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Cascading Effects of Conspecific Aggression on Oxidative Status and Telomere Length in Zebra Finches

Quque Martin [1, *], Ferreira Charly [1, 2], Sosa Sebastian [1], Schull Quentin [3], Zahn Sandrine [1], Criscuolo François [1], Bleu Josefa [1], Viblanc Vincent A. [1]

[1] Université de Strasbourg, CNRS, IPHC UMR 7178, 23 rue du Loess, 67037 Strasbourg Cedex 2, France
[2] Université Claude Bernard Lyon 1, 43 boulevard du 11 Novembre 1918, 69100 Villeurbanne, France
[3] MARBEC, Université Montpellier, IFREMER, IRD, CNRS, Avenue Jean Monnet CS 31071, 34203 Sète Cedex, France

* Corresponding author : Martin Quque, email address : martin.quque@iphc.cnrs.fr

Abstract :

Living in social groups may exacerbate interindividual competition for territory, food, and mates, leading to stress and possible health consequences. Unfavorable social contexts have been shown to elevate glucocorticoid levels (often used as biomarkers of individual stress), but the downstream consequences of socially stressful environments are rarely explored. Our study experimentally tests the mechanistic links between social aggression, oxidative stress, and somatic maintenance in captive zebra finches (Taeniopygia guttata). Over 64 d, we measured the effects of aggression (received or emitted) on the individual oxidative status, body condition, and changes in relative telomere length (rTL) of birds living in high- and low-social-density conditions. Using path analyses, we found that birds living at high social density increased their aggressive behavior. Birds receiving the highest number of aggressions exhibited the strongest activation of antioxidant defenses and highest plasmatic levels of reactive oxygen metabolites. In turn, this prevented birds from maintaining or restoring telomere length between the beginning and the end of the experiment. Received aggression also had a direct negative effect on changes in rTL, unrelated to oxidative stress. In contrast, emitted aggression had no significant effect on individual oxidative stress or changes in rTL. Body condition did not appear to affect the physiological response to aggression or oxidative stress. At low density, we found trends that were similar to those at high density but nonsignificant. Our study sheds light on the causal chain linking the social environment and aggressive behavior to individual oxidative stress and telomere length. The long-term consequences of socially induced stress on fitness remain to be characterized.

Keywords : social environment, competition, behavioral ecology, oxidative stress, path analysis, Taeniopygia guttata

1. Introduction

The advantages of group-living are numerous: from anti-predation benefits (Hamilton 1971) to the sharing of information between conspecifics (Murton 1971), facilitated access to mating partners (Clutton-Brock et al. 2001), or cooperation between group members (Deneubourg et al. 2002; Sachs 2006; Clutton-Brock 2009). By the same token, group-living also incurs costs such as increased transmission rates of parasites and pathogens (Côté and Poulinb 1995; Manlove et al. 2014), the potential for cuckoldry (Hoogland and Foltz 1982; Philipp and Gross 1994; Roux et al. 2013), the necessity to avoid inbreeding by dispersing with associated risks (Lukas and Clutton-Brock 2011), or competition for food, territory, reproduction, or other resources (Craig 1921; Wong and Balshine 2011).

Although the individual fitness benefits of group-living must outweigh its costs for it to evolve, group-living animals are faced with the challenge of balancing individual needs with that of other group members (Jones 1980; Buss 1981; Shrader et al. 2007). Conflicts over resources are often resolved by aggression (Aureli et al. 2002), potentially leading to individuals being injured or even killed (Jones 1980; Hof and Hazlett 2012). It is worth noting that the lack of social interactions – or social isolation – may also have deleterious consequences on individual health and fitness, *e.g.* in fish (Hannes and Franck 1983), insects (Koto et al. 2015), birds (Apfelbeck and Raess 2008; Banerjee and Adkins-Regan 2011; Aydinonat et al. 2014) and mammals (Harlow et al. 1965; Hawkley et al. 2012). Thus, group living (or lack thereof) can present a potent source of stress for social animals.

In vertebrates, the consequences of social environments on individual stress have traditionally been assessed by examinations of the functioning of the hypothalamic-pituitary-adrenal (HPA) axis in response to variation in social contexts, social interactions, or group composition (Sapolsky 1983; Denver 1999; Yao and Denver 2007). For instance, in social groups, both high- and low-ranking individuals may experience substantially elevated levels

of glucocorticoids, as a result of either being kept at the bottom, or fighting to stay on top of the social hierarchy (Fox et al. 1997; Creel 2001; Goymann and Wingfield 2004; Sapolsky 2005). However, the study of glucocorticoid variation alone only provides a partial picture on the stress of group-living, since the consequences of glucocorticoids on fitness may depend on the species or population studied, or on the life span of the individual (Breuner et al. 2008; Bonier et al. 2009). One alternative and complementary framework is to focus on downstream measures of individual stress, such as the ability of individuals to maintain homeostasis in physiologically or ecologically stressful situations. Disruptions in the maintenance of homeostasis may for instance be measured by declines in body condition, or increased oxidative stress. For instance, declines in body condition have been found in various stressful contexts including during dispersal (Maag et al. 2019), or exposure to artificial light (Raap et al. 2016), metal pollutants (Yamamoto and Santolo 2000), or predators (Thomson et al. 2010). Oxidative stress, on the other hand, occurs when individual antioxidant defenses are no longer sufficient to offset the production of oxidizing molecules such as reactive oxygen species (ROS). Because ROS originate mostly from normal cellular respiration (Ott et al. 2007), their presence is ubiquitous in aerobic organisms, and they are known to functionally damage lipids, proteins, and DNA (Wolf 2010, pp. 163–190).

