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**He who dares only wins sometimes: physiological stress and
contest behaviour in *Xiphophorus helleri***

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Keywords: contest behaviour; stress response; stress-coping style; behaviour; experimental
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17 SUMMARY

18 While many factors influence contest outcome and social dominance in animals,
19 there is increasing interest in behavioural-physiological stress-coping styles. Causality,
20 however, is often ambiguous – is physiological state determined by contest outcome or *vice*
21 *versa*? Furthermore, experimental protocols may themselves induce stress responses that
22 impact individual behaviour and thus potentially contest outcome. Here we test whether
23 latency to recover from acute stress, measured both physiologically and behaviourally,
24 predicts who initiates and who wins dyadic contests between pairs of male green swordtails
25 (*Xiphophorus helleri*). In line with our predictions, animals that recovered faster
26 (behaviourally) from disturbance created by the experimental protocol prior to meeting an
27 opponent were more likely to initiate contests; however, they were not more likely to win
28 and, contrary to expectations, had higher pre-contest cortisol levels than their opponents.
29 They also showed greater physiological stress responses to the experiment as determined
30 from the difference between pre- and post-contest cortisol levels. Moreover, stress
31 response was independent of whether a contest escalated. In contradiction to evidence
32 found in other taxa and fish systems, the suite of traits that we measured were not
33 correlated in a manner that allowed classification of the animals into the usual reactive and
34 proactive stress-coping styles. Our results suggest that coping style may play a key role in
35 determining the individual initiates a contest, but that other factors govern contest
36 outcome.

37 INTRODUCTION

38 Competition for resources such as food, mates or territory, often involves contests
39 where winners, or dominant individuals, improve their fitness at the expense of losers

(Brockelman, 1975). Many factors are expected to influence contest outcome and so determine dominance status. While these are known to include size (e.g. Huntingford et al., 1990) and behavioural traits such as aggression (Francis, 1988), individual styles of coping with stress may also be important (Koolhaas et al., 1999; Pottinger & Carrick, 2001; Øverli et al., 2004). Stress threatens homeostasis that is re-established by both physiological and behavioural responses. Importantly, when studying behaviour, experimental protocols may induce stress responses that impact individual behaviour, thus indirectly influencing eventual contest outcome. Here we explore the hypothesis that latency to recover from stress, as measured both behaviourally and physiologically, is a key determinant of contest initiation and outcome. In animals, physiological stress-coping mechanisms are highly conserved and governed by the hypothalamic-pituitary-adrenal (HPA) axis; in fish, this role is assumed by the hypothalamic-pituitary-interrenal (HPI) axis, a good physiological indicator being water-borne cortisol (for a review, see Scott & Ellis, 2007; Scott et al., 2008).

Classically, much research on animal conflict has focused on the concept of resource holding potential (RHP; Parker, 1974). Commonly used measures of RHP (e.g. body size) often predict contest initiation and outcome, although resource ownership, individual motivation and social processes such as eavesdropping and prior fighting experience are also important (Hsu et al., 2006; Arnott & Elwood, 2008). Studies that attempt to control for RHP, for example by size matching and using neutral arenas, have suggested that individuals initiating contests tend to win them (Jackson, 1991). However, this is not always the case (Moretz, 2003), suggesting that factors other than the initial motivation to fight may affect contest outcome especially during escalated contests (Hsu & Wolf, 2001).

The relationship between physiological stress (HPA/HPI axis activity) and social dominance has received increasing attention and has been well studied across many taxa, including rodents (Bronson, 1973), primates (Abbott et al., 2003), birds (Verbeek et al., 1996), mammals (Young et al., 2006), domestic livestock (Bergsma et al., 2008) and fish (Øverli et al., 2007). However, causality is often ambiguous; it is unclear whether physiological state is determined by outcome, or outcome is determined by physiological state. For example, faster recovery of baseline cortisol levels following aggressive contests is associated with dominance (Netherton et al., 2004), while individuals with higher baseline cortisol levels are less likely to win contests or to obtain dominance status in a hierarchy (Hannes, 1984; DiBattista et al., 2005). Other types of behavioural variation may be linked to physiological stress, particularly an individual's coping style (Earley et al., 2006). In a study focusing on both behavioural and neuroendocrinological parameters, Koolhaas et al. (1999) contrasted proactive and reactive coping styles and suggested a proactive/boldness link (boldness is here described as a willingness to explore novel environments, Budaev, 1997). Many studies have demonstrated correlations between boldness and aggression (for example, Bell & Sih, 2007), and of specific interest is that in fishes, empirical measurements of HPI activity, aggression and boldness have been associated with differences in coping style (Schjolden et al., 2005; Aubin-Horth et al., 2012).

The majority of studies investigating contest behaviour and dominance in domestic and wild fishes use experimental designs that require netting individuals to facilitate periods of isolation. This is usually followed by some form of disturbance, such as the removal of partitions between isolated contestants in novel environments (for example, Wilson et al., 2011a). Could it be that contest winners are those that better cope with stressors imposed

by the experimental protocol prior to even encountering an opponent? If so, then aspects of personality (e.g. boldness) and/or stress coping style may predict observed aggression and contest outcome.

