Habituation and individual variation in the endocrine stress response in the Trinidadian guppy (*Poecilia reticulata*)

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1 **Summary statement**

2

3 Trinidadian guppies habituate quickly to repeated stress exposure, and exhibit

4 consistent differences in their endocrine stress response. We provide a

5 framework for analysing individual variation in habituation rate.

6

7 **Abstract**

8

9 The vertebrate stress response enables individuals to react to and cope with

10 environmental challenges. A crucial aspect of the stress response is the elevation of

11 circulating glucocorticoids. However, continued activation of the stress response under

12 repeated (or chronic) stress can be damaging to fitness. Under certain circumstances it

13 may therefore be adaptive to habituate to repeated exposures to a particular stressor by

14 reducing the magnitude of any associated release of glucocorticoids. Here, we

15 investigate whether Trinidadian guppies (*Poecilia reticulata*) habituate to repeated

16 exposure to a mild stressor, using a waterborne hormone sampling approach that has

17 previously been shown to elicit a stress response in small fish. We also test for

18 individual variation in the extent of habituation to this stressor. Concentrating on freely

19 circulating cortisol, we found that the first exposure to the assay induced high cortisol

20 release rates but that guppies tended to habituate quickly to subsequent exposures.

21 There were consistent differences among individuals in their average cortisol release

22 rate (after accounting for effects of variables such as body size) over repeated

23 exposures. Our analyses did not find evidence of individual differences in habituation

24 rate, although limitations in statistical power could account for this finding. We also

25 present data on free 11-ketotestosterone, in addition to conjugated forms of both

26 hormones. We discuss consistent individual differences around the general pattern of

27 habituation in the flexible stress response, and highlight the potential for individual

28 variation in habituation to facilitate selection against the deleterious effects of chronic

29 stress.

30

31 **Introduction**

32 Stress responses involve a complex suite of behavioural, neuroendocrine, and
33 physiological processes that act to maintain organismal health and homeostasis in the
34 face of unpredictable environmental challenges (Selye 1973; Korte *et al.* 2005; Romero,
35 Dickens & Cyr 2009). While disagreements over terminology persist (McEwen and
36 Wingfield, 2010; Romero et al., 2009), stress response mechanisms are broadly seen as
37 underlying a process of "achieving stability through change" (McEwen and Wingfield,
38 2003) and are vital for dealing with acute environmental challenges. However, these
39 same mechanisms can actually be damaging to fitness when organisms are exposed to
40 chronic stressors (Boonstra, 2013; Dallman et al., 1992; Huether, 1996). Given the
41 expectation of such causal links between stress response and fitness, there has been
42 increasing interest in characterising variation among individuals for both behavioural
43 and neuroendocrine stress response phenotypes (e.g., Cockrem 2013; Houslay *et al.*
44 2017). Variation among individuals is a prerequisite for selection to occur, and, if such
45 variation also includes a genetic component, then stress response traits have the
46 potential to evolve under natural selection in wild populations (Atwell *et al.* 2012;
47 Jenkins *et al.* 2014) or be targeted by artificial selection strategies in captive ones
48 (Pottinger & Carrick 1999; Louison *et al.* 2017).
49   Among-individual variation has now been documented in behavioural (Bell et
50 al., 2009) and neuroendocrine (Hau et al., 2016) traits across many taxa. For
51 neuroendocrine traits in particular, a distinction is commonly made between 'baseline'
52 and 'reactivity' phenotypes. The former represents the phenotypic state (e.g. circulating
53 glucocorticoid (GC) level) of an 'unstressed' individual, while the latter is the plastic
54 change in expression (e.g. increase in GC level) when challenged by an acute stressor.
55 Both aspects are likely to vary among individuals within a population (Williams, 2008),
56 and their recognition as distinct – but potentially correlated – traits allows a more
57 nuanced view of how selection might shape the stress response (Bonier and Martin,
58 2016; Cox et al., 2016; Hau and Goymann, 2015; Romero, 2004; Taff and Vitousek,
59 2016). Nonetheless, we suggest this framework will not be sufficient to understand the
60 fitness consequences of chronic stress exposure. This is because plasticity is relevant not
61 just to 'reactivity' when challenged by an acute stressor, but also to longer-term changes
62 in phenotypic state when faced by chronic repeated exposures to stressors. For instance,
63 stress-induced GC release can result in the phenomenon of sensitisation, where the
64 individual enters a state of hyper-responsiveness to the same or novel stimuli (Belda et
65 al., 2015). A more common response to repeated exposure to the same acute stressor,
66 however, is habituation (Martí and Armario, 1998).

67   Habituation can be defined as occurring where repeated, or chronic, application

68   of a stimulus results in a progressively weaker stress response (Thompson and Spencer,

69   1966). Intuitively, habituation to chronic stressors may offer a route to improved health,

70   particularly if simple behavioural responses (e.g. avoidance of the stressor) are

71   curtailed, which will commonly be the case in captive populations. For example, under

72   chronic stress exposure, continued activation of the hypothalamic-pituitary-

73   adrenal/interrenal (HPA/I) axis can occur. This is a metabolically costly response

74   system (Korte et al., 2005) and sustained elevation of circulating glucocorticoids (GCs) is

75   known to reduce growth, suppress immune function and reproduction, and increase

76   mortality (Berga, 2008; Hodgson et al., 2012; Segerstrom and Miller, 2004; Young et al.,

77   2006). As such it should be adaptive for an organism to habituate (via reductions in the

78   magnitude of the HPA/I response) to stressors that are not inherently harmful (Grissom

79   and Bhatnagar, 2009; McEwen, 2001). If present, variation among individuals in their

80   ability to habituate to a repeated stressor thus represents another form of variable

81   plasticity that could facilitate selection to reduce the deleterious effects of chronic stress

82   in natural and captive populations.

