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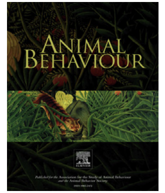
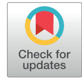


Males with high levels of oxidative damage form weak pair bonds in a gregarious bird species



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The number and quality of social bonds can have major consequences for fitness. For example, in socially monogamous species with biparental care, pair bond quality has been linked to the latency to breed as well as the number and survival of offspring. Given these benefits, what mechanisms prevent some individuals from forming strong pair bonds? Markers of physiological stress and ageing, such as oxidative stress and telomere length, might mediate individual differences in behavioural performance. However, the possibility that physiological stress could also constrain the strength of the pair bond has rarely been investigated. We show that in captive colonies of the socially monogamous, gregarious zebra finch, *Taeniopygia guttata*, individuals with higher levels of plasma lipid oxidative damage formed weaker pair bonds. This effect was sex specific: while males with more oxidative damage spent less time in bodily contact with their prospective breeding partners, no such link was found in females. Although females experienced higher absolute levels of plasma oxidative damage, pair bond investment may have been more constrained in males due to the costly expression of their sexually selected traits. Pair bond strength was not associated with levels of the key antioxidant glutathione or with telomere length. Individuals' ability to form strong pair bonds may thus be constrained by their levels of oxidative damage, with potential downstream effects on fitness.

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A key behavioural trait that impacts fitness in group-living species is the strength or quality of the social bonds between group members. In group-living primates for example, individuals with stronger social bonds to groupmates live longer (Campos et al., 2020; Silk et al., 2010), have more offspring (Schulke et al., 2010) and have higher offspring survival (Silk et al., 2003). In socially monogamous bird species, the strength of the bond with the breeding partner (i.e. the pair bond) has also been found to correlate positively with reproductive success (Griffith, 2019), both in the wild (Mariette & Griffith, 2012) and in captivity (Maldonado-Chaparro et al., 2021; Spoon et al., 2006). Given these potential fitness benefits, why do individuals vary in their social bond strength? Some of the variation in bond strength between group members has been linked to environmental factors such as predation pressure (Heathcote et al., 2017) or the presence of shelter (Jolles et al., 2017), as well as to individual traits such as personality (Aplin et al., 2013). Other studies also suggest that individual levels of corticosterone, a stress-related hormone, may constrain social bond strength (Boogert et al., 2014). However, beyond the hormonal level, the potential for physiological stress and ageing to be mediators specifically of pair bond strength, has been rarely considered.

Oxidative stress, the imbalance between pro-oxidants and antioxidant defences in favour of the former, leading to oxidative

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damage, is inherent to aerobic metabolism and has been identified as a key constraint on the expression of fitness-related traits (Metcalfe & Alonso-Alvarez, 2010; Monaghan et al., 2009). High levels of oxidative stress may limit reproductive investment (Alonso-Alvarez et al., 2017; Costantini et al., 2016; Romero-Haro & Alonso-Alvarez, 2020; Stier et al., 2012) and reduce survival in wild birds and mammals (Noguera et al., 2012; Vitikainen et al., 2016). Oxidative stress also appears to be tightly linked to ageing (Martinez de Toda et al., 2020) and may be involved in the attrition of telomeres (Armstrong & Boonekamp, 2023; Houben et al., 2008; Reichert & Stier, 2017). Telomeres are repetitive noncoding sections of DNA and associated proteins at the end of eukaryotic chromosomes that maintain chromosomal integrity and cellular viability (Blackburn, 1991). Short telomeres have been associated with exposure to harsh environments and different stressors (meta-analysis in Chatelain et al., 2020). Telomere length is often considered to be a marker of physiological ageing in vertebrates (Blackburn, 1991; Lopez-Otin et al., 2013). This is corroborated by an accumulating body of evidence linking telomere length to phenotypic quality, reproductive performance and mortality (Heidinger et al., 2021; Le Vaillant et al., 2015; Wilbourn et al., 2018).

It has been proposed that both oxidative stress and telomere length could be linked to the condition-dependent expression of behavioural traits: high levels of oxidative stress or short telomeres may constrain and/or be a cost of the expression of such traits (Bateson & Nettle, 2018; Metcalfe & Alonso-Alvarez, 2010). There are some examples of these variables being interpreted as the physiological cost of the expression of behavioural traits. In the cichlid fish *Astatotilapia burtoni*, dominant males displaying more aggressive behaviours suffer higher levels of oxidative damage than subordinates (Border et al., 2019). Similarly, in wire-tailed manakins, *Pipra filicauda*, males that interact more frequently and with a greater number of male partners in their lek have shorter telomeres than less social males (Vernasco et al., 2021). There is an example of oxidative stress as a constraint on behavioural performance: in gregarious European starlings, *Sturnus vulgaris*, experimentally reduced levels of the key antioxidant glutathione decreased the social vocalisation rate (Messina et al., 2017). However, there remain relatively few studies on the potential associations between both oxidative stress and telomere length and the expression of social behaviours. Indeed, it remains unclear whether those markers of physiological stress and ageing are linked to fitness outcomes through effects on key social relationships, specifically by constraining the pair bond strength in monogamous species.

Here, we addressed this gap in knowledge and explored whether individuals' levels of oxidative stress and telomere length, as proxies of physiological stress and ageing, constrain prereproductive pair bond strength in captive zebra finches, *Taeniopygia guttata*. Although highly gregarious, zebra finches form the strongest social bonds with their mates, both in the wild (McCowan et al., 2015) and in captivity (Boogert et al., 2014), and the strength of the pair bond has been associated with increased reproductive performance (Maldonado-Chaparro et al., 2021). While the strength of pair bonds is in part determined by having access to preferred mates (Ihle et al., 2015), we observed significant differences in pair bond strength in large groups in captivity where individuals were free to sample and choose their mates (Maldonado-Chaparro et al., 2021). In this study, we quantified lipid oxidative damage levels (malondialdehyde acid, MDA) in plasma, as well as a key intracellular antioxidant (glutathione) in erythrocytes and telomere length in the blood of 111 zebra finches before they were released in groups of 28 birds into four identical aviaries. We then used a state-of-the-art automated behavioural tracking system (Alarcon-Nieto et al., 2018) to record the moment-by-moment social associations across the four replicated colonies. From these behavioural association data, we extracted fine-scale measures of pair bond strength and correlated these with the levels of oxidative damage, glutathione and telomere length. If physiological stress and ageing constrain fitness through mediating effects on social relationships, we predicted that those individuals with higher levels of oxidative damage and/or lower levels of antioxidants, as well as shorter telomeres, would establish weaker pair bonds.

