Evolutionary genetic integration of behavioural and endocrine components of the stress response

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Abstract

The vertebrate stress response comprises a suite of behavioural and physiological traits that must be functionally integrated to ensure organisms cope adaptively with acute stressors. The expectation that natural selection has favoured functional integration leads to a prediction of genetic integration: genetic variation in the stress response should include covariation between its component behavioural and physiological traits. Despite the implications of such genetic integration for our understanding of human and animal health, as well as evolutionary responses to natural and anthropogenic stressors, formal quantitative genetic tests of this prediction are lacking. Here we demonstrate that Trinidadian guppies (*Poecilia reticulata*) show genetic variation in a suite of behavioural and physiological components of the acute stress response, and that these are indeed integrated into a single major axis of genetic variation. This axis appears to reflect continuous variation in the magnitude of integrated stress responsiveness, rather than variation in 'coping style' (a verbal model that postulates equal levels of stress responsiveness will manifest differently across individuals). The genetic integration we find here could either facilitate or constrain evolutionary responses to selection, depending upon the extent to which the direction of selection aligns with this single major axis of genetic covariation among stress response traits. Such integration also suggests that, while stress-related disease typically arises from physiological components of the stress response, selection on the genetically correlated behavioural responses to stress could offer a viable non-invasive route to the genetic improvement of health and welfare in captive animal populations.

45 **Introduction**

46   Stress responses comprise suites of physiological and behavioural traits that

47 enable individuals to cope with adverse conditions (Romero 2004; Øverli *et al.* 2007;

48 McEwen & Wingfield 2010; Taborsky *et al.* 2020). Some individuals are likely better at

49 coping with adverse conditions than others, and understanding the role played by

50 underlying genetic variation could have important implications for managing stress-

51 related disease in captive populations and predicting the evolutionary responses of free-

52 living populations to both natural and anthropogenic stressors (Barton & Iwama 1991;

53 Koolhaas *et al.* 1999; McEwen & Wingfield 2003; Romero 2004; Koolhaas 2008). For

54 instance, if among-individual differences in stress response traits are a product of

55 genetic variation (Koolhaas *et al.* 1999, 2007), then they may be a viable target for

56 artificial selection strategies (Mignon-Grasteau *et al.* 2005). This could be used to reduce

57 stress-related welfare issues in captive populations (e.g. in livestock; Broom & Johnson

58 1993; von Borell 1995; Möstl & Palme 2002). In free-living populations, variation in

59 stress response traits is expected to cause fitness variation under stressful conditions

60 (Wingfield 2003; Koolhaas 2008). Thus, exposure to stressors could lead to evolutionary

61 changes in the distributions of traits that contribute to a population's long-term

62 resilience in the face of natural (e.g. predation risk; Clinchy, Sheriff & Zanette 2013)

63 and/or anthropogenic (Tarlow & Blumstein 2007; Busch & Hayward 2009; Angelier &

64 Wingfield 2012) challenges. However, as the evolutionary response of any trait to

65 selection is determined in large part by its genetic variation, our ability to predict – and

66 potentially harness – evolutionary changes in the stress response is currently hampered

67 by limited understanding of the underlying genetics.

68   Natural selection does not act on single traits in isolation, but rather on

69 multivariate phenotypes (Lande & Arnold 1983; Blows 2007). This is likely to be an

70 important consideration for understanding the evolution of the stress response. For

71 instance, while glucocorticoid (GC) levels are frequently used to measure the stress

72   response (McEwen & Wingfield 2003; Korte *et al.* 2005), an individual's first line of

73   defence against acute environmental challenges will typically be behavioural (Moberg

74   2000). This may include risk avoidance strategies as well as the widely known 'fight-or-

75   flight' responses. Subsequent GC release then serves to mediate physiological (and

76   further behavioural) responses (Wingfield *et al.* 1998; Wingfield & Kitaysky 2002).

77   Natural selection is therefore expected to favour combinations of behavioural and

78   physiological stress response traits that act synergistically to maintain fitness under

79   stressful conditions (Koolhaas *et al.* 1999; Øverli *et al.* 2007). Since evolutionary theory

80   predicts that correlational selection will shape the structure of multivariate quantitative

81   genetic variance (as represented by the genetic covariance matrix **G**; Blows 2007), we

82   should expect genetic – as well as phenotypic – integration of behavioural and

83   physiological stress response traits (McGlothlin & Ketterson 2008; Ketterson, Atwell &

84   McGlothlin 2009; Cox, McGlothlin & Bonier 2016). However, there exists relatively little

85   supporting empirical work for this prediction.

86   The most compelling evidence for genetic integration of behavioural and

87   physiological stress response traits to date comes from artificial selection experiments

88   on domestic animal populations (e.g., rainbow trout (Pottinger and Carrick, 1999),

89   Japanese quail (Jones, Satterlee & Ryder 1994), house mice*,* Veenema *et al.* 2003b). For

90   example, lines of rainbow trout selected for stress-induced plasma cortisol levels

91   (Pottinger and Carrick, 1999) experienced correlated evolutionary changes in behaviour

92   (Øverli, Winberg & Pottinger 2005). In a rare study of a wild-type population (albeit

93   under captive conditions) species, cortisol levels were found to evolve in response to

94   selection on behavioural 'personality' in great tits (*Parus major*; Carere *et al.* 2003). In

95   these examples, the correlated responses of behavioural and physiological stress

96   response traits to selection are consistent with some degree of genetic integration of

97   these behavioural and physiological traits. However, the extent and 'structure' of this

98   integration remains unclear, and some results were inconsistent with a hypothesised

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99   simple axis of genetic (co)variation among behavioural and physiological components of

100   the stress response. For example, in the trout study, the 'low-cortisol response' selected

101   lines actually showed a higher metabolic stress response under confinement (suggestive

102   of opposing responses to selection by different physiological components of the stress

103   response; Trenzado, Carrick & Pottinger 2003). Surprisingly (given the correlated

104   selection response), in the great tit study cortisol levels were found to be phenotypically

105   unrelated to behavioural 'boldness' (the trait selected on) under standardised testing

106   (Thomson *et al.* 2011).

107   While selection experiments illustrate that genetic integration of behaviour and

108   physiology can occur, estimation of the genetic variance-covariance matrix (**G**) through

109   quantitative genetic modelling provides a complementary strategy that also allows

110   investigation of exactly how (multivariate) genetic variation is structured within

111   populations. In the context of the stress response, this should provide insights into both

112   how selection has acted in the past (Ketterson *et al.* 2009), and whether responses to

113   future selection are likely to be constrained (Blows & Walsh 2009; Walsh & Blows

114   2009). The former follows from the fact that strong correlational selection should lead

115   to integration of traits in **G** over the long term, a phenomenon explored most commonly

116   for suites of morphological traits (following, e.g., Cheverud 1982), but that is equally

117   applicable to any aspect of phenotype (see, e.g., Hine, Chenoweth & Blows 2004; Hunt *et*

118   *al.* 2007; Oswald, Singer & Robison 2013 for examples pertaining to behavioural

119   evolution and mate choice). The latter follows from the fact that the direction (in

120   multivariate trait space) and magnitude of a response to contemporary selection is

121   limited by the amount of variance in **G** alignment with the vector of (directional)

122   selection β (Blows & Walsh 2009; Walsh & Blows 2009).

123   Here we estimate **G** for behavioural and physiological components of the acute

124   stress response in Trinidadian guppies (*Poecilia reticulata*). This enables us to

125   determine not only (i) whether these components are genetically integrated into a

126   single major axis of genetic (co)variation, but also (ii) whether the structure and

127   orientation of this axis suggests variation in overall stress responsiveness and/or

128   'coping style' (explained further below; Koolhaas *et al.* 2010; Boulton *et al.* 2015). We

129   use fish from a captive colony of guppies derived from wild ancestors sampled from the

130   Aripo River, Trinidad in 2008 and subsequently maintained at high population size

131   (with no deliberate inbreeding or selection). We have validated the use of standardised

132   'open field trials' (OFTs) for testing (acute) behavioural stress responses in this species

133   (Houslay *et al.* 2018), and demonstrated significant additive genetic (co)variance

134   underpinning variation in risk-taking, exploratory, and 'flight' type components of the

135   behavioural stress response using this testing paradigm (White & Wilson 2018; White,

136   Houslay & Wilson 2018). We have also demonstrated, using a non-invasive waterborne

137   hormone sampling method, that individuals differ significantly in their GC (specifically,

138   free circulating cortisol) response to an acute stressor (handling, coupled to short term

139   isolation and confinement; Houslay *et al.* 2019) and that, on average, this physiological

140   response declines with repeated stressor exposure (consistent with habituation).

141   Nothing is known about the genetic basis of variation in these physiological traits, or

142   about their integration (phenotypically or genetically) with behavioural components of

143   the stress response.

144   First, we combine OFT results with complementary 'emergence trials' (ET) and

145   'shoaling trials' (ST) to characterise among-individual and genetic variation in the

146   behavioural stress response. Second, we characterise the physiological stress response

147   and its rate of habituation by assaying GC levels following first and third exposure to a

148   handling and confinement stressor (see methods). Utilising repeated behavioural and

149   physiological testing of individual fish within a known pedigree structure, we are able to

150   estimate the repeatable (among-individual) component of phenotypic (co)variance in

151   these stress response traits, and then determine the additive genetic contribution to this

152   (**G**; the genetic variance-covariance matrix for this suite of stress response traits). We

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153   predict that individual traits will be heritable and that **G** will contain strong genetic

154   correlation structure between behavioural and physiological components of the stress

155   response consistent with genetic integration. We also predict that **G** will be dominated

156   by a single major axis of genetic variation in multivariate trait space, but are more

157   circumspect about how that might look. The 'stress coping style' model (Koolhaas *et al.*

158   1999) predicts variation in the type of response to stress. Simplistically, as originally

159   proposed this verbal model posits that individuals (or genotypes) perceive equal

160   degrees of stress but differ in how this manifests phenotypically: genotypes at one end

161   of the axis having 'reactive' behavioural phenotype (e.g., freezing behaviour) coupled to

162   lower GC levels, while the 'proactive' end is characterised by more active 'fight or flight'

163   behaviour coupled to higher GC levels. However, previous analyses of this population

164   suggest variation may be more in the stress responsiveness than coping style (Houslay

165   *et al.* 2018; Prentice *et al.* 2020; White, Pascall & Wilson 2020). That is to say, some

166   individuals (or genotypes) perceive the trial as a more severe stimulus and exhibit more

167   characteristic stress behaviours (e.g., flight and/or freezing, thigmotaxis) while others

168   show more typical 'unstressed' behavioural profiles (e.g., exploration of the arena). In

169   this scenario we predict high GC levels to co-occur with characteristic stress behaviours.

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171   **Results**

172   In total we obtained (multivariate) behavioural data from 7,637 trials (3,812 OFTs,

173   1,548 ETs and 1,039 STs) on 1,518 individual fish. The number of individuals

174   phenotyped (OFTs = 1,487, ETs = 806, STs = 532) and the mean number of observations

175   per fish (OFTs = 2.6, ETs = 1.9, STs = 2.0) varied across the behavioural data types. All

176   fish were contained within a genetic pedigree structure comprising maternal full-

177   sibships nested within paternal half-sibships. This structure was produced via multiple

178   round of breeding work and has a maximum depth of 5 'generations'. Some of the OFT

179   data have already been used in studies of the evolutionary genetics of personality

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180   (White & Wilson 2018; White *et al.* 2018), but here we extend that dataset and use it in

181   conjunction with other behavioural and physiological measures for different purposes.