83   In this study we seek to characterise among-individual variation in stress-

84   related endocrine state and habituation in the Trinidadian guppy, *Poecilia reticulata*. We

85   focus principally on HPI activity and thus cortisol, the major GC in teleost fish

86   (Mommsen et al., 1999). We also conduct a parallel analysis of 11-ketotestosterone

87   (subsequently 11KT) from the samples collected. Links between 11KT and stress

88   response have previously been reported in fishes; chronically elevated cortisol can

89   inhibit 11KT synthesis (Consten et al., 2001; Consten et al., 2002), and social/isolation

90   stressors have been shown to reduce 11KT while elevating cortisol (Galhardo and

91   Oliveira, 2014; Haddy and Pankhurst, 1999; Kubokawa et al., 1999). Here we separate

92   both of these target hormones into their 'free' and 'conjugated' fractions for analysis. In

93   the main text we present only results from free hormones, the concentration of which in

94   the water is taken to scale with the 'physiologically active' concentration of hormones in

95   the fish's circulation across the duration of the sampling period (Scott and Ellis, 2007).

96   We refer interested readers to Appendix A for information on the conjugated fractions.

97   Our objectives are not only to determine whether individual fish differ

98   consistently in stress-related endocrine state, but also to determine whether habituation

99   occurs and, if so, whether rates vary among individuals. Our focus on among-individual

100   differences requires repeated sampling of individuals; since this is not feasible using

101   blood sampling in guppies, we adopt a less invasive, waterborne approach to

102   characterising endocrine state. While this method has been widely validated and used

103   for studies of small fishes (reviewed in Scott & Ellis 2007; Scott *et al.* 2008), including

104   guppies (Fischer et al., 2014), the handling required to transfer the fish into the confined

105   space in which waterborne sampling will be conducted causes cortisol release,

106   complicating attempts to measure baseline GC (e.g., Wong et al., 2008). However, in the

107   current context, this is actually advantageous as we use this initial handling and

108   confinement itself as the stressor to which habituation is predicted to occur. For free

109   cortisol we predict that: (i) average levels will decline over repeated handling and

110   sampling events (1 every 48 hours, for a total of 4 repeats), consistent with habituation;

111   (ii) there will be variation among individuals around the average response, with

112   individuals differing in both average 'reactive' endocrine state and their rate of

113   habituation with repeated subsequent exposures; and (iii) that individuals with higher

114   average circulating cortisol levels also habituate at a slower rate. We make no specific

115   predictions about free 11KT levels beyond a general expectation that they will be

116   negatively correlated with free cortisol.

117   **Methods**

118   **Animal husbandry and welfare**

119   The guppies used in this study were taken from our captive population, descended from

120   wild fish collected in 2008 from the lower Aripo River, Trinidad, and housed at the

121   University of Exeter's Penryn campus. We sampled 32 adult fish (17 females, 15 males)

122   from a stock tank haphazardly, and tagged each for identification purposes with

123   coloured elastomer injection under mild MS222 sedation. We housed fish in 2 mixed-sex

124   groups of equal size, using separate tanks that shared a common recirculating water

125   supply. All fish were fed to satiation twice daily (0800-1000h and again at 1600-1800h)

126   using commercial flake food and live *Artemia nauplii*. The experiment described here

127   was carried out in accordance with the UK Animals (Scientific Procedures) Act 1986

128   under licence from the Home Office (UK), and with local ethical approval from the

129   University of Exeter.

130   **Sampling protocol & hormone assay**

131   We used a waterborne sampling method (Scott *et al.* 2008) to obtain 4 repeated

132   measures of hormones from individual fish at 48hr intervals. To control for diel

133   fluctuations in cortisol, we collected all samples between 1200-1400h. In all cases, we

134   first netted an entire group from the housing aquarium quickly using a large net and

135   transferred them to 2 holding tanks for moving to an adjacent quiet room (performed

136 within 20 seconds of the net first hitting the water). We then transferred fish to

137 individual beakers containing 300ml of clean water taken from the main supply (which

138 serves as input to the main housing units) for sample collection, placing beakers within

139 cardboard 'chambers' to prevent fish from seeing each other or being disturbed. 1 fish

140 was transferred every 30 seconds, alternating across holding tanks, such that all fish

141 were in their beakers within 10 minutes of the initial netting (with maximum 11 net

142 entries into the water). Individual ID was noted during transfer to the beaker, and

143 sampling order retained for use in statistical models to control for effects of both the

144 time between first net entry and transfer to the beaker and the number of net entries

145 into the water. After 60 minutes in the beaker, we removed each individual by pouring

146 its sample through a clean net into a second beaker (and returned fish to their home

147 tank immediately). At each time point, we also collected 2 'blank' samples from the

148 water supply.

149   Immediately after sampling, we filtered the water samples (Grade 1 filter paper,

150 Whatman) and then passed them through solid phase C18 extraction columns (Sep-

151 Pak C18 3cc, Waters) that had been primed with 2 x 2ml HPLC-grade methanol followed

152 by 2 x 2ml distilled water, keeping the column moist prior to sample extraction. We used

153 high-purity tubing (Tygon 2475, Saint-Gobain) to pass the water samples to the

154 columns, through which they were drawn slowly under vacuum pressure (see Earley et

155 al., 2006 for detail). Following extraction, we washed columns with 2 x 2ml distilled

156 water to purge salts. We then covered both ends of each column with film (Parafilm M,

157 Bemis) and stored them at -20°C for future analysis. We rinsed all beakers, tubes and

158 funnels with 99% ethanol and distilled water prior to each sampling procedure.

