Behavioral Ecology

The official journal of the
ISBE
International Society for Behavioral Ecology

Behavioral Ecology (2019), 30(2), 490–499. doi:10.1093/beheco/ary189

Original Article

Social rank, color morph, and social network metrics predict oxidative stress in a cichlid fish

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Received 26 April 2018; revised 10 September 2018; editorial decision 21 November 2018; accepted 6 December 2018; Advance Access publication 12 January 2019.

Dominance hierarchies are a fundamental part of social systems in many species and social rank can influence access to resources and impact health and physiology. While social subordination is a profound stressor, few studies consider the social stress experienced by dominant males due to constantly needing to defend their dominance status through costly aggressive displays. Recent studies suggest that in species that use body coloration to signal status, these costs may also be color morph-specific. Our study examines the link between the social rank, intensity of territorial defense, body coloration, and oxidative stress in males of the color polymorphic cichlid fish *Astatotilapia burtoni* where males are either blue or yellow. We studied behavior in naturalistic communities and examined circulating reactive oxygen metabolites (ROMs) and antioxidant defenses. We found that dominant males experience higher concentrations of circulating ROMs without notably increasing their antioxidant defenses, but this effect was not related to color morph. Aggression and social network ties predicted oxidative stress in a morph-specific manner, with yellow but not blue males showing signs of increased oxidative damage with increasing agonistic effort. In contrast to expectation, oxidative stress was not influenced by cortisol or testosterone levels. We conclude that oxidative stress is instrumental to understanding the costs and benefits of high social rank.

Key words: **dominance hierarchy, oxidative stress, social stress.**

INTRODUCTION

Dominance hierarchies are a fundamental aspect of social systems in many species, determining access to resources and mating opportunities, and influencing the health and overall physiology of hierarchy members (Sapolsky 2004; Briffa and Sneddon 2007; Creel et al. 2013). In many hierarchies where rank is enforced by dominant individuals through psychological intimidation or aggression, subordinate individuals have been found to experience heightened glucocorticoid levels, which can result in long-term health consequences (Abbott et al. 2003; Gilmour 2005; Sapolsky 2005), However, in hierarchies where dominant individuals must engage in frequent physical altercations to hold their positions, or where reproductive skew is very high, the situation is often reversed (Creel 2001; Gesquiere et al. 2011). Additionally, while variation in circulating glucocorticoid levels has been characterized across a number of different hierarchy types, other physiological consequences of maintaining high social rank in different social environments are less studied (Gilmour 2005; Creel et al. 2013; Blumstein et al. 2016).

Oxidative stress, defined as an imbalance between the generation of reactive oxygen species (ROS) and their neutralization by antioxidant defenses resulting in higher levels of oxidative damage, appears a likely mechanism mediating life-history strategies (Bize et al. 2008; Metcalfe and Alonso-Alvarez 2010). Because oxidative stress can be influenced by the social environment (Miyashita et al. 2006; Lardy et al. 2016), it has been proposed as one of the costs of holding a high dominance rank that could limit investment in life-history functions such as lifespan and reproductive success (Bize et al. 2008; Speakman et al. 2015). The majority of ROS are generated during normal aerobic metabolism, and although a certain amount are required for cellular signaling and immune function, certain conditions such as reproduction, heightened activity, or increased sexual signaling (all common traits in dominant individuals), may lead to oxidative stress through increased production of ROS or reallocation of resources from antioxidant defense (Bedard and Krause 2007). Studies in birds and primates have found higher oxidative stress in dominant (breeding) individuals (van de Crommenacker et al. 2011; Beaulieu et al. 2014; Cram et al. 2015; Georgiev, Thompson, et al. 2015), however, such studies have mainly been performed in seasonal breeders with relatively stable hierarchies. However, as with glucocorticoid distribution it is important to account for the effects of social environment. Dominance hierarchies can be unstable, and the social stress resulting from increased competition in unstable hierarchies can cause oxidative stress, especially in higher ranked males who maintain

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rank by frequent fighting (Alonso-Alvarez et al. 2008; Metcalfe and Alonso-Alvarez 2010; Beaulieu et al. 2014; Thompson and Georgiev 2014). For example, Fasel et al. (2017) found that in Seba's short-tailed bats (*Carollia perspicillata)*, a species with a dynamic hierarchy where males cycle through both harem and sneaker mating tactics over time, harem males experienced a higher ratio of oxidized to reduced glutathione (an endogenous antioxidant) in blood than sneaker males but not increased lipid peroxidation (oxidative damage), suggesting that the active behavioral patterns required to monopolize access to females may require heightened levels of cellular metabolism and increased compensatory investment to avoid damage. In free-ranging rhesus macaques *(Macaca mulatta*), increased social instability, as measured by rates of attack, led to increased DNA damage, suggesting that social instability can contribute to oxidative stress (Georgiev, Muehlenbein, et al. 2015). In addition, in species that show variation in body coloration, the cost of increased social instability and agonistic effort can be related to color morph. For example, in color polymorphic species, morphs that require more carotenoids in their integument to express yellow–red colors may face different tradeoffs between behavior and oxidative stress relative to morphs that do not express yellow–red color because carotenoids must be obtained via consumption (and may therefore be limited) and also serve important functions for health maintenance (Dijkstra et al. 2011; Garratt and Brooks 2012). These morph-specific physiological costs may help explain the persistence of color variation when morphs represent alternative life-history strategies (Clotfelter et al. 2007; Pryke et al. 2007).

