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

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

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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

40 (Brockelman, 1975). Many factors are expected to influence contest outcome and so
41 determine dominance status. While these are known to include size (e.g. Huntingford et al.,
42 1990) and behavioural traits such as aggression (Francis, 1988), individual styles of coping
43 with stress may also be important (Koolhaas et al., 1999; Pottinger & Carrick, 2001; Øverli et
44 al., 2004). Stress threatens homeostasis that is re-established by both physiological and
45 behavioural responses. Importantly, when studying behaviour, experimental protocols may
46 induce stress responses that impact individual behaviour, thus indirectly influencing
47 eventual contest outcome. Here we explore the hypothesis that latency to recover from
48 stress, as measured both behaviourally and physiologically, is a key determinant of contest
49 initiation and outcome. In animals, physiological stress-coping mechanisms are highly
50 conserved and governed by the hypothalamic-pituitary-adrenal (HPA) axis; in fish, this role is
51 assumed by the hypothalamic-pituitary-interrenal (HPI) axis, a good physiological indicator
52 being water-borne cortisol (for a review, see Scott & Ellis, 2007; Scott et al., 2008).

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

62 The relationship between physiological stress (HPA/HPI axis activity) and social
63 dominance has received increasing attention and has been well studied across many taxa,
64 including rodents (Bronson, 1973), primates (Abbott et al., 2003), birds (Verbeek et al.,
65 1996), mammals (Young et al., 2006), domestic livestock (Bergsma et al., 2008) and fish
66 (Øverli et al., 2007). However, causality is often ambiguous; it is unclear whether
67 physiological state is determined by outcome, or outcome is determined by physiological
68 state. For example, faster recovery of baseline cortisol levels following aggressive contests
69 is associated with dominance (Netherton et al., 2004), while individuals with higher baseline
70 cortisol levels are less likely to win contests or to obtain dominance status in a hierarchy
71 (Hannes, 1984; DiBattista et al., 2005). Other types of behavioural variation may be linked
72 to physiological stress, particularly an individual's coping style (Earley et al., 2006). In a
73 study focusing on both behavioural and neuroendocrinological parameters, Koolhaas et al.
74 (1999) contrasted proactive and reactive coping styles and suggested a proactive/boldness
75 link (boldness is here described as a willingness to explore novel environments, Budaev,
76 1997). Many studies have demonstrated correlations between boldness and aggression (for
77 example, Bell & Sih, 2007), and of specific interest is that in fishes, empirical measurements
78 of HPI activity, aggression and boldness have been associated with differences in coping
79 style (Schjolden et al., 2005; Aubin-Horth et al., 2012).

80 The majority of studies investigating contest behaviour and dominance in domestic
81 and wild fishes use experimental designs that require netting individuals to facilitate periods
82 of isolation. This is usually followed by some form of disturbance, such as the removal of
83 partitions between isolated contestants in novel environments (for example, Wilson et al.,
84 2011a). Could it be that contest winners are those that better cope with stressors imposed

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

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

102 **METHODS**

103 Green swordtails (*Xiphophorus helleri*) obtained from a commercial distributor were
104 housed in heterosexual groups in 152 and 208 L aquaria equipped with gravel substrate
105 (3cm), filtration, and aeration. Water temperature was maintained between 23 - 25° C, pH
106 between 7.2-7.6, and fish were kept on a 12 h light: 12 h dark photoperiod. Stress Coat™
107 (94µl/L) and freshwater aquarium salt (2g/L) were added to the tanks prior to fish arrival to