An individual's oxidative status in response to social environments is an especially interesting metric to consider for at least four reasons. First, chronic stress affects energy mobilization and should increase the rate of oxidative stress (Breuner et al. 2013). Second, oxidative stress and glucocorticoid levels have been positively related in a diversity of vertebrates (Liu and Mori 1999; Almeida et al. 2011; Costantini et al. 2011). Third, ROS are suggested to play an important role in the decline of cellular functions and the process of aging (Liu and Mori 1999; Betteridge 2000; Birben et al. 2012), notably through their deleterious action on telomeres (Saretzki and Zglinicki 2002; Kawanishi and Oikawa 2004; Houben et al. 2008; but Boonekamp et al. 2017). Telomeres are repetitive non-coding DNA

sequences located at the ends of the eukaryotic linear chromosomes that protect DNA from degradation during successive cell divisions (Venkatesan et al. 2017). Because of its sensitivity to environmental and oxidative stress (von Zglinicki 2002; Reichert and Stier 2017; Chatelain et al. 2020), telomere length has been suggested to reflect organismal ability to cope with harsh social and ecological contexts. For instance, increased social competition has been shown to affect telomere loss in birds (Nettle et al. 2015, 2017), whereas high resource abundance (Spurgin et al. 2017) or the presence of helpers at the nest (Hammers et al. 2019; Quque et al. 2021) have, on the contrary, been shown to mitigate telomere shortening. In addition, studies in birds and mammals have found that changes in telomere length are a good predictor of long-term survival (Bize et al. 2009; Whittemore et al. 2019). Fourth, studies have highlighted both positive and negative effects of social contexts on oxidative status (Miyashita et al. 2006; Nation et al. 2008; Hargitai et al. 2009; Jiang et al. 2013; Cram et al. 2015; Lardy et al. 2016) and telomere length (Epel et al. 2004; Cherkas et al. 2006; Lansdorp 2006; Kotrschal et al. 2007; Aydinonat et al. 2014; Lewin et al. 2015; Uchino et al. 2015; Oliveira et al. 2016; Cram et al. 2017). Yet, the direct and indirect pathways linking social environments to telomere length through oxidative stress have seldom been addressed, and results remained equivocal (Epel et al. 2004; Nettle et al. 2015; Nettle and Bateson 2017).

Here, we investigated how social aggression might affect the stress physiology of zebra finches (*Taeniopygia guttata*). Specifically, we considered the cascading effects of aggression (emitted or received) on relative telomere length (rTL) mediated through changes in bird oxidative stress. In zebra finches, adults live in large flocks of 50-100 individuals (Zann 1996) but defend a small area around their nest from conspecifics (Ikebuchi and Okanoya 2006). Their immediate social environment is thus composed of their sexual partner and their chicks. However, they regularly interact with other flock members when competing for food resources, mates, or materials for nest building (Evans 1970; Zann 1996; Yamahachi

et al. 2017). This competition can result in aggressive social behavior between conspecifics (Evans 1970; Caryl 1975; Bonoan et al. 2013).

We used path analyses (Li 1975) to test for the direct and indirect effects of aggression (emitted and received) on changes in individual rTL via cascading effects on oxidative stress. We recorded the aggressive behavior of 36 zebra finches for 64 days and assessed their body condition, oxidative status, and rTL at the start and end of the experiment. Bird oxidative damage was assessed in plasma by measuring an overall marker of oxidative damage (Reactive Oxygen Metabolites; d-ROM test; Costantini 2016), as well as a specific marker of DNA damage (8-Oxo-2'-deoxyguanosine; 8-oxo-dG). We also obtained a marker of circulating antioxidant capacity using the OXY-adsorbent test (Costantini 2011).

Current knowledge suggests that, depending on the species, both subordinates and dominants can suffer from chronic stress (Fox et al. 1997; Creel 2001; Goymann and Wingfield 2004; Sapolsky 2005), often measured through increased levels of glucocorticoids. Whether it is the high- or the low-rank individuals that undergo the negative consequences of hierarchy seems to depend on the stability of social interactions (Sapolsky 2005) and on the daily energy required to maintain them (i.e., allostatic load; Goymann and Wingfield 2004): stable hierarchy benefits the dominants, and the rank (low or high) possessing the higher allostatic load will have higher levels of glucocorticoids. By separately studying emitted and received aggression, we aimed to identify whether stress befell more heavily to dominant or subordinate individuals. We expected to observe positive effects of social aggression on final plasma markers of oxidative damage (reflecting oxidative stress) and antioxidant defenses (reflecting the organism's response to oxidative stress). In addition, we expected birds showing higher levels of oxidative damage to suffer from greater telomere loss throughout the experiment. Finally, we expected birds experiencing chronic stress to also suffer from poor body condition, whereas birds in good body condition should be able to better offset the costs of oxidative stress and exhibit longer telomeres (Le Vaillant et al. 2015;

Criscuolo et al. 2018; Angelier et al. 2019). By combining physiological and behavioral measures, we study how the social environment and aggressive behavior may affect individual physiology and aging.

2. Methods

2.1. Study birds and housing

All birds in the study were individuals of reproductive age (between 1 and 3 years old). Each bird was ringed for identification. Prior to the experiment, birds were kept in unisex groups (6 birds per cage), in cages of the same size as those used during the experiment. Since zebra finches are known to be more aggressive in denser social environments (Poot et al. 2012), we amplified the natural variation in bird aggressive behavior by housing birds either in cages at a low social density (N = 6 birds; 2 cages) or at high social density (12 birds; 2 cages). Wild zebra finches live in bushes housing on average 4-5 nests (8-10 adults), up to a maximum of 21 nests (Zann 1996). Hence, the high-density condition consisting of six pairs per cage was dense but within the natural range. During the experiment, the sex ratio was 1:1, regardless of the cage. Most birds (72.2%) came from pet stores, while the other birds came from our in-house rearing facility. We distributed birds haphazardly between the two density conditions, ensuring that birds from different origins were roughly evenly distributed in both groups (see electronic supplementary material, **ESM Table S1**). However, we ensured that no more than two birds per cage were of the same origin and had lived in the same cage before the experiment began. Pedigree was not available for birds from pet stores, but we made sure that birds from our in-house rearing facility were not related within the same cage. With the effect of confounding factors reduced, the social relationships established depended mainly on the experimental group and social density. Each cage was built to identical dimensions (150 x 100 x 72 cm) and comprised of 6 feeding perches, 2 resting perches, and 3 nest boxes each. On day one of the experiment, males and females

were randomly assigned to a cage. Cages were kept in identical but separate experimental rooms, preventing birds from communicating between different cages. The climatic conditions were set at a temperature of 24°C, 40% hygrometry, and a 14:10 light-dark cycle. Water and food were provided *ad libitum*. Birds were fed with red millet *(Panicum miliaceum)*, yellow millet (*Panicum miliaceum*) and yellow panicum (*Setaria italica*).

