Is aggression costly? Acute aggressive behavior increases oxidative stress independently of testosterone

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Authors' contributions

LM and NMA contributed equally. Both conceived the study, designed it, analyzed the data and wrote the manuscript.

Abstract

Aerobically demanding activities like aggression can lead to an elevated oxidative metabolism influencing the balance between pro-oxidant and antioxidant compounds and result in an overall increase in oxidative stress. Aggression may also generate oxidative stress indirectly through an increase in testosterone levels and in metabolic rate. Given that oxidative stress could represent a physiological cost of aggression we tested the hypothesis that acute aggressive behavior can increase oxidative stress and evaluated the role of testosterone as a potential mediator. To achieve this, we experimentally manipulated the aggressive behavior of wild male and female birds and measured pro-oxidants, enzymatic- and non-enzymatic antioxidants, and testosterone concentrations. After 20 minutes of aggressive behavior, both sexes had higher levels of oxidative stress levels than control individuals. This effect was independent of testosterone levels and much stronger in females. However, only in females but not in males being more aggressive comes at the expense of lower antioxidant capacity. We provide the first experimental evidence that acute aggressive behaviors can generate oxidative stress independently of testosterone in a wild vertebrate, with potential ecological and evolutionary implications given the role of oxidative stress in shaping life-history traits.

Keywords: female aggression, male aggression, oxidative stress, testosterone, rufous hornero, territorial birds.

1. Introduction

Animals frequently engage in aggressive interactions to obtain and secure limited resources that can enhance their reproductive success, like their breeding territory (Beckman & Ames, 1998; Clutton-Brock, 2009; Demas, Cooper, Albers, & Soma, 2007; Duckworth, 2006; Rosvall, 2008; Smith & Blumstein, 2008). However, aggressive interactions entail costs (e.g., territory loss or injuries), which could partially explain why individuals differ in their degree of aggressiveness. A particularly relevant but understudied cost associated with this behavior concerns the oxidative status of an individual (Costantini *et al.* 2008, Isaaksson *et al.* 2011). Oxidative status is the balance between pro-oxidants (i.e., reactive oxygen species, ROS) and antioxidants (i.e., non-enzymatic and enzymatic compounds; Sies 1991; Finkel & Holbrook 2000; Halliwell & Gutteridge 2007; Costantini 2008; Monaghan, Metcalfe & Torres 2009; Skrip & McWilliams 2016). Oxidative stress, defined as a disturbance in the balance in favour of ROS, can lead to damage of biomolecules such as lipids, proteins and DNA (reviewed by Costantini 2008; Monaghan, Metcalfe & Torres 2009). This damage can shape life-history decisions of individuals as well as life history traits such as longevity (e.g., Finkel & Holbrook 2000), this translating into ecological and evolutionary consequences.

Activities that elevate metabolic rate, such as aggression, can lead to elevated production of free radicals since a fixed percentage of oxygen usage in mitochondrial metabolism contributes to ROS formation (Beckman & Ames 1998; Leeuwenburgh & Heinecke 2001; Speakman 2003; Powers & Jackson 2008; Costantini 2008; but see Salin *et al.* 2015). Thus, aggressive individuals, who might have an elevated metabolic rate compared to non-aggressive individuals, have to cope with increased costs associated with oxidative stress. Although our understanding of aggression contributing to a state of oxidative stress is still in its early stages, there is some empirical evidence for such costs in selected lines of mice (*Mus musculus domesticus*), where aggressive males had lower levels of antioxidants than non-aggressive males (Costantini, Carere, Caramaschi, & Koolhaas, 2008). In contrast, aggressive males of wild-caught white skinks (*Egernia whitii)* had higher levels of non-enzymatic antioxidants than less aggressive males, whereas in females no such relationship was apparent (Isaksson et al., 2011). In addition, aggressive behaviors such as territorial fights could incur an oxidative challenge as a consequence of the behavior's aerobic demand. Yet, whether this is the case or not remains unknown.

