidity (swollen abdomens and enlarged 'gravid spots').  
515 Heavily gravid females were then isolated in 2.8L brood tanks to give birth (and were returned to the  
516 breeding tanks either after producing a brood or two weeks of isolation). Any offspring produced in  
517 the breeding tanks were excluded from the experiment as maternal identity could not be positively  
518 identified. For the following generations, after 3 months of isolation from males we moved females  
519 into individual 2.8L tanks, with 1 male then circulated among 3 females. Males were moved between  
520 females every 5-8 days. In this way, females did not have to be moved to brood tanks, and any  
521 offspring could be assigned to mothers definitively. In this setup, offspring were moved to a separate  
522 brood tank on the day of birth. Note that as the gestation period for guppies is approximately 1 month,

523 any brood produced by a female less than one month after exposure to their designated male was  
524 recorded in the pedigree as having unknown paternity.

525         Within 24h of a female producing a brood we recorded her weight (g) and brood size. We kept  
526 juvenile fish in full-sib family groups in 2.8L tanks before moving them to 15L 'growth' tanks at an  
527 average age of 56 days. At an average age of 133 days (range 59-268) we tagged individuals and  
528 placed them into mixed family groups of 16-20 adults (with an even mix of males and females), kept in  
529 15L tanks. Note that variation in tagging age arose largely because groups were necessarily  
530 established sequentially as sufficient individuals from multiple families reached a large enough size  
531 that we deemed the procedure to be safe. Each adult group comprised a mix of fish from different  
532 families, reducing the potential for common environment effects to upwardly bias our genetic  
533 parameter estimation.

534

#### 535 *Overview of behavioural phenotyping*

536 Behavioural phenotyping commenced at least one week after tagging. In all trials, we filmed  
537 movement behaviour of individual fish using a Sunkwang video camera equipped with a 6-60mm  
538 manual focus lens suspended over the tank. We used the tracking software Viewer II (BiObserve) to  
539 extract behavioural data from each recording (detailed below). The tank was lit from below using a  
540 light box, and screened with a cardboard casing to prevent external visual disturbance. After each  
541 behavioural trial, the individual tested was weighed and then moved to a temporary 'holding tank'.  
542 Once a full group (as described above) had been tested, all were moved from the holding tank back to  
543 their home tank. We replaced the water in the testing and holding tanks between groups to reduce the  
544 build-up of hormones or other chemicals. The first offspring generation experienced 4 repeat open  
545 field trials (OFTs) over a 2-week period, with at least 48h between trials. Subsequent generations  
546 experienced 4 repeat behavioural trials, alternating 2 OFTs with 2 emergence trials (ETs). For the final  
547 2 generations, we extended the OFTs by including a shoaling trial (ST) at the end of each OFT.

548

549 *Open field trials (OFT)* followed the methodology described by White et al (2016). Briefly, we assessed  
550 individual behaviour in a 20cm x 30cm tank, filled to a depth of 5cm with room-temperature water

551 from the main supply. We caught fish individually from their home tank, examined them quickly for  
552 identification tags, then placed them immediately into the centre of the OFT tank. After allowing 30s  
553 for acclimation, we filmed behaviour for 4m30s. Behaviours characterised from the tracking software  
554 were *track length* (the total distance the fish moved during the trial; cm), *area covered* (the percentage  
555 of 1cm x 1cm grid squares through which the fish moved during the trial; %), and *time in middle* (time  
556 spent in a rectangular inner zone which was defined as being the same size as an outer area; seconds).

557

558 *Shoaling trials (ST)* were appended to a subset of OFTs, by positioning a small tank containing 10 stock  
559 fish (of same sex as the test subject) next to one end of the OFT tank but with visual cues blocked by a  
560 cardboard divider. At the end of the normal OFT, we removed this divider slowly, allowing the focal  
561 animal to have visual contact with the shoal. We began recording the shoaling trial 30s after removing  
562 the divider in order to limit any artefacts of slight disturbance. (Note that we used a further cardboard  
563 casing around the shoaling tank to avoid any additional external visual stimulus). We then recorded  
564 behaviour of the test fish for an additional 3 minutes. We characterised *shoaling tendency* via the  
565 tracking software by subdividing the tank area into 3 equal-sized rectangular areas: one next to the  
566 tank holding the group of same-sex fish, one farthest from this group, and the central area. We then  
567 calculated *shoaling tendency* as the time spent in the 1/3 area closest to the same-sex group after  
568 subtracting the time spent in the 1/3 area farthest away. The decision to use a single-sex shoal aimed  
569 to reduce any effects of (potential) mate preference and/or avoidance, but also this necessitated  
570 replicate arena setups allowing male and female individuals from each group to be tested in the  
571 OFT/ST concurrently. We randomised which tank was used for each sex in each group and recorded  
572 this information.