182   We also obtained 1,238 waterborne assays of cortisol levels for 629 fish (almost all from

183   the final generation). The handling and confinement stressor applied for this assay was

184   performed 3 times (at 48h intervals) for all fish tested, but the holding water sample

185   was only processed for GC content at two time points (the first and last confinement,

186   subsequently $Cortisol_1$ and $Cortisol_3$). Full details of husbandry, phenotyping and

187   analysis are provided in Materials and Methods.

188

189   **Genetic variance in behavioural components of the stress response**

190   Behavioural data were extracted from OFTs, ETs and STs using video tracking of fish (as

191   described in White, Kells & Wilson 2016; Houslay *et al.* 2018). Time to emerge from the

192   shelter ('*emergence time*') was extracted from ETs and natural log (ln) transformed for

193   analysis, while *shoaling tendency* was calculated from STs as the time spent in the third

194   of the tank closest to a same-sex shoal (which was visible but physically separated)

195   minus the time spent in the third of the tank farthest from the shoal. The OFT, ET and ST

196   testing paradigms are all considered to assay behavioural components of the stress

197   response in the broad sense, as each test starts with the capture and transfer of the focal

198   fish into a novel, brightly lit, arena away from their home tank and familiar tank mates.

199   Three traits were defined from the OFT and measured by videotracking for 4m 30s after

200   an initial 30 second acclimation period on transfer into arena. *Track length* (distance

201   swum), *area covered* (as a proportion of the arena floor area), and *time in the middle*

202   (i.e., in the central area of the open field arena away from the tank walls, which is

203   assumed to be perceived as riskier; e.g., Houslay *et al.* 2018) were recorded. Note low

204   values of *time in the middle* imply thigmotaxis (i.e., tendency to avoid exposure to

205   potential threats by hugging walls). All three of these OFT traits are repeatable and

206   heritable in this population (White *et al.* 2016, 2018; Houslay *et al.* 2018; White &

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207   Wilson 2018). However, the absence of a strong positive correlation between *track*

208   *length* and *area covered* (Fig 1A) is notable and potentially biologically informative; if

209   fish moved randomly with respect to direction in the arena then *area covered* would

210   increase monotonically (to an asymptote at 100%) with *track length*. A possible

211   explanation is that a long *track length* can sometimes arise from a (putatively) less

212   stressed fish exploring the arena (fish 1 in Fig 1B) and sometimes a (putatively) more

213   stressed fish exhibiting a typical 'flight' response (Fish 4 in Fig 1B). These can be

214   discriminated based on whether, in a given trial, high track length is associated with

215   high *area covered* and *time in the middle* (exploration) or the converse (flight response).

216   To quantitatively discriminate between exploratory behaviour and flight

217   responses we derived a new trait, '*relative area covered*'. We used a simple simulation

218   procedure (see Methods) to predict *expected area covered* for any given *track length*

219   under a null 'random swim' within the arena (Fig 1C). *Relative area covered* is then

220   calculated as *observed area covered – expected area covered* given the *track length* (Fig

221   1D) and will be high for fish engaging in exploration, and low for an obvious 'flight'

222   response manifest as rapid swimming around the tank walls.

223   Pedigree-based 'animal models' (Wilson *et al.* 2009) were used to test for and

224   estimate additive genetic variation in each of the five behavioural traits while

225   controlling statistically for social housing group and non-genetic sources of among-

226   individual variance (as well as several fixed effects; see methods for full details). These

227   confirmed the presence of significant additive genetic variation for the *relative area*

228   *covered* trait, as well as for *track length* and *time in the middle* (as expected from

229   previous findings; White & Wilson 2018; White *et al.* 2018) as well as *emergence time*

230   (Table 1). With the exception of *shoaling tendency*, heritabilities (conditional on fixed

231   effects; see methods) are low to moderate (range of 8-17%; Table 1) but within the

232   expected range for behaviours (Stirling, Réale & Roff 2002). We detected no additive

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233   genetic variance for *shoaling tendency* (Table 1), despite there being repeatable

234   differences among individuals; R = 0.19 ± 0.04; $\chi^2_{0,1}$ = 20.01, P < 0.001).

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236   **Genetic variance in physiological components of the stress response**

237   Using a series of nested bivariate animal models, we tested for additive genetic variance

238   in cortisol levels (ln-transformed) following stressor exposure (handling and

239   confinement) and for genotype-by-environment interaction (GxE). In this context, the

240   environment (E) is the trial number in each fish's stress trial series (i.e. cortisol level

241   following stressor trial 1 or 3). Any GxE present can therefore be interpreted as genetic

242   variance for habituation to the stressor, given that the average cortisol level was lower

243   following exposure to the third stressor than the first (ln transformed ng/hr, mean ± SE;

244    $Cortisol_1$ = 8.50 ± 0.05, $Cortisol_3$ = 8.05 ± 0.06, Wald $F_{1,12.9}$ = 120.5, P < 0.001; see

245   methods for explanation of units). We first modelled $Cortisol_1$ and $Cortisol_3$ as distinct

246   response variables in a bivariate framework assuming no GxE (such that we constrain

247    $V_{A\text{-}Cortisol1}$ = $V_{A\text{-}Cortisol3}$ and the cross context additive genetic correlation $r_{A\text{-}Cortisol1,Cortisol3}$ =

248   1). This model revealed a significant additive genetic component to variation among

249   individuals in their cortisol levels following stressor exposure ($\chi^2_{0,1}$ = 6.58, P = 0.005).

250   Expanding the model to allow GxE (by estimating separate genetic variances for

251    $Cortisol_1$ and $Cortisol_3$ and allowing the cross-context genetic correlation to deviate

252   from +1) provides a significantly better fit to the data (χ^2_2 = 9.65, P = 0.008), meaning

253   GxE is present. This can be viewed as genetic variance for habituation to repeated

254   stressor exposure, or as a change in genetic variance for cortisol from the first to the

255   third sampling (Figure 2). These are two perspectives of the same phenomenon; a

256   reduction in additive genetic variance between the first stressor ($V_{A\text{-}Cortisol1}$ = 0.076 ±

257   0.028) and the third ($V_{A\text{-}Cortisol3}$ = 0.047 ± 0.029) arises because genotypes with higher

258   than average levels for *Cortisol_1* habituate more rapidly (i.e. have more negative reaction

259   norm slopes). Note however that the rank order of the genotypes does not appreciably

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260   change across the two contexts (i.e. genetic reaction norms show little crossing; Figure

261   2), so there is a strong positive cross-context genetic correlation ($r_{A\text{-Cortisol1,Cortisol3}} \pm SE =$

262   0.74 ± 0.25).

263   In this model we also find that variance in cortisol explained by housing group

264   effects is similar across contexts ($V_{Group\text{-}Cortisol1} = 0.034 \pm 0.013$, $V_{Group\text{-}Cortisol3} = 0.045 \pm$

265   0.016), but that residual (unexplained) variance is greater after the third stressor

266   exposure ($V_{R\text{-}Cortisol1} = 0.166 \pm 0.021$, $V_{R\text{-}Cortisol3} = 0.229 \pm 0.025$). In combination, the

267   changes in both additive genetic and residual variance between the two contexts lead to

268   appreciably higher heritability for cortisol levels following the first stressor exposure

269   relative to the third ($h^2_{Cortisol1} = 0.275 \pm 0.093$, $h^2_{Cortisol3} = 0.146 \pm 0.088$).

270

271   **Testing for genetic integration and identifying the major axis of genetic**

272   **(co)variance**

273   There is strong evidence for phenotypic integration of *Cortisol* with behaviour at the

274   among-individual levels (see Table S5). To test for and characterise the hypothesised

275   genetic integration between behavioural and physiological components of the stress

276   response, we built a multivariate animal model to estimate **G**. We excluded *shoaling*

277   *tendency* given the absence of detectable genetic variance in the univariate model. We

278   also elected to treat cortisol as a single trait (allowing for a fixed effect of stressor

279   exposure number (1 vs 3) on the mean). Although the above analysis demonstrates GxE

280   for cortisol, the strong positive cross-context genetic correlation justifies collapsing

281   Cortisol$_1$ and Cortisol$_3$ into a single trait to maximise statistical power to detect any

282   genetic covariance with behaviour.

283   Our final model contained 5 response traits: *relative area covered*, *time in the*

284   *middle* and *track length*, (ln-transformed) *emergence time*, and (ln-transformed) *Cortisol*

285   (now treated as two repeats of a single trait). We standardised all (transformed) traits

286   to standard deviation units, to assist multivariate model fitting and to prevent

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287   eigenvectors of **G** (see below) being dominated by traits with higher variance in

288   observed units. To simplify interpretation of **G** we also multiplied *emergence time* by -1

289   after transformation. Thus high values denote rapid emergence from the shelter.

290   The resultant estimate of **G** (Table 2) contains significant additive genetic

291   covariance structure overall (Likelihood Ratio Test of the full model vs. a reduced model

292   **G** that contains variances but not covariances: χ^2_{10} = 36.79, P < 0.001). Strong genetic

293   covariance/correlation estimates, both positive and negative, were found for a number

294   of trait pairs and were deemed statistically significant (based on the bootstrapped 95%

295   confidence intervals not crossing zero). We find strong genetic covariance among all 3

296   OFT traits: *track length* shows significant negative genetic covariance with both *relative*

297   *area covered* and *time in the middle*, and *relative area covered* and *time in the middle*

298   show significant positive genetic covariance with one another. *Track length* also has a

299   significant positive genetic covariance with *–ln(emergence time)*. Ln-transformed

300   *Cortisol* shows significant negative genetic covariance with *time in the middle* (Figure 3).

301   Ln-transformed *Cortisol* also covaries negatively with both *relative area covered* and *–*

302   *ln(emergence time)*, and positively with *track length*, although these covariances were

303   not significantly different from zero (based on 95% confidence interval).

304   Eigen decomposition of **G** provides a more holistic view of the genetic

305   covariance structure and the level of integration among traits. Here the major axis (first

306   principal component, PC1, with 95% confidence intervals from 5000 bootstrap

307   replicates) explains 57.8% (47.3%, 79.6%) of the genetic variance in multivariate

308   phenotype (PC2 = 25.7% [14.3, 37.6]; PC3 = 9.3% [4.7, 16.5]; PC4 = 5.6% [0, 9.2]; PC5 =

309   1.6% [0, 3.6]). All traits except *emergence time* load significantly on this axis (Figure 4).

310   *Relative area covered* and *time in the middle* load in one direction, while *track length* and

311   *Cortisol* load in the other direction. This structure is suggestive of a single major axis of

312   genetic variation in integrated stress response, where genotypes at one end of this axis

313   can be considered to have 'weaker' behavioural stress responses to the OFT assay (i.e.,

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314 swim shorter distances, spend more time in the central area of the tank, and exhibit

315 exploratory swimming patterns that cover greater areas relative to their distance

316 swum) and 'weaker' physiological responses to stress (i.e., produce lower cortisol levels

317 in response to the stressor). Meanwhile, genotypes at the other end of this axis can be

318 considered to have 'stronger' behavioural responses to stress (i.e., swimming further,

319 while spending more time close to the tank edges, and covering less area relative to

320 their distance travelled in OFTs) and 'stronger' physiological responses to stress (i.e.,

321 produce higher cortisol levels in response to the stressor).

322

323 **Discussion**

324 In this study we sought to determine whether – and to what extent – there exists

325 genetic variation for, and integration between, behavioural and physiological

326 (endocrine) components of the stress response. Our results provide three main novel

327 insights. First, we find that genetic variation does underpin individual differences in

328 both behavioural and physiological components of the stress response. Second, we find

329 genetic covariance structure among these behavioural and physiological traits,

330 indicating that they are indeed genetically integrated. Thirdly, having identified the

331 structure of the major axis of **G** we suggest that it is more readily interpreted as an axis

332 of genetic variation in stress responsiveness than in stress coping style (although we

333 acknowledge the distinction may be somewhat subjective). Overall, by estimating the

334 genetic covariance structure among traits we find the first quantitative genetic support

335 to date for the hypothesis of evolutionary integration between behavioural and

336 endocrine components of the stress response.