Aggression can also increase oxidative stress indirectly through an increase in testosterone levels (Schantz *et al.* 1999; Alonso-Alvarez *et al.* 2007). Testosterone is assumed to be the key hormone related to resource-defence aggression and can increase during aggressive interactions in males (Goymann, Landys, & Wingfield, 2007; Hirschenhauser & Oliveira, 2006; J. C. Wingfield, Hegner, Dufty, & Ball, 1990). Testosterone has not only been postulated to have intrinsic oxidative properties (e.g., Chainy *et al.*, 2009, Zhu *et al.*, 1997,

Casagrande *et al.*, 2011) but it can also enhance the metabolic rate of an individual (e.g., Marler & Moore, 1989; Welle et al., 1992; Wikelski *et al.* 1999). Therefore, an increase in aggressive behavior after a territorial intrusion can potentially lead to an increase in testosterone levels (e.g., Wingfield & Wada 1989; Wingfield & Hahn 1994; McGlothlin *et al.* 2008), with testosterone levels influencing the balance between pro-oxidant and antioxidants compounds and resulting in an overall increase in oxidative stress. To date, only one study has looked at the relationship between aggression, oxidative status and testosterone levels in a wild-caught vertebrate (Isaksson et al., 2011): the aggressive phenotype of male white skinks, but not testosterone levels, was positively related to the oxidative condition of individuals. Thus, these findings do not support the hypothesis of testosterone affecting the oxidative status of an individual during aggressive interactions. However, experimental studies directly testing the causal relationship of these patterns are needed.

The main goal of our study was to test the hypothesis that an acute increase in aggressive behavior could impair the oxidative status of wild rufous horneros (Aves: *Furnarius rufus*, hereafter termed hornero) and to test whether this was related to testosterone or not. To elicit an aggressive response, we challenged male and female birds with 20 minutes of simulated territorial intrusions (STI) during the nest-building period. Immediately after the STI we collected blood samples to measure three oxidative status markers and testosterone concentrations. We then compared these parameters with those of birds that were not exposed to acute aggressive behaviors. The hornero represents an excellent model system to study the effects of acute aggression across sexes. They are seasonal breeders and both sexes are involved in territorial defense throughout the year, with males being more aggressive than females (Fraga 1980; Diniz *et al.* 2018). Further, horneros are sexually monomorphic in both body size (i.e., there is no difference in body condition between sexes; Diniz *et al.* 2016) and plumage, and all breeding behaviors studied so far, except for aggressive interactions, are shared and coordinated between the sexes (Massoni *et al.* 2012, Diniz *et al*. 2018).

2. Materials and methods

2.1 Field site and simulated territorial intrusion (STI)

The study was conducted between August 22nd and September 28th of 2016 on the campus of INIA 'Las Brujas' (National Institute of Agricultural Research), department of Canelones, Uruguay (34°40' S, 56°20' W; 0-35 m a.s.l.). During this period horneros were building their nests and females were close to egg laying. In total, we collected behavioral and physiological data from birds defending 51 territories that were subjected to the experimental treatments between 7 am - 1 pm.

We experimentally manipulated hornero aggressive behavior by performing simulated territorial intrusions (STI). STIs are a common method to investigate the aggressive and physiological responses of a territory holder in response to an intruder (e.g. Wingfield, 1994; Gill et al., 2007; Apfelbeck & Goymann, 2011; Villavicencio, Blas, & Goymann, 2014), and STIs are known to be effective in horneros (Adreani, Goymann, & Mentesana, 2018). In particular, a stuffed decoy of a male hornero, a speaker and folded mist nets were placed 5 m away from the nest of focal pairs and solo songs and duets of conspecifics were played (recordings were extracted from the database www.xeno-canto.org and the amplitude of every recording was normalized). For ethical reasons we could not use more than one decoy (i.e., the visual component of the STIs was the same for every territory). However, horneros are sexually indistinguishable in morphological traits (i.e., mass, tarsus and bill) and plumage coloration (i.e., assessed in relation to the avian visual system; Diniz *et al*., 2016). Further, in a comparable STI study done in European great tits (*Parus major*) the decoys used (n=15) did not explain any significant variation in aggressive behavior (Araya-Ajoy & Dingemanse 2013); thus, suggesting that the use of only one decoy did not bias our results. On the other hand, given the lack of information on the function of the different vocal signals in horneros, we decided to form a pool of ten male solo songs and ten duets to make sure that both males and females responded to the STI. We also added ten 'silence' files of 7-15 sec duration to have unpredictable gaps between the different stimuli. The auditory stimuli for each STI were randomly selected from this pool in order to avoid pseudo-replication of the acoustic component across territories (e.g. Apfelbeck et al., 2011). Horneros are suboscines and do not learn their vocalizations (Freeman et al., 2017). Thus, compared to oscines, the acoustic variability of songs and duets across individuals is low (Freeman et al. 2017). Thus, even though the auditory stimuli were randomly chosen from the pool, it is likely that all playbacks elicited a comparable behavioral response across territories. For each territory, the treatment (i.e., control or STI) was randomly determined, and both members of the couple were subjected to same treatment.

