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RESEARCH ARTICLE

Social instability influences rank-specific patterns of oxidative stress in a cichlid fish

Shana E. Border[1,2], Taylor J. Piefke[1], Tyler R. Funnell[1,3], Robert F. Fialkowski[1], Jacob Sawecki[1,3] and Peter D. Dijkstra[1,4,5,*]

ABSTRACT

In many animal societies, dominant individuals have priority access to resources. However, defending high rank can be costly, especially in unstable social hierarchies where there is more intense competition. Oxidative stress has been proposed as a potential cost of social dominance, but few studies have examined this cost in relation to social stability. We studied the cost of social dominance in the cichlid fish *Astatotilapia burtoni* by manipulating social stability among males in replicate naturalistic communities for 22 weeks. We found that our social stability treatment influenced status-specific patterns in 3 out of 6 measurements of oxidative stress. Specifically, dominant males experienced increased plasma oxidative damage (measured as reactive oxygen metabolites, ROMs) compared with subordinate males in stable hierarchies only. Subordinate males in unstable hierarchies had higher ROMs than their stable community counterparts, but we found no effect of social stability treatment for dominant males. However, dominant males tended to have reduced total antioxidant capacity (TAC) in the liver when compared with subordinate males in unstable hierarchies, suggesting that the cost of social dominance is higher in unstable hierarchies. There were no effects of status and treatment on gonad TAC, muscle TAC or oxidative DNA damage. We conclude that the stability of the social environment influences the relative cost of social dominance in a tissue- and marker-specific manner.

KEY WORDS: Social stability, Habitat stability, Dominance, Oxidative stress

INTRODUCTION

In many hierarchical species, social rank has a major impact on health and fitness. High rank is associated with priority access to mates and resources, while lower ranked individuals may suffer from increased disease susceptibility and infertility as a result of chronic stress (Abbott et al., 2003; Briffa and Sneddon, 2007; Creel et al., 2013; Gilmour, 2005; Losdat et al., 2019; Sapolsky, 2004). However, maintaining high social rank comes at a cost (Verhulst et al., 2014), especially when the hierarchy is unstable. Unstable social hierarchies, whether due to environmental changes or changes in group membership, cause an unpredictable social environment with increased competition for dominance (Creel, 2001; Gesquiere et al., 2011; Piefke et al., 2021). Characterization of rank-specific costs and benefits can provide critical insights into the evolution of dominance hierarchies and behavioral variation within hierarchies.

Several studies indicated that oxidative stress is a potential cost of high rank (Dijkstra et al., 2011; Beaulieu et al., 2014; Cram et al., 2015; Georgiev et al., 2015a). Oxidative stress is defined as an imbalance between reactive oxygen species and antioxidant defenses with the balance tipped towards the former (Bize et al., 2008; Metcalfe and Alonso-Alvarez, 2010). There is evidence that oxidative stress can cause cellular level damage and increase the risk for a host of diseases (Cui et al., 2012; Kono et al., 2000; Li et al., 2015; Pohanka, 2013). Oxidative stress has therefore been suggested to play a key role in mediating life history trade-offs, such as between investment in reproduction and longevity (Bize et al., 2008; Dowling and Simmons, 2009; Metcalfe and Monaghan, 2013; Noguera, 2019; Speakman et al., 2015). While social subordination can be a source of chronic stress through elevated glucocorticoid hormones (Abbott et al., 2003; Gilmour, 2005; Sapolsky, 2005), maintaining high dominance status involves potentially costly aggressive interactions that could result in an increase in reactive oxygen species and/or depletion of antioxidant defenses and consequently increased oxidative damage (Creel, 2001; Gesquiere et al., 2011; Silva et al., 2018; but see Isaksson et al., 2011). It appears that the relative cost of rank depends on the social stability of a community and the types and frequency of behaviors used to maintain rank, as well as investment required for reproduction (Alonso-Alvarez et al., 2008; Beaulieu et al., 2014; Georgiev et al., 2015b; Metcalfe and Alonso-Alvarez, 2010; Thompson and Georgiev, 2014). One would expect that increased physical activity associated with agonistic effort during periods of social instability, combined with increased rank uncertainty, places an increased oxidative burden on dominant males relative to subordinate males when the dominance hierarchy is unstable. In line with this reasoning, Beaulieu et al. (2014) found that in one population of male mandrills (*Mandrillus sphinx*), dominant males had the lowest oxidative damage outside the breeding season but experienced an increase in oxidative damage during the breeding season (when both reproductive investment and social competition were highly elevated). However, most studies have relied on natural variation in social stability or agonistic intensity among members of a single community with no experimental manipulation to control the level of social (in)stability. Since dominance hierarchies can be highly variable within a single species, we need to study the link between rank, social stability and oxidative stress in replicate communities (Metcalfe and Monaghan, 2013; Isaksson et al., 2011; Alonso-Alvarez et al., 2008). Finally, most studies rely on a single measure of oxidative stress, and only in blood or a single tissue type. However, oxidative stress can be highly tissue specific. For example, organisms can selectively increase antioxidant defences

[1]Central Michigan University, Department of Biology, Mount Pleasant, MI 48859, USA. [2]School of Biological Sciences, Illinois State University, Normal, IL 61790, USA. [3]Department of Fisheries and Wildlife, Michigan State University, East Lansing, MI 48824, USA. [4]Neuroscience Program, Central Michigan University, Mount Pleasant, MI 48859, USA. [5]Institute for Great Lakes Research, Central Michigan University, Mount Pleasant, MI 48859, USA.

*Author for correspondence (dijks1p@cmich.edu)

P.D.D., 0000-0001-5382-225X

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to limit oxidative stress in specific tissue types when territorial and/or reproductively active (Garratt et al., 2012; Wilson et al., 2014; Xu et al., 2014). In Brandt's voles (*Lasiopodomys brandtii*), when compared with non-reproducing females, lactating females have decreased activity of the antioxidant enzyme superoxide dismutase (SOD) and increased protein damage in serum, but increased SOD activity and decreased protein damage in liver tissue (Xu et al., 2014). In order to fully understand the effects of social rank on oxidative stress, it is imperative that we examine multiple components of oxidative stress in a variety of tissue types.

Here, we investigate how social status and the stability of the social hierarchy impact oxidative damage and antioxidant capacity in the highly social cichlid fish *Astatotilapia burtoni* (Maruska and Fernald, 2018). This species lives in groups consisting of a small number of reproductively active dominant males that hold territory via aggressive behaviors and displays along with non-territorial, reproductively suppressed subordinate males that school together with females (Fernald and Hirata, 1977). Dominant males in this species exhibit bright nuptial coloration (Dijkstra et al., 2017; Korzan et al., 2008) and have lower cortisol levels than subordinate males (Fox et al., 1997). Males can rapidly change between dominant and subordinate status depending on changes in the social environment (Alward et al., 2019; Huffman et al., 2012; Maruska et al., 2013). We previously found that dominant males have higher levels of oxidative damage in plasma compared with subordinate males (Border et al., 2019; Fialkowski et al., 2021) and this pattern was not influenced by manipulation of short-term social stability [consisting of removal of a single male in each experimental tank 3 days prior to blood collection (Border et al., 2019)]. In the current study, we experimentally induced long-term social instability by changing the number and arrangement of flowerpots on a regular basis in mixed-sex replicate communities for 22 weeks, with 'stable' communities where flowerpots were instead removed and replaced in the same position (Fig. 1). Flowerpots are used as a focal point for territorial defense and reproductive behavior, and this manipulation therefore led to rank changes and intense competition over territory in communities with unpredictable environmental changes compared with stable environments. At the end of the experimental period, we collected blood and tissue for oxidative stress measurements. If rank-dependent oxidative stress is tissue specific and influenced by the degree of social instability, we can make the following predictions. (1) Given that dominant males exhibit increased investment in agonistic effort and reproduction, we predicted that dominant males would have higher oxidative damage and/or lower antioxidants than subordinate males, but patterns of oxidative damage and antioxidants would be tissue specific. (2) Social instability generates an environment of low predictability, and potentially higher demands on dominant individuals, who must exert additional effort to maintain dominant status. We therefore predicted that oxidative damage would be higher and/or antioxidants lower in socially unstable communities for both dominant and subordinate males compared with their counterparts in stable communities, but that this effect would be more pronounced in dominant males.