In this study, we use the cichlid fish *Astatotilapia burtoni*, to investigate how social status, agonistic behavior, and the stability of the social community impacts oxidative stress. This species lives in groups consisting of a small number (~10–30%) of reproductively active dominant males that hold territory through a combination of chasing and display behaviors, along with nonterritorial, reproductively suppressed subordinate males that school together with females (Fernald and Hirata 1977; Maruska and Fernald 2018). Dominant males in this species have lower cortisol levels than subordinate males (Fox et al. 1997) and males can rapidly change between dominant and subordinate status depending on changes in the social scene (Huffman et al. 2012; Maruska et al. 2013; Maruska and Fernald 2013). Males exhibit yellow and blue color morphs, and can reversibly switch between morphs (Korzan et al. 2008; Dijkstra et al. 2017). The different morphs have been found to show unique behavioral and physiological profiles. Yellow males are more aggressive than blue males, and yellow tend to have lower cortisol levels than blue males (Korzan et al. 2008; Dijkstra et al. 2017). We quantified the development of social relationships and social network structure in replicated tanks over a period of 19 days, perturbing the social stability after 15 days using male removals. At the end of the experimental period we collected blood for oxidative stress and hormone measurements. We predicted that 1) oxidative stress would be higher in dominant males than subordinate males due to dominant male's increased investment in reproduction and territorial defense, 2) oxidative stress would be higher in socially unstable tanks, especially for dominant males, 3) oxidative stress would be higher in dominant yellow males compared with blue males, and 4) increased agonistic effort and the prominence of a male within a social network would be associated with elevated oxidative stress among dominant males, but more so in yellow males. Given the proposed links between cortisol, testosterone and oxidative stress (Alonso-Alvarez et al. 2007; Hoogenboom et al. 2012), we also predicted that 5) hormones levels would be related to variation in oxidative stress.

METHODS

Animals and housing

For this experiment, adult *A. burtoni* were bred from a laboratory population originally derived from Lake Tanganyika, Africa (Fernald and Hirata 1977). The fish were housed in 120-L tanks, maintained at 28 °C on a 12:12 h light/dark cycle, and fed cichlid flakes (Omega Sea ltd.) every morning. All experimental tanks were setup with partial terra cotta pots placed in each corner to create 4 defendable "caves" per tank. A total of 16 communities (unstable, $n = 9$; stable, $n = 7$) were studied, with one community per tank, each composed of 12 males ($n = 192$) and 14 females ($n = 224$). Fish were approximately 1.5–2 years of age. All fish were individually tagged through the dorsal musculature, using a stainless steel tagging gun and colored beads. After their formation, all communities were given 4 weeks to settle before observation began. All procedures were approved by Central Michigan University Institutional Animal Care and Use Committee.

Behavioral observation

Communities were filmed over a 19-day period (on days: 1, 3, 5, 7, 9, 11, 13, 14, 15, 16, 17, 18, and 19) for 10 min per session for later quantification using a Canon EOS Rebel T5i. All filming was performed at least 10 min after feeding between 8:30 and 10:00 AM (lights turned on at 7:00 AM). Using all-occurrence sampling for each video, the following behaviors were recorded among all members in a community: fleeing, lateral displays, border displays, and chasing (Fernald 1977). For each video, individual males were categorized as either dominant or subordinate. Dominants were categorized as expressing bright coloration, behaving more aggressively than subordinates, and using a cave as the focal point of territorial defense (Desjardins et al. 2012). We also categorized all males as yellow or blue (Korzan et al. 2008).

Experimental manipulation

Communities were randomly assigned to 2 treatments: in the experimental treatment, a dominant male was removed from an experimental community and a mock removal was performed on a randomly chosen subordinate male by netting and immediately releasing him. In the control treatment, a subordinate male was removed from the community and a mock removal was performed on a randomly chosen dominant male. The dominant removal treatment was performed to experimentally alter the social structure by making available a previously occupied territory ("unstable communities"). The subordinate removal controlled for the change in fish density in the dominant removal communities ("stable communities"). Male removals were performed after the filming on day 16.

Sampling methods

Blood was drawn after filming on day 19 for hormone and oxidative stress measurements (removed males were sampled on day 16). All males in a given tank were removed at the same time and placed in several buckets with water. Males were weighed and their standard length was measured before blood was drawn. Blood time for each male was measured as the time from initial disruption of the tank (when the lid was removed) to the completion of blood collection for

that individual (blood collection time ranged from 1.9 to 18.5 min) with individuals processed in a randomized order. Blood was drawn through the dorsal aorta using heparinized 26-gauge butterfly needles (Terumo) and transferred to heparinized centrifuge tubes that were placed on ice until centrifugation was performed for 10 min at 4000*g*. The plasma was stored at −80 °C until used for analysis. We collected approximately 25–100 µL of plasma each from 156 males but were unable to obtain blood from 36 males. We had enough plasma from 96 males to perform both hormone and oxidative stress measurements, after blood was drawn, males were placed in a bucket and sacrificed via spinal cord dislocation within 10 min after blood collection.

Oxidative stress and hormone measurements

Measurement of reactive oxygen metabolites

Concentration of reactive oxygen metabolites (ROMs) (primarily hydroperoxides) in blood plasma was measured using the d-ROM test (Diacrom, Grosseto, Italy) as in previous studies of cichlids (Dijkstra et al. 2011; Dijkstra et al. 2016). ROMs are produced as a result of interactions between ROS and various cellular components (proteins, lipids, and DNA) and so can serve as a general measurement of oxidative damage (Costantini 2016). Each plasma sample (4 µL) was run in duplicate with 200 µL of a master mix (pH 4.8, 0.01M acetic acid/sodium acetate buffer containing N,N-diethylparaphenilendiamine as chromogen). Samples were incubated at 37 °C for 75 min, and absorbance was read at 490 nm by a plate reader (Spectramax M3, Molecular Devices, Sunnyvale, CA). Each plate also included a blank consisting of master mix and ultrapure water, a calibration using 240 mg H_2O_2/dL as a reference standard, and a sample of uniform plasma for interplate variability analysis. The intra-assay CV was 3.8%, and interassay CV was 6.7%.