Challenged birds (hereafter 'STI birds', $N_{males,}$ $N_{females}$ = 18, 9) were exposed to an STI for 20 minutes. During this time, two observers recorded the aggressive behavior of the territorial couple in digital voice recorders (Philips VoiceTraicer DVT1200 and Olympus Digital Recorder VN-733 PC). Each observer followed one member of the focal STI pair, which was decided randomly before the playback begun (to avoid that one observer always recorded the behavior of one specific sex). Observers did not differ in their quantification skills for the behaviors scored (Figure S1 & Table S1; see Supplementary Material section for a detailed description of the inter-observer reliability test). The following parameters were recorded: 1) response latency (time between start of playback and first approach), 2) time spent within 5 m of the decoy, 3) time spent on the nest, 4) number of solo songs, 5) number

of duets, and 6) number of flights over the decoy, which represents both attacks and attempted attacks towards the decoy. After 20 minutes, mist nets were unfolded for ten additional minutes or until capturing both individuals of the pair (mean capture time ± sd = 5.94 ± 4.68 mins for males and 3.11 ± 2.33 mins for females). During a duet, the contributions of each sex are acoustically distinct from each other (Mindlin & Laje 2003). Thus, sex identification in the field was done based on the singing behavior and was later confirmed by molecular sexing (for details see Adreani *et al.*, 2018). Control individuals ($N_{males,}$ $N_{females}$ = 24, 7) were attracted with playback and presentation of the decoy, but because mist nets were open from the beginning of the playback, control birds were caught as soon as they approached the experimental set-up (i.e., they did not perform aggressive behaviors; mean capture time ± sd = 4.50 ± 3.75 mins for males and 4.57 ± 2.97 mins for females). From all captured birds 80 µl of blood were taken from the wing vein using heparinized capillaries (mean sampling time ± sd = 4.75 ± 1.48 mins). In addition, morphometric measurements (i.e. mass, tarsus length and wing length) were recorded. Control and STI individuals did not differ in body condition index (Supplementary materials Table S2). Before releasing them back in their territories, each individual was marked with a numbered aluminum ring and a unique combination of three colored plastic split rings.

Both sexes showed clear behavioural responses towards our treatment. Both control and STIs birds flew immediately towards the stimulus and approached the decoy (N.M. Adreani, personal observations). Further, the simulated territorial intrusions elicited similar behavioural responses as intrusions by real conspecifics or territorial conflicts with neighbours (Diniz *et al.*, 2017; Adreani *et al.*, 2018;). Finally, it is important to note that although both sexes responded to the territorial intrusion, once the male was captured the female would immediately quit the territorial behavior and start alarming. This is the main reason why we caught more than twice the number of males compared to females during our experiments.