METHODS

Study System

Zebra finches were raised in communal breeding aviaries at the Max Planck Institute for Ornithology in Seewiesen, Germany. Once they reached nutritional independence, birds were moved into mixed-sex peer groups until sexual maturity, when they were transferred into unisex indoor aviaries. Zebra finches were transported to the Max Planck Institute of Animal Behavior in Radolfzell, Germany, where they were kept in unisex indoor aviaries other than in the summers of 2017 and 2018, when they were kept in mixed-sex outdoor aviaries as part of this (2018) and a previous study (Maldonado-Chaparro et al., 2021). All birds had previous breeding experience (Maldonado-Chaparro et al., 2021) and were >1.5 years old at the start of our study (mean age ± SE: 677 ± 3.62 days, range 579–732 days).

Our study was carried out in four outdoor aviaries with a natural day:night cycle from May to November 2018. The birds were blood-sampled before they were released into the outdoor aviaries to test whether each bird's oxidative stress and telomere length were correlated with their prereproductive pair bond strength (see timeline, Fig. 1). After blood sampling, we released 14 males and 14 females who had not shared an aviary since reaching sexual maturity, and who had not been in visual or auditory contact for >1.5 years, into each aviary (Maldonado-Chaparro et al., 2021). One aviary contained only 13 males and 14 females. The study population thus consisted of 55 males and 56 females in total. Once the birds were released, our study encompassed two periods: the prebreeding period of 33 days, when birds were not provided with the resources to breed, and the breeding period of 119 days, which started when nestboxes and materials were provided in each aviary.

Birds were individually identified with uniquely coded passive integrated transponder tags (PIT tags) for identification during nestbox visits, a two-dimensional tag (1 × 1 cm, ca. 0.25 g) for automated recognition from digital images (Alarcon-Nieto et al., 2018; Crall et al., 2015) and a combination of three coloured leg bands. The two-dimensional tags and PIT tags were attached to birds using a small three-dimensional printed 'backpack' with elastic wing loops (following Alarcon-Nieto et al., 2018). Each aviary (6 × 8 × 8 m) was equipped with four perch racks monitored with a camera each (Raspberry Pi NoIR 8MP Camera Module V2) and controlled by Raspberry Pis (Raspberry Pi 3 Model B). After 33 days, we introduced 20 nestboxes in each aviary as well as nest-building material (coconut fibre) to induce pair formation and breeding. Each nestbox was fitted with a single radiofrequency identification (RFID) antenna and connected to an RFID logger board (Priority1rfid) for automatic detection of birds using each nestbox (also see Maldonado-Chaparro et al., 2021). Food (mixed finch seed) and drinking water were provided ad libitum. We recorded the social associations among all individuals throughout the prebreeding period and identified breeding pairs (see below) once nesting materials had been supplied (see timeline, Fig. 1).

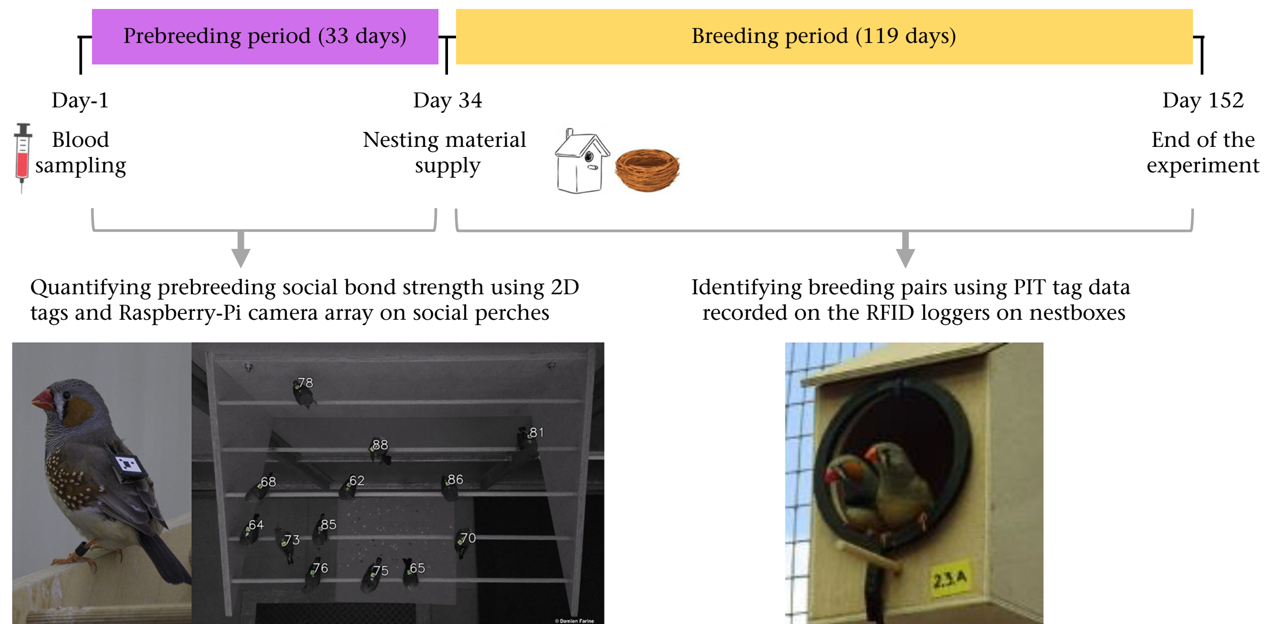

Figure 1. Experiment timeline. Blood sampling was carried out before the birds were released into the aviaries. During the prebreeding period, the birds were not allowed to breed and the social bond strengths were quantified. During the breeding period, the breeding pairs were identified using nest visits detected by radiofrequency identification (RFID) antennas. Pictures by Gustavo Alarcón-Nieto and Adriana Maldonado-Chaparro.

Markers of Physiological Stress and Ageing

To quantify physiological parameters, the day before release into the outdoor aviaries, we collected a small blood sample (ca. 75 μl) from the brachial vein of each bird using heparinized capillaries. Blood samples were stored at 4 °C and centrifuged (5.000 *g*, 5 min) within 3 h, after which the plasma was removed and the buffy coat discarded. A 10 μl aliquot of whole blood was kept for telomere analysis. We stored telomere aliquots, plasma and erythrocyte fractions separately at −80 °C until analyses. The full protocols used for quantification are detailed in the Appendix. In brief, malondialdehyde acid (MDA) is the result of peroxidation of lipids and is a widespread marker of oxidative damage (Monaghan et al., 2009). We quantified plasma MDA levels by high performance liquid chromatography following Agarwal and Chase (2002) with some modifications as described in Romero-Haro and Alonso-Alvarez (2014). A subset of plasma samples was assessed twice and showed high Lessells' repeatabilities (intrasession: $R = 0.96$, $N = 12$, $P < 0.001$; intersession: $R = 0.92$, $N = 15$, $P < 0.001$). Levels of MDA were log-transformed for statistical analyses. Due to plasma sample volume limitations, MDA was not measured for seven females and four males. Glutathione is an endogenous peptide and a key intracellular antioxidant (Wu et al., 2004) that has been shown to modulate the life history of individuals (Costantini et al., 2016; Romero-Haro & Alonso-Alvarez, 2015, 2020; Romero-Haro et al., 2016). Glutathione levels in erythrocytes were quantified by spectrophotometry following Griffith (1980) with some modifications as described in Romero-Haro and Alonso-Alvarez (2014). A subset of samples was assessed twice and showed high Lessells' repeatabilities ($R = 0.91$, $N = 35$, $P < 0.001$). One male's erythrocyte sample was missing and glutathione could not be measured. Blood telomere length was measured by qPCR procedure (Cawthon, 2002) adapted to zebra finches (Criscuolo et al., 2009) and following the protocol of Reichert et al. (2013). All samples were run in duplicate and the intraplate correlation coefficient (ICC) was 0.617. Two male and two female samples failed in telomere analyses. One female telomere length sample represented an extreme outlier (4.23 times the interquartile range) and was removed from the statistical analyses.

