Genetic integration of the stress response

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Abstract

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

Introduction

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

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

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

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

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

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

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

130 responses to this treatment as a proxy for their behavioural stress response (Houslay *et*
131 *al.* 2018). Previous work has demonstrated significant additive genetic (co)variance in
132 risk-taking and exploratory behavioural responses in this context (White & Wilson
133 2018; White, Houslay & Wilson 2018), behaviours that also exhibit strong plastic
134 responses to other acute stressors, including simulated predation events by birds and
135 predatory fish (Houslay *et al.* 2018). Using a non-invasive waterborne hormone
136 sampling method, we have also shown that individuals differ in their GC (specifically
137 free circulating cortisol) response to an acute stressor (handling and short term
138 confinement associated with the sampling itself; Houslay *et al.* 2019) and that, on
139 average, this GC response decreases with repeated exposure to the sampling event
140 stressor. However, nothing is known about the genetic basis of variation in these
141 physiological components of the stress response, or of their integration with genetic
142 variation in stress-related behaviours.
143 Here we test for evidence of genetic integration of the behavioural and
144 endocrine components of the stress response. First, we characterise multiple
145 behavioural components of the stress response, by developing a novel phenotyping
146 approach for OFTs, to better distinguish 'flight' behaviour from potentially confounding
147 variation in activity and exploration traits, and by complementing OFTs with additional
148 'emergence trials' (ET) and 'shoaling trials' (ST). Second, we characterise two key
149 physiological components of the stress response by assaying GC levels following
150 exposure to the first and third handling stress treatments in a stress habituation
151 paradigm (see methods). All behavioural and physiological traits were assayed multiple
152 times per individual using fish within a known genetic pedigree structure, enabling us to
153 partition phenotypic (co)variation in the suite of focal traits into its additive genetic,
154 permanent environment (here, among-individual), housing group, and residual
155 components. We predict that there will be a heritable (genetic) component to each trait
156 measured and that the **G** matrix (the pattern of genetic covariation among traits) will

157 contain significant among-trait correlation structure. More specifically, based on the
158 'stress coping style' model (Koolhaas *et al.* 1999) we predict genetic integration of
159 behavioural and physiological traits in **G** such that a major axis of genetic variation is
160 present: at one end, genotypes will be predisposed to a low-activity, exploratory
161 phenotype with lower GC levels, and at the other a 'flighty' phenotype with high GC
162 levels.
163
164 **Results**
165 Our data set includes behavioural observations from 7,637 trials (3,812 OFTs, 1,548 ETs
166 and 1,039 STs) and physiological measures from 1,238 waterborne assays of cortisol
167 (CORT hereafter). These phenotypic data were collected from 1,518 individual fish
168 within a genetic pedigree structure comprised of maternal full-sibships nested within
169 paternal half-sibships (with some connectedness across 5 'generations', as offspring
170 from one round of breeding were used, where available, as parents in the next). The
171 number of individuals phenotyped (OFTs = 1,487, ETs = 806, STs = 532) and the mean
172 number of observations per fish (OFTs = 2.6, ETs = 1.9, STs = 2.0) varied across the
173 behavioural data types. For the 629 fish that were assayed for cortisol (almost all from
174 the final generation), the handling and confinement stressor applied in this assay was
175 performed 3 times (at 48h intervals) for all fish, with the magnitude of the GC response
176 to the stressor being assessed only for the first and last stressor exposures
177 (subsequently $Cortisol_1$ and $Cortisol_3$). Full details of husbandry, phenotyping and
178 analysis are provided in Materials and Methods.
179
180 **Genetic variance in behavioural components of the stress response**
181 We extracted behavioural data from videos of each trial type using video tracking of fish
182 (as described in White, Kells & Wilson 2016; Houslay *et al.* 2018). Time to emerge from
183 the shelter ('*emergence time*') was extracted from ETs and $\log_n$ transformed for analysis,

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

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

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

coupled to high values of *relative area covered* and *time in the middle,* but a 'flight' response when coupled to low values of *relative area covered* and *time in the middle*.