2.3 Analysis of blood oxidative status parameters

The redox state of the captured individuals was determined from both non-enzymatic and enzymatic antioxidants present in plasma and red blood cells respectively, and from reactive oxygen species present in plasma. Antioxidant plasma concentrations (i.e., OXY) were measured by the OXY-Adsorbent test (Diacron International SRL, Grosseto, Italy) following the protocol described by Costantini *et al.* (2006). Measurements are expressed as mm of HOCl neutralized. The activity of glutathione peroxidase (GPX) present in red blood cells was determined using the Ransel assay (Randox Laboratories, Germany) following Costantini, Monaghan & Metcalfe (2011). Measurements are expressed as U/ml of red blood cells. Oxidative damage was quantified by measuring the levels of reactive oxygen

metabolites (dROMs) with the d-ROM test (Diacron International SRL, Grosseto, Italy) following the protocol described by Costantini *et al.* (2006). Reactive oxygen metabolites are expressed as mm of H_2O_2 equivalents. The absorbance was read using a spectrophotometer (Thermo Scientific Multiskan GO) at a wavelength of 546 nm for OXY and dROMs (endpoint mode) and 340 nm for GPX (kinetic modality).

2.4 Analysis of blood testosterone levels

Testosterone was extracted from plasma and concentrations were determined by radioimmunoassay following Goymann *et al.* (2008). Mean recovery following extraction of the samples was 89 ± 2% (mean ± sd). The lower detection limit of the hormone assay was at 0.39 pg/tube. All samples used in our analyses were above these detection limits and the intra-assay variation was 7.5%. For males, we followed the same procedure as the one previously described. Testosterone data and analyses are already published (for details see Adreani *et al.* 2018). It is important to note that although males and females were analyzed in two separate assays - because the samples collected for males were also part of a complementary study - mean recoveries were similar in both assays (mean recovery ± sd: for males = 87 ± 1.9 % and for females = 89±2%). It is therefore unlikely that differences in testosterone levels obtained for males and females are due to samples measured in separate assays.

2.5 Statistical analysis

All our analyses were performed using the R-packages “lme4” and “arm” in R-3.3.3 (R Core Team 2017) in a Bayesian framework with non-informative priors. For all models, we assumed a Gaussian error distribution, and model fit was assessed by visual inspection of the residuals. We used the “*sim*” function to simulate posterior distributions of the model parameters. Based on 10,000 simulations, we extracted the mean value and 95% credible intervals (CrI) (Gelman & Hill, 2007). Assessment of statistical support was obtained from the posterior distribution of each parameter (Korner-Nievergelt et al., 2015). We considered an effect to be statistically meaningful when the posterior probability of the mean difference (hereafter termed ‘*p(dif.)*’) between compared estimates was higher than 95% or when the estimated CrI did not include zero. For details on this approach see Korner-Nievergelt *et al.* 2015.

First, we ran four linear models fitting OXY, GPX, dROMs, and testosterone as response variables to i) compare the oxidative status and testosterone levels between male and female control birds (i.e., individuals that better represent the baseline status), and to ii) study the effect of 20 minutes of aggressive behavior on the oxidative status and testosterone levels of male and female horneros. To do so, sex (female vs male), and treatment (control vs STI) were included as fixed effects, as well as the interaction between both factors. Although from

Adreani *et al.* (2018) we know that there is no difference between control and STI birds in capture time and handling time we included them as covariates to account for the variation that they might add. Preliminary exploration of our data showed that time of the day as well as observer ID did not explain significant variance, thus they were not included in the final model.

Second, we ran three linear models to investigate the relationship between oxidative status and testosterone levels in control individuals. OXY, GPX, and dROMs were fitted as response variable, and in each model, testosterone was fitted as a covariate. Sex and the interaction of sex with testosterone levels were included as fixed factors. Since males and females differ in their testosterone concentrations (See Figure 1), for each sex we normalized testosterone levels between 0 and 1. This allowed us to compare results between sexes.