Here we test the effect of disturbances imposed by the experimental protocol on contest behaviour and outcome using male green swordtails (*Xiphophorus helleri*), a small, tropical freshwater, live-bearing fish. Due to their readily aggressive nature, species from the *Xiphophorus* genus are commonly used as behavioural models in studies of dominance and many such studies have focused on visual and social cues as explanations for conflict resolution (Earley, 2006). However, we hypothesize that if coping style is important in the determination of observed contest behaviour under experimental situations, then relationships should exist between the behavioural reaction to disturbance prior to meeting an opponent, the likelihood of initiating a contest, contest outcome and the physiological stress response as measured by cortisol levels. Specifically, we predict that a short latency to resume normal swimming behaviour following disturbance will be associated with fish that initiate and win contests; such animals are predicted to be less stressed, i.e., have lower baseline (pre-contest) cortisol levels and a smaller stress response (post contest minus pre-contest cortisol level) than the eventual losers.

METHODS

Green swordtails (*Xiphophorus helleri*) obtained from a commercial distributor were housed in heterosexual groups in 152 and 208 L aquaria equipped with gravel substrate (3cm), filtration, and aeration. Water temperature was maintained between 23 - 25° C, pH between 7.2-7.6, and fish were kept on a 12 h light: 12 h dark photoperiod. Stress Coat™ (94µl/L) and freshwater aquarium salt (2g/L) were added to the tanks prior to fish arrival to

mitigate the loss of fish mucus and to reduce osmotic stress, respectively; each of these is a common response of fish to shipping and handling.

Dyad Establishment

Males were netted from the aquarium and placed in a plastic bag with a small amount of water to keep the gills and body moist and to immobilize the fish for measurement; measurements were taken with Vernier calipers accurate to 0.1 mm. Measurements of standard length (SL, snout tip to caudal peduncle), total body length (snout tip to caudal fin tip), body depth (BD, anterior portion of dorsal fin to origin of gonopodium), and sword length (SwL, caudal fin tip to sword tip) were obtained. Pairs of males for dyadic trials were matched for lateral surface area (LSA; < 20 units difference) because LSA has been shown to be a better predictor of fighting ability than any one measure of size alone (Beaugrand et al., 1996). LSA (mm²) was determined as:

(standard length * body depth) + (sword length * sword depth)

assuming a sword depth of 1.0 mm. Body markings and coloration were also noted for purposes of identification. Macromelanophore patterns and sword characteristics were used to discriminate the two opponents (Franck et al., 2001; Basolo & Trainor, 2002). A total of 30 pairs were formed.

Contests and Hormone Collection

Immediately after measurements, fish were transferred directly from the plastic bag to 1000 ml polypropylene holding beakers containing 1000 ml of aerated freshwater. Stress Coat™ (94µl) and freshwater aquarium salt (2 g) were added to the holding container to replace fish mucus and reduce osmotic stress associated with handling during

measurement. The holding beakers were outfitted with a fine mesh net bottom and placed inside another 1000 ml polypropylene beaker; this design made it possible to transfer the fish between beakers gently, quickly (< 5 seconds) and without the handling typically associated with capture (e.g., chasing, netting). The fish remained in the holding beaker for 2d to acclimate before being transferred to new 1000 ml sampling beakers containing 1000 ml of freshwater (with 4g freshwater salt) for 2 h, with hormones were released into the water during this time (Scott et al., 2008). Stress Coat™ was not added to the hormone collection beaker because it is not known whether the chemical interferes with hormone extraction and assay; freshwater salt, however, can be purged from hormone extraction columns (see below). After 2 h in the pre-fight sampling beaker the fish were transferred using a net to 38 L experimental fight tanks, separated into two equal compartments by an opaque divider. Each compartment was equipped with an aeration device and the water was treated with Stress Coat™ and freshwater aquarium salt. The two fighters were placed on opposite sides of the same fight tank and acclimated for 22 h. After this time the dividers were lifted (remotely) and the air stones were also removed. This physical disturbance typically resulted in frantic swimming behaviour by both fish, characterized by fast, erratic movements both horizontally and vertically before the fish settled to the gravel bottom. We therefore consider it to be a response imposed by the experimental protocol itself. We determined the latency of behavioural recovery from this event as the time (from lifting of partition) to resume normal swimming, defined as swimming slowly in a horizontal orientation with fins often erect or semi-erect.

The fish then interacted until a dominance relationship was established, defined as the point when one individual retreated 10 consecutive times without reciprocating

aggression or displayed typical submissive posturing, such as folding fins upon approach from the opponent (Franck & Ribowski, 1989; Beaugrand, 1997). Contests lasted for an average of 2286 ± 441 seconds and were recorded digitally using a Sony PC110 Digital Video camera then burned to DVD. The identity of the animal that first began swimming normally following partition removal, initiated the contest (approached within one body length of the opponent), and won the contest was recorded using JWatcher version 1.0 (Blumstein & Daniel, 2007; <http://www.jwatcher.ucla.edu/>). Latency to contest initiation, as well as contest duration (from initiation to settlement) was calculated in seconds from partition lifting. Additionally, we classified each contest as being escalated or not. Escalated contests were defined as those involving high intensity reciprocal attacks, where the opponents would alternate attack-bite sequences often while circling one another, and/or mouthwrestling, where contestants would lock jaws in an apparent test of strength. Immediately after contest resolution, fighters were netted and placed in individual 1000 ml sampling beakers for 2 h for a post-fight hormone collection.

