Social dominance and reproduction result in increased integration of oxidative state in males of an African cichlid fish

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Abstract

Oxidative stress is a potential cost of social dominance and reproduction, which could mediate life history trade-offs between current and future reproductive fitness. However, the evidence for an oxidative cost of social dominance and reproduction is mixed, in part because organisms have efficient protective mechanisms that can counteract oxidative insults. Further, previous studies have shown that different aspects of oxidative balance, including oxidative damage and antioxidant function, varies dramatically between tissue types, yet few studies have investigated oxidative cost in terms of interconnectedness and coordination within the system. Here, we tested whether dominant and subordinate males of the cichlid *Astatotilapa burtoni* differ in integration of different components of oxidative stress. We assessed 7 markers of oxidative stress, which included both oxidative damage and antioxidant function in various tissue types (total of 14 measurements). Across all oxidative stress measurements, we found more co-regulated clusters in dominant males, suggesting that components of oxidative state are more functionally integrated in dominant males than they are in subordinate males. We discuss how a high degree of functional integration reflects increased robustness or efficiency of the system (e.g. increased effectiveness of antioxidant machinery in reducing oxidative damage), but we also highlight potential costs (e.g. activation of cytoprotective mechanisms may have unwanted pleiotropic effects). Overall, our results suggest that quantifying the extent of functional integration across different components of oxidative stress could reveal insights into the oxidative cost of important life history events.

Keywords: territoriality, social hierarchy, reproduction, reactive oxygen species, antioxidants, life history trade-offs

1. Introduction

Oxidative cost has been proposed as a potential mediator of life history trade-offs, such as the negative correlation between reproduction and longevity (Alonso-Alvarez et al., 2017; Speakman and Garratt, 2014). Reproduction involves metabolically demanding activities such as egg production, territorial defense, and parental care, leading to increased levels of reactive oxygen species (ROS, also referred to as oxidants). The resulting increased production of ROS, if not sufficiently neutralized by the antioxidant machinery, may lead to increased oxidative stress (Balaban et al., 2005). Given that accumulation of oxidative stress contributes to compromised cell health and disease progression, it is plausible that oxidative stress acts as a constraint in life history decisions, mediating trade-offs between for example fecundity, growth and survival (Dowling and Simmons, 2009; Monaghan et al., 2009; Speakman et al., 2015). Accordingly, studies have shown that reproductive effort can increase oxidative damage and decrease antioxidant capacity in a range of animal species (Christe et al., 2012; Sawecki et al., 2019; Sharick et al., 2015; Stier et al., 2012), and that oxidative stress can negatively affect survival or lifespan (Archer et al., 2013; Bize et al., 2008). In many animals species, social dominance is associated with breeding, and similarly, there is cumulative evidence that social dominance results in an oxidative cost, especially when high dominance is linked to intense agonistic interactions (Beaulieu et al., 2014; Border et al., 2019; van de Crommenacker et al., 2011) and/or increased reproductive effort (Cram et al., 2015; Noguera, 2019; Silva et al., 2018). At the same time, there are also several studies suggesting there is no oxidative cost of social dominance and/or reproduction and that breeding may even reduce oxidative stress (Blount et al., 2016; Costantini et al., 2014; Garratt et al., 2013). This is, in part, due to the fact that organisms have sophisticated defensive mechanisms, such as antioxidant and repair systems, that can avoid or

mitigate the negative consequences of oxidative insults (Hõrak and Cohen, 2010; Pamplona and Costantini, 2011). However, few studies have explored the efficiency or robustness of these defensive mechanisms that maintain redox homeostasis.

Maintaining redox balance requires complex integration of various redox components both within and across interconnected cells, organs, and tissues. For example, oxidative insults may activate the transcription factor nrf1 which regulates many detoxifying enzymes and antioxidant genes (Enomoto et al., 2001). Another example of the highly integrative nature of the antioxidant response is the fact that exogenous antioxidant supplementation often leads to compensatory responses such as reduced production of endogenous antioxidants to maintain oxidative balance (Selman et al., 2006). Therefore, assessing the functional integration and interconnectedness of multiple oxidative stress variables could provide important information about the effectiveness of cytoprotective mechanisms that counteract oxidative damage – such as antioxidant responses – as well as the degree of active regulation of redox balance under stressful conditions. For example, hybrids of the newt species *Triturus* exhibited a lower level of functional integration of the antioxidant system compared to the parental species, which was viewed as a potential oxidative cost of interspecific hybridization (Prokić et al., 2018). Likewise, short-term flight in zebra finches led to reduced integration across blood redox markers, which constitutes a potential oxidative cost (Costantini et al., 2013). However, studies examining the effect of challenging life history events on the degree of functional integration of oxidative stress components across different tissue types are largely lacking, with previous studies focusing on only blood or whole body samples (Costantini et al., 2011; Costantini et al., 2013; Prokić et al., 2018).

The cichlid fish *A. burtoni* lives in a lek-like social system in which dominant males defend a spawning territory while subordinate males are non-territorial and are reproductively suppressed (Fernald, 2017). This social structure is reproducible in a laboratory setting by housing several males and females in an aquarium with flowerpot shards acting as spawning territories to encourage territoriality in several males. Dominance hierarchies in *A. burtoni* are characterized by intense rates of aggression with dominant males engaging in continual rank maintenance using border displays and chases (Piefke et al., 2021). Additionally, dominant males show increased reproductive effort as indicated by larger gonads, brighter coloration, increased courtship displays, and higher androgen levels compared to subordinate males (Alward et al., 2020; Border et al., 2018; O'Connell and Hofmann, 2012). We previously found that in *A. burtoni* dominant males had higher levels of plasma oxidative damage (measured as circulating reactive oxygen metabolites) compared to subordinate males (Border et al., 2019; Border et al., 2021; Fialkowski et al., 2021). In the current study, we tested the effect of social status on oxidative stress levels as well as how the integration between different measurements of oxidative stress varies between social states. Markers of oxidative stress included oxidative DNA damage, NADPH-oxidase (NOX) activity (which influences ROS generation), total antioxidant defense and superoxidative dismutase (SOD) activity (more details about these markers can be found in the method section). Most of these markers were measured in different tissue types including blood, liver, muscle, and gonads. We predict that dominant males will have higher levels of oxidative stress (by way of elevated oxidative damage and/or lower total antioxidant capacity) than subordinate males in most tissue types, but we expect this pattern to be redox marker- and tissue-specific. We also predict that dominant and subordinate males differ in the degree of integration between different measurements of oxidative stress.

2. Methods

2.1 Animals and housing

The cichlid *Astatotilapia burtoni* used in this study were descended from a wild-caught stock population from Lake Tanganyika. Fish were housed in aquaria kept at 28°C with a 12-h light/dark cycle and 10 min each dusk and dawn period to mimic natural settings. Aquaria contained gravel substrate and terracotta shelters. Fish were fed a combination of cichlid flakes (Omega Sea LLC) and granular food (Allied Aqua) each morning. Continuous water flow and central mechanical and biological filtration occurred throughout the entirety of the experiment. All fish used in this experiment were initially housed as larvae and juveniles in 110-L tanks until they were approximately 4 months of age, after which they were transferred to a 407-L tank until randomly selected individuals were transferred to experimental tanks. All fish used in this experiment were adults and had been raised in mixed-sex groups. Experimental males were tagged just below the dorsal fin with colored beads attached to a plastic tag using a stainless-steel tagging gun (Avery-Dennison, Pasadena, CA). All procedures were approved by Central Michigan University Institutional Animal Care and Use Committee (IACUC protocol 15-22).