337 We find heritable (co)variation in and among behaviours assayed in the open

338 field trial (OFT), including the derived trait *relative area covered*. The latter trait, derived

339 by considering an appropriate biological null model of the relationship between *track*

340 *length* and (absolute) *area covered* serves as a useful proxy for exploratory behaviour.

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341   Here we demonstrate an axis of repeatable and heritable variation that spans from less

342   active but more exploratory swimming patterns (lower *track length* but higher *relative*

343   *area covered* and *time in the middle*) through to a 'flight' type response, characterised by

344   higher activity coupled to thigmotaxis and low exploration (higher *track length*, lower

345   *relative area covered* and *time in the middle*). Although the distinction is both subjective

346   and arguable, we interpret this axis as more consistent with variation in the magnitude

347   of stress responsiveness than in coping style. This is because, while low *track lengths*

348   could arise from stressed fish exhibiting 'reactive' freezing behaviour, we would not

349   necessarily expect it to be associated with high *time in the middle* (i.e. reduced

350   thigmotaxis). Given the wide use of OFTs in biomedical research (e.g., Rex *et al.* 1998;

351   von Horsten, Karl & Pabst 2003) as well as in animal behaviour, our phenotyping

352   approach may have broad applicability for discriminating between exploration and

353   stress/anxiety-related behaviours. At the slight risk of introducing semantic confusion,

354   we also note that the OFT paradigm is widely applied to studies of 'shy-bold' type

355   personality variation in fishes (Toms, Echevarria & Jouandot 2010) and other

356   vertebrates (Carter *et al.* 2013; Perals *et al.* 2017). The extent to which behavioural

357   differences deemed characteristic of a 'shy—bold' personality axis (commonly, if not

358   universally defined as repeatable variation in response to perceived risk; Wilson *et al.*

359   1994) should be viewed as equivalent to variance in behavioural stress responsiveness,

360   or coping style, is a matter of debate (see Boulton *et al.* 2015). We view these as

361   overlapping – if not necessarily identical – concepts, and suggest it will generally be

362   prudent to empirically assess the structure of OFT variation for any system rather than

363   assume a priori that it will match a preferred verbal model (see also White *et al.* 2016;

364   Houslay *et al.* 2018; White, Pascall & Wilson 2020).

365   We also find significant heritable variation in *emergence time*, although this is

366   not tightly integrated with OFT traits in **G** in the manner we had expected. Faster

367   emergence in typically interpreted as reflecting a lack of fear of the open arena (i.e.,

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368   greater 'boldness'; see Burns 2008). In our study the qualitative pattern in **G** runs

369   counter to this, if we consider that boldness and behavioural stress responsiveness are

370   broadly analogous. Thus genotypes predisposed to shorter *emergence time* are

371   associated with greater *track length* and, albeit non-significantly, lower *relative area*

372   *covered* and *time in the middle* during OFT trials. That is, shorter *emergence time* is

373   associated with the putatively more stressed 'flight' end of the behavioural axis revealed

374   in the OFT. Our interpretation of this result is that at least some genotypes (and

375   individuals) likely perceive the shelter area as less safe than the open arena. This

376   possibility was also suggested by an earlier finding that some individual guppies

377   decrease (rather than increase) shelter use following simulated predation events

378   (Houslay *et al.* 2018). If general to other systems, unexpected results such as these may

379   have important consequences for interpretation of personality tests, and correlations

380   (or lack thereof) between behaviours assayed across test paradigms (Carter *et al.* 2013).

381   Tendency to shoal varies among individuals but is not detectably heritable.

382   Though not generally considered a stress-response trait *per se*, shoaling is an anti-

383   predator behaviour in guppies (Herbert-Read *et al.* 2017). We had therefore predicted

384   that heightened perception of risk in the open field might also be associated with

385   increased shoaling tendency. This was not the case at the among-individual level (Table

386   S5), while the absence of detectable genetic variance meant that we could not test this

387   prediction in **G**.

388   We find strong evidence of significant additive genetic variance in a key

389   physiological component of the stress response: waterborne cortisol concentrations

390   following exposure of the fish to a handling stressor. Our findings suggest that

391   previously detected differences among individuals in cortisol response to a stressor

392   (Houslay *et al.* 2019) are primarily attributable to genetic effects, with the estimated

393   heritability ($h^2 = 0.26$) being almost 75% of the individual–level repeatability (R = 0.35)

394   for ln-transformed *Cortisol*. In addition, by adopting a reaction norm approach to

15

395   modelling stress physiology, as recently advocated by ourselves (Houslay *et al.* 2019)

396   and others (e.g., Fürtbauer *et al.* 2015; Hau & Goymann 2015; Taff & Vitousek 2016), we

397   detect GxE reflecting genetic differences in the extent of habituation to the stressor over

398   repeated exposures. This result is potentially important since poor habituation of the

399   hypothalamic-pituitary-adrenal/interrenal (HPA/I) response to repeated or ongoing

400   stressors can lead to well documented health problems in human and animal

401   populations (Segerstrom & Miller 2004; Koolhaas 2008; Romero, Dickens & Cyr 2009;

402   Mason 2010). Our detection of heritable variation in the degree of habituation to

403   stressors raises the possibility of developing targeted selection strategies to improve

404   welfare in captive populations (e.g., Frankham *et al.* 1986; Muir & Craig 1995; Oltenacu

405   & Algers 2009).

406   Our findings also highlight that there is greater additive genetic variance (and

407   heritability) for cortisol levels following the first exposure to the stressor than following

408   the third. This pattern, which occurs because genotypes that produce the highest

409   cortisol response at first exposure also show the most marked habituation, is consistent

410   with the idea of cryptic genetic variance (Paaby & Rockman 2014) being 'released' by

411   exposure to novel, and so potentially stressful, environments (Ledón-Rettig, Pfennig &

412   Crespi 2010; Ledón-Rettig *et al.* 2014; Berger *et al.* 2011). All else being equal, it also

413   means that selection on cortisol levels following stressor exposure should induce a

414   stronger evolutionary response in naïve relative to habituated fish. However, the strong

415   positive cross-environment correlation means that the ranking of genotypes with regard

416   to their cortisol responses is consistent across repeated stressor exposures. Thus

417   selection on the (average) GC response would result in a correlated evolutionary

418   response of habituation rate, and *vice versa*.

419   Considering all traits together, **G** shows evidence of genetic integration between

420   behavioural and endocrine components of the stress response. As noted with respect to

421   OFT behaviours, we consider that the major axis of **G** is best interpreted as genetic

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422   variance in the magnitude of stress responsiveness. Accordingly, genotypes tending to

423   show (putatively) more stressed 'flight' type behaviour in the OFT (i.e. thigmotaxis, high

424   *track length*, low *relative area covered*) also produce higher levels of cortisol following

425   the handling and confinement stressor. The fact that the only significant bivariate

426   correlation including cortisol is with *time in the middle* (negative correlation) suggests

427   that thigmotaxis is a particularly strong indicator of high stress responsiveness. We note

428   that our interpretation that **G** is dominated by variation in the magnitude of stress

429   responsiveness does not mean the 'coping style' model has no merit. Indeed, a

430   subsequent 'two-tier' iteration of the coping style model proposed that variation in the

431   magnitude of the stress response (termed 'stress reactivity') could be viewed as a

432   second axis, distinct from variation in the 'type' (or style) of response (Koolhaas *et al.*

433   2010).

434   Here **G** certainly contains substantial variation not explained by its leading axis

435   and this may point towards interesting avenues for further study. For instance, while

436   *relative area* and *tracklength* are negatively correlated and both load significantly on the

437   major axis of **G**, there is also much variation in the former at low to moderate *track*

438   *lengths* (illustrated by e.g., fish 1 and 4 in Figure 1). It seems plausible, if speculative,

439   that this could be caused by a tendency of some individuals to show initial immobility

440   (i.e. freezing) in response to the stressor but then recover quickly and begin explorative

441   swimming. Others may show an initial flight response before relatively rapid recovery

442   to explorative swimming. Such differences, if present, would not be detectable by simple

443   principal components analysis of covariance structures based on whole trial data (as

444   here and, for example, Van Reenen *et al.* 2005). Rather this would require more detailed

445   modelling of within-trial behavioural dynamics.

446   The genetic integration of behaviour and physiology detected here is consistent

447   with the idea that correlational selection in the past has led to the coevolution of these

448   stress response components. Covariance structure in **G** will modify, and potentially

17

449   constrain, evolutionary responses to selection - whether natural or artificial. Here we

450   have no direct knowledge of how contemporary selection is acting in the wild. Nor do

451   we know whether it might be changing as a consequence of anthropogenic stressors.

452   Thus we cannot comment directly on how **G** will shape future evolution of the guppy

453   stress response beyond noting that selection on behaviour will cause correlated

454   evolution of endocrine physiology (and vice versa). Nonetheless, while it seems

455   reasonable to expect that current integration of stress response in natural populations

456   should be broadly adaptive, this seems less likely in captive populations (at least for

457   species without a long history of domestication and opportunity for adaptation to

458   artificial environments). We know that prolonged, chronic activation of stress response

459   pathways (notably the HPA(I) axis) frequently disrupts health and survival in captive

460   animals (Huether 1996; Boonstra 2013). It may be that more stress-responsive

461   genotypes are disadvantaged in novel artificial conditions (e.g., if acute stress

462   responsiveness positively predicts susceptibility to chronic stress). However, even if

463   true this would not imply high (acute) stress-responsiveness was also disadvantageous

464   in the wild. Since natural selection should purge alleles that are universally detrimental,

465   it seems more plausible that genetic variation along the major axis described here is

466   maintained by some form of selective trade-off (as widely hypothesised for maintenance

467   of personality variation; e.g., Stamps 2007; Wolf *et al.* 2007; Réale *et al.* 2010). For

468   instance, genotypes susceptible to harm under chronic stressor exposure will likely

469   persist in populations if they also confer advantages under an acute stress challenge. In

470   natural populations not only is exposure to acute stressors more common than to

471   chronic stressors, but also selection through chronic stress exposure may be conditional

472   on (and subsequent to) surviving acute challenges (such as predator attacks).

473

474   **Conclusions and future directions**

18

475   Here we find evidence for genetic variation in – and integration of – behavioural

476   and physiological (endocrine) components of the stress response. Overall we consider

477   the structure of **G** to be more consistent with a continuous axis of variation in acute

478   stress responsiveness than with the widely invoked 'reactive – proactive' model of

479   variation in stress coping style (Koolhaas *et al.* 2007). This interpretation rests largely

480   on the structure of behavioural variation revealed by the OFT, which is dominated by an

481   axis running from genotypes that are more stress responsive (rapid 'flight' behaviour

482   coupled to thigmotaxis) to those that show more exploratory behaviour expected from a

483   (putatively) unstressed fish. Endocrine traits align with this axis: genotypes exhibiting

484   'flight' behaviour show higher cortisol levels (and exhibit faster habituation of GC

485   physiology) when subject to repeated handling and confinement stressors.

486   Our results suggest that correlational selection in the past has likely shaped the

487   multivariate stress response, and that continued evolution of stress-related behaviour

488   will have consequences for glucocorticoid physiology and *vice versa*. Determining

489   contemporary selection on the stress response, and testing the possibility that genetic

490   variation is maintained by fitness trade-offs, is thus an obvious – if empirically

491   challenging – next step to understanding the functional importance of genetic variation

492   in wild populations. In a more applied context, integration of behavioural and endocrine

493   stress-response components at the genetic level has potential utility for genetic

494   improvement of managed populations. Specifically, it may be possible to identify non-

495   invasive, high throughput, behavioural biomarkers and target them in selection schemes

496   to reduce chronic activation of the HPA/I axis and its attendant deleterious effects.