Hormone Extraction and Radioimmunoassay

C18 SPE columns (Extract-Clean[®], 500 mg, 4.0 ml; Alltech Associates, Inc.) were primed with 2 x 2 ml of 100% ethanol (EtOH) and 2 x 2 ml distilled water. Tygon tubing (formulation 2275) was attached to the C18 columns and placed in a beaker containing a 250 ml water sample taken from the original 1000 ml, the vacuum was engaged and water-borne steroid hormones isolated. Total hormone (free and conjugated fractions) was eluted from the columns with 2 x 2 ml 100% ethanol collected in 6 ml (12 x 75 mm) borosilicate vials. Samples were stored at 4° C overnight and the ethanol was evaporated in a Savant AES 1010 speedvac for 1.5 h (45 min at 40°C) one day prior to radioimmunoassay. Hormone

176 residues were resuspended in 60µl of 0.1 M phosphate buffer. Cortisol radioimmunoassay
177 was conducted using a coat-a-count kit purchased through Diagnostic Products Corporation
178 (Los Angeles, CA). Samples were run in duplicate in three separate assays conducted on
179 three consecutive days. Briefly, 25 µl of each sample was pipetted into antibody-coated
180 polypropylene tubes followed by the addition of 1 ml of I¹²⁵-labeled cortisol. Samples were
181 incubated in a 37°C water bath for 45 min. Liquid in all samples was then decanted, and the
182 tubes were blotted and allowed to air dry for 30 min prior to quantification. The
183 sensitivities of the three assays were 0.0268 µg/dl, 0.033 µg/dl, and 0.0624 µg/dl. Pooled
184 low-, medium- and high-level human serum (CON6 Multivalent Control Module, Diagnostic
185 Products Corporation) were used as intra-assay controls; intra-assay coefficients of variation
186 (assay 1, 2, and 3) were: tri-level low (6.2%, 3.8%, 2.1%), tri-level medium (2.8%, 12.0%,
187 4.3%), and tri-level high (4.8%, 5.0%, 7.2%). Inter-assay coefficients of variation were 6.4%,
188 7.5%, and 7.3% for tri-level low, tri-level medium and tri-level high, respectively.

189 The kit was validated for *X. helleri* by assessing parallelism and by calculating
190 expected versus observed cortisol concentrations from known samples cold-spiked with
191 standards. Twenty non-experimental swordtails (males and females) were transferred to
192 collection beakers filled with 400 ml freshwater for 8h (0800-1600 h). Hormones were
193 extracted and processed as described above, except that they were resuspended in 120µl
194 and combined to form a pool of 2.4 ml stored as 55µl aliquots at -80 °C. 240 µl of the
195 pooled control was used for serial dilutions. Briefly, 120 µl of this sample was transferred to
196 a 1.5 ml Eppendorf tube and mixed by vortexing with 120µl of 0.1 M phosphate buffer to
197 create a 1:2 dilution; 120µl of 1:2 dilution was mixed with an equal volume of 0.1 M
198 phosphate buffer to create a 1:4 dilution, and so on until 1:16. The serial dilutions were run

in quadruplicate using the RIA protocol described above with the Cortisol Coat-a-Count kit from DPC. The log-logit transformed dilution curve was parallel to the standard curve (comparison of slopes: $t_7 < 0.01$, $p > 0.05$; (Zar, 1996), p. 355). A 385 μ l sample of pooled hormone extract was used to assess recovery. 110 μ l was pipetted into a tube to constitute the 'neat' (1:1) control. 55 μ l of the large sample was then pipetted into 5 additional tubes and mixed with an equal volume of each standard provided with the DPC Cortisol coat-a-count kit (1, 5, 10, 20, 50 μ g/dl). Expected recovery concentrations were based on the known amount of cortisol in the *X. helleri* control sample. Minimum recovery was 90.3% and the slope of the observed vs. expected curve was 0.97, demonstrating a highly linear relationship between observed and expected recovery.

One fish died during the period of post-contest cortisol collection and therefore data relating to the trial that it participated were excluded from analysis. A further pair was eliminated because they did not interact on any level. A total of 28 contests from the original 30 pairs of fish were therefore observed, where 25 produced clear winners and losers and 15 were classified as escalated. The first individual to swim normally following partition removal and the individual that initiated the contest was unambiguously determined in all 28 cases (see supplemental material Table S1 for raw data on all contests).

Data analysis

In order to summarize associations among the full set of morphological, behavioural, and endocrine traits measured we generated a correlation matrix using Genstat 14.1 (Payne et al., 2005). Correlations between morphological and physiological traits were estimated using the full set of observations (i.e. one record per individual, n=56) for body depth (BD), standard length (SL), sword length (SwL), lateral surface area (LSA), pre-contest (PreCORT)

222 and post-contest (PostCORT) cortisol levels and physiological stress response (SR).
223 Endocrine assays before and after the trial were $\log_{10}$ transformed to yield PreCORT and
224 PostCORT respectively, while we defined SR as the change in cortisol expression on a $\log_{10}$
225 scale (i.e., $SR = \text{PostCORT} - \text{PreCORT}$). For those traits where the phenotypic value of one
226 individual within a trial necessarily determines that of the second, we used observations
227 from one randomly chosen focal individual per trial only ($n = 28$). These traits include the
228 binary variables of Swimfirst (whether the focal fish was first to resume normal swimming
229 after disturbance), Initiate (whether the focal fish initiated the contest) and Status (whether
230 the focal fish was the winner). For these randomly chosen focal individuals we also
231 determined a relative measure of size difference (LSAdiff), defined as the difference in
232 phenotypic values (focal LSA – opponent LSA). Correlations with two further traits, latency
233 to swim (LatSwim) and latency to initiate (LatInit) were also estimated. However, these
234 traits are only meaningfully observed for the individual within each trial that either swims
235 first or initiates the contest, respectively. Thus estimated correlations with these variables
236 are conditional on moving first or initiating the contest as appropriate ($n=28$).

