Genetic integration of behavioural and endocrine components of the stress response

T.M. Houslay*[1], R.L. Earley[2], S.J. White[1], W. Lammers[1], A.J. Grimmer[1], L.M. Travers[1,3], E.L. Johnson[2,4], A.J. Young[1] and A.J. Wilson[1]

[1] Centre for Ecology and Conservation, University of Exeter (Penryn Campus), Cornwall, TR10 9FE, UK.

[2] Department of Biological Sciences, University of Alabama, 300 Hackberry Lane, Box 870344, Tuscaloosa, AL 35487, USA.

[3] Current address: School of Biological Sciences, University of East Anglia, Norwich, Norfolk, NR4 7TJ, UK.

[4] Current address: Southern Research, 2000 Ninth Avenue South, Birmingham, AL 35205, USA.

* Corresponding author: houslay@gmail.com

Keywords: stress; coping styles; animal behaviour; quantitative genetics; physiology; endocrinology; genetic integration; heritability

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. Natural selection should favour functional integration, leading to a prediction of genetic integration of these 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 acute stress response components in Trinidadian guppies are both heritable and integrated on the major axis of genetic covariation. This integration could either facilitate or constrain evolutionary responses to selection, depending upon the alignment of selection with this axis. Such integration also suggests artificial selection on the genetically correlated behavioural responses to stress could offer a viable non-invasive route to the improvement of health and welfare in captive animal populations.

Introduction

Stress responses comprise suites of physiological and behavioural traits that enable individuals to cope with adverse conditions (Romero 2004; Øverli *et al.* 2007; McEwen & Wingfield 2010; Taborsky *et al.* 2020). Some individuals are likely better at coping with adverse conditions than others, and understanding the role played by underlying genetic variation could have important implications for managing stress-related disease in captive populations and predicting the evolutionary responses of free-living populations to both natural and anthropogenic stressors (Barton & Iwama 1991; Koolhaas *et al.* 1999; McEwen & Wingfield 2003; Romero 2004; Koolhaas 2008). For instance, if among-individual differences in stress response traits are a product of genetic variation (Koolhaas *et al.* 1999, 2007), then they may be a viable target for artificial selection strategies (Mignon-Grasteau *et al.* 2005). This could be used to reduce stress-related welfare issues in captive populations (e.g. in livestock; Broom & Johnson 1993; von Borell 1995; Möstl & Palme 2002; Kasper *et al.* 2020). In free-living populations, variation in stress response traits is expected to cause fitness variation under stressful conditions (Wingfield 2003; Koolhaas 2008). Thus, exposure to stressors could lead to evolutionary changes in the distributions of traits that contribute to a population's long-term resilience in the face of natural (e.g. predation risk; Clinchy, Sheriff & Zanette 2013) and/or anthropogenic (Tarlow & Blumstein 2007; Busch & Hayward 2009; Angelier & Wingfield 2012; Sadoul *et al.* 2021) challenges. However, as the evolutionary response of any trait to selection is determined in large part by its genetic variation, our ability to predict – and potentially harness – evolutionary changes in the stress response is currently hampered by limited understanding of the underlying genetics.

Natural selection does not act on single traits in isolation, but rather on multivariate phenotypes (Lande & Arnold 1983; Blows 2007). This is likely to be an important consideration for understanding the evolution of the stress response. For

instance, while glucocorticoid (GC) levels are frequently used to measure the stress response (McEwen & Wingfield 2003; Korte *et al.* 2005), an individual's first line of defence against acute environmental challenges will typically be behavioural (Moberg 2000). This may include risk avoidance strategies as well as the widely known 'fight-or-flight' responses. Subsequent GC release then serves to mediate physiological (and further behavioural) responses (Wingfield *et al.* 1998; Wingfield & Kitaysky 2002). Natural selection is therefore expected to favour combinations of behavioural and physiological stress response traits that act synergistically to maintain fitness under stressful conditions (Koolhaas *et al.* 1999; Øverli *et al.* 2007). Evolutionary theory predicts that correlational selection will shape the structure of multivariate quantitative genetic variance (as represented by the genetic covariance matrix **G;** Cheverud 1982; Blows 2007; Roff & Fairbairn 2012), contingent on the distribution of new pleiotropic mutations that generate multivariate genetic variance as well as the selection that depletes it (Blows & Walsh 2009; Walsh & Blows 2009). In general, correlational selection should (under certain assumptions) have direct and strong effects on genetic covariances (Lande 1980; Jones, Arnold & Bürger 2003; Arnold *et al.* 2008). In the context of the stress response we should therefore expect genetic – as well as phenotypic – integration of behavioural and physiological traits (McGlothlin & Ketterson 2008; Ketterson, Atwell & McGlothlin 2009; Cox, McGlothlin & Bonier 2016). By genetic integration, we mean genetic correlation structure among suites of traits. Genetic integration of the stress response components is hypothesised to underpin adaptive variation in multivariate phenotypes in many fields of evolutionary biology (e.g., life history (Stearns 1989; Roff 1992), behavioural ecology (Sih & Bell 2008)), but has not been tested for explicitly using quantitative genetic approaches.

The most compelling evidence for genetic integration of behavioural and physiological stress response traits to date comes from artificial selection experiments on domestic animal populations (e.g., rainbow trout *Oncorhynchus mykiss* (Pottinger and

Carrick, 1999), Japanese quail *Coturnix coturnix japonicus* (Jones, Satterlee & Ryder 1994), house mice *Mus musculus domesticus* (Veenema *et al.* 2003b)). For example, lines of rainbow trout selected for stress-induced plasma cortisol levels (Pottinger and Carrick, 1999) experienced correlated evolutionary changes in behaviour (Øverli, Winberg & Pottinger 2005). In a rare study of a wild-type population (albeit under captive conditions), cortisol levels were found to evolve in response to selection on behavioural 'personality' in great tits (*Parus major*; Carere *et al.* 2003). In these examples, the correlated responses of behavioural and physiological stress response traits to selection are consistent with some degree of genetic integration of these behavioural and physiological traits. However, the extent and 'structure' of this integration remains unclear, and some results were inconsistent with a hypothesised simple axis of genetic (co)variation among behavioural and physiological components of the stress response. For example, in the trout study, the 'low-cortisol response' selected lines actually showed a higher metabolic stress response under confinement (suggestive of opposing responses to selection by different physiological components of the stress response; Trenzado, Carrick & Pottinger 2003). In the same selection lines, there was also no link between 'boldness' (an aspect of personality related to risk-taking behaviour) and cortisol response under standardised testing (Thomson *et al.* 2011).

While selection experiments illustrate that genetic integration of behaviour and physiology can occur, estimation of the genetic variance-covariance matrix (**G**) through quantitative genetic modelling provides a complementary strategy that also allows investigation of exactly how multivariate genetic variation is structured within populations. In the context of the stress response, this should provide insights into both how selection has acted in the past (Ketterson *et al.* 2009), and whether responses to future selection are likely to be constrained (Blows & Walsh 2009; Walsh & Blows 2009). Insight into past selection follows from the fact that strong correlational selection should lead to integration of traits in **G** over the long term, a phenomenon explored

114 most commonly for suites of morphological traits (following, e.g., Cheverud 1982), but
115 that is equally applicable to any aspect of the phenotype (see, e.g., Hine, Chenoweth &
116 Blows 2004; Hunt *et al.* 2007; Oswald, Singer & Robison 2013 for examples pertaining to
117 behavioural evolution and mate choice). Insight into future responses to selection
118 follows from the fact that the direction (in multivariate trait space) and magnitude of a
119 response to selection is limited by the amount of variance in **G** that is in alignment with
120 the vector of (directional) selection β (Blows & Walsh 2009; Walsh & Blows 2009).
121 Integration in **G** is manifest as genetic correlations between trait pairs, and also at the
122 level of the whole matrix by an overdispersion of eigenvalues—indicating that there are
123 fewer effective dimensions of genetic variation than there are traits measured (Blows
124 2007).
125 Here we estimate **G** for behavioural and physiological components of the acute
126 stress response in Trinidadian guppies (*Poecilia reticulata*). This enables us to
127 determine not only (i) whether these components are genetically integrated on the
128 major axis of genetic (co)variation (i.e., the first eigen vector of **G**, $\mathbf{g}_{max}$), but also (ii)
129 whether the structure and orientation of this axis suggests variation in overall stress
130 responsiveness and/or 'coping style' (explained further below; Koolhaas *et al.* 2010;
131 Boulton *et al.* 2015). We use fish from a captive colony of guppies derived from wild
132 ancestors sampled from the Aripo River, Trinidad in 2008 and subsequently maintained
133 at high population size (with no deliberate inbreeding or selection). We have validated
134 the use of standardised 'open field trials' (OFTs) for testing (acute) behavioural stress
135 responses in this species (Houslay *et al.* 2018), and demonstrated significant additive
136 genetic (co)variance underpinning variation in risk-taking, exploratory, and 'flight' type
137 components of the behavioural stress response using this testing paradigm (White &
138 Wilson 2018; White, Houslay & Wilson 2018). We have also demonstrated, using a non-
139 invasive waterborne hormone sampling method, that individuals differ significantly in
140 their GC (specifically, free circulating cortisol) response to an acute stressor (handling,

141 coupled with short term isolation and confinement; Houslay *et al.* 2019) and that, on
142 average, this physiological response declines with repeated stressor exposure
143 (consistent with habituation). Nothing is known about the genetic basis of variation in
144 these physiological traits, or about their integration (phenotypically or genetically) with
145 behavioural components of the stress response.

146 First, we combine OFT results with complementary 'emergence trials' (ET) and
147 'shoaling trials' (ST) to characterise among-individual and genetic variation in the
148 behavioural stress response. Second, we characterise the physiological stress response
149 and its rate of habituation by assaying GC levels following first and third exposure to a
150 handling and confinement stressor (see methods). Utilising repeated behavioural and
151 physiological testing of individual fish within a known pedigree structure, we are able to
152 estimate the repeatable (among-individual) component of phenotypic (co)variance in
153 these stress response traits, and then determine the additive genetic contribution to this
154 (**G**; the genetic variance-covariance matrix for this suite of stress response traits). We
155 predicted that individual traits will be heritable and that **G** will contain strong genetic
156 correlation structure between behavioural and physiological components of the stress
157 response consistent with genetic integration. We also predicted that both behavioural
158 and endocrine components of the stress response would load on the major axis of
159 genetic variation in multivariate trait space, $\mathbf{g}_{max}$. The 'stress coping style' model
160 (Koolhaas *et al.* 1999) predicts variation in the type of response to stress. Simplistically,
161 as originally proposed this verbal model posits that individuals (or genotypes) perceive
162 equal degrees of stress but differ in how this manifests phenotypically: genotypes at one
163 end of the axis having 'reactive' behavioural phenotype (e.g., freezing behaviour)
164 coupled to lower GC levels, while the 'proactive' end is characterised by more active
165 'fight or flight' behaviour coupled to higher GC levels. However, previous analyses of this
166 population suggest variation may be more in terms of 'stress responsiveness' than stress
167 coping style (Houslay *et al.* 2018; Prentice *et al.* 2020; White, Pascall & Wilson 2020).

168 That is to say, some individuals (or genotypes) perceive the trial as a more severe
169 stimulus and exhibit more characteristic stress behaviours (e.g., flight and/or freezing,
170 thigmotaxis) while others show more typical 'unstressed' behavioural profiles (e.g.,
171 exploration of the arena). In this scenario we predicted high GC levels to co-occur with
172 characteristic stress behaviours, and low GC levels with 'unstressed' behavioural
173 profiles.
174
175 **Results**
176 In total we obtained multivariate behavioural data from 5,966 trials (3,379 OFTs, 1,548
177 ETs and 1,039 STs) on 1,384 individual fish. The number of individuals phenotyped
178 (OFTs = 1,365, ETs = 806, STs = 532) and the mean number of observations per fish
179 (OFTs = 2.5, ETs = 1.9, STs = 2.0) varied across the behavioural data types. All fish were
180 contained within a genetic pedigree structure comprising maternal full-sibships nested
181 within paternal half-sibships. This structure was produced via multiple rounds of
182 breeding work and has a maximum depth of 5 'generations'. Some of the OFT data have
183 already been used in studies of the evolutionary genetics of personality (White & Wilson
184 2018; White *et al.* 2018), but here we extend that dataset and use it in conjunction with
185 other behavioural and physiological measures for different purposes. We also obtained
186 1,238 waterborne assays of cortisol levels for 629 fish (almost all from the final
187 generation). The handling and confinement stressor applied for this assay was
188 performed 3 times (at 48h intervals) for all fish tested, but the holding water sample
189 was only processed for GC content at two time points (the first and last confinement,
190 subsequently $Cortisol_1$ and $Cortisol_3$). Full details of husbandry, phenotyping and
191 analysis are provided in Materials and Methods.
192
193 **Genetic variance in behavioural components of the stress response**

194 Behavioural data were extracted from OFTs, ETs and STs using video tracking of fish (as
195 described in White, Kells & Wilson 2016; Houslay *et al.* 2018). Time to emerge from the
196 shelter ('*emergence time*') was extracted from ETs and natural log (ln) transformed for
197 analysis, while *shoaling tendency* was calculated from STs as the time spent in the third
198 of the tank closest to a same-sex shoal (which was visible but physically separated)
199 minus the time spent in the third of the tank farthest from the shoal. The OFT, ET and ST
200 testing paradigms are all considered to assay behavioural components of the stress
201 response in the broad sense, as each test starts with the capture and transfer of the focal
202 fish into a novel and brightly lit arena away from their home tank and familiar tank
203 mates.

204 Four traits were defined from the OFT and measured by videotracking for 4m
205 30s after an initial 30 second acclimation period upon transfer into the arena: *track*
206 *length* (distance swum), *area covered* (as a proportion of the arena floor area), the
207 number of *freezings* (the number of times a fish's speed dropped below the minimum
208 velocity threshold of 4cm/s for at least 2.5s, Houslay *et al.* 2018; this trait was square
209 root transformed for analysis) and *time in the middle* (i.e., in the central area of the open
210 field arena away from the tank walls, which is assumed to be perceived as riskier, e.g.,
211 Houslay *et al.* 2018). Note low values of *time in the middle* imply thigmotaxis (i.e.,
212 tendency to avoid exposure to potential threats by hugging walls), and were very highly
213 correlated with a measure of average distance from the wall at the observation level (r =
214 0.94, $t_{1,3368}$ = 159.2, P < 0.0001; see Supplementary File 1 for further discussion of the
215 selection of OFT behavioural traits). All of these OFT traits are repeatable and heritable
216 in this population (White *et al.* 2016, 2018; Houslay *et al.* 2018; White & Wilson 2018).