2.2 Experimental design

We divided experimental 110-L tanks in half widthwise with clear, perforated acrylic barriers to create two compartments as described elsewhere (Fialkowski et al., 2021). In each experimental compartment we placed a group comprised of three males and five females (n=31 groups). Fish were between 10 – 12 months of age. Males were weighed and their standard length (SL) was

139 measured before adding them to the experimental tanks. We provided one flowerpot shard in
140 each compartment which was occupied by the dominant male in each group. In most groups (27
141 out of 31) the largest male (at least 0.1 g bigger than the other males) attained social dominance
142 while the other two males in each group became subordinate. Hence, social status was mostly,
143 albeit not perfectly, assigned based on size asymmetry. The initial body mass of dominant males
144 was 5.2 ± 0.2 g (range 2.6 – 6.8, *n*=31) and subordinate males was 4.2 ± 0.1 g (range 3.0 – 6.0,
145 *n*=31). Given the considerable size overlap between social states *across* groups, size is unlikely a
146 confounding factor. Fish were able to interact physically with members in their own group and
147 visually with those in the adjacent compartment, allowing for the full expression of dominant
148 behaviors, including aggressive interactions between dominant males between compartments.
149 We collected tissue and blood from the dominant male and one randomly selected subordinate
150 male after housing fish in this arrangement for 6 – 7 weeks.
151
152 *2.3 Behavioral observations*
153 We recorded male social status (dominant or subordinate) three times per week following a two
154 week stabilization period as described previously (Border et al., 2019). In brief, dominant males
155 were brightly colored, defended a flowerpot and had a dark eye bar while subordinate males are
156 cryptically colored and shoal with females. We filmed each group in the morning before 10 a.m.
157 weekly during the final 4 weeks of the experiment, with the final recording made on the morning
158 of tissue collection. For each group, we quantified the behaviors of the focal males (dominant
159 male and subordinate male for which we collected tissue) over a five-minute period. One person
160 scored the frequency of chases, lateral displays, border displays, cave visits, courtship displays,
161 and flees as previously described (Fialkowski et al., 2021). We were unable to carry out the

observations blind with respect to social status due to the readily apparent differences in coloration and behaviors observed between dominant and subordinate males. Behavioral coding was completed using Behavioral Observation Research Interactive Software (BORIS)(Friard and Gamba, 2016).

2.4 Tissue sampling methods

Immediately following the final behavioral observation, we removed focal males (one dominant and one subordinate male) to collect blood and tissue. 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 ranging from 2.5 -11.75 minutes, with individuals processed in a randomized order with respect to social status. We collected approximately 25-100 μL of blood from each male (n=31 each). 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.

Immediately after blood was drawn, males were euthanized via rapid cervical transection and tissues (gonads, liver, muscle) were collected and flash frozen in liquid nitrogen. Gonads were weighed prior to freezing. Frozen tissue samples were immediately placed in 2 mL tubes on a M15 Coolrack block (Biocision, Larkspur, CA, USA) on dry ice. Tissue collection time (measured as the time from initial disruption of the tank to freezing tissue) ranged from 3.1 – 23.0 minutes. Blood samples were centrifuged for 10 minutes at 4000g before plasma red blood cells were separated. Blood cells, plasma and tissue samples were stored at -80°C until used for analysis.

185

186 *2.5 Measurement of oxidative stress*

187

188 *2.5.1 Choice of markers of oxidative stress*

189 As an overall marker of oxidative stress, we selected plasma reactive oxygen metabolites
190 (ROMs). ROMs are primarily organic hydroperoxides which include a range of oxidized
191 substrates, such as polyunsaturated fatty acids, proteins and nucleic acids, and thus represents a
192 comprehensive measure of oxidative damage (Costantini, 2016). We measured plasma
193 antioxidant capacity in three ways: total antioxidant capacity (TAC), the OXY-adsorbent Test
194 (OXY), and the Biological Antioxidant Potential Test (BAP). TAC is a cumulative measure of
195 total antioxidants based on the ability of antioxidants with both low molecular weight and high
196 molecular weight (such as enzymatic antioxidants) to scavenge peroxyl radicals using the oxygen
197 radical absorbance capacity (ORAC) assay (Marrocco et al., 2017)). Like TAC, OXY also
198 measures total antioxidant capacity but it is based on a different biochemical reaction (it
199 measures the ability of plasma to withstand oxidative insult from hypochlorous acid) and tends
200 not to measure larger, enzymatic antioxidants. The OXY assay was selected because it is a
201 widely used marker for systemic levels of total antioxidant capacity including in haplochromine
202 cichlid fish (Dijkstra et al., 2011; Dijkstra et al., 2016). The BAP test is based on iron oxidation
203 and provides insight into more dynamic antioxidant activity of substances with lower molecular
204 weight. In addition to plasma TAC, we measured TAC in liver, gonad, and muscle using the
205 ORAC assay. We also measured superoxide dismutase (SOD) activity, an important enzymatic
206 antioxidant that removes oxygen radicals via conversion to molecular oxygen and uric acid. SOD
207 was measured it in the liver and gonads. Oxidative DNA damage was evaluated by measuring

levels of 8-hydroxy-2'-deoxyguanosine (8-OhDG) in red blood cells, liver, and gonads. As an indirect marker of ROS production, we measured nicotinamide adenine dinucleotide phosphate-oxidase (NADPH-oxidase or NOX) activity in the liver and gonads. NOX are a major source of superoxide (a primary reactive oxygen species) in various tissue cell types (Bedard and Krause, 2007). We were unable to measure muscle NOX and muscle SOD activity due to technical difficulties (NOX) or lack of sample (SOD). We measured several redox markers in gonadal tissue since we expected social status differences in the reproductive system. The liver is particularly vulnerable to oxidative damage and is frequently included in tissue oxidative stress analyses. Muscle was also selected because of its importance for physical activities.

Here we consider increases in (potential) oxidative damage and/or lower antioxidant capacity as indicative of increased oxidative stress. More specifically, increased DNA damage and NOX activity may reflect elevated oxidative stress while lower total antioxidant protection (TAC, OXY, and BAP) could reflect antioxidant depletion under high oxidative stress levels. However, it is important to note that increased ROS production can lead to compensatory mechanisms, such as upregulated SOD. It is therefore difficult to interpret SOD findings in relation to the level of oxidative stress an organism experiences (Monaghan et al., 2009).

All samples were run in duplicate and included a pooled sample to evaluate interplate variability with the exception of SOD. For all assays we used clear flat-bottom 96-well plates unless indicated otherwise. For all assays, the intra-assay CV (coefficient of variation) and the inter-assay CV were typically below 5% and 13%, respectively.

2.5.2 Protein quantification

The protein concentration of each prepared tissue sample (tissue TAC, SOD, and NOX activity) was measured with a Bicinchoninic Acid (BCA) Protein Assay kit (Pierce, Rockford IL) following the manufacturer's protocol. Frozen supernatant was thawed on ice and diluted 1:4 with buffer used in their respective sample preparation. We used 10 µL of this diluted supernatant in the assay. Absorbance was read by a plate reader (Epoch2T, Biotech Instruments, Winooski, VT, USA).

2.5.3 Circulating Reactive Oxygen Metabolites (ROMs)

We measured the concentration of plasma ROMs (primarily organic hydroperoxides) in blood plasma using the widely used d-ROM test (Diacron, Grosseto, Italy) using 4 µL plasma per well as previously described (Border et al., 2019). We calculated values in Carratelli units which was then converted to H_2O_2 mg/dL. Absorbance was read by a plate reader (Epoch2T, Biotech Instruments, Winooski, VT, USA).

2.5.4 Circulating Antioxidants

Total Antioxidant Capacity (TAC): Circulating TAC was determined in diluted blood plasma (1:100) in 7.4 pH PBS using 20 µL of diluted sample via an oxygen radical absorbance capacity (ORAC) assay as previously described (Border et al., 2019). For circulating TAC, samples were reported as µmol TE/dL of the sample. Absorbance was read by a plate reader (Spectramax M3, Molecular Devices, Sunnyvale, CA, USA).