MATERIALS AND METHODS

Experimental design and sampling

Animals and housing

For this experiment, adult *Astatotilapia burtoni* (Günther 1894) were bred from a laboratory population originally derived from Lake Tanganyika, Africa (Fernald and Hirata, 1977), and all fish used were kept in 450-liter tanks prior to the start of the experiment. All aquaria were maintained at 28°C on a 12 h:12 h light:dark cycle and fed cichlid flakes (Omega Sea Ltd) and granular food (Allied Aqua) every morning. All fish were individually tagged through the dorsal musculature using a stainless steel tagging gun and colored beads. Animal were approximately one year old. Experimental tanks (114 liters) were set up with partial terracotta pots used to create defendable territories (details below). After their formation, communities were given 4 weeks to settle before the habitat manipulation began. All procedures were approved by Central Michigan University Institutional Animal Care and Use Committee.

Habitat manipulation

In order to examine how relationships between social status and oxidative stress are affected by community stability, we set up communities (n=15) consisting of 10 males and 14 females. Communities initially had one flowerpot placed in each corner of the tank and one flowerpot placed in the center of the tank (5 total). Communities were randomly assigned stable treatment (n=7) or unstable treatment (n=8), which experienced weekly changes in flowerpot number and arrangement for a total of 22 weeks (Fig. 1). Territorial males typically defend one flowerpot and removing or rearranging the flowerpots can lead to loss of territory (for paradigm, see Hofmann et al., 1999). During the final week (week 22), both stable and unstable communities had the same number and arrangement of available territories before the animals were euthanized (details below). In stable communities, flowerpots were removed and then immediately placed back in the same location following the same schedule as used for the socially unstable communities. This treatment controlled for disturbance stress to the communities that is not related to changing the arrangement and number of flowerpots. During the 22-week experiment, a total of 6 out of 150 males died (mortality rate of 4%), which did not appear to be driven by treatment. Males that died were replaced by similar sized males, except for one male who died just prior to tissue collection. Replacement males were excluded from the analysis.

Observations of status

We recorded social status for each male three times per week, with the first observation starting after the 4-week settling period (hereafter, week 1). Observations were performed in the mornings after feedings. Social status was determined by characterizing males as dominant or subordinate (Korzan et al., 2008). Dominant males were defined as guarding at least one flowerpot and engaging in regular aggressive behaviors with other males (chasing, border displays, and lateral displays) along with chasing and courtship towards females (Fernald, 1977). Subordinate males were characterized as not guarding a flowerpot and rarely engaging in aggressive behaviors. We also recorded each time a male transitioned from dominant to subordinate status or vice versa. Transitions in status over time, measured as shifts from subordinate to dominant or dominant to subordinate between two consecutive observations, were counted for each male. More details about the statistical analysis are given below.

Behavioral quantification

To allow for behavioral analysis, tanks were filmed weekly for 5 min just prior to flowerpot manipulation. All filming was performed at least 10 min after feeding. Aggressive behaviors (chasing, border displays and lateral displays), courtship display, and fleeing were quantified for each male from the final video for their community as previously described in Border et al. (2019), taken just prior to sampling (see below).

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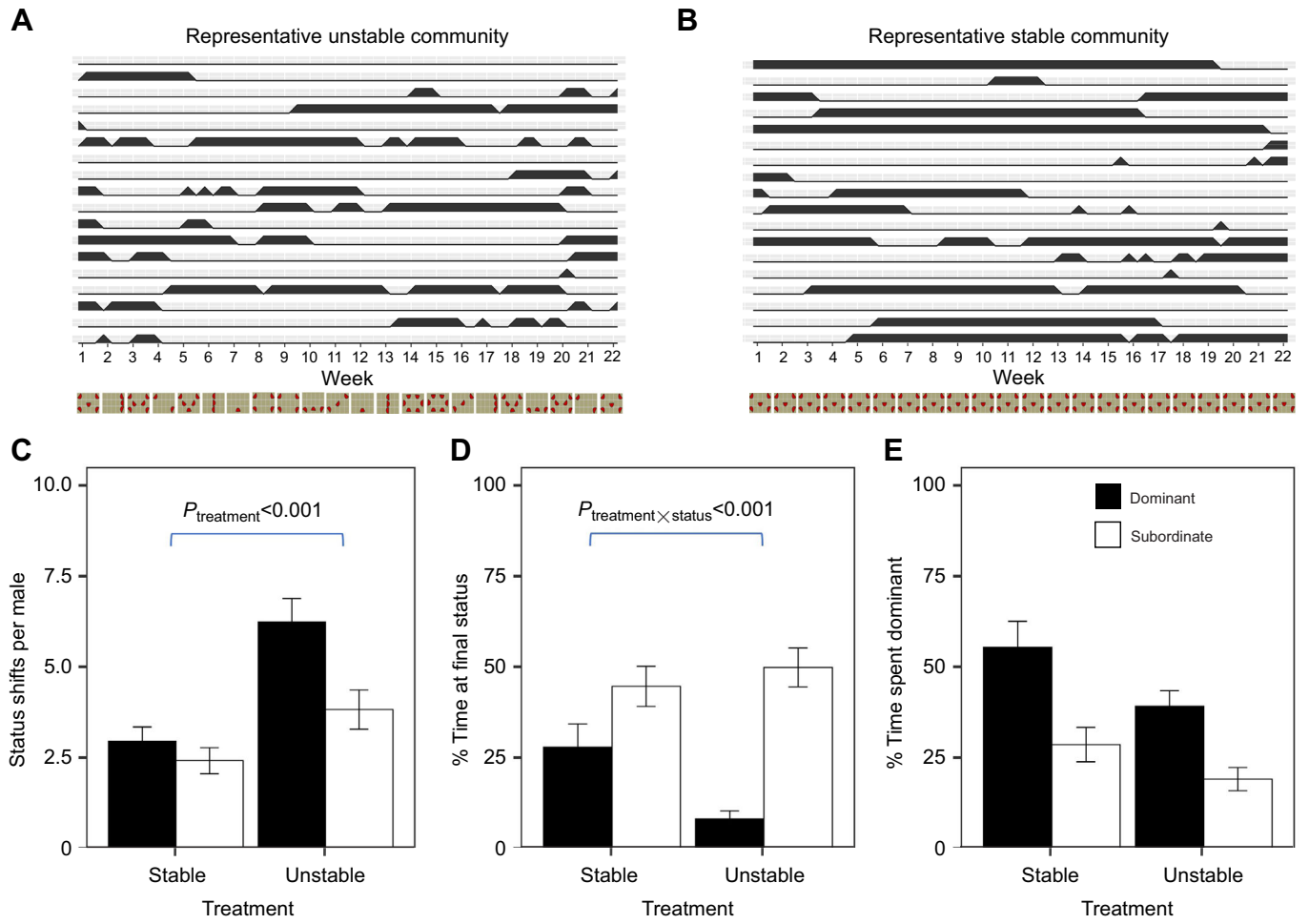

Fig. 1. Flowerpot arrangements in home tanks, male status shifts and dominance tenure in *Astatotilapia burtoni*. Schematic drawings of weekly flowerpot number and arrangement within 'unstable' (A, *n*=8) and 'stable' (B, *n*=7) community tanks during weeks 1 through 22. Small brown rectangles indicate the aquarium floor with red circles representing flowerpots that males use as the focal point for territorial defense. We recorded social status for each male three times per week, with the third observation occurring just prior to the flowerpot manipulation. The first manipulation took place at the end of week 1 (after the third status recording) moving three flowerpots to one side of the aquarium in the socially unstable communities as shown under week 2. Social status is shown in line charts as a function of time in 10 representative males from an unstable community (A) and 10 from a stable community (B). Each row represents an individual male over the 22 weeks of the experiment. Shaded areas indicate periods of time where the male was dominant, while unshaded regions represent periods of subordination. (C) Total number of status shifts during the 22-week experiment defined as a transition between dominant and subordinate between two consecutive observations. (D) Final status tenure defined as the tenure of a male's status at the end of the experiment. (E) Total time spent dominant during the experiment. Values are means±s.e.m. Sample sizes: stable communities, dominant males, *n*=24, subordinate males *n*=43; unstable communities, dominant males, *n*=25, subordinate males, *n*=52.