217 The absence of a strong positive correlation between *track length* and *area*
218 *covered* (Fig 1A) is notable and potentially biologically informative; if fish moved
219 randomly with respect to direction in the arena then *area covered* would increase
220 monotonically (to an asymptote at 100%) with *track length*. A possible explanation is

221 that a long *track length* arises sometimes from a (putatively) less stressed fish exploring
222 the arena (fish 1 in Fig 1B) and sometimes from a (putatively) more stressed fish
223 exhibiting a typical 'flight' response (fish 4 in Fig 1B). These two types of response can
224 be discriminated based on whether, in a given trial, higher track length is associated
225 with higher *area covered* and *time in the middle* (exploration) or the converse (flight
226 response).

227 To quantitatively discriminate between exploratory behaviour and flight
228 responses we derived a new trait, '*relative area covered*'. We used a simple simulation
229 procedure (see Methods) to predict *expected area covered* for any given *track length*
230 under a null 'random swim' within the arena (Fig 1C). *Relative area covered* is then
231 calculated as observed *area covered – expected area covered* given the *track length* (Fig
232 1D) and will be high for fish engaging in exploration, and low for an obvious 'flight'
233 response manifest as rapid swimming around the tank walls. Our subsequent analyses
234 then used this derived *relative area covered* trait in place of *area covered*.

235 Pedigree-based 'animal models' (Wilson *et al.* 2009) were used to test for and
236 estimate additive genetic variation in each of the six behavioural traits (*emergence time*,
237 *shoaling tendency*, and the four OFT traits) while controlling statistically for social
238 housing group and non-genetic sources of among-individual variance (as well as several
239 fixed effects; see methods for full details). These confirmed the presence of significant
240 additive genetic variation for the *relative area covered* trait, as well as for *track length,*
241 *time in the middle* (as expected from previous findings; White & Wilson 2018; White *et*
242 *al.* 2018), $\sqrt{freezings}$ and *ln emergence time* (Table 1). With the exception of *shoaling*
243 *tendency*, heritabilities (conditional on fixed effects; see methods) are low to moderate
244 (range of 9-20%; Table 1) and within the expected range for behaviours (Stirling, Réale
245 & Roff 2002). We detected no additive genetic variance for *shoaling tendency* (Table 1),
246 despite there being repeatable differences among individuals; R = 0.19 ± 0.04; $\chi^2_{0,1}$ =
247 20.01, P < 0.001).

Table 1: Estimated variance components, along with adjusted heritability, for each trait as estimated in a univariate model (± standard error). Chi-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	42.87 ± 12.89	56.16 ± 10.92	19.17 ± 4.92	203.17 ± 6.26	0.13 ± 0.04	31.2	<0.001
Time in the middle	554.78 ± 144.18	473.24 ± 114.77	196.94 ± 53.54	2002.06 ± 62.38	0.17 ± 0.04	47.2	<0.001
Track length	23584.54 ± 5534.90	26587.74 ± 4556.87	3173.34 ± 1396.84	76140.49 ± 2376.32	0.18 ± 0.04	88.7	<0.001
√Freezings	0.34 ± 0.08	0.18 ± 0.06	0.08 ± 0.02	1.13 ± 0.04	0.20 ± 0.05	48.2	<0.001
ln Emergence time	0.12 ± 0.05	0.06 ± 0.06	0.05 ± 0.02	1.07 ± 0.05	0.09 ± 0.02	22.5	<0.001
Shoaling tendency	0 ± 0	2457.36 ± 570.96	708.87 ± 316.30	9900.95 ± 622.10	0 ± 0	0	0.5
ln Cortisol	0.08 ± 0.02	0.02 ± 0.02	0.01 ± 0.01	0.15 ± 0.01	0.31 ± 0.09	24.4	<0.001

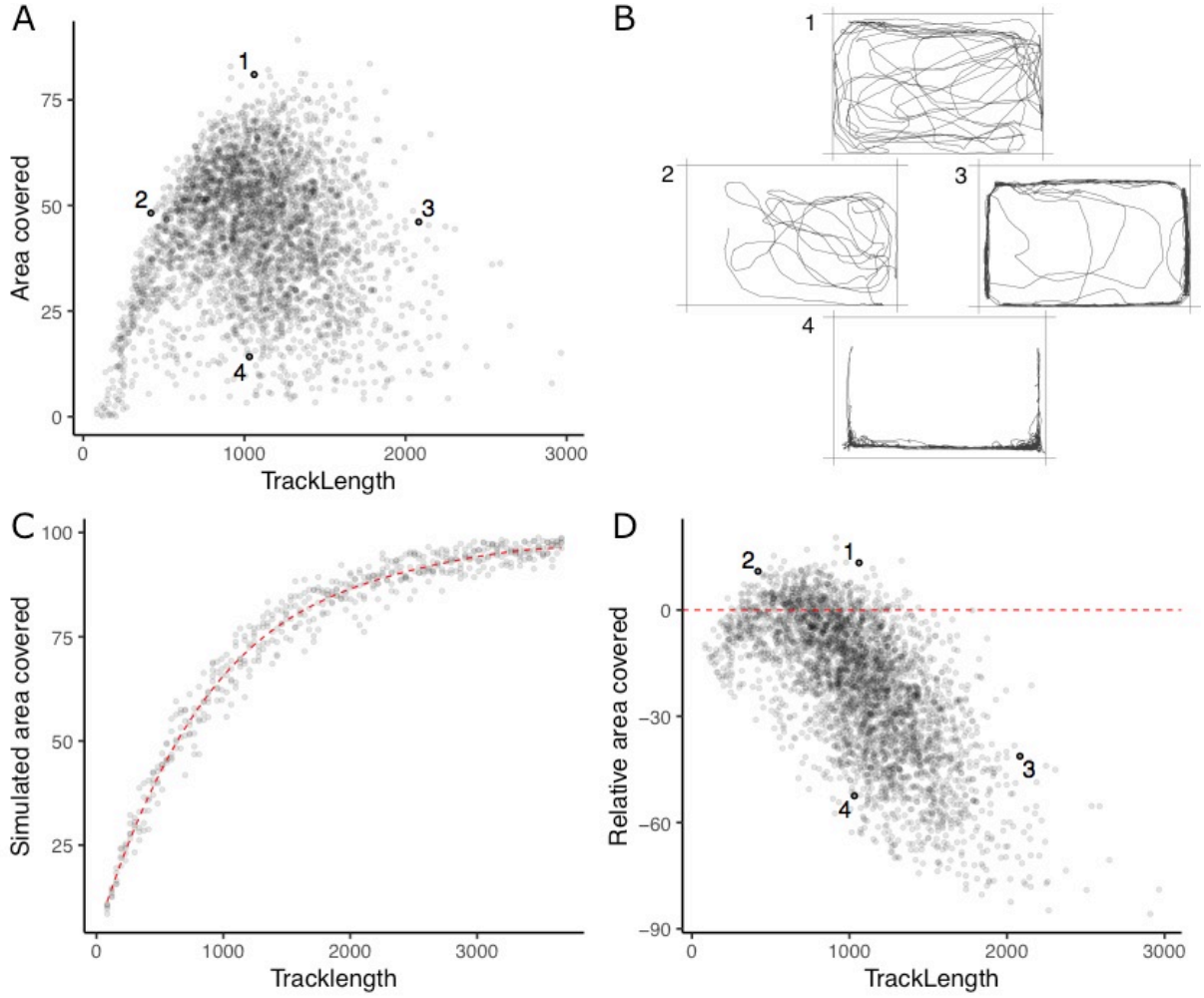


Figure 1: The lack of a strong positive relationship between observed *track length* and *area covered* (panel A), is initially puzzling given expected autocorrelation and that both are used as positive indicators of exploratory (or 'bold') behaviour. Inset examples of OFT tracks from 4 individuals (panel B) shed light on this. Fish 1 and 2 appear to be exploring the tank, while 3 and 4 are engaging in stereotypical 'flight' behaviour characterised by strong thigmotaxis (remaining close to tank walls) and/or rapid movement along tank walls. As a consequence, individuals 2 and 3 have similar *area covered* during the OFT, but very different *track lengths*. We simulated random movements to define an expected null relationship between *area covered* and *track length* (panel C; dashed red line shows the fourth order polynomial model fit; see Appendix A). The polynomial regression was then used to predict the expected area covered

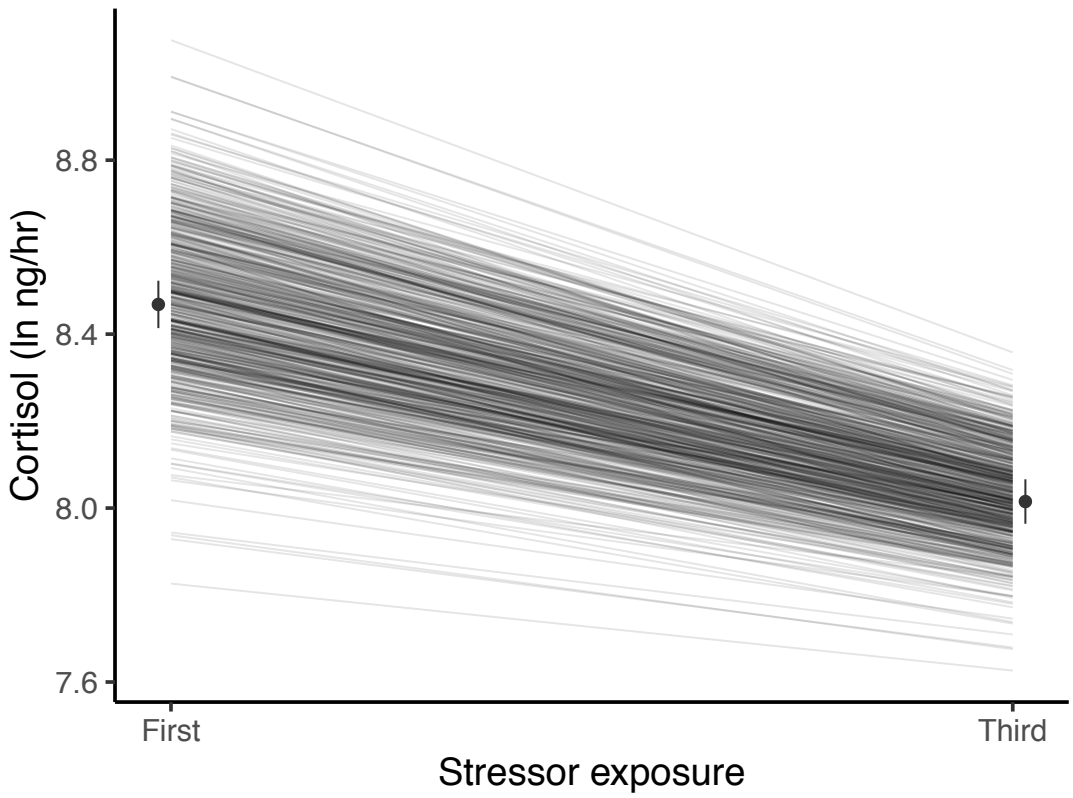
under random movement for each trial's observed *track length*, and the '*relative area covered*' was calculated as the observation minus this prediction. Panel D shows the resultant *relative area covered* plotted against *track length* for all trials (dashed red line at *relative area covered* = 0, shows where individuals of any *track length* are expected to lie if they move randomly with respect to direction). From this it is apparent that fish 1 and 2 have high *relative area covered*, while 3 and 4 do not.

Genetic variance in physiological components of the stress response

Using a series of nested bivariate animal models, we tested for additive genetic variance in cortisol levels (ln-transformed) following stressor exposure (handling and confinement) and for genotype-by-environment interaction (GxE). In this context, the environment (E) is the trial number in each fish's stress trial series (i.e., cortisol level after stressor trial 1 or 3). Any GxE present can therefore be interpreted as genetic variance for habituation to the stressor, given that the average cortisol level was lower following exposure to the third stressor than the first (ln transformed ng/hr, mean ± SE; $Cortisol_1 = 8.47 \pm 0.05$, $Cortisol_3 = 8.02 \pm 0.05$, Wald $F_{1,38.8} = 110.0$, $P < 0.001$; see methods for explanation of units). We first modelled $Cortisol_1$ and $Cortisol_3$ as distinct response variables in a bivariate framework assuming no GxE (such that we constrain $V_{A\text{-}Cortisol1} = V_{A\text{-}Cortisol3}$ and the cross context additive genetic correlation $r_{A\text{-}Cortisol1,Cortisol3} = 1$). This model revealed a significant additive genetic component to variation among individuals in their cortisol levels following stressor exposure ($\chi^2_{0,1} = 18.2$, $P < 0.001$).

Expanding the model to allow GxE by estimating separate genetic variances for $Cortisol_1$ and $Cortisol_3$ provides a significantly better fit to the data ($\chi^2_{0,1} = 3.8$, $P = 0.03$), meaning GxE is present. This can be viewed as genetic variance for habituation to repeated stressor exposure, or as a change in genetic variance for cortisol from the first to the third sampling (Figure 2). These are two perspectives of the same phenomenon; a

290 reduction in additive genetic variance between the first stressor ($V_{A\text{-}Cortisol1}$ = 0.081 ±
291 0.029) and the third ($V_{A\text{-}Cortisol3}$ = 0.041 ± 0.025) arises because genotypes with higher-
292 than-average levels for *Cortisol₁* habituate more rapidly (i.e., have more negative
293 reaction norm slopes). Note however that allowing the cross-context genetic correlation
294 to deviate from +1 does not significantly improve the model fit (χ^2_1 = 0.0, P = 1): thus
295 the rank order of the genotypes does not appreciably change across the two contexts
296 (i.e., genetic reaction norms show little crossing; Figure 2), so there is a strong positive
297 cross-context genetic correlation ($r_{A\text{-}Cortisol1,Cortisol3}$ ± SE = 0.83 ± 0.21).
298 In this model we also find that variance in cortisol explained by housing group
299 effects is similar across contexts ($V_{Group\text{-}Cortisol1}$ = 0.038 ± 0.014, $V_{Group\text{-}Cortisol3}$= 0.032 ±
300 0.012), but that residual (unexplained) variance is greater after the third stressor
301 exposure ($V_{R\text{-}Cortisol1}$ = 0.164 ± 0.022, $V_{R\text{-}Cortisol3}$ = 0.237 ± 0.023). In combination, the
302 changes in both additive genetic and residual variance between the two contexts lead to
303 appreciably higher heritability for cortisol levels following the first stressor exposure
304 relative to the third ($h^2_{Cortisol1}$= 0.285 ± 0.094, $h^2_{Cortisol3}$= 0.131 ± 0.078).
305



306

307 **Figure 2**: Guppies habituate to the waterborne sampling procedure, as shown
308 by a decline in average ln-transformed cortisol level (ng/hr) following stressor
309 exposure between first and third exposures. Black circles and associated bars
310 denote predicted population means (± standard error) from mixed model
311 analysis. Grey lines depict the predicted genetic reaction norms across repeated
312 stressor exposure for each individual. Weak, but statistically significant GxE is
313 manifest as variance in the genetic reaction norm slopes (i.e. lack of parallelism)
314 and results in a slight reduction of genetic variance for cortisol at the third
315 exposure relative to the first.