237 To more directly test the hypothesized causal relationships between behavioural
238 recovery from disturbance, contest initiation and outcome (i.e., status) and stress response,
239 we formulated a set of linear models that were solved by restricted maximum likelihood
240 using ASReml (Version 3, Gilmour et al., 2009). In particular this allowed us to test our
241 hypotheses while properly accounting for any influence of body size (LSA) on endocrine
242 traits and/or contest behaviour. Note therefore that our phenotypic measures of the
243 endocrine traits (PreCORT, PostCORT, SR) are not corrected in any way for the expected

244 influence of fish size (Scott & Ellis, 2007) prior to analysis; rather, the linear model
245 framework allows us to control for these effects statistically within the analysis.

246 As described above, each contest provides only a single phenotypic observation for
247 the binary traits of Initiate (Model 1) and Status (Model 2) and these response variables
248 were analysed using generalized linear models (with logit link function). Thus we modelled
249 probability (on the logit scale) of initiating a contest as a function of being first to adjust to
250 normal swimming behaviour following removal of the partition (*Swimfirst*), as well as
251 baseline cortisol (*PreCORT*), size (*LSA*), and all two-way interactions of these explanatory
252 variables such that:

$$\begin{aligned} 253 \quad \text{Initiate}_{ik} = & \mu + \text{Swimfirst} + \text{PreCORT} + \text{LSA} + \text{Swimfirst.PreCORT} + \text{Swimfirst.LSA} + \\ 254 \quad & \text{PreCORT.LSA} + \varepsilon_k \end{aligned}$$

255 (Model 1)

256 Where Initiate_{ik} is the probability (on the logit scale) of individual i initiating contest k , μ is
257 an overall mean, and ε is a residual error term (assumed to be uncorrelated across trials).
258 The probability of winning a contest (*Status*, 0/1) was modelled in a similar way, but with
259 the addition of fight *Escalation* (as a two-level categorical variable, i.e. whether a fight did or
260 did not escalate) fitted as a factor, and its interaction terms as additional explanatory
261 effects. Escalation is included here because Swimfirst may only predict contest winners
262 when fights do not escalate (e.g., see Hsu & Wolf, 2001).

$$\begin{aligned} 263 \quad \text{Status}_{ik} = & \mu + \text{Swimfirst} + \text{PreCORT} + \text{Escalation} + \text{LSA} + \text{Swimfirst.PreCORT} + \text{Swimfirst.LSA} + \\ 264 \quad & \text{PreCORT.Escalation} + \text{PreCORT.LSA} + \text{Escalation.LSA} + \varepsilon_k \end{aligned}$$

265 (Model 2)

Finally we modelled stress response (SR) to test the hypothesis that it would be lower for those individuals that had won contests, and particularly so in the absence of contest escalation. Values of SR can be assigned to both individuals within a trial but may not be fully independent. We therefore analysed SR using a linear mixed effect model (with normal error structure) that included a random effect of trial to account for non-independence (Model 3).

$$SR_{ik} = \mu + Swimfirst + Status + Escalation + LSA + Swimfirst.LSA + Swimfirst.Status + Swimfirst.Escalation + Status.LSA + Status.Escalation + Escalation.LSA + Trial_k + \varepsilon_k$$

(Model 3)

For each of the models shown above we adopted a model reduction strategy where explanatory terms were dropped if they were statistically non-significant at $p \geq 0.1$ under a two-tailed conditional F - test. Main effects were retained in the model if one or more of their interactions were retained on this basis. Note that we chose to use a threshold of $\alpha=0.1$ rather than 0.05 in our model reduction strategy and therefore our final models can contain marginally non-significant explanatory terms (i.e. $0.1 \leq p \leq 0.05$). We adopted this strategy as, since available sample sizes are fairly small we expect power will be limiting. However, we deem that it is instructive to consider whether marginally non-significant terms are at least qualitatively consistent with hypothesized biological processes, i.e. it may not be sensible to equate non-significance with an effect size of zero.

RESULTS

Among trait correlations

The estimated correlation structure provided evidence of significant associations among a number of the traits measured (Table 1). Phenotypic correlations were close to unity among the morphological traits of BD, SL and LSA ($r_{BD.SL} = 0.95$, $r_{BD.LSA} = 0.99$, $r_{SL.LSA} = 0.98$; all $p < 0.001$), an unsurprising result given that these all capture aspects of body size. Sword length (SwL) was also positively correlated with body size traits although less strongly. Body size traits were significantly and positively correlated with both pre- and post-contest cortisol levels (r ranging from 0.42 - 0.48, all $p \leq 0.001$; Table 1) although again the correlation between PostCORT and sword length (SwL) was lower ($r = 0.30$, $p = 0.03$). Given that endocrine traits are not standardised for size variation prior to analysis these results are consistent with the expectation of a positive association between body size and cortisol release into the water (Scott et al., 2008), controlled for in our model based hypothesis testing (as discussed above). Note that stress response (SR) is auto-correlated with pre- and post-contest cortisol levels as a consequence of its definition (i.e. SR = PreCORT – PostCORT; $r_{SR.PreCORT} = -0.43$, and $r_{SR.PostCORT} = 0.43$, both $p = 0.001$). Cortisol levels before and after the contest were also significantly correlated within individuals ($r_{PreCORT, PostCORT} = 0.64$, $p = < 0.001$). However, correlations between SR and size (as measured by the various morphology traits) are weak and non-significant.