Quantifying Prebreeding Social Bond Strength

We tracked individuals visiting the perch racks (social perches) using the Raspberry-Pi camera array (see above). Each social perch had a camera mounted above it, facing downwards. Each camera took a photograph every 2–3 s from sunrise to sunset. Photographs were converted into videos and processed using the PinPoint library in Python (Graving, 2017). We then extracted the identity and position (*x, y* coordinates) of each bird by automatically detecting its two-dimensional tag in each frame of the video. This procedure produced a large amount of data: across 28 individuals in an aviary, we recorded between 73 893 and 305 427 detections on social perches each day (mean = 192 656, median = 192 464), which produced a mean number of daily potential co-observations (i.e. both birds were on the same perch) of 1356 (range 0–13 915) and a mean number of clumping events (see below) of 19 (range 0–11 331) per dyad.

We used the extracted data on individuals' positions to infer clumping, a behaviour that involves two birds perching in bodily contact, and calculated the propensity for each dyad to clump together given that the two birds were on the same perch. We defined two birds as 'clumped' when they were detected at a distance of <80 pixels, which our measurements suggested to correspond to body contact (i.e. a zebra finch was 80 pixels wide at the camera mounting height, and the centre of the barcodes was along the centre line of each bird; Maldonado-Chaparro et al., 2021). We then defined the association strength (i.e. edge weight) for each dyad as the probability of observing individuals *i* and *j* clumping together, given that both *i* and *j* were detected at the perch (a stricter version of the simple ratio index, sSRI; Farine & Whitehead, 2015). This stricter SRI was implemented because when using automated detection methods, the absence of an individual from a perch could be due to a true absence or due to a missing detection, meaning that absences are not informative of social separation (He et al., 2023).

sSRI values could vary from 0, where two individuals were never observed to clump together when on the same perch, to 1, where two individuals were always clumped together and were never observed on a perch without being clumped together. We created aviarywide networks for each of the 33 days of prebreeding.

The average strength of each bird's social association with each aviary member was then calculated as the mean weighted dyadic sSRI averaged over the length of the prebreeding season. From these association strengths, we extracted three main variables for each individual: (1) the ‘pair bond strength’, which was the same value for the two members of the breeding pair (see Breeding Pair Identification below); (2) ‘overall sociability’, i.e. each individual's weighted mean sSRI averaged across all its dyads; (3) ‘nonpair sociability’, i.e. each individual's weighted mean sSRI averaged across all its dyads as in overall sociability but excluding the dyad with the prospective breeding partner. Note that previous studies on this system have shown that social bond parameters obtained using this approach are robust and repeatable (Ogino et al., 2023). These three variables were log-transformed for statistical analyses to fulfil normality requirements.

Breeding Pair Identification

To identify the breeding pairs after the introduction of nest material, we quantified the number of times that each individual visited each nestbox based on the PIT tag data recorded on the RFID loggers. The daily number of visits per individual per nestbox was standardized by the total number of visits recorded on the same day at that nest. For each incubation day, we identified the male and female that had the highest percentage of visits to the nest (>80 %). If the same male and female were identified to be visiting the nest >50 % of the incubation period, they were considered to be the ‘breeding pair’. Nine males and nine females did not reproduce and were excluded from analyses relating pair bond strength to physiological variables. The likelihood to reproduce was not predicted by individuals' physiological variables or by their ‘overall sociability’ (all P values > 0.205, see Appendix, Table A1). In addition, a pair's latency to breed (mean ± SE = 15.3 ± 2.6 days; range 1–61 days) was not predicted by the strength of the pair bond ($\chi^2 = 3.211$, $P = 0.073$, LMM with aviary identity (ID) as random effect, latency log-transformed).

Ethical Note

This study was conducted under permit 35–9185.81/G16/73 approved by Ethical Committee of Baden-Wurttemberg, Germany, following the ASAB/ABS guidelines for the treatment of animals for research and considering the three Rs principles. The birds used in this study were bred in aviaries of the Max Planck Institute of Ornithology, and the study was conducted in outdoor aviaries of the Max Planck Institute of Animal Behavior. The health status of all individuals was checked before the start of the study and the breeding period, as well as after the study. Birds were provided with food and water ad libitum and disturbance was kept to a minimum due to the behavioural tracking system being automated. The birds were regularly monitored to identify any negative effects of the tags on the behaviour of the individuals. The tags were removed at the end of the experiment and birds were transferred back to the aviaries of the Max Planck Institute of Ornithology.

Statistical Analyses

First, we ran two linear mixed-effect models (one for males and one for females) to test whether individuals' physiological markers of stress and ageing before being released into the aviaries was correlated with their prebreeding pair bond strength. Analyses were run separately by sex due to the nonindependence of their pair bond strength values (Firth et al., 2018). We fitted the prebreeding pair bond strength with the breeding partner as the response variable and aviary ID as a random effect. The predictor variables were plasma oxidative damage in lipids (MDA), erythrocytes' glutathione and telomere length values (corrected for laboratory session, see Appendix). The physiological variables were not correlated (see Appendix, Table A2). To control for individual differences in propensity to associate with aviary members (Firth et al., 2018), ‘nonpair sociability’ (as defined above) was also included as a predictor variable. Because individuals were limited by the perch set-up in being able to clump with only two others at any one time, we expected that higher sociability scores would correspond to a lower pair bond strength. Models that also included individuals' body mass as a proxy of quality are presented in the Appendix (Table A3).

To ratify the results from these models suggesting a sex-specific effect of physiological markers of stress and ageing on pair bond strength (see Results), we also fitted a pair-focused linear mixed-effects model including the bond strength of the pair as the response variable. We included the levels of oxidative damage of the female and the male of the pair and their interaction as predictor variables and aviary ID as a random factor. Since sociability could affect pair bond strength (see Results), we also included the sociability of the male and female of the pair and their interaction as predictor variables (see Appendix, Table A4).