Sampling methods

Blood was drawn at the end of week 22 for oxidative stress measurements. All males in a given tank were removed at the same time and placed in 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 (range: 1.5–18.0 min) with individuals processed in a randomized order. We collected approximately 25–100 µl of blood from each male (*n*=147) but were unable to obtain blood from 2 males (1 additional male died overnight just prior to sampling). Blood was drawn through the caudal vein using heparinized 26-gauge butterfly needles (Terumo) and transferred to heparinized centrifuge tubes that were placed on ice until centrifugation was performed (10 min at 4000 ***g***). Following centrifugation, plasma and packed red blood cells (PRBCs) were separated. Immediately after blood draw, males were killed via spinal cord dislocation and tissue (gonads, liver, muscle) was collected and flash frozen in liquid nitrogen, with gonads weighed prior to freezing. Tissue collection time (measured as the time from initial disruption of the tank to the removal of each organ from the male) ranged from 3.2 to 38.3 min. Frozen tissue samples were immediately placed in 2 ml tubes on a cold block on dry ice before transfer to long-term storage. Plasma, PRBCs and tissue were stored at −80°C until used for analysis.

To determine which oxidative stress markers to use for our experiment, we performed a preliminary study in which we measured several markers of oxidative stress in various tissues using dominant and subordinate males housed in more isolated conditions (S.E.B., R.J.F. and P.D.D., unpublished results). Based on this preliminary study, we selected the following markers: plasma reactive oxygen metabolites (ROMs, an overall measurement of oxidative damage) and plasma total antioxidant capacity (TAC) as used previously in our species (Border et al., 2019); TAC in liver, muscle and gonad tissue,

and oxidative DNA damage in liver tissue. We also measured circulating testosterone levels to help verify our assignment of male status since dominant males are expected to have higher levels of testosterone than subordinate males as a result of an activated reproductive system in the former. All measurements were done in duplicate. For each assay, we used a pooled sample to calculate the inter-assay coefficient of variation (CV).

Measurement of oxidative stress

Protein quantification

Protein concentration of tissue samples for TAC assays was measured in duplicate with the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Cell lysates were used to standardize concentrations for oxidative stress tissue assays. Absorbance was read by a plate reader (Epoch2T, Biotech Instruments, Winooski, VT, USA).

Circulating oxidative damage

ROMs (primarily organic hydroperoxides) were measured in blood plasma using the d-ROM test (Diacron, Grosseto, Italy) as previously described (Border et al., 2019). The d-ROM test is a widely used assay for the measurement of oxidative damage based on blood plasma (Costantini, 2016) and although it has some caveats (Kilk et al., 2014), it is considered a good measurement of circulating organic peroxidation products as a measure of overall oxidative damage in the organism. Absorbance was read by a plate reader (Epoch2T, Biotech Instruments). The intra-assay CV was 3.57% and the inter-assay CV was 4.66%.

Circulating total antioxidant capability

An oxygen radical absorbance capacity (ORAC) assay was used to measure total antioxidant capability (TAC), a cumulative measure of hydrophilic antioxidants (Marrocco et al., 2017; Taylor et al., 2016) in blood plasma as previously described (Border et al., 2019). In brief, blood plasma was added to wells in a 96-well plate containing a fluorescent marker, incubated at 37°C for 30 min, then an oxidizing agent was added to the wells and fluorescence was measured over time. The area under the curve was calculated and compared with a set of antioxidant (Trolox) standards included on the same plate. Fluorescence was read by a plate reader (Spectramax M3, Molecular Devices, Sunnyvale, CA, USA) and the results were reported in µmol TE dl^{-1} plasma, where TE is Trolox equivalent. The intra-assay CV was 1.24% and the inter-assay CV was 22.52%. This high inter-assay CV was due to one plate with a pooled sample TAC value that was 49.78% lower than the other pooled sample values (the other samples on that plate were normal). Excluding this low value resulted in an inter-assay CV of 12.61%.

Tissue total antioxidant capability

TAC was measured in tissue (liver, gonad, and muscle) following the same procedure as described for plasma with the following exceptions. Tissues were removed from −80°C and homogenized on ice in 0.250 ml lysis buffer (20 mmol l^{-1} Tris–HCl, 137 mmol l^{-1} NaCl, 1% NP-40, 10% glycerol, 2 mmol l^{-1} EDTA) using an Omni Tissue Master (Omni International, Kenosha, WI, USA), then centrifuged at 4°C at 17,000 ***g*** for 10 min. Lysate was collected and used to run protein assays and ORAC. Concentrations obtained from the BCA protein assays were used to standardize concentrations used in the ORAC assay (used to measure TAC) to ∼150 µg ml^{-1}. Fluorescence was read by a plate reader (Spectramax M3, Molecular Devices) and the results reported in µmol TE $µg^{-1}$ protein. For the liver, the intra-assay CV was 2.03% and the inter-assay CV was 9.56%. For the gonad, the intra-assay CV was 0.92% and the inter-assay CV was 3.17%. For the muscle, the intra-assay CV was 1.37% and the inter-assay CV was 3.94%.

DNA damage

We measured oxidative DNA damage by estimating 8-hydroxy-2′-deoxyguanosine (8-OHdG) content. DNA was extracted from frozen liver samples using a commercially available DNA extraction kit (Zymo quick-DNA miniprep plus kit). Extracted samples were stored at 4°C until digestion. Samples were digested at a standardized concentration of 400–500 ng $µl^{-1}$ using a modified version of Quinlivan and Gregory (2008) and stored at −20°C until use. Digested samples were tested for 8-OhDG damage using a DNA damage ELISA kit (StressMarq Biosciences Inc.) at 12× dilution, with all samples run in duplicate following the manufacturer's instructions as described previously (Fialkowski et al., 2021). Absorbance was measured using a microplate reader (Epoch2T, Biotech Instruments). 8-OhDG concentration was standardized relative to total DNA concentration added and reported in ng 8-OHdG ng^{-1} DNA. The intra-assay CV was 4.60% and the inter-assay CV was 9.31%.

Measurement of testosterone

To confirm that dominant and subordinate males are physiologically distinct relative to activation of the reproductive system, we quantified circulating testosterone levels using competitive ELISA kits (Enzo Life Sciences) as previously described (Border et al., 2019). Absorbance was read by a plate reader (Epoch2T, Biotech Instruments). The intra-assay CV was 3.3% and the inter-assay CV was 2.9%.

Statistical analysis

We identified and excluded outliers based on Tukey's rule (Hoaglin and Iglewicz, 1987) and did not have enough tissue/plasma to include all males in all analyses. Final sample sizes ranged from 120 to 144 (see Table S1 with sample sizes for each oxidative stress measurement). Samples were processed by researchers blinded in respect to treatment and tracked by community and individual code. Neither testosterone nor any of our oxidative stress measurements were found to be significantly related to restraint time or tissue collection time, so we did not include restraint/collection time in our models. Gonadosomatic index (GSI) was calculated as (gonad mass/total body mass)×100. We calculated body condition as body mass/$(\text{length})^3$×100 (Bolger and Connolly, 1989). Total number of status shifts (defined as a transition from subordinate to dominant or vice versa between consecutive observations) for each male and duration of final status were calculated from status observations. Male status (dominant or subordinate) was defined for all analyses as the social status a male held at the time of sampling. The duration of each male's final status was calculated based on days from last status shift to tissue/blood sampling date.