Among behavioural traits we found a significant positive correlation between swimming first and initiating the contest as we hypothesized ($r = 0.56$, $p = 0.004$). For the set of individuals that both swam first and initiated the contest, latency to swim was also strongly correlated with latency to initiate ($r = 0.64$, $p = 0.003$). However, swimming first was not positively correlated with status (i.e. winning, $r = -0.16$, $p = 0.58$), and among those fish that did swim first the correlation between latency to swim and status was close to zero

($r = 0.16$, $p = 0.45$). Thus the correlation structure is consistent with our hypothesis that individuals more rapidly resuming normal swimming after partition removal are more likely to initiate contests. However, these individuals are not more likely to win the subsequent contest.

The correlation structure provided only limited statistical support for relationships between behavioural and endocrine traits. Contrary to our expectation that individuals exhibiting lower baseline cortisol, i.e., presumably less stressed prior to the trial, would move first, we actually found a positive, albeit weak and non-significant, correlation between preCORT and Swimfirst ($r = 0.006$, $p = 0.98$). Higher PreCORT was significantly associated with an increased tendency to initiate the contest ($r = 0.45$, $p = 0.025$). Both PreCORT and PostCORT levels were negatively correlated with latency to swim (among fish that swam first) and the relationship was significant in both cases ($r_{\text{PreCORT.LatSwim}} = -0.45$, $p = 0.024$, $r_{\text{PostCORT.LatSwim}} = -0.70$, $p < 0.001$). Negative correlations of similar magnitude were found between PreCORT and PostCORT and the latency to initiate a contest; however, only the PostCORT correlation was significant ($r_{\text{PreCORT.LatInit}} = -0.39$, $p = 0.10$, $r_{\text{PostCORT.LatInit}} = -0.47$, $p = 0.04$) (Table 1).

Model based hypothesis testing

Model 1 supported our hypothesis that individuals that swim first would also initiate contests more often ($p = 0.029$); however, contrary to our *a priori* expectation that contest initiators would have lower levels of pre-contest cortisol, higher PreCORT levels were in fact associated with contest initiators ($p = 0.036$, Table 2). These patterns are qualitatively consistent with the significant correlation structure among initiate, PreCORT and Swimfirst as reported above. The estimated effect of PreCORT on tendency to initiate was more convincing in the reduced model (3.03 ± 1.37 $\mu\text{g/dl}$) than in the full model (-7.34 ± 15.64

μg/dl). This could reflect the fact that the latter estimate of the PreCORT effect is conditioned on the putative dependence on body size (although neither LSA nor its interactions were statistically significant). Model 2 provided no evidence that contest winning is predicted by swimming first or by baseline physiological stress (i.e. PreCORT). These findings are counter to our second *a priori* hypothesis, but again consistent with the simple correlation analysis. Although we also tested for dependency of these effects on contest escalation and/or size effects, in fact no explanatory variables were retained in the reduced version of Model 2. Thus we were unable to predict contest outcome from size, behaviour, or baseline physiological stress. Finally, although stress response was lower in contest winners as we had predicted, the difference between losers and winners was not significant in the full model (-0.40 ± 0.46 μg/dl, $p = 0.90$) and therefore status was not retained in our reduced model (Model 3). However, based on a marginally non-significant interaction of Swimfirst and size (LSA) ($p = 0.071$, Table 2) both variables were retained in the reduced model. Under the full model for stress response, 5 (± 23) % of the observed variance not explained by fixed effects was explained by Trial. Under the reduced model, the corresponding estimate was 14 (± 19) % of the variance. The random effect of trial is not significant in either the full ($p = 0.83$) or the reduced ($p = 0.49$) models.

DISCUSSION

The primary goals of this study were to determine firstly whether the latency to recover behaviourally from an acutely stressful event commonly employed in behavioural experiments – lifting partitions - could explain variation in contest behaviour and outcome. Secondly, we wanted to test whether this latency was related to endocrine measures of physiological stress obtained from water-borne cortisol assays. Our first prediction was that

fish more rapidly resuming normal swimming behaviour following removal of a partition in a dyadic behavioural trial would tend to initiate and win contests. These relationships among behavioural traits were not supported by our data, suggesting that a proactive coping style is associated with readjusting to experimental protocol disturbances; however, it is not associated with initiating or winning contests. Although many studies on fish have found a strong positive association between initiating and winning contests (e.g., Jackson, 1991; Hsu et al., 2009), our data suggest that we should be careful in assuming this pattern will always hold.

Both the correlation analysis and the linear models, where potentially confounding effects of body size could be statistically accounted for (Scott & Ellis, 2007), revealed some associations between behavioural and endocrine traits. However, these associations were not consistent with our *a priori* predictions. For example, we predicted that behavioural recovery following a partition being lifted would be faster for fishes with lower baseline (pre-contest) cortisol levels; however, the reverse pattern was seen. While this effect was non-significant, pre-contest cortisol level was significantly and positively associated with tendency to initiate contests. Pre-contest cortisol level did not predict contest outcome, and there was no significant effect of status on stress response. Although SR was lower in winners as we predicted the effect size was small and non-significant.

Overall our results do not fit comfortably into the proactive-reactive framework that has been used to interpret suites of correlated traits as reported in mammalian, avian, and other fish systems (Koolhaas et al., 1999; Øverli et al., 2007; Carere et al., 2010). Some recent studies provide evidence consistent with this framework, testing the hypothesis that differences in behaviour are associated with differences in stress response (Øverli et al.,

2002; Øverli et al., 2005; Øverli et al., 2007). These studies found that those individuals more rapidly resuming normal behaviour in novel environments or following acute stress were socially more dominant and in addition, had lower baseline cortisol levels and stress-responsive cortisol levels than those taking longer to resume normal behaviour. Thus, individuals have been argued to lie along a continuum of coping styles ranging from proactive to reactive, respectively. It should be noted that these fish studies were carried out using lines of domestic rainbow trout (*Oncorhynchus mykiss*) specifically selected for divergent cortisol responses; however, more recent work focussing on variation within populations has reached similar conclusions in a range of wild and domestic fish species (see Conrad et al., 2011 for a comprehensive review).