159   We later shipped the frozen columns on dry ice from the University of Exeter's

160 Penryn campus to the University of Alabama. We thawed each column and purged with

161 2 x 2ml distilled water before eluting both free and conjugated (sulphated and

162 glucuronidated) hormones. We used 2 x 2ml ethyl acetate and 2 x 2 ml HPLC-grade

163 methanol in successive, separate washes to elute free and conjugated hormones

164 respectively into separate 13 x 100 mm borosilicate vials. For the free fraction, ethyl

165 acetate was evaporated under nitrogen in a water bath at 37°C, leaving a hormone

166 residue, which was resuspended in 600 μl of 5% EtOH: 95% EIA kit buffer (the latter

167 supplied with Cayman Chemicals, Inc. EIA kits) and vortexed for 20 minutes. For the

168 conjugated hormones, methanol was evaporated under nitrogen and the hormone

169 residue was resuspended in 600 μl of 5% EtOH: 95% EIA kit buffer. The resuspended

170 conjugates were then diluted with 8 ml distilled water, re-extracted over primed solid

171 phase C18 extraction columns (Sep-Pak C18 3cc, Waters) and eluted into 13 x 100 mm

172   borosilicate vials with 2 x 2 ml successive washes of methanol, which was evaporated

173   under nitrogen. We processed these samples further according to previously published

174   protocols with some modification (Canario and Scott, 1989; Scott and Canario, 1992;

175   Scott and Vermeirssen, 1994).

176   Briefly, 1 ml trifluoroacetic acid [TFA]:ethyl acetate (1:100) was added to the

177   hormone residue and incubated overnight in a water bath at 37°C. The TFA:ethyl acetate

178   was evaporated under nitrogen at 37°C followed by addition of 0.5 ml 0.1M sodium

179   acetate buffer (pH 4.5). We vortexed the sample for 5 minutes, followed by addition of 4

180   ml diethyl ether and a further 5 minutes of vortexing. We then allowed samples to sit for

181   approximately 30 minutes to enable separation of the organic and aqueous phases, and

182   then immersed the samples in a methanol-dry ice bath to freeze the aqueous phase. The

183   organic phase containing the sulphated fraction was poured off into a new 13 x 100 mm

184   borosilicate vial. 10μl of β-glucuronidase (Sigma Aldrich, Cat No. G-7017) was added to

185   the remaining aqueous phase and incubated overnight in a water bath at 37°C, followed

186   by the diethyl ether extraction as described above. The organic phase containing the

187   glucuronidated fraction was poured into the same vial as the ether containing the

188   sulphated fraction to produce a sample with total conjugated hormone. Diethyl ether

189   was evaporated under nitrogen in a 37°C water bath and the hormone residue was

190   resupended in 200μl of 5% EtOH: 95% EIA kit buffer followed by vortexing for 20 min.

191   We ran assays in strict accordance with the manufacturer's instructions, and all samples

192   were run in duplicate.

193   We generated a pooled waterborne hormone extract for males and females

194   separately by combining 20 μl taken from each of the 128 samples. These pools were

195   serially diluted to assess parallelism with the standard curves from the Cayman

196   Chemicals, Inc cortisol and 11KT kits. Two serial dilution curves were run in duplicate

197   for each hormone, and each was parallel to the standard curve (slope comparison test,

198   Zar, 1996, p. 355; Cortisol-1: $t_{12} = 0.204$, P = 0.84; Cortisol-2: $t_{12} = 0.013$, P=0.99; 11KT-1:

199     $t_{12} = 0.02$, P = 0.98; 11KT-2: $t_{12} = 0.005$, P = 0.99). We also ran the pooled samples in

200   duplicate at both the beginning and end of each of the four 96-well plates for each

201   hormone (free fraction) to assess intra- and inter-assay coefficients of variation. Intra-

202   assay coefficients of variation were 6.9%, 11.5%, 4.9%, and 9.5% for each of the cortisol

203   plates and 7.8%, 3.4%, 2.9%, and 1.6% for each of the 11KT plates; the inter-assay

204   coefficients for cortisol and 11KT were 10.7% and 7.3%, respectively. We generated a

205   separate pool for the conjugated hormone, which was run in duplicate at the beginning

206   and end of an additional four 96-well plates for each hormone (conjugated fraction).

207   Intra-assay coefficients of variation were 4.9%, 7.7%, 6.6% and 5.6% for cortisol and

208   5.6%, 15.2%, 7.6% and 10.1% for 11KT; the inter-assay coefficients for cortisol and

209   11KT were 6.5% and 11.3%, respectively.

210   **Modelling approach and statistical analysis**

211   In what follows we conceptualise habituation as plasticity in endocrine state with

212   respect to repeated stressor exposure. This allows us to test for and characterise among-

213   individual variation in habituation using the well-known 'character state' and 'reaction

214   norm' frameworks for analysing plasticity (Henderson, 1982; Nussey et al., 2007; Van

215   Noordwijk, 1989; Via et al., 1995). In the reaction norm framework, which we illustrate

216   graphically in Fig. 1, each individual's endocrine state is modelled as a linear function of

217   sampling repeat number. This allows us to characterise among-individual variation in

218   habituation (i.e. plasticity in endocrine state) using random regression (i.e. random

219   slope) linear mixed effect models. Under the alternative character state approach,

220   endocrine state at each sampling repeat is treated as a distinct (but potentially

221   correlated) sub-trait. When more than 2 such sub-traits exist, the character state

222   approach provides more information than the simple linear formulation of random

223   regression, although this additional flexibility does require the estimation of additional

224   parameters (Roff and Wilson, 2014). Using this approach, positive (within-individual)

225   correlation across observations provides evidence of repeatable variation in endocrine

226   state. If variation in habituation also occurs then this will lead to changes in observed

227   endocrine state variance over sampling repeats, and/or declining (within-individual)

228   correlation with increasing time between observations (such that $r_{1,2} > r_{1,3} > r_{1,4}$).