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

Genetic variation in physiological components of the stress response

Using a series of nested bivariate animal models, we tested for the presence of additive genetic variation in cortisol levels ($\log_n$ transformed) following stressor exposure (handling and confinement), and for genotype-by-environment interaction (GxE) in cortisol levels across the two samples assayed per fish (i.e. following stressors 1 and 3). In this context, the environment (E) is the time point at which the fish was exposed to the stressor. 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 ($\log_n$ transformed mean ± SE; $Cortisol_1 = 8.50 \pm 0.05$, $Cortisol_3 = 8.05 \pm 0.06$, Wald $F_{1,12.9} = 120.5$, $P < 0.001$). We first modelled $Cortisol_1$ and $Cortisol_3$ as distinct response variables in a bivariate framework but assuming no GxE (such that we assume $V_{A\text{-}Cortisol1} = V_{A\text{-}Cortisol3}$ and the cross context

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

241 Expanding the model to allow GxE (by freely estimating separate genetic
242 variances for $Cortisol_1$ and $Cortisol_3$ as well as the cross-context genetic correlation)
243 provides a significantly better fit to the data ($\chi^2_2 = 9.65$, P = 0.008). This means that
244 GxE is present, or (equivalently) that the magnitude of change in cortisol levels from the
245 first to the third sampling (i.e., the degree of habituation to the stressor) varies among
246 genotypes. The cross-context genetic correlation is strongly positive ($r_{A\text{-}Cortisol1,Cortisol3}$ ± SE
247 = 0.74 ± 0.25), while genetic variance in cortisol levels is greater following exposure to
248 the first stressor than the third ($V_{A\text{-}Cortisol1}$ = 0.076 ± 0.028, $V_{A\text{-}Cortisol3}$ = 0.047 ± 0.029).
249 Both findings are apparent when visualising model predictions (Figure 2): while there is
250 genetic variation in habituation rates (indicated by variation in the slopes of genotypic
251 reaction norms; Figure 2), there is still positive cross-context genetic correlation
252 (indicated by little change in the rank order of individuals across the two contexts;
253 Figure 2). The reduction in genetic variance for cortisol levels between the first and
254 third stressor exposure arises because genotypes with higher than average cortisol
255 levels following the first exposure habituate more rapidly following subsequent
256 exposures (reflected in the model as GxE). In this model we also find that variance in
257 cortisol explained by average differences among groups is similar across contexts
258 ($V_{Group\text{-}Cortisol1}$ = 0.034 ± 0.013, $V_{Group\text{-}Cortisol3}$= 0.045 ± 0.016), but that residual variance is
259 greater following exposure to the third stressor than the first ($V_{R\text{-}Cortisol1}$ = 0.166 ± 0.021,
260 $V_{R\text{-}Cortisol3}$ = 0.229 ± 0.025). This means that there is no change in variance explained
261 among groups across contexts, but variance within groups is greater after the third
262 stressor relative to the first. Overall, this pattern creates a higher adjusted heritability
263 (the ratio of additive genetic variance to the sum of additive genetic, group and residual

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

Testing for genetic integration and identifying the major axis of genetic variance

Lastly, to test for the hypothesised genetic integration between behavioural and physiological components of the stress response, we built a multivariate animal model to estimate patterns of genetic covariances among these traits (**G**). We excluded *shoaling tendency* given the absence of detectable genetic variance in the univariate model of this trait. 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 analysis described above shows evidence of GxE, the strong positive genetic correlation between $Cortisol_1$ and $Cortisol_3$ justifies combining into a single trait here in order to maximise power to detect overall genetic integration of behaviour and GC expression.