Measurement of antioxidant capability

Total antioxidant capability (TAC) was measured in blood plasma via an ORAC (Oxygen Radical Absorbance Capacity) assay (Lucas-Abellán et al. 2008; Wilson et al. 2012; Bodera et al. 2013). Fluorescence was measured with an excitation wavelength of 485 nm, an emission wavelength of 515 nm, and a cutoff filter wavelength of 495 nm. Assays were run on black-sided 96-well plates, using a plate reader maintained at 37 °C. Each well in the outer ring contained 200 µL of pure water to increase stability. The other wells contained 120 µL fluorescein in 75 mM potassium phosphate (3.82 µM final concentration), and 20 µL of either diluted plasma (1:100 plasma:buffer), blank (75 mM potassium phosphate, pH 7.4), or standard (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid in 75 mM potassium phosphate (Trolox); 0–400 µM initial concentration). All measures were done in duplicate. Each plate was incubated at 37 °C for 30 min, then 60 µL of 2.2′-azobis (2-amidinopropane) dihydrochloride in 75 mM potassium phosphate (AAPH, 79.83 mM final concentration) was rapidly added via multichannel pipette and fluorescence was read every 35 s for 60 min using a Spectramax M3 (Molecular Devices). Area under the curve (AUC) was calculated for each well by dividing the fluorescence at each time point by the initial fluorescence for that well, then summing the results for all data points. Net AUC for samples and standards was found by subtracting the AUC of the blank from the AUC for the sample/standard; then the standards were used to determine the TAC of each sample (reported in Trolox equivalents). The intra-assay CV was 1.4%, and interassay CV was 8.4%.

Measurement of cortisol and testosterone

We quantified circulating cortisol and testosterone levels using competitive ELISA kits (Enzo Life Sciences) as done previously in *A. burtoni* (O'Connell and Hofmann 2012; Dijkstra et al. 2017). Plasma samples were diluted 1:30 in assay buffer according to Kidd et al. (Kidd et al. 2010) and processed according to the manufacturer's instructions. Absorbance was measured using the SpectraMax M3 (Molecular Devices). Overall, the intra-assay CV were 3.5% and 3.3% for cortisol and testosterone, respectively. The interassay CV (coefficient of variation) were 5.4% and 7.7% for cortisol and testosterone, respectively.

Statistics

All analyses were conducted in R v3.4.3 (2017) (R Core Team, 2012). Social network analysis is a quantitative framework to describe social relationships and dynamics (Dey et al. 2013; Pinter-Wollman et al. 2014; Solomon-Lane et al. 2015). We reconstructed social networks based on chase and display rates using the package igraph (Csárdi and Nepusz 2006). Using these networks, we extracted weighted OutDegree (WO, the sum total of an individual's behavior each day) for both chase and display networks (see Figure 1 for example networks). We also calculated weighted degree centrality (WDC, Candeloro et al. 2016) for both chase and display

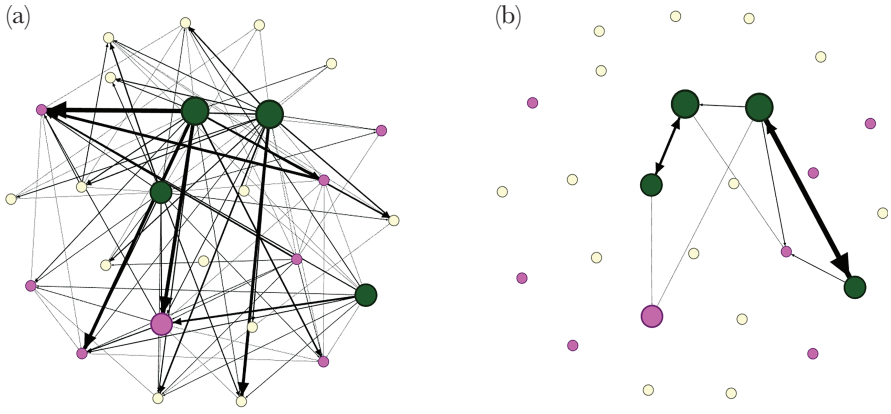

Figure 1
Weighted social networks for (a) chase behavior and (b) display behavior. Each node represents an individual, and individuals are in the same place in both graphs. Green nodes are dominant males, purple nodes are subordinate males, and tan nodes are females. Edges are weighted based on the number of behaviors (chases or displays) directed at a specific target, nodes are weighted based on the total number of behaviors that node produced.

networks. WDC is a combined measure of agonistic intensity and number of interaction partners, allowing us to test how prominence of a given male within a social network affected his oxidative stress. We used WO and WDC for each male from their network on the day they were sampled. We used cosine similarity (a measure of consistency in behavioral patterns; see Jarrett et al. 2016), based on chase networks and averaged across the duration of the experiment, as a measure of individual stability for each male to test whether individual changes in agonistic relationships influences oxidative stress. We used linear mixed models (LMMs, with tank as a random effect) with a maximum-likelihood protocol (R package lme4, Bates et al. 2015) to model our data and all models within 2 AICc units of the top model are reported. We report *P*-values and 95% confidence intervals of parameter estimates of all fixed effects. We square-root transformed cortisol and log transformed testosterone, chase, and display data to ensure normality, and calculated the residuals for both ROM and cortisol levels (to control for restraint stress from time from netting the male to the blood draw) which were used for analyses. We fitted LMM with ROM or TAC as dependent variables and social status (subordinate or dominant), male removal treatment (stable or unstable), color morph (yellow or blue), chased-based WO, display-based WO, chase-based WDC, display-based WDC, cosine similarity, cortisol, and testosterone, as predictors. As testosterone and cortisol were strongly correlated with social status, they were never included in models examining the effects of status on oxidative stress measurements, and relationships between hormones and oxidative stress were evaluated independently in dominants and subordinates. In all model summaries, the reference category for fixed effects were determined alphabetically or numerically: blue (vs. yellow, for color morph), dominant (vs. subordinate for status), stable (vs. unstable communities). Only males with oxidative stress measurements (n = 96) were included in the analyses. For all analyses, model residuals were examined to ascertain assumptions of normality and homogeneity of variances were met.