Below we report the full model estimates of predictor variables and their significance extracted from likelihood ratio tests, including parametric P values (‘P (model)’). However, to confirm that the significance values obtained from the regression models were not sensitive to modelling assumptions, we also calculated the significance of predictor variables using a randomization or ‘null models’ approach (Farine, 2017). We compared the estimated coefficient of the slope for the predictor variables to the ‘null distribution’ of coefficients generated by running the same statistical model on 1000 permutations of the observed values for the predictor of interest, while keeping the structure of the data and model otherwise constant. Node permutations have been shown to perform reliably in the absence of major nuisance effects (Farine & Carter, 2022), and since observation bias was low (see Quantifying Prebreeding Social Bond Strength), our groups were of known size and had a stable membership, the observed preferred associations can be assumed to emerge from social motivation (Puga-Gonzalez et al., 2021). Here we report the significance of the predictor variables as compared to that expected under this null distribution as ‘P (permutations)’ (see Results and Appendix, Tables A3–A4). We concluded that there was a biologically meaningful effect when P (model) and P (permutations) for the same analysis were both <0.05. We describe results for which the data suggest a trend (e.g. if one or both tests show $0.05 < P < 0.10$) as warranting closer investigation in future studies.

Second, we tested whether males and females showed differences in physiological traits that could help to explain the sex differences observed in the previously described analyses. We ran two linear mixed-effect models using levels of plasma oxidative damage or erythrocytes' glutathione as response variables, sex as a predictor variable and laboratory session as a random factor. Similarly, we ran a linear model using telomere length as response variable and sex as a predictor. The aviary ID was not included in these models since birds were blood-sampled before they were released into the aviaries.

Finally, to explore whether males and females mated assortatively with regard to physiological variables, or whether, in contrast, there was heterophily, we ran three linear mixed-effect

Table 1
Full linear mixed-effect model results for males and females testing the association between the prebreeding pair bond strength and physiological variables (levels of plasma oxidative damage in lipids (malondialdehyde acid, MDA), erythrocytes' glutathione and blood telomere length) before the birds' release into the aviaries

Response variable	Predictors	Slope	SE	χ^2	*P* (model)	*P* (permutations)
Males ($N = 44$)						
Pair bond strength	Oxidative damage	−0.446	0.174	6.560	**0.010**	**0.046**
	Glutathione	0.040	0.061	0.424	0.515	0.500
	Telomere length	0.229	0.267	0.731	0.393	0.342
	Nonpair sociability	−0.504	0.364	1.912	0.167	0.148
Females ($N = 40$)						
Pair bond strength	Oxidative damage	0.176	0.142	1.538	0.215	0.792
	Glutathione	0.082	0.056	2.168	0.141	0.326
	Telomere length	−0.009	0.321	0.001	0.978	0.740
	Nonpair sociability	−0.740	0.410	3.250	0.071	**0.049**

To control for individual differences in propensity to associate with aviary members, nonpair sociability was also included as a predictor. *P* values extracted from likelihood ratio tests (*P* (model)) and calculated from 'null models' (*P* (permutations)) are shown. Significant *P* values are indicated in bold.

models for each physiological variable (i.e. plasma oxidative damage, erythrocytes' glutathione and telomere length). The male physiological values (oxidative damage, glutathione or telomere length) were used as response variables and the corresponding female partner physiological values as predictor variables. Aviary ID was included as a random factor in all models. Laboratory session was included as another random factor in the oxidative damage and glutathione models.

We performed all statistical analyses in R version 4.2.1 (R Core Team, 2021), using the package lme4 (Bates et al., 2015) for mixed-effect models. In addition to the null models, we calculated the significance of predictors using the 'Anova' function from the car package, performing likelihood ratio tests for linear mixed-effect models and *F* tests for linear models (Fox & Weisberg, 2019), without removing any of the nonsignificant effects. Normality of the residuals and homoscedasticity were confirmed by visual inspection and Shapiro–Wilk tests. Data are deposited in the Open Science Framework (OSF) Repository (https://doi.org/10.17605/OSF.IO/2TMXZ) (Romero-Haro et al., 2023).

RESULTS

We found that males with higher levels of oxidative damage (MDA) prior to their release into the aviaries with females formed weaker pair bonds during the prebreeding period (Table 1, Fig. 2a). In contrast, there was no significant relationship between oxidative damage levels and pair bond strength in females (Table 1, Fig. 2b). The results of the pair-focused model corroborated the effect of male oxidative damage levels and showed no evidence for an interaction between male and female levels of oxidative damage on pair bond strength (Appendix, Table A4). The pair bond strength was not associated with glutathione levels or telomere length in males or females (Appendix, Fig. A1). Finally, although more sociable females appeared to form weaker pair bonds (Fig. 2c and d), these results did not withstand our sensitivity analysis (Table 1).

Females had significantly higher levels of oxidative damage in lipids than males (mean ± SE: females: $N = 41$, 7.69 ± 0.659 μM; males: $N = 44$, 6.08 ± 0.641 μM, $\chi^2 = 5.934$, $P = 0.015$; Fig. 3a) and longer telomeres than males (mean ± SE: females: $N = 46$, 0.765 ± 0.027; males: $N = 46$, 0.665 ± 0.027, $F_{1,90} = 5.919$, $P = 0.017$; Fig. 3b). However, the sexes did not differ in glutathione levels (females: $N = 47$, 5.76 ± 0.343 mM; males: $N = 46$, 5.65 ± 0.342 mM, $\chi^2 = 0.270$, $P = 0.604$; Fig. 3c). We found no indication for assortative mating or heterophily according to individuals' levels of oxidative damage, antioxidant glutathione or telomere length. In other words, female physiological variables were not associated with the corresponding male physiological variables (oxidative damage: $\chi^2 = 0.999$, $N = 42$, $P = 0.318$; glutathione: $\chi^2 = 0.543$, $N = 50$, $P = 0.461$; telomere length: $\chi^2 = 0.062$, $N = 49$, $P = 0.804$). Models including the female physiological values as response variables and male values as predictor variables showed similar results.

DISCUSSION

Here, we tested whether proxies of physiological stress and ageing (i.e. oxidative stress and telomere length) constrain individuals' ability to form pair bonds. We found correlative support in males, where individuals with high levels of plasma oxidative damage formed weak pair bonds with their future breeding partners. In contrast, there was no significant association between oxidative damage, antioxidant levels or telomere length and pair bond strength in females. These correlative results may suggest a causal link, where individual variation in oxidative damage levels may drive interindividual differences in the development of pair bond strength. If it exists, this mechanism seems to be sex specific.