All analyses were conducted in R v4.0.3 (https://www.r-project.org/). We analyzed our data using the R packages lme4 (https://CRAN.R-project.org/package=lme4, lmerTest (https://CRAN.R-project.org/package=lmerTest), MASS (https://CRAN.R-project.org/package=MASS) and glmmTMB (https://CRAN.R-project.org/package=glmmTMB). We used linear mixed models (LMMs) with a maximum-likelihood protocol and generalized linear mixed models (GLMMs), with treatment and status as fixed effects, an interaction term for treatment × status, and community as a random effect. GLMMs were only used for variables where LMM model

assumptions were not met (see below). We analyzed TAC, ROMs, DNA damage (8-OHdG) and GSI using LMMs. Testosterone was analyzed using a GLMM assuming a gaussian distribution with log link. Final status tenure and total time spent dominant were expressed in percentage relative to the total duration of the experiment and were analyzed using GLMMs assuming a beta distribution. Number of status shifts, chases, fleeing events and courtship displays were analyzed as count data using GLMMs assuming a Poisson or negative binomial distribution. In some cases, we found a better model fit when using a hurdle model with zero-truncated Poisson or negative binomial distribution (for more details, see Supplementary Materials and Methods).

There were several significant correlations across the different oxidative stress measurements and all correlations were positive (Fig. S1). Principal component analysis (PCA) was used to summarize oxidative status of each male by fewer axes. Before analysis, we standardized all measurements by calculating z-scores, which were obtained by subtracting the entire sample mean from each observation and dividing by the standard deviation. The first three principal components (PCs) accounted for 67% of the variation in the original dataset. We then assessed whether PC1 and PC2 were influenced by status and treatment using LMM.

All models within two AICc units of the top model were considered best fit models. Body mass was included as a fixed effect in all models but only reported it was retained in a best-fit model. We report *P*-values, estimates and standard error for each model. In all model summaries, the reference category for fixed effects was determined alphabetically or numerically: dominant (versus subordinate for status), stable (versus unstable communities). To evaluate the validity of our models, we examined the residuals, qqplots and plots of predicted values versus residuals.

RESULTS

Habitat variability leads to social instability

To establish the effectiveness of our habitat disruption in creating an unstable social environment, we examined several measures of male behavior. As anticipated, males in unstable communities experienced considerably more status shifts than males in stable communities (GLMM, treatment: 0.75±0.21, $P<0.001$; Fig. 1C) regardless of status at the end of the experiment (GLMM, treatment×status: −0.30±0.35, $P=0.40$). The duration of a male's final status prior to sampling was influenced by both treatment and male status (GLMM, treatment×status: 1.43±0.44, $P=0.001$, Fig. 1D), driven by significantly shorter final dominance tenure for dominant males in unstable communities relative to stable communities (GLMM, treatment: −1.60±0.33, $P<0.0001$; Fig. 1D) as subordinate males in both treatments had similar final subordinance tenure (GLMM, treatment: −0.02±0.27, $P=0.94$). Despite males in unstable communities experiencing a greater number of status shifts as a result of fluctuations in territory availability (Fig. 1A,C), there was no significant difference in the total time males were dominant between treatments (GLMM, treatment: −0.24±0.19, $P=0.214$; Fig. 1E). The same model retained status as a significant effect, suggesting that males who were dominant at the end of the experiment had spent more time being dominant compared with subordinate males (GLMM, status: −1.78±0.23, $P<0.00001$).

To confirm that dominant and subordinate males differ in agonistic and sexual behavior, we analyzed aggression (chase rates), courtship and fleeing behavior. For chase rate, we found a significant interaction between treatment and status (GLMM, hurdle model, truncated Poisson, treatment×status: −1.24±0.18, $P<0.0001$; Fig. 2A) after controlling for the effect of body mass (GLMM, mass: 0.08±0.02, $P<0.0001$). Subordinate males did not significantly differ in aggression between treatments (GLMM, negative binomial, treatment: −0.09±0.41, $P=0.824$); however, dominant males in unstable communities were significantly more aggressive than dominant males in stable communities (GLMM, negative binomial, treatment: 0.46±0.18, $P=0.011$). As a result, males in unstable communities showed more fleeing behavior (GLMM, hurdle model, truncated negative binomial, treatment: 0.25±0.13, $P=0.048$; Fig. 2C). In the same model, status was retained as a highly significant effect (GLMM, status: 1.57±0.24, $P<0.0001$), confirming that subordinate males showed more fleeing behavior than dominant males. As expected, dominant males performed more courtship behavior than subordinate males (GLMM, hurdle model, truncated negative binomial, zero-inflation model: 3.17±0.59, $P<0.0001$, Fig. 2B) and this effect was not influenced by treatment (GLMM, treatment×status: 1.75±1.31, $P=0.181$).

As expected, testosterone levels were higher in dominant males compared with subordinate males (LMM, GLMM, testosterone: −0.95±0.07, $P<0.0001$, Fig. 2D). Additionally, the gonadosomatic index (GSI, the ratio of gonad mass to total body mass) tended to be higher in dominant males compared with subordinate males, although this effect was not significant (LMM, −0.05±0.03, $P=0.055$; Fig. 2E). None of these status effects depended on treatment (all $P>0.1$).

Fish details regarding body mass, standard length and body condition are shown in Table 1. At the end of the experiment, dominant males were significantly bigger than subordinate males in both treatments, but this effect was greater in stable communities (LMM, treatment×status, body mass: 1.37±0.68, $P=0.047$, standard length: 3.20±1.32, $P=0.017$). Body condition was not affected by status (LMM, status: $1.99\times10^{-5}\pm3.94\times10^{-5}$, $P=0.614$) or treatment (LMM, treatment: $8.63\times10^{-5}\pm4.76\times10^{-5}$, $P=0.090$).

Rank-specific patterns in oxidative stress are influenced by social stability

There was a significant interaction effect between status and treatment on plasma ROM levels (LMM, treatment×status: 1.44±0.53, $P=0.007$; Fig. 3A), suggesting that the degree of social stability influenced the status-dependent patterns in ROMs. Dominant males in stable communities had significantly higher levels of ROMs than subordinate males (LMM, status: −0.91±0.41, $P=0.029$), consistent with our earlier study (Border et al., 2019). We predicted that oxidative stress would be higher in socially unstable communities and that the cost of social instability would be higher in dominant males compared with subordinate males. This prediction was somewhat supported for subordinate males: subordinate males had a tendency towards higher ROM levels in unstable communities compared with their stable community counterparts (LMM, treatment: 0.62±0.30, $P=0.043$). However, in contrast to expectation, we found that dominant males in unstable communities had similar ROM levels to those in stable communities (LMM, treatment: −0.82±0.44, $P=0.071$). In addition, in unstable communities, dominant and subordinate males had similar ROM levels to each other (LMM, status: 0.50±0.34, $P=0.138$). Because dominant males in unstable communities had shorter final dominance tenure than dominant males in stable communities, we examined whether dominance tenure related to ROMs. However, final dominance tenure had no significant effect on ROM levels (LMM, final dominance tenure: 0.008±0.0006, $P=0.154$) and it is therefore unlikely to explain the similarity in ROM levels between stable and unstable community dominants.