RESULTS

Effects of status, color morph, and social stability on oxidative stress

Neither male removal treatment nor average cosine similarity had a significant effect on ROMs (LMM, effect of treatment: −0.09, 95% confidence interval [CI]: −0.56–0.39, P = 0.767, Figure 2a; LMM, effect of individual stability: 0.23, 95% CI: −0.80–1.26, P = 0.715, Figure 2b) or TAC (LMM, effect of treatment: −21.37, 95% CI: −91.24–48.49, P = 0.624, Figure 2c; LMM, effect of individual stability: 99.91, 95% CI: −45.96–245.77, P = 0.263, Figure 2d) contrary to expectation. In addition, dominant males who ascended (n = 4) or expanded their territory to encompass 2 flowerpots (n = 1) within the final 3 days were compared to their

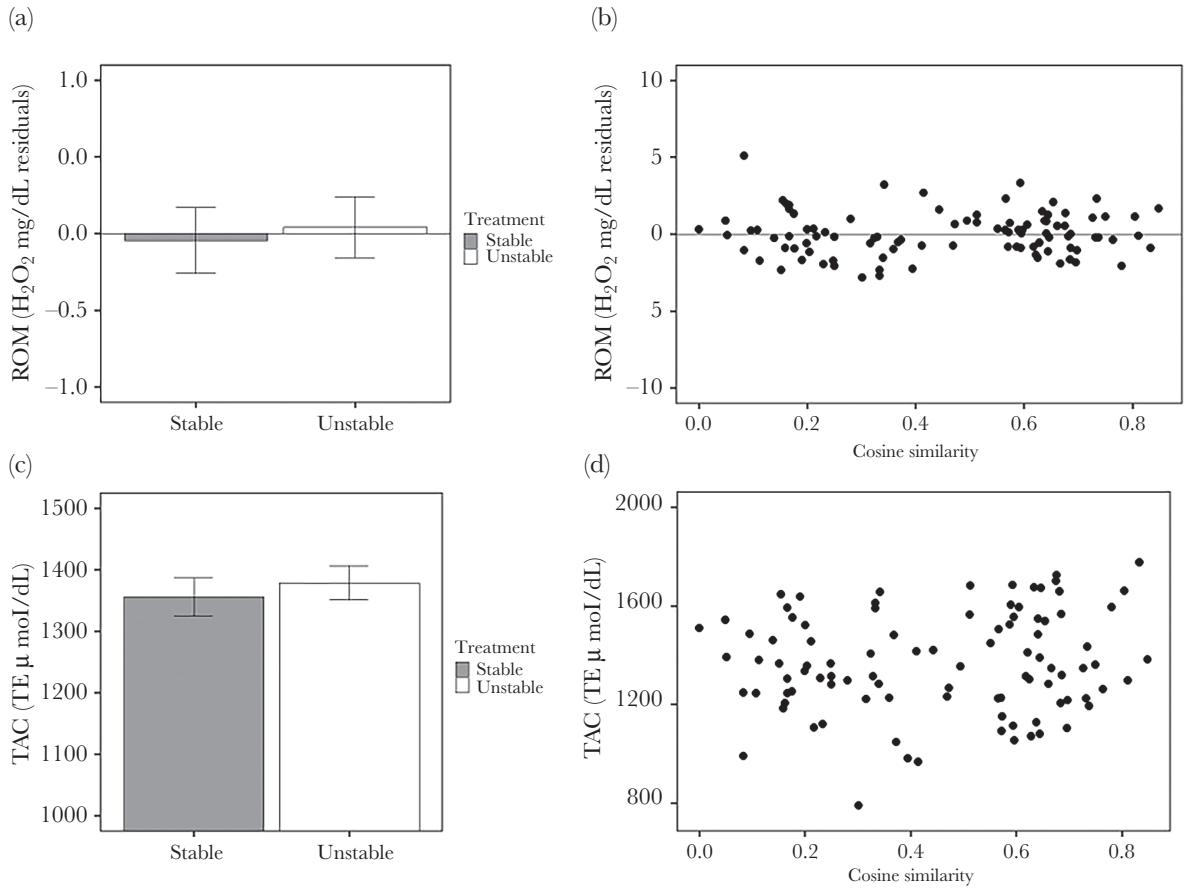

Figure 2
Neither removal treatments nor individual stability influenced ROMs or TAC. (a) mean ± SE ROM residuals and removal treatment, (b) ROM residuals and average individual cosine similarity (a measurement of stability), (c) mean ± SE TAC and removal treatment, and (d) TAC and average individual cosine similarity. Sample size: stable, n = 47, unstable, n = 49.

stable dominant tankmates, but we found no differences in either ROMs (LMM, effect of status change: 0.20, 95% CI: −0.90–1.30, $P = 0.764$) or TAC (LMM, effect of status change: 62.33, 95% CI:−112.00–236.67, $P = 0.564$).

Dominant males had higher levels of circulating ROMs than subordinate males (LMM, effect of status: −0.67, 95% CI: −1.23–−0.11, $P = 0.02$, Figure 3a), indicating that they experienced higher overall oxidative damage. TAC was found to be primarily influenced by weight rather than status (LMM, effect of weight: 46.17, 95% CI: 14.30–78.04, $P = 0.005$) but a model containing weight and status as covariates was also retained (LMM, effects of status: −47.62, 95% CI: −127.03–31.78, $P = 0.240$, Figure 3b; effect of weight: 43.08, 95% CI: 11.02–75.13, $P = 0.008$). Neither ROMs nor TAC were significantly different between blue and yellow dominant males (both $P > 0.4$)

Effects of color morph and aggression on oxidative stress

We did not find any difference in chase-based WO, ROMs, or TAC between yellow and blue dominant males ($P > 0.4$). However, in dominant males chase-based WO had morph-specific effects on ROMs (LMM, chase-based WO × morph: 1.76, 95% CI: 0.81–2.70, $P = 0.004$, Figure 4a) driven by the significant negative correlation between chase-based aggression rate and ROMs for blue males and an opposing significant positive trend in yellow males (LMM, effect of chase-based WO in blue dominants: −0.79, 95% CI: −1.29–−0.48, $P = 0.008$; effect of chase-based WO in yellow dominants: 0.86, 95% CI: 0.14–1.59, $P = 0.062$). In addition, there was a marginally nonsignificant interaction effect between chase-based WDC and morph on ROMs in dominant males (LMM, chase-based WDC × morph: 0.25, 95% CI: 0.04–0.47, $P = 0.058$, Figure 4b). We found no effects of display behavior (either display-based WO or display-based WDC) on ROMs, with or without morph interactions (all $P > 0.5$, Figure 4c,d).

