The Stress Response of the Highly Social African Cichlid *Neolamprologus pulcher*

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ABSTRACT

In group-living species, dominant individuals are frequently aggressive toward subordinates, and such dominant aggression can lead to chronic stress, higher glucocorticoid levels, and decreased fitness for subordinates. However, in many cooperatively breeding species, it is surprisingly the dominants rather than the subordinates that exhibit higher levels of glucocorticoids, a possible consequence of the demands of maintaining high social rank and socially suppressing the reproduction of other group members. This study investigates the relationship between social status and circulating plasma cortisol in groups of the cooperatively breeding African cichlid *Neolamprologus pulcher*. Baseline (resting) levels of cortisol were quantified, as was the cortisol response following an acute stressor. Dominants had the higher cortisol concentrations, and these were not related to their social behavior. Cortisol concentrations correlated (positively) with social behaviors and general activity levels only in subordinate males, arguably the individuals with the least stability in the social group. No status-dependent differential responses to acute stress were detected, suggesting that the status-induced chronic stress has little effect on the capacity to mount a full stress response to large-scale, life-threatening risk.

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Introduction

The sight of a predator, the need for shelter, the lack of food, or the interaction with a more dominant or aggressive conspecific may all cause significant stress in individuals. Across vertebrates, an integral component of the stress response is mediated by the hypothalamic-pituitary-adrenal (HPA) axis, or the hypothalamic-pituitary-interrenal (HPI) axis in fish. This axis helps the animal to regain homeostasis by releasing glucocorticoid (GC) hormones (i.e., cortisol or corticosterone) into the bloodstream. In turn, GCs mobilize fatty acids and liver glycogen to provide the energy needed to deal with the stressor. While such stress responses are adaptive in the short-term, chronic stress can lead to decreases in immune function, reproduction, growth, and ultimately fitness (for reviews, see Wendelaar Bonga 1997; Chrousos 1998).

Subordinate individuals within many species exhibit chronically elevated GC levels, while dominants commonly have lower levels (e.g., olive baboons *Papio Anubis*: Virgin and Sapolsky 1997; rainbow trout *Oncorhynchus mykiss* and brown trout *Salmo trutta*: Sloman et al. 2001; for review, see Creel 2001). However, in many cooperatively breeding species, the opposite pattern has been observed, with dominants exhibiting higher GC levels than subordinates (e.g., Florida scrub jay *Aphelocoma coerulescens*: Schoech et al. 1991; but see Schoech et al. 1997; African wild dogs *Lycaon pictus* and dwarf mongoose *Helogale parvula*: Creel et al. 1996; common marmoset *Callithrix jacchus* and cotton top tamarin *Saguinus oedipus*: reviewed in Abbott et al. 2003; for review, see Creel 2001). In cooperatively breeding species, subordinates help raise the offspring of dominants, often forgoing reproduction themselves, either by design or by force.

In most social species, both dominants and subordinates can realize some reproductive success, while in many cooperatively breeding species, it is only the dominants that breed (despotic or skewed reproduction), and subordinate reproduction is suppressed. In the few cooperatively breeding species in which both dominants and subordinates reproduce within a group (egalitarian reproduction), subordinates tend to have higher cortisol levels, just as in the vast majority of noncooperatively breeding social and nonsocial species. These patterns are apparent in Creel's (2001) comparative review of GCs across cooperatively breeding species. More recently, Goymann and Wingfield (2004) have proposed that allostatic load (or the relative physiological costs of achieving and maintaining dominance status) best explains the observed species differences in status-related stress responses. Note that the concept of allostatic load and its quantification can easily and naturally encompass the degree to which subordinates are prevented from reproducing. In gen-

eral, when the allostatic load is high, dominants will have higher cortisol levels. When allostatic load is low, subordinates are expected to have higher cortisol levels, or no status-related differences in cortisol levels are expected.

The main goals of this study were to elucidate the interactions between circulating cortisol levels, sex, and social status in relation to allostatic load under baseline, or resting, conditions as well as following an acute stressor in the cooperatively breeding cichlid fish *Neolamprologus pulcher* from Lake Tanganyika. This fish lives in permanent social groups consisting of a dominant breeding male and female together with anywhere from one to 20 subordinate helpers (on average, groups have five subordinates; Taborsky and Limberger 1981; Balshine et al. 2001; Heg et al. 2005). *Neolamprologus pulcher* subordinate helpers assist dominant breeders in territory maintenance, defense, and brood care (Taborsky 1984; Brouwer et al. 2005). *Neolamprologus pulcher* dominants are frequently aggressive toward subordinate group members (laboratory data: 0.7 ± 0.01 aggressive acts min^{-1}), while subordinates rarely show aggression toward dominants (laboratory data: 0.04 ± 0.01 aggressive acts min^{-1}), and dominant individuals claim the majority, if not all, of the reproductive opportunities in natural social groups in the wild (Dierkes et al. 2005; Fitzpatrick et al. 2006; Stiver et al. 2009; but see Dierkes et al. 1999 and Heg et al. 2006 for some contrary evidence based on captive laboratory groups). In addition, achieving and maintaining a dominant breeding position is extremely challenging in *N. pulcher*, where there are many more subordinate helpers than vacant breeding positions (Balshine-Earn et al. 1998; Stiver et al. 2006; Fitzpatrick et al. 2008), and thus the allostatic load for dominant breeders is expected to be higher than for subordinate helpers (see "Results" and Table 1 for our allostatic load calculation for dominant and subordinate *N. pulcher*). Moreover, a new model by Rubenstein and Shen (2009) suggests that the allostatic load of dominants will increase with increased numbers of subordinates. On the basis of the allostatic load hypothesis and consistent with the reproductive skew cortisol framework, we predicted that dominant *N. pulcher* would have higher cortisol levels than subordinates. We also predicted that dominant breeders in large groups with more subordinates would have higher cortisol levels than dominant breeders in smaller groups.