316

317 **Testing for genetic integration and identifying the major axis of genetic**
318 **(co)variance**

319 There is strong evidence for phenotypic integration of *Cortisol* with behaviour at the
320 among-individual levels (see Table S5). To test for and characterise the hypothesised

genetic integration between behavioural and physiological components of the stress response, we built a multivariate animal model to estimate **G**. We excluded *shoaling tendency* given the absence of detectable genetic variance in the univariate model. We also elected to treat cortisol as a single trait (allowing for a fixed effect of stressor exposure number (1 vs 3) on the mean). Although the above analysis demonstrates GxE for cortisol, the strong positive cross-context genetic correlation justifies collapsing $Cortisol_1$ and $Cortisol_3$ into a single trait to maximise statistical power to detect any genetic covariance with behaviour.

Our final model contained six response traits: *relative area covered*, *time in the middle, track length*, (square root-transformed) *freezings,* (ln-transformed) *emergence time*, and (ln-transformed) *Cortisol* (now treated as two repeats of a single trait). We standardised all traits to standard deviation units (after transformation where applicable) to assist multivariate model fitting and to prevent eigenvectors of **G** (see below) being dominated by traits with higher variance in observed units. To simplify interpretation of **G** we also multiplied *emergence time* by -1 after transformation. Thus high values denote rapid emergence from the shelter.

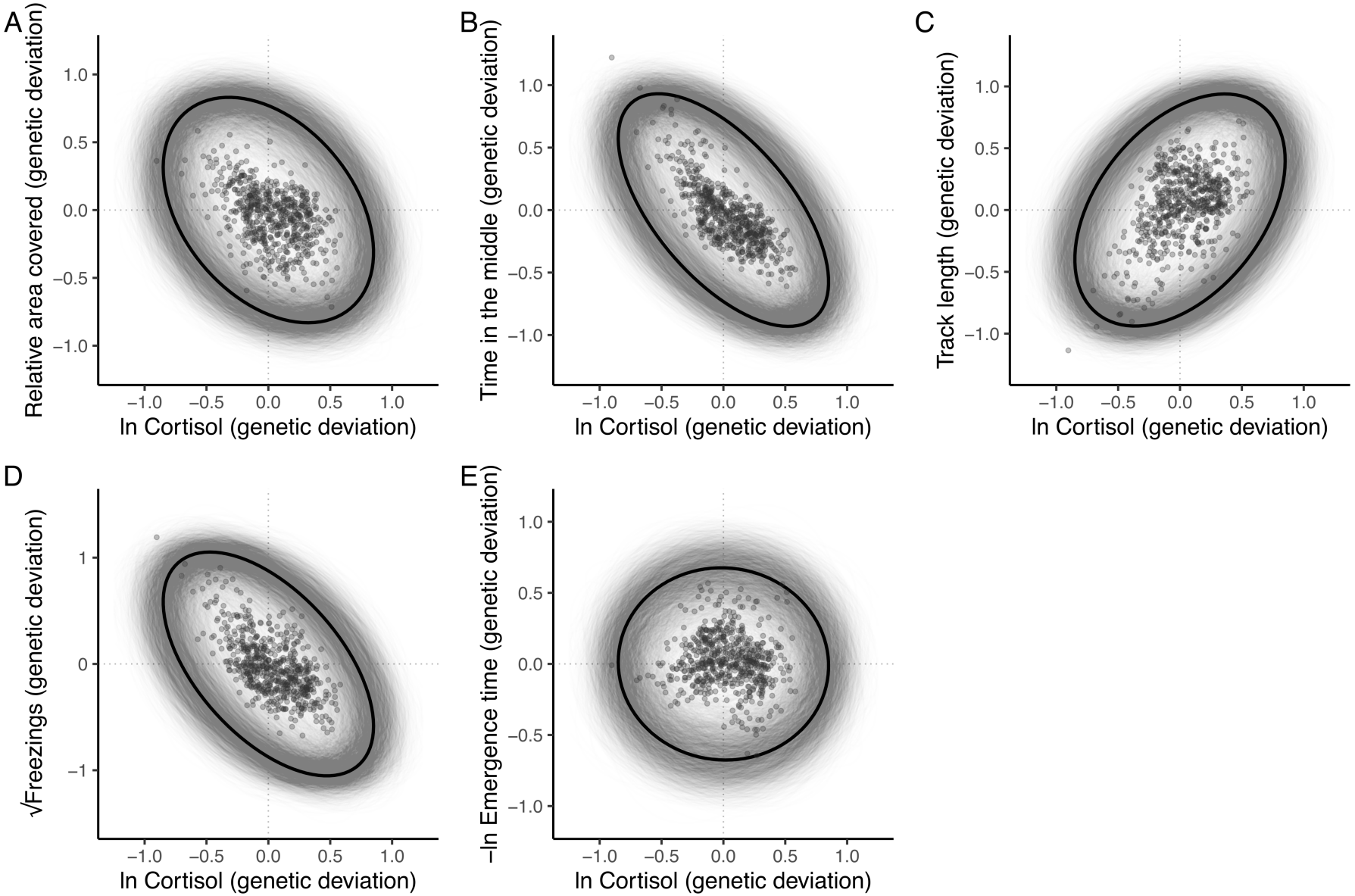
The resultant estimate of **G** (Table 2) contains significant additive genetic covariance structure overall (Likelihood Ratio Test of the full model vs. a reduced model **G** that contains variances but not covariances: $\chi^2_{15} = 91.06$, $P < 0.001$). We found a large number of strong genetic covariance/correlation estimates between trait pairs that were deemed statistically significant (based on the bootstrapped 95% confidence intervals not crossing zero). The 4 OFT traits (*relative area covered, time in the middle, track length,* and $\sqrt{}$*freezings*) show strong patterns of pairwise genetic correlation. *Track length* shows significant negative genetic correlation with *relative area covered, time in the middle*, and $\sqrt{}$*freezings* (such that genotypes predisposed to higher values of *track length* are also predisposed to lower values of *relative area covered, time in the middle,* and $\sqrt{}$*freezings*, and vice versa). *Relative area covered* and *time in the middle* show

348 significant positive genetic correlation with one another, and √*freezings* shows
349 significant positive correlation with *time in the middle*. The only genetic correlation
350 among OFT traits that is not significant is between √*freezings* and *relative area covered.*
351 The speed at which individuals emerge from the shelter during the emergence trials, -*ln*
352 *emergence time*, shows significant positive genetic correlation with *track length* (i.e.,
353 genotypes predisposed to higher values of *track length* in the OFT are also predisposed
354 to faster *emergence* in the ET). There are no significant pairwise genetic correlations
355 between –*ln emergence time* and the other OFT traits, but all correlation estimates are
356 negative. Pairwise genetic correlations between *ln cortisol* and all behavioural traits are
357 illustrated in Figure 3. Ln-transformed *cortisol* shows significant negative genetic
358 correlations with both *time in the middle* and √*freezings*. Genetic correlations between
359 *ln cortisol* and other OFT traits were not significantly different from zero (based on 95%
360 confidence intervals), with correlations estimated as negative with *relative area covered*
361 and positive with *track length*. The genetic correlation between –*ln emergence time* and
362 *ln cortisol* is also not significant, and is estimated at very close to zero.
363 Eigen decomposition of **G** provides a more holistic view of the genetic
364 covariance structure and the level of integration among traits. Here the major axis $\mathbf{g}_{\mathbf{max}}$
365 (first principal component, PC1, with 95% confidence intervals from 5000 bootstrap
366 replicates) explains 59.5% (47.5%, 75.7%) of the genetic variance in multivariate
367 phenotype. Subsequent axes necessarily capture declining proportions of the
368 multivariate genetic variance (PC2 = 20.2% [14.8, 32.1]; PC3 = 14.1% [5.6, 19.6]; PC4 =
369 4.5% [0.9, 8.2]; PC5 = 1.0% [0, 2.8], PC6 = 0.7% [0,0.1]; see Table S6, Figure 4—figure
370 supplement 1). All traits except -*ln emergence time* load significantly on $\mathbf{g}_{\mathbf{max}}$ (Figure 4).
371 *Relative area covered, time in the middle* and √*freezings* load in one direction, while *track*
372 *length* and *ln Cortisol* load in the other direction. This structure indicates a major axis of
373 genetic variation in integrated stress response (Figure 4—figure supplement 2), where
374 genotypes at one end of this axis can be considered to have a 'freeze' (or possibly

375 'weaker'; discussed later) type of behavioural stress response to the OFT assay (i.e.,
376 freezing more frequently, swimming shorter distances, spending more time in the
377 central area of the tank, and exhibiting exploratory swimming patterns that cover
378 greater areas relative to their distance swum). This behavioural profile is associated
379 with 'weaker' physiological responses to stress (i.e., producing lower cortisol levels in
380 response to the stressor). Meanwhile, genotypes at the other end of $\mathbf{g}_{\mathbf{max}}$ have a more
381 'flight' (or arguably 'stronger') type of behavioural stress response to the OFT assay (i.e.,
382 freezing less frequently, swimming further, spending more time close to the tank edges,
383 and covering less area relative to their distance travelled in OFTs). This behavioural
384 profile is associated with 'stronger' physiological responses to stress (i.e., producing
385 higher cortisol levels in response to the stressor).

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

	Relative area covered	*Time in the middle*	*Track length*	√*Freezings*	-ln *Emergence time*	ln *Cortisol*
Relative area covered	**0.115 (0.050,0.182)**	**0.795 (0.601,0.952)**	**-0.549 (-0.789,-0.239)**	0.139 (-0.260,0.511)	-0.438 (-1.305,0.239)	-0.376 (-0.959,0.272)
Time in the middle	**0.103 (0.044,0.165)**	**0.145 (0.070,0.215)**	**-0.658 (-0.86,-0.414)**	**0.363 (0.024,0.659)**	-0.153 (-0.815,0.538)	**-0.617 (-1.139,-0.155)**
Track length	**-0.071 (-0.127,-0.017)**	**-0.096 (-0.154,-0.035)**	**0.147 (0.080,0.219)**	**-0.801 (-0.931,-0.647)**	**0.61 (0.064,1.328)**	0.425 (-0.027,0.968)
√*Freezings*	0.020 (-0.034,0.076)	0.059 (-0.001,0.120)	**-0.132 (-0.200,-0.060)**	**0.185 (0.091,0.269)**	-0.483 (-1.103,0.146)	**-0.556 (-1.069,-0.067)**
-ln *Emergence time*	-0.041 (-0.103,0.012)	-0.016 (-0.073,0.041)	**0.065 (0.008,0.121)**	-0.057 (-0.126,0.009)	**0.076 (0.009,0.148)**	-0.020 (-0.807,0.790)
ln *Cortisol*	-0.044 (-0.105,0.023)	**-0.082 (-0.145,-0.018)**	0.057 (-0.002,0.119)	**-0.083 (-0.155,-0.01)**	-0.002 (-0.058,0.053)	**0.121 (0.036,0.206)**


A
Relative area covered (genetic deviation)
ln Cortisol (genetic deviation)
B
Time in the middle (genetic deviation)
ln Cortisol (genetic deviation)
C
Track length (genetic deviation)
ln Cortisol (genetic deviation)
D
√Freezings (genetic deviation)
ln Cortisol (genetic deviation)
E
−ln Emergence time (genetic deviation)
ln Cortisol (genetic deviation)

391

Figure 3: The additive genetic relationship between ln-transformed cortisol (x-axis) and five behaviours (a, *relative area covered*; b, *time in the middle*; c, *track length*; d, √*freezings;* e, -*ln emergence time*). Points show (predicted) bivariate genetic deviations from the population means, plotted for those individuals in the pedigree with cortisol data. In each case the black ellipse depicts the 'shape' of the relationship as given by the point estimate of **G**. Specifically it encompasses the area expected to contain 95% of the bivariate genetic distribution for the population. Grey ellipses denote the corresponding areas defined from 5000 bootstrapped replicates of **G**, and so highlight the uncertainty around these bivariate distributions.

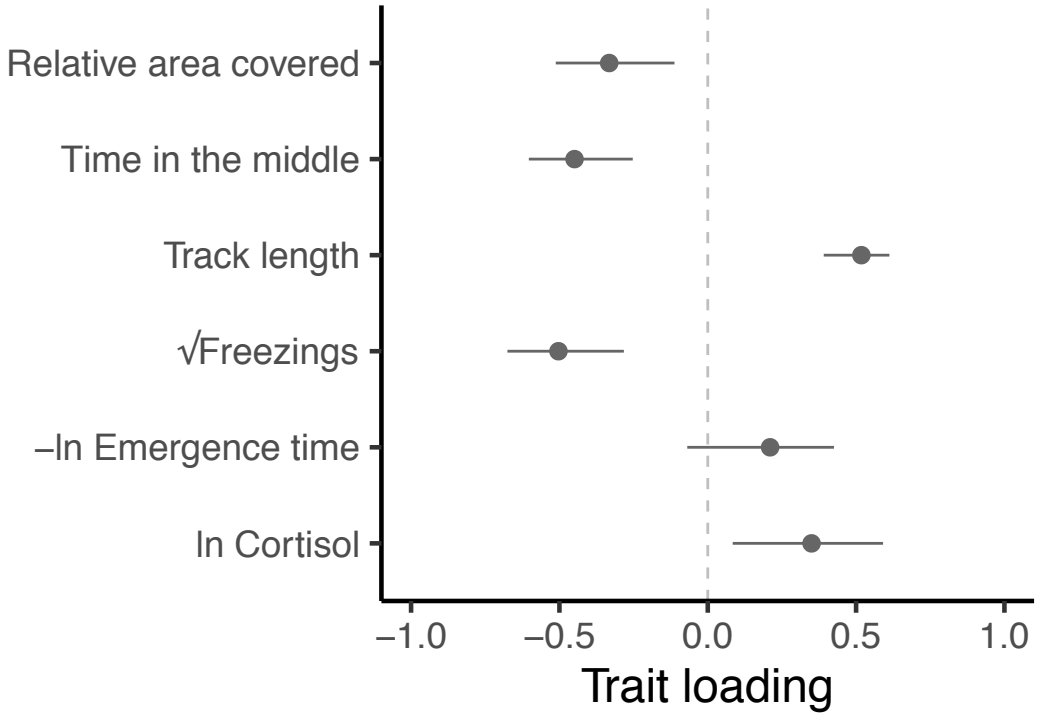


Figure 4: Trait loadings from $\mathbf{g}_{max}$, the first eigen vector (principal component) of **G**. This axis explains 59.5% of the genetic (co)variation found in the focal behavioural and physiological components of the stress response in our guppy population. Points show trait loadings from the first eigen vector of our estimate of G, with bars representing 95% confidence intervals on each loading (calculated from 5000 bootstrapped replicates of the model).