2.2. Behavioral and physiological monitoring

Bird behavior was filmed using three GoPro Hero© (GoPro, Inc., USA) cameras per cage. Cameras were placed from above the cages, located 37 cm from the nests or perches, and set to film at a wide angle so that the entire width of the cage could be seen on the videos. One camera was placed above the nests, one above the central perches and one above the feeders. The combination of the three cameras made it possible to record the whole cage. Birds could be individually recognized by a small, numbered label paper tag that was glued to a feather on the top of their head. We balanced filming hours between morning 08:30-12:30 and afternoon 12:30-18:30. We recorded bird behavior every five days for 2.1 hours continuously (range: min = 1.9, max = 2.2), leading to 14 recording sessions distributed from the start to the end of the experiment. The slight variation between recording durations was due to differences in the battery capacity and specific models of the cameras. Over the 64 days of the experiment, we collected an average of 28.31 hours of video per bird.

On the first and final days of the experiment, we weighed birds on a precision scale (± 0.1 g) and took a blood sample of ca. 120 µL from the brachial vein using two 100 µL heparinized capillary tubes (see below). Blood sampling sessions involved all birds and took place between 10:00 am and 12:00 pm. All birds in a cage were captured at the same time, then placed in individual boxes in the dark to rest for a few minutes. Then, we rapidly drew blood from each bird (usually within a few minutes) before letting them rest again in their individual boxes. Sampling the entire aviary took approximately 30 minutes. All birds were

then released back into the main cage. Bird structural size was measured (tarsus length, ± 0.1 mm) at the start of the experiment using digital calipers, since tarsus length does not change in adults (average length: 15.1 mm ± 0.8, min = 13.1 mm, max = 16.6 mm). The study was conducted over a total period of 64 days.

2.3. Behavior analysis

Videos analyses were conducted using the Behavioral Observation Research Interactive Software (BORIS, v 7.4 Friard and Gamba 2016). We quantified the occurrence of aggressive behaviors emitted and received by specific individuals over the 2-hour recording periods, on each image frame of the video. The videos were scored by two separate observers with relatively high inter-observer repeatability (0.82, *i.e.* above the 0.80 threshold typically used in behavioral studies; Hartmann, 1977; Watkins and Pacheco, 2000). Both observers watched the same number of videos. Aggressive behaviors included '*pecking*', *'chase',* and '*displacement*' behavior. '*Pecking'* occurred when a bird emitted a single rapid or sequence of beak strikes directed towards another bird (beak, head, body, or tail). '*Chases*' occurred when a perched bird rapidly initiated a hoping movement towards another bird on the perch, forcing it to flee to another perch, without contact. '*Displacements*' occurred when a bird flew from a different perch to the location of a perched bird forcing it to flee to another perch with or without contact. In the analysis, a sequence of events was considered to be a single event (multiple fast bill hits were considered as *'pecking*'; we did not count every single peck).

2.4. Effects of social density treatment on bird aggressive behavior

We controlled for the fact that higher density increases the number of aggressive social interactions by calculating the frequency of agonistic interactions per bird and per hour of observation in both social density conditions. As the number of aggressive behaviors per bird

per hour was derived from count data, we performed a GLMM with a quasi-Poisson distribution. We used group density and sex as independent variables and added cage ID as a random factor.

2.5 Oxidative stress measurements

Blood samples (120 µL) were kept on crushed ice from the time of sampling and centrifuged within the hour (10 min, 3500 rpm, 4°C) to separate red blood cells (used for telomere measurements) from plasma (used for oxidative stress measurements). Samples were immediately frozen and stored at -80°C, until analyses.

We assessed birds' oxidative status *via* final measurement of global antioxidant defenses and oxidative damage in blood plasma. Specifically, oxidative damage and non-enzymatic antioxidant defenses in plasma were measured through colorimetric assays, using an infinite M200 microplate reader (Tecan Group Ltd. Männedorf, Switzerland). All measurements were performed in duplicate, and we controlled for interplate variation with a duplicated point repeated on each plate containing a goose plasma standard (sampled and provided by colleagues). Inter- and intra-plate coefficients of variation (CVs) are given below for each oxidative status marker.

First, the antioxidant capacity (Costantini 2011) of bird plasma was assessed using the OXY-Adsorbent test (DIACRON Labs, Grosseto, Italy). This test measures the ability of antioxidant defenses to buffer the action of the highly oxidative hypochlorous acid (HOCl). We used 5µL of 1:100 diluted plasma and measures are expressed as µM of HOCl neutralized per mL. The absorbance, measured at 505 nm, decreases when the antioxidant concentration increases. For the Oxy-Adsorbent assays, the mean intraplate CV was 7.3 % and the mean interplate CV was 8.2 %. Second, plasma oxidative damage was evaluated using the d-ROM test (DIACRON Labs, Grosseto, Italy) that measures the concentration of Reactive Oxygen Metabolites (ROMs, see Costantini 2016). This test utilizes the organic molecules oxidized

by free radicals in a chain oxidative reaction, the final oxidized molecule of which is a chromogen, which turns pink when oxidized. The more free radicals the sample contains, the higher the absorbance (505 nm). We used 4 µL of non-diluted plasma and measures are expressed in mg H_2O_2.dL^{-1}. For the d-ROM assays, mean intra- and inter-plate CV were respectively 7.5 % and 7.9 %. Finally, to obtain an oxidative damage metric related to telomeres, we measured the plasma concentration of 8-oxo-dG, an oxidized derivative of deoxyguanosine after DNA oxidation and repair (Cooke et al. 2000, 2002; Lunec et al. 2002). We assessed 50 µL of 1:20 diluted plasma samples in 8-oxo-dG ($ng.mL^{-1}$) through the Damage DNA (8-oxo-dG) ELISA Kit (StressMarq Biosciences, Victoria, Canada) as previously described in birds (Stier et al. 2014; Marasco et al. 2017). The absorbance (450 nm) is inversely proportional to 8-oxo-dG concentrations. For the 8-oxo-dG assays, mean intraplate CV was 8.3% and mean interplate CV was 6.7 %.