We further predicted that high levels of aggression and social policing (often observed in more dominant individuals) would be associated with higher baseline cortisol levels. This prediction was shaped by previous work on the physiological costs of social living in *N. pulcher*. For example, Buchner et al. (2004) reported that among size-matched and sex-matched *N. pulcher* subordinates, the more dominant subordinates tended to have higher plasma cortisol concentrations than the subordinates lower down in the hierarchy. Bender et al. (2006) found that more submissive male subordinates exhibited lower cortisol levels. We also examined the status-dependent effect of an acute stressor (confining fish in a net for 10 min) on the cortisol response, an approach never before utilized in *N. pulcher* and only rarely examined in cooperative breeders (but for some illuminating exceptions, see Schoech et al. 1997, 2007; Creel 2005; Rubenstein 2007). The pattern found in noncooperative species suggests that a high preexisting cortisol level would attenuate an individual's ability to mount an additional acute cortisol response (for examples, see Barton et al. 1986; Rotllant et al. 2000; Reeder et al. 2004). Similarly, using the cooperative superb starling (*Lamprotornis superbus*), Rubenstein (2007) found that subordinates had higher handling stress GC responses but only in times of low rainfall and presumably food shortage. In more favorable years, dominant and subordinate superb starlings had similar responses to handling. Schoech et

Table 1: Allostatic loads and cortisol levels of male and female *Neolamprologus pulcher*

Social Status	Dominance Acquisition Costs	Dominance Maintenance Costs	Subordinance Costs: Threats from Dominants	Subordinance Coping Costs	Strong Bias in Resource Control	Sum of Allostatic Load	Ratio of Allostatic Load (Dominant/ Subordinate)	GC Levels in Dominants and Subordinates (Mean)	Ratio of GC Concentration (Dominant/ Subordinate)
Male:									
Dominant	2	3				5	1.66	25.92	1.83
Subordinate			2	1	0	3		16.12	
Female:									
Dominant	1	3				4	1.33	42.0	1.38
Subordinate			2	1	0	3		30.41	

Note. This table was adapted from a table by Goymann and Wingfield (2004) for other cooperative breeders, and the assignment of allostatic loads followed their criteria. Dominance acquisition costs for males are higher than for females because males are unlikely to inherit a territory (Stiver et al. 2006; Balshine and Buston, forthcoming), must disperse farther to breed (Stiver et al. 2004, 2007), and have shorter tenure as breeders compared with females (Stiver et al. 2004). The costs of maintaining dominance are high and roughly equivalent for both males and females since they both defend the territory and "police" within a territory (i.e., keep subordinates in check; Fitzpatrick et al. 2008). Dominant female breeders are usually more active in territory defense against heterospecific predators and space competitors (Balshine et al. 2001; Desjardins et al. 2008); however, dominant males constantly defend territories from takeovers by other conspecific males (Desjardins et al. 2008; J. Fitzpatrick, unpublished data). Both female and male subordinates within a territory receive moderate threats from dominants, but because there are several helpers in each group (mean = 5, range 1–20; Balshine et al. 2001; Heg et al. 2005), the aggression received from dominants is diluted. Also, subordinates can hide in shelters and/or leave the group to avoid aggression (Bergmüller et al. 2005). Thus, we assume that the coping costs for subordinates are low. No effective bias exists in the control of resources in *N. pulcher* because dominants and subordinates both feed in the water column and hence do not compete for food (Balshine et al. 2001) and because shelter, another key resource on the territory, is plentiful and shared (Balshine-Earn et al. 1998). Because of year differences in cortisol levels, subordinate males from 2007 were compared with dominant males from 2007, while subordinate females from 2006 were compared with dominant females from 2006. GC = glucocorticoid.

al. (1997) also found no difference in mounting of a stress response in dominant breeder versus subordinate helper Florida scrub jays, another cooperative breeder. Thus, on the basis of these results, we predicted either no difference in dominant and subordinate responses to an acute stressor or a larger cortisol response to an acute stressor in the social class with the lower preexisting cortisol levels (i.e., in our predictions, subordinate helpers).

