

Social interactions tune aggression and stress responsiveness in a territorial cichlid fish (*Archocentrus nigrofasciatus*)

Ryan L. Earley^{a,c,*}, Jonathan T. Edwards^a, Obaidullah Aseem^b, Kathryn Felton^a,
Lawrence S. Blumer^{b,c}, Mary Karom^a, Matthew S. Grober^{a,c}

^a Department of Biology, Georgia State University, 24 Peachtree Center Ave. NE, Atlanta, GA 10 30303, USA

^b Department of Biology, Morehouse College, 830 Westview Drive, Atlanta, GA 30314, USA

^c Center for Behavioral Neuroscience, USA

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Abstract

We examined the relative influences of pre-fight housing condition, contest intensity, and contest outcome in modulating post-fight stress hormone concentrations in territorial male convict cichlids (*Archocentrus nigrofasciatus*). Individuals were housed either in isolation or in semi-natural communal tanks. Pairs of male cichlids that differed considerably in body mass were selected from the same housing regime. Pre-fight water-borne cortisol levels were obtained before allowing the dyad to interact until contest resolution, after which time post-fight cortisol levels were obtained from the winner and loser. There were no outcome-related differences in post-fight cortisol concentrations following escalated or non-escalated contests, a result that held true for both housing regimes. Pre-fight cortisol levels were significantly higher than post-fight cortisol levels, suggesting that initial confinement in a beaker for the water-borne hormone samples was a stressor, but that the animals acclimated quickly to confinement. Fights involving previously isolated participants were significantly more intense than those involving group-housed animals, which we explain as being a function of established relationships between social isolation, heightened acute cortisol responsiveness, and the expression of excessive aggressive behavior. Only group-housed losers demonstrated the ability to modulate aggression or hypothalamic–pituitary–interrenal (HPI) activity in a graded fashion to acute increases in cortisol or changes in contest intensity, respectively. We discuss a variety of factors that could disrupt the ability of isolates to appropriately modulate interactions between social behavior and the HPI axis, and we examine a number of functional hypotheses underlying the sensitivity of group-housed losers to changes in contest dynamics. Crown Copyright © 2006 Published by Elsevier Inc. All rights reserved.

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1. Introduction

Agonistic encounters constitute a potent, socially relevant stressor [1,2] as indicated by manifold changes in physiological and neuroendocrine processes that accompany social interaction [3–6]. Stress associated with social subordination has attracted particular attention. Ample evidence in many animal systems indicates that chronic losers, or individuals that occupy low ranks in a dominance hierarchy exhibit elevated corticosteroid levels relative to dominant animals [7–9]. Several recent field-

based and comparative studies, however, have cast doubt on the rigid association between social status and peripheral neuroendocrine stress axis activity in social groups and have shown, for instance, that corticosteroid profiles are either unrelated to social status or that corticosteroid levels map onto social status with varying success depending on the characteristics of the social system [6,10–12].

Many territorial species engage in pairwise aggressive interactions during the breeding season, the outcomes of which do not necessarily contribute to an overarching social structure (e.g., well established hierarchy) but rather facilitate territory acquisition. Social stability, opportunity for social support, and degree of reproductive skew, factors that appear to explain variability in corticosteroid levels in strictly group-living animals [10,11,13], may not contribute to differences in

* Corresponding author. Department of Biology, Georgia State University, 24 Peachtree Center Ave. NE, Atlanta, GA 10 30303, USA. Tel.: +1 404 651 4347 or 404 463 9578; fax: +1 404 651 3929.

E-mail address: biorle@langate.gsu.edu (R.L. Earley).

corticosteroid concentrations between winners and losers of species that do not establish persistent hierarchies. Yet substantial variation in post-fight corticosteroid concentrations still exists among and between winners and losers of these species [14–16].

One potential source of variation in corticosteroid concentrations that has received less attention is the dynamics of the social milieu or, in the case of pairwise interactions, the intensity of a single interaction [9,17–19]. What many of these studies suggest is that the hypothalamic–pituitary–adrenal axis (or HP–interrenal axis in fishes) may respond more on a continuous scale to the finer characteristics of aggressive interactions (e.g., number of aggressive acts received) than in a binary or categorical fashion to states of dominance or subordination, winner or loser.

Remarkably few studies in fishes have measured cortisol release in winners and losers immediately following contest settlement [14,20], and only a modest number of studies have explored this issue in rodents [21–23] and reptiles [15,16,24,25]. Many studies allow the competitors to cohabitate for extended periods (from hours to weeks) following fight resolution, which is important for exploring the time course of neuroendocrine modifications that occur from introduction of the opponents through stabilization of a dominance relationship and beyond [1]. This type of paradigm, however, does not allow one to identify how animals respond in the short-term to a single fighting experience. Corticosteroid concentrations following extended cohabitation could thus represent a summation of the psychosocial or metabolic effects of the initial fighting experience (winner versus loser) coupled with physical or social stressors associated with continued cohabitation, e.g., performance of additional aggressive acts to reinforce status, persistent threat by the dominant, and sustained injury [26]. Furthermore, studies seldom attempt to correlate variation in corticosteroid concentrations with the dynamics of the aggressive dispute [16,24,27], an important indicator of the intensity of the social stressor [9,17,20].

Superimposed on dominance outcome and contest intensity are a number of additional factors that can affect stress hormone profiles going into a contest, cortisol responses to agonistic encounters, and aggressive behavior displayed during a fight. These factors include heritable or environmentally induced individual differences in stress responsiveness [28,29], experience-dependent or baseline differences in corticosteroid levels between contesting animals [30], and housing regime (e.g., extended social isolation tends to enhance aggressive behavior; see [31] for a review in fishes, and [3] for neuroendocrine perspectives).