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

110 *Dyad Establishment*

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

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

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

125 *Contests and Hormone Collection*

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

130 measurement. The holding beakers were outfitted with a fine mesh net bottom and placed
131 inside another 1000 ml polypropylene beaker; this design made it possible to transfer the
132 fish between beakers gently, quickly (< 5 seconds) and without the handling typically
133 associated with capture (e.g., chasing, netting). The fish remained in the holding beaker for
134 2d to acclimate before being transferred to new 1000 ml sampling beakers containing 1000
135 ml of freshwater (with 4g freshwater salt) for 2 h, with hormones were released into the
136 water during this time (Scott et al., 2008). Stress Coat™ was not added to the hormone
137 collection beaker because it is not known whether the chemical interferes with hormone
138 extraction and assay; freshwater salt, however, can be purged from hormone extraction
139 columns (see below). After 2 h in the pre-fight sampling beaker the fish were transferred
140 using a net to 38 L experimental fight tanks, separated into two equal compartments by an
141 opaque divider. Each compartment was equipped with an aeration device and the water
142 was treated with Stress Coat™ and freshwater aquarium salt. The two fighters were placed
143 on opposite sides of the same fight tank and acclimated for 22 h. After this time the
144 dividers were lifted (remotely) and the air stones were also removed. This physical
145 disturbance typically resulted in frantic swimming behaviour by both fish, characterized by
146 fast, erratic movements both horizontally and vertically before the fish settled to the gravel
147 bottom. We therefore consider it to be a response imposed by the experimental protocol
148 itself. We determined the latency of behavioural recovery from this event as the time (from
149 lifting of partition) to resume normal swimming, defined as swimming slowly in a horizontal
150 orientation with fins often erect or semi-erect.

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

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

167 *Hormone Extraction and Radioimmunoassay*

168 C18 SPE columns (Extract-Clean[®], 500 mg, 4.0 ml; Alltech Associates, Inc.) were
169 primed with 2 x 2 ml of 100% ethanol (EtOH) and 2 x 2 ml distilled water. Tygon tubing
170 (formulation 2275) was attached to the C18 columns and placed in a beaker containing a
171 250 ml water sample taken from the original 1000 ml, the vacuum was engaged and water-
172 borne steroid hormones isolated. Total hormone (free and conjugated fractions) was eluted
173 from the columns with 2 x 2 ml 100% ethanol collected in 6 ml (12 x 75 mm) borosilicate
174 vials. Samples were stored at 4° C overnight and the ethanol was evaporated in a Savant
175 AES 1010 speedvac for 1.5 h (45 min at 40°C) one day prior to radioimmunoassay. Hormone

176 residues were resuspended in 60 μ l 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 μ l 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 μ g/dl, 0.033 μ g/dl, and 0.0624 μ g/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 120 μ l
194 and combined to form a pool of 2.4 ml stored as 55 μ l aliquots at -80 °C. 240 μ l of the
195 pooled control was used for serial dilutions. Briefly, 120 μ l of this sample was transferred to
196 a 1.5 ml Eppendorf tube and mixed by vortexing with 120 μ l of 0.1 M phosphate buffer to
197 create a 1:2 dilution; 120 μ l 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

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

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

216 *Data analysis*

217 In order to summarize associations among the full set of morphological, behavioural,
218 and endocrine traits measured we generated a correlation matrix using Genstat 14.1 (Payne
219 et al., 2005). Correlations between morphological and physiological traits were estimated
220 using the full set of observations (i.e. one record per individual, n=56) for body depth (BD),
221 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:

$$253 \quad \textit{Initiate}_{ik} = \mu + \textit{Swimfirst} + \textit{PreCORT} + \textit{LSA} + \textit{Swimfirst.PreCORT} + \textit{Swimfirst.LSA} + \\ 254 \quad \textit{PreCORT.LSA} + \varepsilon_k$$

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).

$$263 \quad \textit{Status}_{ik} = \mu + \textit{Swimfirst} + \textit{PreCORT} + \textit{Escalation} + \textit{LSA} + \textit{Swimfirst.PreCORT} + \textit{Swimfirst.LSA} + \\ 264 \quad \textit{PreCORT.Escalation} + \textit{PreCORT.LSA} + \textit{Escalation.LSA} + \varepsilon_k$$

265 (Model 2)