Material and Methods

Study Population and Holding Conditions

The study was conducted using a colony of *Neolamprologus pulcher* held at McMaster University, Hamilton, Ontario, Canada. Fish in this experiment were descendants of fish caught in Lake Tanganyika, Zambia, in 2001 and 2002. Genetic relatedness in these laboratory social groups is thought to be roughly similar to field populations for two reasons. (1) In the wild, these fish are highly philopatric. (2) New breeding pairs are regularly formed in the laboratory. In total, 30 social groups were observed and sampled, with each group containing a dominant breeding pair and subordinate helpers (mean group size ± SE was 10.8 ± 0.8 individuals). In each tank, the two dominant fish and two large subordinate helpers (>4 cm standard length) were selected for focal observations and eventual cortisol sampling. The dorsal fins of these individuals were clipped in a unique pattern 3–7 d before observations began so that all four fish could be clearly distinguished from one another. Fin clipping does not adversely affect behaviors, and the fish return to normal social behavior within 1–2 min of being clipped (Stiver et al. 2004). Also, in other fish species, following an acute stressor such as handling or marking stress, cortisol levels are known to return to baseline after 24 h (Pickering and Pottinger 1989).

A total of 115 fish were used for the two experiments: 30 dominant males, 30 dominant females, and 55 subordinate helpers. In experiment 1, one group did not have a second large subordinate, while in experiment 2, four groups did not have a second large subordinate. Across all groups, the dominant breeder males were on average 70.3 ± 1.2 mm (mean ± SE) in standard length, dominant breeder females were 64.5 ± 1.1 mm long, and subordinates were on average 50.8 ± 0.8 mm long. Each social group was housed individually in a 189-L tank containing ~2 cm of coral sand, two foam filters, air stones, a heater, a thermometer, two upside-down flowerpot halves (to provide shelter and a place to lay and fertilize eggs), and two mirrors (used to alleviate excessive aggression toward group members). Water temperature was kept constant at 26° ± 2°C, and a 13L : 11D photoperiod was maintained. Groups were fed ad lib. daily with Nutrafin Basix large flake commercial cichlid food.

Experiment 1: Baseline Cortisol Levels

This experiment was conducted in August 2006 and in August and September of 2007. Ten male and 10 female dominant breeders were used in each year. In 2006, all but one of the subordinates removed from a tank to measure baseline cortisol levels were female ($n = 20$), and in 2007 all subordinates observed and measured were male ($n = 19$). Three to 7 d after (fin) clipping the fish, three focal watches were performed on the four marked fish (dominant breeder male, dominant breeder female, and two large subordinate helpers). Focal observations on each fish in each social group were made for a total of 30 min (3 × 10-min watches), and behaviors were then averaged among the three watches to yield the number of observed behaviors per 10 min for each fish. Observers sat still for 3–5 min before recording behavior to habituate the fish to their presence, and they always remained at a distance of 1.5 m from the tank. Observations were conducted between 0800 and 1300 hours to control for observed diurnal fluctuations in behavior in this species (J. K. Desjardins, J. L. Fitzpatrick, K. A. Stiver, G. Van Der Kraak, and S. Balshine, in preparation). To minimize possible time of day variation in plasma cortisol levels (Davis et al. 1984; Barton et al. 1986), all blood sampling was performed between 1330 and 1530 hours.

On the basis of detailed ethograms that have been published elsewhere for this species (Taborsky 1984; Buchner et al. 2004; Sopinka et al. 2009), we documented all behaviors, including aggression (ramming, chasing, biting, etc.), submission (submissive postures and displays), affiliative behaviors (soft touches and parallel swims, follows), and workload or care behaviors (visiting the brood chamber, micronipping eggs, defense and guarding of young, fanning, territory maintenance activities such as carrying and digging, etc.). Additionally, time spent by the focal fish behind filters and in the brood chambers as well as the frequency that each individual performed locomotive (swimming, darting) and maintenance (feeding, yawning, and scraping) activities were recorded.

Following the final observational period, all four fish were quickly netted and killed (as described in the "Ethical Note"), and blood was collected in 10-μL heparinized microcapillary tubes following caudal severance. Multiple microcapillary tubes were used depending on the size of the fish in order to collect as much blood as possible (typically, 10–25 μL of blood were collected). All blood was collected within 2 min of the initial approach toward the tank (mean ± SE = 112 ± 2 s). During dissection, sex was confirmed. Blood samples were centrifuged at 8,000 g for 10 min, and plasma was separated and stored at −80°C for later analysis of cortisol concentration.

Experiment 2: Acute Stress Induction

This experiment was conducted in 2007. We used 10 social groups for this study (total of 36 fish observed and processed: 10 breeder males, 10 breeder females, 10 female helpers, and 6 male helpers). All conditions and procedures in this experiment mimicked those used in experiment 1. However, one important addition was made. At the end of the last observational watch, all four fish were individually netted as quickly as possible, except that the fish remained confined in the net in water for 10 min. We chose 10 min of confinement for *N*.

pulcher on the basis of similar approaches used in other species (see Gamperl et al. 1994). Confinement for 30 min was enough to elicit a maximal stress response in salmonids (Gamperl et al. 1994), which are cold-water fish that are burst swimmers, whereas *N. pulcher* is a tropical fish that does not normally swim for long periods or distances. Additionally, a recent study (Alderman and Bernier 2009) used a 15-min stressor for another small freshwater tropical fish species, the zebrafish *Danio rerio*. Following this acute stress, fish were killed (see "Ethical Note"), and blood was sampled (within 111 ± 2 s following the removal of fish from the net).