TAC was not affected by chase-based WO (LMM, effect of chase-based WO: 60.54, 95% CI: −20.52–141.60, $P = 0.227$, Figure 5a) but positively correlated with chase-based WDC (LMM, effects of chase-based WDC: 20.29, 95% CI: 4.08–36.51, $P = 0.047$, Figure 5b). Display-based WO was positively correlated with TAC (LMM, effects of display-based WO: 176.67, 95% CI: 39.65–313.70, $P = 0.041$, Figure 5c) but display-based WDC was not (LMM, effects of display-based WDC: 21.75, 95% CI: −63.19–106.69, $P = 0.676$, Figure 5d). An additional best-fit model for chase-based WDC retained weight, and in this model chase-based WDC was found to be marginally nonsignificant (LMM, weight: 33.23, 95% CI: −14.05–80.51, $P = 0.256$, chase-based WDC: 18.30, 95% CI: 2.14–34.46, $P = 0.071$). Likewise, an additional best-fit model for display-based WDC retained weight and a marginally nonsignificant effect of display-based WO (LMM, weight: 39.88, 95% CI: −7.23–86.99, $P = 0.172$, display-based WO: 159.99, 95% CI: 25.06–294.91, $P = 0.059$). We found no morph-specific effects of aggression on TAC (all $P > 0.2$).

Hormones and oxidative stress

As expected, cortisol was higher in subordinate than dominant males (LMM, status: 34.59, 95% CI: 5.42–63.76, $P = 0.022$, Table 1), while testosterone was higher in dominant males (LMM, effect of status: −40.89, 95% CI: −48.83–−32.95, $P < 0.001$, Table 1), although neither was influenced by the male removal treatment ($P > 0.1$). Contrary to expectations, testosterone levels did not predict ROMs in either dominant (LMM, testosterone: −0.66, 95% CI: −1.39–0.07, $P = 0.148$) or subordinate males (LMM, testosterone: −0.17, 95% CI: −0.67–0.32, $P = 0.569$). Testosterone levels also did not predict TAC in either dominant (LLM, testosterone: 14.30, 95% CI: −108.19–136.79, $P = 0.849$) or subordinate males (LLM, testosterone: 10.54, 95% CI: −55.71–76.80, $P = 0.794$). Likewise, cortisol levels did not predict ROMs in dominant (LMM, cortisol: −0.09, 95% CI: −0.21–0.04, $P = 0.262$) or subordinate males (LMM, cortisol: −0.06, 95% CI: −0.13–0.01, $P = 0.159$). Similarly, cortisol did not predict TAC in dominant (LMM, cortisol: 14.89, 95% CI: −5.03–34.80, $P = 0.226$) or subordinate males (LMM, cortisol: −1.37, 95% CI: −11.16–8.42, $P = 0.818$).

DISCUSSION

Oxidative stress has been suggested as a potential cost of maintaining high social status. We expected that oxidative stress would be a cost of dominance in *A. burtoni* in both stable and unstable hierarchies because dominant males in this species engage in costly territorial defense and have an upregulated reproductive axis (Maruska and Fernald 2011; O'Connell and Hofmann 2012). We expected

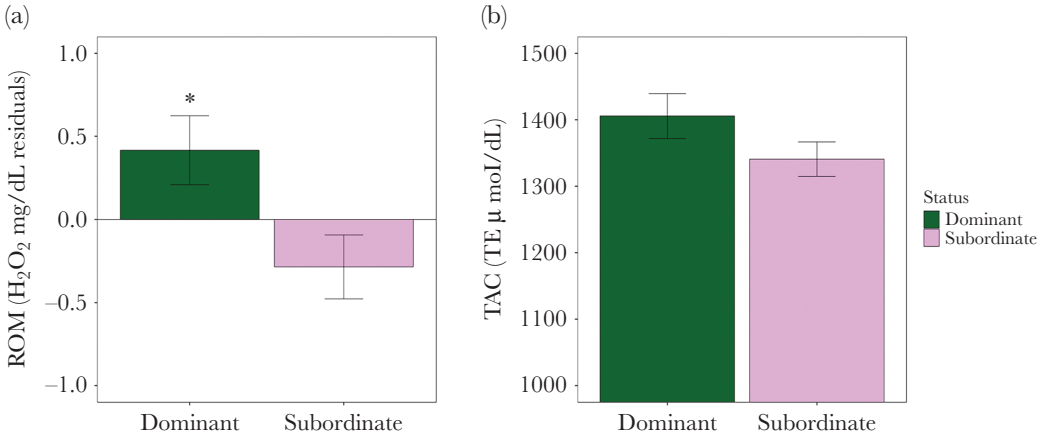

Figure 3
Dominant males have higher ROMs than subordinates, but there is no difference in TAC. (a) Mean ± SE ROM residuals after controlling for restraint time; dominants, $n = 42$, subordinates, $n = 65$, (b) mean ± SE TAC measured in Trolox equivalents (TE); dominants, $n = 39$, subordinates, $n = 57$. Asterisks indicate significance. *$P < 0.05$.