Finally, within the STI birds, we studied the effect that aggressive behavior had on the oxidative status and testosterone levels. To do so, we fitted OXY, GPX, dROMs and testosterone concentrations as the response variable in a linear model. While GPX and dROMS were square-root transformed for a better fit of each model, testosterone was log-transformed for the same purpose. Although we measured 6 behavioral responses as proxies of aggressiveness (detailed above), to avoid multiple testing, here we only used "flights over the decoy" as a covariate in the model since this is the best proxy for aggression during a territorial intrusion in horneros (Adreani *et al.* 2018; Adreani *et al.* under review). In addition, since males and females differ in their aggressive behaviors (i.e., males are more aggressive than females; Diniz *et al.* 2018), for each sex we normalized the behavioral variables between 0 and 1 in order to capture the aggression gradient in each sex and to be able to compare the effects between the sexes in one model. Therefore, we tested the effect of aggression on the different physiological parameters by including "normalized flights over the decoy" as a covariate in the model. Sex, together with the interaction between aggressive behavior and sex, were fitted as fixed factors. Given that we had a limited sample size and that neither capture nor handling time had an effect on any of the oxidative parameters or testosterone levels (see Table S3), these variables were not included in the final models.

3. Results

3.1 Sex differences in oxidative status and testosterone levels of control birds

Control males had a higher non-enzymatic antioxidant capacity (OXY) than control females (Fig. 1A; *p(dif.)* > 99.99%). Both sexes had similar enzymatic antioxidant concentrations (GPX; Fig. 1B; *p(dif.)* = 79.03%). We found a trend for control males having lower levels of pro-oxidants than females (dROMs; Fig. 1C; *p(dif.)* = 94.67%). Control males had higher

testosterone levels than control females (Fig. 1D; *p(dif.)* = 99.86%). For details on model estimates see Table S3. There was no relationship between any oxidative marker and testosterone level in either sex (Fig. S2, see Table S4 for the model estimates).

3.2 Effect of acute aggressive behavior on oxidative status and testosterone levels

Both sexes had lower levels of OXY after 20 minutes of simulated territorial intrusion (Fig. 1A; $p(dif.)_{females}$ > 99.99%; $p(dif.)_{males}$ > 99.99%) compared to control individuals. However, the treatment affected males and females differently: the difference in OXY concentrations between control and STI females was much stronger than that in males (females' OXY differed by 39.62% between treatments, while in males this difference was 6.18%; *p(dif.)* = 99.50%). No difference in GPX ($p(dif.)_{females}$ = 76.62%; $p(dif.)_{males}$ = 44.85%), dROMs ($p(dif.)_{females}$ = 31.35%; $p(dif.)_{males}$ = 44.14%) or testosterone ($p(dif.)_{females}$ = 45.56%; $p(dif.)_{males}$ = 79.65%) concentration was observed between groups (Fig. 1 B-D). For details on model estimates see Table S3.