Analysis of Cortisol Concentrations

Assessment of cortisol concentration was performed on 10-μL plasma samples using a commercial ^{125}I RIA kit (CA-1529, DiaSorin) as per the manufacturer's instructions. Each fish had very little plasma (typically 5–15 μL of blood plasma could be obtained from a single fish, and the cortisol assay requires 10 μL), and thus we were unable to analyze samples in duplicate. Where plasma samples were lower than 10 μL, a dilution factor was taken into account in calculating cortisol concentration. The sensitivity of the RIA is listed as 2.1 ng mL^{-1}, while the interassay variation (coefficient of variation) is 8.8%–9.8%. In our hands, the intra-assay variation (coefficient of variation) was 1.5%–8.8%. Cortisol analysis by this method has been validated for *N. pulcher* in previous studies (Buchner et al. 2004) and in other cichlid species (e.g., *Astatotilapia burtoni*; Fox et al. 1997).

Statistical Analyses

An aggression composite, a submission composite, and a dominance index were calculated as follows. The aggression composite was based on the formula Σ(aggressive acts given) + Σ(submissive acts received). The submission composite was calculated for each fish using the formula Σ(aggressive acts received) + Σ(submissive acts given). A dominance index for each focal fish was created using the sum of aggressive/dominant behaviors minus the sum of submissive behaviors (for an example of this well-used index, see Aubin-Horth et al. 2007). Overall activity levels were scored as all aggressive, submissive, social, and care/workload behaviors performed.

All statistical analyses were performed using JMP IN 5.0 and Microsoft Excel 2007. Data were tested for normality and transformed when necessary; where transformation did not normalize the data, an alternative nonparametric test was used. ANOVAs were employed to examine the effect of sex and status on cortisol concentrations; interactions that were not significant were removed from the models. Because we predicted a priori that dominant individuals would have higher cortisol levels than subordinates, we used one-tailed tests to compare baseline cortisol levels of dominant versus subordinate individuals. All other tests were two-tailed.

To fully explore the influence of status, year, and group size on cortisol levels, we performed a general linear model (GLM) analysis and removed all nonsignificant interactions. We observed a strong year effect despite the fact that the fish were held under identical conditions in the same lab, were fed the same food, and were held under the same temperatures and light regimes. Fish were handled in an identical manner by the same group of researchers. We do not know what caused the year differences, so we statistically controlled for year effects by using year as a covariate in a GLM and by using paired Wilcoxon and *t*-tests to examine cortisol concentrations. These controlled for year effects and for potential group/tank effects in cortisol concentrations by comparing individuals differing only in status within the same social group (see "Results"). Note that cortisol values for acutely stressed fish were compared with baseline cortisol levels for fish from the same year.

One fish (a dominant female) was excluded from analysis of baseline cortisol as an extreme outlier (see outlier analysis in Sokal and Rohlf 1995, pp. 406–407). Inclusion of data for this fish would have increased the significance of results (to $P = 0.027$ for the status and cortisol analysis; see "Results"). Note that body mass and length were strongly linked with social status; dominants were always bigger than subordinates in their group (Wilcoxon rank sum test: body mass, $\chi_1^2 = 69.79$, $P < 0.0001$; body length, $\chi_1^2 = 70.77$, $P < 0.0001$). However, body size was not used as a covariate in any analyses because circulating cortisol concentrations are expected to be independent of mass (Hartman et al. 1943).

Ethical Note

To kill fish quickly, individuals were given a firm cephalic blow. The procedures described conform to the protocols approved by the Animal Research Ethics Board of McMaster University (AUP 06-10-59) and the Canadian Council for Animal Care guidelines.

Results

Experiment 1: Cortisol Concentrations at Baseline: The Influence of Status and Sex