The swordtails used for our study were captive bred and, although they had wild-type colours, have an unknown history of artificial selection under conditions of high resource availability with environmental stressors likely to differ substantially from those of wild fish. We certainly acknowledge that relaxed natural selection in captivity might result in increased phenotypic variance and/or behaviour-physiology correlations that are either unexpected or that would be maladaptive in the wild (e.g., Lee & Berejikian, 2008; Conrad & Sih, 2009). We also acknowledge that our sample size was relatively small, thus limiting statistical power, and that control experiments to examine physiological responses to barrier removal without a subsequent dyadic contest would be useful. Nevertheless, it is equally true that other studies conducted under both laboratory and field conditions have reported deviations from the expected trait correlation structure among proactive – reactive coping style extremes, suggesting that the categorization is too simplistic (Brelvi et al., 2008; Archard & Braithwaite, 2011; Vaz-Serrano et al., 2011; Archard et al., 2012).

Environmental context can dissolve or generate trait correlations (e.g, Bell & Sih, 2007), and even completely reverse relationships between behaviour and physiology (Ruiz-Gomez et al. 2008). These studies suggest considerable plasticity in trait associations and the involvement of multiple, perhaps independently operating mechanisms that shape associations between behaviour and endocrine state.

Evidence from studies of behaviour in male tree lizard morphs (Thaker et al., 2009) suggests that animals with elevated cortisol levels are more prepared for an immediate response to predators. Koolhaas et al., (1997) suggested that elevations of glucocorticoids at appropriate times can be adaptive, in that they prepare the animal for immediate environmental unpredictability. Speculatively, it is possible that in our study we have uncovered a similar finding: animals with already elevated cortisol levels recover more quickly from stressors and therefore behave, at least initially, in a proactive manner. Similarly, contest winners may simply be reacting more quickly on a physiological level both to the disturbance from the experimental protocol and the attack from the proactive opponent. If this were indeed the case then a higher overall stress response for the reactive individual would seem to be appropriate.

Variation in endocrine traits did not match all our *a priori* expectations. *Post hoc* analyses revealed significant variance among-individuals that may have important functional consequences. Specifically, a *post hoc* mixed model analysis showed that after conditioning on size (LSA) and sampling point (i.e., pre- or post-trial) $\log_{10}$ transformed cortisol levels were repeatable (interclass correlation of $0.26 (\pm 0.13)$, $\chi^2_{1DF} = 6.16$, $P = 0.013$). This highlights the fact that there is among-individual variation (and within individual consistency) in assayed cortisol levels, beyond that attributable to size variation). This

model also confirmed the expected increase in cortisol levels with LSA ($0.002 (\pm 0.0004)$, $F_{1,54DF} = 11.38$, $p = 0.002$), and also that average cortisol levels were higher post-trial (difference of $0.125 (\pm 0.046)$ on the $\log_{10}$ scale, $F_{1,55DF} = 7.52$, $p = 0.008$) consistent with a positive physiological reaction, i.e., stress response, to the contest and/or experimental protocol. However, there was variation in SR and indeed 18 of 56 fish actually had lower cortisol release rates (i.e., $SR < 0$) in response to barrier removal and social challenge.

Furthermore, neither the causes nor the consequences of this among-individual variance are known at present. Such differences could emerge if individuals experience size- and status-dependent shifts in gill permeability to steroid hormones (e.g., Scott et al., 2008), i.e., a change in stress responsive release rates reflects the ability of steroids to leak across the gills for water-borne hormone measurement. Alternatively, given the inherent lag between spikes in plasma and water-borne hormones, we could be observing the confluence of status- and size-dependent differences in within-contest cortisol production. Acute elevations of stress hormone have been associated with increased aggression during social interactions (e.g., Kruk et al., 2004; Earley et al., 2006). Although we do not know if the association between acute stress responses, aggression, and social dominance is size-dependent, it is possible that large winners mounted a stronger within-contest stress response than is detectable in the water-borne sample.

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Table 1.

Phenotypic trait correlation matrix. The full data set was used to estimate correlations between the morphology and physiology traits of body depth (BD), standard length (SL), sword length (SwL), lateral surface area (LSA), Pre- (PreCORT) and post-contest (PostCORT) cortisol levels, and stress response (SR). The randomly selected half data set was used to calculate correlations between traits with only one phenotypic observation per trial: Status, Swimfirst, Initiate and differences in lateral surface area between opponents in the same contest (LSAdiff). Correlations for the traits latency to swim (LatSwim) and latency to initiate (LatInit) are calculated using one observation per trial, conditional on swimming first or initiating the contest. Bold font denotes a significant correlation (2-tailed $p < 0.05$). Bold italic font denotes a marginally non-significant correlation (2-tailed $p < 0.1$).