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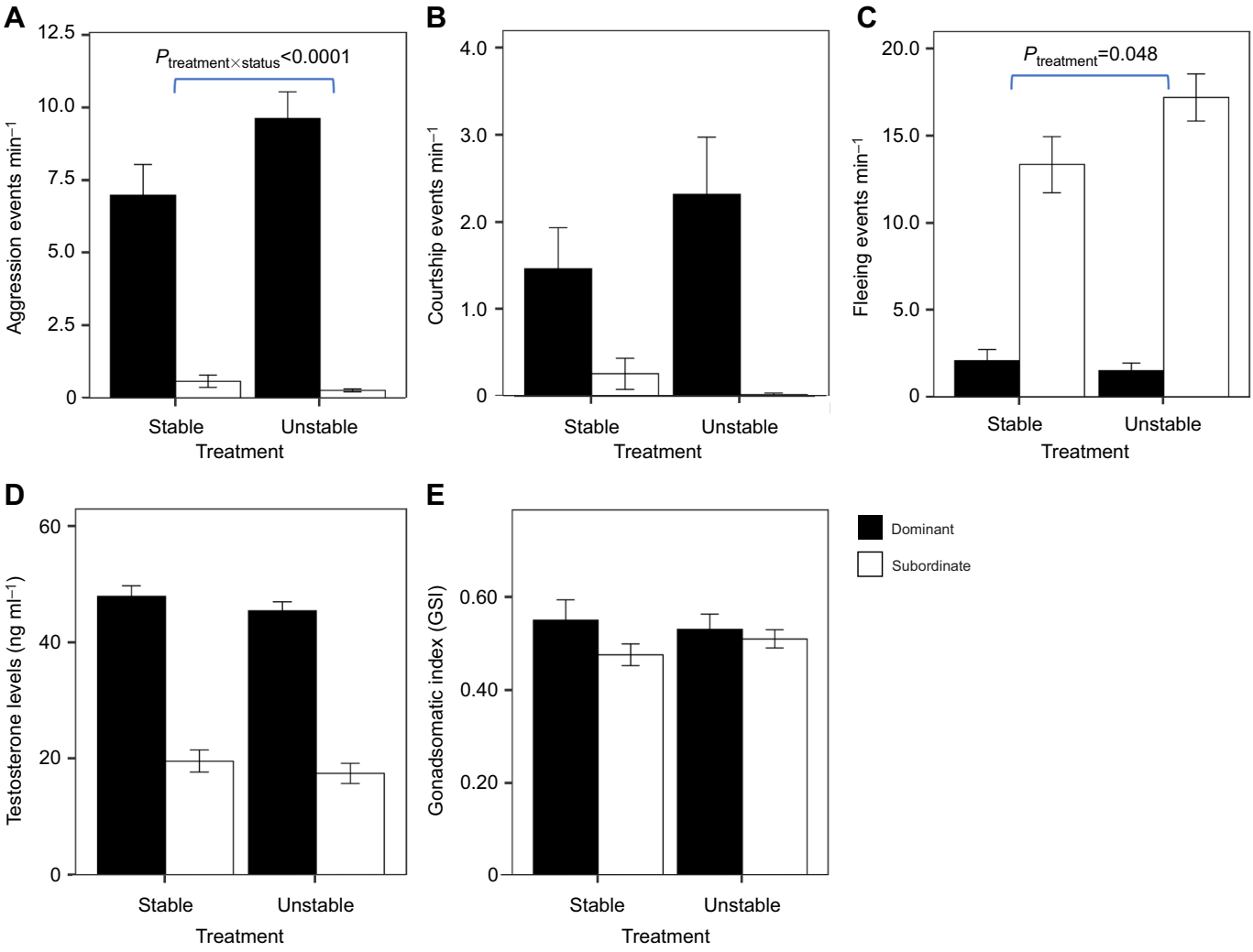

Fig. 2. Behavior, testosterone levels and gonadosomatic index (GSI) in dominant and subordinate males in stable and unstable communities. Behavioral differences in aggression (A), courtship behavior (B) and fleeing behavior (C) between dominant and subordinate males. Behavior was quantified on the day of tissue collection. Circulating testosterone levels (D) and GSI (E) in dominant and subordinate males. Values are means±s.e.m. Sample sizes: (A–C,E) stable communities, dominant males, *n*=24, subordinate males *n*=43; unstable communities, dominant males, *n*=25, subordinate males, *n*=52; (D) stable communities, dominant males, *n*=23, subordinate males *n*=43; unstable communities, dominant males, *n*=25, subordinate males, *n*=50.

Liver DNA damage was not influenced by status (LMM, status: −0.009±0.010, *P*=0.343; Fig. 3F) or treatment (LMM, treatment: −0.0002±0.0187, *P*=0.99). For plasma TAC, there was a significant interaction effect between status and treatment (LMM, treatment×status: 258.41±123.04, *P*=0.0378; Fig. 3B), suggesting that the degree of social stability influenced the status-dependent patterns in plasma TAC. However, a model with body mass alone was a better fit, indicating that plasma TAC was positively related to body mass (LMM, mass: 40.58±14.27, *P*=0.00515). For liver TAC, there was a significant interaction between treatment and status (LMM, treatment×status: 0.096±0.043, *P*=0.027; Fig. 3D). In line with an increased cost of social dominance in unstable environments, we found that there was a significant tendency for lower levels of liver TAC in dominant males compared to subordinate males in unstable communities (LMM, status: 0.071±0.031, *P*=0.0267) but not in stable communities (LMM, status: −0.025±0.029, *P*=0.386). In addition, dominant males in unstable communities tended to have lower liver TAC than their counterparts in stable communities, although this effect was not significant even after controlling for body mass (LMM, treatment: −0.141±0.079, *P*=0.0904; mass: 0.020± 0.011, *P*=0.0816). For muscle TAC, one model was retained with mass as a borderline nonsignificant predictor (LMM, mass: 0.0101± 0.0053, *P*=0.0568; Fig. 3E). For gonad TAC, three best-fit models were found, all retaining nonsignificant effects for status and treatment (and its interaction) and significant effects for body mass

Table 1. Body mass, standard length and body condition based on male status and stability treatment

Variable	Treatment	Dominant	Subordinate
Body mass (g)	Stable	15.49±0.34	13.28±0.422
	Unstable	16.10±0.28	15.19±0.26
Standard length (mm)	Stable	82.42±0.78	77.62±0.82
	Unstable	82.46±0.64	80.76±0.50
Body condition	Stable	0.00277±3.7×10^{-5}	0.00280±3.9×10^{-5}
	Unstable	0.00288±4.5×10^{-5}	0.00288±3.3×10^{-5}

We calculated body condition as mass/(length)^3×100. Dominant/subordinate status indicates the status at the end of the experimental period just prior to tissue sampling. Values are means±s.e.m. Sample sizes: stable communities, dominant males, *n*=24, subordinate males *n*=43; unstable communities, dominant males, *n*=25, subordinate males, *n*=52.

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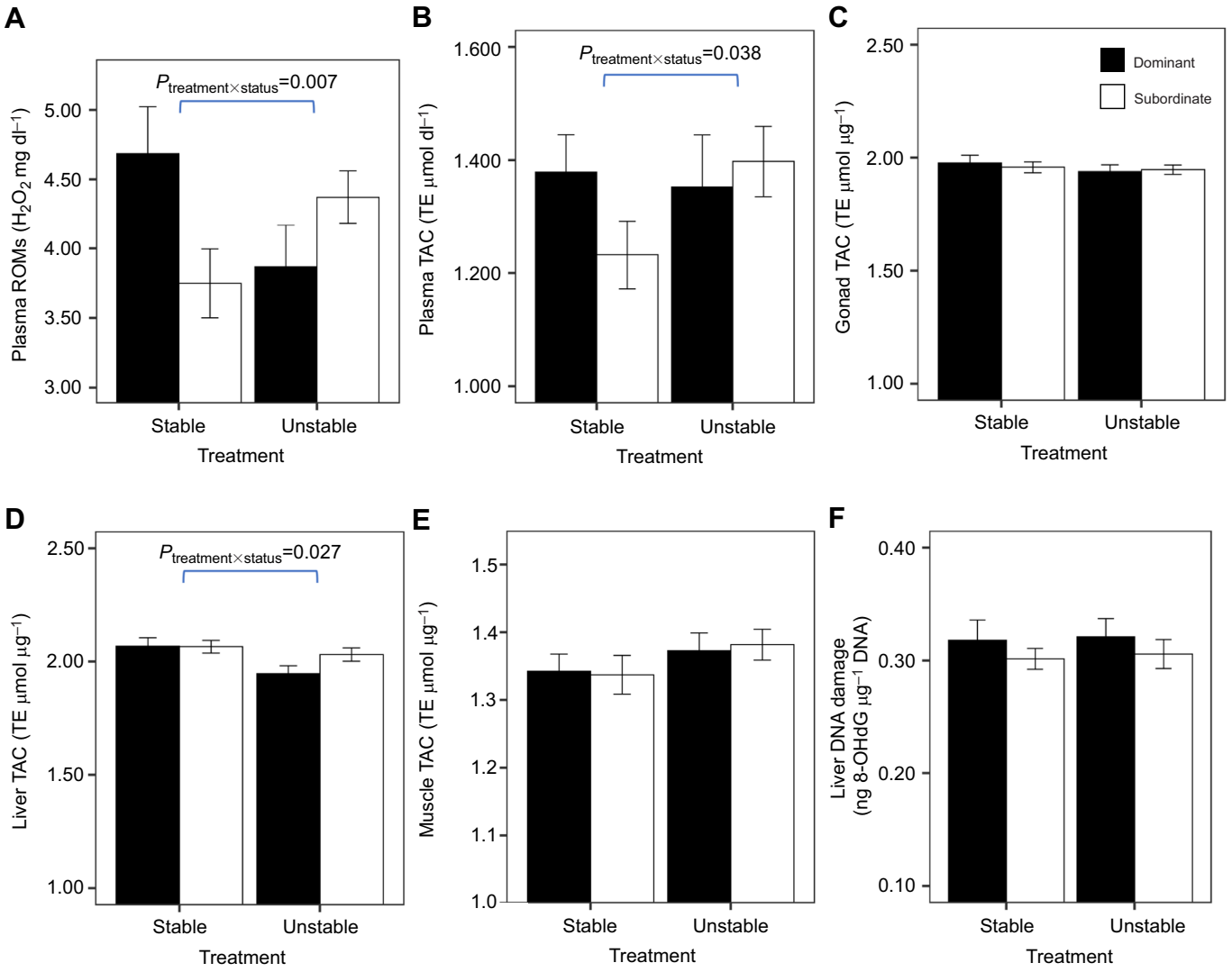