2.6 Relative telomere length measurements

In zebra finches, erythrocytes are nucleated and rTL in these cells correlates with rTL measured in other tissues, including liver, bone marrow, brain, and pectoral muscle (Reichert et al. 2013). The DNA extracted from red blood cells (DneasyBlood and Tissue kit, Qiagen) was used to measure rTL by quantitative real-time PCR (qPCR) based on a previously implemented protocol (Cawthon 2002; Criscuolo et al. 2009). rTL was then calculated from the T/S ratio, where 'T' is the copy number of the telomeric sequence and 'S' is the copy number of the control sequence. As a control gene, we used glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Smith et al., 2011). We amplified the GAPDH gene using the following forward and reverse primers: 5′-AACCAGCCAAGTACGATGATGACAT-3′ (GAPDH-F) and 5′-CCATCAGCAGCAGCAGCCGCCTTCA-3′ (GAPDH-R). Telomere primers were Tel1b (5′-

CGGTTTGTTTGGTTTGGTTTGGTTTGGTTGTTGTTTGTTGTTGTTGTT-3′) and Tel2b (5′-GGCTTGCCTTACTTACTTACCCTTACTTACTTACTTACTTCCTTCCTT-3′).

qPCR amplification was performed with 5 ng of DNA, 200 nmol.L-1 of primers (Tel1b/Tel2b or GAPDH-F/GAPDH-R) and 5 µl of Power SYBR Green PCR Master Mix (AppliedBiosystems) in a final volume of 10 µl. Telomere and GAPDH sequences were amplified on one 384 welled plate. The qPCR conditions for telomeres were 10 min at 95°C, then 30 cycles of 1 min at 56°C and 1 min at 95°C. The qPCR cycles for the GAPDH sequence were as follows: 10 min at 95°C, followed by 40 cycles of 1min at 60°C and 1min at 95°C. All samples were measured in one run of qPCR, containing one plate for the telomere sequence amplification and one plate for the GAPDH gene amplification. Samples were duplicated. A reference curve was drawn from a serial dilution (10, 5, 2.5, 2.5, 1.25 ng) of a reference individual's DNA (chosen haphazardly) to evaluate the amplification efficiency. Amplification efficiency was calculated as the slope of the dilution curve, and was 100.8% and 99.9 % for telomere and GAPDH sequences respectively. We took into account the slight variation of efficiency between telomere and GAPDH amplification by calculating the T/S ratio according to Pfaffl's recommendations (Pfaffl 2001): $[(E_{telomere})^{\Delta Ct_{telomere}}]/[(E_{GAPDH})^{\Delta Ct_{GAPDH}}]$. 'E' is 1 + calculated amplification efficiency and 'ΔCt' is the difference in time required to reach the fluorescence detectability threshold between control and sample ($Ct_{control} - Ct_{sample}$). Mean intraplate coefficient of variation was 2.67 ± 0.24 % for the Ct values of the telomere assay and 0.43 ± 0.04 % for the Ct values of the GAPDH assay. Intraplate coefficient of variation was 13.61 ± 0.99 % for the relative T/S ratios. Following Nettle *et al.* (2019), we also calculate the Intra-class correlation (ICC) to assess the repeatability of T/S ratio, which was 0.902. We checked the absence of non-specific amplification with a well containing PCR-grade water instead of DNA and by drawing the melting curve (primer-dimer artifact).

2.7. Data analyses

We used standardized partial regression analyses, also called covariance-based path analysis (i.e., path analysis; Li 1975) to explore the causal relationships between social aggression, oxidative status, and somatic maintenance over the course of the experiment (**Fig. 1**). Such a path analysis is structured as a sequence of multiple linear regressions and correlations that can be visualized on a path diagram, the putative causation patterns of which depend on *a priori* hypotheses. Path analyses allow for the exploration of both direct and indirect pathways through which social aggression may affect individual physiology and body condition. An indirect path is considered significant when all direct paths that make it up are significant (Cohen et al. 2013). All variables in the path analyses were mean centered and standardized to unit variance, so that *ß* path coefficients could be directly compared. Statistics were performed in R v. 3.6. (R Core Team 2019), and tests based on *a priori* hypotheses were done at an alpha level of 0.05. Path analysis-related statistics have been performed through the lavaan package in R (v 0.6.11; Rosseel 2012).

2.7.1 Variable involved in the path models

Separate path analyses were performed for birds living at high (12 birds in about $1m^3$) or low (6 birds in about $1m^3$) social density conditions. The time budget spent in aggression for each bird (N = 36 birds in total, 18 males and 18 females) was calculated as the number of aggressive events (separated in aggression received or emitted) divided by the total recording time (hours) of the cage over the entire experiment. One might expect subordinate birds to be aggressed more often and to initiate aggression less often than dominant birds (Bonoan et al. 2013). Hence, we specifically tested for a negative correlation between received and emitted aggressions, but we did not find any (electronic supplementary material available online **ESM Fig. S1**). In addition, we checked the independent variables for collinearity using variance inflation factors (VIFs) The dependent and independent variables

showed no concerning sign of collinearity (highest VIF = 1.99, suggested cut-off of 3: Zuur et al. 2010). Finally, when emitted and received aggressions were used in the same model we calculated statistical values from permutation tests to ensure that the potential dependence of these two variables did not bias the results (Croft et al. 2011; Farine 2017). To maintain realistic agonistic structures in the network, permutations were done to conserve the relationships between received and emitted aggression in our data set. For each model, we ran 10 000 permutations with the 'ANTs' package in R (v.0.0.13; Sosa et al. 2018).

Oxidative status (ROM, OXY, 8-oxo-dG) was assessed from the final blood sample at the end of the 64-day experiment. Since some points were missing due to low plasma volume (26/288 = 9.03% of data), we used an iterative PCA algorithm to estimate missing values ('missMDA' package v.1.14, Josse and Husson 2016). This method is preferred to classical completion of missing data by the group's mean, leading to underestimating the true variance within the population (Little and Rubin 2019).