The primary objective of our study was to determine whether differences in cortisol profiles exist between winners and losers of contests involving territorial male convict cichlid fish (*Archocentrus nigrofasciatus*) that had been housed either in isolation or in communal tanks prior to the interaction. Convict cichlids are native to Central America and form monogamous pair bonds during a discrete breeding season [32,33]. The male and female of a pair participate in the establishment and defense of a territory, but males typically assume a more active role in

defense against conspecific intruders [34]. During territorial disputes between monogamous pairs, fights normally occur between individuals of the same sex (M. Draud, personal communication). As such, we focused on intrasexual fights between males, the dynamics of which have been well characterized in dyadic contests [35–39]. Importantly, neuroendocrine changes that accompany fighting experience remain largely unexplored in this species despite its history as a model system for understanding territorial aggression. We explored the relative roles of contest outcome and contest dynamics in mediating neuroendocrine stress axis activity, with particular emphasis on differences between winners and losers in cortisol responsiveness to natural variation in contest intensity. We also capitalized on the different housing regimes (group versus isolated) employed in this study, and on pre-fight hormone sampling to determine the effects of housing on both stress responsiveness and aggression levels during a contest.

2. Materials and methods

2.1. Study organism and maintenance

Convict cichlids were obtained commercially from Sun Pet Ltd. in Atlanta, Georgia (USA). Individuals were housed either communally in three, 590-l holding tanks ($N=40$ fish used for 20 pairings) or individually isolated in 19-l aquariums bisected into two compartments of equal size ($N=60$ fish used for 30 pairings). Individuals from the same holding tank never were paired. All holding tanks had a shallow gravel substrate, large cylindrical tubing or broken terra cotta pots as refuge, and biological and chemical filtration. The water temperature in the holding and experimental tanks was maintained at 27 °C and the photoperiod at 12-h light/12-h dark. Stress Coat® (Aquarium Pharmaceuticals, Inc.) was added to the holding tanks following water changes or fish measurement to reduce osmotic stress and replenish the slime coating. The fish were fed flake food, cichlid pellets, or frozen brine shrimp (*Artemia*) twice daily. Prior to experimentation and housing, isolated individuals were anesthetized slightly with tricaine methanesulfonate, and sexed by observation of the genital papilla with methylene blue (males have a pointed genital papilla while females have an ovate papilla) and presence of ventral coloration patterns [40]. While anesthetized, the fish also were measured (total body length, standard length, and body depth, cm), and weighed (wet mass, g). Total body length was measured from the tip of the snout to the end of the caudal fin; standard length was measured from the tip of the snout to the end of the caudal peduncle; body depth was measured from the anterior end of the dorsal fin to the origin of the genital papilla. Verification of sex by gonadal analysis was conducted after the trials finished.

2.2. Experimental protocol

Forty-eight pairs of size-mismatched males were established; only male–male pairings were used for statistical analyses in this study ($N=2$ group-housed pairings contained one female). Body mass asymmetries were, on average, $32.2 \pm 1.85\%$ to

increase the likelihood of unambiguous contest settlement in favor of the larger animal (mean±S.E.; large, mass=5.25±0.25 g, standard length=52.06±0.88 mm, body depth=22.57±0.38 mm; small, mass=3.56±0.19 g, standard length=46.00±0.86 mm, body depth=19.64±0.36 mm). Body size asymmetries were distributed homogeneously between the two housing regimes (group housed and isolated; Levene's test: $F_{1,45} < 0.80$, $P > 0.38$).

Group-housed fish were netted from the communal tank ($N=18$ pairs), sampled for pre-fight cortisol levels (see below), and isolated behind opaque partitions on opposite ends of a 38-l tank with filtration for 24 h prior to fighting to allow for acclimation to the experimental arena and to minimize the effects of previous social experience in the holding tanks on subsequent behaviour or physiological measurements. Individually housed cichlids ($N=30$ pairs) were netted from their home tank, sampled for pre-fight cortisol levels (see below), and placed behind opaque plastic partitions on opposite ends of experimental tank arranged identically as described previously for a 24-h acclimation period. All experimental tanks were equipped with a terra cotta shelter, a gravel substrate at 6 cm depth, and an external basket filtration system. The position of small and large contestants in the aquarium was reversed on a trial-by-trial basis to control for any tank effects.

Following acclimation, the opaque partitions were lifted and the small and large fish were allowed to interact until a dominance relationship was established, which we defined as the point at which the winner directed aggression toward the loser 10 consecutive times without reciprocation [41] or when the winner secured exclusive access to the refuge (mean contest duration±S.E.: 12.08±1.64 min; range: 0.42–41.77 min). If a clear dominance relationship was not established within 1 h, the contest was terminated ($N=1$). Rates of total aggression (lateral and head-on threat display, approach, attack, bite) and submission (avoid, retreat) were recorded for each contestant (see [42] for detailed descriptions of agonistic behaviour in cichlid fishes). The total number of reciprocal interactions, instances where an aggressive act by one individual is countered by an aggressive act by its opponent, also was recorded. Low intensity (LI) reciprocal interactions refer to mutual threat displays or approach-threat display sequences and high intensity (HI) reciprocal interactions refer to attack-bite sequences or circle biting (i.e., 'carouselling'; [43]). The total time spent mouthwrestling (seconds; MW) was documented and contest duration (minutes; DUR) was measured from the commencement of aggression to fight settlement. All interactions were recorded on video for data collection. Methods and animal care and maintenance procedures were approved by the Center for Laboratory Animal Research at the Morehouse School of Medicine (IACUC Protocol No. 02-13) and by Georgia State University (IACUC Protocol No. A02017).

2.3. Hormone collection and radioimmunoassay

One day before, and immediately following the establishment of a dominance relationship, individuals of a pair were transferred by netting to separate 400-ml beakers filled with

200 ml of clean water at a temperature of 27 °C for hormone collection; hormone collection water was the same used to fill the experimental and holding tanks. Pre-fight hormones were not obtained for individuals in two of the group-housed contests. The order in which winners and losers were removed from the experimental tank for post-fight hormone collection was randomized across trials. Subjects remained in the hormone collection beakers for 2 h. Transfers occurred between 0829 and 1038 h. The water samples were filtered (Whatman filters) to remove particulate matter, and either stored as four 50-ml aliquots at -20 °C or extracted immediately following collection; freeze storage of water samples has been determined not to affect cortisol concentrations [44]. Cortisol was extracted from the water samples using C18 solid phase extraction (SPE) columns (Extract-Clean®, 500 mg, 4.0 ml; Alltech Associates, Inc.) fitted to a 12-port vacuum manifold. Columns were primed using two consecutive washes with 2 ml 100% EtOH followed by two consecutive washes with 2 ml ddH₂O. The 200 ml water samples were then pushed through the columns using the vacuum manifold. Hormone was eluted from the columns into 12×75 mm borosilicate vials by two consecutive 2 ml washes with 100% EtOH. The 4 ml of eluted solvent was evaporated in a vacuum centrifuge (Savant AES 1010 Speedvac), leaving a pellet of hormone. The pellet was resuspended in 60 µl of 0.1 M phosphate buffer and stored at 4 °C overnight.