407 **Discussion**

408 In this study we sought to determine whether – and to what extent – there exists genetic
409 variation for, and integration between, behavioural and physiological (endocrine) components of the
410 stress response. Our results provide three main novel insights. First, we find that genetic variation
411 does underpin individual differences in both behavioural and physiological components of the stress
412 response. Second, we find genetic covariance structure among these behavioural and physiological
413 traits, indicating that they are indeed genetically integrated. Thirdly, having identified the structure of
414 the major axis $\mathbf{g}_{max}$ of the genetic variance-covariance matrix **G** we suggest that it is more readily
415 interpreted as an axis of genetic variation in stress coping style than in stress responsiveness, although
416 we acknowledge that any distinction may be somewhat subjective. Overall, by estimating the genetic
417 covariance structure among traits we find the first quantitative genetic support to date for the
418 hypothesis of evolutionary integration between behavioural and endocrine components of the stress
419 response.

420 We find heritable variation in, and covariation among, behaviours assayed in the open field
421 trial (OFT), including the derived trait *relative area covered*. The latter trait, derived by considering an
422 appropriate biological null model of the relationship between *track length* and (absolute) *area covered*
423 serves as a useful proxy for exploratory behaviour. Here we demonstrate an axis of repeatable and
424 heritable variation that spans from 'freeze' type responses characterised by less active but more
425 exploratory swimming patterns (more *freezings* and lower *track length,* coupled with higher *relative*
426 *area covered* and *time in the middle*) through to a 'flight' type response characterised by higher activity
427 coupled to thigmotaxis and low exploration (fewer *freezings* and higher *track length*, coupled with
428 lower *relative area covered* and *time in the middle*). Given the wide use of OFTs in biomedical research
429 (e.g., Rex *et al.* 1998; von Horsten, Karl & Pabst 2003) as well as in animal behaviour, our phenotyping
430 approach (including the derivation of *relative area*) may have broad applicability for discriminating
431 between exploration and stress/anxiety-related patterns of behaviour. The OFT paradigm is also
432 widely applied to studies of 'shy—bold' type personality variation in fishes (Toms, Echevarria &
433 Jouandot 2010) and other vertebrates (Carter *et al.* 2013; Perals *et al.* 2017). The extent to which
434 behavioural differences deemed characteristic of a 'shy—bold' personality axis (commonly, if not

435 universally, defined as repeatable variation in response to perceived risk; Wilson *et al.* 1994) should
436 be viewed as equivalent to variance in 'stress coping style' and/or 'stress responsiveness', is a matter
437 of debate (see Boulton *et al.* 2015). While we view these as overlapping concepts, our findings do
438 highlight reasons for caution in considering them interchangeable. For example, while the structure of
439 the major axis of **G**, $\mathbf{g}_{max}$, is broadly consistent with it reflecting an axis of integrated genetic variation
440 in 'stress coping style' (see below), *emergence time*, an OFT trait that is commonly considered to reflect
441 'boldness' (Burns 2008), was found to be heritable but did not load significantly on $\mathbf{g}_{max}$ (see Figure 4).
442 This could indicate that the shy—bold continuum does not align with the stress coping style
443 continuum in our model organism. However, it could also reflect complexities with interpreting
444 *emergence time*. For example, at least some genotypes (and individuals) could perceive the shelter
445 area as less safe than the open arena (i.e., counter to the assumption that fast emergence reflects a lack
446 of fear of the open arena, and thus greater boldness, e.g., Burns 2008). Indeed, in an earlier study some
447 guppies decreased (rather than increased) shelter use following simulated predation events (Houslay
448 *et al.* 2018).

449 Tendency to shoal varies among individuals but is not detectably heritable. Though not
450 generally considered a stress-response trait *per se*, shoaling is an anti-predator behaviour in guppies
451 (Herbert-Read *et al.* 2017). We had therefore predicted that heightened perception of risk in the open
452 field might also be associated with increased shoaling tendency. This was not the case at the among-
453 individual level (Table S5), while the absence of detectable genetic variance meant that we could not
454 test this prediction in **G**.

455 We find strong evidence of significant additive genetic variance in a key physiological
456 component of the stress response: waterborne cortisol concentrations following exposure of the fish
457 to a handling stressor. Our findings suggest that previously detected differences among individuals in
458 their cortisol response to a stressor (Houslay *et al.* 2019) are primarily attributable to genetic effects,
459 with the estimated heritability ($h^2 = 0.31$) constituting over 80% of the individual–level repeatability
460 ($R = 0.37$) for ln-transformed *Cortisol*. In addition, by adopting a reaction norm approach to modelling
461 stress physiology, as recently advocated by ourselves (Houslay *et al.* 2019) and others (e.g., Fürtbauer
462 *et al.* 2015; Hau & Goymann 2015; Taff & Vitousek 2016; Guindre-Parker 2020; Malkoc *et al.* 2021), we

463 detect GxE reflecting genetic differences in the extent of habituation to the stressor over repeated
464 exposures. This result is potentially important because poor habituation of the hypothalamic-
465 pituitary-adrenal/interrenal (HPA/I) response to repeated or ongoing stressors can lead to well
466 documented health problems in human and animal populations (Segerstrom & Miller 2004; Koolhaas
467 2008; Romero, Dickens & Cyr 2009; Mason 2010). Our detection of heritable variation in the degree of
468 habituation to stressors raises the possibility of developing targeted selection strategies to improve
469 welfare in captive populations (e.g., Frankham *et al.* 1986; Muir & Craig 1995; Oltenacu & Algers
470 2009).

471 Our findings also highlight that there is greater additive genetic variance (and heritability) for
472 cortisol levels following the first exposure to the stressor than following the third. This pattern, which
473 occurs because genotypes that produce the highest cortisol response at first exposure also show the
474 most marked habituation, is consistent with the idea of cryptic genetic variance being 'released' by
475 exposure to novel, and so potentially stressful, environments (Ledón-Rettig, Pfennig & Crespi 2010;
476 Ledón-Rettig *et al.* 2014; Berger *et al.* 2011; Paaby & Rockman 2014). All else being equal, it also
477 means that selection on cortisol levels following stressor exposure should induce a stronger
478 evolutionary response in naïve relative to habituated fish. However, the strong positive cross-
479 environment correlation means that the ranking of genotypes with regard to their cortisol responses
480 is consistent across repeated stressor exposures. Thus selection on the (average) GC response would
481 result in a correlated evolutionary response of habituation rate, and *vice versa*. With regard to drawing
482 inferences from these GC levels about the extent to which an organism is in a 'stressed' state, it is
483 worth noting the potential complexity that may arise from GCs serving numerous functions. For
484 example, in addition to mobilising energy and down-regulating non-essential processes in order to
485 facilitate coping with an acute stressor (Romero & Beattie 2021), GCs such as cortisol are also thought
486 to play a key role in reproductive processes (Wingfield & Sapolsky 2003; Breuner, Patterson & Hahn
487 2008; Bonier *et al.* 2009b; Ouyang *et al.* 2011; but see also Bonier *et al.* 2009a). Thus, there is a need
488 for some caution when equating a high GC level with a highly stressed 'state'. Notably, studies of GC
489 levels alone will naturally not capture a complete picture of among-individual variation in stress
490 physiology. The HPA/I axis (which culminates in GC secretion) reflects just one of several important

491 neuro-endocrine stress response pathways (see MacDougall-Shackleton *et al.* 2019) whose wider
492 integration, while logistically challenging to study at present, strongly merits attention going forward
493 (Gormally & Romero 2020).

494 Considering all traits together, **G** shows evidence of genetic integration between behavioural
495 and endocrine components of the stress response. The ends of the major axis of **G** ($\mathbf{g}_{max}$) are largely
496 consistent with expectations given genetic variance in 'stress coping styles' (Koolhaas *et al.* 1999).
497 Genotypes showing (putatively) more proactive 'flight' type behaviour in the OFT (i.e. thigmotaxis,
498 high *track length*, low *relative area covered,* few *freezings*) produce higher levels of cortisol following
499 the handling and confinement stressor, while genotypes showing (putatively) more reactive 'freezing'
500 type behaviour in the OFT (i.e., low *track length* and many *freezings*, in addition to higher *relative area*
501 *covered* and more *time in the middle*) produce lower levels of cortisol following the same stressor.
502 Were one to accept this view, the trait loadings on $\mathbf{g}_{max}$ would suggest that thigmotaxis and freezing
503 behaviour are strong indicators of stress coping style. Notably, here we find continuous variation
504 along this major axis of genetic variation (see Figure 4—figure supplement 2), rather than the bimodal
505 distribution suggested by some of the coping styles literature (Koolhaas *et al.* 1999, 2007, 2010). An
506 alternative interpretation, however, is that $\mathbf{g}_{max}$ represents variance in stress response 'magnitude'
507 rather than coping style *per se*. This is because the putatively 'reactive' end of $\mathbf{g}_{max}$ might simply reflect
508 a low magnitude stress response rather than a particular style of 'coping' with a stressor. Such an
509 interpretation would help explain why fish (or genotypes) that produce lower levels of cortisol
510 following handling and confinement also have more 'exploratory' movement (higher *relative area*
511 *covered*) and reduced thigmotaxis (i.e., increased *time in the middle*) in the OFT. We note that the
512 distinction between 'style' and 'responsiveness' may be rather moot if, for example, fish with proactive
513 styles are also more responsive.

514 In fact, a subsequent 'two-tier' iteration of the coping style model proposed that variation in
515 the magnitude of the stress response (termed 'stress reactivity') could be viewed as a second
516 dimension of variation, distinct from differences in the 'type' (or style) of response (Koolhaas *et al.*
517 2010). It is not clear to us that these two dimensions, if both present, can be tested for and
518 disentangled empirically in the current data—for example, there is no *a priori* expectation that style

519 and magnitude should manifest as orthogonal axes in **G** among traits analysed here (and so both could
520 conceivably align on $\mathbf{g}_{max}$). In principle, disentangling these dimensions should be possible by
521 characterising stress response through within-subject comparison of behaviour and endocrine state
522 between stressed and 'baseline' (unstressed) states. In practice, we found here that genetic variation
523 in GC responsiveness (i.e., the slope across states, accepting that habituation represents a form of this)
524 is very strongly correlated with the average (or intercept). We found similar results for behavioural
525 responses to increased stressor severity in another study of this population, where there exist strong
526 among-individual correlations between the intercept and slope of the plastic response (Houslay *et al.*
527 2018). While we cannot claim a true 'baseline' state in either case, these results suggest that stress
528 coping style and the magnitude of response do both exist as axes of variation, but may also be tightly
529 correlated.

530 The genetic integration of behaviour and physiology detected here is consistent with (but not
531 proof of) the hypothesis that correlational selection in the past has led to the coevolution of these
532 components of the stress response. This hypothesis assumes that correlational selection favours any
533 combination of trait values that yield higher fitness, creating a genetic correlation as mutations that
534 generate such combinations will be recruited into the population while those that do not will be
535 selected out (Lande 1980; Roff & Fairbairn 2012). While reflections on the role of past correlational
536 selection are necessarily speculative, we can say that the structure of **G** should shape (and potentially
537 constrain) future evolutionary responses to selection—whether natural or artificial. Here we have no
538 direct knowledge of how contemporary selection is acting in the wild or whether it might be changing
539 as a consequence of anthropogenic stressors. Nor do we know exactly how well our estimate of **G** will
540 match that which may be found in the wild (though our study animals are all recent descendants of
541 wild-collected animals). Thus we cannot comment directly on how **G** will shape future evolution of the
542 guppy stress response beyond noting that selection on behaviour will cause correlated evolution of
543 endocrine physiology (and vice versa). Nonetheless, while it seems reasonable to expect that current
544 integration of stress response in natural populations should be broadly adaptive, this seems less likely
545 in captive populations (at least for species without a long history of domestication and opportunity for
546 adaptation to artificial environments). We know that prolonged, chronic activation of stress response

547 pathways (notably the HPA/I axis) frequently disrupts health and survival in captive animals (Huether
548 1996; Boonstra 2013). It may be that more stress-responsive genotypes are disadvantaged in novel
549 artificial conditions (e.g., if acute stress responsiveness positively predicts susceptibility to chronic
550 stress). However, even if true this would not imply high (acute) stress-responsiveness was also
551 disadvantageous in the wild. Since natural selection should purge alleles that are universally
552 detrimental, it seems more plausible that genetic variation along the major axis described here is
553 maintained by some form of selective trade-off (as widely hypothesised for maintenance of personality
554 variation; e.g., Stamps 2007; Wolf *et al.* 2007; Réale *et al.* 2010). For instance, genotypes susceptible to
555 harm under chronic stressor exposure will likely persist in populations if they also confer advantages
556 under an acute stress challenge. In natural populations not only is exposure to acute stressors more
557 common than to chronic stressors, but also selection through chronic stress exposure may be
558 conditional on (and subsequent to) surviving acute challenges (such as predator attacks).
559

560 **Conclusions and future directions**

561 Here we find evidence for genetic variation in – and integration of – behavioural and physiological
562 (endocrine) components of the stress response. Overall we consider the structure of **G** to be broadly
563 consistent with the widely invoked 'reactive—proactive' model of variation in stress coping style
564 (Koolhaas *et al.* 2007). This interpretation rests largely on the structure of behavioural variation
565 revealed by the OFT, which is dominated by a major axis running from genotypes with more proactive
566 'flight' type stress responses to those with more reactive 'freeze' type stress responses. Endocrine
567 traits align with this axis: genotypes exhibiting 'flight' type responses show higher cortisol levels (and
568 exhibit faster habituation of GC physiology) when subjected to repeated handling and confinement
569 stressors than those exhibiting more 'freeze' type responses. However, as these latter genotypes with
570 more 'freeze' type responses and low cortisol responses to stressors also tend – on average – to
571 display space use patterns characteristic of exploration and reduced thigmotaxis (potentially
572 indicative of being relatively unstressed), an alternative interpretation is that $\mathbf{g}_{\mathbf{max}}$ primarily reflects
573 differences in stress responsiveness. Further distinguishing between 'style' and 'responsiveness' may
574 depend on their association, for example if fish with proactive styles are also more responsive.