We investigated variation in bird somatic maintenance by measuring the variation (final value – initial value) of relative telomere length (rTL) and body condition. Body condition was calculated as the residuals of the regression between tarsus length and mass ($r = 0.83$, $t = 1.99$, p-value = 0.03) at the start and end of the experiment. We calculated the change in body condition and rTL over the course of the experiment, correcting for potential regression to the mean effects (Blomqvist 1987; Kelly and Price 2005; Verhulst et al. 2013), before inserting them into the path model. On average the change in rTL was slightly positive (mean ± SE = 0.17 ± 0.12), suggesting that some birds partly reconstructed their telomeres over the course of the experiment (**ESM Fig. S2**). The structure of a path analysis does not allow for a direct test of the effect of sex in the model. To overcome this problem, we first tested males and females in two separate models (see online **ESM Fig. S3**). The magnitude and direction of statistically significant effects were similar, regardless of sex. We thus pooled sexes in the path analyses.

2.7.2 Choice of causal models

As recommended by Henseler and Sarstedt for covariance-based structural equation modeling, we estimated the goodness-of-fit of our path models by measuring the Akaike's Information Criterion (AIC) of each, since it discriminates between acceptable models with high proficiency and parsimony (Henseler and Sarstedt 2013). A lower AIC indicated a better fit. First, we built the most complete causal model including all observed variables (**Fig. S4**). The hypotheses underlying this model are summarized in **Fig. 1**. The direct effects of density on oxidative status and somatic maintenance were not evaluated because we had only two replicates per density, which prevented us from rigorous statistical analysis. However social density can modify the frequency and intensity of social interactions. We performed separate analyses in low and high social density conditions to address causal links through which social aggression might affect individual stress, all while taking advantage of the experimental density design. However, it should be noted that the sample size (N = 24) in the high-density condition was twice as large as that in the low-density condition (N=12), so that statistical power was lower in the latter analyses. Thus, whereas we report statistical tests and *P-values*, we also report standardized path coefficients as effect sizes and focus on the meaning of biological effects rather than statistical thresholds (Nakagawa and Cuthill 2007). Finally, since the birds were fed ad-libitum, the experimental conditions might have only had a limited effect on body condition. To test this hypothesis, we ran all models with and without this variable.

2.8. Ethics statement

This project was approved by an independent ethics committee and authorized by French Ministry for research (authorization reference: APAFIS#12019-2018012511525879).

3. Results

3.1. Exploring causal models

First, we found that the signs (negative or positive) of path coefficients were identical between the causal models in low and high social density. The AIC was higher in the full causal model (700.5), compared to both models without density (low: 219.9, high: 482.8). Regarding body condition, all causal models without body condition had a lower AIC (**see Table S2**). We thus chose to present the relationships among our variables for each causal model separately (one per density) without body condition (**Fig. 2**). Correlations among observed variables were similar between both sexes, which were thus pooled in the analyses. Details about the selection procedure of causal models can be found in online supplementary material (**ESM 4**).

3.2. Effects of social density treatment on bird aggressive behavior

Bird aggressive behavior was more closely related within a density condition than among different conditions (intra-class coefficient of correlation = 46%, p = 0.004, CI95 = [0, 0.772]), suggesting that density is the main factor explaining behavioral variability in this study. At high social density, the total number of aggressive behaviors expressed by birds was 2.2-fold higher than at low social density (GLMM: $t = -3.79$, $p < 0.001$). The number of 'pecks' (GLMM: $t = -3.25$, $p = 0.002$) and 'chases' (GLMM: $t = -2.59$, $p = 0.012$) increased in the high-density group, while the number of 'displacements' (GLMM: $t = -0.12$, $p = 0.905$) did not differ significantly. Since we only had two cages per density condition, we could not draw statistically robust conclusions on density effects. However, the overall distribution of the data suggests that higher social density might have been associated with more aggression (see **Fig. 3**). The magnitude and significance of relationships between behavior, oxidative status, and changes in rTL might also have been influenced by social density, with higher social density possibly leading to stronger relationships than lower social density environments (**Fig. 2**).

3.3. Path analysis

At high social density, the path model revealed both direct and indirect effects of received aggression on changes in bird rTL throughout the experiment (**Table 1, Fig. 2, Fig. 4**). Received (but not emitted) aggression had a significant direct negative effect on rTL change (standardized β coefficient = -0.428, **Table 1, Fig. 2**). Received aggression also had a significant indirect effect on rTL change via the activation of bird antioxidant defenses (indirect effect = -0.307). Specifically, when received aggression increased, so did plasmatic antioxidant defenses (direct effect = 0.456), causing a decrease in rTL change over the course of the experiment (direct effect of OXY on rTL = -0.674). The concentration of reactive oxygen metabolites (ROM) also caused a decrease in rTL change (direct effect = -0.517). However, aggressive behaviors did not appear to result in higher ROM values. All other direct and indirect effects tested were not significant (see **Table 1** for statistics). Independent variables were not significantly correlated.

At low social density, although relationships were not significant, the path model revealed overall similar direct and indirect effects of received aggression on changes in bird rTL over the course of the experiment (**Table 1, Fig. 2**). Received (but not emitted) aggression had a direct negative effect on rTL change (standardized β coefficient = -0.469) of similar magnitude than in the high social density group. Received aggression also appeared to increase plasmatic antioxidant defenses (direct effect = 0.439) and had positive direct effects on plasma oxidative damage (direct effects = 0.336 and 0.435). In turn, plasma levels of oxidative damage had direct negative effects on changes in rTL (direct effects -0.280 and -0.294, non-significantly). In contrast to the high social density group, however, the effect of plasma antioxidant levels on changes in rTL was minute (direct effect = -0.004). Here again, independent variables were not significantly correlated.

4. Discussion

We analyzed the consequences of aggressive behavior of 18 male and 18 female zebra finches on bird oxidative status and changes in body condition and relative telomere length (rTL) over 64 days (**Fig. 1**). On one hand, the amount of aggression received by birds over the study period affected rTL change both directly, and indirectly, through an imbalance of oxidative stress regulation processes. On the other hand, the amount of emitted aggression had no significant effect on rTL change or oxidative status. Similarly, variation in bird body condition over the experiment did not appear to be influenced by aggression or oxidative status (**Fig. 2, Table 1**).