19

497 Table 1: Estimated variance components, along with adjusted heritability, for each trait as estimated in a univariate model (± standard error). Chi-

498 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
Relative area covered	26.35 ± 9.37	72.42 ± 9.28	33.33 ± 6.86	205.22 ± 5.90	0.08 ± 0.03	20.7	<0.001
Time in the middle	588.48 ± 139.61	554.58 ± 109.57	203.29 ± 53.41	2070.06 ± 60.08	0.17 ± 0.04	53.7	<0.001
Track length	26832.64 ± 5925.25	32204.05 ± 4868.88	9956.4 ± 2626.41	93921.54 ± 2711.28	0.16 ± 0.03	86.3	<0.001
log Emergence time	0.12 ± 0.05	0.06 ± 0.06	0.05 ± 0.02	1.07 ± 0.05	0.09 ± 0.04	23.2	<0.001
Shoaling tendency	0 ± 0	2457.36 ± 570.96	708.87 ± 316.30	9900.95 ± 622.10	0 ± 0	0	0.5
log Cortisol	0.07 ± 0.02	0.02 ± 0.02	0.01 ± 0.01	0.15 ± 0.01	0.26 ± 0.08	22.0	<0.001

499

500

502 Table 2: Additive genetic covariance-correlation matrix (G) from the full multivariate animal model. Genetic variances provided on the shaded

503 diagonal, with genetic covariances below and genetic correlations above. 95% confidence intervals in parentheses are estimated from 5000

504 bootstrapped replicates. Where the confidence intervals for any estimate do not cross zero the estimate is considered statistically significant (at the

505 0.05 alpha level) and are shown in bold.

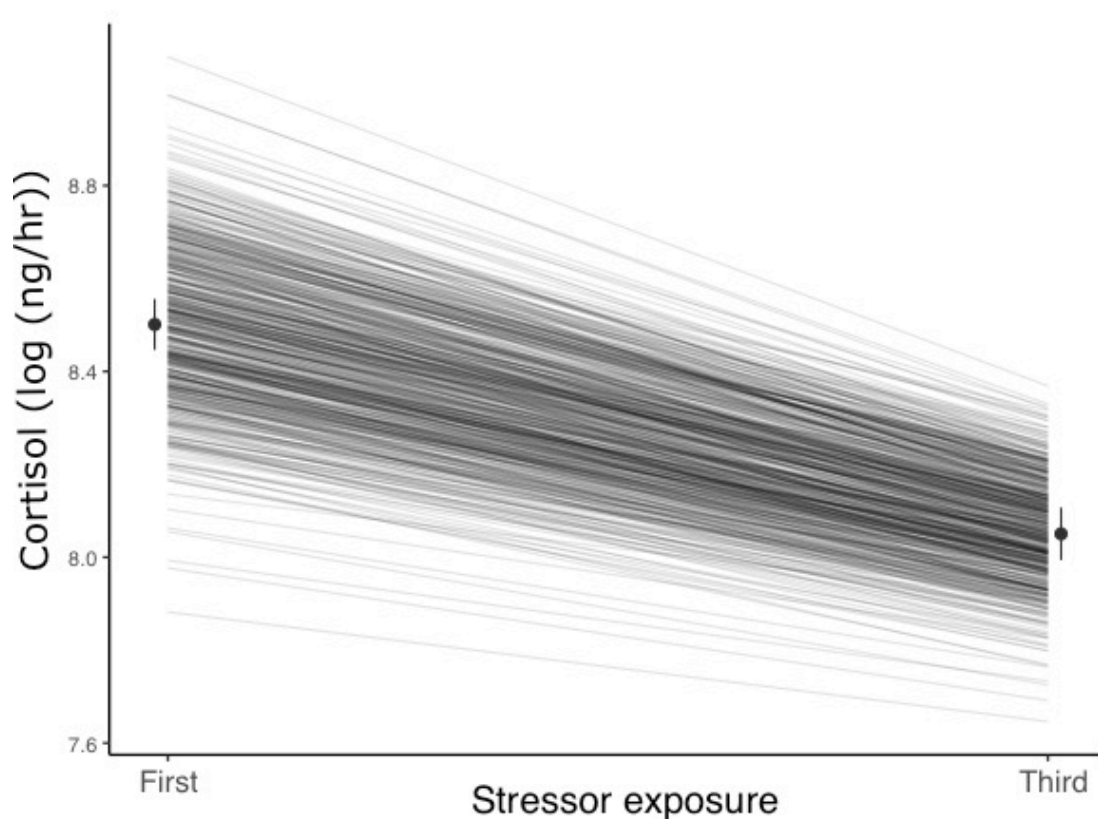|	Relative area covered	Time in the middle	Track length	-log Emergence time	log Cortisol
Relative area covered	**0.074 (0.029,0.122)**	**0.761 (0.549,0.955)**	**-0.506 (-0.758,-0.184)**	-0.503 (-1.394,0.256)	-0.414 (-1.035,0.225)
Time in the middle	**0.075 (0.031,0.124)**	**0.130 (0.062,0.191)**	**-0.554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

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507

508   Figure 1: The lack of a strong positive relationship between observed *track length* and *area covered*

509   (panel A), is initially puzzling given expected autocorrelation and that both are used as positive

510   indicators of exploratory (or 'bold') behaviour. Inset examples of OFT tracks from 4 individuals (panel

511   B) shed light on this. Fish 1 and 2 appear to be exploring the tank, while 3 and 4 are engaging in

512   stereotypical 'flight' behaviour characterised by strong thigmotaxis (remaining close to tank walls)

513   and/or rapid movement along tank walls. As a consequence, individuals 2 and 3 have similar *area*

514   *covered* during the OFT, but very different *track lengths*. We simulated random movements to define

515   an expected null relationship between *area covered* and *track length* (panel C; dashed red line shows

516   the fourth order polynomial model fit; see Appendix A). The polynomial regression was then used to

517   predict the expected area covered under random movement for each trial's observed *track length*, and

518   the '*relative area covered*' was calculated as the observation minus this prediction. Panel D shows the

519   resultant *relative area covered* plotted against *track length* for all trials (dashed red line at *relative area*

520   *covered* = 0, shows where individuals of any *track length* are expected to lie if they move randomly

521   with respect to direction). From this it is apparent that fish 1 and 2 have high *relative area covered*,

522   while 3 and 4 do not.

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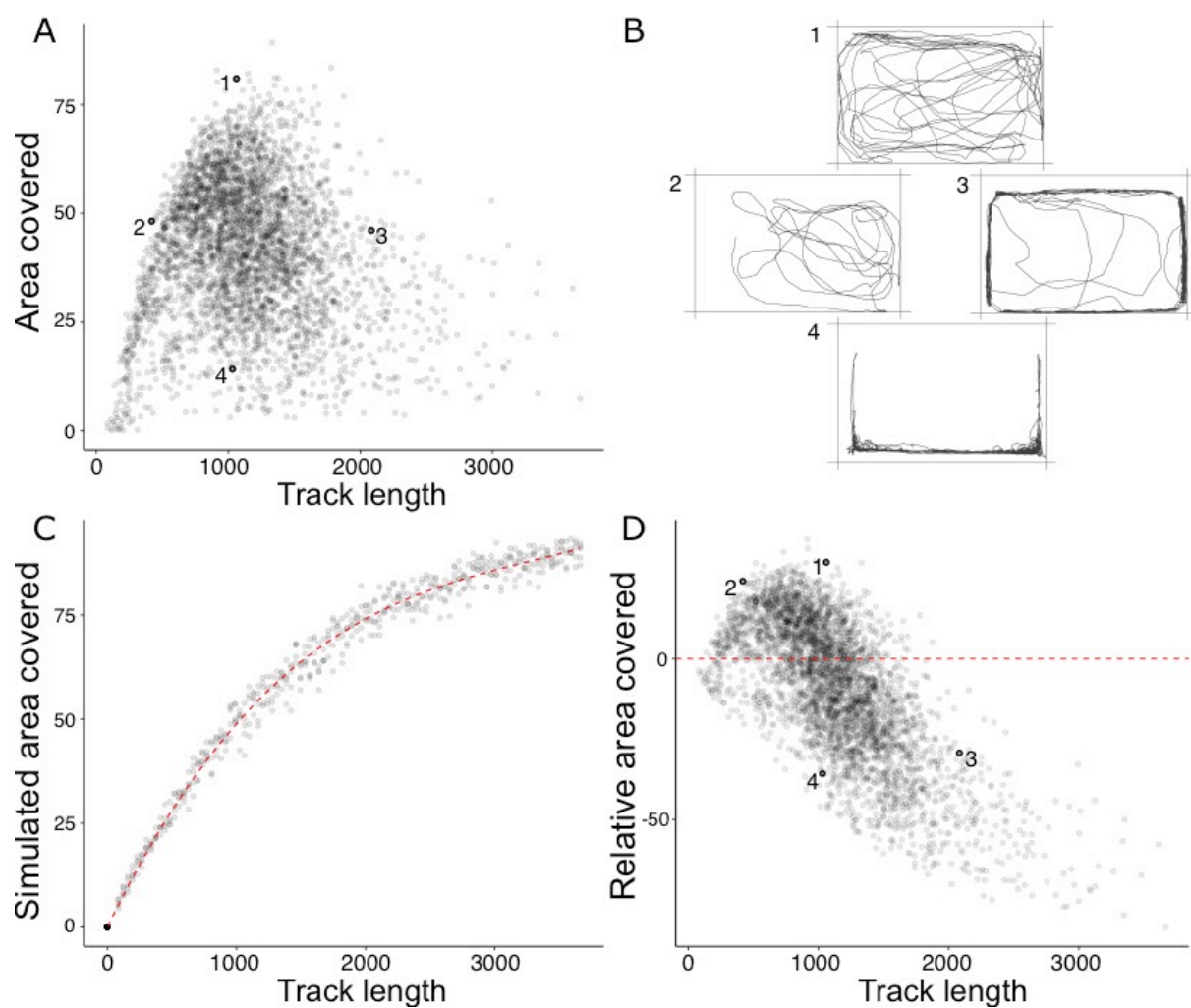
530   Figure 2: Guppies habituate to the waterborne sampling procedure, as shown by a decline in average

531   log-transformed cortisol level (ng/hr) following stressor exposure between first and third exposures.

532   Black circles and associated bars denote predicted population means (± standard error) from mixed

533   model analysis. Grey lines depict the predicted genetic reaction norms across repeated stressor

534   exposure for each individual. Weak, but statistically significant GxE is manifest as variance in the

535   genetic reaction norm slopes (i.e. lack of parallelism) and results in a slight reduction of genetic

536   variance for cortisol at the third exposure relative to the first.

537

538

539   Figure 1: The additive genetic relationship between log-transformed cortisol (y-axis) and four

540   behaviours (a, *relative area covered*; b, *time in the middle*; c, *track length*; d, *-log emergence time*).