229   The analysis procedure described below was performed separately for both free

230   and conjugated forms of cortisol (log nanograms) and 11-ketotestosterone (log

231   picograms). We present only the results for free hormones below, with results and

232   discussion of conjugated forms available in Appendix A. All analyses were performed in

233   R version 3.4.1 (R Core Team, 2017), using the package ASreml-R 3.0 (Butler, 2009) to

234   test for hypothesised changes in mean endocrine state with repeated samplings, and to

235   characterise variation among-individuals around this mean state. We used visual

236   representations of model residuals to check that the assumptions of regression

237   modelling were met in all cases. A single measurement of conjugated 11KT was

238   excluded from all relevant analyses as it was far outside the range of all other values,

239   and exerted large influence on the fitted model when included. Note that we control for

240   any effects of sex in the statistical models and also include body mass as a covariate to

241   account for the expected relationship between size and hormone release rates. We do

242   not adjust observed endocrine state for size prior to modelling since this causes

8

243   problems of interpretation for repeatability estimates (Wilson, 2018). Data handling and

244   visualisation in R was carried out using the 'tidyverse' packages (Wickham, 2017). R

245   code for performing the analyses is provided in the supplementary materials.

246   *Habituation effects on mean hormone levels*

247   For each of the endocrine response traits ($Cort_{free}$, $11KT_{free}$, $Cort_{conjugate}$, $11KT_{conjugate}$) in

248   turn, we tested for the hypothesised effects of habituation on hormone levels across

249   repeated assays by fitting assay repeat (as a categorical variable with levels 1 to 4). We

250   also included sex, and the interaction of sex and assay repeat to account for any sex

251   difference in mean endocrine state or in average habituation. We specified assay repeat

252   as a factor rather than as a continuous predictor to allow maximum flexibility (i.e., to

253   avoid imposing a linear response in the mean). Tank (2-level categorical variable),

254   sampling order (the order transferred from the holding tank to the individual beakers,

255   fitted as a mean-centred linear effect), and body mass were included as further

256   covariates to control for potential sources of variance not relevant to our present

257   hypotheses. We standardised body mass measurements by centring and scaling

258   (subtracting the population mean and dividing by the standard deviation) to improve

259   their interpretability (Gelman and Hill, 2007; Schielzeth, 2010). We used conditional

260   Wald F-tests to test all fixed effects, and reduced the model by dropping the sex × assay

261   repeat interaction if not significant. For fixed effects inference we use a 'random slopes'

262   model (described below) where possible, which groups repeated measures to avoid

263   pseudoreplication and also allows individuals to vary as a continuous function of

264   stressor exposure, preventing inflation of Type I and Type II errors when estimating

265   population-level effects (Schielzeth and Forstmeier, 2009). In cases where the random

266   slopes model did not converge, we used a random intercepts model instead.

267   *Among-individual variation*

268   First, for each response trait in turn we tested for among-individual variance within the

269   reaction norm framework. For each response trait we use the simplified fixed effects

270   structure as determined by the model used to test for habituation described above. To

271   test for repeatable differences in average hormone levels (i.e., among-individual

272   variance in reaction norm intercept) across all four repeats, we compared a 'null' model

273   (with no random effects) to one in which we fit a random effect of individual ID. We then

274   extended the random intercepts model to include among-individual differences in

275   habituation rate (i.e., reaction norm slopes), by fitting both a random ID effect and a

276   random interaction of ID with assay repeat number (this time as a continuous

277   covariate), as well as the intercept-slope covariance. We compared nested models using

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278 likelihood ratio tests (LRTs), in which we assume that twice the difference in model log-

279 likelihoods conforms to a chi-square distribution where the degrees of freedom are set

280 by the number of additional parameters in the more complex model. When testing the

281 effect of a single variance term, the test statistic distribution is assumed to correspond

282 to a 50:50 mix of chi-squared distributions having 0 and 1 degrees of freedom

283 respectively (Self and Liang, 1987; Visscher, 2006).

284   Second, for each response variable we formulated a multivariate (4-'trait') model

285 to test hypotheses about variance in – and covariance among – the four repeat-specific

286 observations. Rather than using the raw data, we estimated (co)variances conditional on

287 the repeat-specific means as well as fixed effects as described above. We fitted a series

288 of nested models to test hypotheses about the structure of individual variation. The first

289 model estimated no covariances, and constrained the repeat-specific variances to be

290 equal. Model 2 allowed the repeat-specific variances to differ, and model 3 extended

291 model 2 by also estimating all covariances. We compare nested models using LRTs as

292 detailed above. Model 2 vs model 1 therefore tests whether phenotypic variance

293 (conditional on fixed effects) changes significantly across repeats (i.e., that the amount

294 of variation among individuals in hormone state changes over the repeated exposures,

295 suggesting variation in habituation), and model 3 vs model 2 tests for the existence of

296 significant within-individual covariance structure (i.e., that some degree of repeatability

297 exists). Model 3 estimates the within-individual covariance-correlation matrix

298 (conditional on fixed effects), which we used as the basis for a parametric bootstrap

299 method (described in Boulton *et al.* 2014; Houslay *et al.* 2017) to generate approximate

300 95% confidence intervals on all parameters. Inspection of these parameters and their

301 associated 95% CIs can also be used to diagnose variation in habituation (again,

302 evidenced by changes in observed endocrine state variance over sampling repeats,

303 and/or declining (within-individual) correlation with increasing time between

304 observations (such that $r_{1,2}>r_{1,3}>r_{1,4}$)).

305 # Results

306 ## Changes in hormone concentrations following repeated stress exposures

307 Both free cortisol and free 11KT show significant changes in their mean waterborne

308 concentrations (i.e., immediately following transfer-related stress exposure) across the

309 four successive stress exposures. Free cortisol concentration follows a pattern that

310 largely conforms to our expectations of habituation, the mean declining significantly

311 from stress exposures 1 to 2 and remaining stable at 3, although then showing a slight

10

312 increase at exposure 4 (Fig. 2A, Table 1A). Females produce higher levels of cortisol than

313 do males across all repeats. We find no sex differences in habituation rate for free

314 cortisol (sex × stressor number interaction: $F_{3, 63.3} = 0.63$, $P = 0.60$). In contrast, male and

315 female guppies do differ in how their 11KT levels change across repeated assays (sex ×

316 stressor number interaction: $F_{3, 65.9} = 5.11$, $P = 0.003$; Fig. 3, Table 1B). Males show fairly

317 stable levels of 11KT across all repeats, while females show increased 11KT after the

318 first assay. Males consistently produce greater levels of 11KT in comparison to females.