Why would only males be constrained in their pair bond formation by oxidative damage? High levels of oxidative damage may have impaired males' ability to fly (Costantini et al., 2013) and/or communicate vocally (Casagrande et al., 2016; Messina et al., 2017). These behaviours may be critical to develop and maintain strong pair bonds, since mates keep in contact, synchronize movements and commonly use distance calls throughout the year, including outside breeding periods (Loning et al., 2023; McCowan et al., 2015; Zann, 1996). However, females' ability to form strong prebreeding pair bonds did not appear to be similarly constrained by their oxidative damage levels, even though females experienced higher absolute levels of oxidative damage, a sex difference reported previously (e.g. Romero-Haro & Alonso-Alvarez, 2015; Romero-Haro et al., 2016). Although male zebra finches selectively pair with high-quality females (Monaghan et al., 1996), females are thought to be the choosier sex (Forstmeier, 2004; Zann, 1996). Consequently, males may be under stronger sexual selection pressures, and oxidative damage may have had a larger negative impact on males' ability to maintain strong pair bonds. Indeed, males with high levels of oxidative damage that bond weakly to their mates may be a consequence of females' mate choice behaviour. In this species, the time a female spends close to a male is thought to indicate her preference for that male as a reproductive partner (Witte, 2006). Females may have spent less time clumping with males suffering high levels of oxidative damage because they considered these males to be less attractive (Witte, 2006); more attractive males with lower levels of oxidative damage may have been paired up already, leaving some females no other mate options. Although body mass, a potential quality indicator, did not predict pair bond strength, oxidative stress may have caused males

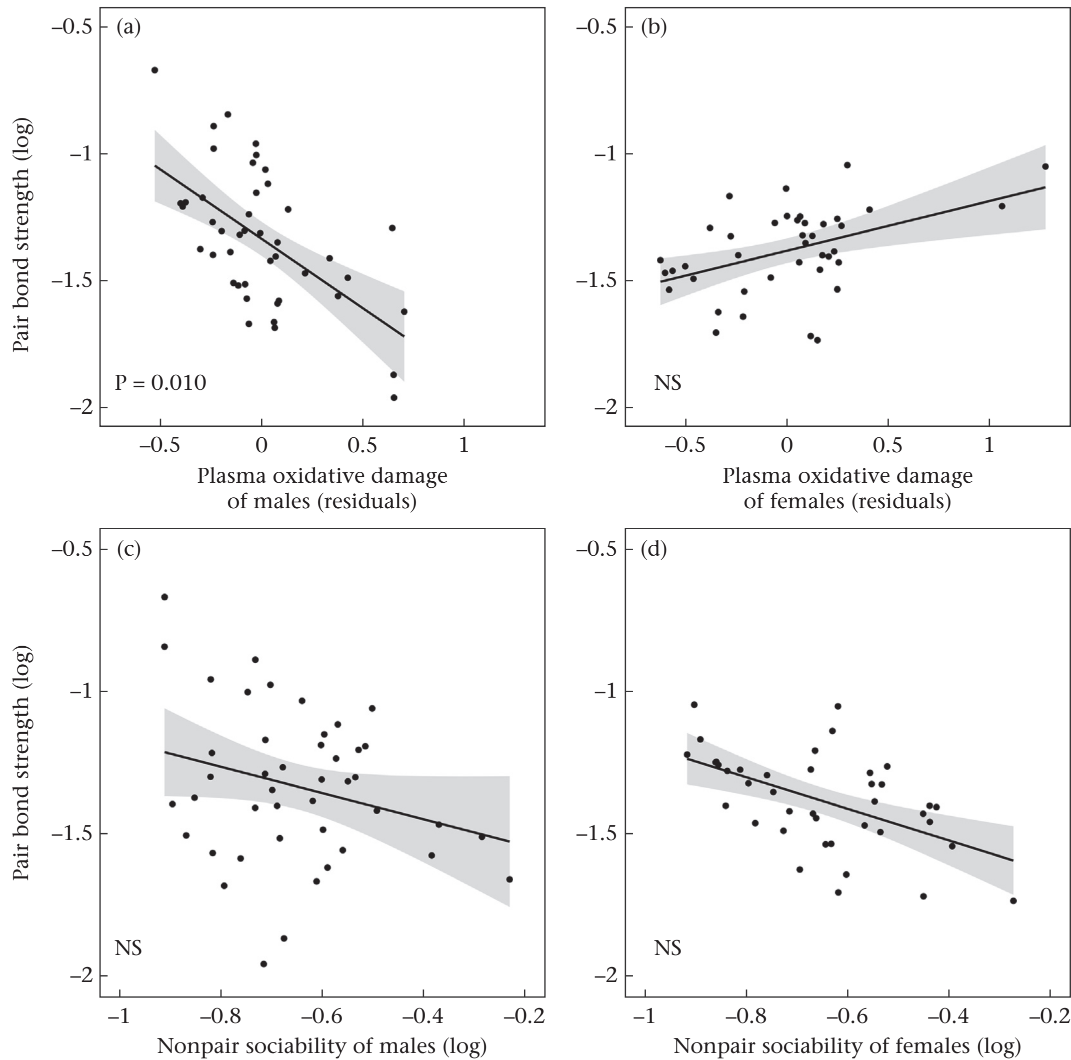

Figure 2. The relationship between log-transformed pair bond strength (average proportion of time spent clumping) during the prebreeding period and plasma oxidative damage in lipids (malondialdehyde acid, MDA) prior to the prebreeding period in (a) males and (b) females, and the relationship between pair bond strength and nonpair sociability (propensity to associate with aviary members excluding the breeding partner) in (c) males and (d) females, respectively. The regression line shows the model prediction, while the grey ribbon shows the 95 % confidence interval.

to display paler bills (Alonso-Alvarez et al., 2004; Noguera, 2017), where bill redness may be a sexually selected trait in this species (Simons et al., 2012; Simons & Verhulst, 2011). Unfortunately, we did not measure sexual traits in our study population, and the two to three second gaps between images obtained in this study did not allow us to determine which sex approached or avoided the other sex, thus leaving these questions open for future studies to address.

Pair bond strength was not clearly associated with glutathione level or telomere length, in contrast to previous studies that have linked both variables with the expression of fitness traits in birds (Costantini et al., 2016; Haussmann & Vleck, 2002; Heidinger et al., 2012, 2021; Romero-Haro & Alonso-Alvarez, 2015, 2020). For example, female canaries, *Serinus canaria*, whose glutathione levels were experimentally decreased laid smaller clutches than controls (Costantini et al., 2016). In zebra finches, telomere length predicts life span (Heidinger et al., 2012). However, the toxicity and rapid reactivity of MDA (Ayala et al., 2014), our marker of oxidative damage, may better explain the importance of this variable in the behavioural, short-term context of this study. In other words, oxidative damage might represent the current physiological status of the males, directly affecting the pair bond-related traits (see above), as compared to the longer-term effects on fitness traits that glutathione level and telomere length seem to exert (Heidinger et al., 2012, 2021; Romero-Haro & Alonso-Alvarez, 2015, 2020). For example, in zebra finches, the experimental decrease of glutathione levels showed clear negative effects only in the next generation, where offspring showed a reduction in body size (Romero-Haro & Alonso-Alvarez, 2020).