OXY-adsorbent Test (OXY): For the OXY assay (Diacron, Grosseto, Italy), we diluted blood plasma (1:100) with distilled water using 2 µL of diluted sample per well following the protocol as described previously (Dijkstra et al., 2016). Plasma OXY concentration was

253 expressed as μmol of HClO/mL of sample. Absorbance was read at 550 nm by a plate reader
254 (Spectramax M3, Molecular Devices, Sunnyvale, CA, USA).
255 *Biological Antioxidant Potential Test (BAP):* For the BAP assay (Diacron, Grosseto,
256 Italy), we used 2 μL of plasma for each well and followed the manufacturer instructions. Plasma
257 BAP concentration was expressed as μmol/mL of sample. Absorbance was read at 505 nm by a
258 plate reader (Spectramax M3, Molecular Devices, Sunnyvale, CA, USA).
259
260 *2.5.5 Tissue Total Antioxidant Capacity (TAC)*
261 TAC was determined in tissue (liver, gonad, and muscle) following the same ORAC procedure
262 as described for plasma with the following exceptions for all tissues. Tissues were removed from
263 -80°C and homogenized on ice in 0.250 mL lysis buffer (20 mM Tris–HCl, 137 mM NaCl, 1%
264 NP-40, 10% glycerol, 2 mM EDTA) using an Omni Tissue Master (Omni International,
265 Kenosha, Wisconsin), then centrifuged at 4°C at 17,000g for 10 minutes. Supernatant was
266 collected and used to run BCA and ORAC. For ORAC, protein concentrations were standardized
267 to ~150 μg/mL and reported as μmol TE/μg protein. Absorbance was read by a plate reader
268 (Spectramax M3, Molecular Devices, Sunnyvale, CA, USA).
269
270 *2.5.6 Superoxide dismutase (SOD)*
271 SOD was measured using Water Soluble Tetrazolium Salts (WSTs) via a modified competitive
272 assay (Peskin and Winterbourn 2000) in liver and gonads. Samples were taken from -80°C and
273 homogenized on ice in a PBS (pH 7.4, 75mM). Tissue homogenates were then centrifuged at 4
274 °C at 10,000 g for 10 min. The supernatant was then transferred to a new tube and stored at -80
275 °C until used for analysis. Protein concentration was measured using BCA before preparing each

supernatant at multiple protein concentrations (liver: 0.375 µg/µL, 0.25 µg/µL, 0.125 µg/µL, and 0.05 µg/µL; gonads: 0.4 µg/µL, 0.3 µg/µL, 0.2 µg/µL, and 0.1 µg/µL). SOD activity was measured as described previously (Fialkowski et al., 2021). The amount of SOD content was reported per µg of protein (one unit of SOD is defined as the amount of enzyme in 20 µL of the sample solution that inhibits the reduction reaction of WST-1 with superoxide anion by 50%). Absorbance was read by a plate reader (Epoch2T, Biotech Instruments, Winooski, VT, USA).

2.5.7 NADPH-Oxidase (NOX) activity

NOX activity was measured in the liver and the gonads via a lucigenin-based chemiluminescence assay using a microplate luminometer (Fialkowski et al., 2021). Liver samples were taken from -80°C and homogenized on ice in a Krebs-HEPES buffer. The homogenized samples (before centrifugation) were subjected to a freeze-thaw cycle to ensure total cell lysis. After thawing, samples were centrifuged at 4°C at 600g for 10 minutes and supernatant was transferred and stored at -80°C. For gonad samples, the supernatant was subjected to an additional freeze-thaw cycle to ensure total cell lysis. For each sample, an aliquot of supernatant was used to run BCA. To measure NOX activity, samples containing 30 µg (liver) or 15 µg (gonad) of protein were placed in a solid black 96-well plate (Corning 3912), then the reaction was initiated out of direct light by the addition of buffer containing lucigenin and NADPH, with a final concentration of 5uM lucigenin and 100uM NADPH. The plate was kept covered for transport to luminometer (Tecan infinite F200 Pro, Tecan Life Sciences, Männedorf, Zürich, Switzerland) and readings of Relative Light Units (RLU) were taken every 2 minutes for 20 minutes. The area under the curve was calculated for minutes 2-20, and the results were expressed as RLU per minute per µg protein after subtraction of background chemiluminescence.

299

300 *2.5.8 Oxidative DNA damage*

301 Oxidative DNA damage was evaluated for 8-OhDG damage using a DNA damage ELISA kit
302 (StressMarq Biosciences Inc.) (Fialkowski et al., 2021). DNA was extracted from packed red
303 blood cells (PRBCs) and frozen tissue samples (liver, gonad) using a commercially available
304 DNA extraction kit (Zymo quick-DNA miniprep plus kit) as described previously. Extracted
305 samples were stored at 4 ℃ until digestion. Samples were digested at a standardized
306 concentration of ~200 ng/ul (PRBCs, gonad) or 400-500 ng/ul (liver) using a modified digest
307 mix protocol by Quinlivan and Gregory (2008) and stored at -20 ℃ until use in DNA damage
308 plate (Quinlivan and Gregory, 2008). Digested samples were tested for 8-OhDG damage using a
309 DNA damage ELISA kit at 12x dilution for blood and gonad samples and a 10x dilution for liver
310 samples, with all samples run in duplicate following the manufacturer's instructions. Absorbance
311 was measured using a microplate reader (Epoch2T, Biotech Instruments, Winooski, VT, USA).
312 8-OhDG concentration was standardized relative to total DNA concentration and reported in
313 ng/µL.

314

315 *2.5.9 Testosterone levels*

316 To confirm that dominant males upregulated their reproductive system, we quantified circulating
317 testosterone levels using competitive ELISA kits (Enzo Life Sciences) as previously described
318 (Border et al., 2019). Absorbance was read by a plate reader (Epoch2T, Biotech Instruments,
319 Winooski, VT, USA).

320

321 *2.6 Statistical analysis*

We calculated a dominance index score for each weekly 5-minute focal observation as the sum of (aggressive behavior + reproductive behaviors) – fleeing events per min, as done previously in *A. burtoni* (Maruska et al., 2013). Gonadosomatic index (GSI) was calculated as (gonad weight/total body weight)*100. Specific growth rate was expressed as the daily percentage weight change from the beginning to the end of the experiment relative to the initial weight (calculated as [ln(body $weight_{final}$) – ln(body $weight_{initial}$)] x 100/days) (Ricker, 1975). Since males that were initially smaller grew more during the experiment, we calculated the residuals of specific growth rate using a linear regression and used this as our growth variable in the analysis.

All analyses were conducted in R v3.4.3. We analyzed our data using the R packages lme4, lmerTest, MASS (Bates et al., 2015), and glmmTMB (Brooks et al., 2017). We identified and excluded outliers based on Tukey's rule (between 0 and 2 values were excluded per measurement). In addition, samples size for oxidative stress measurements varied depending on availability of tissue or technical constraints (Table 1). We used linear mixed models (LMMs) with a maximum-likelihood protocol. For count and proportional data, we used generalized linear mixed models (GLMMs). In each model, we used 'pair code' as random effect to account for fish that were housed in the same group. To evaluate the validity of our LMM and GLMM models, we examined the residuals, qqplots, and plots of predicted values versus residuals. We report mean ± SE for our model estimates.

2.6.2 Analysis of behavior, GSI, and testosterone.

To confirm the assigned social status of each male, we compared behavior, GSI, and testosterone levels between dominant and subordinate males. We tested for differences between social states in dominance index using a LMM. Shoaling duration was analyzed using a GLMM assuming a

Gaussian distribution with log link function and foraging bouts were analyzed using GLMM assuming a negative binomial distribution. In addition to 'pair code', we used 'fish code' nested within 'pair code' as random effect to account for the 4 weekly measurements of behavior for each focal male. GSI was analyzed using a LMM and testosterone levels were analyzed using a GLMM assuming a Gaussian distribution with log link function.