575 Although future studies could certainly target separation of these dimensions further, we think greater
576 insights may come from expanding the set of traits (in particular, to include other components of the
577 physiological stress response, as noted above) and/or stress contexts (i.e. exposing the subjects to
578 different types of stressor). With respect to the latter, here we observed behavioural and GC responses
579 to two different stressors separated in time. We think that our demonstration of genetic correlation
580 structure between behaviours tested in one context and physiology assayed in a different one adds at
581 least some support to the idea that the integration characterised here may ultimately prove
582 generalisable across stress contexts.

583 Our results suggest that continued evolution of stress-related behaviour will have
584 consequences for glucocorticoid physiology and *vice versa*. Determining contemporary selection on
585 the stress response, and testing the possibility that its underlying genetic variation is maintained by
586 fitness trade-offs, is thus an obvious – if empirically challenging – next step to understanding the
587 functional importance of genetic variation in the stress response in wild populations. In a more
588 applied context, integration of behavioural and endocrine stress response components at the genetic
589 level has potential utility for artificial selection to improve resilience to chronic stressors in managed
590 populations (Gebauer *et al.* 2021). Specifically, it may be possible to identify non-invasive, high
591 throughput, behavioural biomarkers and target them in selection schemes to reduce chronic activation
592 of the HPA/I endocrine axis and its attendant deleterious effects.
593

Materials and methods

Husbandry and breeding

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

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

623 brood tank on the day of birth. Note that as the gestation period for guppies is approximately 1 month,
624 any brood produced by a female less than one month after exposure to their designated male was
625 recorded in the pedigree as having unknown paternity.
626 Within 24h of a female producing a brood we recorded her weight (g) and brood size. We kept
627 juvenile fish in full-sib family groups in 2.8L tanks before moving them to 15L 'growth' tanks at an
628 average age of 56 days. At an average age of 133 days (range 59-268) we tagged individuals and
629 placed them into mixed family groups of 16-20 adults (with an even mix of males and females), kept in
630 15L tanks. Note that variation in tagging age arose largely because groups were necessarily
631 established sequentially as sufficient individuals from multiple families reached a large enough size
632 that we deemed the procedure to be safe. Each adult group comprised a mix of fish from different
633 families, reducing the potential for common environment effects to upwardly bias our genetic
634 parameter estimation.
635
636 *Overview of behavioural phenotyping*
637 Behavioural phenotyping commenced at least one week after tagging. In all trials, we filmed
638 movement behaviour of individual fish using a Sunkwang video camera equipped with a 6-60mm
639 manual focus lens suspended over the tank. We used the tracking software Viewer II (BiObserve) to
640 extract behavioural data from each recording (detailed below). The tank was lit from below using a
641 light box and screened with a cardboard casing to prevent external visual disturbance. After each
642 behavioural trial, the individual tested was weighed and then moved to a temporary 'holding tank'.
643 Once a full group (as described above) had been tested, all were moved from the holding tank back to
644 their home tank. We replaced the water in the testing and holding tanks between groups to reduce the
645 build-up of hormones or other chemicals. The first offspring generation experienced 4 repeat open
646 field trials (OFTs) over a 2-week period, with at least 48h between trials. Subsequent generations
647 experienced 4 repeat behavioural trials, alternating 2 OFTs with 2 emergence trials (ETs). For the final
648 2 generations, we extended the OFTs by including a shoaling trial (ST) at the end of each OFT.
649

Open field trials (OFT) followed the methodology described by White et al (2016). Briefly, we assessed individual behaviour in a 20cm x 30cm tank, filled to a depth of 5cm with room-temperature water from the main supply. We caught fish individually from their home tank, examined them quickly for identification tags, then placed them immediately into the centre of the OFT tank. After allowing 30s for acclimation, we filmed behaviour for 4m30s. Behaviours characterised from the tracking software were *track length* (the total distance the fish moved during the trial; cm), *area covered* (the percentage of 1cm x 1cm grid squares through which the fish moved during the trial; %), *time in middle* (time spent in a rectangular inner zone which was defined as being the same size as an outer area; seconds), and number of *freezings* (defined in practice as the number of times that an individual's velocity dropped below 4cm/s for a minimum of 2.5 seconds; White & Wilson 2018).

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

677 *Emergence trials (ET)* followed the methodology described by White *et al.* (2016). Briefly, we tested
678 individuals in a 20cm x 40cm tank, filled to a depth of 8cm with room-temperature water from the
679 main supply. A 10cm section of the tank length was walled off creating a shelter area (20cm x10cm),
680 the walls and floor of which were painted black. The focal fish was placed into the shelter area and
681 allowed to acclimate for 30s, at which point we opened a sliding door to allow access to the rest of the
682 tank, which was brightly lit from below and otherwise bare. *Time to emerge* (in seconds) was recorded
683 by the tracking software automatically as the fish exited the shelter area and emerged into the open
684 tank section. Trials were ended either at emergence or at 15 min if the fish had not emerged by that
685 point; in the case of non-emergence, fish were given the maximum value (i.e., 900s).
686
687 *Derivation of 'relative area' from OFT trials*
688 The '*area covered'* variable assayed in the OFT is calculated in BiObserve by dividing the arena (i.e., the
689 total area of the tank as viewed from the camera) into 1cm x 1cm grid squares. The path taken by the
690 fish during observation is then used to determine what proportion of these grid squares the fish
691 entered. However, we sought to derive a measure of '*relative area*' that describes whether a fish covers
692 a large, or small area relative to its observed *track length.*
693 To do this we simulated 'random swims' within the arena across the observed range of *track*
694 *lengths*. We first selected 40 OFT results at random from our total data set and extracted the
695 coordinates of the fish in each frame from the raw tracking file, creating a set of x and y movements
696 and their associated distances. As original coordinates were recorded in pixels we used the calibration
697 of the software to convert to cm units. We then use a 'random walk' algorithm to select a movement
698 (i.e., step size and direction) from this observed distribution at random and calculate the new
699 coordinates. If the movement keeps the 'fish' within the bounds of the 'tank' (i.e., defined as a 20cm x
700 30cm arena), the movement is accepted and coordinates added to a movement matrix; if not, a new
701 movement is drawn from the distribution. If the movement is greater than 1cm in distance, we break
702 the movement into a number of smaller parts to be added to the matrix (such that we capture the
703 coordinates of grid squares through which the 'fish' moved along the way). Once the total distance of
704 the random walk reached or exceeded the *track length* set as the simulation objective, the path is

705 terminated and the area covered is calculated by counting the number of unique grid squares in the
706 matrix of coordinates and dividing by the total number possible.
707 After simulating random walks across 500 values of *track length* (using a vector of 100 values
708 evenly spaced across the range of true data, repeated 5 times), we modelled (simulated) area covered
709 as a fourth order polynomial function of *track length*. Using this regression model (which explained
710 97.8% of the variance in simulated data), we calculated the *relative area* for each actual OFT trial as
711 the observed area covered minus the area covered under a random swim, as predicted from our
712 regression model and the observed *track length*.
713
714 *Waterborne hormone sampling*
715 On completion of behavioural data collection, individuals entering the endocrine testing program were
716 left undisturbed for a minimum of two weeks. Waterborne hormone sampling was then conducted
717 over a 5-day period that included three handling and confinement stressor exposures with 48h
718 between each. We followed the method described by Houslay *et al*. (2019) to obtain repeated non-
719 invasive GC measures of individuals using holding water samples from the first and third
720 confinements. Note that only two samples per fish were analysed because the financial and time costs
721 of doing three was deemed prohibitive. We nonetheless applied the stressor stimulus three times as
722 our prior study showed this was sufficient to produce a strong habituation response, i.e., a significant
723 decrease in water-borne cortisol over the three sampling periods (Houslay *et al.* 2019).
724 We collected samples between 1200 – 1400h to control for diel fluctuations in GC levels. For
725 each sample, we netted an entire group from their home tank quickly using a large net, transferring
726 them to 2 holding tanks (containing water from the home tank supply) for moving to an adjacent quiet
727 room (performed within 20s of the net first hitting the water). We then transferred fish to individual
728 Pyrex beakers containing 300ml of clean water from the main supply (which serves the main housing
729 units), which had been warmed to the appropriate temperature (mean = 24.1°C, range 23-24.9°C).
730 Beakers were placed within cardboard 'chambers' to prevent fish from seeing each other or
731 experiencing outside disturbance. One fish was transferred every 30s, alternating across holding
732 tanks, such that all fish were in their beakers within 10min of the initial netting. After 60 mins in the

733 beaker, each fish was removed by pouring its sample through a clean net into a second beaker, with
734 the fish then quickly checked to confirm ID and returned to the holding tank until the entire group
735 could be returned to its home tank.

736 We immediately filtered each water sample using Grade 1 filter paper (Whatman), then passed
737 them slowly through solid phase C18 extraction columns (Sep-Pak C18, 3cc, Waters) via high-purity
738 tubing (Tygon 2474, Saint Gobain) under vacuum pressure (Earley *et al.* 2006). Columns were primed
739 beforehand with 2 x 2ml HPLC-grade methanol followed by 2 x 2ml distilled water and were washed
740 afterwards with a further 2 x 2ml distilled water to purge salts. We then covered both ends of each
741 column with film (Parafilm M, Bemis) and stored them at -20C for future analysis. We washed all
742 beakers, tubes and funnels with 99% ethanol and rinsed them with distilled water prior to each
743 sampling procedure. The remainder of the endocrine assay procedure involved elution, resuspension,
744 separation and quantification of free cortisol by enzyme immunoassay (EIA) using Cayman Chemicals,
745 Inc EIA kits. Detailed methods are exactly as described by Houslay *et al.* (2019) and so not repeated
746 here (note that here we assayed the free fraction of cortisol only). To validate the cortisol kits, we
747 examined whether the kit standard curve was parallel to a serial dilution curve derived from pooled
748 guppy water-borne hormone extract. 20µl was taken from each of the male samples and pooled; 20µl
749 was taken from each of the female samples and combined into a separate pool. 400µl of the pools was
750 serially diluted from 1:1 to 1:128 and these samples were assayed alongside the kit standard curve on
751 two occasions (June and December 2017, marking the start and finish of sample processing). All
752 dilution curves were parallel to the standard curve (slope comparison test, Zar 1996, p.355; June,
753 male: $t_{12} = 0.029$, $P = 0.97$; June, female: : $t_{12} = 0.343$, $P = 0.74$; December, male: : $t_{12} = 0.119$, $P = 0.91$;
754 December, female: : $t_{12} = 0.224$, $P = 0.83$). The serial dilution curves also identified 1:32 as an
755 appropriate dilution to ensure that all samples fell on the linear phase of the standard curve. A total of
756 37 96-well plates were used, and the pooled sample was included at the beginning and end of each
757 plate. Intra-assay coefficients of variation ranged from 0.12-19.83% with a median of 3.08%; the inter-
758 assay coefficient of variation was 19.22%. Cortisol is presented and modelled in (ln-transformed)
759 units of ng/hr to reflect the 1 hour sampling duration.
760

761 **Statistical methods**

762

763 All data handling and analysis was performed in R version 3.6.3 (Team 2020). We used the 'tidyverse'
764 set of packages for data handling and visualisation (Wickham 2017), and ASreml-R v4 (Butler 2021)
765 for fitting linear mixed effects models (as described in full below). We also used 'nadiv' for pedigree
766 preparation (Wolak 2012). All models fitted assumed (multivariate) Gaussian error structures, and we
767 visually assessed residuals to verify this was reasonable (after data transformation in some cases). To
768 test for significance of among individual and/or genetic (co)variance components, we fitted nested
769 models with different random effects structures and compared them using likelihood ratio tests
770 (LRTs). We calculated $\chi^2_{n\mathrm{DF}}$ as twice the difference in model log likelihoods, with the number of
771 degrees of freedom (n) equivalent to the number of additional parameters in the more complex model.
772 When testing a single random effect (variance component), we assumed the difference to be
773 asymptotically distributed as an equal mix of χ^2_0 and χ^2_1 (denoted $\chi^2_{0,1}$; Self and Liang, 1987;
774 Visscher, 2006).