319 **Reaction norm analyses of among-individual variation**

320 LRT comparison of the univariate mixed models with and without a random effect of

321 individual identity) provides statistical support for repeatable differences in average

322 free cortisol concentrations across the repeated stress exposures. That said, the adjusted

323 repeatability (i.e., the ratio of among-individual variation to phenotypic variation

324 conditional on fixed effects) for free cortisol is relatively low (R=0.15 SE 0.10, $\chi^2_{0,1} =$

325 3.11, $P = 0.04$). Individuals do not show significant differences in their average level of

326 free 11KT (R=0.03 SE 0.08, $\chi^2_{0,1} = 0.14$, $P = 0.36$).

327   Addition of random reaction norm slope terms to the mixed models does not

328 lead to a significantly better model for either free cortisol or free 11KT. For free cortisol,

329 visual inspection of model predictions is certainly suggestive of an improved fit to the

330 observed data (Figs 2B,C), though the apparent improvement is not significant ($\chi^2_2 =$

331 4.12, $P = 0.13$). Under this random slope model, the correlation between individual

332 intercepts and slopes is strongly positive (such that individuals with higher average

333 levels of cortisol also had a more positive slope), although the standard errors around

334 the estimate are large ($r = 0.88$ SE 0.56). For free 11KT we were unable to detect any

335 estimable variance in random slope ($\chi^2_2 = 0$, $P = 1$).

336 **Character state analyses of among-individual variation**

337 For free cortisol, there is a significant change in repeat-specific variances, such that

338 variance increases over successive repeats (model 2 vs model 1: $\chi^2_3 = 8.2$, $P = 0.03$; Fig.

339 4). This increase in variance over stress exposures suggests individuals may differ in

340 their patterns of habituation. We also found significant within-individual covariance

341 structure between repeats (model 3 vs model 2: $\chi^2_6 = 14.0$, $P = 0.03$), indicative of some

342 repeatable variation among individuals. However, examination of the covariance-

343 correlation matrix (Table 2A) shows that the correlations, though largely positive (5 out

344 of 6) as predicted, are relatively weak (ranging from -0.239 to 0.345). Furthermore,

345 there is no clear pattern of decline in the strength of the correlations as the interval

346   between pairs of exposures increases. For free 11KT, we found no significant changes in
347   repeat-specific variances for (model 2 vs model 1: χ^2_3 = 0.6, P = 0.89), and no evidence
348   for significant within-individual covariance structure (model 3 vs model 2: χ^2_6 = 3.1, P =
349   0.62; correlations ranging from -0.184 to 0.197, Table 2B).

Discussion

351   Our results show a striking pattern of change in the mean stress-induced free cortisol
352   concentrations expressed by guppies across repeated stress exposures. This pattern was
353   consistent with our predictions regarding habituation to the sampling protocol stressor
354   following repeated exposure to it, with fish showing significantly higher free cortisol
355   concentrations during the first exposure than during the following three. Free 11KT
356   concentrations also change on average across the repeated stress exposures, most
357   clearly in females which produce relatively low 11KT levels on the first (nominally more
358   stressful) exposure relative to later ones. Our analyses of among-individual variation –
359   conducted within both random regression and multivariate "character state"
360   frameworks – also provide clear evidence that individuals differ in their average stress-
361   induced hormone levels across sampling events. While we find some limited support for
362   our final prediction that individuals would also differ in their rates of habituation with
363   regard to stress-induced free cortisol release, statistical evidence is equivocal
364   (potentially due to limited statistical power).
365
366   *Habituation in stress-induced free hormone concentrations*
367   The high levels of (average) free cortisol during the first stress exposure, coupled with
368   the decreased levels during subsequent exposures, are consistent with our prediction
369   that habituation should occur with repeated stressor exposure. The population-level
370   habituation dynamics seen here echo largely those reported previously in guppies
371   (Fischer et al., 2014) and other small fish (e.g., Wong *et al.* 2008; Fürtbauer *et al.* 2015).
372   This pattern constitutes a form of plasticity in stress responsiveness that is widely
373   considered adaptive in the context of 'coping' with environmental challenges: while the
374   stress response enables individuals to react appropriately to acute environmental
375   change, habituation may be important to protect against the wear and tear that would
376   arise from continued activation of the HPA/I axis. Less clear, however, is the reason for
377   the apparent increase in the stress-induced free cortisol concentration levels between
378   the third and fourth stress exposures (Fig. 2A). While the average level of free cortisol is
379   still lower during the fourth stress exposure than the first – indicating that individuals