2.6.3 Analysis of oxidative stress

Measurements of oxidative stress were compared between dominant and subordinate males using LMMs. To examine whether covariance patterns across the different oxidative stress measurements varied by social status, we created clustered correlation matrices for dominant and subordinate males separately. We then carried out a hierarchical cluster analysis to identify clusters of oxidative stress measurements that were coregulated. We obtained *P*-values using multiscale bootstrap resampling from the pvclust package (Suzuki and Shimodaira, 2006). Since growth rate and investment in territorial defense and reproduction could influence oxidative state, we also carried out the same hierarchical cluster analysis after adding GSI, specific growth rate, dominance index (based on the final five-minute focal observation, closest to tissue sampling) and testosterone to the oxidative stress dataset.

3. Results

3.1 Males become dominant or subordinate

Males did not change social status a during the entire duration of the experiment (Fig. 1A). The dominance index (the difference between aggressive behavior and fleeing events) was

368 significantly higher in dominant males (LMM, -12.6871±0.4730, t_{62}=-26.82, *P*<0.00001).
369 Subordinate males spent more time shoaling than dominant males (GLMM, 2.59±0.50, z=5.19,
370 *P*<0.00001). Social dominance was linked to more reproductive behavior, with dominant males
371 showing more courtship behavior than subordinate males (GLMM, zero-inflation model,
372 3.27±0.61, z=5.34, *P*<0.00001). We observed mouthbrooding females in all groups during the
373 entire duration of the experiment, suggesting that all dominant males spawned (females typically
374 spawn with dominant males, PDD pers. obs.).
375 Dominant males had higher gonadosomatic index (LMM, -0.125±0.050, t_{31}=-2,
376 *P*=0.0189) and circulating testosterone levels (GLMM, -0.595±0.102, z=-5.85, P<0.00001) than
377 subordinate males, confirming that the former had an activated reproductive system (Fig. 1B).
378 Finally, dominant males grew faster than subordinate males (specific growth rate, dominant
379 males: 1.044±0.067, subordinate males: 1.019±0.063) and after correcting for the effect of initial
380 body weight this effect of social status on growth rate was significant (LMM, -0.196±0.067, t_{31}=-
381 2.94, *P*=0.006).
382
383 *3.2 Dominant males experienced greater circulating oxidative damage than subordinate males*
384 We compared a total of 14 oxidative stress measures between dominant and subordinate males
385 (Fig. 2, Table 1). Plasma reactive oxygen metabolites (ROMs), a marker of overall oxidative
386 damage, was higher in dominant males than in subordinate males (Table 1), consistent with
387 previous findings in the same species (Border et al., 2019; Fialkowski et al., 2021). Dominant
388 males displayed higher NOX activity in the gonads and liver, although this effect was only
389 significant in the gonads (Table 1). There were no significant status differences in the other
390 measurements of oxidative stress (Table 1).

391

392 *3.3 Dominant males express greater covariance patterns of oxidative stress profile than*
393 *subordinate males*

394 We tested whether dominant and subordinate males vary in co-variance patterns across the
395 different markers of oxidative stress and tissue types by examining clustered correlation matrices
396 for dominant and subordinate males separately (Fig. 3A). In dominant males, significant clusters
397 included both antioxidant function and oxidative damage across multiple tissue types while in
398 subordinate males only one plasma antioxidant cluster was significant. Specifically, there were
399 three significant clusters in dominant males, one involving plasma BAP, liver TAC, and gonad
400 SOD, a second cluster involving muscle TAC and liver SOD, and a third cluster comprised of the
401 remaining variables (all $P<0.05$). By contrast, there was only one small cluster that was
402 significant in subordinate males comprised of plasma BAP and plasma OXY ($P<0.05$).

403 Given that investment in growth, social dominance, and reproduction may be linked to
404 oxidative stress, we also tested for co-variance patterns in oxidative stress datasets that included
405 GSI, specific growth rate, testosterone, and dominance index. This analysis revealed two
406 significant clusters in dominant males versus only one in subordinate males (Fig. 3, all $P<0.05$).
407 Similar to the analysis that only included measurements of oxidative stress, there were more
408 clusters in dominant males than subordinate males. Clusters in dominant males included both
409 antioxidant function and oxidative damage across tissue types, supporting that social dominance
410 is causally linked to a higher degree of functional integration of oxidative state.

411

412 **4. Discussion**

We found clear dominance hierarchies with dominant males having upregulated GSI and higher testosterone levels than subordinate males. However, we found limited evidence that dominant males had higher levels of oxidative stress than subordinate males when comparing independent oxidative stress measurements. Importantly, there were more significant clusters of coregulated oxidative stress variables in dominant males than in subordinate males, suggesting that dominant males have more efficient or more active regulation of oxidative balance than subordinate males. Below, we discuss these findings in more detail.

4.1 Limited cost of social dominance when markers are evaluated in isolation

Our findings suggest that social status-specific differences in oxidative balance is highly tissue- and marker-specific. Organisms are comprised of a complex set of integrated organs performing unique functions. As a result, energetically demanding activities such as reproduction and defending high rank likely affect parts of the body differently (Costantini, 2019; Speakman and Garratt, 2014). It is therefore unsurprising that reproduction may elevate, reduce, or have no impact on oxidative damage and/or antioxidant function depending on which macromolecules and tissues are considered, which our findings here support (Garratt et al., 2011; Garratt et al., 2013; Ołdakowski et al., 2015; Yang et al., 2013). However, it was surprising that when comparing markers of oxidative stress in isolation, dominant and subordinate males only differed in two measurements of oxidative stress. We also note that increased NOX activity in the gonads of dominant males is consistent with higher NOX signaling in mature sperm, which does not necessarily constitute a 'cost' given the important role of redox signaling in sperm function (Tremellen, 2012). Furthermore, there was no social status effect on oxidative DNA damage, even though we measured it in three different tissue types.

There are several explanations for the limited status-dependent differences in oxidative stress, including the ability to effectively manage or minimize oxidative stress and the fact that our animals were housed under benign lab conditions. Since oxidative stress is an ever present problem, organisms have evolved multi-faceted highly-regulated cytoprotective mechanisms to mitigate oxidative damage (Balaban et al., 2005). We recently induced social status transition from subordinate to dominant position in *A. burtoni* and found that social ascent was associated with dynamic changes in plasma ROMs, plasma TAC, liver TAC and liver SOD activity (Fialkowski et al., 2021). Specifically, plasma TAC was rapidly depleted while liver SOD and liver TAC were increased during social ascent. However, after 2 weeks of social dominance, dominant males did not have different levels of liver SOD or TAC and had higher plasma ROMs than subordinate males, consistent with the current study.

The fact that in the current study we did not detect social status differences in oxidative balance in most tissue types could be due to a limited cost of social dominance when the hierarchy is stable (for a study comparing stable hierarchies and social ascension in another cichlid species, see (Culbert et al., 2022)). Our findings are consistent with the notion that animals undergo behavioral and physiological adjustments to cope with demanding life history activities. For example, the onset of reproduction is often associated with dramatic metabolic and morphological remodeling to cope with the energetic demands of reproduction (Reiff et al., 2015) and these changes may also involve upregulated antioxidant defense (Blount et al., 2016). Further, our experiments were carried out in captivity under benign conditions providing ad libitum food and protection. It is possible that the cost of social dominance is more pronounced when dominant individuals face additional challenges, such as parental care, food shortage, temperature stress, parasites, or social instability (Speakman et al., 2015). For example, in the

459 white-browed sparrow weaver (*Plocepasser mahali*), highly ranked females but not highly
460 ranked males experienced increased oxidative stress after breeding, presumable due to the fact
461 that females exert more effort during reproduction in relation to egg laying, incubation and egg
462 provisioning (Cram et al., 2015).
463
464 *4.2 Social status differences in integration of oxidative stress components*
465 Social status predicted the extent of functional integration across different components of
466 oxidative stress. Across all 14 measurements of oxidative stress (i.e. all markers and tissue
467 types), we observed three significant co-regulated clusters in dominant males and only one
468 cluster in subordinate males. In dominant males, all oxidative stress variables were part of one of
469 these clusters. In addition, in dominant males all clusters contained variables from different
470 tissue types, in contrast to the situation in subordinate males (in the latter, plasma BAP and
471 plasma OXY formed a cluster, which is not surprising given that both measure overlapping
472 components of antioxidant capacity in blood). Further, in dominant males there was coregulation
473 between different measurements within the same tissue as well as the same measurement across
474 different tissue. It was particularly interesting to note that all oxidative DNA damage
475 measurements were included in this cluster. The other two smaller clusters in dominant males
476 linked different measures of antioxidant function (TAC and SOD) across different tissue types,
477 both containing measures in liver linked to either gonads or muscle. It is difficult to interpret the
478 functional significance of these smaller clusters in dominant males, but they suggest a relatively
479 high level of coordination between different components of the antioxidant defense system
480 across completely different organs.