Beyond physiological stress and ageing, we also investigated social trade-offs in the formation of pair bonds. Specifically, we expected that spending more time in close contact with other flockmates would reduce the opportunities to spend time in bodily contact with specific partners, thereby creating a negative relationship between pair bond strength and sociability beyond the social partner. Extrapair sociality could provide benefits of nonmate associations, including extrapair mating opportunities (Beck et al., 2020; Maldonado-Chaparro et al., 2018) and greater social buffering of physiological stress (Hennessy et al., 2009). Although visualization of our data suggests that such a trade-off may exist (Fig. 2), the results of the different statistical approaches did not concur (Table 1). We expect that this trade-off between pair bond strength and sociability will be more evident in future studies

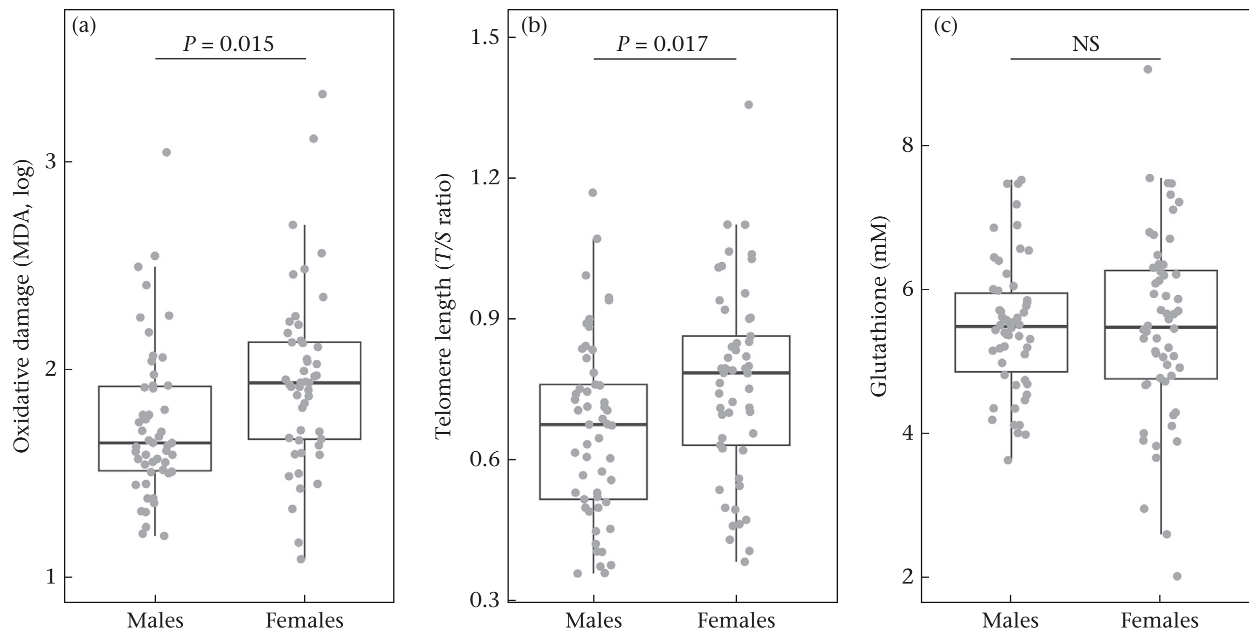

Figure 3. (a) Lipid oxidative damage (malondialdehyde acid, MDA) levels in plasma, (b) blood telomere length and (c) erythrocytes' glutathione levels in males and females. The midlines of the boxes represent median values, and the lower and upper edges represent the first and third quartiles, respectively. Individuals' values are represented by grey dots.

specifically designed to address this phenomenon. Thus, the existence and generality of such a trade-off is an avenue for further investigation.

In conclusion, our study suggests that individuals' ability to form strong pair bonds may be constrained by their levels of physiological damage. Unravelling the causality of such relationships is challenging, but there are some promising approaches that could definitively answer this question. For example, future studies could manipulate the oxidative status of the individuals prior to mating (Koch & Hill, 2017), for instance decreasing antioxidants levels (e.g. Costantini et al., 2016; Romero-Haro & Alonso-Alvarez, 2015) or by experimentally forming pairs with known levels of oxidative damage. Combining such studies with capturing complete movements of individuals in space (e.g. Wang et al., 2022) would show who is approaching or avoiding whom, revealing how differences in pair bond strengths emerge.

Author Contributions

A. A. Romero-Haro: Conceptualization; Methodology; Investigation; Laboratory analyses; Visualization; Data analyses and Writing-original draft. **A. A. Maldonado-Chaparro**: Conceptualization; Investigation; Data curation; Software; Methodology; Validation; Writing – review & editing. **L. Pérez-Rodríguez**: Resources; Laboratory analyses; Writing – review & editing. **J. Bleu**: Resources; Laboratory analyses; Writing – review & editing. **F. Criscuolo**: Resources; Laboratory analyses; Writing – review & editing. **S. Zahn**: Laboratory analyses; Writing – review & editing. **D. R. Farine**: Conceptualization; Funding acquisition; Data curation; Resources; Software; Validation; Writing – review & editing. **N. J. Boogert**: Conceptualization; Methodology; Funding acquisition; Investigation; Resources; Writing – review & editing.

Data Availability

We provide the complete data to replicate the results described in this study, available to download from the Open Science Framework (OSF) Repository (https://doi.org/10.17605/OSF.IO/2TMXZ) (Romero-Haro et al., 2023).

Declaration of Interests

We declare no competing interests.

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Appendix

Methods Used for Quantification of Physiological Parameters

Oxidative damage in plasma lipids, malondialdehyde acid (MDA)

We followed published protocols (Agarwal & Chase, 2002; Nussey et al., 2009; Romero-Haro & Alonso-Alvarez, 2014) to quantify MDA in plasma. We prepared the standard curve for calibration using a 1,1,3,3-tetraethoxypropane (TEP) stock solution (5 μM in 40 % ethanol) serially diluted using 40 % ethanol. We added 15 μl of a butylated hydroxytoluene (BHT) solution (0.05 % w/v in 95 % ethanol), 120 μl phosphoric acid solution (0.44 M) and 30 μl thiobarbituric acid (TBA) solution (42 mM) to 10 μl of plasma and 5 μl of water or 15 μl of standards. The samples were capped and vortexed for 5 s. We then heated the standards and samples at 100 °C for 1 h in a dry bath incubator to allow formation of MDA-TBA adducts. We stopped the reaction by placing samples and standards on ice for 5 min. Subsequently, we added 75 μl n-butanol to samples and standards to extract the MDA-TBA complex. Next, we vortexed tubes for 60 s and centrifuged them at 18000 *g* at 4 °C for 3 min. We then moved 50 μl of the samples and standards of the upper (n-butanol) phase to HPLC vials, which were immediately saturated with N_2 to avoid oxidation. We injected samples and standards into an Agilent 1100 Series HPLC system (Agilent, Waldbronn, Germany) fitted with a fluorescence detector set and a 5 μm ODS-2 C-18 4.0 × 250 mm column maintained at 37 °C. The mobile phase was MeOH:KH_2PO_4 (50 mM) (40:60 v/v), running isocratically for 10 min at a flow rate of 1 ml/min. We collected data at 515 nm (excitation) and 553 nm (emission). A subset of plasma samples were assessed twice and showed high Lessells' repeatabilities (intrasession: $R = 0.96$, $N = 12$, $P < 0.001$; intersession: $R = 0.92$, $N = 15$, $P < 0.001$). Plasma samples of eight females and two males were too small to be analysed.