573

574 *Emergence trials (ET)* followed the methodology described by White *et al.* (2016). Briefly, we tested  
575 individuals in a 20cm x 40cm tank, filled to a depth of 8cm with room-temperature water from the  
576 main supply. A 10cm section of the tank length was walled off creating a shelter area (20cm x 10cm),  
577 the walls and floor of which were painted black. The focal fish was placed into the shelter area and  
578 allowed to acclimate for 30s, at which point we opened a sliding door to allow access to the rest of the

579 tank, which was brightly lit from below and otherwise bare. *Time to emerge* (in seconds) was recorded  
580 by the tracking software automatically as the fish exited the shelter area and emerged into the open  
581 tank section. Trials were ended either at emergence or at 15 min if the fish had not emerged by that  
582 point; in the case of non-emergence, fish were given the maximum value (i.e., 900s).

583

#### 584 *Derivation of 'relative area' from OFT trials*

585 The '*area covered*' variable assayed in the OFT is calculated in BiObserve by dividing the arena (i.e., the  
586 total area of the tank as viewed from the camera) into 1cm x 1cm grid squares. The path taken by the  
587 fish during observation is then used to determine what proportion of these grid squares the fish  
588 entered. However, we sought to derive a measure of '*relative area*' that describes whether a fish covers  
589 a large, or small area relative to its observed *tracklength*.

590 To do this we simulated 'random swims' within the arena across the observed range of  
591 tracklengths. We first selected 40 OFT results at random from our total data set and extracted the  
592 coordinates of the fish in each frame from the raw tracking file, creating a set of x and y movements  
593 and their associated distances. As original coordinates were recorded in pixels we used the calibration  
594 of the software to convert to cm units. We then use a 'random walk' algorithm to select a movement  
595 (i.e., step size and direction) from this observed distribution at random, and calculate the new  
596 coordinates. If the movement keeps the 'fish' within the bounds of the 'tank' (i.e., defined as a 20cm x  
597 30cm arena), the movement is accepted and coordinates added to a movement matrix; if not, a new  
598 movement is drawn from the distribution. If the movement is greater than 1cm in distance, we break  
599 the movement into a number of smaller parts to be added to the matrix (such that we capture the  
600 coordinates of grid squares through which the 'fish' moved along the way). Once the total distance of  
601 the random walk reached or exceeded the *tracklength* set as the simulation objective, the path is  
602 terminated and the area covered is calculated by counting the number of unique grid squares in the  
603 matrix of coordinates and dividing by the total number possible.

604 After simulating random walks across 500 values of *tracklength* (using a vector of 100 values  
605 evenly spaced across the range of true data, repeated 5 times), we modelled (simulated) area covered  
606 as a fourth order polynomial function of *tracklength*. Using this regression model (which explained

607 97.8% of the variance in simulated data), we calculated the *relative area* for each actual OFT trial as  
608 the observed area covered minus the area covered under a random swim, as predicted from our  
609 regression model and the observed *tracklength*.

610

611

### 612 *Waterborne hormone sampling*

613 On completion of behavioural data collection, individuals entering the endocrine testing program were  
614 left undisturbed for a minimum of two weeks. Waterborne hormone sampling was then conducted  
615 over a 5-day period that included three handling and confinement stressor exposures with 48h  
616 between each. We followed the method described by Houslay et al (2019) to obtain repeated non-  
617 invasive GC measures of individuals using holding water samples from the first and third  
618 confinements. Note that only two samples per fish were analysed because the financial and time costs  
619 of doing three was deemed prohibitive. We nonetheless applied the stressor stimulus three times as  
620 our prior study showed this was sufficient to produce a strong habituation response, i.e., a significant  
621 decrease in water-borne cortisol over the three sampling periods (Houslay *et al.* 2019)).

622 We collected samples between 1200 – 1400h to control for diel fluctuations in GC levels. For  
623 each sample, we netted an entire group from their home tank quickly using a large net, transferring  
624 them to 2 holding tanks (containing water from the home tank supply) for moving to an adjacent quiet  
625 room (performed within 20s of the net first hitting the water). We then transferred fish to individual  
626 Pyrex beakers containing 300ml of clean water from the main supply (which serves the main housing  
627 units), which has been warmed to the appropriate temperature (mean = 24.1°C, range 23-24.9°C).  
628 Beakers were placed within cardboard ‘chambers’ to prevent fish from seeing each other or  
629 experiencing outside disturbance. One fish was transferred every 30s, alternating across holding  
630 tanks, such that all fish were in their beakers within 10min of the initial netting. After 60 mins in the  
631 beaker, each fish was removed by pouring its sample through a clean net into a second beaker, with  
632 the fish then quickly checked to confirm ID and returned to the holding tank until the entire group  
633 could be returned to its home tank.