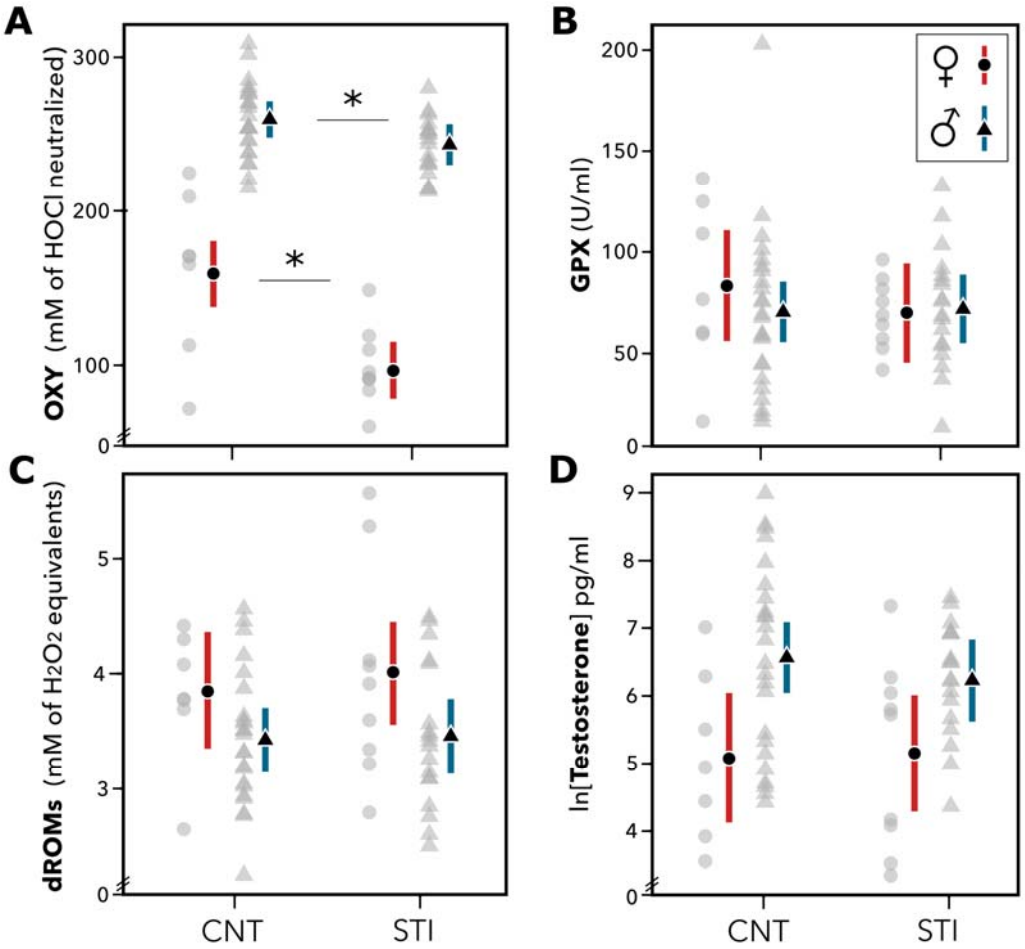

Fig. 1. Concentrations of testosterone and three oxidative markers of female (♀) and male (♂) horneros that were exposed to simulated territorial aggression (STI) and controls (CNT). (A) Non-enzymatic

antioxidant concentrations (OXY); (B) enzymatic antioxidant concentrations (GPX); (C) pro-oxidant concentrations (dROMs) and (D) testosterone levels. Grey circles and triangles show the individual data for females and males respectively. Mean estimates of the model (black symbols) and 95% credible intervals (colored vertical bars) are also shown. Statistically meaningful differences are marked with an asterisk. Testosterone data of males were already published by Adreani *et al.* 2018.

3.3 Relationship between aggression and oxidative status

In males, aggressive behavior and OXY concentrations were unrelated (Figure 2A; posterior probability for the slope being < 0: 52.42%), but more aggressive females had lower concentrations of antioxidants than less aggressive females (Fig. 2A; posterior probability for the slope being < 0: 99.64%). Aggression levels were unrelated to testosterone levels (Fig. 3B), GPX or dROMs in either sex (the posterior probability for the slope being < 0 was smaller than 90 % for each). For details in these models' estimates see Table S5.