Fig. 3. Oxidative stress measurements in dominant and subordinate males in stable and unstable communities. (A) Plasma reactive oxygen metabolite (ROM) levels. (B–E) Total antioxidant capacity (TAC) in indicated tissues. (F) Liver oxidative DNA damage (8-OHdG levels). Values are means±s.e.m. Sample size ranges: stable communities, dominant males, *n*=22–24, subordinate males *n*=37–43; unstable communities, dominant males, *n*=21–25, subordinate males, *n*=44–52.

(Fig. 3C). The model with only body mass had the lowest AIC value (LMM, mass: 0.01126±0.00541, *P*=0.0429).

To summarize the six measurements of oxidative stress, we applied PCA analysis and tested whether status and treatment explained variation in PC1 or PC2. PC1, which represents 28% of the variation from the original dataset, was characterized by negative loadings for all measurements (Table 2). PC2, which represented 20% of the variation from the original dataset, was characterized by high positive loadings on liver DNA damage and plasma ROMs and high negative loadings on muscle and gonad TAC (Table 2). PC2 thus reflects overall oxidative stress level, although increasing PC2 values was also associated with increasing plasma TAC (Table 2, Fig. 4C). PC1 was not influenced by status (LMM, status: −0.175±0.258, *P*=0.500; Fig. 4A) or treatment (LMM, treatment: −0.117±0.402, *P*=0.775). However, for PC2 there was a significant interaction between treatment and status (LMM, treatment×status: 1.199±0.449, *P*=0.00936; Fig. 4B), consistent with the notion that overall, status-specific oxidative balance is impacted by social stability. A second best-fit model also retained body mass as an additional (nonsignificant) predictor of PC2 (LMM, treatment×status: 1.357±0.457, *P*=0.00401; mass: −0.258±0.131, *P*=0.0523). There was a nonsignificant tendency of dominant males to have higher PC2 values than subordinate males in stable communities (LMM, status effect: −0.610±0.324, *P*=0.0682) whereas the reverse pattern was observed in unstable communities (LMM, status effect: 0.585±0.311, *P*=0.0677).

Table 2. Measurement of oxidative stress loadings for the principal component analysis (PCA)

Oxidative stress variable	PC1	PC2
Plasma TAC	−0.354	**0.431**
Gonad TAC	−0.576	−0.191
Liver TAC	−0.435	**0.010**
Muscle TAC	−0.547	−0.402
Plasma ROMs	−0.194	**0.470**
DNA damage	−0.130	**0.628**

Shown are the loadings for plasma reactive oxygen metabolites (ROMs), total antioxidant capacity (TAC) and liver oxidative DNA damage based on 8-OHdG level. Positive loadings are shown in bold.

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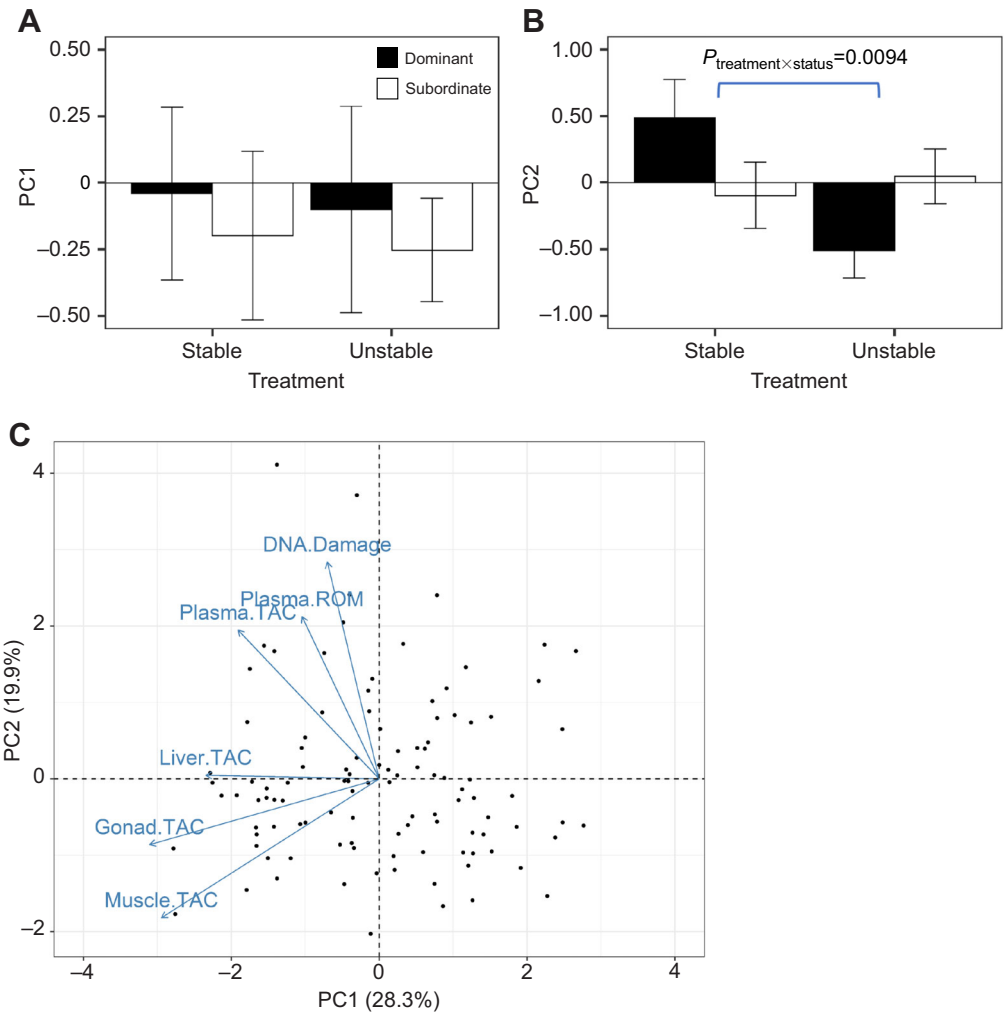

Fig. 4. Principal component analysis (PCA) of six measurements of oxidative stress. (A,B) Effect of status and treatment on principal component (PC) axis 1 and 2. Values are means± s.e.m. Sample sizes: stable communities, dominant males, *n*=18, subordinate males, *n*=22; unstable communities, dominant males, *n*=13, subordinate males, *n*=31. (C) Biplot showing the influence of the different measurements of oxidative stress. Circles represent coordinates of individual males, and indicated with arrows are the influence of plasma ROM levels, TAC and liver oxidative DNA damage (8-OHdG levels).

Dominant males in stable communities had higher PC2 than dominant males in unstable communities (LMM, treatment: −0.994±0.408, P=0.0267), which is inconsistent with a higher cost of social dominance in unstable hierarchies.