380 are still habituated to repeated stress exposure – there is a small increase in the final
381 stress exposure compared to the second and third. This pattern is suggestive of a more
382 complex change in HPA/I axis performance in response to repeated stress exposure
383 than initially envisaged, and could conceivably reflect a number of different
384 physiological changes acting in isolation or concert. For example, while this pattern
385 could reflect an increase in the magnitude of stress-induced cortisol release between
386 exposures 3 and 4 (consistent with some form of attenuation of habituation), it could
387 also arise in part from impacts of habituation on baseline cortisol concentrations prior
388 to stress exposure (if baseline cortisol concentrations rose over the course of
389 habituation, this too could contribute to an increase in the stress-induced cortisol
390 concentrations). Teasing apart the relative contributions of these proximate
391 explanations for the observed pattern would be fruitful prior to speculation on ultimate
392 explanations.
393   We also find evidence of clear sexual dimorphism in levels of stress-induced free
394 cortisol, with females producing more on average across all repeats. This endocrine sex
395 difference cannot be attributed simply to sex differences in body mass (female guppies
396 being much larger on average than males, and also more variable), as a linear effect of
397 body mass was not significant in our model of free cortisol. This sex difference in stress
398 responsive cortisol levels could therefore reflect different selection pressures acting on
399 the male and female stress response, possibly due to sex differences in life histories,
400 rather than simple associations with body size (Ricklefs and Wikelski, 2002). Male
401 guppies have higher free 11KT concentrations than females across all stress exposures,
402 in line with the androgenic role of this hormone. However, while the cortisol habituation
403 dynamics are remarkably similar between males and females, 11KT shows sex-specific
404 trajectories across repeat stress exposures. Male levels remain stable (on average) while
405 females tend to have a depressed initial response before stabilising at a higher level.
406 While increased cortisol concentrations have been shown to depress circulating 11KT
407 concentrations in other (male) teleost fish (e.g. male *Salmo trutta,* Pickering et al., 1987),
408 we see no evidence of a link between the stress-induced concentrations of the two
409 hormones in male guppies. 11KT is important for male mating behaviour in teleosts
410 generally (Borg, 1994); given the well-characterised sexual selection through female
411 choice on male guppies (Brooks and Endler, 2001; Head and Brooks, 2006; Luyten and
412 Liley, 1991), it seems plausible that a lack of stress-sensitivity reflects strong selection
413 on males to maintain mating behaviours even in the face of high environmental stress. In
414 females, by contrast, the low 11KT concentrations during the first stress exposure
415 relative to the following exposures could indeed be consistent with GC-related inhibition

13

416   of circulating 11KT concentrations: the strongest cortisol response in the first stress

417   exposure could be depressing 11KT levels to a greater extent than the weaker cortisol

418   responses to the subsequent exposures.

419

420   *Individual variation in stress-induced hormone levels and habituation*

421   In addition to the population-level evidence of habituation reported above, we also find

422   evidence of consistent differences among individuals in their average stress-induced

423   free cortisol concentrations (after controlling statistically for variation in body size).

424   Whether there are differences among individuals in their rate of habituation to the

425   stressor is less clear. Plotting fitted reaction norms shows that individuals seem to

426   diverge over repeated stress exposures, yet allowing for the existence of individual

427   differences in these habituation gradients did not yield a model of significantly better fit

428   to the data than assuming that all individuals shared the same habituation gradient.

429   Similarly, the character state approach shows that individual variance increases over

430   successive repeats; however, the covariance structure is not consistently positive,

431   suggesting that this is not a simple case of individuals habituating at different rates. That

432   these patterns are suggestive of individual variation in habituation, but without yielding

433   conclusive evidence for it, points to a lack of sufficient power in our study. Mixed effects

434   models – particularly those with complex random effects structures – can be data

435   hungry, and it appears likely that a greater number of individuals would need to be

436   assayed in order to demonstrate that there are significant differences among individuals

437   in their rate of to habituation to a repeated stressor (Martin et al., 2011). Similar (but

438   higher-powered) studies are required to shed light upon the causes and consequences of

439   individual variation in endocrine traits.

440   One of the main drivers behind the interest in among-individual variation in

441   labile traits is that its presence means there is an opportunity for selection to act on

442   them. Such variation can stem from a variety of different sources, which have been

443   discussed widely in the behavioural literature (e.g., Bierbach et al., 2017; Fisher et al.,

444   2018; Reale et al., 2010) and are liable to apply to the endocrine stress response as well.

445   For example, previous studies in guppies alone have shown effects on the GC response

446   due to developmental plasticity (Chouinard-Thuly et al., 2018) and environmental

447   stressors (Fischer et al., 2014). Recent studies of the population used here have shown

448   that stress-related behaviours also respond plastically to environmental change

449   (perceived level of predation risk) but show limited among-individual variation in this

450   plasticity (Houslay et al., 2018). We also know that that these behaviours are under

451   some degree of genetic control (White and Wilson, 2018; White et al., 2018), which may

14

452   or may not be the case for cortisol expression. In fact, some debate exists as to whether

453   hormone levels themselves can be viewed as heritable 'traits' under selection (Hau and

454   Goymann, 2015; Zera et al., 2007), but there is certainly increasing evidence of a genetic

455   basis to GC variation (Jenkins et al., 2014; Stedman et al., 2017). Links between GC

456   baseline and/or reactivity and individual fitness have also been documented in a

457   number of species (Blas et al., 2007; Cabezas et al., 2007; Patterson et al., 2014; Vitousek

458   et al., 2014; Vitousek et al., 2018). Williams (2008) advocated looking beyond the

459   'tyranny of the Golden Mean' to embrace individual variation in endocrine systems; this

460   challenge is starting to be taken up, with exciting possibilities for our understanding of

461   the evolution of flexible phenotypes and hormonally mediated suites of traits (discussed

462   further in Cockrem, 2013; Hau et al., 2016; Hau and Goymann, 2015; Taff and Vitousek,

463   2016).

464

465   *Conclusions*

466   As predicted, we find that guppies habituate quickly to repeated exposure to a stressor.

467   We also provide examples of a framework for characterising GC variability and

468   flexibility within and among individuals using both reaction norm and character state

469   approaches. Our results show individual variation in stress-induced cortisol levels, in

470   addition to some limited evidence of individual variation in habituation to the stressor.

471   Such variation provides material for selection to act upon, and could (given underlying

472   genetic variation in habituation rate) enable a population to mount an evolutionary

473   response – for example, to the deleterious effects of a failure to habituate to chronic

474   stress. Given the current lack of consensus around how to diagnose chronic stress in

475   populations (Dickens and Romero, 2013), one fruitful line of future research could be to

476   estimate covariance at the among-individual or genetic level between failure to

477   habituate to repeated exposure to a predictable, homotypic stressor (such as the

478   sampling process itself) and a loss of body mass, changes in behaviour, or even survival.