The more modular redox responses in dominant males relative to subordinate males could be an indication of a more efficient and/or active management of oxidative balance. This finding suggests that dominant males pay a reduced cost to maintaining oxidative balance due to more effective neutralization of oxidative insults by antioxidant defense systems or dominant males benefiting from a more robust, stable system guarding oxidative balance relative to subordinate males. The lack of modularity in subordinate males could suggest that social subordination is associated with increased dysregulation of oxidative balance, and perhaps increased (and costly) investment into maintaining redox balance. This notion that a low level of integration reflects an oxidative cost is consistent with lower integration of antioxidant parameters observed in hybridizing newts as a cost of interspecific hybridization (Prokić et al., 2018) and exercise-induced loss of integration in zebra finches (Costantini et al., 2013). It is also supported by proteomic and metabolomic studies suggesting that the degree of integration or connectivity in metabolic networks may reveal information about the robustness or efficiency of systems that maintain stability and homeostasis. Integration of these systems may decline with age due to failure in communication between interacting units (Hoffman et al., 2017).

However, increased modularity observed in dominant males may also entail costs. More integration may be a manifestation of more active management of oxidative balance. Although the relative energetic cost of upregulating antioxidant enzymes is probably low, active regulation of redox balance is not cost-free due to 'physiological constraints' or pleiotropic effects of activating antioxidant response systems (Pamplona and Costantini, 2011). For example, in addition to being a damaging by-product, ROS also have important cell signaling functions, and mounting an antioxidant response could also quench ROS that have beneficial effects (Linnane et al., 2007). Consequently, upregulation of antioxidant enzymes may lead to detrimental side

effects (Barajas et al., 2011). The interpretation of increased integration of oxidative stress in dominant males relative to the oxidative cost of social dominance/reproduction is complicated, and future studies should shed more light on functional significance of variation in integration in the context of life history trade-offs. Specifically, to what extent does the degree of integration reflect robustness and efficiency of interacting systems that maintain redox homeostasis? How is the relationship between integration and robustness/efficiency modulated by the type, duration, and magnitude of stressors associated with social dominance and reproduction? And based on this information on the link between integration and efficiency/activation of the system, what are the long-term fitness consequences? These are interesting questions that are not always easy to tackle (e.g. addressing some of these questions requires longitudinal sampling of the same individuals, which is challenging unless non-invasive sampling techniques are used (Alonso-Alvarez et al., 2017)) but may prove useful in future studies.

To conclude, we found that dominant *A. burtoni* males experienced more oxidative stress in only two oxidative stress markers (plasma ROMs and gonad NOX activity) out of a total of 14 different oxidative stress measurements. We found evidence for more integrated redox regulation, and hence more active or efficient management of oxidative balance in dominant males. Whether this supports the oxidative cost of social dominance remains to be tested in future studies. We propose that studies on the oxidative cost of demanding life history events more generally (e.g. migration: (Eikenaar et al., 2020); parental care: (Guindre-Parker and Rubenstein, 2018)) should include analyses of how different components of oxidative stress are interconnected. Even in the absence of difference in mean values in redox markers between for examples breeders and nonbreeders, there might be differences in correlation structure that could provide important insights into the role of oxidative stress as a mediator of life history trade-offs.

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Author's contributions

Authors PD and SB contributed to the conception and design of the experiment. RF, SB, and PD contributed material preparation and data collection with IB performing behavioral coding. The first draft of the manuscript was written by PD and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC, protocols #15-22 and #18-10) prior to conducting the experiment. All applicable international, national, and/or institutional guidelines for the use of animals were followed.

Declaration of competing interests

The authors declare no competing interests.

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References

Alonso-Alvarez, C., Canelo, T. and Romero-Haro, A. Á. (2017). The oxidative cost of reproduction: Theoretical questions and alternative mechanisms. *Bioscience* **67**, 258–270.

552 **Alward, B. A., Laud, V. A., Skalnik, C. J., York, R. A., Juntti, S. A. and Fernald, R. D.**
553 (2020). Modular genetic control of social status in a cichlid fish. *Proc. Natl. Acad. Sci. U. S.*
554 *A.* **117**, 28167–28174.
555 **Archer, C. R., Sakaluk, S. K., Selman, C., Royle, N. J. and Hunt, J.** (2013). Oxidative stress
556 and the evolution of sex differences in life span and ageing in the decorated cricket,
557 Gryllodes sigillatus. *Evolution (N. Y).* **67**, 620–634.
558 **Balaban, R. S., Nemoto, S. and Finkel, T.** (2005). Mitochondria, oxidants, and aging. *Cell* **120**,
559 483–95.
560 **Barajas, B., Che, N., Yin, F., Rowshanrad, A., Orozco, L. D., Gong, K. W., Wang, X.,**
561 **Castellani, L. W., Reue, K., Lusis, A. J., et al.** (2011). NF-E2-related factor 2 promotes
562 atherosclerosis by effects on plasma lipoproteins and cholesterol transport that overshadow
563 antioxidant protection. *Arterioscler. Thromb. Vasc. Biol.* **31**, 58–66.
564 **Bates, D., Mächler, M., Bolker, B. and Walker, S.** (2015). Fitting linear mixed-effects models
565 using lme4. *J. Stat. Softw.* **67**,.
566 **Beaulieu, M., Mboumba, S., Willaume, E., Kappeler, P. M. and Charpentier, M. J. E.**
567 (2014). The oxidative cost of unstable social dominance. *J. Exp. Biol.* **217**, 2629–32.
568 **Bedard, K. and Krause, K. H.** (2007). The NOX family of ROS-generating NADPH oxidases:
569 Physiology and pathophysiology. *Physiol. Rev.* **87**, 245–313.
570 **Bize, P., Devevey, G., Monaghan, P., Doligez, B. and Bize, P.** (2008). Fecundity and survival
571 in relation to resistance to oxidative stress in a free-living bird. *Ecology* **89**, 2584–2593.
572 **Blount, J. D., Vitikainen, E. I. K., Stott, I. and Cant, M. A.** (2016). Oxidative shielding and
573 the cost of reproduction. *Biol. Rev.* **91**, 483–497.
574 **Border, S. E., Piefke, T. J., Fialkowski, R. J., Tryc, M. R., Funnell, T. R., Deoliveira, G. M.**
575 **and Dijkstra, P. D.** (2018). Color change and pigmentation in a color polymorphic cichlid
576 fish. *Hydrobiologia* **832**, 175–191.
577 **Border, S. E., Deoliveira, G. M., Janeski, H. M., Piefke, T. J., Brown, T. J. and Dijkstra, P.**
578 **D.** (2019). Social rank , color morph , and social network metrics predict oxidative stress in
579 a cichlid fish. *Behav. Ecol.* **30**, 490–499.
580 **Border, S. E., Piefke, T. J., Funnell, T. R., Fialkowski, R. F., Sawecki, J. and Dijkstra, P. D.**
581 (2021). Social instability influences rank-specific patterns of oxidative stress in a cichlid
582 fish. *J. Exp. Biol.* **224**,.
583 **Brooks, M. E., Kristensen, K., van Benthem, K. J., Magnusson, A., Berg, C. W., Nielsen, A.,**
584 **Skaug, H. J., Mächler, M. and Bolker, B. M.** (2017). glmmTMB balances speed and
585 flexibility among packages for zero-inflated generalized linear mixed modeling. *R J.* **9**,
586 378–400.
587 **Christe, P., Glaizot, O., Strepparava, N., Devevey, G. and Fumagalli, L.** (2012). Twofold
588 cost of reproduction: An increase in parental effort leads to higher malarial parasitaemia and
589 to a decrease in resistance to oxidative stress. *Proc. R. Soc. B Biol. Sci.* **279**, 1142–1149.
590 **Costantini, D.** (2016). Oxidative stress ecology and the d-ROMs test: facts, misfacts and an
591 appraisal of a decade's work. *Behav. Ecol. Sociobiol.* **70**, 809–820.
592 **Costantini, D.** (2019). Understanding diversity in oxidative status and oxidative stress: The
593 opportunities and challenges ahead. *J. Exp. Biol.* **222**,.
594 **Costantini, D., Monaghan, P. and Metcalfe, N. B.** (2011). Biochemical integration of blood
595 redox state in captive zebra finches (Taeniopygia guttata). *J. Exp. Biol.* **214**, 1148–52.
596 **Costantini, D., Monaghan, P. and Metcalfe, N. B.** (2013). Loss of integration is associated
597 with reduced resistance to oxidative stress. *J. Exp. Biol.* **216**, 2213–20.