541   Points show (predicted) bivariate genetic deviations from the population means, plotted for those

542   individuals in the pedigree with cortisol data. In each case the black ellipse depicts the 'shape' of the

543   relationship as given by the point estimate of **G**. Specifically it encompasses the area expected to

544   contain 95% of the bivariate genetic distribution for the population. Grey ellipses denote the

545   corresponding areas defined from 5000 bootstrapped replicates of **G**, and so highlight the uncertainty

546   around these bivariate distributions.
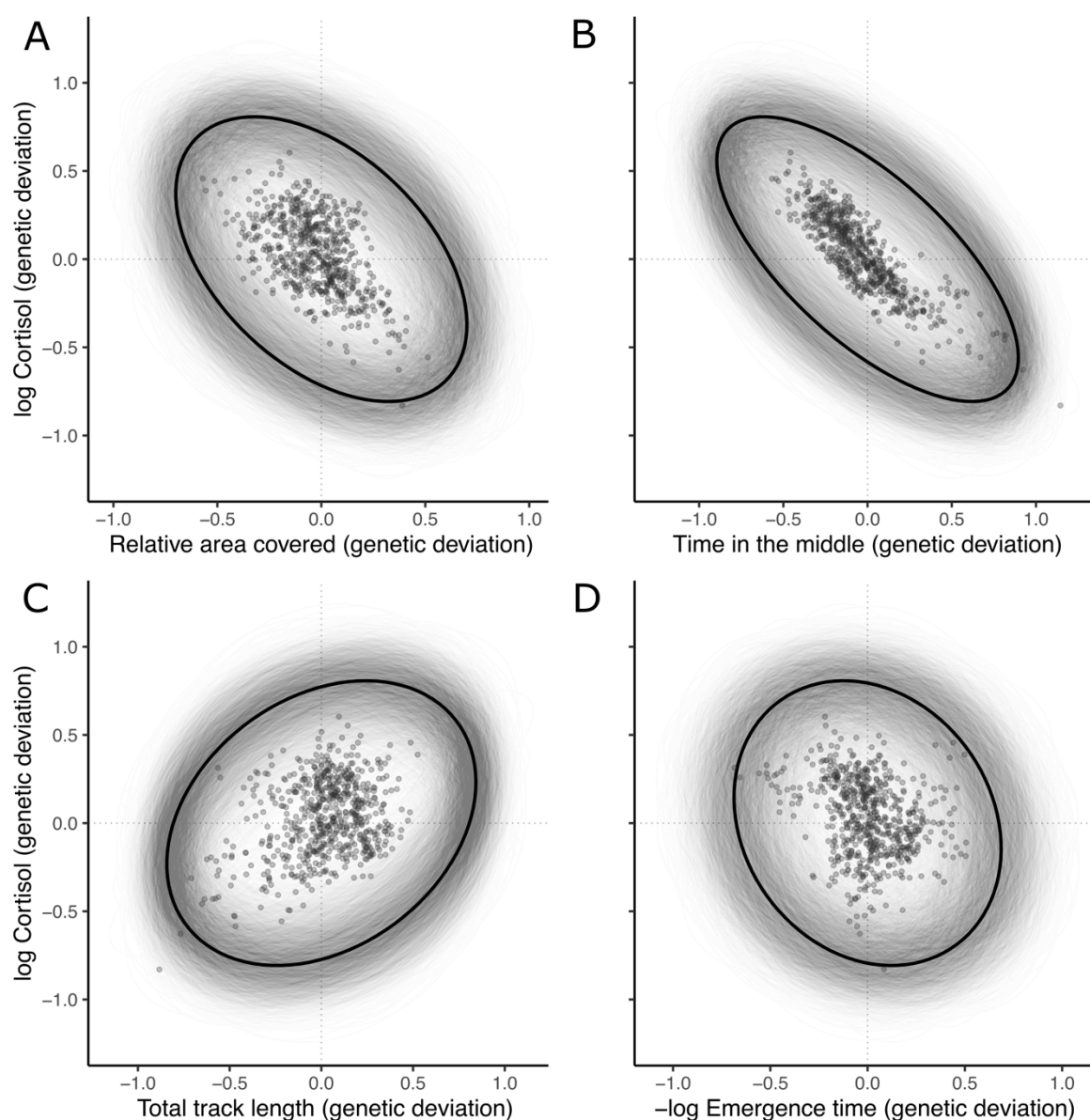
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549   Figure 2: Trait loadings from the first eigen vector (principal component) of **G**. This axis explains

550   57.8% of the genetic (co)variation found in the focal behavioural and physiological components of the

551   stress response in our guppy population. Points show trait loadings from the first eigen vector of our

552   estimate of G, with bars representing 95% confidence intervals on each loading (calculated from 5000

553   bootstrapped replicates of the model).
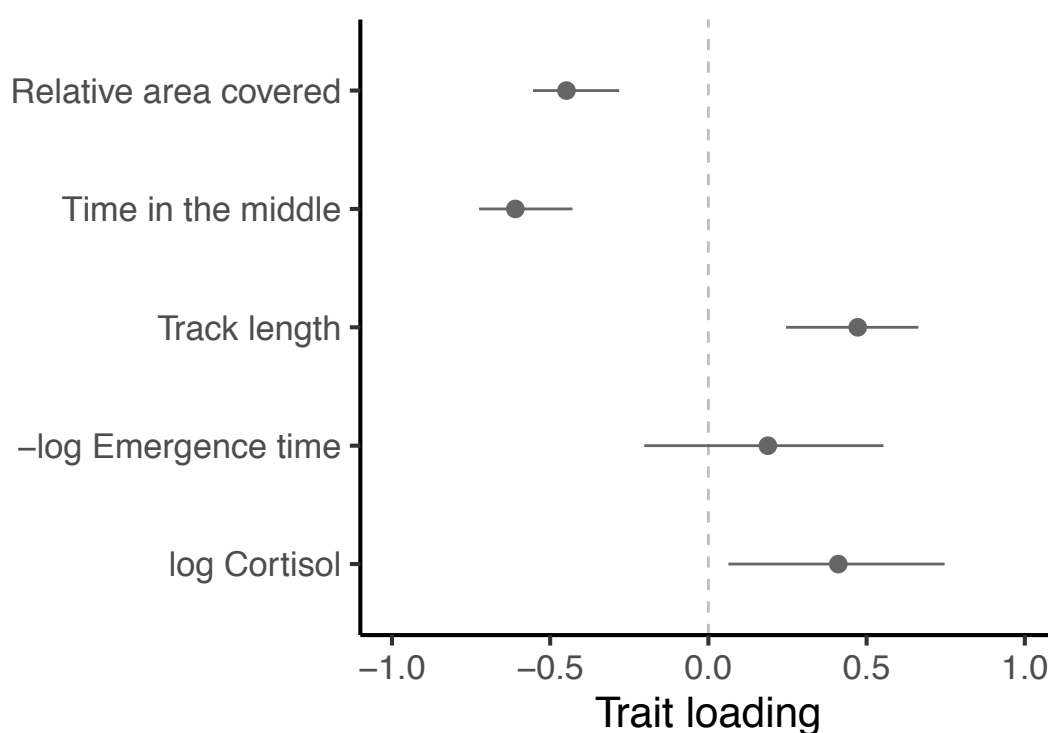
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555 **Materials and methods**

556

557 *Husbandry and breeding*

558 We used fish taken from our captive population housed at the University of Exeter's Penryn campus,

559 which is descended from wild fish collected in 2008 from the lower Aripo River in Trinidad. This

560 population has been maintained at a population size of several thousand, and has undergone no

561 deliberate selection or inbreeding. All fish are fed to satiation twice daily (0800 – 1000h and again at

562 1600 – 1800h) using commercial flake food and live *Artemia nauplii*. Water temperature is maintained

563 at 23-24°C in well-aerated closed system tank stacks that undergo 25% water changes each week and

564 with weekly tests for ammonia, nitrate and nitrite levels. Lighting is kept at a 12:12 light/dark cycle.

565 The experiment described in this study was carried out in accordance with the UK Animals (Scientific

566 Procedures) Act 1986 under licence from the Home Office (UK), and with local ethical approval from

567 the University of Exeter.

568 To create our pedigreed sub-population, female fish were sampled haphazardly from the stock

569 tanks and kept in female-only groups for 3 months. Isolation from male contact minimised the chance

570 of females carrying viable sperm from previous matings. For the first generation of offspring, we used

571 a group breeding design (as detailed in White and Wilson, 2018); briefly, females were tagged under

572 anaesthetic (buffered MS222 solution) using visible implant elastomer (VIE) to allow individual

573 identification. We then assigned groups of 4 females to 1 male in 15L breeding tanks (18.5cm x 37cm x

574 22cm), and inspected females daily for high gravidity (swollen abdomens and enlarged 'gravid spots').

575 Heavily gravid females were then isolated in 2.8L brood tanks to give birth (and were returned to the

576 breeding tanks either after producing a brood or two weeks of isolation). Any offspring produced in

577 the breeding tanks were excluded from the experiment as maternal identity could not be positively

578 identified. For the following generations, after 3 months of isolation from males we moved females

579 into individual 2.8L tanks, with 1 male then circulated among 3 females. Males were moved between

580 females every 5-8 days. In this way, females did not have to be moved to brood tanks, and any

581 offspring could be assigned to mothers definitively. In this setup, offspring were moved to a separate

582 brood tank on the day of birth. Note that as the gestation period for guppies is approximately 1 month,

27

583   any brood produced by a female less than one month after exposure to their designated male was

584   recorded in the pedigree as having unknown paternity.

585   Within 24h of a female producing a brood we recorded her weight (g) and brood size. We kept

586   juvenile fish in full-sib family groups in 2.8L tanks before moving them to 15L 'growth' tanks at an

587   average age of 56 days. At an average age of 133 days (range 59-268) we tagged individuals and

588   placed them into mixed family groups of 16-20 adults (with an even mix of males and females), kept in

589   15L tanks. Note that variation in tagging age arose largely because groups were necessarily

590   established sequentially as sufficient individuals from multiple families reached a large enough size

591   that we deemed the procedure to be safe. Each adult group comprised a mix of fish from different

592   families, reducing the potential for common environment effects to upwardly bias our genetic

593   parameter estimation.

594

595   *Overview of behavioural phenotyping*

596   Behavioural phenotyping commenced at least one week after tagging. In all trials, we filmed

597   movement behaviour of individual fish using a Sunkwang video camera equipped with a 6-60mm

598   manual focus lens suspended over the tank. We used the tracking software Viewer II (BiObserve) to

599   extract behavioural data from each recording (detailed below). The tank was lit from below using a

600   light box, and screened with a cardboard casing to prevent external visual disturbance. After each

601   behavioural trial, the individual tested was weighed and then moved to a temporary 'holding tank'.

602   Once a full group (as described above) had been tested, all were moved from the holding tank back to

603   their home tank. We replaced the water in the testing and holding tanks between groups to reduce the

604   build-up of hormones or other chemicals. The first offspring generation experienced 4 repeat open

605   field trials (OFTs) over a 2-week period, with at least 48h between trials. Subsequent generations

606   experienced 4 repeat behavioural trials, alternating 2 OFTs with 2 emergence trials (ETs). For the final

607   2 generations, we extended the OFTs by including a shoaling trial (ST) at the end of each OFT.

608

609   *Open field trials (OFT)* followed the methodology described by White et al (2016). Briefly, we assessed

610   individual behaviour in a 20cm x 30cm tank, filled to a depth of 5cm with room-temperature water

611   from the main supply. We caught fish individually from their home tank, examined them quickly for

612   identification tags, then placed them immediately into the centre of the OFT tank. After allowing 30s

613   for acclimation, we filmed behaviour for 4m30s. Behaviours characterised from the tracking software

614   were *track length* (the total distance the fish moved during the trial; cm), *area covered* (the percentage

615   of 1cm x 1cm grid squares through which the fish moved during the trial; %), and *time in middle* (time

616   spent in a rectangular inner zone which was defined as being the same size as an outer area; seconds).