Overall, cortisol levels were higher in dominants than subordinates (Fig. 1*A*). Along with the status difference, there was also a significant year effect, but group size had no effect on cortisol levels (GLM, overall model: $F_{3,74} = 8.08$, $P < 0.0001$; year: $F_{1,74} = 17.13$, $P < 0.0001$; status: $F_{1,74} = 4.22$, $P = 0.04$; group size: $F_{1,74} = 2.64$, $P = 0.11$). When we controlled for the year effect by pairing dominants and subordinates within a social group, again dominants had higher cortisol levels (Wilcoxon signed-ranks test, $W_{19} = 46.00$, $P = 0.045$). Within a social status category, cortisol concentrations did not differ with rank. For example, the two dominants from the same social group (male and female of a breeding pair) had similar cortisol levels (Wilcoxon signed-ranks test, $W_{19} = -18.5$, $P = 0.75$), despite the fact that male breeders are dominant to female breeders. Also, in each group, the subordinates of higher rank (H1) had cortisol concentrations similar to those of the subordinates of lower rank (H2; paired *t*-test, $t_{18} = 0.05$, $P = 0.96$). In gen-
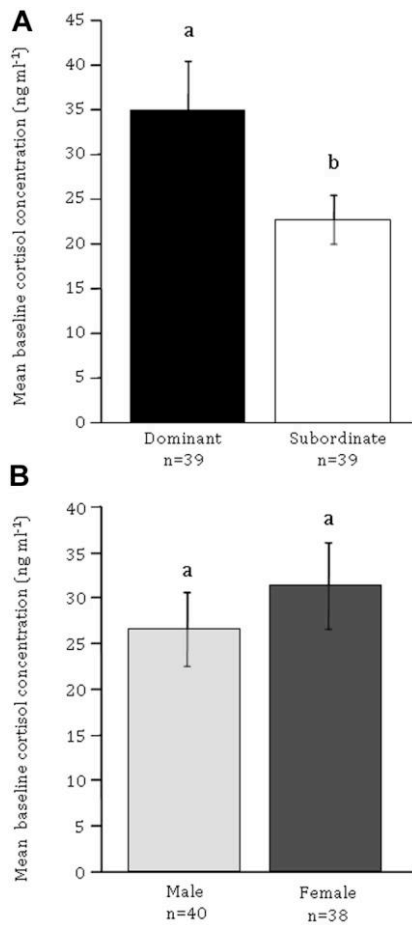
Figure 1. *A*, Mean baseline cortisol concentration (±SE) in dominant and subordinate individuals pooled across both experimental years. *B*, Mean baseline cortisol concentrations (±SE) in male and female fish in both experimental years. Letters denote significant differences at $\alpha = 0.05$ after controlling for year effects.

eral, males and females did not differ in cortisol concentrations (two-way ANOVA, effect of sex: $F_{1,75} = 0.82$, $P = 0.37$; Fig. 1*B*).

Experiment 2: Cortisol Following an Acute Stressor

Acutely stressed fish exhibited markedly higher cortisol concentrations than fish for which baseline cortisol levels were determined (Mann-Whitney *U*-test, $\chi_1^2 = 54.72$, $P < 0.0001$; Fig. 2*A*). However, stressed dominants and stressed subordinates elevated cortisol concentrations to a similar extent (ANOVA, $F_{1,34} = 0.59$, $P = 0.45$; within group paired *t*-tests, $t_9 = -1.15$, $P = 0.28$; Fig. 2*B*). Following a 10-min acute stressor, males tended to have higher cortisol concentrations than females, but this difference did not reach significance (ANOVA, $F_{1,34} = 3.20$, $P = 0.08$; Fig. 2*C*).

Social Behaviors

Dominant breeders (both sexes) had higher dominance indexes than subordinate helpers (Wilcoxon rank sum test, $\chi^2 =$

43.43, $P < 0.0001$; Fig. 3). Dominants were more aggressive and received more submission (had higher aggression composite scores) than male and female subordinates, while subordinates were more submissive and received more aggression (see Table 2). Dominant breeders of both sexes exhibited more workload/
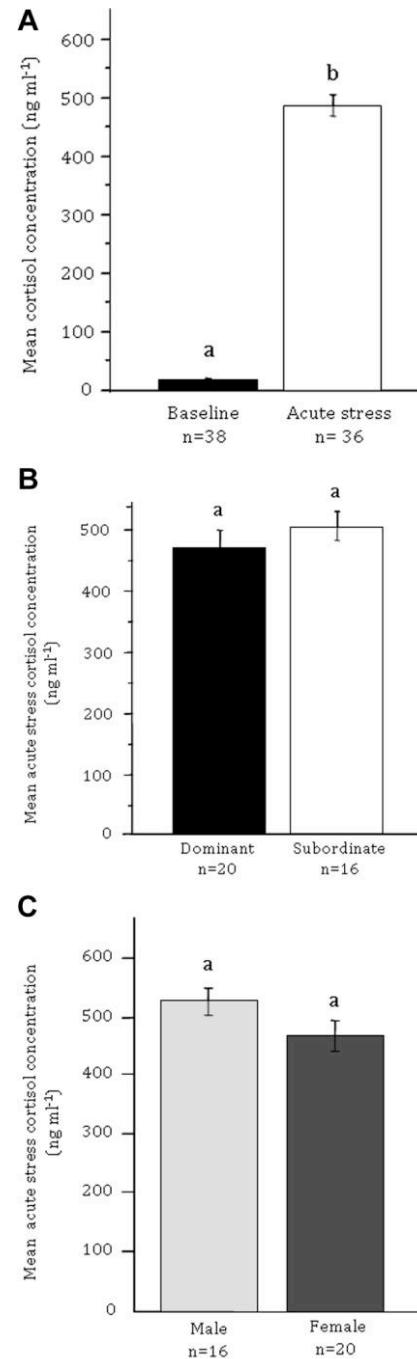

Figure 2. *A*, Mean cortisol concentration (±SE) of all baseline and acutely stressed fish in 2007. *B*, Mean cortisol concentration (±SE) of acutely stressed dominant and subordinate *Neolamprologus pulcher* of either sex. *C*, Mean cortisol concentration (±SE) of acutely stressed male and female *N. pulcher* of both status classes. Letters denote significant differences at $\alpha = 0.05$.