Effect of number of status changes on oxidative stress

To further explore the effect of social instability on oxidative stress, we took advantage of variation among males in the number of status shifts experienced throughout the experiment. If social instability increases the oxidative burden, we hypothesized that the number of status shifts a male had experienced would be positively related to oxidative stress. The total number of status shifts had a negative effect on plasma ROM levels (LMM, number of status shifts −0.081±0.0385, P=0.0383; Fig. 5A), and this effect was even stronger when restricting the analysis to the number of status shifts a male had experienced in the recent past (LMM, number of status shifts over the final 4 weeks, −0.3837±0.01145, P=0.001). This means that males who had experienced a greater number of recent changes in status had lower levels of ROMs in plasma. Neither liver DNA damage nor any antioxidant measurement were related to the number of (recent) status shifts (all P>0.1; Fig. 5B–F). There were no significant interactions with final status or treatment (all P>0.1) for any measurement. PC1 and PC2 were not impacted by the number of (recent) status shifts with or without final status or treatment (data not shown, all P>0.1).

DISCUSSION

Oxidative stress has been hypothesized to be an important potential cost of social dominance, especially in socially unstable environments. However, experimental support for this hypothesis is scarce. In our previous study, we found that an oxidative damage marker in plasma was elevated in dominant males but that this was not influenced by a single short-term social disruption (Border et al., 2019). We posited that a more intensive and long-term social disruption, which mimicked potential environmental disturbances in natural communities (Maruska and Fernald, 2018), would result in increased oxidative stress, especially in dominant males. This is because when territory availability frequently changes, dominant males need to exert more aggression to maintain social dominance, and the resulting heightened metabolic rate could increase oxidative stress (Dijkstra et al., 2011, 2013). Moreover, dominant males experience increased rank uncertainty during periods of instability, and the resulting social stress could also lead to an increased oxidative burden. We first confirmed that the habitat manipulation led to social instability, demonstrated by increased frequency of status shifts, reduced final dominance tenure, and increased aggression in dominant males in unstable communities relative to stable communities. Since oxidative balance can vary by tissue type and redox marker, we measured several aspects of oxidative damage and antioxidant defense in various tissue types. We hypothesized that oxidative stress would be elevated in unstable

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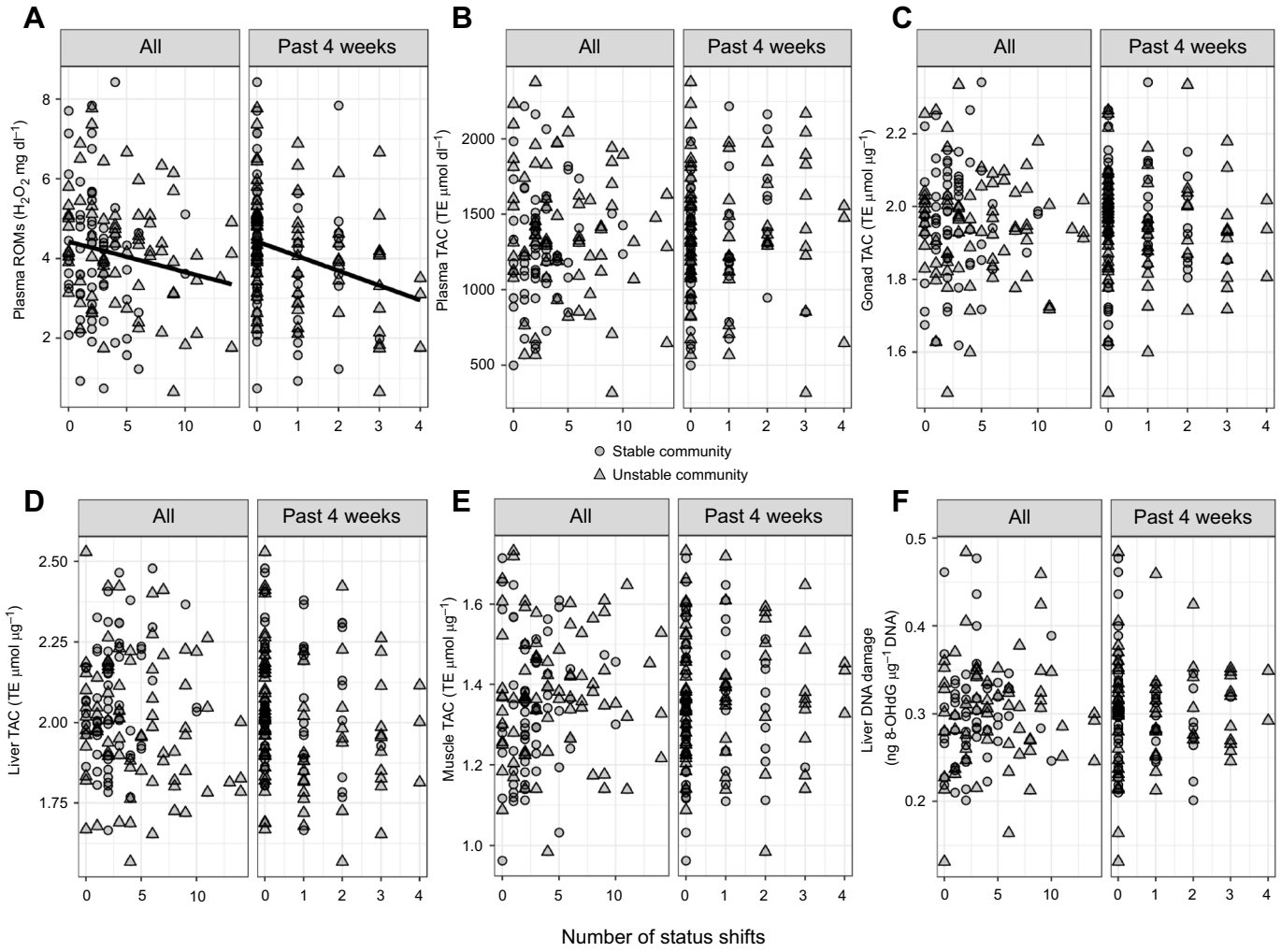

Fig. 5. Effect of number of status shifts on markers of oxidative stress. Number of status shifts a male experienced during the entire 22-week experiment (All) and during the final 4 weeks prior to tissue collection (Past 4 weeks) are shown on the *x*-axis. Circles are males from stable communities and triangles males from unstable communities. (A) Plasma ROM level. (B–E) TAC in indicated tissues. (F) Liver oxidative DNA damage (8-OHdG levels). Regression lines are only shown for (borderline) significant effects.

communities, and that dominant males would experience a greater increase in oxidative stress in unstable communities relative to subordinate males. In contrast to our expectation, while dominant males had higher circulating ROMs (which represent a cumulative effect of oxidative damage across the body) than subordinate males in stable communities as expected, in contrast to our hypothesis, dominant males did not experience elevated ROMs in unstable communities. In fact, there was a significant difference in circulating ROMs between dominant and subordinate males only in stable communities. Overall, we found significant treatment×status interaction effects in three out of six measurements of oxidative stress (plasma ROM level, plasma TAC and liver TAC) and PC2, suggesting that social instability influences status-dependent patterns in oxidative stress. Liver TAC levels were lower in dominant males compared to subordinate males in unstable communities only, which could lend support to the notion that social dominance is more costly in unstable hierarchies. Collectively, our findings provide some support for the idea that while oxidative stress may be elevated in dominant males relative to subordinates, social stability also plays an important role on this relationship.

Male status and tissue-specific oxidative stress in stable communities

Given the increased investment in both aggression and reproduction in dominant males, we expected that dominant males would experience heightened oxidative stress compared with subordinates. However, much of the previous work on oxidative stress and dominance has measured oxidative stress exclusively in the blood (Beaulieu et al., 2014; Cram et al., 2015; Dijkstra et al., 2016; Georgiev et al., 2015a,b), and other studies have found that patterns in oxidative stress vary based on both tissue and specific oxidative balance measurement (Garratt et al., 2012; Taylor et al., 2015; Tkachenko et al., 2014; Xu et al., 2014). In this study, we examined tissue-specific patterns in oxidative damage and antioxidant activity in relation to dominance status.