Bird levels of plasmatic antioxidant defenses (OXY) were positively related to received aggression at the end of the experiment, and bird oxidative status (OXY and ROM) was negatively related to changes in telomere length over the 64 days. This suggests that birds the most targeted by aggressive conspecifics suffered from increased oxidative damage and increased plasmatic antioxidant defenses in response, and were less able to maintain or reconstruct their telomeres over the course of the experiment as a result. The increase in antioxidant defenses might have been sufficient to mitigate the deleterious effect of aggression on oxidative stress, perhaps explaining why none of oxidative stress markers (8-oxo-dG and ROM) were significantly associated with aggressive behavior. Capturing the entire dynamics of oxidative stress over the study period would have required more frequent blood sampling, which we decided against to limit handling stress. The lack of relationship between 8-oxo-dG and rTL change could be due to the fact that this measure is known to reflect both DNA damage (Cooke et al. 2000), *and* repair processes (Cooke et al. 2002; Lunec et al. 2002). These results are consistent with studies showing that aggression is often associated with increased oxidative stress in vertebrates (Costantini et al. 2008; Rammal et al. 2010; Wapstra et al. 2011; Beaulieu et al. 2014b; Nettle et al. 2017), that telomeres are reduced under stressful conditions (reviewed in Oliveira et al. 2016; Louzon et al. 2019; Chatelain et al. 2020), and that social stress can interfere with the maintenance or restoration

of telomere length that can occur in the absence of socially stressful conditions (Kotrschal et al. 2007).

Using path analysis, we were able to disentangle and compare direct and indirect effects of social interactions on changes in telomere length. In addition to indirect effects of aggressive interactions on rTL mediated by changes in birds' oxidative status, received aggression had a direct negative effect affecting bird rTL. The path coefficient for this direct effect (-0.428) was slightly larger than the indirect effect (-0.307), suggesting that alternative, non-mutually exclusive, processes linking received aggression to changes in rTL are at play. First, this direct effect may reflect other latent processes not measured in our study. For instance, received aggression is likely to affect glucocorticoid hormones (Creel et al. 1996; Creel 2001; Wapstra et al. 2011), capable of negatively affecting telomere dynamics independently of oxidative stress, by affecting telomerase activity or gene expression (Carrero et al. 2008; Choi et al. 2008; Paul 2011; Kratschmar et al. 2012; Angelier et al. 2017). Second, a direct effect might be explained by inter-individual variation in quality. High-quality individuals usually perform well in a suite of phenotypic traits displaying higher foraging performance, higher body condition, higher antibody levels, higher reproductive success, and longer telomeres (Le Vaillant et al. 2015; Criscuolo et al. 2018; Angelier et al. 2019). High-quality individuals are also often dominant in social hierarchies (Haley et al. 1994; Zucker and Murray 2010; Chelliah and Sukumar 2013; Georgiev et al. 2015; Francis et al. 2018), which implies they receive fewer aggressions and have better access to resources (Evans 1970; Caryl 1975). Access to resources is known to be associated with longer telomeres (Paul 2011; Mizutani et al. 2013; Spurgin et al. 2017; Young et al. 2017). Thus, the negative correlation between received aggression and change in rTL may also reflect low quality individuals with poor performances in a suite of phenotypic traits.

Contrary to our expectation, there was no direct or indirect effect of emitted aggression on oxidative stress and telomere attrition. Our results therefore suggest that in zebra finches,

the costs of social aggression are paid by individuals receiving, rather than emitting, aggressive behavior. Similar results have been found in species with stable hierarchical relationships (*e.g.*, Barnett 1955; Eberhart et al. 1983). On the contrary, in species with an unstable hierarchical structure, social stress seems to befall more dominant individuals (*e.g.*, Masataka et al. 1990; Creel et al. 1996; Cavigelli 1999; Wapstra et al. 2011). Thus, our results suggest that zebra finches conform more to a stable social hierarchy (Ardia et al. 2010; Bonoan et al. 2013; but Evans 1970), though a specific experiment is needed to test this.

Finally, the full causal model (**ESM 4**) found no significant direct or indirect effects of received or emitted aggressions on changes in bird body condition over the course of the experiment. Oxidative status markers had also no effect on body condition. Moreover, all tested causal models had a lower AIC once body condition was removed. These findings indicate that body condition was not strongly affected by our experiment, regardless of social density. Although copulation attempts occurred, no female laid eggs. In addition, all individuals were adults and therefore had no growth-related costs. Because food was provided *ad libitum*, if deleterious consequences of aggression on bird body condition occurred, our results suggest that these were probably offset by food availability or favorable energy trade-offs (no investments in growth nor reproduction).

Our results highlight a mechanistic pathway relating the social environment to impaired telomere restoration processes, establishing a causal link between received aggression, oxidative stress, and rTL. However, our analysis also shows that important alternative mechanisms remain to be tested, as is evident by the relative contribution of direct and indirect effects relating social aggression to changes in rTL (see Nettle and Bateson 2017 for a discussion about causative links between telomere length and behavior). It would be of particular interest to test for additional physiological mechanisms thought to mediate telomere loss in adverse situations (*e.g.* glucocorticoids, inflammation) in order to clarify the underlying pathways involved. To deal with oxidative stress and its potentially harmful

consequences, living organisms have evolved either (or both) endogenous antioxidant mechanisms and specific behavioral patterns allowing for the compensatory consumption of exogenous antioxidants. To date, the extent to which social stress can be buffered by an active selection of antioxidants-rich food has only sparsely been investigated in non-human animals (Senar et al. 2010; Beaulieu and Schaefer 2013, 2014; Roode et al. 2013; Beaulieu et al. 2014a) and deserves greater attention.