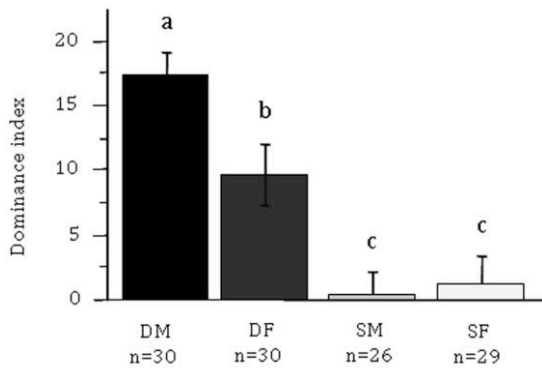Figure 3. Mean dominance indexes ($\pm$ SE), calculated as the sum of dominant behaviors minus the sum of submissive behaviors for dominant males (*DM*), dominant females (*DF*), subordinate males (*SM*), and subordinate females (*SF*). Letters denote significant differences at $\alpha = 0.05$.

care behaviors than subordinate helpers ($\chi^2 = 13.35$, $P = 0.0003$), a pattern that has been reported previously in the field (Desjardins et al. 2008). Female and male subordinates had similar workload/care scores ($\chi^2 = 1.15$, $P = 0.28$), as did dominant female and male breeders ($\chi^2 = 0.002$, $P = 0.96$). There were no differences in the total number of affiliative interactions performed between dominants and subordinates ($\chi^2 = 2.084$, $P = 0.15$) or between males and females ($\chi^2 = 0.02$, $P = 0.90$).

Social Behavior and Baseline Cortisol

In subordinate males, overall activity levels were significantly positively correlated with cortisol levels (Spearman's rank correlation test, $\rho = 0.76$, $P = 0.0002$). Following sequential Bonferonni corrections for multiple comparisons (Rice 1989), of all the social behaviors examined, only affiliative behaviors (nonaggressive social interactions with other group members) remained significantly (positively) correlated with cortisol levels ($\rho = 0.75$, $P = 0.0002$; Fig. 4), although before the correction for multiple comparisons, aggression and submission performed were also positively correlated with cortisol levels (aggression, $\rho = 0.57$, $P = 0.01$; submission, $\rho = 0.53$, $P = 0.02$). None of the behavioral categories were significantly related to baseline cortisol concentrations when all fish were examined together. This was also the case when we examined dominant breeding males, dominant breeding females, and female subordinates separately (all $P > 0.05$).

Social Behavior and Cortisol Levels Following Acute Stress

As observed in the baseline condition, social behaviors were not correlated with cortisol levels following an acute stressor when we examined all fish together. Within dominant breeding males and dominant breeding females, fish that performed more care had lower cortisol levels following a stressor (males: $\rho = -0.76$, $P = 0.01$; females: $\rho = -0.64$, $P = 0.05$). Inter-

estingly, although subordinate males that were more active and affiliative had higher cortisol levels in the unstressed condition, following an acute stressor, the opposite trend was observed, with subordinate males that performed a high frequency of affiliative displays having lower cortisol levels ($\rho = -0.81$, $P = 0.05$). However, stringent Bonferroni corrections for multiple comparisons removed significance from all of these analyses.

Discussion

In this study, we have documented one aspect of the stress response in an African cichlid fish with a cooperative breeding system and have shown that cortisol differences are based on the position a fish occupies in a social hierarchy but not on its sex. Dominant *Neolamprologus pulcher* exhibited higher baseline cortisol concentrations than did subordinate helpers. Additionally, we discovered a relationship between behavior and cortisol (in subordinate males), and these patterns may be important when examining the consequences of status and sex differences in stress responses, general physiology, and reproductive success in complex social groups. After a 10-min acute stressor, cortisol levels did not reflect the social status–dependent cortisol differences observed in unstressed animals, broadly suggesting that mounting of a stress response is similar in dominants and subordinates.

This study provides a teleost example supporting the allostatic load hypothesis. In *N. pulcher*, as seen in many other cooperative breeders, a high allostatic load in dominants compared with subordinates was associated with higher circulating GCs in dominants (Goymann and Wingfield 2004; Table 1). Dominance status in *N. pulcher* is difficult to achieve, and few fish manage to inherit a position or successfully take over a breeding position via competition (Balshine-Earn et al. 1998; Balshine et al. 2001; Stiver et al. 2006; Desjardins et al. 2008; Fitzpatrick et al. 2008). Perhaps somewhat surprisingly given the allostatic load concept, group size did not influence cortisol levels of dominants (for predictions, see Rubenstein and Shen 2009).