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

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

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

Discussion

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

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

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

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

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

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

367 previously detected differences among individuals in cortisol response to a stressor
368 (Houslay *et al.* 2019) are primarily attributable to genetic effects, with the estimated
369 heritability ($h^2 = 0.26$) being >75% of the individual–level repeatability for log(Cortisol).
370 Moreover, by adopting a reaction norm approach to modelling stress physiology, as
371 recently advocated by ourselves and others (e.g., Fürtbauer *et al.* 2015; Hau & Goymann
372 2015; Taff & Vitousek 2016), we detect GxE reflecting genetic differences in the extent of
373 habituation to the stressor over repeated exposures. This result is potentially important
374 since poor habituation of the hypothalamic-pituitary-adrenal/interrenal (HPA/I)
375 response to repeated or ongoing stressors can lead to well documented health problems
376 in human and animal populations (Segerstrom & Miller 2004; Koolhaas 2008; Romero,
377 Dickens & Cyr 2009; Mason 2010). Heritable variation in habituation to stressors raises
378 the possibility of developing targeted selection strategies to improve welfare in captive
379 populations (e.g., Frankham *et al.* 1986; Muir & Craig 1995; Oltenacu & Algers 2009).
380 These findings also highlight the possibility that habituation could be important for
381 adaptation in the wild, as many agents of natural selection can be viewed as
382 environmental stressors (Bijlsma & Loeschcke 2005).
383 Our findings also highlight that there is greater additive genetic variance (and
384 heritability) for cortisol levels following the first exposure to the stressor than following
385 the third. This pattern, which occurs because genotypes that produce the highest
386 cortisol response at first exposure also show the most marked habituation, is consistent
387 with the idea of cryptic genetic variance (Paaby & Rockman 2014) being 'released' by
388 exposure to novel, and so potentially stressful, environments (Ledón-Rettig, Pfennig &
389 Crespi 2010; Ledón-Rettig *et al.* 2014; Berger *et al.* 2011). All else being equal, it also
390 means that selection on cortisol levels following stressor exposure should induce a
391 stronger evolutionary response in naïve relative to habituated fish. However, the strong
392 positive cross-environment correlation means that the ranking of genotypes is
393 consistent across repeated stressor exposures. It also means that we would expect a

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

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

Conclusions and future directions

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

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

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

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

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

Figure 1: The lack of a strong positive relationship between observed *tracklength* and *area covered* (panel 1), 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 2) shed light on this. Fish A and B appear to be exploring the tank, while C and D are engaging in a stereotypical 'flight' behaviour characterised by strong thigmotaxis (remaining close to tank walls) and/or rapid movement along tank walls. As a consequence, individuals B and C have similar *area covered* during the OFT as a consequence, but very different *track lengths*. We simulated random movements to define an expected null relationship between *area covered* and *track length* (panel 3; dashed red line shows the third 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 4 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 A and B have high *relative area covered*, while C and D do not.