617

618   *Shoaling trials (ST)* were appended to a subset of OFTs, by positioning a small tank containing 10 stock

619   fish (of same sex as the test subject) next to one end of the OFT tank but with visual cues blocked by a

620   cardboard divider. At the end of the normal OFT, we removed this divider slowly, allowing the focal

621   animal to have visual contact with the shoal. We began recording the shoaling trial 30s after removing

622   the divider in order to limit any artefacts of slight disturbance. (Note that we used a further cardboard

623   casing around the shoaling tank to avoid any additional external visual stimulus). We then recorded

624   behaviour of the test fish for an additional 3 minutes. We characterised *shoaling tendency* via the

625   tracking software by subdividing the tank area into 3 equal-sized rectangular areas: one next to the

626   tank holding the group of same-sex fish, one farthest from this group, and the central area. We then

627   calculated *shoaling tendency* as the time spent in the 1/3 area closest to the same-sex group after

628   subtracting the time spent in the 1/3 area farthest away. The decision to use a single-sex shoal aimed

629   to reduce any effects of (potential) mate preference and/or avoidance, but also this necessitated

630   replicate arena setups allowing male and female individuals from each group to be tested in the

631   OFT/ST concurrently. We randomised which tank was used for each sex in each group and recorded

632   this information.

633

634   *Emergence trials (ET)* followed the methodology described by White *et al.* (2016). Briefly, we tested

635   individuals in a 20cm x 40cm tank, filled to a depth of 8cm with room-temperature water from the

636   main supply. A 10cm section of the tank length was walled off creating a shelter area (20cm x10cm),

637   the walls and floor of which were painted black. The focal fish was placed into the shelter area and

638   allowed to acclimate for 30s, at which point we opened a sliding door to allow access to the rest of the

639   tank, which was brightly lit from below and otherwise bare. *Time to emerge* (in seconds) was recorded

640   by the tracking software automatically as the fish exited the shelter area and emerged into the open

641   tank section. Trials were ended either at emergence or at 15 min if the fish had not emerged by that

642   point; in the case of non-emergence, fish were given the maximum value (i.e., 900s).

643

644   *Derivation of 'relative area' from OFT trials*

645   The '*area covered'* variable assayed in the OFT is calculated in BiObserve by dividing the arena (i.e., the

646   total area of the tank as viewed from the camera) into 1cm x 1cm grid squares. The path taken by the

647   fish during observation is then used to determine what proportion of these grid squares the fish

648   entered. However, we sought to derive a measure of '*relative area*' that describes whether a fish covers

649   a large, or small area relative to its observed *track length.*

650   To do this we simulated 'random swims' within the arena across the observed range of *track*

651   *lengths*. We first selected 40 OFT results at random from our total data set and extracted the

652   coordinates of the fish in each frame from the raw tracking file, creating a set of x and y movements

653   and their associated distances. As original coordinates were recorded in pixels we used the calibration

654   of the software to convert to cm units. We then use a 'random walk' algorithm to select a movement

655   (i.e., step size and direction) from this observed distribution at random, and calculate the new

656   coordinates. If the movement keeps the 'fish' within the bounds of the 'tank' (i.e., defined as a 20cm x

657   30cm arena), the movement is accepted and coordinates added to a movement matrix; if not, a new

658   movement is drawn from the distribution. If the movement is greater than 1cm in distance, we break

659   the movement into a number of smaller parts to be added to the matrix (such that we capture the

660   coordinates of grid squares through which the 'fish' moved along the way). Once the total distance of

661   the random walk reached or exceeded the *track length* set as the simulation objective, the path is

662   terminated and the area covered is calculated by counting the number of unique grid squares in the

663   matrix of coordinates and dividing by the total number possible.

664   After simulating random walks across 500 values of *track length* (using a vector of 100 values

665   evenly spaced across the range of true data, repeated 5 times), we modelled (simulated) area covered

666   as a fourth order polynomial function of *track length*. Using this regression model (which explained

30

667   97.8% of the variance in simulated data), we calculated the *relative area* for each actual OFT trial as

668   the observed area covered minus the area covered under a random swim, as predicted from our

669   regression model and the observed *track length*.

670

671   *Waterborne hormone sampling*

672   On completion of behavioural data collection, individuals entering the endocrine testing program were

673   left undisturbed for a minimum of two weeks. Waterborne hormone sampling was then conducted

674   over a 5-day period that included three handling and confinement stressor exposures with 48h

675   between each. We followed the method described by Houslay et al (2019) to obtain repeated non-

676   invasive GC measures of individuals using holding water samples from the first and third

677   confinements. Note that only two samples per fish were analysed because the financial and time costs

678   of doing three was deemed prohibitive. We nonetheless applied the stressor stimulus three times as

679   our prior study showed this was sufficient to produce a strong habituation response, i.e., a significant

680   decrease in water-borne cortisol over the three sampling periods (Houslay *et al.* 2019).

681   We collected samples between 1200 – 1400h to control for diel fluctuations in GC levels. For

682   each sample, we netted an entire group from their home tank quickly using a large net, transferring

683   them to 2 holding tanks (containing water from the home tank supply) for moving to an adjacent quiet

684   room (performed within 20s of the net first hitting the water). We then transferred fish to individual

685   Pyrex beakers containing 300ml of clean water from the main supply (which serves the main housing

686   units), which has been warmed to the appropriate temperature (mean = 24.1°C, range 23-24.9°C).

687   Beakers were placed within cardboard 'chambers' to prevent fish from seeing each other or

688   experiencing outside disturbance. One fish was transferred every 30s, alternating across holding

689   tanks, such that all fish were in their beakers within 10min of the initial netting. After 60 mins in the

690   beaker, each fish was removed by pouring its sample through a clean net into a second beaker, with

691   the fish then quickly checked to confirm ID and returned to the holding tank until the entire group

692   could be returned to its home tank.

693   We immediately filtered each water sample using Grade 1 filter paper (Whatman), then passed

694   them slowly through solid phase C18 extraction columns (Sep-Pak C18, 3cc, Waters) via high-purity

31

695   tubing (Tygon 2474, Saint Gobain) under vacuum pressure (Earley *et al.* 2006). Columns were primed

696   beforehand with 2 x 2ml HPLC-grade methanol followed by 2 x 2ml distilled water, and were washed

697   afterwards with a further 2 x 2ml distilled water to purge salts. We then covered both ends of each

698   column with film (Parafilm M, Bemis) and stored them at -20C for future analysis. We washed all

699   beakers, tubes and funnels with 99% ethanol and rinsed them with distilled water prior to each

700   sampling procedure. The remainder of the endocrine assay procedure involved elution, resuspension,

701   separation and quantification of free cortisol by enzyme immunoassay (EIA) using Cayman Chemicals,

702   Inc EIA kits. Detailed methods are exactly as described by Houslay et al (2019) and so not repeated

703   here (note that here we assayed the free fraction of cortisol only). To validate the cortisol kits, we

704   examined whether the kit standard curve was parallel to a serial dilution curve derived from pooled

705   guppy water-borne hormone extract. 20µl was taken from each of the male samples and pooled; 20µl

706   was taken from each of the female samples and combined into a separate pool. 400µl of the pools was

707   serially diluted from 1:1 to 1:128 and these samples were assayed alongside the kit standard curve on

708   two occasions (June and December 2017, marking the start and finish of sample processing). All

709   dilution curves were parallel to the standard curve (slope comparison test, Zar 1996, p.355; June,

710   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;

711   December, female: : $t_{12} = 0.224$, P = 0.83). The serial dilution curves also identified 1:32 as an

712   appropriate dilution to ensure that all samples fell on the linear phase of the standard curve. A total of

713   37 96-well plates were used and the pooled sample was included at the beginning and end of each

714   plate. Intra-assay coefficients of variation ranged from 0.12-19.83% with a median of 3.08%; the inter-

715   assay coefficient of variation was 19.22%. Cortisol is presented and modelled in (ln-transformed)

716   units of ng/hr to reflect the 1 hour sampling duration.

717

718   **Statistical methods**

719

720   All data handling and analysis was performed in R version 3.4.1 (R Core Team 2017). We used the

721   'tidyverse' set of packages for data handling and visualisation (Wickham 2017), and ASreml-R 3.0

722   (Butler 2009) for fitting linear mixed effects models (as described in full below). We also used 'nadiv'

32

723 for pedigree preparation and to estimate (approximate) standard errors on linear functions of

724 variance components as estimated from the mixed models (Wolak 2012). All models fitted assumed

725 (multivariate) Gaussian error structures, and we visually assessed residuals to verify this was

726 reasonable (after data transformation in some cases). To test for significance of among individual

727 and/or genetic (co)variance components, we fitted nested models with different random effects

728 structures and compared them using likelihood ratio tests (LRTs). We calculated χ^2_{nDF} as twice the

729 difference in model log likelihoods, with the number of degrees of freedom (n) equivalent to the

730 number of additional parameters in the more complex model. When testing a single random effect

731 (variance component), we assumed the difference to be asymptotically distributed as an equal mix of

732 χ^2_0 and χ^2_1 (denoted $\chi^2_{0,1}$; Self and Liang, 1987; Visscher, 2006).

733 For each OFT and ST behaviour in turn (*relative area, time in middle, track length, shoaling

734 tendency,* and *emergence time*), we used the random effects specification to partition phenotypic

735 variation (V_p, conditional on fixed effects as described below) into the effects of additive genetics (V_a),

736 permanent environment defined as the non-(additive) genetic component of among-individual

737 differences, V_{pe}), and housing group (V_{group}), as well as residual variation ($V_{residual}$). We natural log-

738 transformed *emergence time* prior to analysis to meet assumptions of residual normality and

739 homoscedasticity. For all behavioural traits, we included fixed effects of assay *repeat*, the *order within*

740 *each group* in which the fish was trialled (mean-centred continuous predictor), *temperature* (mean-

741 centred and scaled to standard deviation units), *time* (in minutes from midnight, mean-centred and

742 scaled to standard deviation units), *age* (mean-centred and scaled to standard deviation units), *sex*,

743 and the *generation* from the breeding population. For *shoaling tendency* only, we incorporated an

744 additional fixed effect of *setup* (as detailed above). We tested the significance of genetic variance for

745 each behaviour by LRT comparison of the corresponding full model to one in which the (additive)

746 genetic random effect was excluded.

747 Cortisol data were also natural log (ln) transformed for analysis. We formulated a bivariate

748 model to test for both additive genetic variation and genotype-by-environment interaction (GxE) in

749 cortisol levels across the two 'contexts' (i.e. samples retained for each individual at first and third

750 confinement, denoted Cortisol$_1$, Cortisol$_3$). Random effects were first used to partition phenotypic

33

751 (co)variance (conditional on fixed effects) into among-group and residual components. Fixed effects

752 included the context-specific means, and overall effects of the *order* in which the fish was caught from

753 each group for assay (mean-centred continuous predictor), *temperature* (mean-centred and scaled to

754 standard deviation units), *time of day* (mean-centred and scaled to standard deviation units), *age*

755 (mean-centred and scaled to standard deviation units), and *sex*. In addition, we included fixed

756 covariates of *body mass* (mean-centred and scaled to standard deviation units) and a *sex* by *body mass*

757 interaction (see Houslay *et al.* 2019 for rationale of controlling for body size effects on waterborne

758 hormone levels in this way). Note that modelled in this way each individual is sampled only once for

759 each context-specific cortisol trait so no random effect of individual identity is included. To test for

760 additive genetic variation (V_a) we compared this first bivariate model to a second formulation that also

761 included the (additive) genetic merit, but under the assumption that this is invariant with context

762 within an individual (such that $V_{a1}=V_{a3}$ and $r_{a1,3}=1$ and there is no GxE). We then test for the GxE by

763 comparing the second model to a third in which we allow GxE (i.e., the context-specific genetic

764 variances are free to differ and the cross-context genetic correlation can be <+1).