Ellis et al. [44] recently conducted a thorough validation of this non-invasive hormone collection technique in rainbow trout. Two aspects of their validation are of particular relevance to our study. First, they showed concomitant increases in water-borne and plasma cortisol concentrations following exposure to an acute stressor, indicating that hormones collected from water are reflective of plasma hormone concentrations. Indeed, the authors showed highly significant correlations between plasma and water-borne cortisol concentrations at 2 h following the acute stressor. Second, Ellis et al. [44] showed pronounced increases in water-borne cortisol concentrations as soon as 30 min after the acute stressor, indicating little delay between increases in plasma cortisol concentrations and their detection in water samples. It should be noted also that Ellis et al. [44] pumped water directly from large holding tanks housing multiple individuals, whereas our procedure isolates hormones from one animal in a relatively small volume. Preliminary analyses in the convict cichlids demonstrated that 2 h of continuous hormone collection per individual was necessary to obtain cortisol concentrations consistently above the detection limit of the manufacturer's kit (see below). The size of the beaker (400 ml) and the 200 ml volume of collection water were chosen because this approximates the size/volume of the shelters provided to the fish in the housing tanks; male convict cichlids regularly occupy these shelters unless disturbed or feeding.

Cortisol radioimmunoassay was conducted using a coat-a-count kit purchased through Diagnostic Products Corporation (Los Angeles, CA). Pre- and post-fight samples were run in duplicate in three separate assays ($N=2$ pairs in first assay; $N=14$ pairs in second assay; $N=32$ pairs in third assay). Briefly, 25 µl of each sample was pipetted into antibody-coated

polypropylene tubes followed by the addition of 1 ml of 125 -labeled cortisol. The samples were incubated in a 37 °C water bath for 45 min. The liquid in all samples was decanted, and the tubes were blotted and allowed to air dry for 30 min prior to quantification. The sensitivities of the three assays were 0.028 µg/dl, 0.072 µg/dl, and 0.055 µg/dl. The intra-assay coefficient of variation for the first assay was 2.46% (pooled hamster serum). Pooled cichlid water extract, and low- and high-level human serum (CON6 Multivalent Control Module, Diagnostic Products Corporation) were used as intra-assay controls in the second and third assays. The intra-assay coefficients of variation for pooled cichlid extract, low-level and high-level human serum in the second/third assays were 1.79/3.94%, 3.30/2.78%, and 4.99/5.83%, respectively. The inter-assay coefficient of variation was 11.9% for tri-level human serum low, 10.5% for tri-level human serum high, and 15.9% for pooled cichlid extract.

The kit was validated for *A. nigrofasciatus* by assessing parallelism and by calculating expected versus observed cortisol concentrations from known samples spiked with standards (e.g., recovery). Water-borne hormones were obtained from 32 non-experimental fish using a method similar to that described above, extracted, dried, resuspended in 90 µl of 0.1 M phosphate buffer, and combined to form a 2.88-ml pool. Serial dilutions from 1:1 to 1:32 were conducted on a subset of the pooled sample; the curve generated from the linear dilutions was parallel to the standard curve (comparison of slopes: $t_6=0.33$, $P=0.75$; [45]). Recovery was assessed by spiking 110 µl of the pooled sample with an equal volume of the 1, 5, 10, 20, and 50 µg/dl standards supplied with the radioimmunoassay kit. Expected recovery concentrations were based on the known amount of cortisol in the *A. nigrofasciatus* pooled sample (e.g., known concentration + 20 µg/dl divided by 2). Minimum observed recovery was 96.7%. The slope of the observed versus expected curve was 0.953, indicating a linear relationship between observed and expected recovery ($F=1981.35$, $p<0.0001$, $R^2=0.997$). All cortisol values are reported as ng/sample.

2.4. Statistical analyses

The mean (\pm S.E.) time of day to finish the 2 h hormone collection was 1102 h \pm 5.3 min (median: 1107 h, interquartile range: 1055–1129 h, min/max: 1029–1238 h). Most hormone collection times fell within a 1-h window. There was no significant effect of slight variation in sampling time on cortisol concentrations (ANCOVA: $F_{1, 90}=0.01$, $P=0.95$), and this relationship was not dependent on status (ANCOVA, outcome \times time: $F_{1, 90}=3.23$, $P=0.08$) or housing (ANCOVA, housing \times time: $F_{1, 90}=0.03$, $P=0.87$). There were no significant effects of post-fight handling time (transfer from experimental apparatus to hormone collection beaker by netting) on cortisol levels in 90 (of 96) individuals for which handling time (seconds) was recorded (ANCOVA: $F_{1, 86}=0.87$, $P=0.35$; mean \pm S.E.: 7.9 \pm 0.28 s; range: 3–22 s). The effect of handling also was not different for winners and losers (ANCOVA, outcome \times handling: $F_{1, 86}=1.33$, $p=0.25$)

or between housing regimes (ANCOVA, housing \times handling: $F_{1, 86}=0.04$, $p=0.85$).