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

$$272 \quad SR_{ik} = \mu + Swimfirst + Status + Escalation + LSA + Swimfirst.LSA + Swimfirst.Status + \\ 273 \quad Swimfirst.Escalation + Status.LSA + Status.Escalation + Escalation.LSA + Trial_k + \varepsilon_k$$

274 (Model 3)

275 For each of the models shown above we adopted a model reduction strategy where
276 explanatory terms were dropped if they were statistically non-significant at $p \geq 0.1$ under a
277 two-tailed conditional *F* - test. Main effects were retained in the model if one or more of
278 their interactions were retained on this basis. Note that we chose to use a threshold of
279 $\alpha=0.1$ rather than 0.05 in our model reduction strategy and therefore our final models can
280 contain marginally non-significant explanatory terms (i.e. $0.1 \leq p \leq 0.05$). We adopted this
281 strategy as, since available sample sizes are fairly small we expect power will be limiting.
282 However, we deem that it is instructive to consider whether marginally non-significant
283 terms are at least qualitatively consistent with hypothesized biological processes, i.e. it may
284 not be sensible to equate non-significance with an effect size of zero.

285 **RESULTS**

286 *Among trait correlations*

287 The estimated correlation structure provided evidence of significant associations
288 among a number of the traits measured (Table 1). Phenotypic correlations were close to
289 unity among the morphological traits of BD, SL and LSA ($r_{BD.SL} = 0.95$, $r_{BD.LSA} = 0.99$, $r_{SL.LSA} =$
290 0.98 ; all $p < 0.001$), an unsurprising result given that these all capture aspects of body size.
291 Sword length (SwL) was also positively correlated with body size traits although less
292 strongly. Body size traits were significantly and positively correlated with both pre- and
293 post-contest cortisol levels (r ranging from 0.42 - 0.48, all $p \leq 0.001$; Table 1) although again
294 the correlation between PostCORT and sword length (SwL) was lower ($r = 0.30$, $p = 0.03$).
295 Given that endocrine traits are not standardised for size variation prior to analysis these
296 results are consistent with the expectation of a positive association between body size and
297 cortisol release into the water (Scott et al., 2008), controlled for in our model based
298 hypothesis testing (as discussed above). Note that stress response (SR) is auto-correlated
299 with pre- and post-contest cortisol levels as a consequence of its definition (i.e. SR =
300 PreCORT – PostCORT; $r_{SR.PreCORT} = -0.43$, and $r_{SR.PostCORT} = 0.43$, both $p = 0.001$). Cortisol levels
301 before and after the contest were also significantly correlated within individuals ($r_{PreCORT,$
302 $PostCORT} = 0.64$, $p = < 0.001$). However, correlations between SR and size (as measured by the
303 various morphology traits) are weak and non-significant.

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

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

314 The correlation structure provided only limited statistical support for relationships between
315 behavioural and endocrine traits. Contrary to our expectation that individuals exhibiting
316 lower baseline cortisol, i.e., presumably less stressed prior to the trial, would move first, we
317 actually found a positive, albeit weak and non-significant, correlation between preCORT and
318 Swimfirst ($r = 0.006, p = 0.98$). Higher PreCORT was significantly associated with an
319 increased tendency to initiate the contest ($r = 0.45, p = 0.025$). Both PreCORT and PostCORT
320 levels were negatively correlated with latency to swim (among fish that swam first) and the
321 relationship was significant in both cases ($r_{PreCORT.LatSwim} = -0.45, p = 0.024, r_{PostCORT.LatSwim} = -$
322 $0.70, p < 0.001$). Negative correlations of similar magnitude were found between PreCORT
323 and PostCORT and the latency to initiate a contest; however, only the PostCORT correlation
324 was significant ($r_{PreCORT.LatInit} = -0.39, p = 0.10, r_{PostCORT.LatInit} = -0.47, p = 0.04$) (Table 1).