An alternative explanation for the observed higher cortisol detected in dominant fish is that cortisol levels may simply increase when a fish reproduces. Cortisol levels are known to be associated with spawning and the production of gametes in other species (brown trout *Salmo trutta*: Pickering and Christie 1981; catfish *Heteropneustes fossilis*: Lamba et al. 1983). In *N. pulcher*, subordinates are either reproductively immature or reproductively suppressed (see Fitzpatrick et al. 2006). Therefore, reproduction itself may drive the social status–related difference observed in circulating cortisol concentration. The most dominant pair in the group breeds in *N. pulcher*, whereas other more subordinate individuals do not; therefore, it remains possible that either or both factors may be driving the relationship between circulating cortisol levels and status.

No sex differences in cortisol concentrations were detected in this study. To date, the vast majority of studies on status-related stress have not examined sex differences. This may be

Table 2: Behavioral composite scores (mean $\pm$ SE; number of acts per 10 min) for all fish used

| Behavior | Dominant | | Subordinate | | χ^2 | P |
	Males ($n = 30$)	Females ($n = 30$)	Males ($n = 26$)	Females ($n = 29$)		
Aggression composite	18.4 $\pm$ 2.00	15.7 $\pm$ 1.8	8.0 $\pm$ 1.3	10.4 $\pm$ 1.8	24.7	<.0001
Submission composite	.9 $\pm$ .3	5.8 $\pm$ 1.0	7.5 $\pm$ 1.00	8.9 $\pm$ .8	58.7	<.0001
Affiliative:						
Given	1.8 $\pm$ .5	2.7 $\pm$ .4	1.6 $\pm$ .3	1.3 $\pm$ .3	7.4	.06
Received	1.7 $\pm$ .3	1.6 $\pm$ .3	.7 $\pm$ .3	1.1 $\pm$ .4	15.1	.002
Workload composite	5.1 $\pm$ 1.1	12.1 $\pm$ 7.4	2.1 $\pm$ .7	3.7 $\pm$ 1.0	14.5	.002

Note. Dominant male and female breeders had significantly higher levels of aggression and lower levels of submission than subordinate individuals. There were no differences in the frequency of the behavioral categories across experiments and years, and therefore the behavioral observations were pooled for these analyses. The behaviors that are included in each category are described in detail in "Material and Methods." Wilcoxon statistic (χ^2) is reported as well as its corresponding P value.

because in many cooperatively breeding species, subordinate helpers are only one sex (but see Jarvis 1981; Komdeur 1994; Carlson et al. 2004). No corticosterone-level differences were found between subordinate males and subordinate females in the cooperatively breeding African striped mouse *Rhabdomys pumilio* during either the breeding or the nonbreeding season (Schradin 2008). However, dominant males had significantly lower corticosterone levels during the breeding season than dominant females (Schradin 2008). In the dwarf mongoose, dominant females had higher baseline cortisol levels than subordinates (Creel et al. 1996; Creel 2005). Additionally, acutely stressed dominant females showed the highest cortisol rise, while dominant males showed little increase (Creel et al. 1996; Creel 2005). In both wild dogs and wolves, dominant individuals had higher baseline cortisol levels than subordinates, but males and females within a social rank had similar cortisol levels (Creel 2005). Among noncooperatively breeding species too, sex differences in cortisol levels are variable, with some studies finding females having higher baseline cortisol levels, even after acute stressors (Siberian hamsters *Phodopus sungorus*: Bilbo and Nelson 2003), and other studies reporting higher baseline cortisol levels in males (the variable flying fox *Pteropus hypomelanus*: Reeder et al. 2004). There are also studies that report no sex differences in either baseline or acutely stressed conditions (for review, see Touma and Palme 2005). Diurnal fluctuations, the time of the reproductive cycle (rats *Rattus norvegicus*: Critchlow et al. 1963), and female versus male HPA-axis differences (humans *Homo sapiens*: Kudielka and Kirschbaum 2005) have been put forth to explain these discrepancies. Previous findings in salmonids suggest that androgens suppress the stress response while estrogen can enhance it (Pottinger et al. 1996). In *N. pulcher*, testosterone was higher in females, while the 11-ketotestosterone/testosterone ratio was higher in males (Taves et al. 2009). The high levels of androgens in both males and females may partially explain the lack of sex differences observed.

Why did male but not female subordinates show correlations between social behavior and cortisol levels? In *N. pulcher*, female subordinates are likely to inherit a territory, while male

subordinates tend to disperse into new territories (Stiver et al. 2004; Bergmüller et al. 2005; Stiver et al. 2006, 2008). Hence, because males eventually leave their natal groups and are less related to the same sex breeder, male subordinates may be experiencing more stress compared with female subordinates because males may be under a greater threat of eviction, and this might explain the links between social behaviors and plasma cortisol in subordinate males.