Simple linear regression was used to assess whether size asymmetries affected contest dynamics. ANOVA was used to determine differences between housing regimes in contest dynamics; in these analyses, MW and DUR were natural-log transformed, LI was $y^{1/3}$ -transformed, and HI was $y^{1/6}$ -transformed to achieve normality (non-parametric Kruskal–Wallis tests yielded the same results). Simple linear regression also was used to examine relationships between pre- and post-fight cortisol levels, and various measures of contest intensity (time spent mouthwrestling (MW), number of low-intensity (LI), high intensity (HI) and total reciprocal interactions, and contest duration (DUR)). P -values from the regressions were adjusted with sequential Dunn–Sidak adjustments to control for Type I error rate ($k=6$ analyses). The effects of housing, status, and escalation (defined as contests in which mouthwrestling did or did not occur) on cortisol levels were evaluated with ANCOVA on natural-log transformed post-fight cortisol values. Natural-log transformed pre-fight cortisol levels and untransformed body mass were used as covariates, and did not interact significantly with the independent variables in explaining post-fight cortisol levels (pre-cortisol \times housing: $F_{1, 74}=0.41$, $P=0.52$; pre-cortisol \times status: $F_{1, 74}=1.87$, $P=0.18$; pre-cortisol \times escalation: $F_{1, 74}=2.86$, $P=0.10$; mass \times housing: $F_{1, 74}=4.09$, $P=0.05$; mass \times status: $F_{1, 74}=0.14$, $P=0.71$; mass \times escalation: $F_{1, 74}=0.23$, $P=0.64$). Linear contrasts were conducted on the three-way interaction term (housing \times status \times escalation), and P -values were subject to sequential Dunn–Sidak adjustments ($k=12$ comparisons). To account for non-independence of contest participants, the status effects for each housing regime were confirmed with paired t -tests. Degrees of freedom for the denominator of the F -value for some analyses are expressed as a range. All statistics were conducted on SAS v8.2 or JMP v5.01 (SAS Institute, Cary, NC). All trends described in the results remained the same when mass-specific cortisol (cortisol g^{-1}) was analyzed in lieu of absolute cortisol concentrations.

3. Results

3.1. General contest dynamics and outcome

Larger individuals won a significant overall proportion of contests that were settled (41 of 47; $\chi^2_1=15.24$, $P<0.0001$; Table 1); in one trial, the contestants rarely interacted and the fight was terminated after 1 h of observation. The probability that larger individuals would win was not affected by housing regime (large wins; group: 14 of 18, isolate: 27 of 29; $\chi^2_2=2.28$, $P=0.19$). Small and large individuals initiated aggression in an equal proportion of settled contests (24 versus 21 of 45; $\chi^2_1=0.4$, $P=0.53$), and this trend held true for both housing regimes (small initiates; group: 10 of 18, isolate: 11 of 27; $\chi^2_2=0.95$, $P=0.37$); in two contests, there was no clear initiator (i.e., contestants approach simultaneously).

All measures of contest intensity except LI and HI ($r_{46}=0.12$, $P=0.44$) were significantly positively correlated

Table 1
Measurement details for winners and losers, including the percent difference in body measurements between paired contestants

Identification	Measurement	Value	Range
Losers	Total length (mm)	57.96±1.30	44.5–81.6
	Standard length (mm)	46.71±0.94	36.3–63.1
	Body depth (mm)	19.98±0.42	15.2–28.1
	Body mass (g)	3.75±0.22	1.77–7.68
Winners	Total length (mm)	63.54±1.28	45.5–83.6
	Standard length (mm)	51.35±0.90	38.6–66.9
	Body depth (mm)	22.23±0.37	17.3–29.6
	Body mass (g)	5.06±0.25	2.19–11.4
Percent difference	Total length (%)	11.75±0.86	1.32–25.89%
	Standard length (%)	12.05±0.76	1.94–23.20%
	Body depth (%)	13.25±0.91	0.00–27.88%
	Body mass (%)	32.19±1.85	4.56–59.03%

All values represent means±S.E. (N=47 dominant–subordinate pairs).

(Pearson product moment; LI-MW: $r_{33}=0.31$, $P=0.07$; LI-DUR: $r_{46}=0.59$, $P<0.0001$; HI-MW: $r_{33}=0.48$, $P=0.004$; HI-DUR: $r_{46}=0.43$, $P=0.003$; MW-DUR: $r_{33}=0.58$, $P=0.0004$). Of the body size measures, asymmetries in body depth ($F_{1, 45}=4.13$, $P=0.048$, $R^2=0.08$) and body mass ($F=3.86$, $P=0.05$, $R^2=0.08$) predicted contest duration. Percent difference in body depth and mass were significantly negatively correlated with contest duration. No other relationship between body size asymmetries and contest dynamics were significant ($F<2.83$, $P>0.1$, $R^2<0.06$), and these relationships were not differentially affected by housing regime (ANCOVA; housing×body asymmetry: $F_{1, 29-44}<1.4$, $P>0.24$).

3.2. Pre-fight housing and contest dynamics

Contests involving previously isolated individuals escalated to mouthwrestling significantly more often than contests involving group-housed contestants (number escalated; group: 9 of 18;

isolated 24 of 29; $\chi_2=5.64$, $P=0.018$). In contests that escalated, however, there was no difference in mouthwrestling duration between housing regimes (isolated: 3.04 ± 1.03 min; group: 2.95 ± 1.68 min; $F_{1, 31}=0.015$, $P=0.91$). Previously isolated contestants engaged in significantly more high intensity reciprocal aggression than group-housed contestants (isolated: 28.64 ± 11.03 acts/contest; group: 2.67 ± 13.76 acts/contest; $F_{1, 44}=7.72$, $P=0.008$). There was no difference between housing regimes in contest duration (isolates: 11.40 ± 2.11 min; group: 13.17 ± 2.68 min; $F_{1, 45}=0.11$, $P=0.74$) or low intensity reciprocal aggression (isolates: $31.71\pm 318.6.15$ acts/contest; group: 29.39 ± 7.67 acts/contest; $F_{1, 44}=0.03$, $P=0.86$).