765   Lastly, we built a multivariate animal model to estimate **G** and to test the hypothesised genetic

766 integration among behavioural and physiological stress components. We retained only response traits

767 that harboured significant V_a as shown in univariate models, and so the final model comprised

768 response traits *relative area, time in middle, track length, emergence time* (log transformed)*,* and

769 *Cortisol* (log transformed). We multiplied (transformed) *emergence time* by -1 to simplify

770 interpretation of estimated correlation structures (i.e., higher values for all behavioural traits then

771 represent nominally 'bolder' behaviours). We also scaled all (transformed) response variables to

772 standard deviation units. This was to facilitate model fitting, and also prevent scale effects

773 complicating interpretation of eigenvectors of **G**. Fixed and random effects were fitted on each trait as

774 specified for the univariate models. Note that one exception to this is that we elected to treat *Cortisol*

775 as a single repeated-measures trait here (with two repeats, one per context) such that a permanent

776 environment effect was now included. Fixed effects estimates are reported in the supplementary

777 information (Table S1).

34

778   We specified additive genetic (**G**), permanent environment (**PE**), group (**GROUP**), and residual

779   (**R**) covariance structures as unstructured matrices to be estimated. Note that **R** partitions

780   observation-level covariances (as opposed to individual-level in **PE**) that are not definable or

781   statistically identifiable if traits are not measured at the same time (i.e., all covariances relating to

782   *emergence time* or *Cortisol*). Where this was the case we constrained specific covariance terms in **R** to

783   equal zero. Estimates of **PE**, **GROUP** and **R** are provided in the supplementary information (Tables S2-

784   S4). We tested for overall additive genetic covariance among the traits by comparing this model

785   against a reduced one in which **G** was specified as a diagonal matrix (i.e., additive variances are

786   estimated but covariances are assumed to equal zero). To aid the interpretation of covariance terms

787   contained in **G,** we calculated the corresponding genetic correlations r_a from the full model. For any

788   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

789   decomposition to determine the proportion of additive genetic variation captured by each principal

790   component and assess whether a single major axis of variation could indeed explain most of the

791   genetic variance in the multivariate phenotype (consistent with a simple proactive-reactive coping

792   style model). We estimated uncertainty on the trait loadings associated with each principal component

793   (eigenvector) using a parametric bootstrap approach as described by Boulton et al (2014).

794   For visualisation of bivariate relationships at the additive genetic level, we used the R package

795   'ellipse' (Murdoch & Chow 2018) to determine the coordinates of an ellipse representing the

796   approximate 95% confidence region of deviations based on the point estimate of **G**. We repeated this

797   procedure for the corresponding regions defined from 5000 bootstrapped values of **G** (i.e., to indicate

798   uncertainty arising from estimation of the genetic covariance structure itself). Best linear unbiased

799   predictors (BLUPs) are used for visualisation only, not for any statistical analysis (Houslay & Wilson

800   2017).

801   To test for associations between all traits (i.e., including *shoaling tendency*) at the among-

802   individual level, we also built a multivariate model as above with the addition of *shoaling tendency* and

803   without estimating additive genetic effects. The estimates of all among-individual (co)variances are

804   provided in the supplementary information (Table S5).

805

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806

807 **SUPPLEMENTARY MATERIAL**

808

809   Table S1: Fixed effects estimates from the full multivariate animal model.
810   Table S2: Permanent environment (co)variance matrix from the full multivariate animal
811   model.
812   Table S3: Group (co)variance matrix from the full multivariate animal model.
813   Table S4: Residual variance-correlation matrix from the full multivariate animal model.
814   Table S5: Among-individual (co)variance matrix from the multivariate model that excluded
815   genetic effects.
816

REFERENCES

Angelier, F. & Wingfield, J.C. (2012) Importance of the glucocorticoid stress response in a changing world: Theory, hypotheses and perspectives. *General and Comparative Endocrinology*, **190**, 118–128.

Barton, B.A. & Iwama, G.K. (1991) Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases*, **1**, 3–26.

Berger, D., Bauerfeind, S.S., Blanckenhorn, W.U. & Schäfer, M.A. (2011) HIGH TEMPERATURES REVEAL CRYPTIC GENETIC VARIATION IN A POLYMORPHIC FEMALE SPERM STORAGE ORGAN. *Evolution*, **65**, 2830–2842.

Bijlsma, R. & Loeschcke, V. (2005) Environmental stress, adaptation and evolution: an overview. *Journal of evolutionary biology*, **18**, 744–9.

Blows, M.W. (2007) A tale of two matrices: multivariate approaches in evolutionary biology. *Journal of evolutionary biology*, **20**, 1–8.

Blows, M.W. & Walsh, B. (2009) Spherical cows grazing in Flatland: Constraints to selection and adaptation. *Adaptation and Fitness in Animal Populations*, pp. 83–101. Springer, Dordrecht.

Boonstra, R. (2013) Reality as the leading cause of stress: Rethinking the impact of chronic stress in nature. *Functional Ecology*, **27**, 11–23.

von Borell, E. (1995) Neuroendocrine integration of stress and significance of stress for the performance of farm animals. *Applied Animal Behaviour Science*, **44**, 219–227.

Boulton, K., Couto, E., Grimmer, A.J., Earley, R.L., Canario, A.V.M., Wilson, A.J. & Walling, C.A. (2015) How integrated are behavioral and endocrine stress response traits? A repeated measures approach to testing the stress-coping style model. *Ecology and Evolution*, **5**, 618–633.

Boulton, K., Grimmer, A.J., Rosenthal, G.G., Walling, C.A. & Wilson, A.J. (2014) How stable are personalities? A multivariate view of behavioural variation over long and short timescales in the sheepshead swordtail, Xiphophorus birchmanni. *Behavioral Ecology and Sociobiology*, **68**, 791–803.

Broom, D.M. & Johnson, K.G. (1993) Assessing welfare: short-term responses. *Stress and Animal*

38

845   *Welfare*, pp. 87–110. Springer Netherlands, Dordrecht.

846   Burns, J.G. (2008) The validity of three tests of temperament in guppies (Poecilia reticulata). *Journal of*

847   *comparative psychology*, **122**, 344–356.

848   Busch, D.S. & Hayward, L.S. (2009) Stress in a conservation context: A discussion of glucocorticoid

849   actions and how levels change with conservation-relevant variables. *Biological Conservation*, **142**,

850   2844–2853.

851   Butler, D. (2009) asreml: asreml() fits the linear mixed model.

852   Carere, C., Groothuis, T.G.G., Möstl, E., Daan, S. & Koolhaas, J.M. (2003) Fecal corticosteroids in a

853   territorial bird selected for different personalities: Daily rhythm and the response to social stress.

854   *Hormones and Behavior*, **43**, 540–548.

855   Carter, A.J., Feeney, W.E., Marshall, H.H., Cowlishaw, G. & Heinsohn, R. (2013) Animal personality: What

856   are behavioural ecologists measuring? *Biological Reviews*, **88**, 465–475.

857   Cheverud, J.M. (1982) Phenotypic, Genetic, and Environmental Morphological Integration in the

858   Cranium. *Evolution*, **36**, 499.

859   Clinchy, M., Sheriff, M.J. & Zanette, L.Y. (2013) Predator-induced stress and the ecology of fear.

860   *Functional Ecology*, **27**, 56–65.

861   Cox, R.M., McGlothlin, J.W. & Bonier, F. (2016) Hormones as Mediators of Phenotypic and Genetic

862   Integration: An Evolutionary Genetics Approach. *Integrative and Comparative Biology*, **56**, 126–

863   137.

864   Earley, R.L., Edwards, J.T., Aseem, O., Felton, K., Blumer, L.S., Karom, M. & Grober, M.S. (2006) Social

865   interactions tune aggression and stress responsiveness in a territorial cichlid fish (Archocentrus

866   nigrofasciatus). *Physiology and Behavior*, **88**, 353–363.

867   Frankham, R., Hemmer, H., Ryder, O.A., Cothran, E.G., Soulé, M.E., Murray, N.D. & Snyder, M. (1986)

868   Selection in captive populations. *Zoo Biology*, **5**, 127–138.

869   Fürtbauer, I., Pond, A., Heistermann, M. & King, A.J. (2015) Personality, plasticity and predation:

870   linking endocrine and behavioural reaction norms in stickleback fish. *Functional Ecology*, n/a-

871   n/a.

872   Hau, M. & Goymann, W. (2015) Endocrine mechanisms, behavioral phenotypes and plasticity: known

873   relationships and open questions. *Frontiers in Zoology*, **12**, S7.

874   Herbert-Read, J.E., Rosén, E., Szorkovszky, A., Ioannou, C.C., Rogell, B., Perna, A., Ramnarine, I.W.,

875   Kotrschal, A., Kolm, N., Krause, J. & Sumpter, D.J.T. (2017) How predation shapes the social

876   interaction rules of shoaling fish. *Proceedings of the Royal Society B: Biological Sciences*, **284**,

877   20171126.

878   Hine, E., Chenoweth, S.F. & Blows, M.W. (2004) Multivariate quantitative genetics and the lek paradox:

879   genetic variance in male sexually selected traits of Drosophila serrata under field conditions.

880   *Evolution*, **58**, 2754–62.

881   von Horsten, S., Karl, T. & Pabst, R. (2003) Behavioral phenotyping of mice in pharmacological and

882   toxicological research. *Experimental and Toxicologic Pathology*, **55**, 69–83.

883   Houslay, T.M., Earley, R.L., Young, A.J. & Wilson, A.J. (2019) Habituation and individual variation in the

884   endocrine stress response in the Trinidadian guppy (Poecilia reticulata). *General and*

885   *Comparative Endocrinology*, **270**, 113–122.

886   Houslay, T.M., Vierbuchen, M., Grimmer, A.J., Young, A.J. & Wilson, A.J. (2018) Testing the stability of

887   behavioural coping style across stress contexts in the Trinidadian guppy. *Functional Ecology*, **32**,

888   424–438.

889   Houslay, T.M. & Wilson, A.J. (2017) Avoiding the misuse of BLUP in behavioural ecology. *Behavioral*

890   *Ecology*, **00**, 1–5.

891   Huether, G. (1996) The central adaptation syndrome: Psychosocial stress as a trigger for adaptive

892   modification of brain structure and brain function. *Progress in Neurobiology*, **48**, 569–612.

893   Hunt, J., Blows, M.W., Zajitschek, F., Jennions, M.D. & Brooks, R.C. (2007) Reconciling strong stabilizing

894   selection with the maintenance of genetic variation in a natural population of black field crickets

895   (Teleogryllus commodus). *Genetics*, **177**, 875–80.

896   Jones, R.B., Satterlee, D.G. & Ryder, F.H. (1994) Fear of Humans in Japanese-Quail Selected for Low or

897   High Adrenocortical-Response. *Physiology & Behavior*, **56**, 379–383.

898   Ketterson, E.D., Atwell, J.W. & McGlothlin, J.W. (2009) Phenotypic integration and independence:

899   Hormones, performance, and response to environmental change. *Integrative and Comparative*

900   *Biology*, **49**, 365–379.

901 Koolhaas, J.M. (2008) Coping style and immunity in animals: Making sense of individual variation.

902   *Brain, Behavior, and Immunity*, **22**, 662–667.

903 Koolhaas, J.M., de Boer, S.F., Buwalda, B. & Van Reenen, K. (2007) Individual variation in coping with

904   stress: A multidimensional approach of ultimate and proximate mechanisms. *Brain, Behavior and*

905   *Evolution*, **70**, 218–226.

906 Koolhaas, J.M., de Boer, S.F., Coppens, C.M. & Buwalda, B. (2010) Neuroendocrinology of coping styles:

907   Towards understanding the biology of individual variation. *Frontiers in Neuroendocrinology*, **31**,

908   307–321.

909 Koolhaas, J.M., Korte, S.M., De Boer, S.F., Van Der Vegt, B.J., Van Reenen, C.G., Hopster, H., De Jong, I.C.,

910   Ruis, M.A.W. & Blokhuis, H.J. (1999) Coping styles in animals: Current status in behavior and

911   stress- physiology. *Neuroscience and Biobehavioral Reviews*, **23**, 925–935.