479   More broadly, our study adds to the burgeoning body of research showing that

480   individuals differ in flexible endocrine traits that are likely to influence fitness. Future

481   challenges include the investigation of whether individual habituation rates are

482   themselves repeatable, and whether genetic links exist between GC variation and other

483   aspects of the phenotype on which selection acts.

484

Competing interests

No competing interests declared.

Data statement

The data used in this study will be uploaded to Dryad upon completion. R code for analyses is provided in the supplementary materials.

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715

716 ## Tables

717

	Hormone	Effect	Coefficient (SE)	DF	F	P
(A)	Cortisol (free)	(Intercept)	8.951 (0.116)	1,27.3	8845.0	<0.001
		Assay 1	0 (-)	3,64.7	14.2	<0.001
		Assay 2	-0.617 (0.106)			
		Assay 3	-0.578 (0.111)			
		Assay 4	-0.318 (0.120)			
		Sex (Male)	-0.799 (0.168)	1,27.5	37.8	<0.001
		Body mass	-0.120 (0.084)	1,27.5	2.1	0.164
		Order	-0.014 (0.009)	1,100.0	2.6	0.115
		Tank A	0 (-)	1,27.3	2.5	0.129
		Tank B	0.153 (0.098)			
(B)	11-ketotestosterone (free)	(Intercept)	1.308 (0.073)	1,65.9	1178.0	<0.001
		Assay 1	0 (-)	3,65.9	10.5	<0.001
		Assay 2	0.475 (0.088)			
		Assay 3	0.330 (0.089)			
		Assay 4	0.436 (0.090)			
		Sex (Male)	0.897 (0.115)	1,65.9	71.7	<0.001
		Assay 1 x Sex	0 (-)	3,65.9	5.1	0.003
		Assay 2 x Sex	-0.402 (0.128)			
		Assay 3 x Sex	-0.029 (0.130)			
		Assay 4 x Sex	-0.331 (0.131)			
		Body mass	0.062 (0.042)	1,65.9	2.2	0.155
		Order	-0.003 (0.005)	1,65.9	0.3	0.595

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Tank A	0 (-)	1,65.9	3.1	0.078
Tank B	-0.086 (0.049)			

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719　Table 1: Fixed effect estimates from mixed-effects model analyses of free

720　circulating (A) cortisol and (B) 11-ketotestosterone levels in individual guppies

721　over four repeated measures.

A) Free cortisol	Repeat 1	Repeat 2	Repeat 3	Repeat 4
Repeat 1	0.127 (0.058,0.189)	0.121 (-0.505,0.327)	0.345 (-0.074,0.486)	0.141 (-0.45,0.352)
Repeat 2	0.018 (-0.036,0.075)	0.182 (0.087,0.28)	0.34 (-0.099,0.479)	-0.239 (-1.262,0.077)
Repeat 3	0.06 (-0.006,0.126)	0.071 (-0.01,0.151)	0.237 (0.114,0.353)	0.265 (-0.217,0.429)
Repeat 4	0.03 (-0.046,0.113)	-0.062 (-0.159,0.03)	0.078 (-0.031,0.189)	0.364 (0.183,0.549)

B) Free 11KT	Repeat 1	Repeat 2	Repeat 3	Repeat 4
Repeat 1	0.076 (0.036,0.114)	-0.184 (-1.214,0.113)	-0.017 (-0.79,0.251)	0.197 (-0.382,0.367)
Repeat 2	-0.013 (-0.039,0.012)	0.063 (0.03,0.096)	0.143 (-0.463,0.338)	-0.048 (-0.781,0.205)
Repeat 3	-0.001 (-0.028,0.028)	0.01 (-0.015,0.035)	0.076 (0.036,0.112)	0.103 (-0.551,0.313)
Repeat 4	0.013 (-0.013,0.037)	-0.003 (-0.023,0.019)	0.007 (-0.018,0.031)	0.059 (0.03,0.09)

Table 2: Covariance-correlation matrix (conditional on fixed effects) for free circulating (A) cortisol, (B) 11KT. Variances are on shaded diagonals, covariances below and correlations above. In parentheses are the 95% confidence intervals on calculated from 5000 bootstrapped replicates.

Figures

Figure 1: Characterising individual variation in habituation.

Main panel (A) shows how habituation across repeated stressor exposures is predicted to affect average levels of cortisol (adapted from Fig. 3 of Fischer et al., 2014). Inset panels show examples of three individuals sampled haphazardly from such a population, indicating (B) no consistent differences among individuals, (C) among-individual differences in intercept only (average cortisol levels differ but rate of habituation does not), (D) among-individual differences in both intercept and slope (variation in both average cortisol levels and the rate of habituation). Note that while absolute cortisol levels depicted here do not follow linear (i.e., straight line) reaction norms, this assumption is not made in our analysis. Rather we assume only that a straight line is adequate to describe each individual's deviation from the replicate-specific mean, as is the case in this depiction (E-G; dotted black line indicates zero deviation from the population mean).

Figure 2: Variation in free circulating cortisol (log-transformed nanograms) in guppies.

Panel (A) shows changes in cortisol as a function of stressor exposure (here, sampling repeat) separately for each sex; points are predictions from a linear mixed effects model (with 95% confidence intervals), with raw individual-level data in faint lines. Panel (B) shows predictions for each individual from a random regression model (coloured by sex; shaded area gives 95% confidence interval around each prediction), and (C) shows individual deviations from the population mean after accounting for all fixed effects (including sex and repeat).

Figure 3: Variation in free circulating 11-ketotestosterone (log-transformed picograms) in guppies.