Costantini, D., Casasole, G. and Eens, M. (2014). Does reproduction protect against oxidative stress? *J. Exp. Biol.* **217**, 4237–4243.

Cram, D. L., Blount, J. D. and Young, A. J. (2015). Oxidative status and social dominance in a wild cooperative breeder. *Funct. Ecol.* **29**, 229–238.

Culbert, B. M., Dijkstra, P. D., Gilmour, K. M. and Balshine, S. (2022). Antioxidant capacity differs across social ranks and with ascension in males of a group-living fish. *Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol.* **265**, 111126.

Dijkstra, P. D., Wiegertjes, G. F., Forlenza, M., van der Sluijs, I., Hofmann, H. A., Metcalfe, N. B. and Groothuis, T. G. G. (2011). The role of physiology in the divergence of two incipient cichlid species. *J. Evol. Biol.* **24**, 2639–52.

Dijkstra, P. D., Pierotti, M. E. R., Seehausen, O. and Metcalfe, N. B. (2016). Metabolism, oxidative stress and territorial behaviour in a female colour polymorphic cichlid fish. *Behav. Ecol. Sociobiol.* **70**, 99–109.

Dowling, D. K. and Simmons, L. W. (2009). Reactive oxygen species as universal constraints in life-history evolution. *Proc. Biol. Sci.* **276**, 1737–45.

Eikenaar, C., Winslott, E., Hessler, S. and Isaksson, C. (2020). Oxidative damage to lipids is rapidly reduced during migratory stopovers. *Funct. Ecol.* **34**, 1215–1222.

Enomoto, A., Itoh, K., Nagayoshi, E., Haruta, J., Kimura, T., O'Connor, T., Harada, T. and Yamamoto, M. (2001). High Sensitivity of Nrf2 Knockout Mice to Acetaminophen Hepatotoxicity Associated with Decreased Expression of ARE-Regulated Drug Metabolizing Enzymes and Antioxidant Genes. *Toxicol. Sci.* **59**, 169–177.

Fernald, R. D. (2017). Cognitive skills and the evolution of social systems. *J. Exp. Biol.* **220**, 103–113.

Fialkowski, R., Aufdemberge, P., Wright, V. and Dijkstra, P. (2021). Radical change: temporal patterns of oxidative stress during social ascent in a dominance hierarchy. *Behav. Ecol. Sociobiol.* **75**, 43.

Friard, O. and Gamba, M. (2016). BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. *Methods Ecol. Evol.* **7**, 1325–1330.

Garratt, M., Vasilaki, A., Stockley, P., McArdle, F., Jackson, M. and Hurst, J. L. (2011). Is oxidative stress a physiological cost of reproduction? An experimental test in house mice. *Proc. R. Soc. B Biol. Sci.* **278**, 1098–1106.

Garratt, M., Pichaud, N., King, E. D. A. and Brooks, R. C. (2013). Physiological adaptations to reproduction. I. Experimentally increasing litter size enhances aspects of antioxidant defence but does not cause oxidative damage in mice. *J. Exp. Biol.* **216**, 2879–2888.

Guindre-Parker, S. and Rubenstein, D. R. (2018). No short-term physiological costs of offspring care in a cooperatively breeding bird. *J. Exp. Biol.* **221**,.

Hoffman, J. M., Lyu, Y., Pletcher, S. D. and Promislow, D. E. L. (2017). Proteomics and metabolomics in ageing research: from biomarkers to systems biology. *Essays Biochem.* **61**, 379–388.

Hõrak, P. and Cohen, A. (2010). How to measure oxidative stress in an ecological context: Methodological and statistical issues. *Funct. Ecol.* **24**, 960–970.

Linnane, A. W., Kios, M. and Vitetta, L. (2007). Healthy aging: Regulation of the metabolome by cellular redox modulation and prooxidant signaling systems: The essential roles of superoxide anion and hydrogen peroxide. *Biogerontology* **8**, 445–467.

Marrocco, I., Altieri, F. and Peluso, I. (2017). Measurement and clinical significance of biomarkers of oxidative stress in humans. *Oxid. Med. Cell. Longev.* **2017**,.