775 For each OFT, ST and ET behaviour in turn (*relative area, time in middle, track length, freezings,*
776 *shoaling tendency,* and *emergence time*), we used the random effects specification to partition
777 phenotypic variation (V_p, conditional on fixed effects as described below) into the effects of additive
778 genetics (V_a), permanent environment defined as the non-(additive) genetic component of among-
779 individual differences, V_pe), and housing group (V_group), as well as residual variation (V_residual). We
780 natural log-transformed *emergence time* prior to analysis to meet assumptions of residual normality
781 and homoscedasticity. For all behavioural traits, we included fixed effects of assay *repeat*, the *order*
782 *within each group* in which the fish was trialled (mean-centred continuous predictor), *temperature*
783 (mean-centred and scaled to standard deviation units), *time* (in minutes from midnight, mean-centred
784 and scaled to standard deviation units), *age* (mean-centred and scaled to standard deviation units),
785 *sex*, and the *generation* from the breeding population. The *order caught* predictor in particular was
786 used to control statistically for variation in disturbance over the course of measuring a group (White &
787 Wilson 2018). For *shoaling tendency* only, we incorporated an additional fixed effect of *setup* (as

788 detailed above). We tested the significance of genetic variance for each behaviour by LRT comparison
789 of the corresponding full model to one in which the (additive) genetic random effect was excluded.
790 Cortisol data were also natural log (ln) transformed for analysis. We formulated a bivariate
791 model to test for both additive genetic variation and genotype-by-environment interaction (GxE) in
792 cortisol levels across the two 'contexts' (i.e. samples retained for each individual at first and third
793 confinement, denoted $Cortisol_1$, $Cortisol_3$). Random effects were first used to partition phenotypic
794 (co)variance (conditional on fixed effects) into among-group and residual components. Fixed effects
795 included the context-specific means, and overall effects of the *order* in which the fish was caught from
796 each group for assay (mean-centred continuous predictor), *temperature* (mean-centred and scaled to
797 standard deviation units), *time of day* (mean-centred and scaled to standard deviation units), *age*
798 (mean-centred and scaled to standard deviation units), and *sex*. In addition, we included fixed
799 covariates of *body mass* (mean-centred and scaled to standard deviation units) and a *sex* by *body mass*
800 interaction (see Houslay *et al.* 2019 for rationale of controlling for body size effects on waterborne
801 hormone levels in this way). Note that modelled in this way each individual is sampled only once for
802 each context-specific cortisol trait so no random effect of individual identity is included. To test for
803 additive genetic variation (V_a) we compared this first bivariate model to a second formulation that also
804 included the (additive) genetic merit, but under the assumption that this is invariant with context
805 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
806 comparing the second model to a third in which we allow GxE (i.e., the context-specific genetic
807 variances are free to differ and the cross-context genetic correlation can be <+1).
808 Lastly, we built a multivariate animal model to estimate **G** and to test the hypothesised genetic
809 integration among behavioural and physiological stress components. We retained only response traits
810 that harboured significant V_a as shown in univariate models, and so the final model comprised
811 response traits *relative area, time in middle, track length, freezings* (square root transformed),
812 *emergence time* (ln transformed), and *Cortisol* (ln transformed). We multiplied (transformed)
813 *emergence time* by -1 to simplify interpretation (higher values represent faster emergence). We also
814 scaled all (transformed) response variables to standard deviation units. This was to facilitate model
815 fitting, and also prevent scale effects complicating interpretation of eigenvectors of **G**. Fixed and

random effects were fitted on each trait as specified for the univariate models. Note that one exception to this is that we elected to treat *Cortisol* as a single repeated-measures trait here (with two repeats, one per context) such that a permanent environment effect was now included. Fixed effects estimates are reported in the supplementary information (Table S1).

We specified additive genetic (**G**), permanent environment (**PE**), group (**GROUP**), and residual (**R**) covariance structures as unstructured matrices to be estimated. Note that **R** partitions observation-level covariances (as opposed to individual-level in **PE**) that are not definable or statistically identifiable if traits are not measured at the same time (i.e., all covariances relating to *emergence time* or *Cortisol*). Where this was the case we constrained specific covariance terms in **R** to equal zero. Estimates of **PE**, **GROUP** and **R** are provided in the supplementary information (Tables S2-S4). We tested for overall additive genetic covariance among the traits by comparing this model against a reduced one in which **G** was specified as a diagonal matrix (i.e., additive variances are estimated but covariances are assumed to equal zero). To aid the interpretation of covariance terms contained in **G,** we calculated the corresponding genetic correlations r_a from the full model. For any 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 decomposition to determine the proportion of additive genetic variation captured by each principal component and assess whether the major axis of variation ($\mathbf{G}_{\max}$) could indeed explain most of the genetic variance in the multivariate phenotype. We estimated uncertainty on the trait loadings associated with each principal component (eigenvector), as well as on the variance explained by each eigenvector, using a parametric bootstrap approach as described by Boulton et al (2014).

For visualisation of bivariate relationships at the additive genetic level, we used the R package 'ellipse' (Murdoch & Chow 2018) to determine the coordinates of an ellipse representing the approximate 95% confidence region of deviations based on the point estimate of **G**. We repeated this procedure for the corresponding regions defined from 5000 bootstrapped values of **G** (i.e., to indicate uncertainty arising from estimation of the genetic covariance structure itself). Best linear unbiased predictors (BLUPs) are used for visualisation only, not for any statistical analysis (Houslay & Wilson 2017).

To test for associations between all traits (i.e., including *shoaling tendency*) at the among-individual level, we also built a multivariate model as above with the addition of *shoaling tendency* and without estimating additive genetic effects. The estimates of all among-individual (co)variances are provided in the supplementary information (Table S5).

Acknowledgements

We are grateful to P. Sharman, D. Maskrey and C. Mnatzagian for help in the lab, and to J. Martin, J. Styga, P. Vullioud and all members of the Wilson group for discussion. We also thank J. McGlothlin and two anonymous reviewers for comments and suggestions that greatly improved this manuscript.

852 **REFERENCES**

853

854 Angelier, F. & Wingfield, J.C. (2012) Importance of the glucocorticoid stress response in a changing
855 world: Theory, hypotheses and perspectives. *General and Comparative Endocrinology*, **190**, 118–
856 128.
857 Arnold, S.J., Bürger, R., Hohenlohe, P.A., Ajie, B.C. & Jones, A.G. (2008) Understanding the evolution and
858 stability of the G-matrix. *Evolution*, **62**, 2451–61.
859 Barton, B.A. & Iwama, G.K. (1991) Physiological changes in fish from stress in aquaculture with
860 emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases*, **1**, 3–26.
861 Berger, D., Bauerfeind, S.S., Blanckenhorn, W.U. & Schäfer, M.A. (2011) HIGH TEMPERATURES REVEAL
862 CRYPTIC GENETIC VARIATION IN A POLYMORPHIC FEMALE SPERM STORAGE ORGAN.
863 *Evolution*, **65**, 2830–2842.
864 Blows, M.W. (2007) A tale of two matrices: multivariate approaches in evolutionary biology. *Journal of*
865 *evolutionary biology*, **20**, 1–8.
866 Blows, M.W. & Walsh, B. (2009) Spherical cows grazing in Flatland: Constraints to selection and
867 adaptation. *Adaptation and Fitness in Animal Populations*, pp. 83–101. Springer, Dordrecht.
868 Bonier, F., Martin, P.R., Moore, I.T. & Wingfield, J.C. (2009a) Do baseline glucocorticoids predict fitness?
869 *Trends in Ecology and Evolution*, **24**, 634–642.
870 Bonier, F., Moore, I.T., Martin, P.R. & Robertson, R.J. (2009b) The relationship between fitness and
871 baseline glucocorticoids in a passerine bird. *General and Comparative Endocrinology*, **163**, 208–
872 213.
873 Boonstra, R. (2013) Reality as the leading cause of stress: Rethinking the impact of chronic stress in
874 nature. *Functional Ecology*, **27**, 11–23.
875 von Borell, E. (1995) Neuroendocrine integration of stress and significance of stress for the
876 performance of farm animals. *Applied Animal Behaviour Science*, **44**, 219–227.
877 Boulton, K., Couto, E., Grimmer, A.J., Earley, R.L., Canario, A.V.M., Wilson, A.J. & Walling, C.A. (2015)
878 How integrated are behavioral and endocrine stress response traits? A repeated measures
879 approach to testing the stress-coping style model. *Ecology and Evolution*, **5**, 618–633.
880 Boulton, K., Grimmer, A.J., Rosenthal, G.G., Walling, C.A. & Wilson, A.J. (2014) How stable are
881 personalities? A multivariate view of behavioural variation over long and short timescales in the
882 sheepshead swordtail, Xiphophorus birchmanni. *Behavioral Ecology and Sociobiology*, **68**, 791–
883 803.
884 Breuner, C.W., Patterson, S.H. & Hahn, T.P. (2008) In search of relationships between the acute
885 adrenocortical response and fitness. *General and Comparative Endocrinology*, **157**, 288–295.
886 Broom, D.M. & Johnson, K.G. (1993) Assessing welfare: short-term responses. *Stress and Animal*
887 *Welfare*, pp. 87–110. Springer Netherlands, Dordrecht.
888 Busch, D.S. & Hayward, L.S. (2009) Stress in a conservation context: A discussion of glucocorticoid
889 actions and how levels change with conservation-relevant variables. *Biological Conservation*, **142**,
890 2844–2853.
891 Butler, D. (2021) asreml: Fits the Linear Mixed Model.
892 Carere, C., Groothuis, T.G.G., Möstl, E., Daan, S. & Koolhaas, J.M. (2003) Fecal corticosteroids in a
893 territorial bird selected for different personalities: Daily rhythm and the response to social stress.
894 *Hormones and Behavior*, **43**, 540–548.
895 Carter, A.J., Feeney, W.E., Marshall, H.H., Cowlishaw, G. & Heinsohn, R. (2013) Animal personality: What
896 are behavioural ecologists measuring? *Biological Reviews*, **88**, 465–475.
897 Cheverud, J.M. (1982) Phenotypic, Genetic, and Environmental Morphological Integration in the
898 Cranium. *Evolution*, **36**, 499.
899 Clinchy, M., Sheriff, M.J. & Zanette, L.Y. (2013) Predator-induced stress and the ecology of fear.
900 *Functional Ecology*, **27**, 56–65.
901 Cox, R.M., McGlothlin, J.W. & Bonier, F. (2016) Hormones as Mediators of Phenotypic and Genetic
902 Integration: An Evolutionary Genetics Approach. *Integrative and Comparative Biology*, **56**, 126–
903 137.
904 Earley, R.L., Edwards, J.T., Aseem, O., Felton, K., Blumer, L.S., Karom, M. & Grober, M.S. (2006) Social

interactions tune aggression and stress responsiveness in a territorial cichlid fish (Archocentrus nigrofasciatus). *Physiology and Behavior*, **88**, 353–363.
Frankham, R., Hemmer, H., Ryder, O.A., Cothran, E.G., Soulé, M.E., Murray, N.D. & Snyder, M. (1986) Selection in captive populations. *Zoo Biology*, **5**, 127–138.
Fürtbauer, I., Pond, A., Heistermann, M. & King, A.J. (2015) Personality, plasticity and predation: linking endocrine and behavioural reaction norms in stickleback fish. *Functional Ecology*, n/a-n/a.
Gebauer, T., Gebauer, R., Palińska-Żarska, K., Císař, P., Movchan, O., Tomášek, O., Prokešová, M., Matoušek, J., Hliwa, P., Król, J., Żarski, D., Rebl, A. & Stejskal, V. (2021) Assessment of behavioural and physiological traits as indicators of suitability for European perch aquaculture. *Aquaculture*, **544**.
Gormally, B.M.G. & Romero, L.M. (2020) What are you actually measuring? A review of techniques that integrate the stress response on distinct time-scales. *Functional Ecology*, **34**, 2030–2044.
Guindre-Parker, S. (2020) Individual variation in glucocorticoid plasticity: Considerations and future directions. *Integrative and Comparative Biology*, **60**, 79–88.
Hau, M. & Goymann, W. (2015) Endocrine mechanisms, behavioral phenotypes and plasticity: known relationships and open questions. *Frontiers in Zoology*, **12**, S7.
Herbert-Read, J.E., Rosén, E., Szorkovszky, A., Ioannou, C.C., Rogell, B., Perna, A., Ramnarine, I.W., Kotrschal, A., Kolm, N., Krause, J. & Sumpter, D.J.T. (2017) How predation shapes the social interaction rules of shoaling fish. *Proceedings of the Royal Society B: Biological Sciences*, **284**, 20171126.
Hine, E., Chenoweth, S.F. & Blows, M.W. (2004) Multivariate quantitative genetics and the lek paradox: genetic variance in male sexually selected traits of Drosophila serrata under field conditions. *Evolution*, **58**, 2754–62.
von Horsten, S., Karl, T. & Pabst, R. (2003) Behavioral phenotyping of mice in pharmacological and toxicological research. *Experimental and Toxicologic Pathology*, **55**, 69–83.
Houslay, T.M., Earley, R.L., Young, A.J. & Wilson, A.J. (2019) Habituation and individual variation in the endocrine stress response in the Trinidadian guppy (Poecilia reticulata). *General and Comparative Endocrinology*, **270**, 113–122.
Houslay, T.M., Vierbuchen, M., Grimmer, A.J., Young, A.J. & Wilson, A.J. (2018) Testing the stability of behavioural coping style across stress contexts in the Trinidadian guppy. *Functional Ecology*, **32**, 424–438.
Houslay, T.M. & Wilson, A.J. (2017) Avoiding the misuse of BLUP in behavioural ecology. *Behavioral Ecology*, **00**, 1–5.
Huether, G. (1996) The central adaptation syndrome: Psychosocial stress as a trigger for adaptive modification of brain structure and brain function. *Progress in Neurobiology*, **48**, 569–612.
Hunt, J., Blows, M.W., Zajitschek, F., Jennions, M.D. & Brooks, R.C. (2007) Reconciling strong stabilizing selection with the maintenance of genetic variation in a natural population of black field crickets (Teleogryllus commodus). *Genetics*, **177**, 875–80.
Jones, A.G., Arnold, S.J. & Bürger, R. (2003) Stability of the G-matrix in a population experiencing pleiotropic mutation, stabilizing selection, and genetic drift. *Evolution*, **57**, 1747–1760.
Jones, R.B., Satterlee, D.G. & Ryder, F.H. (1994) Fear of Humans in Japanese-Quail Selected for Low or High Adrenocortical-Response. *Physiology & Behavior*, **56**, 379–383.
Kasper, C., Ribeiro, D., Almeida, A.M. De, Larzul, C., Liaubet, L. & Murani, E. (2020) Emphasis on Stress Response and Damaging Behaviour in Pigs. *Genes*, **11**, 1–24.
Ketterson, E.D., Atwell, J.W. & McGlothlin, J.W. (2009) Phenotypic integration and independence: Hormones, performance, and response to environmental change. *Integrative and Comparative Biology*, **49**, 365–379.
Koolhaas, J.M. (2008) Coping style and immunity in animals: Making sense of individual variation. *Brain, Behavior, and Immunity*, **22**, 662–667.
Koolhaas, J.M., de Boer, S.F., Buwalda, B. & Van Reenen, K. (2007) Individual variation in coping with stress: A multidimensional approach of ultimate and proximate mechanisms. *Brain, Behavior and Evolution*, **70**, 218–226.
Koolhaas, J.M., de Boer, S.F., Coppens, C.M. & Buwalda, B. (2010) Neuroendocrinology of coping styles: Towards understanding the biology of individual variation. *Frontiers in Neuroendocrinology*, **31**,