3.3. Pre-fight cortisol concentrations and contest dynamics

There was no relationship between pre-fight cortisol and contest initiation in fights between group housed (8 of 16 initiated by individual with higher pre-fight cortisol) or isolated (15 of 27 initiated by individual with higher pre-fight cortisol) animals. Isolated animals had significantly higher pre-fight cortisol levels than group-housed animals (mean±S.E. ng/sample; isolates: 28.38 ± 1.92 , group: 17.79 ± 1.91 ; $F_{1, 86}=15.73$, $P=0.0002$). Eventual winners and losers had similar pre-fight cortisol levels ($F_{1, 86}=0.42$, $P=0.52$) regardless of housing regime (housing×status: $F_{1, 86}=2.55$, $P=0.11$). In contests between group-housed animals, pre-fight cortisol levels of the eventual losers predicted the number of low-intensity reciprocal interactions ($F_{1, 14}=8.83$, $P=0.01$, $R^2=0.39$, $\beta=1.97$, $\alpha_{adj}=0.013$), total number of reciprocal interactions ($F_{1, 14}=11.28$, $P=0.005$, $R^2=0.45$, $\beta=2.54$, $\alpha_{adj}=0.009$), and mouthwrestling duration ($F_{1, 14}=9.42$, $P=0.008$, $R^2=0.40$, $\beta=0.25$, $\alpha_{adj}=0.01$) (Fig. 1). Pre-fight cortisol levels of group-housed winners, isolated winners, and isolated losers did not predict any aspect of contest dynamics (group, winners: $F_{1, 14}<0.69$, $P>0.42$, $R^2<0.05$; isolates: $F_{1, 26}$

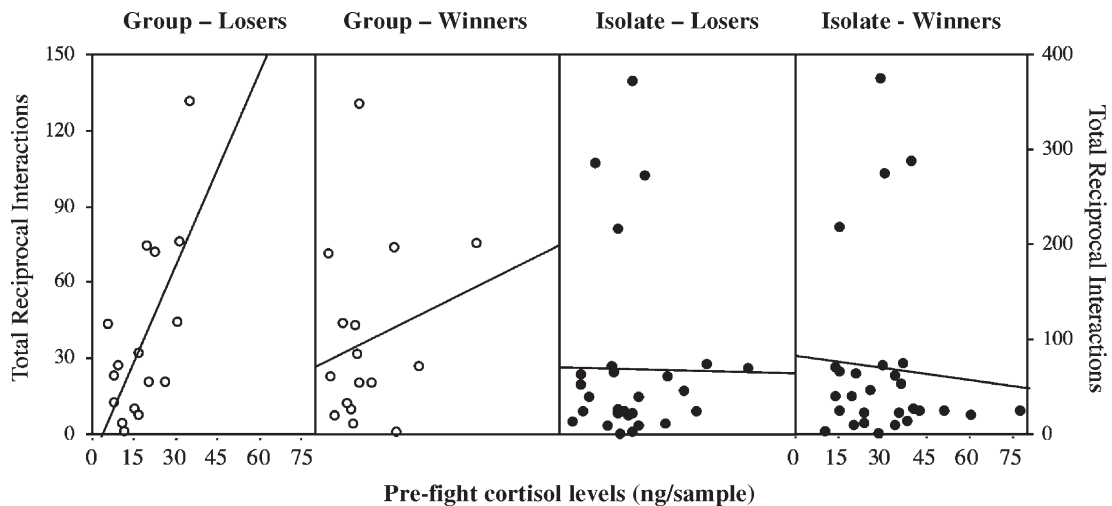


Fig. 1. Relationship between pre-fight cortisol levels and contest intensity (total number of reciprocal interactions) in eventual winners and losers from the group-housed (open circles) and isolated (dark circles) conditions; the same trends held true for the relationship between pre-fight cortisol levels and the number of low-intensity reciprocal interactions and mouthwrestling duration. Y-axis scales are the same for winners and losers within each housing regime, but are displayed for only one status class. Relationships for isolated winners ($F_{1,22}=0.68$, $P=0.42$) and losers ($F_{1,22}=1.83$, $P=0.19$) remained non-significant after removal of the largest four values for total reciprocal interactions, which were discontinuous from the rest of the distribution.

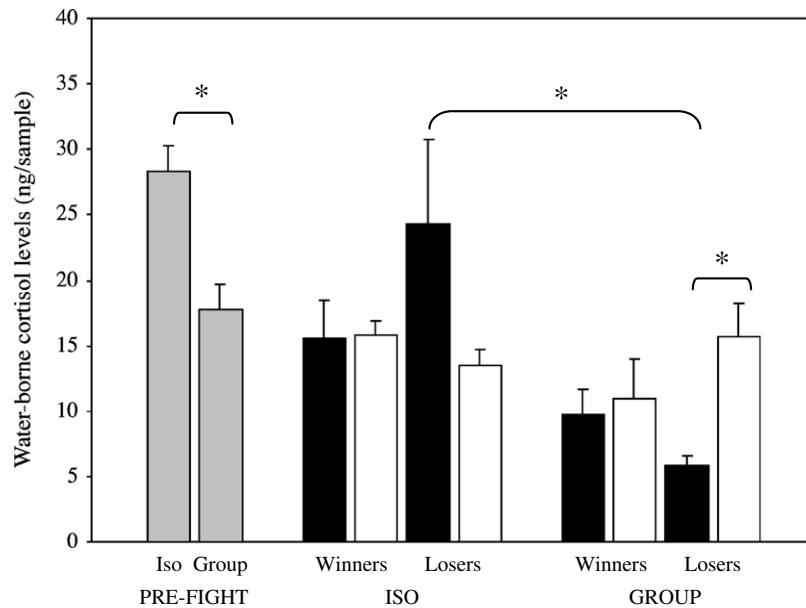


Fig. 2. Representation of the three-way housing \times status \times escalation interaction with black bars depicting non-escalated contests (without mouthwrestling) and white bars depicting escalated contests (with mouthwrestling), and pre-fight cortisol levels in group-housed and isolated animals (grey bars). Untransformed mean (\pm S.E.) pre-fight cortisol levels of isolated (iso; 28.38 ± 1.92) and group-housed (group; 17.79 ± 1.91) animals expressed in ng/sample. Untransformed mean (\pm S.E.) post-fight cortisol levels of winners and losers from both housing conditions involved in non-escalated (isolated—winners: 15.52 ± 3.01 , losers: 24.3 ± 6.46 , $N=5$; group-housed—winners: 9.74 ± 1.93 , losers: 5.82 ± 0.78 , $N=9$) and escalated contests (isolated—winners: 15.75 ± 1.10 , losers: 13.5 ± 1.20 , $N=24$; group-housed—winners: 10.97 ± 3.03 , losers: 15.69 ± 2.49 , $N=9$). Pairs of histogram bars denoted with asterisks were significantly different from one another ($P < 0.05$).

< 2.12 , $P > 0.16$, $R^2 < 0.08$; Fig. 1). There was a significant positive relationship between pre- and post-fight cortisol levels (ANCOVA; $F_{1, 78} = 4.13$, $P = 0.045$), and this relationship was independent of housing regime and post-fight status (see Statistical analyses).