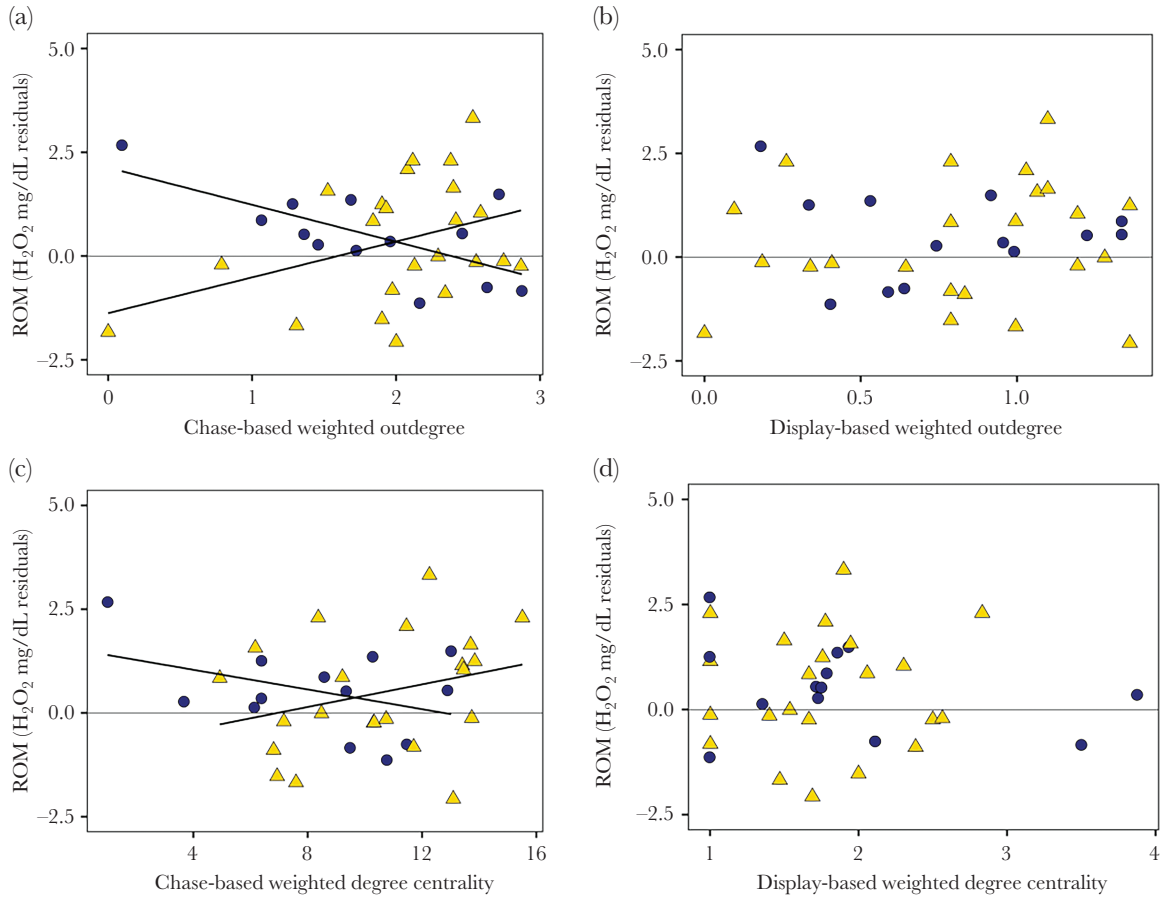

Figure 4
ROMs have a morph-specific effect relative to active aggression but not display behavior in dominant males. Panels a and b: ROMs and (a) chase-based WO (log transformed chases/min) and (b) display-based WO (log transformed displays/min) in dominant males. Panels c and d: ROMs and (c) chase-based WDC (number of individuals chased weighted by distribution of chases) and (d) display-based WDC (number of individuals displayed at weighted by distribution of displays). Trend lines indicate significant (a) or near significant (c) interaction effects. Sample size: blue, $n = 13$, yellow, $n = 23$.

the cost of dominance to be higher in unstable hierarchies as they require dominant males to exert more effort in maintaining their status. Our results demonstrated that dominant males, regardless of treatment, endured increased levels of ROMs relative to subordinates, representing increased oxidative damage. Increased oxidative damage may be caused by an increase in reactive species generation, a decrease in investment in antioxidant defenses, or a combination of both (Monaghan et al. 2009). We found that the major factor in determining circulating antioxidant capacity was body weight, however, and that dominant males did not have significantly different antioxidant levels than subordinates. This suggests that dominant individuals experienced an increase in production of reactive species without increasing their investment in antioxidant defenses. Our study is consistent with the observation that oxidative stress or damage is increased in dominant males that engage in territorial aggression in the mandrill (*Mandrillus sphinx*) (Beaulieu et al. 2014) and in the house mouse (Garratt et al. 2011). In the white-browed sparrow weaver (*Plocepasser mahali*), oxidative damage was not related to rank alone, although it was increased in dominant, breeding females (Cram et al. 2015).

The cost of social dominance could result from investment in a variety of traits involved in the upregulation of the reproductive axis and in maintaining high social status (Alonso-Alvarez et al. 2007; Metcalfe and Alonso-Alvarez 2010). In haplochromine cichlids, dominant males are highly aggressive and upregulate their reproductive axis (Maruska and Fernald 2011; O'Connell and Hofmann 2012) resulting in elevated metabolic rate (Dijkstra et al. 2013), increased nuptial coloration (Dijkstra et al. 2007), and increased gonadal size (White et al. 2002; Maruska and Fernald 2010). Since metabolic rate can be linked to oxidative stress, we predicted that increased social competition—as measured by both rate of aggression (WO) and our combined measure of agonistic intensity and the way aggression was distributed towards community members (WDC)—would be linked to increased oxidative stress in dominant males. We did not find an effect of the rate of aggression or prominence in the social network in dominant males on their oxidative damage, but TAC was positively related to some social network measures.

We also predicted that because of their carotenoid-based nuptial coloration (which is presumably more costly to produce), yellow dominant males would display higher levels of oxidative stress than blue dominant males. However, neither oxidative damage (ROMs) nor antioxidant capacity (TAC) differed between color morphs. This finding is not consistent with the observation that oxidative stress was higher in the territorial males of the cichlid *Pundamilia nyererei*, a cichlid with red, carotenoid-based nuptial coloration, compared