	BD	SL	SwL	LSA	Pre CORT	Post CORT	SR	Status	Swim first	Initiate	LSA diff	Lat Swim	Lat Init
BD	-												
SL	0.949	-											
SwL	0.308	0.429	-										
LSA	0.987	0.984	0.391	-									
PreCORT	0.432	0.477	0.418	0.453	-								
PostCORT	0.422	0.434	0.296	0.425	0.639	-							
SR	-0.013	-0.050	-0.144	-0.033	-0.425	0.425	-						
Status	0.021	0.044	-0.200	0.037	-0.157	-0.191	-0.009	-					
Swimfirst	0.040	0.014	0.001	0.040	0.006	0.273	0.315	-0.116	-				
Initiate	0.016	0.059	0.172	0.042	0.449	0.370	-0.181	-0.131	0.559	-			
LSAdiff	0.104	0.164	-0.164	0.128	-0.181	-0.133	0.092	0.344	0.016	-0.202	-		
LatSwim	-0.163	-0.204	-0.382	-0.187	-0.450	-0.695	-0.086	0.157	*	-0.178	0.344	-	
LatInit	-0.228	-0.321	-0.464	-0.286	-0.392	-0.474	-0.035	0.359	*	*	0.412	0.642	-

*Correlation not available

604 **Table 2.** ANOVA table of fixed effects fitted in full and reduced linear models of Initiate, Status and Stress Response. Indicated are estimated effect sizes for explanatory
605 terms fitted (with SE in parentheses), and conditional F tests. Initiate and status are modelled as binary response variables while a normal error structure was fitted for stress
606 response. Where used as explanatory variables Swimfirst, Status and EscalF were fitted as two level factors with the estimated coefficients denoting the effect of factor level 1
607 (fish swam first, fish won the contest, contest was escalated) relative to factor level 0. Models of stress response also included a random effect of trial (see text for details).

Trait	Fixed Effect	FULL MODEL				REDUCED MODEL			
		Coefficient (SE)	DF	F	p	Coefficient (SE)	DF	F	p
Initiate	mu	-2.37 (8.28)	1,21	0.59	0.449	-1.52 (0.802)	1,25	0.04	0.838
	Swimfirst	9.35 (9.92)	1,21	3.71	0.068	2.48 (1.07)	1,25	5.35	0.029
	PreCORT	-7.38 (15.6)	1,21	4.61	0.044	3.03 (1.37)	1,25	4.90	0.036
	LSA	0.001 (0.019)	1,21	1.49	0.235				
	PreCORT.LSA	0.033 (0.036)	1,21	0.84	0.368				
	Swimfirst.LSA	-0.015 (0.023)	1,21	0.42	0.522				
	Swimfirst.PreCORT	-0.781 (6.50)	1,21	0.01	0.906				
Status	mu	-7.33 (9.19)	1,14	0.84	0.375	-0.080 (0.400)	1,24	0.04	0.843
	Swimfirst	4.27 (8.90)	1,14	0.08	0.778				
	PreCORT	-2.42 (7.21)	1,14	1.43	0.252				
	EscalF	2.32 (7.66)	1,14	0.06	0.804				
	LSA	0.018 (0.021)	1,14	0.86	0.371				
	Swimfirst.PreCORT	3.72 (3.94)	1,14	0.89	0.361				
	Swimfirst.EscalF	1.68 (2.07)	1,14	0.66	0.431				
	Swimfirst.LSA	-0.013 (0.021)	1,14	0.38	0.548				
	PreCORT.LSA	-0.007 (0.014)	1,14	0.25	0.625				
	PreCORT.EscalF	1.91 (3.18)	1,14	0.36	0.557				
	EscalF.LSA	-0.007 (0.018)	1,14	0.15	0.707				
Stress Response	mu	0.094 (0.455)	1,39	1.99	0.167	-0.220 (0.295)	1,26.2	0.62	0.438
	Swimfirst	0.824 (0.456)	1,19	0.13	0.720	0.758 (0.383)	1,26	0.42	0.525
	Status	-0.402 (0.456)	1,19	0.02	0.903				

EscalF	-0.467 (0.500)	1,20	0.42	0.522				
LSA	-0.002 (0.001)	1,20.1	0.02	0.885	0.001 (0.001)	1,26.2	0.05	0.824
Swimfirst.Status	0.038 (0.211)	1,20	0.03	0.858				
Swimfirst.EscalF	-0.081 (0.202)	1,19	0.16	0.694				
Swimfirst.LSA	-0.002 (0.001)	1,19.1	3.31	0.085	-0.002 (0.001)	1,26.1	3.56	0.071
Status.EscalF	-0.120 (0.202)	1,19	0.35	0.559				
Status.LSA	0.001 (0.001)	1,19.1	1.10	0.307				
EscalF.LSA	0.001 (0.001)	1,20.1	1.15	0.295				

609 **Supplemental material.**

610 **Table S1**

611 Detail of morphological and physiological measurements for individual fish, where: Trial is the competition that
 612 the individual participated in; Fish is the identity assumed in the particular trial; SL is Standard Length; BD is
 613 Body Depth; SwL is sword length; LSA is Lateral Surface Area; Lat Swim is latency to swim; Lat Init is latency to
 614 initiate; Status is W, win and L, lose; PreCORT is pre-contest cortisol level; PostCORT is Post-contest cortisol
 615 level; SR is stress response; Escal denotes fight escalation (Y) per trial.