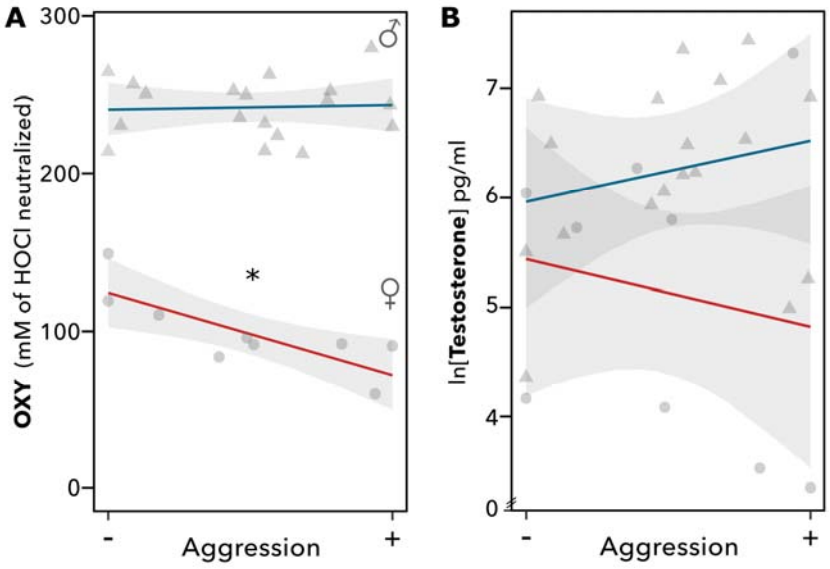

Fig. 2. Relationship between aggressive behavior and non-enzymatic antioxidant concentration (OXY) and testosterone in female (♀; circles; red line) and male (♂; triangles; blue line) horneros. (A) More aggressive females had lower OXY concentrations than less aggressive females; no relationship was found in males. (B) No relationship between aggression and testosterone in male or female birds. Triangles and circles show the individual data for males (♂) and females (♀) respectively. Mean estimates of the model (solid lines) and 95% credible intervals (shaded area) are also shown. Testosterone data from the males were already published by Adreani *et al*. 2018.

4. Discussion

We experimentally tested whether aggressive behavior can induce a change in oxidative stress and whether this effect is mediated by testosterone in a wild bird. The results provide the first

evidence that an acute increase in aggressive behavior (i.e., for 20 mins) elicits an increase in oxidative stress in males and females independently of testosterone concentrations. This effect of STI was much stronger in females than males. Moreover, being more aggressive during the nest-building period was related to higher levels of oxidative stress in females, but not in males.

4.1. Sex differences in the oxidative status and testosterone concentrations in control birds

Control females had higher levels of oxidative stress (i.e., lower concentrations of non-enzymatic antioxidants and a trend for higher concentrations of reactive oxygen species) than control males. In both sexes, we found no relationship between testosterone and any of the markers of oxidative stress measured. These results were unexpected given that female horneros are less aggressive (Diniz *et al*., 2018), have lower levels of testosterone and have similar body condition compared to males (Supplementary Materials Table S2). Also, estrogens, which are generally present at higher concentrations in females close to egg laying compared to males, can increase antioxidant defenses (Halifeoglu, Karatas, Canatan, Colak, & Karadas, 2003). One possible explanation for the sex difference in oxidative status might be that males and females face different reproductive challenges during the early reproductive period, when females are about to lay eggs. In birds, the resting metabolic rate can increase by up to 27% during the period of egg production (Nilsson & Råberg, 2001). Such an increase in metabolic rate could potentially explain the lower levels of non-enzymatic antioxidant protection (OXY) and their tendency to have higher levels of reactive oxygen species (*p(dif.)* = 94.75%; Fig. 1C) compared to males (Fig. 1A). The egg laying period is also physiologically demanding for females since some components mothers transmit into their eggs are limited in their availability (Hulbert & Abbott, 2011; Møller et al., 2000; Peter F Surai, 2002). Antioxidants, such as vitamin E and carotenoids, cannot be synthesized *de novo* (e.g., Surai, Speake & Sparks 2001) and so are of dietary origin. Therefore, another possible and non-exclusive explanation for the difference in the oxidative status between sexes, could be associated with females allocating non-enzymatic antioxidant components into the egg yolk (Royle *et al.* 1999, Mentesana *et al.*, 2019) to protect the developing embryo (Peter F Surai, 2002).

4.2. Effect of aggressive behavior on oxidative status

An increase in aggressive behavior and in associated activity patterns can lead to an increase of free radicals as a consequence of the aerobic demand (Beckman & Ames, 1998; Leeuwenburgh & Heinecke, 2001; Speakman, 2003). However, there was no difference in pro-oxidant concentrations (dROMs) between control and STI individuals in either sex, while

STI individuals had lower levels of OXY. Further, the concentration of enzymatic antioxidants (i.e., GPX) was not different between control and STI birds. The latter could be explained because GPX plays an important role in cellular detoxification of dROMs (Ji 2008, Casagrande & Hau 2018), which did not change after the STI, or by the fact that an up- or down- regulation of enzymatic defenses takes more than 20 minutes. On the other hand, aggressive behaviors can increase oxidative stress indirectly through an increase in testosterone levels (Schantz *et al.* 1999; Alonso-Alvarez *et al.* 2007; Isaksson *et al.* 2011). However, STI horneros had similar testosterone levels compared to control birds indicating that the difference in oxidative status observed between experimental groups is not mediated by this steroid hormone. In line with our results, the relationship between aggressive phenotypes and oxidative status was also independent of plasma testosterone levels in both male and female wild-caught lizards (Isaksson et al., 2011).