In previous experiments with short-term communities, we found that plasma ROM levels were higher in dominant males than in subordinate males (Border et al., 2019; Fialkowski et al., 2021; Funnell et al., 2021); here we replicate that finding in long-term stable communities. Our findings are consistent with studies on birds (Cram et al., 2015; Silva et al., 2018), primates (Beaulieu et al., 2014; Georgiev et al., 2015a) and other cichlid species

(Dijkstra et al., 2011, 2013) where dominant or breeding individuals were found to have increased oxidative stress. However, we only found increased ROMs in dominant males in stable communities, where environmental disturbance was at a minimum. Previous work has often found that the cost of social dominance or breeding is subject to environmental or social influences (Dijkstra et al., 2011; Beaulieu et al., 2014; Georgiev et al., 2015b; van de Crommenacker et al., 2012). For example, Van de Crommenacker et al. (2012) found that Seychelles warblers experienced an increased level of oxidative damage during the high-energy provisioning stage of breeding, but only when also experiencing a malaria infection. A study on rhesus macaques (Georgiev et al., 2015b) found that only males who had been highly aggressive in a recent breeding season endured increased levels of oxidative DNA damage. These results suggest that relationships between oxidative stress and social dominance may be determined in part by the degree of effort required to either maintain rank or engage in reproductive activities, and that this effort can be influenced by multiple factors with varying effects on oxidative stress.

The effects of reproductive effort on liver oxidative stress have been found to vary considerably between studies (Blount et al., 2016; Xu et al., 2014). Dominant male *A. burtoni* expressed greater levels of aggression compared with subordinate males and a likely consequence of this is elevated metabolic rates as shown in other haplochromine cichlids (Dijkstra et al., 2013). The combined increase in reproductive investment and metabolism might predict higher levels of liver DNA damage in dominant males, but we found no effect of status on liver oxidative damage. In addition, male status was not a significant predictor of total antioxidant capacity in tissue or plasma. However, dominant males had higher PC2 values (which reflects overall oxidative stress level) than subordinate males, supporting the notion that there is an oxidative cost to social dominance.

While male status did not significantly predict total antioxidant capacity, body mass was positively correlated to plasma and gonad TAC. This corresponds with our previous findings in plasma from males in short-term communities (Border et al., 2019). Body mass is a key factor contributing to overall condition in many fish species, and an individual's size relative to community members directly influences that individual's chances of ascending to dominant status (Maruska and Fernald, 2010; Maruska et al., 2013). However, it should be noted that our antioxidant measure was a general measure of total antioxidants capacity (mostly hydrophilic antioxidants) and we did not distinguish between different kinds of antioxidants. Enzymatic antioxidants (such as superoxide dismutase, catalase, and glutathione peroxidase) may be differentially regulated in different tissues and/or affected by male status (Fialkowski et al., 2021).

Influence of community stability on oxidative stress patterns

We expected higher levels of oxidative stress in socially unstable hierarchies, especially in dominant males. We instead observed this effect only in subordinate males, with higher levels of plasma-based oxidative damage (ROMs) in subordinate males in unstable communities compared with their stable community counterparts. While previous studies such as that of Beaulieu et al. (2014) found that social instability resulted in increased oxidative stress among the most dominant individuals, we found that dominant males in unstable hierarchies did not experience an increase in plasma ROM levels compared with dominant males in stable hierarchies. This data suggests that increased social instability and increased aggression in dominant males did not directly influence their oxidative damage. Since plasma ROMs are not significantly elevated in males that recently ascended in *A. burtoni* (Fialkowski et al., 2021), we hypothesized that the lack of increased plasma ROMs in dominant males in unstable communities was caused by their having shorter dominance tenure at the end of the experiment relative to dominant males in stable communities. However, this idea was not supported because final dominance tenure did not significantly impact plasma ROMs. Social instability was linked to more frequent status shifts, and if acquiring and maintaining social dominance is costly, a history of more frequent status shifts is predicted to be associated with more oxidative stress. In contrast to expectation, we found that the total number of status shifts negatively predicted plasma ROM levels but had no impact on the other measures of oxidative stress. As has been noted elsewhere (Nussey et al., 2009; Xu et al., 2014), measures of oxidative damage using plasma might only reflect relatively recent oxidative state as turnover of plasma constituents is high. We therefore restricted our analysis to the number of recent status shifts (past 4 weeks), and the negative relationship with plasma ROMs became even stronger. Longitudinal studies in which status is experimentally assigned combined with repeated plasma measurements could provide more insight into the causal relationship between rank tenure, social stability (as determined by number of rank shifts), and oxidative stress.

Various lines of evidence did not support our hypothesis that social instability increases oxidative stress in dominant individuals. First, dominant individuals did not experience the expected increase in oxidative damage or decrease in antioxidants in unstable communities in most tissue types. Second, PC2 (which could reflect general oxidative stress) was significantly lower (and not higher) for dominant males in unstable communities relative to their counterparts in stable communities. Third, the number of total status shifts a male experienced was not related to most measurements of oxidative stress; it was for ROM levels, but in the opposite direction, as discussed earlier. However, there was a significant interaction between treatment and male status on liver TAC: liver TAC was significantly lower in dominant males compared with subordinate males in unstable communities only. Although a lower antioxidant capacity does not necessarily reflect more oxidative stress (Costantini and Verhulst, 2009), our finding is consistent with an increased cost to maintain higher social rank in a socially unstable environment. Reduced antioxidant capacity could occur by dominant individuals depleting more antioxidant resources as they engage in more costly agonistic interactions (Mentesana and Adreani, 2021). Although speculative, it is possible that the lack of liver oxidative damage for dominant males in unstable communities may be due to a mitigating effect of antioxidants, resulting in lower liver TAC as they are depleted for defense against ROS. As such, while we did not see increases in damage in the bounds of our experiment, in a more natural setting, where antioxidants may be more limited and additional stressors are present (i.e. infections, parasites, risk of predation), dominant males may be unable to maintain defenses against oxidative stress (see also literature on reproduction affecting oxidative balance under challenging conditions: Van de Crommenacker et al., 2012; Beaulieu et al., 2015). Studies that more closely mimic natural conditions and environmental challenges are necessary to determine how they influence the relationship between oxidative stress and social instability.

Our findings support the notion that oxidative stress in a dominance hierarchy is influenced by community stability. However, we found limited support for the hypothesis that increased social instability may raise oxidative stress in dominant males, with a significant reduction in overall liver antioxidant

capacity found in dominant males relative to subordinates in socially unstable communities, but no observed increase in oxidative damage. While we have begun to tease apart the complex relationships regulating variation in oxidative stress among members of a dominance hierarchy, future research will attempt to further broaden our understanding by measuring variation in specific enzymatic antioxidants and additional oxidative damage markers. In addition, longitudinal studies that better mimic natural conditions are critical to further our understanding of changes in oxidative stress relative to an individual's changing social position over time. Finally, studies incorporating experimentally induced rank changes and measuring both immediate effects and long-term consequences (such as changes in telomere length, see Monaghan et al., 2009) could help elucidate the long-term fitness consequences of living in a highly competitive environment.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: S.E.B., T.J.P., P.D.D.; Methodology: S.E.B., T.J.P., T.R.F., P.D.D.; Software: S.E.B.; Validation: S.E.B.; Formal analysis: S.E.B., P.D.D.; Investigation: S.E.B., T.J.P., T.R.F., R.F.F., J.S., P.D.D.; Resources: P.D.D.; Data curation: S.E.B., R.F.F., P.D.D.; Writing - original draft: S.E.B., P.D.D.; Writing - review & editing: S.E.B., T.J.P., R.F.F., J.S., P.D.D.; Visualization: S.E.B., P.D.D.; Supervision: P.D.D.; Project administration: P.D.D.; Funding acquisition: P.D.D.

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Data availability
Data are available from the Dryad Digital Repository (Dijkstra, 2021): https://doi.org/10.5061/dryad.m63xsj3x7

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