644 **Maruska, K. P., Becker, L., Neboori, a. and Fernald, R. D.** (2013). Social descent with
645 territory loss causes rapid behavioral, endocrine and transcriptional changes in the brain. *J.*
646 *Exp. Biol.* **216**, 3656–3666.
647 **Monaghan, P., Metcalfe, N. B. and Torres, R.** (2009). Oxidative stress as a mediator of life
648 history trade-offs: mechanisms, measurements and interpretation. *Ecol. Lett.* **12**, 75–92.
649 **Noguera, J. C.** (2019). Crickets increase sexual signalling and sperm protection but live shorter
650 in the presence of rivals. *J. Evol. Biol.* **32**, 49–57.
651 **O'Connell, L. A. and Hofmann, H. A.** (2012). Social status predicts how sex steroid receptors
652 regulate complex behavior across levels of biological organization. *Endocrinology* **153**,
653 1341–51.
654 **Ołdakowski, Ł., Wasiluk, A., Sadowska, E. T., Koteja, P. and Taylor, J. R. E.** (2015).
655 Reproduction is not costly in terms of oxidative stress. *J. Exp. Biol.* **218**, 3901–3910.
656 **Pamplona, R. and Costantini, D.** (2011). Molecular and structural antioxidant defenses against
657 oxidative stress in animals. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* **301**,.
658 **Piefke, T. J., Bonnell, T. R., DeOliveira, G. M., Border, S. E. and Dijkstra, P. D.** (2021).
659 Social network stability is impacted by removing a dominant male in replicate dominance
660 hierarchies of a cichlid fish. *Anim. Behav.* **175**, 7–20.
661 **Prokić, M. D., Despotović, S. G., Vučić, T. Z., Petrović, T. G., Gavrić, J. P., Gavrilović, B.**
662 **R., Radovanović, T. B. and Saičić, Z. S.** (2018). Oxidative cost of interspecific
663 hybridization: A case study of two Triturus species and their hybrids. *J. Exp. Biol.* **221**, 1–7.
664 **Quinlivan, E. P. and Gregory, J. F.** (2008). DNA digestion to deoxyribonucleoside: A
665 simplified one-step procedure. *Anal. Biochem.* **373**, 383–385.
666 **Reiff, T., Jacobson, J., Cognigni, P., Antonello, Z., Ballesta, E., Tan, K. J., Yew, J. Y.,**
667 **Dominguez, M. and Miguel-Aliaga, I.** (2015). Endocrine remodelling of the adult intestine
668 sustains reproduction in drosophila. *Elife* **4**,.
669 **Ricker, W.** (1975). Computation and interpretation of biological statistics of fish populations.
670 *Bull. Fish. Res. Board Canada* **191**, 1–382.
671 **Sawecki, J., Miros, E., Border, S. E. and Dijkstra, P. D.** (2019). Reproduction and maternal
672 care increase oxidative stress in a mouthbrooding cichlid fish. *Behav. Ecol.*
673 **Selman, C., McLaren, J. S., Meyer, C., Duncan, J. S., Redman, P., Collins, A. R., Duthie, G.**
674 **G. and Speakman, J. R.** (2006). Life-long vitamin C supplementation in combination with
675 cold exposure does not affect oxidative damage or lifespan in mice, but decreases
676 expression of antioxidant protection genes. *Mech. Ageing Dev.* **127**, 897–904.
677 **Sharick, J. T., Vazquez-Medina, J. P., Ortiz, R. M. and Crocker, D. E.** (2015). Oxidative
678 stress is a potential cost of breeding in male and female northern elephant seals. *Funct.*
679 *Ecol.* **29**, 367–376.
680 **Silva, L. R., Lardy, S., Ferreira, A. C., Rey, B., Doutrelant, C. and Covas, R.** (2018).
681 Females pay the oxidative cost of dominance in a highly social bird. *Anim. Behav.* **144**,
682 135–146.
683 **Speakman, J. R. and Garratt, M.** (2014). Oxidative stress as a cost of reproduction: Beyond
684 the simplistic trade-off model. *BioEssays* **36**, 93–106.
685 **Speakman, J. R., Blount, J. D., Bronikowski, A. M., Buffenstein, R., Isaksson, C.,**
686 **Kirkwood, T. B. L., Monaghan, P., Ozanne, S. E., Beaulieu, M., Briga, M., et al.**
687 (2015). Oxidative stress and life histories: Unresolved issues and current needs. *Ecol. Evol.*
688 **5**, 5745–5757.
689 **Stier, A., Reichert, S., Massemin, S., Bize, P. and Criscuolo, F.** (2012). Constraint and cost of

690 oxidative stress on reproduction: Correlative evidence in laboratory mice and review of the
691 literature. *Front. Zool.* **9**,.
692 **Suzuki, R. and Shimodaira, H.** (2006). Pvclust: An R package for assessing the uncertainty in
693 hierarchical clustering. *Bioinformatics* **22**, 1540–1542.
694 **Tremellen, K.** (2012). Oxidative stress and male infertility: A clinical perspective. *Stud. Men's*
695 *Heal. Fertil.* **14**, 325–353.
696 **van de Crommenacker, J., Komdeur, J. and Richardson, D. S.** (2011). Assessing the cost of
697 helping: The roles of body condition and oxidative balance in the seychelles warbler
698 (acrocephalus sechellensis). *PLoS One* **6**,.
699 **Yang, D. B., Xu, Y. C., Wang, D. H. and Speakman, J. R.** (2013). Effects of reproduction on
700 immuno-suppression and oxidative damage, and hence support or otherwise for their roles
701 as mechanisms underpinning life history trade-offs, are tissue and assay dependent. *J. Exp.*
702 *Biol.* **216**, 4242–4250.
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Tables

Table 1. Statistical results comparing measurements of oxidative stress between dominant and subordinate males. Significant effects are shown in bold.

					Sample sizes	
Marker	**Estimate**	**df**	**t value**	**P value**	**Dominant**	**Subordinate**
Plasma ROMs	-0.603±0.191	26.46	-3.167	**0.00385****	29	29
Plasma TAC	-95.52±62.03	58.00	-1.54	0.129	28	30
Plasma OXY	-35.24±42.13	38.00	-0.836	0.408	19	19
Plasma BAP	-1812.9±1083.6	26.0	-1.673	0.106	13	13
Liver TAC	0.005±0.068	31.00	0.07	0.944	31	31
Gonad TAC	-0.058±0.051	30.28	-1.151	0.259	31	30
Muscle TAC	0.069±0.068	61.00	1.021	0.311	30	31
Blood DNA damage	0.011±0.011	0.25	1.008	0.323	29	28
Liver DNA damage	-0.0034±0.0060	0.18	-0.565	0.579	26	19
Gonad DNA damage	-0.0135±0.011	0.27	-1.246	0.223	29	26
Liver NOX	-4.36±2.48	28.88	-1.755	0.0899	29	29
Gonad NOX	-6.68±3.05	30.96	-2.19	**0.0362***	30	30
Liver SOD	-0.011±0.036	49.00	-0.313	0.755	26	23
Gonad SOD	-0.023±0.029	27.40	-0.79	0.436	27	20