307–321.
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., Ruis, M.A.W. & Blokhuis, H.J. (1999) Coping styles in animals: Current status in behavior and stress- physiology. *Neuroscience and Biobehavioral Reviews*, **23**, 925–935.
Korte, S.M., Koolhaas, J.M., Wingfield, J.C. & McEwen, B.S. (2005) The Darwinian concept of stress: Benefits of allostasis and costs of allostatic load and the trade-offs in health and disease. *Neuroscience and Biobehavioral Reviews*, **29**, 3–38.
Lande, R. (1980) The genetic covariance between characteres maintained by pleiotropic mutations. *Genetics*, **94**, 203–215.
Lande, R. & Arnold, S.J. (1983) The measurement of selection on correlated characters. *Evolution*, **37**, 1210–1226.
Ledón-Rettig, C.C., Pfennig, D.W., Chunco, A.J. & Dworkin, I. (2014) Cryptic genetic variation in natural populations: a predictive framework. *Integrative and comparative biology*, **54**, 783–793.
Ledón-Rettig, C.C., Pfennig, D.W. & Crespi, E.J. (2010) Diet and hormonal manipulation reveal cryptic genetic variation: Implications for the evolution of novel feeding strategies. *Proceedings of the Royal Society B: Biological Sciences*, **277**, 3569–3578.
MacDougall-Shackleton, S.A., Bonier, F., Romero, L.M. & Moore, I.T. (2019) Glucocorticoids and "Stress" Are Not Synonymous. *Integrative Organismal Biology*, **1**.
Malkoc, K., Mentesana, L., Casagrande, S. & Hau, M. (2021) Quantifying Glucocorticoid Plasticity Using Reaction Norm Approaches: There Still is So Much to Discover! *Integrative and Comparative Biology*, **00**, 1–13.
Mason, G.J. (2010) Species differences in responses to captivity: Stress, welfare and the comparative method. *Trends in Ecology and Evolution*, **25**, 713–721.
McEwen, B.S. & Wingfield, J.C. (2003) The concept of allostasis in biology and biomedicine. *Hormones and Behavior*, **43**, 2–15.
McEwen, B.S. & Wingfield, J.C. (2010) What is in a name? Integrating homeostasis, allostasis and stress. *Hormones and Behavior*, **57**, 105–111.
McGlothlin, J.W. & Ketterson, E.D. (2008) Hormone-mediated suites as adaptations and evolutionary constraints. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, **363**, 1611–1620.
Mignon-Grasteau, S., Boissy, A., Bouix, J., Faure, J.M., Fisher, A.D., Hinch, G.N., Jensen, P., Le Neindre, P., Mormède, P., Prunet, P., Vandeputte, M. & Beaumont, C. (2005) Genetics of adaptation and domestication in livestock. *Livestock Production Science*, **93**, 3–14.
Moberg, G.P. (2000) Biological response to stress: implications for animal welfare. *The biology of animal stress: basic principles and implications for animal welfare*, pp. 1–21.
Möstl, E. & Palme, R. (2002) Hormones as indicator of stress. *Domestic animal and endocrinology*, **23**, 67–74.
Muir, W.M. & Craig, J. V. (1998) Improving animal well-being through genetic selection. *Poultry Science*, **77**, 1781–1788.
Murdoch, D. & Chow, E.D. (2018) ellipse: Functions for Drawing Ellipses and Ellipse-like Confidence Regions.
Oltenacu, P.A. & Algers, B. (2009) Selection for Increased Production and the Welfare of Dairy Cows: Are New Breeding Goals Needed? *AMBIO: A Journal of the Human Environment*, **34**, 311–315.
Oswald, M.E., Singer, M. & Robison, B.D. (2013) The Quantitative Genetic Architecture of the Bold-Shy Continuum in Zebrafish, Danio rerio. *PLoS ONE*, **8**, 1–10.
Ouyang, J.Q., Sharp, P.J., Dawson, A., Quetting, M. & Hau, M. (2011) Hormone levels predict individual differences in reproductive success in a passerine bird. *Proceedings of the Royal Society B: Biological Sciences*, **278**, 2537–2545.
Øverli, Ø., Sørensen, C., Pulman, K.G.T., Pottinger, T.G., Korzan, W., Summers, C.H. & Nilsson, G.E. (2007) Evolutionary background for stress-coping styles: Relationships between physiological, behavioral, and cognitive traits in non-mammalian vertebrates. *Neuroscience and Biobehavioral Reviews*, **31**, 396–412.
Øverli, Ø., Winberg, S. & Pottinger, T.G. (2005) Behavioral and Neuroendocrine Correlates of Selection for Stress Responsiveness in Rainbow Trout—a Review. *Integrative and Comparative Biology*, **45**, 463–474.

Paaby, A.B. & Rockman, M. V. (2014) Cryptic genetic variation: Evolution's hidden substrate. *Nature Reviews Genetics*, **15**, 247–258.
Perals, D., Griffin, A.S., Bartomeus, I. & Sol, D. (2017) Revisiting the open-field test: what does it really tell us about animal personality? *Animal Behaviour*, **123**, 69–79.
Pottinger, T.G. & Carrick, T.R. (1999) Modification of the plasma cortisol response to stress in rainbow trout by selective breeding. *General and Comparative Endocrinology*, **116**, 122–132.
Prentice, P.M., Houslay, T.M., Martin, J.G.A. & Wilson, A.J. (2020) Genetic variance for behavioural 'predictability' of stress response. *Journal of Evolutionary Biology*, 1–11.
Réale, D., Garant, D., Humphries, M.M., Bergeron, P., Careau, V. & Montiglio, P.-O. (2010) Personality and the emergence of the pace-of-life syndrome concept at the population level. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, **365**, 4051–4063.
Rex, A., Voigt, J.P., Voits, M. & Fink, H. (1998) Pharmacological evaluation of a modified open-field test sensitive to anxiolytic drugs. *Pharmacology Biochemistry and Behavior*, **59**, 677–683.
Roff, D.A. (1992) *The Evolution of Life Histories: Theory and Analysis.* Chapman & Hall.
Roff, D.A. & Fairbairn, D.J. (2012) A test of the hypothesis that correlational selection generates genetic correlations. *Evolution*, **66**, 2953–60.
Romero, L.M. (2004) Physiological stress in ecology: Lessons from biomedical research. *Trends in Ecology and Evolution*, **19**, 249–255.
Romero, L.M. & Beattie, U.K. (2021) Common myths of glucocorticoid function in ecology and conservation. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*, 1–8.
Romero, L.M., Dickens, M.J. & Cyr, N.E. (2009) The reactive scope model - A new model integrating homeostasis, allostasis, and stress. *Hormones and Behavior*, **55**, 375–389.
Sadoul, B., Blumstein, D.T., Alfonso, S. & Geffroy, B. (2021) Human protection drives the emergence of a new coping style in animals. *PLoS Biology*, **19**, 1–11.
Segerstrom, S.C. & Miller, G.E. (2004) Psychological stress and the human immune system: a meta-analytic study of 30 years of inquiry. *Psychological bulletin*, **130**, 601–30.
Self, S.G. & Liang, K. (1987) Asymptotic properties of maximum likelihood estimators and likelihood ratio tests under nonstandard conditions. *J. Am. Stat. Assoc.*, **82**, 605–610.
Sih, A. & Bell, A.M. (2008) Insights for behavioral ecology from behavioral syndromes. *Advances in the Study of Behavior*
Stamps, J.A. (2007) Growth-mortality tradeoffs and "personality traits" in animals. *Ecology Letters*, **10**, 355–363.
Stearns, S.C. (1989) Trade-Offs in Life-History Evolution. *Functional Ecology*, **3**, 259–268.
Stirling, D.G., Réale, D. & Roff, D.A. (2002) Selection, structure and the heritability of behaviour. *Journal of Evolutionary Biology*, **15**, 277–289.
Taborsky, B., English, S., Fawcett, T.W., Kuijper, B., Leimar, O., McNamara, J.M., Ruuskanen, S. & Sandi, C. (2020) Towards an Evolutionary Theory of Stress Responses. *Trends in Ecology and Evolution*, 1–10.
Taff, C.C. & Vitousek, M.N. (2016) Endocrine Flexibility: Optimizing Phenotypes in a Dynamic World? *Trends in Ecology and Evolution*, **31**, 476–488.
Tarlow, E.M. & Blumstein, D.T. (2007) Evaluating methods to quantify anthropogenic stressors on wild animals. *Applied Animal Behaviour Science*, **102**, 429–451.
Team, R.C. (2020) R: A Language and Environment for Statistical Computing.
Thomson, J.S., Watts, P.C., Pottinger, T.G. & Sneddon, L.U. (2011) Physiological and genetic correlates of boldness: Characterising the mechanisms of behavioural variation in rainbow trout, Oncorhynchus mykiss. *Hormones and Behavior*, **59**, 67–74.
Toms, C.N., Echevarria, D.J. & Jouandot, D.J. (2010) A Methodology Review of Personality-Related Studies in Fish: Focus on the Shy-Bold Axis of Behaviour. *Berkeley Planning Journal*, **26**, 12.
Trenzado, C.E., Carrick, T.R. & Pottinger, T.G. (2003) Divergence of endocrine and metabolic responses to stress in two rainbow trout lines selected for differing cortisol responsiveness to stress. *General and Comparative Endocrinology*, **133**, 332–340.
Veenema, A.H., Meijer, O.C., De Kloet, E.R., Koolhaas, J.M. & Bohus, B.G. (2003) Differences in basal and stress-induced HPA regulation of wild house mice selected for high and low aggression. *Hormones and Behavior*, **43**, 197–204.
Visscher, P.M. (2006) A Note on the Asymptotic Distribution of Likelihood Ratio Tests to Test Variance

Components. *Twin Research and Human Genetics*, **9**, 490–495.
Walsh, B. & Blows, M.W. (2009) Abundant Genetic Variation + Strong Selection = Multivariate Genetic Constraints: A Geometric View of Adaptation. *Annual Review of Ecology, Evolution, and Systematics*, **40**, 41–59.
White, S.J., Houslay, T.M. & Wilson, A.J. (2018) Evolutionary genetics of personality in the Trinidadian guppy II: sexual dimorphism and genotype-by-sex interactions. *Heredity*.
White, S.J., Kells, T.J. & Wilson, A.J. (2016) Metabolism, personality and pace of life in the Trinidadian guppy, Poecilia reticulata. *Behaviour*, **153**, 1517–1543.
White, S.J., Pascall, D.J. & Wilson, A.J. (2020) Towards a comparative approach to the structure of animal personality variation. *Behavioral Ecology*, **31**, 340–351.
White, S.J. & Wilson, A.J. (2018) Evolutionary genetics of personality in the Trinidadian guppy I: maternal and additive genetic effects across ontogeny. *Heredity*.
Wickham, H. (2017) tidyverse: Easily Install and Load "Tidyverse" Packages.
Wilson, D.S., Clark, A.B., Coleman, K. & Dearstyne, T. (1994) Shyness and boldness in humans and other animals. *Trends in Ecology and Evolution*, **9**, 442–446.
Wilson, A.J., Réale, D., Clements, M.N., Morrissey, M.M., Postma, E., Walling, C.A., Kruuk, L.E.B. & Nussey, D.H. (2009) An ecologist's guide to the animal model. *Journal of Animal Ecology*, **79**, 13–26.
Wingfield, J.C. (2003) Control of behavioural strategies for capricious environments. *Animal Behaviour*, **66**, 807–816.
Wingfield, J.C. & Kitaysky, A.S. (2002) Endocrine responses to unpredictable environmental events: Stress or anti-stress hormones? *Integr Comp Biol*, **42**, 600–609.
Wingfield, J.C., Maney, D.L., Breuner, C.W., Jacobs, J.D., Lynn, S., Ramenofsky, M. & Richardson, R.D. (1998) Ecological Bases of Hormone-Behavior Interactions: The "Emergency Life History Stage." *American Zoologist*, **38**, 191–206.
Wingfield, J.C. & Sapolsky, R.M. (2003) Reproduction and resistance to stress: when and how. *Journal of neuroendocrinology*, **15**, 711–724.
Wolak, M.E. (2012) Nadiv: An R package to create relatedness matrices for estimating non-additive genetic variances in animal models. *Methods in Ecology and Evolution*, **3**, 792–796.
Wolf, M., van Doorn, G.S., Leimar, O. & Weissing, F.J. (2007) Life-history trade-offs favour the evolution of animal personalities. *Nature*, **447**, 581–4.
Zar, J.H. (1996) *Biostatistical Analysis*. Prentice-Hall International, Inc., London.

1104 **Supplementary materials**

1105

1106 Table S1: Fixed effects estimates from the full multivariate animal model.

1107 Table S2: Permanent environment (co)variance matrix from the full multivariate animal model.

1108 Table S3: Group (co)variance matrix from the full multivariate animal model.

1109 Table S4: Residual variance-correlation matrix from the full multivariate animal model.

1110 Table S5: Among-individual (co)variance matrix from the multivariate model that excluded genetic

1111 effects.

1112 Table S6. Table showing full description of eigen decomposition of the **G** matrix.

1113 Figure 4—figure supplement 1: Scree plot showing the proportion of total variance explained by each

1114 eigen vector, with vertical bars indicating 95% confidence intervals as estimated from 5000

1115 bootstrapped replicates. The horizontal dotted line indicates where equal variance across all

1116 eigen vectors would lie.

1117 Figure 4—figure supplement 2: Histogram of PC scores for $\mathbf{g}_{max}$ calculated for each individual that was

1118 assayed for both behaviour and endocrine responses. The distribution is skewed but clearly not

1119 bimodal, as is predicted by some verbal models of 'stress coping style'.