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Figure legends for online figures (color)

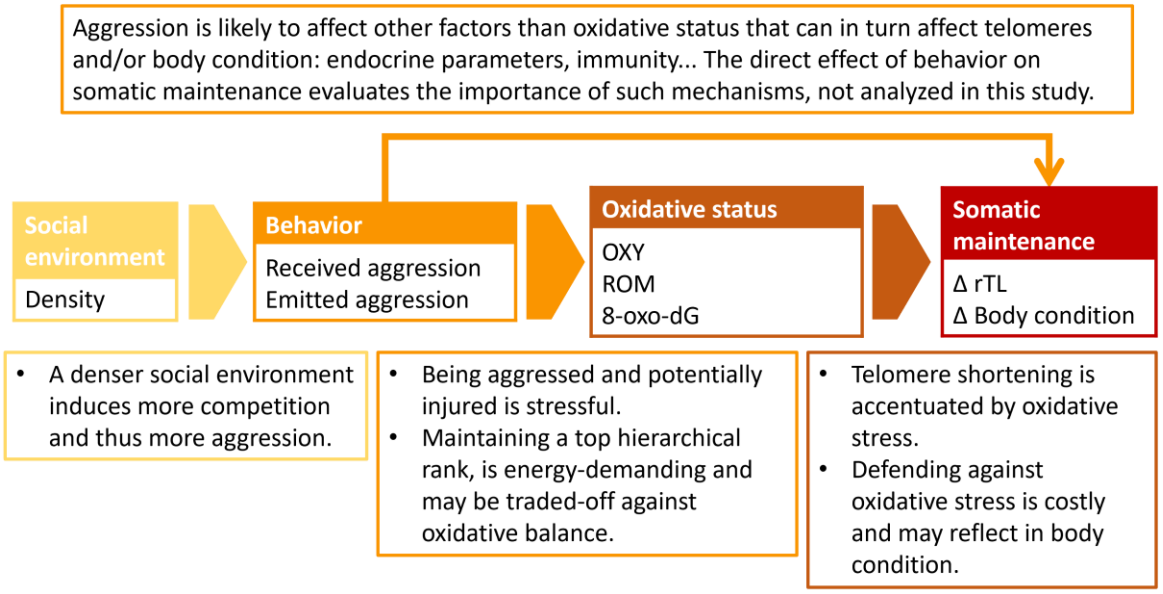

Fig. 1 Global path diagram of all variables involved in the study and underlying hypotheses. The rationale and related references for those hypotheses can be found in the 'Introduction' and 'Methods' sections.

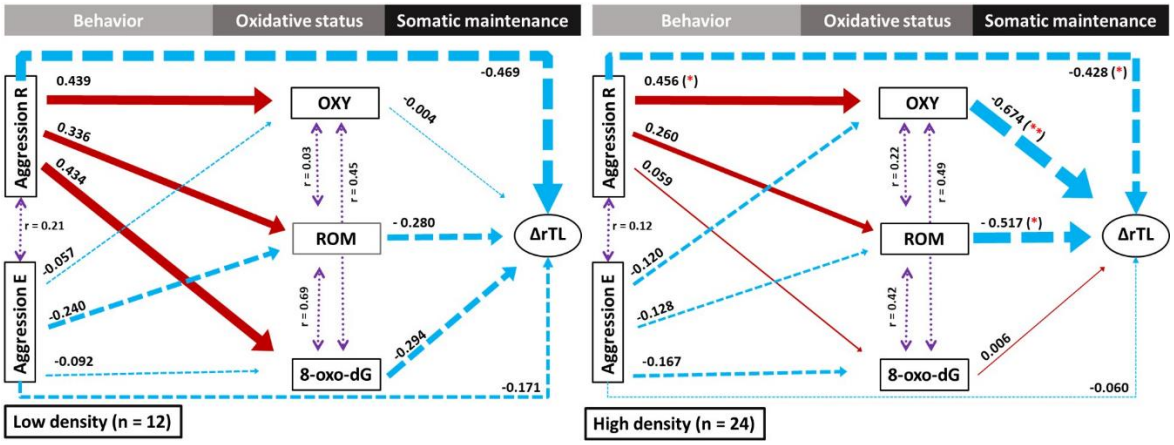

Fig. 2 Separated path analysis according to density. Values above the solid arrows are β path coefficients (standardized estimates) of the regressions detailed in Table 1. Correlation coefficients (Pearson's r) are given above double-headed dashed arrows. Table 1. Correlation coefficients (Pearson's r) are given above double-headed dashed arrows. The thickness of the arrows is proportional to the path coefficient (absolute value). Red arrows indicate positive relationships, while blue arrows indicate negative relationships. ΔrTL is the variation (final-initial) in relative telomere length. Aggression R and Aggression E are respectively the number of aggressive behaviors received and emitted, divided by the duration a bird was observed. VIFmax = 1.99. Significance thresholds: "*": $p<0.05$, "**" < 0.01.