634 We immediately filtered each water sample using Grade 1 filter paper (Whatman), then passed them  
635 slowly through solid phase C18 extraction columns (Sep-Pak C18, 3cc, Waters) via high-purity tubing  
636 (Tygon 2474, Saint Gobain) under vacuum pressure (Earley *et al.* 2006). Columns were primed  
637 beforehand with 2 x 2ml HPLC-grade methanol followed by 2 x 2ml distilled water, and were washed  
638 afterwards with a further 2 x 2ml distilled water to purge salts. We then covered both ends of each  
639 column with film (Parafilm M, Bemis) and stored them at -20C for future analysis. We washed all  
640 beakers, tubes and funnels with 99% ethanol and rinsed them with distilled water prior to each  
641 sampling procedure. The remainder of the endocrine assay procedure, involved elution, resuspension,  
642 separation and quantification of free cortisol by enzyme immunoassay (EIA) using Cayman Chemicals,  
643 Inc EIA kits). Detailed methods are exactly as described by Houslay *et al* (2019), and so not repeated  
644 here (note that here we assayed the free fraction of cortisol only). To validate the cortisol kits, we  
645 examined whether the kit standard curve was parallel to a serial dilution curve derived from pooled  
646 guppy water-borne hormone extract. 20µl was taken from each of the male samples and pooled; 20µl  
647 was taken from each of the female samples and combined into a separate pool. 400µl of the pools was  
648 serially diluted from 1:1 to 1:128 and these samples were assayed alongside the kit standard curve on  
649 two occasions (June and December 2017, marking the start and finish of sample processing). All  
650 dilution curves were parallel to the standard curve (slope comparison test, Zar 1996, p.355; June,  
651 male:  $t_{12} = 0.029$ ,  $P = 0.97$ ; June, female: :  $t_{12} = 0.343$ ,  $P = 0.74$ ; December, male: :  $t_{12} = 0.119$ ,  $P = 0.91$ ;  
652 December, female: :  $t_{12} = 0.224$ ,  $P = 0.83$ ). The serial dilution curves also identified 1:32 as an  
653 appropriate dilution to ensure that all samples fell on the linear phase of the standard curve. A total of  
654 37, 96-well plates were used and pooled sample was included at the beginning and end of each plate.  
655 Intra-assay coefficients of variation ranged from 0.12-19.83% with a median of 3.08%; the inter-assay  
656 coefficient of variation was 19.22%.

657

## 658 **Statistical methods**

659

660 All data handling and analysis was performed in R version 3.4.1 (R Core Team 2017). We used the  
661 'tidyverse' set of packages for data handling and visualisation (Wickham 2017), and ASreml-R 3.0

662 (Butler 2009) for fitting linear mixed effects models (as described in full below). We also used ‘nativ’  
663 for pedigree preparation and to estimate (approximate) standard errors on linear functions of  
664 variance components as estimated from the mixed models (Wolak 2012). All models fitted assumed  
665 (multivariate) Gaussian error structures, and we visually assessed residuals to verify this was  
666 reasonable (after data transformation in some cases). To test for significance of among individual  
667 and/or genetic (co)variance components, we fitted nested models with different random effects  
668 structures and compared them using likelihood ratio tests (LRTs). We calculated  $\chi^2_{nDF}$  as twice the  
669 difference in model log likelihoods, with the number of degrees of freedom ( $n$ ) equivalent to the  
670 number of additional parameters in the more complex model. When testing a single random effect  
671 (variance component), we assumed the difference to be asymptotically distributed as an equal mix of  
672  $\chi^2_0$  and  $\chi^2_1$  (denoted  $\chi^2_{0,1}$ ; Self and Liang, 1987; Visscher, 2006).

673

674 For each OFT and ST behaviour in turn (*relative area, time in middle, track length, shoaling tendency,*  
675 *and emergence time*), we used the random effects specification to partition phenotypic variation ( $V_p$ ,  
676 conditional on fixed effects as described below) into the effects of additive genetics ( $V_a$ ), permanent  
677 environment defined as the non-(additive) genetic component of among-individual differences ( $V_{pe}$ ),  
678 and housing group ( $V_{group}$ ), as well as residual variation ( $V_{residual}$ ). We natural log-transformed  
679 *emergence time* prior to analysis to meet assumptions of residual normality and homoscedasticity. For  
680 all behavioural traits, we included fixed effects of *assay repeat*, the *order within each group* in which  
681 the fish was trialled (mean-centred continuous predictor), *temperature* (mean-centred and scaled to  
682 standard deviation units), *time* (in minutes from midnight, mean-centred and scaled to standard  
683 deviation units), *age* (mean-centred and scaled to standard deviation units), *sex*, and the *generation*  
684 from the breeding population. For *shoaling tendency* only, we incorporated an additional fixed effect of  
685 *setup* (as detailed above). We tested the significance of genetic variance for each behaviour by LRT  
686 comparison of the corresponding full model to one in which the (additive) genetic random effect was  
687 excluded.