3.4. Housing and post-fight cortisol concentrations

Pre-fight cortisol levels were significantly higher than post-fight cortisol levels (overall: paired $t_{89} = 8.13$, $P < 0.0001$;

isolates: $t_{57} = 7.27$, $P < 0.0001$; group: $t_{31} = 3.97$, $P = 0.0004$). There was a significant three-way interaction between housing, status, and escalation on post-fight cortisol concentrations (ANCOVA: $F_{1, 78} = 6.45$, $P = 0.01$; Fig. 2); the only significant main or two-way interaction effects were housing (ANCOVA: $F_{1, 78} = 11.36$, $P = 0.001$) and housing \times escalation (ANCOVA: $F_{1, 78} = 7.45$, $P = 0.008$). Linear contrasts demonstrated that previously isolated losers had significantly higher cortisol levels than previously group-housed losers following non-escalated contests ($F_{1, 74} = 20.01$, $P < 0.0001$, $\alpha_{\text{adj}} = 0.004$; Fig. 2). In

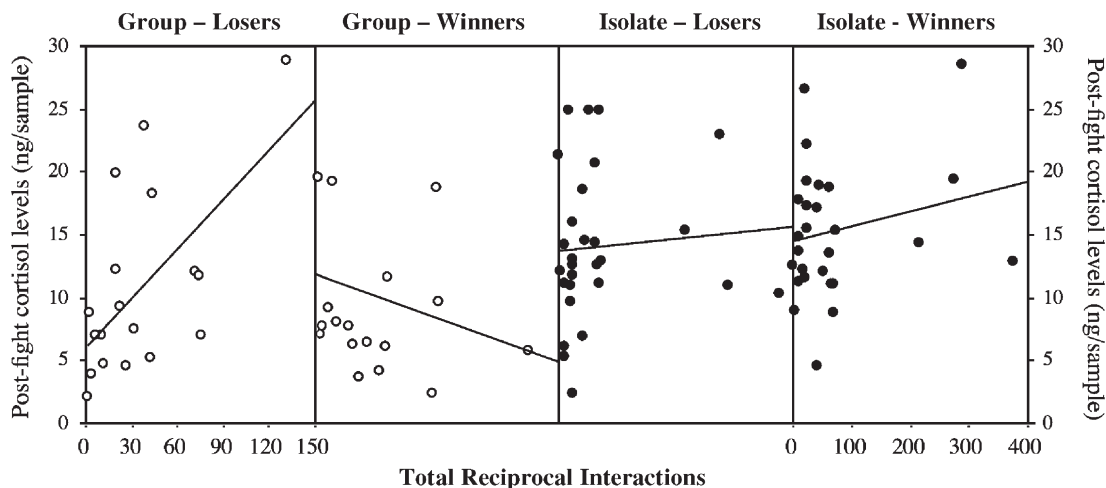


Fig. 3. Relationship between contest intensity (total number of reciprocal interactions) and post-fight cortisol levels in winners and losers from the group-housed (open circles) and isolated (dark circles) conditions; the same trends held true for the relationship between contest duration and post-fight cortisol levels. X-axis scales are the same for winners and losers within each housing regime, but are displayed for only one status class. Relationships for isolated winners ($F_{1, 22} = 0.52$, $P = 0.48$) and losers ($F_{1, 22} = 1.79$, $P = 0.19$) remained non-significant after removal of the largest four values for total reciprocal interactions, which were discontinuous from the rest of the distribution.

addition, group-housed losers responded with significantly higher cortisol levels to escalated versus non-escalated contests ($F_{1, 74}=9.82$, $P=0.0025$, $\alpha_{\text{adj}}=0.0047$; Fig. 2). No other comparison met significance criteria with sequential Dunn–Sidak adjustments.

The lack of a status effect was confirmed with paired t -tests conducted for participants in each contest (overall: $t_{46}=0.045$, $P=0.97$, mean difference=0.065 ng/sample; isolated: $t_{28}=0.21$, $P=0.84$; group: $t_{17}=0.15$, $P=0.88$). There was no significant correlation between post-fight cortisol levels of winners and losers (overall: $r_{47}=0.17$, $P=0.26$; isolates: $r_{29}=0.22$, $P=0.25$; group: $r_{18}=0.14$, $P=0.58$). The number of total reciprocal interactions and contest duration were significantly positively correlated with post-fight cortisol levels of group-housed losers ($F=9.19$, $P=0.0079$, $R^2=0.36$, $\beta=0.13$, $\alpha_{\text{adj}}=0.0085$, and $F_{1, 14}=8.80$, $P=0.0091$, $R^2=0.35$, $\beta=0.38$, $\alpha_{\text{adj}}=0.010$, respectively; Fig. 3). Contest dynamics did not predict post-fight cortisol levels of group-housed winners ($F<3.82$, $P>0.07$, $R^2<0.19$) or isolated winners or losers ($F<5.08$, $P>0.03$, $R^2<0.16$, $\alpha_{\text{adj}}=0.0085$; Fig. 3).

4. Discussion

4.1. Contest outcome, cortisol, and methodological considerations

Resolution of aggressive contests did not elicit detectable differences in cortisol release in winners and losers of territorial male convict cichlids within the time frame of our sampling protocol (2 h). In both housing regimes, and irrespective of contest type (escalated versus non-escalated), winners and losers showed statistically equivalent post-fight cortisol levels (Fig. 2). At first glance, our findings appear to contradict much of the existing literature on outcome-related corticosteroid profiles following dyadic conflicts in fishes [9,14,46,47], reptiles [16,24], and mammals [8,27]. Each of these studies indicated that post-fight corticosteroid hypersecretion is exclusive to or at least more pronounced in losers. Nevertheless, our results are consistent with recent work on the relationship between corticosteroids and fighting experience in other cichlid species [48,49] and in some lizards [15]. Correa et al. [48], for instance, found no difference in plasma cortisol concentrations between winners and losers after 6 h of social interaction, while Buchner et al. [49] showed a similar trend after 48 h of cohabitation. Øverli et al. [20] not only found no significant differences in plasma cortisol concentrations of juvenile rainbow trout (*Oncorhynchus mykiss*) that had won or lost a contest at 5 min post-fight, but also found that losers exhibited delayed cortisol elevation relative to winners at 3 h post-fight.