912 Korte, S.M., Koolhaas, J.M., Wingfield, J.C. & McEwen, B.S. (2005) The Darwinian concept of stress:

913   Benefits of allostasis and costs of allostatic load and the trade-offs in health and disease.

914   *Neuroscience and Biobehavioral Reviews*, **29**, 3–38.

915 Lande, R. & Arnold, S.J. (1983) The measurement of selection on correlated characters. *Evolution*, **37**,

916   1210–1226.

917 Ledón-Rettig, C.C., Pfennig, D.W., Chunco, A.J. & Dworkin, I. (2014) Cryptic genetic variation in natural

918   populations: a predictive framework. *Integrative and comparative biology*, **54**, 783–793.

919 Ledón-Rettig, C.C., Pfennig, D.W. & Crespi, E.J. (2010) Diet and hormonal manipulation reveal cryptic

920   genetic variation: Implications for the evolution of novel feeding strategies. *Proceedings of the*

921   *Royal Society B: Biological Sciences*, **277**, 3569–3578.

922 Mason, G.J. (2010) Species differences in responses to captivity: Stress, welfare and the comparative

923   method. *Trends in Ecology and Evolution*, **25**, 713–721.

924 McEwen, B.S. & Wingfield, J.C. (2003) The concept of allostasis in biology and biomedicine. *Hormones*

925   *and Behavior*, **43**, 2–15.

926 McEwen, B.S. & Wingfield, J.C. (2010) What is in a name? Integrating homeostasis, allostasis and stress.

927   *Hormones and Behavior*, **57**, 105–111.

928 McGlothlin, J.W. & Ketterson, E.D. (2008) Hormone-mediated suites as adaptations and evolutionary

41

929   constraints. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*,

930   **363**, 1611–1620.

931   Mignon-Grasteau, S., Boissy, A., Bouix, J., Faure, J.M., Fisher, A.D., Hinch, G.N., Jensen, P., Le Neindre, P.,

932   Mormède, P., Prunet, P., Vandeputte, M. & Beaumont, C. (2005) Genetics of adaptation and

933   domestication in livestock. *Livestock Production Science*, **93**, 3–14.

934   Moberg, G.P. (2000) Biological response to stress: implications for animal welfare. *The biology of

935   animal stress: basic principles and implications for animal welfare*, pp. 1–21.

936   Möstl, E. & Palme, R. (2002) Hormones as indicator of stress. *Domestic animal and endocrinology*, **23**,

937   67–74.

938   Muir, W.M. & Craig, J. V. (1998) Improving animal well-being through genetic selection. *Poultry Science*,

939   **77**, 1781–1788.

940   Murdoch, D. & Chow, E.D. (2018) ellipse: Functions for Drawing Ellipses and Ellipse-like Confidence

941   Regions.

942   Oltenacu, P.A. & Algers, B. (2009) Selection for Increased Production and the Welfare of Dairy Cows:

943   Are New Breeding Goals Needed? *AMBIO: A Journal of the Human Environment*, **34**, 311–315.

944   Oswald, M.E., Singer, M. & Robison, B.D. (2013) The Quantitative Genetic Architecture of the Bold-Shy

945   Continuum in Zebrafish, Danio rerio. *PLoS ONE*, **8**, 1–10.

946   Øverli, Ø., Sørensen, C., Pulman, K.G.T., Pottinger, T.G., Korzan, W., Summers, C.H. & Nilsson, G.E. (2007)

947   Evolutionary background for stress-coping styles: Relationships between physiological,

948   behavioral, and cognitive traits in non-mammalian vertebrates. *Neuroscience and Biobehavioral

949   Reviews*, **31**, 396–412.

950   Øverli, Ø., Winberg, S. & Pottinger, T.G. (2005) Behavioral and Neuroendocrine Correlates of Selection

951   for Stress Responsiveness in Rainbow Trout—a Review. *Integrative and Comparative Biology*, **45**,

952   463–474.

953   Paaby, A.B. & Rockman, M. V. (2014) Cryptic genetic variation: Evolution's hidden substrate. *Nature

954   Reviews Genetics*, **15**, 247–258.

955   Perals, D., Griffin, A.S., Bartomeus, I. & Sol, D. (2017) Revisiting the open-field test: what does it really

956   tell us about animal personality? *Animal Behaviour*, **123**, 69–79.

957   Pottinger, T.G. & Carrick, T.R. (1999) Modification of the plasma cortisol response to stress in rainbow

958   trout by selective breeding. *General and Comparative Endocrinology*, **116**, 122–132.

959   Prentice, P.M., Houslay, T.M., Martin, J.G.A. & Wilson, A.J. (2020) Genetic variance for behavioural

960   'predictability' of stress response. *Journal of Evolutionary Biology*, 1–11.

961   R Core Team. (2017) R: A language and environment for statistical computing.

962   Réale, D., Garant, D., Humphries, M.M., Bergeron, P., Careau, V. & Montiglio, P.-O. (2010) Personality

963   and the emergence of the pace-of-life syndrome concept at the population level. *Philosophical*

964   *transactions of the Royal Society of London. Series B, Biological sciences*, **365**, 4051–4063.

965   Van Reenen, C.G., O'Connell, N.E., Van der Werf, J.T.N., Korte, S.M., Hopster, H., Jones, R.B. & Blokhuis,

966   H.J. (2005) Responses of calves to acute stress: Individual consistency and relations between

967   behavioral and physiological measures. *Physiology & Behavior*, **85**, 557–570.

968   Rex, A., Voigt, J.P., Voits, M. & Fink, H. (1998) Pharmacological evaluation of a modified open-field test

969   sensitive to anxiolytic drugs. *Pharmacology Biochemistry and Behavior*, **59**, 677–683.

970   Romero, L.M. (2004) Physiological stress in ecology: Lessons from biomedical research. *Trends in*

971   *Ecology and Evolution*, **19**, 249–255.

972   Romero, L.M., Dickens, M.J. & Cyr, N.E. (2009) The reactive scope model - A new model integrating

973   homeostasis, allostasis, and stress. *Hormones and Behavior*, **55**, 375–389.

974   Segerstrom, S.C. & Miller, G.E. (2004) Psychological stress and the human immune system: a meta-

975   analytic study of 30 years of inquiry. *Psychological bulletin*, **130**, 601–30.

976   Self, S.G. & Liang, K. (1987) Asymptotic properties of maximum likelihood estimators and likelihood

977   ratio tests under nonstandard conditions. *J. Am. Stat. Assoc.*, **82**, 605–610.

978   Stamps, J.A. (2007) Growth-mortality tradeoffs and "personality traits" in animals. *Ecology Letters*, **10**,

979   355–363.

980   Stirling, D.G., Réale, D. & Roff, D.A. (2002) Selection, structure and the heritability of behaviour. *Journal*

981   *of Evolutionary Biology*, **15**, 277–289.

982   Taborsky, B., English, S., Fawcett, T.W., Kuijper, B., Leimar, O., McNamara, J.M., Ruuskanen, S. & Sandi,

983   C. (2020) Towards an Evolutionary Theory of Stress Responses. *Trends in Ecology and Evolution*,

984   1–10.

985 Taff, C.C. & Vitousek, M.N. (2016) Endocrine Flexibility: Optimizing Phenotypes in a Dynamic World?

986   *Trends in Ecology and Evolution*, **31**, 476–488.

987 Tarlow, E.M. & Blumstein, D.T. (2007) Evaluating methods to quantify anthropogenic stressors on wild

988   animals. *Applied Animal Behaviour Science*, **102**, 429–451.

989 Thomson, J.S., Watts, P.C., Pottinger, T.G. & Sneddon, L.U. (2011) Physiological and genetic correlates of

990   boldness: Characterising the mechanisms of behavioural variation in rainbow trout,

991   Oncorhynchus mykiss. *Hormones and Behavior*, **59**, 67–74.

992 Toms, C.N., Echevarria, D.J. & Jouandot, D.J. (2010) A Methodology Review of Personality-Related

993   Studies in Fish: Focus on the Shy-Bold Axis of Behaviour. *Berkeley Planning Journal*, **26**, 12.

994 Trenzado, C.E., Carrick, T.R. & Pottinger, T.G. (2003) Divergence of endocrine and metabolic responses

995   to stress in two rainbow trout lines selected for differing cortisol responsiveness to stress.

996   *General and Comparative Endocrinology*, **133**, 332–340.

997 Veenema, A.H., Meijer, O.C., De Kloet, E.R., Koolhaas, J.M. & Bohus, B.G. (2003) Differences in basal and

998   stress-induced HPA regulation of wild house mice selected for high and low aggression.

999   *Hormones and Behavior*, **43**, 197–204.

1000 Visscher, P.M. (2006) A Note on the Asymptotic Distribution of Likelihood Ratio Tests to Test Variance

1001   Components. *Twin Research and Human Genetics*, **9**, 490–495.

1002 Walsh, B. & Blows, M.W. (2009) Abundant Genetic Variation + Strong Selection = Multivariate Genetic

1003   Constraints: A Geometric View of Adaptation. *Annual Review of Ecology, Evolution, and*

1004   *Systematics*, **40**, 41–59.

1005 White, S.J., Houslay, T.M. & Wilson, A.J. (2018) Evolutionary genetics of personality in the Trinidadian

1006   guppy II: sexual dimorphism and genotype-by-sex interactions. *Heredity*.

1007 White, S.J., Kells, T.J. & Wilson, A.J. (2016) Metabolism, personality and pace of life in the Trinidadian

1008   guppy, Poecilia reticulata. *Behaviour*, **153**, 1517–1543.

1009 White, S.J., Pascall, D.J. & Wilson, A.J. (2020) Towards a comparative approach to the structure of

1010   animal personality variation. *Behavioral Ecology*, **31**, 340–351.

1011 White, S.J. & Wilson, A.J. (2018) Evolutionary genetics of personality in the Trinidadian guppy I:

1012   maternal and additive genetic effects across ontogeny. *Heredity*.

44

1013 Wickham, H. (2017) tidyverse: Easily Install and Load "Tidyverse" Packages.

1014 Wilson, D.S., Clark, A.B., Coleman, K. & Dearstyne, T. (1994) Shyness and boldness in humans and other

1015   animals. *Trends in Ecology and Evolution*, **9**, 442–446.

1016 Wilson, A.J., Réale, D., Clements, M.N., Morrissey, M.M., Postma, E., Walling, C.A., Kruuk, L.E.B. & Nussey,

1017   D.H. (2009) An ecologist's guide to the animal model. *Journal of Animal Ecology*, **79**, 13–26.

1018 Wingfield, J.C. (2003) Control of behavioural strategies for capricious environments. *Animal Behaviour*,

1019   **66**, 807–816.

1020 Wingfield, J.C. & Kitaysky, A.S. (2002) Endocrine responses to unpredictable environmental events:

1021   Stress or anti-stress hormones? *Integr Comp Biol*, **42**, 600–609.

1022 Wingfield, J.C., Maney, D.L., Breuner, C.W., Jacobs, J.D., Lynn, S., Ramenofsky, M. & Richardson, R.D.

1023   (1998) Ecological Bases of Hormone-Behavior Interactions: The "Emergency Life History Stage."

1024   *American Zoologist*, **38**, 191–206.

1025 Wolak, M.E. (2012) Nadiv: An R package to create relatedness matrices for estimating non-additive

1026   genetic variances in animal models. *Methods in Ecology and Evolution*, **3**, 792–796.

1027 Wolf, M., van Doorn, G.S., Leimar, O. & Weissing, F.J. (2007) Life-history trade-offs favour the evolution

1028   of animal personalities. *Nature*, **447**, 581–4.

1029 Zar, J.H. (1996) *Biostatistical Analysis*. Prentice-Hall International, Inc., London.

1030