688



689 Cortisol data were also natural log-transformed for analysis. We formulated a bivariate model to test  
690 for both additive genetic variation and genotype-by-environment interaction (GxE) in cortisol levels  
691 across the two 'contexts' (i.e. samples retained for each individual at first and third confinement,  
692 denoted Cortisol<sub>1</sub>, Cortisol<sub>3</sub>). Random effects were first used to partition phenotypic (co)variance  
693 (conditional on fixed effects) into among-group and residual components. Fixed effects included the  
694 context-specific means, and overall effects of the *order* in which the fish was caught from each group  
695 for assay (mean-centred continuous predictor), *temperature* (mean-centred and scaled to standard  
696 deviation units), *time of day* (mean-centred and scaled to standard deviation units), *age* (mean-centred  
697 and scaled to standard deviation units), and *sex*. In addition, we included fixed covariates of *body mass*  
698 (mean-centred and scaled to standard deviation units) and a *sex* by *body mass* interaction (see Houslay  
699 *et al.* 2019 for rationale of controlling for body size effects on waterborne hormone levels in this way).  
700 Note that modelled in this way each individual is sampled only once for each context-specific cortisol  
701 trait so no random effect of individual identity is included. To test for additive genetic variation ( $V_a$ )  
702 we compared this first bivariate model to a second formulation that also included the (additive)  
703 genetic merit, but under the assumption that this is invariant with context within an individual (such  
704 that  $V_{a1}=V_{a3}$  and  $r_{a1,3}=1$  and there is no GxE). We then test for the GxE by comparing the second model  
705 to a third in which we allow GxE (i.e., the context-specific genetic variances are free to differ and the  
706 cross-context genetic correlation can be  $<+1$ ).

707

708 Lastly, we built a multivariate animal model to estimate  $\mathbf{G}$  and to test the hypothesised genetic  
709 integration among behavioural and physiological stress components. We retained only response traits  
710 that harboured significant  $V_a$  as shown in univariate models, and so the final model comprised  
711 response traits *relative area*, *time in middle*, *track length*, *emergence time* (log transformed), and  
712 *Cortisol* (log transformed). We multiplied (transformed) *emergence time* by -1 to simplify  
713 interpretation of estimated correlation structures (i.e., higher values for all behavioural traits then  
714 represent nominally 'bolder' behaviours). We also scaled all (transformed) response variables to  
715 standard deviation units. This was to facilitate model fitting, and also prevent scale effects  
716 complicating interpretation of eigenvectors of  $\mathbf{G}$ . Fixed and random effects were fitted on each trait as



717 specified for the univariate models. Note that one exception to this is that we elected to treat *Cortisol*  
718 as a single repeated-measures trait here (with two repeats, one per context) such that a permanent  
719 environment effect was now included.

720

721 We specified additive genetic genetic (**G**), permanent environment (**PE**), group (**GROUP**), and residual  
722 (**R**) covariance structures as unstructured matrices to be estimated. Note that **R** partitions  
723 observation-level covariances (as opposed to individual-level in **PE**) that are not definable or  
724 statistically identifiable if traits are not measured at the same time (i.e., all covariances relating to  
725 *emergence time* or *Cortisol*). Where this was the case we constrained specific covariance terms in **R** to  
726 equal zero. We tested for overall additive genetic covariance among the traits by comparing this model  
727 against a reduced one in which **G** was specified as a diagonal matrix (i.e., additive variances are  
728 estimated but covariances are assumed to equal zero). To aid the interpretation of covariance terms  
729 contained in **G**, we calculated the corresponding genetic correlations  $r_a$  from the full model. For any  
730 pair of traits (x,y),  $r_{a(x,y)} = COV_{a(x,y)} / (\sqrt{V_{a(x)}} \times \sqrt{V_{a(y)}})$ . We also subjected our estimate of **G** to eigen  
731 decomposition to determine the proportion of additive genetic variation captured by each principal  
732 component and assess whether a single major axis of variation could indeed explain most of the  
733 genetic variance in the multivariate phenotype (consistent with a simple proactive-reactive coping  
734 style model). We estimated uncertainty on the trait loadings associated with each principal component  
735 (eigenvector) using a parametric bootstrap approach as described by Boulton et al (2014).

736

737 For visualisation of bivariate relationships at the additive genetic level, we used the R package 'ellipse'  
738 (Murdoch & Chow 2018) to determine the coordinates of an ellipse representing the approximate 95%  
739 confidence region of deviations based on the point estimate of **G**. We repeated this procedure for the  
740 corresponding regions defined from 5000 bootstrapped values of **G** (i.e., to indicate uncertainty arising  
741 from estimation of the genetic covariance structure itself).

742

743

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