Our data thus suggest that winning or losing, independent of other variables, is not a particularly stressful experience for the cichlids. An interesting alternative explanation is that winners and losers show differential responses to the hormone sampling procedure (i.e., confinement in a beaker for 2 h) such that the post-fight cortisol levels that we report represent an interaction between social status and stress responsiveness. A conservative assumption based on the available literature in fishes

[9,14,46,47] would be that losers should have exhibited higher post-fight cortisol levels, and that a more significant response of winners to confinement might have equalized post-fight cortisol levels between the status classes. Data from the present study, and from studies on salmonids, however, provide little support for this alternative. In salmonids, eventual winners exhibit lower, or equivalent cortisol responses to pre-fight confinement relative to eventual losers [28,29,50], suggesting that individuals with a predisposition for winning do not show elevated responses to non-social stressors. In our study, pre-fight cortisol levels of the cichlids were significantly higher than post-fight cortisol levels. The vast majority of literature reports corticosteroid increases after fighting typically in losers or in both winners and losers, effectively discounting the possibility that fighting itself is responsible for the decline from pre-fight to post-fight sampling. It also is possible that cohabitation with another animal reduces cortisol levels. If this was the case, however, we would expect a correspondence between pre- and post-fight cortisol levels in group-housed animals and a significant post-fight decline in isolated but not group-housed animals, neither of which occurred. Taken together, these data support the thesis that cichlids show a pronounced cortisol response to a novel non-social confinement stressor (pre-fight) and also that one exposure to this stressor is sufficient for acclimation (i.e., the post-fight samples were significantly lower).

Eventual winners and losers of the cichlid fights did not differ in their response to the initial confinement stressor, which supports the contention that the absence of status differences in post-fight cortisol levels in our study was not due to differential responses of the individuals to the 2 h confinement/hormone sampling procedure. Neither the salmonid nor the cichlid results, however, can eliminate the possibility that acute winning or losing experiences could substantially alter stress responsiveness to a non-social stressor. Two important studies in salmonids and rats have demonstrated that chronically subordinated individuals show a blunted cortisol response to ACTH perfusion of the interrenal cells, and peripheral CRH injection, respectively [51,52]. Other studies have reported no differences between dominant and subordinate animals from a stable social hierarchy in the cortisol response to immobilization [53]. Whether acute, experience-dependent blunting or enhancement of the cortisol response to confinement stress occurs on the order of hours remains uncertain.

We sampled water-borne cortisol continuously for 2 h following contest settlement in both winners and losers. Thus, hormone concentrations represent the summation of cortisol release over that collection interval. Although sampling water-borne hormones is relatively non-invasive and the concentrations obtained are indicative of plasma cortisol concentrations [44], this technique did not allow for temporal resolution on the scale of minutes post-fight for small fishes with the assays employed in this study. It is therefore possible that we missed transient peaks and troughs in cortisol release, which could have distinguished winners and losers at various points between 0 and 2 h after contest resolution. Øverli et al. [20] demonstrated clearly that the relative cortisol profiles of winners and losers

change from 5 min to 3 h post-fight but they also did not resolve differences between winners and losers within this time frame. Data on domestic pigs, however, indicate that in the absence of further interactions with an opponent, winners (but not losers) recover baseline corticosterone levels within 1 h [27]; see also [14] and [25] for similar trends in cohabitating opponents. If winners and losers show similar elevations in cortisol immediately following an aggressive interaction [27,54], then faster recovery on the part of winners should have been reflected in our water extracts.

Fine-scale temporal analyses of post-fight cortisol concentrations are important for understanding more fully the neuroendocrine response patterns of winners and losers [1,14,20,25,27,55]. In practice, however, such analyses can be difficult whether employing non-invasive water collection techniques or more invasive techniques. For instance, handling during a first blood collection could exacerbate the stress response, making interpretation of cortisol concentrations from subsequent samples more difficult with respect to the relative influence of the target stressor (e.g., aggressive contests) versus the collection technique. An advantage to the water extraction technique, at least for cichlids, is that individuals appear to acclimate quickly to being held in a beaker, as indicated by the dramatic reduction in cortisol levels on the second, post-fight sample despite some individuals having engaged in highly escalated interactions. Future studies will verify that a reduction in cortisol levels does occur between a first and second hormone collection (in beakers) in the absence of social interaction. This will allow us to examine the relative contribution of stress responsiveness to a non-social stressor (first sample; [28,29,51]) and basal pre-fight cortisol levels (second sample; [47,56]) to variation in contest dynamics and outcome. Because excessive handling is not necessary for collection of water-borne hormones, the limitation for temporal analyses using this technique is the sensitivity of commercially available assays. More sensitive enzyme immunoassays have been developed since this study was conducted (Cayman Chemicals, Inc.) and should allow for the collection of detectable concentrations of hormones within 30 min post-fight (R.L. Earley, unpublished data), which might be informative with respect to quantifying differential recovery rates of winners and losers following agonistic interaction.

4.2. Housing, pre-fight cortisol, and contest dynamics

Individuals housed in social isolation showed significantly higher pre-fight cortisol levels than group-housed animals, which could be indicative of enhanced responsiveness of isolates to the initial confinement stress (see previous section), higher basal cortisol levels, or both. A number of studies show higher basal corticosteroid levels in socially isolated relative to group-housed animals [57,58], although the relationship between housing regimes remains somewhat equivocal [59,60] and is likely to depend on a number of variable factors associated with group-housing, such as density, sex ratio, and social stability. There is far greater concordance among studies that examine housing effects on corticosteroid responsiveness,

and the general consensus is that long-term social isolation leads to hyperactive corticosteroid responses to non-social stressors ([61–63], but see [59]).

Contests between individuals housed in isolation escalated to mouthwrestling significantly more often, and were characterized by more high-intensity reciprocal acts (attack-bite sequences) than contests between group-housed animals. Instigation of aggression by social isolation is well documented in fishes [31] and other vertebrates (e.g., [64]), and is more consistent with the hypothesis that isolated cichlids experience increased stress responsiveness, as indicated by the pre-fight cortisol sample, rather than chronic elevations in corticosteroid levels. Chronic corticosteroid treatment tends to inhibit aggressive behavior and to potentiate submissive behaviour [30,65–67] whereas acute increases in corticosteroids facilitate aggression in a variety of taxa including cichlid fish [68], lizards [67], and mammals [69].