Trial	Fish	SL (mm)	BD (mm)	SwL (mm)	LSA (mm ²)	Lat Swim (secs)	Lat Init secs)	Status	Pre CORT (µg/dl)	Post CORT (µg/dl)	SR	Escal
1	A	46.50	14.00	23.40	674.40	10	63	L	3.800	1.223	-2.576	N
1	B	45.05	14.80	13.00	679.74	*	*	W	0.809	2.962	2.153	-
2	A	43.60	13.20	17.30	592.82	*	*	W	0.895	5.065	4.171	Y
2	B	43.80	13.30	13.40	595.94	67	98	L	2.258	3.807	1.549	-
4	A	40.10	11.90	12.70	489.89	7	11	L	3.049	2.630	-0.419	Y
4	B	39.80	12.20	11.90	497.46	*	*	W	1.044	1.440	0.396	-
5	A	35.05	11.00	14.20	399.75	3	*	L	1.337	4.554	3.218	Y
5	B	35.65	10.70	13.30	394.76	*	14	W	1.227	4.717	3.491	-
7	A	37.20	10.65	18.20	414.38	*	*	L	1.242	1.826	0.585	Y
7	B	37.20	10.75	17.75	417.65	103	109	W	3.350	1.695	-1.655	-
8	A	35.10	10.30	16.05	377.58	6	24	W	2.110	1.634	-0.476	Y
8	B	35.15	10.00	15.65	367.15	*	*	L	1.663	2.281	0.619	-
9	A	42.20	12.05	24.70	533.21	85	92	W	2.909	2.322	-0.587	Y
9	B	42.50	12.20	18.25	536.75	*	*	L	1.450	2.046	0.596	-
10	A	39.95	11.75	20.30	489.71	*	*	L	1.534	1.840	0.306	Y
10	B	40.35	12.10	18.50	506.74	51	196	W	1.315	2.394	1.078	-
11	A	48.80	13.90	19.00	697.32	242	*	W	0.889	1.369	0.480	N
11	B	46.60	14.15	20.55	679.94	*	254	L	3.292	4.259	0.967	-
12	A	32.60	9.25	9.30	310.85	127	184	W	0.159	0.805	0.646	Y
12	B	33.85	9.30	7.95	322.76	*	*	L	0.740	0.986	0.246	-
13	A	38.40	11.50	21.85	463.45	*	*	W	1.623	1.338	-0.285	Y
13	B	39.20	11.50	20.20	471.00	14	26	L	1.958	4.684	2.726	-
14	A	37.50	10.80	16.00	421.00	*	*	L	1.337	1.820	0.483	Y
14	B	37.40	10.90	17.60	425.26	240	465	W	1.514	0.673	-0.841	-
15	A	41.40	12.25	17.60	524.75	246	*	*	0.593	0.256	-0.337	N
15	B	40.10	12.35	17.50	512.74	*	304	*	1.060	0.321	-0.739	-
16	A	39.70	11.20	15.00	459.64	*	181	L	1.371	1.030	-0.340	N
16	B	38.70	11.30	15.10	452.41	101	*	W	0.380	0.889	0.508	-
17	A	34.85	10.00	12.05	360.55	66	1275	W	0.952	1.104	0.151	N
17	B	35.60	10.00	11.75	367.75	*	*	L	1.868	3.177	1.308	-
18	A	33.90	9.90	9.35	344.96	*	*	W	0.638	0.516	-0.122	Y
18	B	33.30	10.00	13.45	346.45	640	641	L	0.617	0.163	-0.454	-
19	A	39.85	10.90	20.05	454.42	*	1640	*	1.258	1.102	-0.156	Y
19	B	39.75	11.15	22.60	465.81	116	*	*	0.567	2.120	1.554	-
20	A	35.80	10.10	22.35	383.93	109	155	L	0.742	2.279	1.537	N
20	B	36.75	10.00	22.40	389.90	*	*	W	0.977	1.246	0.269	-
21	A	33.60	9.60	15.15	337.71	*	*	W	0.350	0.241	-0.108	N
21	B	33.30	9.25	16.90	324.93	27	30	L	0.205	0.892	0.687	-
22	A	35.60	10.30	14.50	381.18	278	*	L	0.102	0.327	0.224	N
22	B	34.60	10.40	13.70	373.54	*	354	W	1.132	1.151	0.019	-
23	A	41.90	12.30	11.00	526.37	*	*	W	0.578	0.596	0.018	Y

23	B	41.90	12.35	13.45	530.92	421	460	L	1.621	2.267	0.646	-
24	A	39.50	11.25	14.60	458.98	17	121	L	0.722	2.704	1.981	Y
24	B	40.05	10.95	16.75	455.30	*	*	W	1.993	2.179	0.186	-
25	A	33.40	9.60	18.00	338.64	61	96	L	2.876	2.271	-0.606	N
25	B	35.00	9.80	17.30	360.30	*	*	W	0.536	0.744	0.207	-
26	A	39.20	10.40	19.50	427.18	46	48	*	2.499	1.700	-0.799	N
26	B	37.65	10.50	16.30	411.63	*	*	*	1.686	1.814	0.128	-
27	A	34.15	10.05	10.80	354.01	142	*	L	0.120	0.642	0.522	N
27	B	34.85	10.00	10.50	359.00	*	189	W	0.429	0.329	-0.100	-
28	A	35.00	9.50	13.40	345.90	*	*	W	0.300	0.303	0.003	N
28	B	33.40	9.40	11.60	325.56	83	108	L	1.120	0.516	-0.604	-
29	A	34.40	10.12	11.30	359.43	540	*	W	0.069	0.358	0.290	N
29	B	34.42	9.80	18.85	356.17	*	586	L	0.468	2.099	1.630	-
30	A	32.85	9.26	17.50	321.69	*	*	L	0.179	0.094	-0.085	Y
30	B	33.15	10.00	9.30	340.80	554	3202	W	0.308	0.412	0.104	-