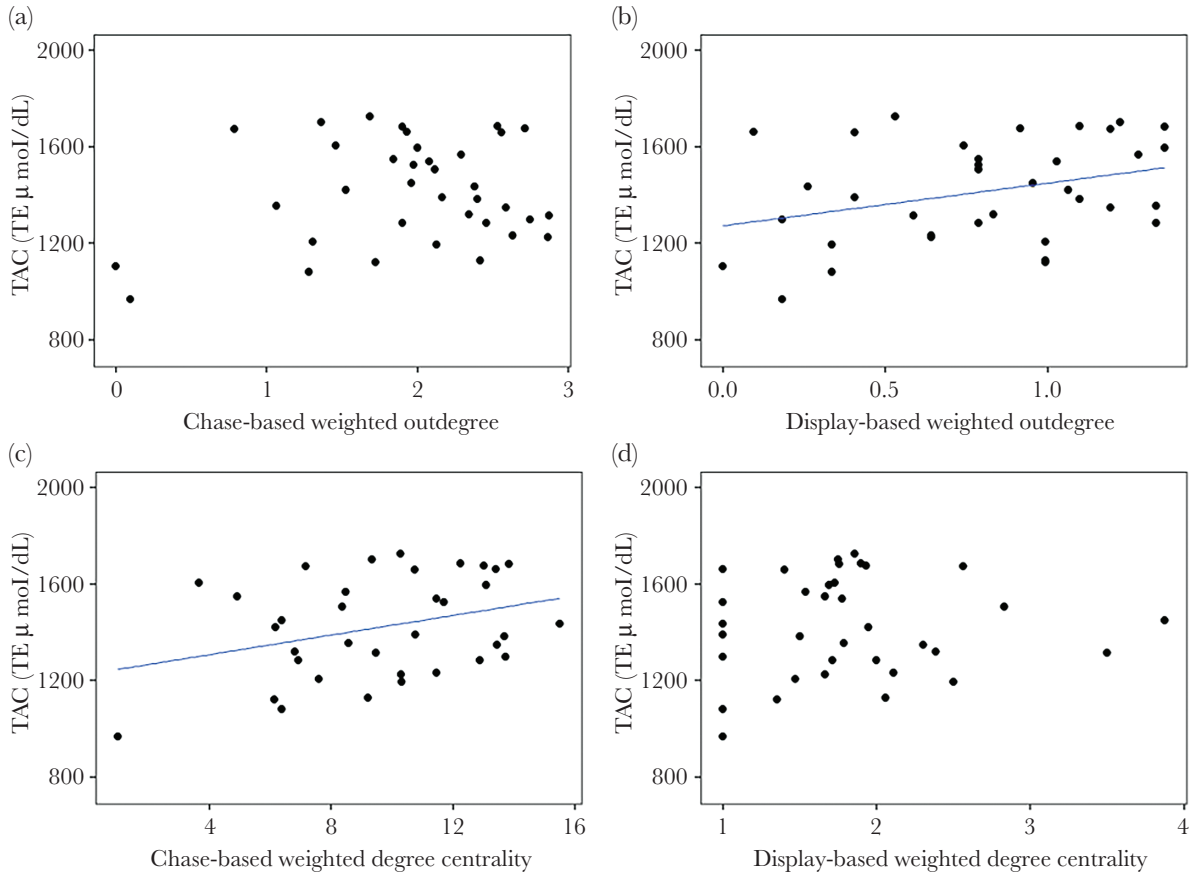

Figure 5

TAC is related to aggression distribution and display rate in dominant males. Panels a and b: TAC and (a) chase-based WO (log transformed chases/min) and (b) display-based WO (log transformed displays/min) in dominant males. Panels c and d: TAC and (c) chase-based WDC (number of individuals chased weighted by distribution of chases) and (d) display-based WDC (number of individuals displayed at weighted by distribution of displays). Trend lines indicate significant correlations. $N = 39$.

Table 1

Raw circulating cortisol, testosterone, and ROM levels, and residual cortisol levels, in dominant and subordinate males

	Dominant	Subordinate
Cortisol (raw) (ng/mL)	65.01 ± 10.15	124.09 ± 15.81
Cortisol (residuals) (ng/mL)	−1.024 ± 0.44	0.70 ± 0.57
Testosterone (ng/mL)	3.84 ± 0.07	2.18 ± 0.08
d-ROM (raw) H_2O_2 (mg/dL)	5.72 ± 0.22	4.79 ± 0.21

Shown are mean ± SE.

with territorial males of *Pundamilia pundamilia*, a closely related cichlid species with blue coloration (Dijkstra et al. 2011). Instead, to our surprise we found that there was a significant morph-dependent effect of the intensity of competition (measured as chase-based WO) on oxidative damage. In yellow males, increased aggression was associated with increased ROMs. However, in blue males, increased aggression was negatively correlated with ROMs. The positive relationship between aggression and oxidative damage in yellow males suggests that territorial defense is more costly for yellow males than for blue males. Von Schantz et al. (1999) suggested that sexually selected traits produced by carotenoids (many yellow–red ornaments) could enforce honest signaling of male quality to females due to the alternative use of carotenoids as antioxidants combined with their limited availability (as carotenoids cannot be synthesized de novo). According to this hypothesis, animals face a trade-off between allocating carotenoids to sexual signaling or antioxidant defense (Metcalfe and Alonso-Alvarez 2010). Our observation that yellow males were more sensitive to the effect of social competition than blue males is somewhat consistent with the *Pundamilia* example discussed earlier (Dijkstra et al. 2011). As in this study, carotenoid intake was not limited because fish were fed a cichlid specific flake food with several components containing carotenoids. However, the negative relationship between blue male aggression and oxidative damage is less easily explained. Blue males have been found to differ physiologically from yellow males in previous studies (Korzan et al. 2008; Dijkstra et al. 2017), and the factor mediating the link between oxidative stress and aggression may be related to these physiological differences. It is important to note that while carotenoids are believed to be the main source of yellow–red coloration in cichlid species (Sefc et al. 2014), pteridines (which are able to be synthesized de novo) are also able to be used as yellow–red pigments (Leclercq et al. 2010). Certain pteridine derivatives are known to influence oxidative balance and behavior (Hoekstra and Fekkes 2002; Oettl and Reibnegger 2002). Experiments are required to determine the role of these pigments in the morph-specific trade-off between oxidative balance and agonistic behavior.

Previous studies indicated that the yellow–blue color polymorphism is linked to unique behavioral and endocrine profiles (Korzan

et al. 2008; Dijkstra et al. 2017). Here, we did not detect differences in cortisol or testosterone levels between morphs, which are inconsistent with previous studies. Some of these differences in color effects between studies could be explained by differences between labs. Our study adds to the idea that the 2 color morphs are physiologically different, and it is tempting to hypothesize that they represent different strategies through which males could derive equal fitness. However, it is important to emphasize that color has no detectable heritability in *A. burtoni* (Dijkstra, unpublished data) and that males can change color repeatedly (Piefke, unpublished data). Future studies should investigate potentially adaptive strategies of the 2 color morphs and their ability to change color. Most studies on plastic color phenotypes focus on camouflage, background color adaptation, or ontogenetic color changes. However, we do not have evidence that the color polymorphism in *A. burtoni* is related to background coloration or ontogenetic changes. Rather, we believe color is linked to unique physiological setups that might be sensitive to environmental influences. The color morph-dependent effect on behavior and oxidative stress supports the idea that yellow and blue are physiologically unique phenotypes.