Total glutathione levels in red blood cells

We quantified levels of the antioxidant glutathione as described in Griffith (1980) and Romero-Haro and Alonso-Alvarez (2014). Briefly, we thawed the erythrocytes and immediately diluted (1:10 w/v) and homogenized them in a stock buffer (0.01 M PBS and 0.02 M EDTA), working on ice to avoid oxidation. We created three working solutions in the same stock buffer as follows: 0.3 mM NADPH (solution I), 6 mM DTNB (solution II) and 50 units of glutathione reductase/ml (solution III). We vortexed an aliquot (0.2 ml) of homogenate of blood cells with 0.2 ml of diluted trichloroacetic acid (10 % in H_2O) three times, for 5 s each time, within a 15 min period. In the meantime, samples were protected from light and refrigerated to prevent oxidation. We then centrifuged the mixture (1125 *g* for 15 min at 6 °C) and removed the supernatant. We performed subsequent steps in an

automated spectrophotometer (A25-Autoanalyzer, Biosystems). We mixed solutions I and II at a ratio of 7:1 v/v, respectively, and automatically added 160 μl of this new mixture to 40 μl of sample (i.e. supernatant) in a cuvette. Then, we added 20 μl of solution III after 15 s and monitored the absorbance at 405 nm after 30 s and 60 s. We used the change in absorbance to determine total glutathione levels by comparing the output with the results from a standard curve generated by serial dilution of glutathione from 1 mM to 0.031 mM. Results are given in mM per gram of pellet. A subset of plasma samples was assessed twice and showed high Lessells' repeatabilities ($R = 0.91$, $N = 35$, $P < 0.001$).

Telomere length

Telomere length was analysed by quantitative real-time amplification (qPCR) procedure (Cawthon, 2002) adapted to zebra finches (Criscuolo et al., 2009). Details about DNA extraction, DNA quality assessments and amplification conditions are detailed elsewhere (Reichert et al., 2013). qPCR amplifications produce cycle numbers representative of the telomere length of the sample (*T*), and to the genomic DNA quantity present in the sample (number of copies of a nonvariable copy number gene, control gene *S*, glyceraldehyde-3-phosphate dehydrogenase (GAPDH)). Relative telomere length for each sample is expressed as the ratio (*T/S*) following (Pfaffl, 2001). Primer sequences and amplification conditions are as described in Reichert et al. (2013). Telomere and GAPDH real time amplifications were performed on separate plates. The adult samples used in the present paper ($N = 111$) were part of a larger sampling group ($N = 593$) that were randomly assigned on a total of five plates of 384 wells and run in duplicate. All adults were run on the same plate. Each plate (telomere and GAPDH) included serial dilutions (40 ng, 20 ng, 10 ng, 5 ng, 2.5 ng, 1.25 ng) of DNA of a pooled reference mix of individual DNAs, also run in duplicate, enabling the generation of a reference curve and the calculation of the amplifying efficiency of the qPCRs. Mean r^2 of the reference curves were within 0.956 and 0.998 for telomere amplifications and within 0.993 and 0.997 for the control gene amplifications. Mean amplification efficiency of the qPCR runs ranged between 99.5 % and 100.5 % for telomere and between 99.4 % and 100.1 % for the control gene. Both a negative control (water) and melting curves were run for each plate to control for the absence of (1) nonspecific amplification and of (2) primer–dimer artefacts. Repeatability of *T/S* ratio was calculated following the intraclass correlation coefficient (ICC, R package ICC; Wolak et al., 2012) as suggested by Nettle et al. (2019). Based on the whole sample size ($N = 593$), intraplate ICC = 0.918 and interplate ICC = 0.842. For the 109 samples presently used, intraplate ICC = 0.617. All ICC values are ranked as good or excellent by Cicchetti (1994). We controlled for any plate position effect bias on *T/S* values, using a mixed-model, with *T/S* value as the response variable and sample plate position as a random factor ($T/S \sim (1|\text{plate position})$). The plate position random factor explained only a negligible part of the variance of *T/S* values (0.00093 ± 0.031) compared to the residual variance (0.057 ± 0.24).

Statistical analyses

First, to include the physiological variables in the mixed-effect models described in the main text while controlling for the potential effect of the different laboratory sessions, we calculated the residuals from two linear mixed-effect models including log-transformed values of plasma oxidative damage and raw values of erythrocytes' glutathione levels as the response variables, respectively, and the corresponding laboratory session as a random effect. Since all telomere length values were analysed in the same plate, the correction for laboratory session was not necessary for this variable. In agreement with the analyses described in the main text, we conducted analyses separately for males and females.

Second, to check for potential associations between the physiological variables, which could cause collinearity issues when they are all included together in the same model, we calculated Pearson's correlation coefficients separately by sex for each pairwise combination of laboratory session-corrected oxidative damage, laboratory session-corrected glutathione and telomere length.

Finally, we tested whether the likelihood to establish a breeding pair was predicted by physiological biomarkers or overall sociability. We ran one generalized linear mixed-effect model with binomial distribution for each sex. To establish a breeding pair or not was the response variable, aviary ID was a random factor and laboratory session-corrected oxidative damage, glutathione and telomere length, as well as sociability values, were included as predictor variables.

We performed all statistical analyses in R version 4.2.1 (R Core Team, 2021) and the package lme4 (Bates et al., 2015) for mixed-effect models. Significance of predictors was determined using the 'Anova' function from the car package, performing likelihood ratio tests for mixed-effect models and *F* tests for linear models (Fox & Weisberg, 2019). The removal of nonsignificant effects was not performed. Normality of the residuals and homoscedasticity were confirmed by visual inspection and Shapiro–Wilk tests.

Table A1
Full generalized mixed-effect models for males and females testing the association between the likelihood to establish a breeding pair during the breeding period and the physiological variables (levels of plasma oxidative damage in lipids (MDA), erythrocytes' glutathione and blood telomere length) before the birds' release into the aviaries and their overall sociability during the prebreeding period

Response variable	Predictors	Slope	SE	χ^2	*P*
Males ($N = 51$)					
Likelihood to establish a breeding pair	Oxidative damage	−1.536	1.292	1.414	0.235
	Glutathione	−0.255	0.512	0.249	0.618
	Telomere length	−1.595	2.241	0.506	0.477
	Overall sociability	2.356	3.509	0.451	0.502
Females ($N = 47$)					
Likelihood to establish a breeding pair	Oxidative damage	−0.792	1.041	0.580	0.447
	Glutathione	−0.571	0.451	1.602	0.206
	Telomere length	0.043	2.012	0.001	0.983
	Overall sociability	3.383	3.178	1.133	0.287

The likelihood to establish a breeding pair was not predicted by any physiological variables or overall sociability in males or females.