If we assume that enhanced stress responsiveness to the initial confinement can be generalized to aggressive contests between previously isolated participants, then it is possible that acute anticipatory increases in cortisol occur when confronted with an opponent (the social stressor). A cortisol surge may then be responsible for elevating aggression of both opponents and, as a consequence, contributing to the development of highly escalated fights. This hypothesis is inspired by work on the hypothalamic attack area in rodents (e.g., [69]) wherein confronting an opponent stimulates acute corticosteroid increases, which in turn activates hypothalamic brain regions, within which there are a multitude of candidate molecular alterations that exert positive feedback on neuroendocrine stress axis activity. Many of these candidate mechanisms also are related to social isolation, including changes in aminergic (e.g., increased brain dopamine levels, decreased serotonergic metabolism [70]), and GABAergic (e.g., decreased GABA_A receptor function [71]) systems, production of neuroactive steroids (e.g., allopregnanolone [72]), and activation of other neuropeptide systems (CRF [73]). Given that all of these systems interact with corticosteroids, some in a reciprocal fashion, and also modulate aggression [67], it is likely that isolation-induced aggression in cichlids results from acutely elevated cortisol in combination with one or more of these factors.

Although enhanced stress responsiveness in isolated animals might explain wholesale increases in contest intensity, variation in pre-fight cortisol levels of isolated winners/losers did not explain variation in contest intensity (Fig. 1). Pre-fight cortisol levels of group-housed losers, but not winners, however, were significantly positively correlated with contest intensity (Fig. 1). These data raise two questions regarding the interrelationships between housing, stress responsiveness, and contest intensity. When a correlation exists between stress responsiveness and fight intensity, why is it evident only for losers? And, why is it that isolates show no relationship? Eventual losers ultimately dictate contest intensity; fights will escalate until one animal, the loser, opts to withdraw. Thus, traits exhibited by eventual losers that influence its willingness to persist in an encounter should be the most salient predictors of contest intensity. As described in

the previous two paragraphs, anticipatory elevations in cortisol at the start of a contest may be tightly linked to the expression of aggressive behavior during a contest [67,69]. Our results support the notion that variation in stress responsiveness of eventual losers can predict a substantial portion of the variation in fight intensity in contests involving previously group-housed animals. It is tempting to attribute the absence of such a relationship in contests involving previously isolated participants to a ‘ceiling effect’. In this case, one could posit that above a threshold level of stress responsiveness, extreme aggression by the loser, and intense contests, are enabled independent of any variation in anticipatory cortisol release. Given that the ranges of pre-fight cortisol concentrations in group-housed and isolated animals overlapped considerably (Fig. 1), it is unlikely that such a ‘ceiling effect’ is operating. Rather, it is possible that long-term social isolation disables another element of the circuit, such as the serotonergic system, responsible for modulating aggression in a graded fashion in response to acute increases in cortisol.

4.3. Housing, contest dynamics, and post-fight cortisol

Group-housed losers exhibited a more pronounced response to escalated (involving mouthwrestling) versus non-escalated contests (Fig. 2), and appeared to respond in a continuous manner to changes in contest intensity (Fig. 3). Group-housed winners, and previously isolated winners and losers showed no such response to fight escalation. These data raise two important questions about the relationships among housing, contest dynamics, status, and post-fight cortisol concentrations. Why is the cortisol response to contest intensity abolished in previously isolated animals? And, in the group-housed condition, why is such a response evident only in losers?

We posited in the previous section that long-term social isolation might disrupt the ability of animals to modulate aggression levels in response to acute increases in cortisol levels. Similarly, long-term social isolation might disable changes in hypothalamic–pituitary–interrenal (HPI) activity in response to subtle perturbations in their social environment such as increased fight intensity, or in general (see [74] for disruption of diurnal patterns in rats). Because various aspects of the serotonergic system have been implicated in the regulation of aggression [9,20,66,75] and HPI activity [76,77] in fishes, isolation-induced changes to this monoaminergic system could account for both of the phenomena described above, i.e. inability of isolates to modulate aggression and/or neuroendocrine stress axis activity. Indeed, Höglund et al. [77] demonstrated in Arctic charr that the effects of 5-HT_{1A} receptor activation on plasma cortisol depended on whether the animals were undisturbed or stressed, suggesting state-dependent interactions between the serotonergic systems and HPI axis.

There are several non-mutually exclusive explanations for why group-housed losers, not winners, exhibit a graded post-fight cortisol response to contest intensity. We will focus on the relationship between corticosteroids and metabolic costs accrued during a contest. Aggressive contests are metabolically

costly [78,79], and losers accumulate metabolic costs (e.g., depletion of glycogen stores) associated with fighting at a more rapid rate than winners [24,42,43]. Several recent studies suggest that corticosteroids impede metabolic recovery following physical exertion [24,80], indicating that sustained cortisol elevations in losers after intense interactions might reflect within-contest energetic demands rather than facilitate post-fight recovery. Fighters often exhibit decreased muscle and liver glycogen levels, and increased glycolytic activity [43,81,82]. Furthermore, muscle sugar content is negatively correlated with escalated fight duration, suggesting that glycogen stores are mobilized to a greater extent during intense contests [43,83]. Cortisol plays an important role in the liberation of glucose from fish muscle tissue both in vitro and in vivo [84,85]. Thus, graded elevations in cortisol concentrations may be necessary to meet the greater energetic demands imposed on losers by intense contests. Two other plausible alternatives are that elevated cortisol levels operate in concert with other neuropeptide (e.g., CRF) or neurotransmitter (e.g., GABA) systems to alter behavior, particularly agonistic behavior, in a manner consistent with recovery from physical challenge [65,86–88], and/or to facilitate the consolidation of memories associated with escalated fighting experiences [89–91]. In all, these alternatives suggest that the sensitivity of losers to subtle changes in contest intensity might have a variety of functions, all of which might help to explain the persistence of loser effects relative to winner effects in a range of taxa [92].

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