Figures

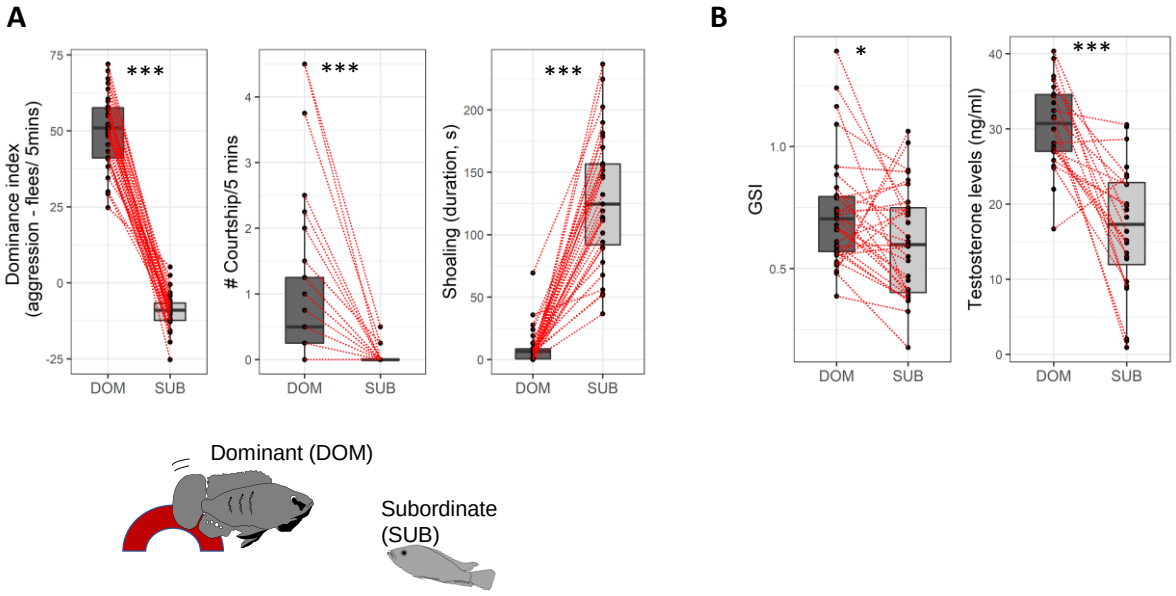
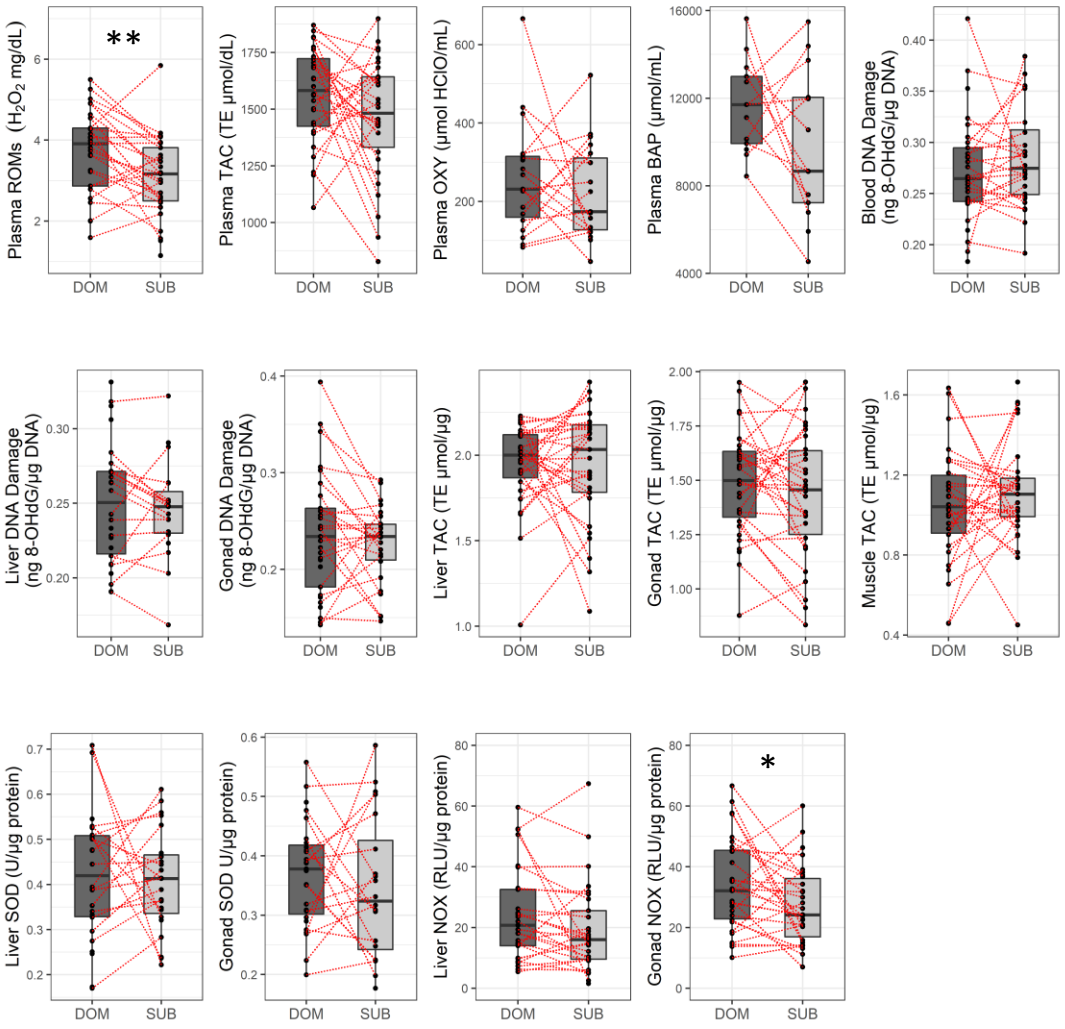


Fig. 1. Levels of behavior, testosterone, and gonadosomatic index by social status. (A) Dominant males show more aggressive and courtship behaviors while subordinate males show more subordinate behaviors such as fleeing and shoaling during four weekly observations. Shown are the rates (dominant index per minute, courtship per 5 minutes, shoaling amount of time spent) averaged across the final four weeks prior to tissue sampling. (B) Dominant males also had higher gonadal somatic index (GSI) and higher circulating testosterone levels than subordinate males. Bold lines indicate medians. Boxes enclose 25th to 75th percentiles. Error bars enclose data range, excluding outliers. Dots are data points and red lines connect data for males that were housed together. * $P<0.05$, *** $P<0.001$



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742 **Fig. 2.** Measurements of oxidative stress by social status. Reactive oxygen metabolites (ROMs)
743 and NOX (NADPH-oxidase) activity were higher in dominant males than in subordinate males.
744 The other markers did not vary by social status. Boxes enclose 25th to 75th percentiles. Error
745 bars enclose data range, excluding outliers. Dots are data points and red lines connect data for
746 males that were housed together. For statistics, see table 1. * $P<0.05$, ** $P<0.01$

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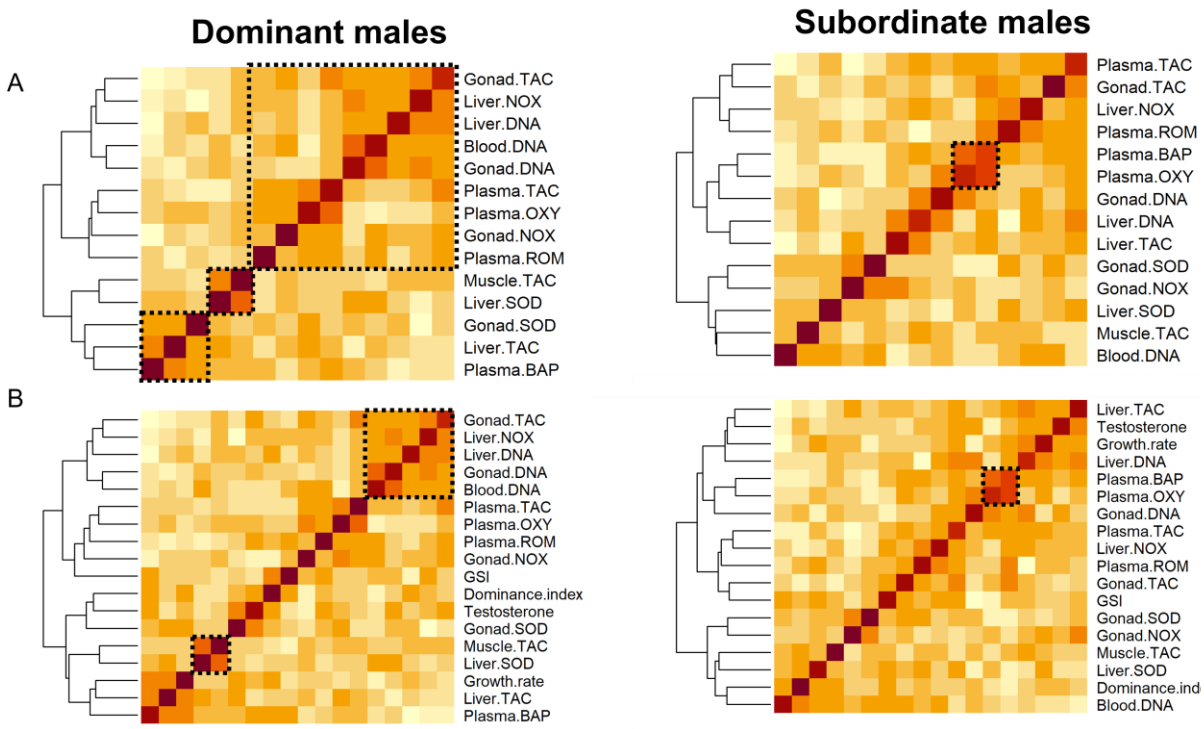

ROMs: reactive oxygen metabolites); TAC and OXY: total antioxidant capacity; BAP: biological antioxidant capacity (low molecular weight antioxidant capacity); SOD: superoxide dismutase activity, NOX: NADPH-oxidase activity. DNA: oxidative DNA damage (8-OHdG).

749 **Fig. 3.** Covariances patterns across markers of oxidative stress by status. (A) Covariance across
750 14 different oxidative stress measurements for dominant and subordinate males. (B) Covariance
751 patterns for the same oxidative stress measurements combined with indicators of reproduction
752 and social dominance (dominance index, gonadosomatic index (GSI), and testosterone).
753 Hierarchical clustering revealed more significant clusters (indicated by dashed box) in dominant
754 males than in subordinate males. Each cell represents the correlation value, with hotter colors
755 representing a more positive correlation.