Points show predictions from a linear mixed effects model (with 95% confidence intervals) as a function of stressor exposure (sampling repeat), plotted separately for each sex. Raw individual-level data is shown in faint lines.

Figure 4: Change in individual variation in cortisol over increasing stressor exposures.

Variance estimate and 95% confidence intervals (from 5000 bootstrapped replicates) for each sampling repeat, calculated from a multivariate mixed effects model fit with ASreml-R.

Appendix A: Analysis of conjugated fractions of cortisol and

11-ketotestosterone

Mean effects

We find significant effects of repeated stress exposure on conjugated hormone fractions. For the conjugated form of cortisol this appears to be due largely to increased concentrations at exposures 3 and 4 relative to earlier exposures (Table A1a). We find no sex differences in how these levels changed over the repeated measures (sex × stressor number interaction: $F_{3, 62.9} = 0.91$, $P = 0.44$). We also find no clear sex differences in mean conjugated cortisol concentrations, although there is a positive effect of mass. Concentrations of the conjugated form of 11KT also change over repeats, rising to a peak at the third exposure before decreasing in the final exposure (Table A1b). Males exhibit consistently higher levels of conjugated 11KT, and there are no sex differences in the response to repeated assays ($F_{3, 88.7} = 0.86$, $P = 0.46$).

Among-individual variance: random regression

Under the random regression approach, there is significant variance among individuals in the conjugated forms of both hormones. We find moderate adjusted repeatability for conjugated cortisol (R=0.26 SE 0.10, $\chi^2_{0,1} = 8.91$, $P = 0.001$) and for 11KT (R=0.27 (SE 0.10), $\chi^2_{0,1} = 9.30$, $P = 0.001$). For conjugated cortisol the comparison of random intercept and random slope models is non-significant ($\chi^2_2 = 1.64$, $P = 0.44$). For conjugated 11KT we do not detect any estimable variance in random slope ($\chi^2_2 = 0$, $P = 1$).

Among-individual variance: character state

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Using the character state approach, conjugated cortisol shows no significant change in variance over repeats, but there is significant within-individual covariance structure. The covariance-correlation matrix shows that correlations between pairs of assays are weakly positive (ranging from 0.131 to 0.411, Table A2a), and we also find a qualitative pattern of declining strength as the inter-observation time increases. Conjugated 11KT does show changes in variance across repeats (χ^2_3 = 33.3, P < 0.001), as well as within-individual covariance structure (χ^2_6 = 19.8, P = 0.003) and a pattern of positive correlations among repeats (ranging from 0.124 to 0.561, Table A2b), which tend to decline in strength with increasing inter-observation interval.

Hormone	Effect	Coefficient (SE)	DF	F	P
Cortisol (conjugated)	(Intercept)	-1.125 (0.146)	1,26.9	94.7	<0.001
	Assay 1	0 (-)	3,64.3	3.3	0.026
	Assay 2	-0.035 (0.105)			
	Assay 3	0.277 (0.110)			
	Assay 4	0.105 (0.117)			
	Sex (Male)	0.207 (0.205)	1,27.1	1.0	0.322
	Body mass	0.557 (0.103)	1,27.2	29.3	<0.001
	Order	0.010 (0.010)	1,115.6	1.1	0.295
	Tank A	0 (-)	1,26.9	2.4	0.132
	Tank B	-0.186 (0.119)			
11-ketotestosterone (conjugated)	(Intercept)	1.764 (0.163)	1,27.3	217.1	<0.001
	Assay 1	0 (-)	3,91.1	9.8	<0.001
	Assay 2	0.132 (0.126)			
	Assay 3	0.646 (0.126)			
	Assay 4	0.176 (0.126)			
	Sex (Male)	1.381 (0.241)	1,27.4	32.9	<0.001
	Body mass	0.165 (0.120)	1,27.4	1.9	0.182
	Order	0.037 (0.012)	1,118.0	10.2	0.002
	Tank A	0 (-)	1,27.4	1.3	0.269
	Tank B	-0.158 (0.140)			

Table A2: Fixed effect estimates from mixed-effects model analyses of conjugated (a) cortisol and (b) 11-ketotestosterone levels in individual guppies over four repeated measures.

c) Conjugated cortisol	Repeat 1	Repeat 2	Repeat 3	Repeat 4
Repeat 1	0.238 (0.116,0.367)	0.411 (-0.008,0.519)	0.202 (-0.394,0.356)	0.131 (-0.499,0.327)
Repeat 2	0.126 (-0.001,0.245)	0.396 (0.198,0.609)	0.379 (-0.04,0.492)	0.162 (-0.376,0.368)
Repeat 3	0.045 (-0.042,0.122)	0.109 (-0.005,0.217)	0.208 (0.097,0.321)	0.367 (-0.052,0.508)
Repeat 4	0.029 (-0.053,0.11)	0.046 (-0.052,0.16)	0.075 (-0.005,0.16)	0.201 (0.098,0.309)

d) Conjugated 11KT	Repeat 1	Repeat 2	Repeat 3	Repeat 4
Repeat 1	0.231 (0.105,0.346)	0.477 (0.113,0.582)	0.176 (-0.404,0.371)	0.124 (-0.6,0.31)
Repeat 2	0.157 (0.018,0.292)	0.471 (0.246,0.729)	0.267 (-0.184,0.439)	0.372 (-0.034,0.517)
Repeat 3	0.1 (-0.105,0.313)	0.215 (-0.074,0.537)	1.381 (0.646,2.053)	0.561 (0.273,0.645)
Repeat 4	0.029 (-0.062,0.109)	0.123 (-0.005,0.264)	0.319 (0.07,0.553)	0.233 (0.102,0.357)

Table A2: Covariance-correlation matrix (conditional on fixed effects) for conjugated circulating (a) cortisol, (b) 11KT. Variances are on shaded diagonals, covariances below and correlations above. In parentheses are the 95% confidence intervals on calculated from 5000 bootstrapped replicates.