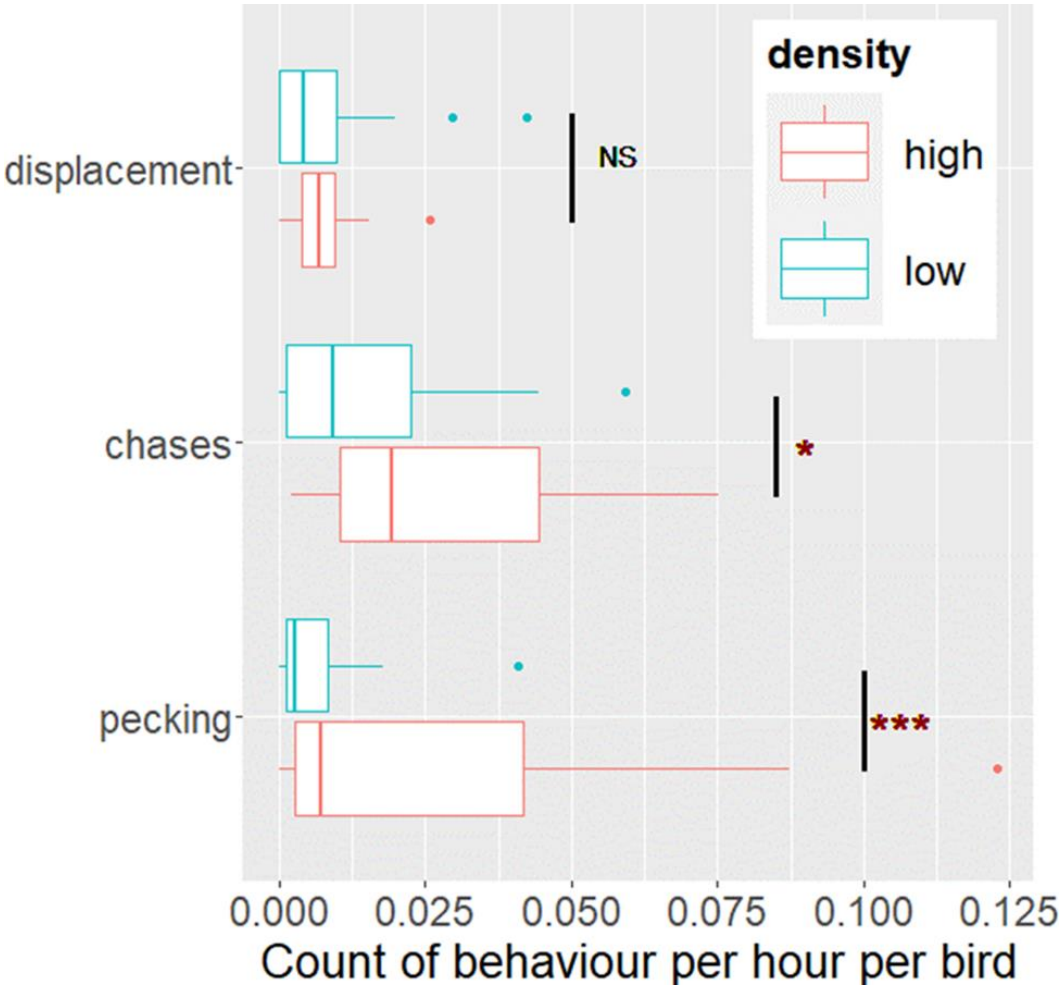

Fig. 3 Effects of social density on bird aggressive behavior. Boxplot showing the number of the three aggressive behaviors recorded in our study per hour per bird: pecking (t = -3.25, p = 0.002), chases (t = -2.59, p = 0.012) and displacement (t = -0.12, p = 0.905) according to social density: **blue boxplots = low** (n=6 birds per cage, 2 cages) or **red box plots = high** (n=12 birds per cage, 2 cages).

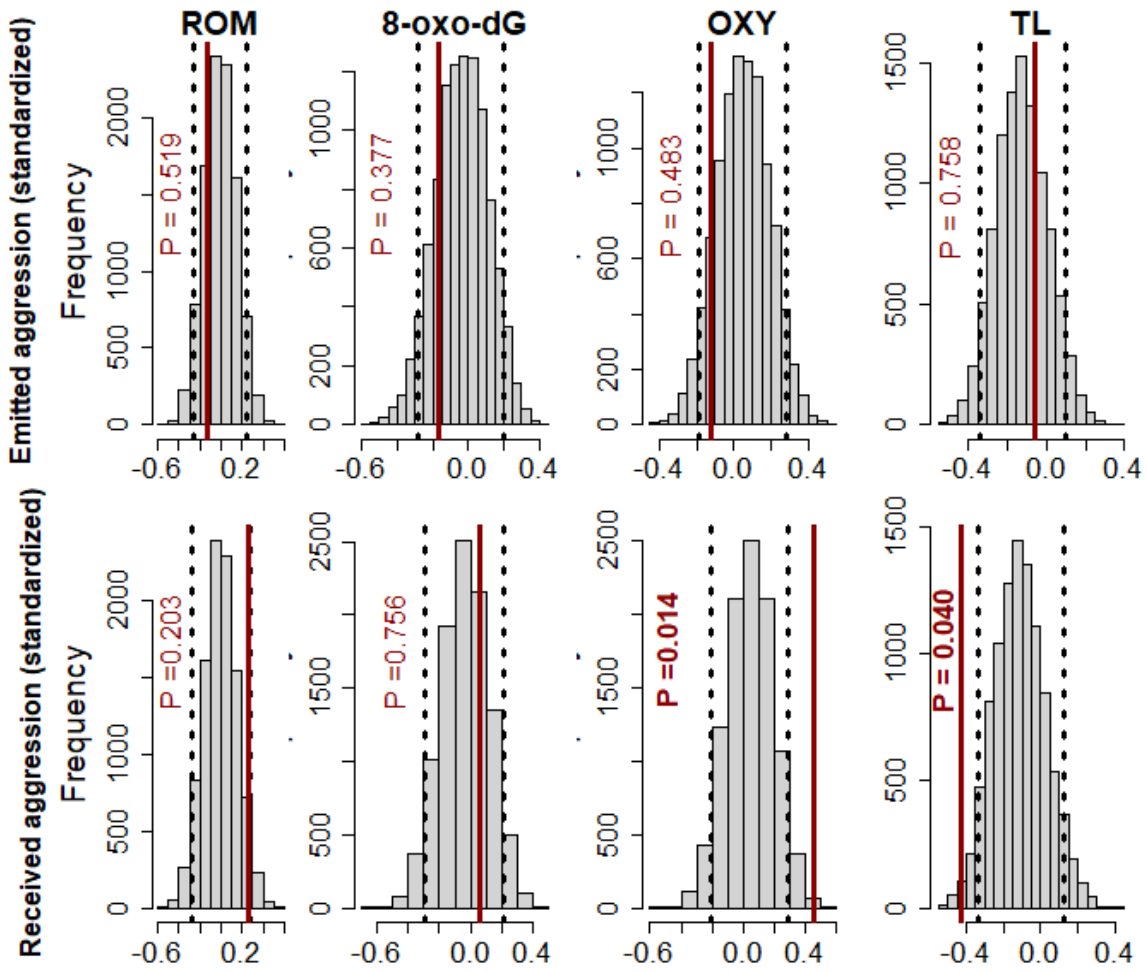

Fig. 4 Distribution of the estimated slopes from 10 000 randomized networks. Slopes are estimated from the models used in the path analysis in the high-density group (Fig. 2, right). They involve standardized emitted and received aggressions per hour as independent variables and oxidative status markers, or variation in body condition, or variation in relative telomere length (rTL) as dependent variables. The dotted black lines give the significance threshold (α/2) and the solid red line gives the position of the model estimate. P-values are calculated from permutation tests.

Figure legends for printed figures (grayscale)

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Fig. 4 Distribution of the estimated slopes from 10 000 randomized networks. Slopes are estimated from the models used in the path analysis in the high-density group (Fig. 2, right). They involve standardized emitted and received aggressions per hour as independent variables and oxidative status markers, or variation in body condition, or variation in relative telomere length (rTL) as dependent variables. The dotted black lines give the significance threshold (α/2) and the solid red line gives the position of the model estimate. P-values are calculated from permutation tests.