In our current study, we examined natural variation in the intensity of competition, making it difficult to determine the causal relationship between territorial aggression and oxidative stress (Isaksson et al. 2011). While increased aggression can result in increased oxidative stress via an uptick in production of reactive species, there is some evidence that individuals facing an increased oxidative challenge may increase aggression either as a direct behavioral response (a form of terminal investment) or due to changes in brain chemistry caused by oxidative damage (Garratt and Brooks 2015). A study using SOD1 (superoxide dismutase-1, an enzyme involved in the neutralization of superoxide) knockout mice found that SOD1$^{-/-}$ mice exhibited significantly more aggression than heterozygous and wild-type mice (Garratt and Brooks 2015). Additionally, a recent study found that isolation and L-DOPA (L-3,4-dihydroxyphenylalanine) induced aggression in mice could be significantly reduced by injection of antioxidants (Hira et al. 2018). This is consistent with the studies on the relationship between oxidative stress and anxiety, which suggest that increased oxidative stress can result in significant changes in brain chemistry and function in areas related to behavioral determination (Bouayed et al. 2009; Salim 2017). Previous studies suggest that in our species, yellow and blue males have unique neuroendocrine profiles (Korzan et al. 2008; Dijkstra et al. 2017). Disentangling the causal relationship between oxidative stress and aggression using manipulative studies where, for example, the rate of aggression is experimentally altered, is an exciting avenue for future research.

In contrast to expectation, neither the stability manipulation nor individual social stability had a significant effect on oxidative stress. Our manipulation involved removal of a single male which led to a transient increase of social competition over the vacant territory (Piefke et al. in preparation). Males of *A. burtoni* modulate their social behavior based on learning from past social interactions (Desjardins et al. 2012; Fernald and Maruska 2012), and so males in an established community may be able to settle ownership of a newly available territory and change social rank with minimal social stress. While the social manipulation led to changes in the social relationships and rank amongst males, the social hierarchy in most manipulated communities stabilized within 1 day (Piefke et al. in preparaton). In addition, previous studies suggest that males that ascend in status rapidly become established as a dominant males (Maruska and Fernald 2010), although Huffman et al. 2015 found that cortisol was still elevated 3 days after ascent (but see Maruska (2015) where cortisol was low the day after ascent). Hence, the physiological effects of the social manipulation may have already subsided by the time we collected blood 3 days after the male removal. We also emphasize that our experiments were carried out in a social community setting with more opportunities for buffering of social stress. In more isolated settings, social defeat can be extremely stressful. In a group setting, which more closely resembles the situation in the wild, these effects can be dramatically different (Williamson et al. 2016). Future experiments should examine how more dramatic changes in social structure impact oxidative balance, preferably using a longitudinal approach.

In accordance with previously published data in *A. burtoni* (Fox et al. 1997; Parikh et al. 2006), we found lower cortisol and higher testosterone in dominant males, however, patterns in circulating cortisol levels were not influenced by the male removal treatment. When we accounted for status-specific differences in hormone levels, we did not find a correlation between either of our hormone measurements and oxidative damage or antioxidant activity, contrary to expectations. Glucocorticoids may represent an important link between chronic stress and oxidative stress and damage, such that heightened cortisol may be expected to increase oxidative stress (Aschbacher et al. 2013). Male sex hormones, such as testosterone, may be expected to play an important role in modulating oxidative stress in dominant males by shifting the balance between ROS generation and antioxidants (Alonso-Alvarez et al. 2007). Testosterone may increase oxidative stress either indirectly by raising metabolic rate, or by directly promoting pro-oxidant mechanisms (Ros et al. 2004; Reckelhoff 2005; Metcalfe and Alonso-Alvarez 2010). However, other papers have shown that testosterone can also help to reduce oxidative stress by increasing circulating levels of carotenoids (Blas et al. 2006; Peters 2007; Alonso-Alvarez et al. 2008). In addition, oxidative stress in gonadal tissue can itself limit testosterone production (Glade and Smith 2015). In this study, both our oxidative damage marker and our antioxidant marker were broad scope measurements, and oxidative stress can vary widely between tissues (Tkachenko et al. 2014; Xu et al. 2014; Taylor et al. 2015). Future experiments should focus on isolating specific components of antioxidant defense and ROS generation in both whole-body and tissue-specific assays to accurately assess the relationship between oxidative stress, cortisol, and testosterone in this system.

Males of *A. burtoni* can change status reversibly and may ascend or descend depending on the social situation and their physiological state. Our findings support the notion that individuals pay a price for the reproductive benefits of territoriality in the form of increased oxidative damage. Dominant and descending males have reduced somatic growth compared to subordinates and ascending males (Hofmann et al. 1999) likely as a combined result of their heightened metabolic activity, investment in reproduction, and larger somatostatin-containing neurons (Hofmann et al. 1999; Hofmann and Fernald 2000). It is likely that the metabolic demands of upregulation of the reproductive axis and territorial defense increases the production of pro-oxidants resulting in increased oxidative damage which may prevent dominant males from maintaining dominant status for extended periods of time. Several males who lost status during the experiment were able to become dominant again, suggesting that males can recover and rise again. Oxidative stress has been linked to accelerated aging, decline of reproductive function, and increased susceptibility to infection and disease (Beckman and Ames 1998; Cui et al. 2012; Pohanka 2013). Although subordinate males are rarely able to reproduce and experience higher levels of cortisol, their lower level of oxidative damage may benefit future reproductive opportunities. Further

research will attempt to experimentally alter oxidative stress and will characterize tissue-specific oxidative damage and antioxidant allocation to clarify the link between oxidative stress and social rank, as well as the potential impact of male color morph on the relationship between aggression and oxidative stress.

FUNDING

The authors have no support or funding to report.

We thank members of the Dijkstra lab for providing helpful comments to earlier drafts of the manuscript. We would also like to thank Tyler Bonnell for his assistance with the cosine similarity measure.

Data accessibility: Analyses reported in this article can be reproduced using the data provided by Border et al. (2018).

Handling editor: John Fitzpatrick

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