Bender et al. (2006) found that more submissive subordinate *N. pulcher* males had the lowest cortisol concentrations, which is effectively the opposite of our observations. However, it is possible that this discrepancy is due to the different procedure used for measuring cortisol between studies. We used plasma cortisol (a concentration measurement). Bender et al. (2006) removed each focal fish from its tank (a potential stressor), placed it in a set volume of water, and assayed the cortisol excreted by the fish into the water (a production measurement)
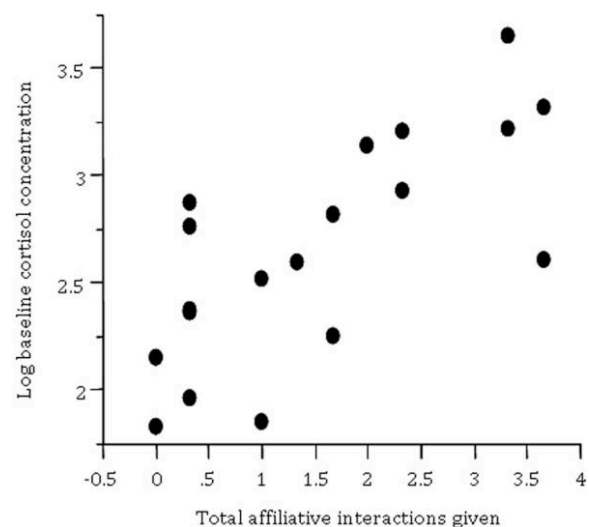

Figure 4. Correlation between baseline cortisol concentration (ng mL^{-1}; log transformed) and total affiliative interactions given as an average of three 10-min watches by subordinate males.

during a 1-h period. If the fish examined by Bender et al. (2006) were indeed stressed, then our results align. Following a stressor, the more affiliative subordinate males in our experiment did have the lower cortisol levels.

Exposure to a standardized stressor (confinement in a net for 10 min) did not differentially affect circulating cortisol levels in dominants versus subordinates or in males versus females. It is possible that the mechanism of stress used in this experiment elicited a stronger cortisol response than would occur with threats encountered in this species' natural environment. As reviewed by Gamperl et al. (1994), following a prolonged confinement stressor ($\sim$30 min), all salmonid species studied had circulating levels of cortisol below those of N. pulcher. After 30 min of severe confinement, plasma cortisol levels in salmonids ranged from $\sim$100 to $\sim$260 ng mL^{-1} (Gamperl et al. 1994), while in N. pulcher, a 10-min confinement caused cortisol to rise to 491 ng mL^{-1} (Fig. 2A). This suggests that net confinement is a very strong stressor for N. pulcher, and correspondingly, any status or sex differences in cortisol may have been "washed out" because of the severity of this stressor.

To further explore the relationship between social status and cortisol responses to a perceived threat, a follow-up experiment could be to introduce naturalistic intruders that represent differing levels of risk and danger (for a similar protocol, see Desjardins et al. 2008) and then measure changes in circulating cortisol levels. Arguably, a same-sex intruding conspecific would pose a greater threat to a dominant individual of the group than to a subordinate (in terms of group stability, offspring survival, etc.), but a heterospecific predator (of adult fish) would be equally stressful and dangerous to each social class. Also, we measured the effect of the stressor only at one time point (10 min). It is possible that status (or sex) differences in cortisol levels could have emerged if the stress response were followed over a longer time course. It is also possible that while cortisol concentrations rise to the same levels, they may rise (or decrease following a stressor) more rapidly in one social class or sex than the other, a possibility that could be addressed by assessing cortisol turnover rates. Unfortunately, because of N. pulcher's small size and the need to perform a terminal bleed to examine plasma cortisol levels, we currently do not have a stress response curve for this species, another obvious area for future study.

It is also important to note that while dominant N. pulcher exhibit higher baseline cortisol levels, their similar responses to an acute stressor provide evidence that they do not have an attenuated cortisol response. Such an attenuated stress response has been shown in juvenile rainbow trout (Barton et al. 1986) and gilthead sea bream Sparus aurata (Rotllant et al. 2000); in both cases, acute stress in chronically stressed individuals did not lead to higher cortisol levels. In yellow perch Perca flavescens, individuals that have been chronically exposed to contaminants (Hontela et al. 1995; Hontela 1998) do not respond to acute stress with higher cortisol levels, perhaps because of the negative feedback action of cortisol on the HPI axis (Barton 2002) or because of the action of the contaminant itself. The addition of an acute stressor in this study provides a "snapshot"

of the stress response exhibited by dominant and subordinate N. pulcher, but a fuller picture of HPI-axis function would be beneficial. Future study could involve characterizing corticosteroid receptors and the time course and magnitude of cortisol responses to various acute stressors. Additionally, measuring levels of gonadal hormones may be important to elucidating the interplay between the HPI and HPG axes.

In conclusion, the concept of allostatic load appears to be a useful indicator of stress levels and visa versa, especially in cooperative breeders. With more empirical evidence, the concept will be further tested and refined, and it promises to be an important tool in determining the interplay between sociality and stress.

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