Table A2
Pearson's correlations between plasma oxidative damage levels (malondialdehyde acid, MDA), erythrocytes' glutathione levels and blood telomere length in each sex

Variables	Males ($N = 44$)		Females ($N = 40$)	
	R	*P*	*R*	*P*
Oxidative damage − Glutathione	0.206	0.180	0.018	0.912
Oxidative damage − Telomere length	−0.052	0.737	0.021	0.899
Glutathione − Telomere length	−0.166	0.281	0.018	0.913

The three physiological biomarkers were not correlated with each other in males or females, reducing the possibility of collinearity issues in the models.

Table A3
Full linear mixed-effect models for males and females testing the association between the prebreeding pair bond strength and physiological variables (levels of plasma oxidative damage in lipids (malondialdehyde acid, MDA), erythrocytes' glutathione and blood telomere length) before the birds' release into the aviaries

Response variable	Predictors	Slope	SE	χ^2	*P* (model)	*P* (permutations)
Males ($N = 44$)						
Pair bond strength	Oxidative damage	−0.437	0.175	6.226	**0.013**	**0.049**
	Glutathione	0.031	0.063	0.243	0.622	0.768
	Telomere length	0.200	0.271	0.525	0.461	0.238
	Nonpair sociability	−0.593	0.383	2.392	0.122	0.092
	Body mass	0.035	0.045	0.589	0.443	0.072
Females ($N = 40$)						
Pair bond strength	Oxidative damage	0.157	0.162	0.941	0.332	0.610
	Glutathione	0.085	0.057	2.163	0.141	0.398
	Telomere length	-0.024	0.331	0.005	0.942	0.540
	Nonpair sociability	-0.746	0.417	3.203	0.074	0.070
	Body mass	0.008	0.031	0.059	0.808	0.774

Body mass was included as an additional predictor variable. *P* values extracted from likelihood ratio tests (*P* (model)) and calculated from 'null models' (*P* (permutations)) are shown. Significant *P* values are indicated in bold.

Table A4
Pair-focused full linear mixed-effect model testing the effects of male and female plasma oxidative damage levels, and their interaction, on the pair's bond strength

Response variable	Predictors	Slope	SE	χ^2	*P* (model)	*P* (permutations)
Pair bond strength ($N = 40$)	Oxidative damage of the male	−0.226	0.210	3.413	0.065	0.063
	Oxidative damage of the female	0.268	0.160	2.051	0.153	0.114
	Nonpair sociability of the male	−1.793	1.595	2.400	0.121	0.230
	Nonpair sociability of the female	−1.750	1.694	1.805	0.179	0.222
	Oxidative damage of the male*Oxidative damage of the female	0.684	0.776	0.776	0.378	0.470
	Nonpair sociability of the male*Nonpair sociability of the female	−1.998	2.592	0.594	0.441	0.232

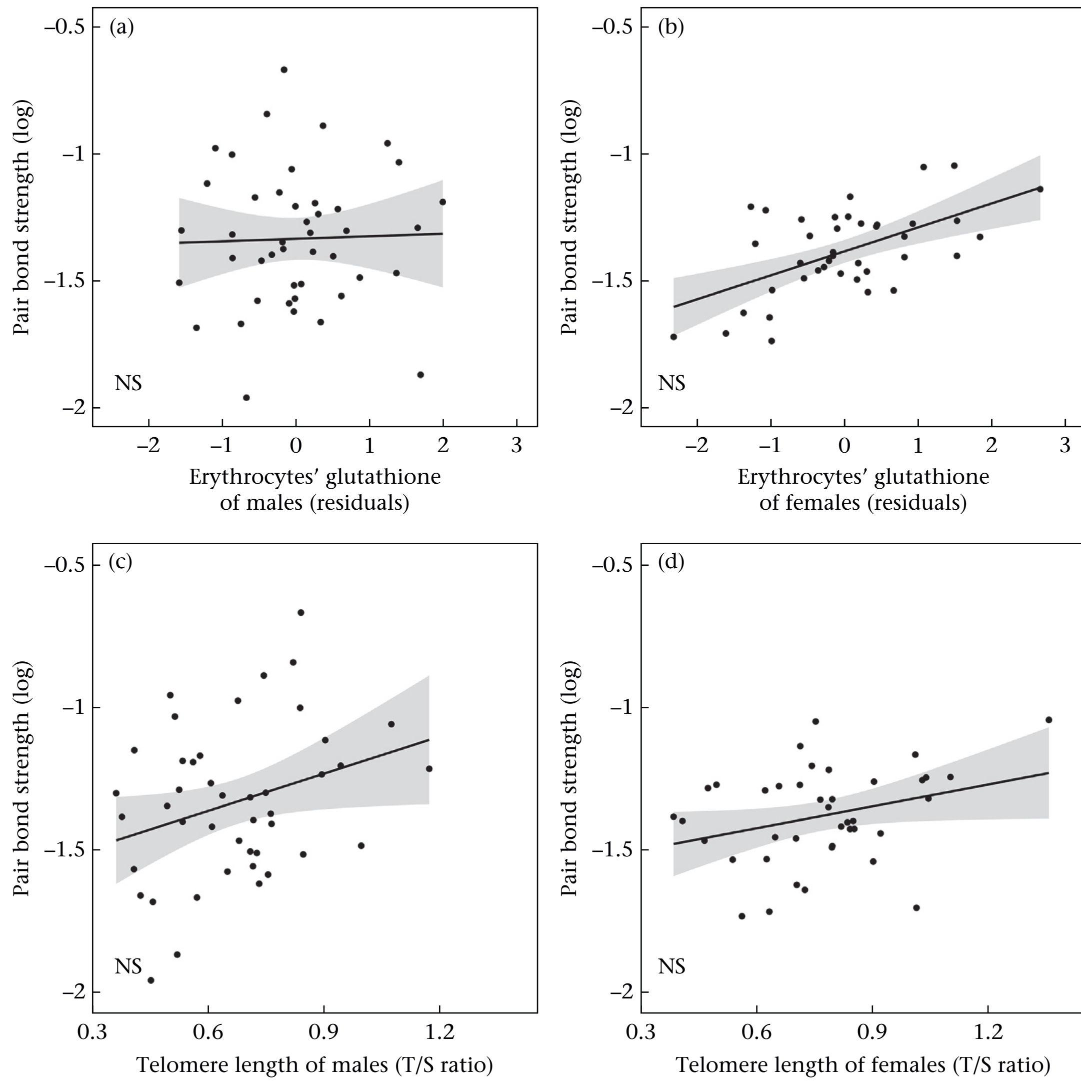

Figure A1. The relationship between log-transformed pair bond strength (average proportion of time spent clumping) during the prebreeding period and glutathione levels in erythrocytes and blood telomere length in (a, c) males and (b, d) females, respectively. The regression line shows the model prediction, while the grey ribbon shows the 95 % confidence interval.