325 *Model based hypothesis testing*

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

333 $\mu\text{g/dl}$). This could reflect the fact that the latter estimate of the PreCORT effect is
334 conditioned on the putative dependence on body size (although neither LSA nor its
335 interactions were statistically significant). Model 2 provided no evidence that contest
336 winning is predicted by swimming first or by baseline physiological stress (i.e. PreCORT).
337 These findings are counter to our second *a priori* hypothesis, but again consistent with the
338 simple correlation analysis. Although we also tested for dependency of these effects on
339 contest escalation and/or size effects, in fact no explanatory variables were retained in the
340 reduced version of Model 2. Thus we were unable to predict contest outcome from size,
341 behaviour, or baseline physiological stress. Finally, although stress response was lower in
342 contest winners as we had predicted, the difference between losers and winners was not
343 significant in the full model ($-0.40 \pm 0.46 \mu\text{g/dl}$, $p = 0.90$) and therefore status was not
344 retained in our reduced model (Model 3). However, based on a marginally non-significant
345 interaction of Swimfirst and size (LSA) ($p = 0.071$, Table 2) both variables were retained in
346 the reduced model. Under the full model for stress response, $5 (\pm 23)$ % of the observed
347 variance not explained by fixed effects was explained by Trial. Under the reduced model,
348 the corresponding estimate was $14 (\pm 19)$ % of the variance. The random effect of trial is
349 not significant in either the full ($p = 0.83$) or the reduced ($p = 0.49$) models.

350 **DISCUSSION**

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

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

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

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

379 2002; Øverli et al., 2005; Øverli et al., 2007). These studies found that those individuals
380 more rapidly resuming normal behaviour in novel environments or following acute stress
381 were socially more dominant and in addition, had lower baseline cortisol levels and stress -
382 responsive cortisol levels than those taking longer to resume normal behaviour. Thus,
383 individuals have been argued to lie along a continuum of coping styles ranging from
384 proactive to reactive, respectively. It should be noted that these fish studies were carried
385 out using lines of domestic rainbow trout (*Oncorhynchus mykiss*) specifically selected for
386 divergent cortisol responses; however, more recent work focussing on variation within
387 populations has reached similar conclusions in a range of wild and domestic fish species (see
388 Conrad et al., 2011 for a comprehensive review).

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

402 Environmental context can dissolve or generate trait correlations (e.g, Bell & Sih, 2007), and
403 even completely reverse relationships between behaviour and physiology (Ruiz-Gomez et al.
404 2008). These studies suggest considerable plasticity in trait associations and the
405 involvement of multiple, perhaps independently operating mechanisms that shape
406 associations between behaviour and endocrine state.

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

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

425 model also confirmed the expected increase in cortisol levels with LSA ($0.002 (\pm 0.0004)$,
426 $F_{1,54DF} = 11.38, p = 0.002$), and also that average cortisol levels were higher post-trial
427 (difference of $0.125 (\pm 0.046)$ on the \log_{10} scale, $F_{1,55DF} = 7.52, p = 0.008$) consistent with a
428 positive physiological reaction, i.e., stress response, to the contest and/or experimental
429 protocol. However, there was variation in SR and indeed 18 of 56 fish actually had lower
430 cortisol release rates (i.e., $SR < 0$) in response to barrier removal and social challenge.

431 Furthermore, neither the causes nor the consequences of this among-individual
432 variance are known at present. Such differences could emerge if individuals experience
433 size- and status-dependent shifts in gill permeability to steroid hormones (e.g., Scott et al.,
434 2008), i.e., a change in stress responsive release rates reflects the ability of steroids to leak
435 across the gills for water-borne hormone measurement. Alternatively, given the inherent
436 lag between spikes in plasma and water-borne hormones, we could be observing the
437 confluence of status- and size-dependent differences in within-contest cortisol production.
438 Acute elevations of stress hormone have been associated with increased aggression during
439 social interactions (e.g., Kruk et al., 2004; Earley et al., 2006). Although we do not know if
440 the association between acute stress responses, aggression, and social dominance is size-
441 dependent, it is possible that large winners mounted a stronger within-contest stress
442 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	-

602 *Correlation not available

603

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	-