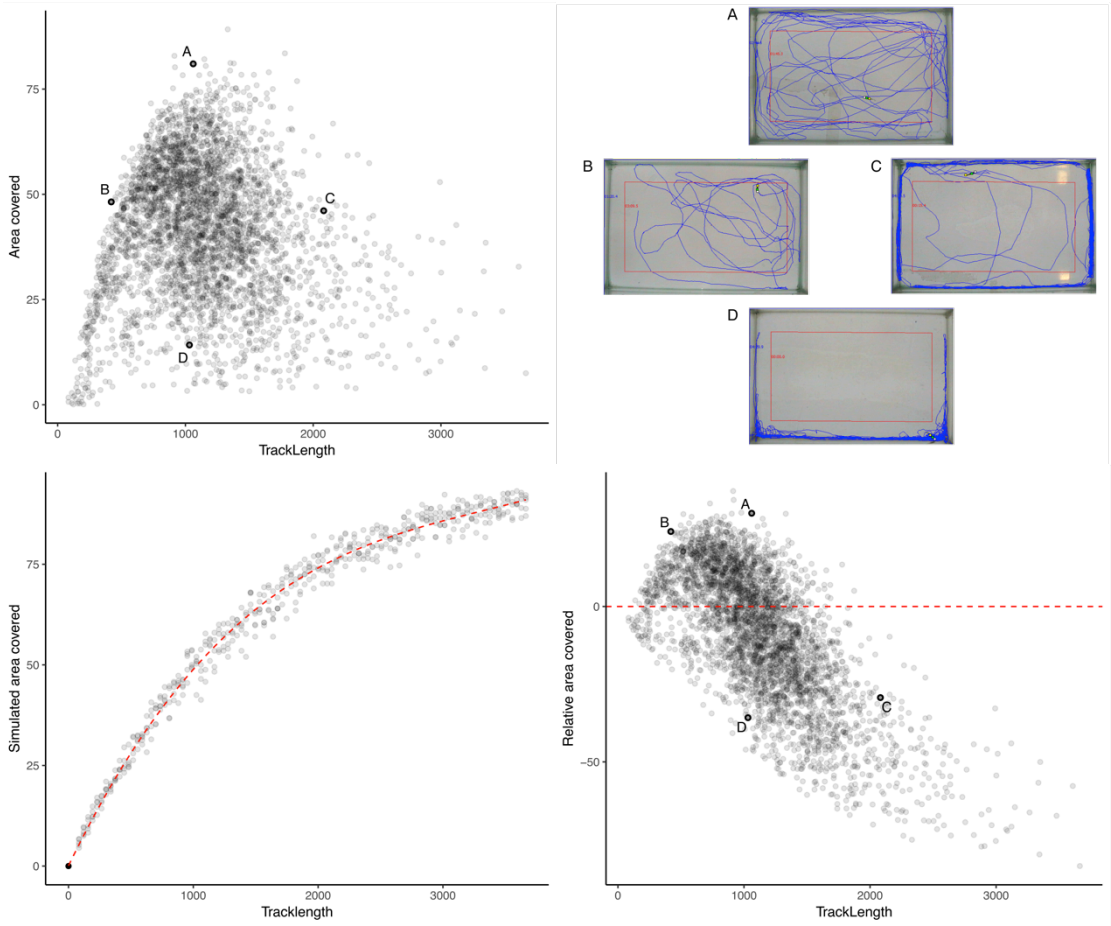


Figure 2: Guppies habituate to the waterborne sampling procedure, as shown by a decline in log-transformed cortisol levels following stressor exposure declining from their first stressor exposure to their third. Black circles and associated bars denote predicted population means (± standard error) from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