1120 Supplementary File 1: Selection of OFT behaviours.

1121 Table S1. Fixed effects estimates from the full multivariate animal model.
1122
1123

Trait	Parameter	Estimate	SE	z
Relative area	(Intercept)	-0.198	0.153	-1.291
	Age	0.04	0.04	1.003
	Generation (1)	-0.118	0.107	-1.103
	Generation (2)	0.268	0.115	2.327
	Generation (3)	0.657	0.282	2.331
	Generation (S F1)	0.868	0.274	3.166
	Order	-0.004	0.004	-1.067
	Repeat	-0.07	0.015	-4.584
	Sex (Male)	0.142	0.039	3.614
	Temperature	-0.026	0.023	-1.161
	Time of day	0.18	0.122	1.47
Time in the middle	(Intercept)	-0.344	0.156	-2.209
	Age	0.084	0.041	2.047
	Generation (1)	0.064	0.11	0.578
	Generation (2)	0.359	0.12	3
	Generation (3)	0.582	0.287	2.028
	Generation (S F1)	1.077	0.279	3.865
	Order	-0.004	0.004	-0.943
	Repeat	-0.098	0.015	-6.344
	Sex (Male)	0.346	0.04	8.721
	Temperature	0.025	0.023	1.109
	Time of day	0.271	0.124	2.184
Track length	(Intercept)	-0.694	0.143	-4.861
	Age	-0.167	0.038	-4.436
	Generation (1)	0.567	0.097	5.871
	Generation (2)	0.469	0.106	4.41
	Generation (3)	0.703	0.266	2.646
	Generation (S F1)	0.233	0.257	0.907
	Order	-0.009	0.004	-2.419
	Repeat	0.147	0.015	10.05
	Sex (Male)	-0.155	0.04	-3.862
	Temperature	0.04	0.021	1.894
	Time of day	0.028	0.115	0.247
√Freezings	(Intercept)	0.431	0.158	2.731
	Age	0.157	0.041	3.862
	Generation (1)	-0.236	0.105	-2.236
	Generation (2)	-0.252	0.116	-2.163
	Generation (3)	-0.193	0.293	-0.66
	Generation (S F1)	0.088	0.284	0.311
	Order	-0.002	0.004	-0.518
	Repeat	-0.138	0.016	-8.45
	Sex (Male)	-0.022	0.04	-0.535
	Temperature	0.037	0.023	1.616
	Time of day	0.058	0.127	0.456
-ln Emergence time	(Intercept)	0.087	0.334	0.26
	Age	0.002	0.045	0.046
	Generation (3)	-0.713	0.668	-1.068
	Generation (S F1)	-0.593	0.668	-0.889
	Order	-0.004	0.005	-0.737
	Repeat	0.073	0.045	1.597
	Sex (Male)	0.259	0.052	4.988
	Temperature	0.08	0.039	2.048
	Time of day	-0.265	0.326	-0.811
ln Cortisol	(Intercept)	1.054	0.334	3.154
	Age	0	0.029	0.007
	Order	-0.001	0.004	-0.318
	Sex (Male)	-0.846	0.079	-10.668
	Stressor exposure (first)	0.672	0.035	19.316
	Temperature	-0.1	0.048	-2.1
	Time of day	-1.337	0.465	-2.876
	Weight	0.261	0.055	4.74
	Weight x Sex (Male)	-0.158	0.102	-1.554

Table S2. Permanent environment (co)variance matrix from the full multivariate animal model.

	Relative area	Time in the middle	Track length	√Freezings	-ln(Emergence time)	ln Cortisol
Relative area	0.15 ± 0.03					
Time in the middle	0.10 ± 0.03	0.14 ± 0.03				
Track length	-0.14 ± 0.02	-0.10 ± 0.02	0.18 ± 0.03			
√Freezings	0.10 ± 0.02	0.10 ± 0.03	-0.10 ± 0.03	0.10 ± 0.03		
-ln(Emergence time)	0.05 ± 0.03	-0.01 ± 0.03	-0.02 ± 0.03	-0.01 ± 0.03	0.07 ± 0.04	
ln Cortisol	0.03 ± 0.03	0.09 ± 0.03	-0.04 ± 0.03	0.05 ± 0.03	-0.03 ± 0.03	0.08 ± 0.04

Table S3. Group (co)variance matrix from the full multivariate animal model.

	Relative area	Time in the middle	Track length	√Freezings	-ln Emergence time	ln Cortisol
Relative area	0.05 ± 0.01					
Time in the middle	0.03 ± 0.01	0.06 ± 0.02				
Track length	-0.02 ± 0.01	-0.03 ± 0.01	0.03 ± 0.01			
√Freezings	0.01 ± 0.01	0.03 ± 0.01	-0.02 ± 0.01	0.05 ± 0.01		
-ln Emergence time	-0.00 ± 0.01	-0.02 ± 0.01	0.01 ± 0.01	-0.03 ± 0.01	0.03 ± 0.01	
ln Cortisol	-0.01 ± 0.01	0.02 ± 0.01	-0.03 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.07 ± 0.03

Table S4. Residual variance-correlation matrix from the full multivariate animal model.

	Relative area	Time in the middle	Track length	√Freezings	-ln Emergence time	ln Cortisol
Relative area	0.55 ± 0.02	0.65 ± 0.01	-0.64 ± 0.01	0.41 ± 0.02	-	-
Time in the middle		0.56 ± 0.02	-0.59 ± 0.01	0.51 ± 0.02	-	-
Track length			0.50 ± 0.02	-0.68 ± 0.01	-	-
√Freezings				0.63 ± 0.02		
-ln Emergence time					0.79 ± 0.04	-
ln Cortisol						0.35 ± 0.02

1139

Table S5. Among-individual (co)variance matrix from the multivariate model excluding genetic effects.

	Relative area	Time in the middle	Track length	√Freezings	-ln Emergence time	Shoaling tendency	ln Cortisol
Relative area	0.25 (0.211,0.288)	0.747 (0.692,0.801)	-0.728 (-0.78,-0.674)	0.49 (0.392,0.576)	0.032 (-0.219,0.273)	0.198 (-0.054,0.436)	-0.002 (-0.186,0.16)
Time in the middle	0.19 (0.154,0.223)	0.258 (0.216,0.298)	-0.645 (-0.702,-0.577)	0.601 (0.522,0.68)	-0.134 (-0.373,0.113)	0.025 (-0.22,0.287)	0.123 (-0.05,0.299)
Track length	-0.203 (-0.237,-0.167)	-0.182 (-0.218,-0.148)	0.31 (0.265,0.35)	-0.76 (-0.808,-0.709)	0.168 (-0.046,0.399)	-0.053 (-0.286,0.168)	-0.003 (-0.159,0.156)
√Freezings	0.123 (0.09,0.155)	0.153 (0.118,0.187)	-0.212 (-0.248,-0.173)	0.251 (0.21,0.297)	-0.333 (-0.605,-0.101)	-0.066 (-0.307,0.185)	-0.038 (-0.231,0.131)
-ln Emergence time	0.006 (-0.038,0.044)	-0.024 (-0.066,0.017)	0.033 (-0.01,0.073)	-0.059 (-0.101,-0.02)	0.127 (0.06,0.193)	0.319 (0.001,0.666)	-0.236 (-0.502,0.015)
Shoaling tendency	0.043 (-0.009,0.093)	0.006 (-0.048,0.058)	-0.013 (-0.067,0.036)	-0.014 (-0.066,0.036)	0.049 (0.004,0.098)	0.185 (0.102,0.267)	-0.01 (-0.243,0.237)
ln Cortisol	0 (-0.038,0.033)	0.026 (-0.011,0.063)	-0.001 (-0.037,0.035)	-0.008 (-0.046,0.028)	-0.035 (-0.069,0.002)	-0.002 (-0.045,0.037)	0.176 (0.133,0.222)

Shoaling tendency is included as there is significant among-individual variance in this trait. Among-individual variances provided on the shaded diagonal, with among-individual covariances below and among-individual correlations above. 95% confidence intervals in parentheses are estimated from 5000 bootstrapped replicates.

Table S6. Table showing full description of eigen decomposition of the **G** matrix.

	Eigen vectors					
	1	2	3	4	5	6
Eigen value	0.47	0.16	0.11	0.04	0.01	0.01
Percentage of total	59.5%	20.2%	14.1%	4.5%	1.0%	0.7%
	Trait loadings					
Relative area covered	-0.33	-0.53	-0.33	0.32	-0.24	0.58
Time in the middle	-0.45	-0.50	0.10	-0.41	0.57	-0.20
Track length	0.52	-0.17	0.26	0.40	0.61	0.32
√*Freezings*	-0.50	0.62	0.17	-0.03	0.28	0.51
-ln *Emergence time*	0.21	-0.20	0.58	-0.54	0.35	0.42
ln *Cortisol*	0.35	0.11	-0.67	-0.53	0.21	0.30

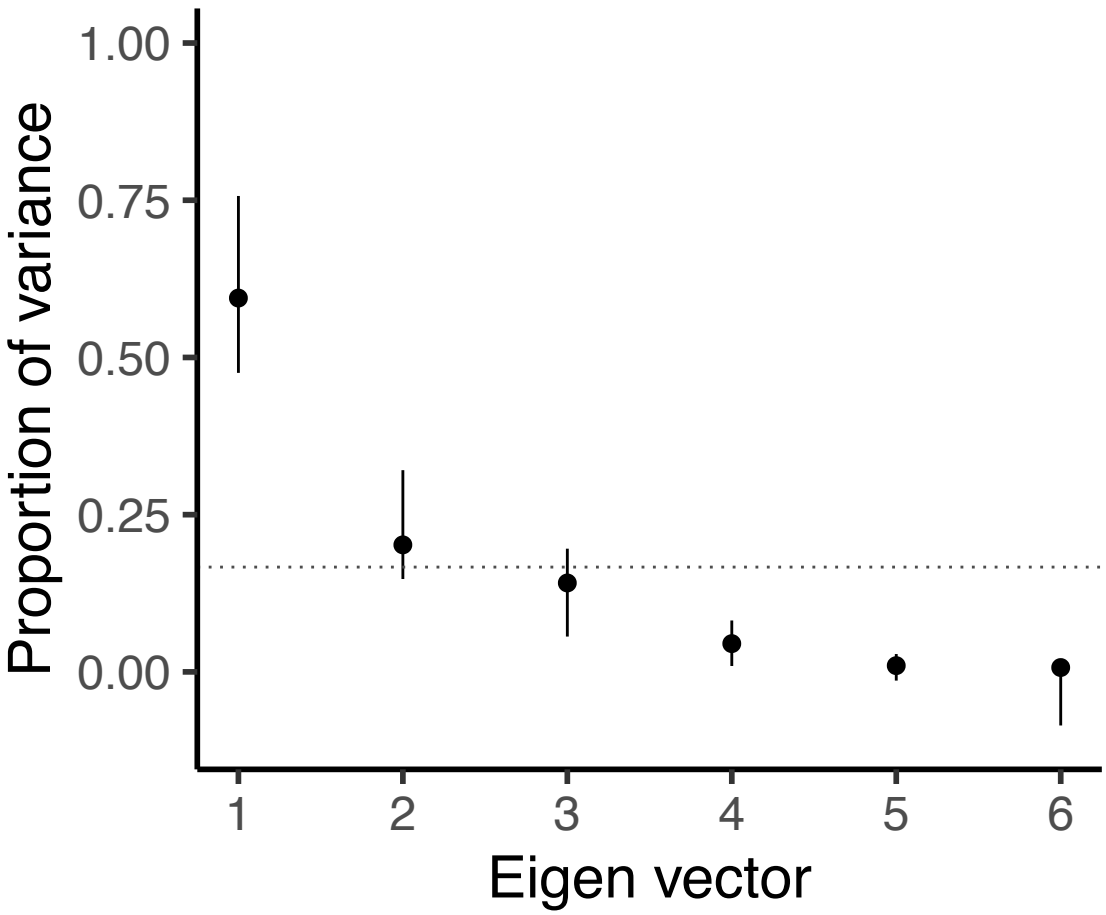


Figure 4—figure supplement 1: Scree plot showing the proportion of total variance explained by each eigen vector, with vertical bars indicating 95% confidence intervals as estimated from 5000 bootstrapped replicates. The horizontal dotted line indicates where equal variance across all eigen vectors would lie.

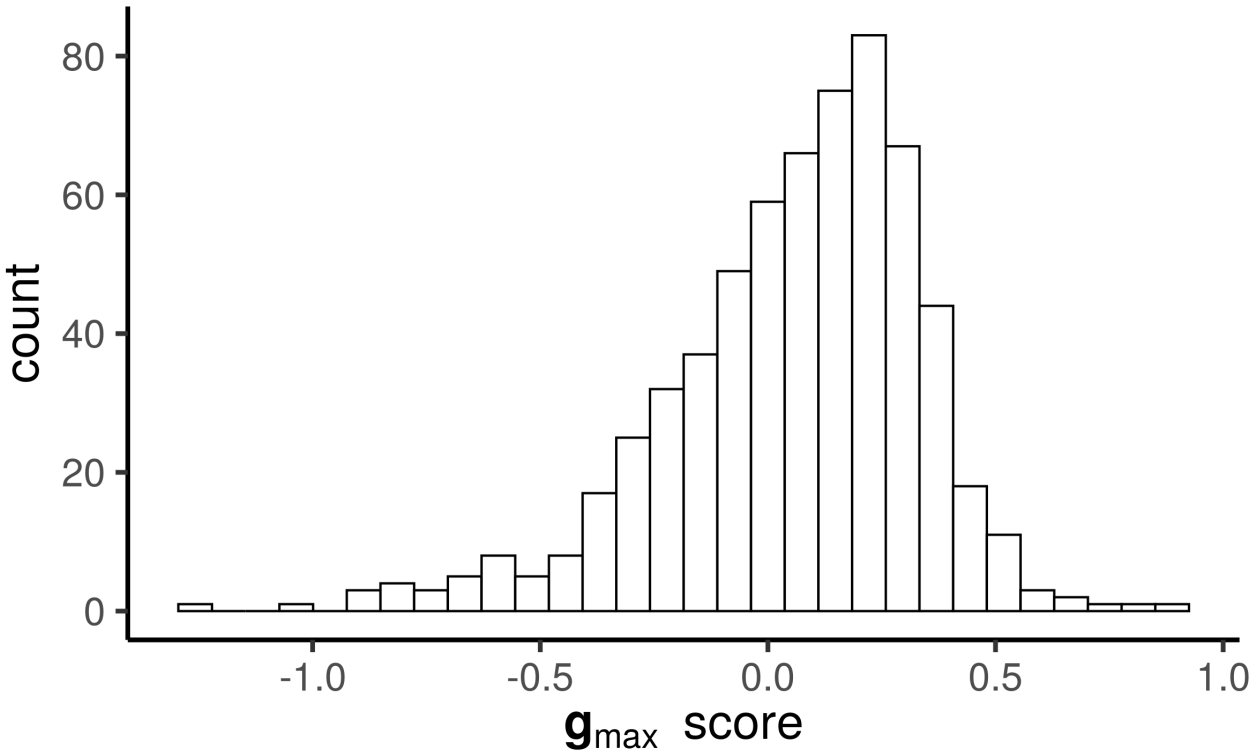


Figure 4—figure supplement 2: Histogram of PC scores for $\mathbf{g}_{max}$ calculated for the subset of individuals that were assayed for both behaviour and endocrine responses. The distribution is clearly not bimodal, as is predicted by some verbal models of 'stress coping style'.