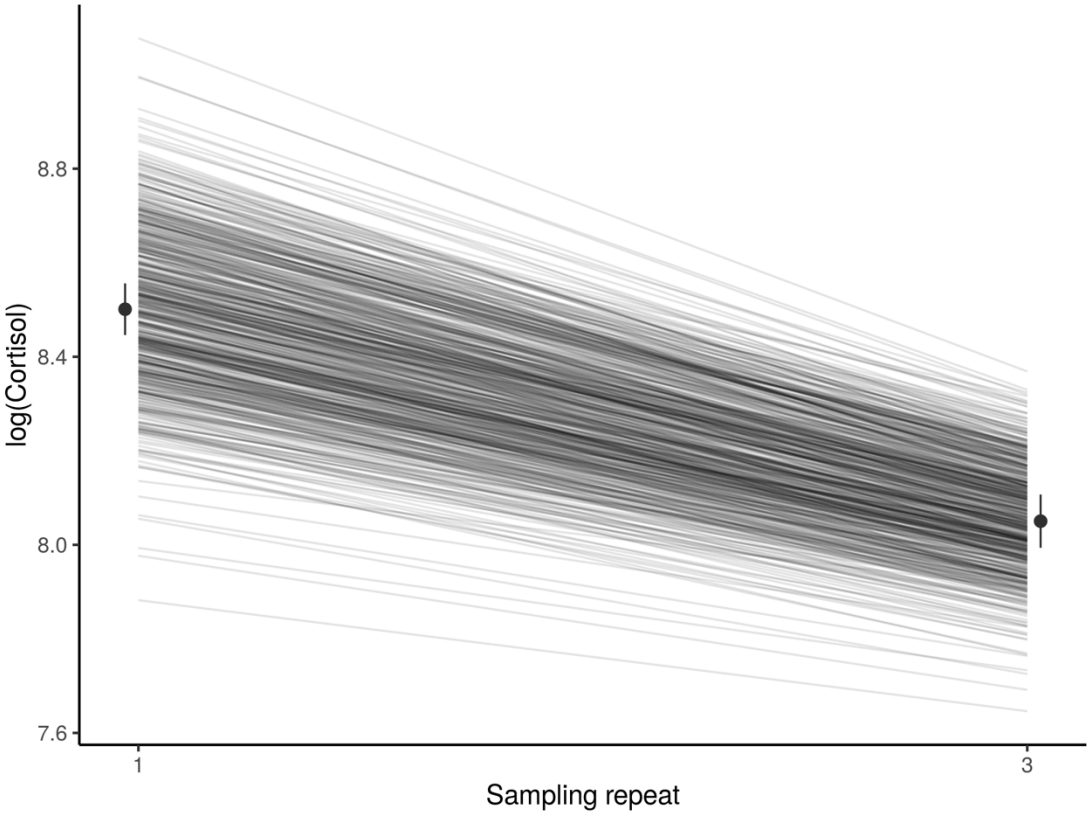


Figure 1: The additive genetic relationship between log-transformed cortisol (y-axis) and four behaviours (a, *relative area covered*; b, *time in the middle*; c, *track length*; d, *-log emergence time*). Points show (predicted) bivariate genetic deviations from the population means, plotted for those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

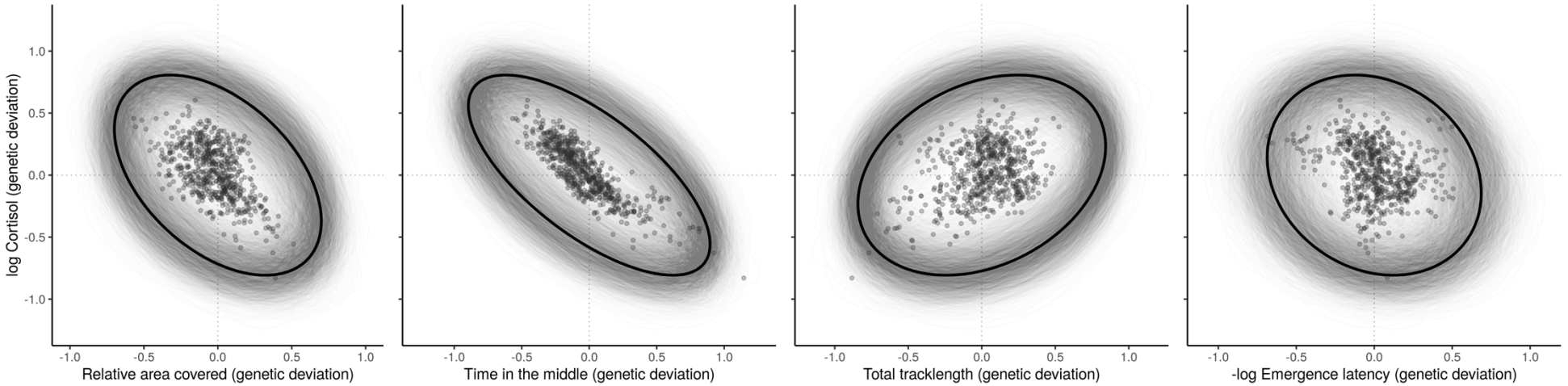
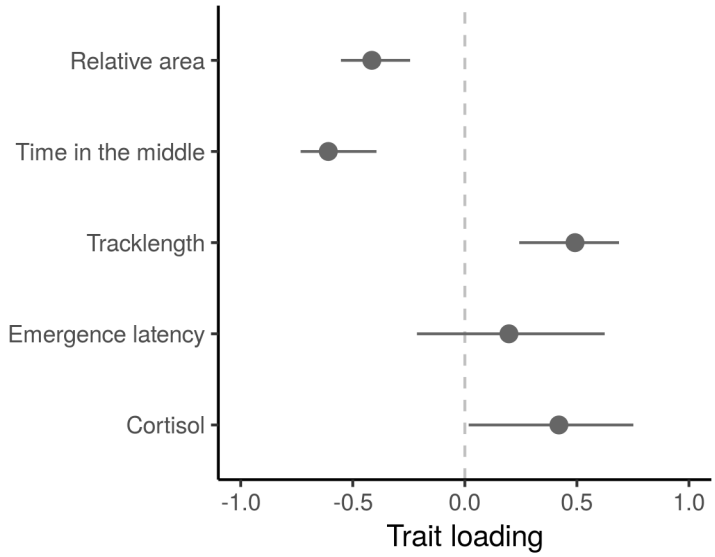


Figure 2: Trait loadings from the first eigen vector (principal component) of **G**. This axis explains 57.8% of the genetic (co)variation found in the focal behavioural and physiological components of the stress response in our guppy population. Points show trait loadings from the first eigen vector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



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

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

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

Overview of behavioural phenotyping

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

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

551 from the main supply. We caught fish individually from their home tank, examined them quickly for
552 identification tags, then placed them immediately into the centre of the OFT tank. After allowing 30s
553 for acclimation, we filmed behaviour for 4m30s. Behaviours characterised from the tracking software
554 were *track length* (the total distance the fish moved during the trial; cm), *area covered* (the percentage
555 of 1cm x 1cm grid squares through which the fish moved during the trial; %), and *time in middle* (time
556 spent in a rectangular inner zone which was defined as being the same size as an outer area; seconds).
557
558 *Shoaling trials (ST)* were appended to a subset of OFTs, by positioning a small tank containing 10 stock
559 fish (of same sex as the test subject) next to one end of the OFT tank but with visual cues blocked by a
560 cardboard divider. At the end of the normal OFT, we removed this divider slowly, allowing the focal
561 animal to have visual contact with the shoal. We began recording the shoaling trial 30s after removing
562 the divider in order to limit any artefacts of slight disturbance. (Note that we used a further cardboard
563 casing around the shoaling tank to avoid any additional external visual stimulus). We then recorded
564 behaviour of the test fish for an additional 3 minutes. We characterised *shoaling tendency* via the
565 tracking software by subdividing the tank area into 3 equal-sized rectangular areas: one next to the
566 tank holding the group of same-sex fish, one farthest from this group, and the central area. We then
567 calculated *shoaling tendency* as the time spent in the 1/3 area closest to the same-sex group after
568 subtracting the time spent in the 1/3 area farthest away. The decision to use a single-sex shoal aimed
569 to reduce any effects of (potential) mate preference and/or avoidance, but also this necessitated
570 replicate arena setups allowing male and female individuals from each group to be tested in the
571 OFT/ST concurrently. We randomised which tank was used for each sex in each group and recorded
572 this information.
573
574 *Emergence trials (ET)* followed the methodology described by White *et al.* (2016). Briefly, we tested
575 individuals in a 20cm x 40cm tank, filled to a depth of 8cm with room-temperature water from the
576 main supply. A 10cm section of the tank length was walled off creating a shelter area (20cm x10cm),
577 the walls and floor of which were painted black. The focal fish was placed into the shelter area and
578 allowed to acclimate for 30s, at which point we opened a sliding door to allow access to the rest of the

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

Derivation of 'relative area' from OFT trials

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

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

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

607 97.8% of the variance in simulated data), we calculated the *relative area* for each actual OFT trial as
608 the observed area covered minus the area covered under a random swim, as predicted from our
609 regression model and the observed *tracklength*.
610
611
612 *Waterborne hormone sampling*
613 On completion of behavioural data collection, individuals entering the endocrine testing program were
614 left undisturbed for a minimum of two weeks. Waterborne hormone sampling was then conducted
615 over a 5-day period that included three handling and confinement stressor exposures with 48h
616 between each. We followed the method described by Houslay et al (2019) to obtain repeated non-
617 invasive GC measures of individuals using holding water samples from the first and third
618 confinements. Note that only two samples per fish were analysed because the financial and time costs
619 of doing three was deemed prohibitive. We nonetheless applied the stressor stimulus three times as
620 our prior study showed this was sufficient to produce a strong habituation response, i.e., a significant
621 decrease in water-borne cortisol over the three sampling periods (Houslay *et al.* 2019)).
622 We collected samples between 1200 – 1400h to control for diel fluctuations in GC levels. For
623 each sample, we netted an entire group from their home tank quickly using a large net, transferring
624 them to 2 holding tanks (containing water from the home tank supply) for moving to an adjacent quiet
625 room (performed within 20s of the net first hitting the water). We then transferred fish to individual
626 Pyrex beakers containing 300ml of clean water from the main supply (which serves the main housing
627 units), which has been warmed to the appropriate temperature (mean = 24.1°C, range 23-24.9°C).
628 Beakers were placed within cardboard 'chambers' to prevent fish from seeing each other or
629 experiencing outside disturbance. One fish was transferred every 30s, alternating across holding
630 tanks, such that all fish were in their beakers within 10min of the initial netting. After 60 mins in the
631 beaker, each fish was removed by pouring its sample through a clean net into a second beaker, with
632 the fish then quickly checked to confirm ID and returned to the holding tank until the entire group
633 could be returned to its home tank.

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

Statistical methods

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

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

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

Cortisol data were also natural log-transformed for analysis. We formulated a bivariate model to test for both additive genetic variation and genotype-by-environment interaction (GxE) in cortisol levels across the two 'contexts' (i.e. samples retained for each individual at first and third confinement, denoted $Cortisol_1$, $Cortisol_3$). Random effects were first used to partition phenotypic (co)variance (conditional on fixed effects) into among-group and residual components. Fixed effects included the context-specific means, and overall effects of the *order* in which the fish was caught from each group for assay (mean-centred continuous predictor), *temperature* (mean-centred and scaled to standard deviation units), *time of day* (mean-centred and scaled to standard deviation units), *age* (mean-centred and scaled to standard deviation units), and *sex*. In addition, we included fixed covariates of *body mass* (mean-centred and scaled to standard deviation units) and a *sex* by *body mass* interaction (see Houslay *et al.* 2019 for rationale of controlling for body size effects on waterborne hormone levels in this way). Note that modelled in this way each individual is sampled only once for each context-specific cortisol trait so no random effect of individual identity is included. To test for additive genetic variation (V_a) we compared this first bivariate model to a second formulation that also included the (additive) genetic merit, but under the assumption that this is invariant with context 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 comparing the second model to a third in which we allow GxE (i.e., the context-specific genetic variances are free to differ and the cross-context genetic correlation can be <+1).

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

717 specified for the univariate models. Note that one exception to this is that we elected to treat *Cortisol*
718 as a single repeated-measures trait here (with two repeats, one per context) such that a permanent
719 environment effect was now included.
720
721 We specified additive genetic genetic (**G**), permanent environment (**PE**), group (**GROUP**), and residual
722 (**R**) covariance structures as unstructured matrices to be estimated. Note that **R** partitions
723 observation-level covariances (as opposed to individual-level in **PE**) that are not definable or
724 statistically identifiable if traits are not measured at the same time (i.e., all covariances relating to
725 *emergence time* or *Cortisol*). Where this was the case we constrained specific covariance terms in **R** to
726 equal zero. We tested for overall additive genetic covariance among the traits by comparing this model
727 against a reduced one in which **G** was specified as a diagonal matrix (i.e., additive variances are
728 estimated but covariances are assumed to equal zero). To aid the interpretation of covariance terms
729 contained in **G,** we calculated the corresponding genetic correlations r_a from the full model. For any
730 pair of traits (x,y), $r_{a(x,y)} = COV_{a(x,y)} / (\sqrt{}(V_{a(x)}) \times \sqrt{}(V_{a(y)}))$. We also subjected our estimate of **G** to eigen
731 decomposition to determine the proportion of additive genetic variation captured by each principal
732 component and assess whether a single major axis of variation could indeed explain most of the
733 genetic variance in the multivariate phenotype (consistent with a simple proactive-reactive coping
734 style model). We estimated uncertainty on the trait loadings associated with each principal component
735 (eigenvector) using a parametric bootstrap approach as described by Boulton et al (2014).
736
737 For visualisation of bivariate relationships at the additive genetic level, we used the R package 'ellipse'
738 (Murdoch & Chow 2018) to determine the coordinates of an ellipse representing the approximate 95%
739 confidence region of deviations based on the point estimate of **G**. We repeated this procedure for the
740 corresponding regions defined from 5000 bootstrapped values of **G** (i.e., to indicate uncertainty arising
741 from estimation of the genetic covariance structure itself).
742
743

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