The effect that acute aggressive behaviors had on OXY concentrations was much stronger in females than in males (i.e., STI females had 39.62% lower OXY concentrations than control females while STI males had only 6.18% lower OXY concentrations than controls). This sex difference in sensitivity could be given by a differential re-allocation of antioxidants to: protect gametes (Rojas Mora *et al.* 2017), the developing embryo (see above; Schantz *et al.* 1999; Surai, Speake & Sparks 2001; Parolini *et al.* 2017), and/or those tissues with the greatest oxygen consumption (e.g., muscles; Ji 2008). Sex differences could also be explained by other physiological pathways besides an increase in activity levels (see below for further discussion). The implications of these sex differences will depend on the life-history stage that the individuals are in (Speakman *et al.*, 2015). Repeated aggressive interactions experienced by females during egg laying may come at a cost to the deposition of substances into the egg that could affect her fitness. For example, an exercised group of female zebra finches (*Taeniopygia guttata*) that had higher oxidative stress than non-exercised birds after flight training, decreased the deposition of antioxidants (i.e., lutein) into the egg yolk (Skrip, Seeram, Yuan, Ma, & McWilliams, 2016). However, birds can also recover their circulating non-enzymatic antioxidant capacity by consuming antioxidant-rich foods (e.g., Skrip & McWilliams 2016) to avoid negative fitness consequences. Thus, to understand if acute aggressive interactions have long-lasting and sex-specific effects, future studies should ideally measure the oxidative condition of an individual not only immediately after facing aggressive interactions but also over repeated interactions, in relation to fitness traits and across different life-history stages.

To the best of our knowledge this is the first study that demonstrates an effect of an acute increase in aggressive behavior (i.e., 20 minutes) on the oxidative status of wild animals. To date, only two other studies investigated the relationship between aggressive behavior and oxidative status by looking at the correlation between the personality trait

"aggression" (i.e., over a long-term scale) and the redox status of a selected line of mice (Costantini *et al.* 2008) and white skinks (Isaksson *et al.* 2011). Interestingly, these two studies support our findings: they also found a relationship between aggression and OXY concentration, but not with dROMs as previously suggested. Altogether, this suggests that aggression has both short- and long-term consequences on the oxidative status of an individual, and that this occurs through a change in non-enzymatic antioxidants. On a broader scale, these studies show that the relationship between aggression and oxidative status is present across different vertebrate groups (i.e., reptiles, mammals and birds). The oxidative status of an individual might therefore be a key physiological mechanism underlying different life-history strategies (e.g., longevity) in individuals that show different levels of aggression.

4.3. Relationship between aggression and oxidative status

Whereas more aggressive females had lower levels of OXY than less aggressive females, no such relationship was found in males. There was also no relationship between testosterone levels and aggressive behavior in either sex. Together with the previous results, this indicates that mechanisms other than testosterone determine the levels of oxidative stress observed in males and females after aggressive interactions. Aggression is often regulated by other hormones, such as progesterone (e.g., Goymann *et al.* 2008) or glucocorticoids (e.g., Wingfield *et al.* 1990; Woodley & Moore 1999). Progesterone has indeed been linked with the generation of oxidative stress in studies done *in vitro* (e.g., Zhu *et al.*, 1997; Itagaki *et al.*, 2008). To test for the potential effect that this hormone might have on the oxidative status of control and STI horneros in this study we measured, besides testosterone, plasma progesterone levels (see Supplementary Materials). In horneros, progesterone (as testosterone) seems to be decoupled from aggression-induced oxidative stress (Sup. Table 6-8, Supplementary Material). We did not include these results in the main text given the lack of clear hypotheses about the relationship between progesterone and oxidative status in living organisms.

Engaging in aggressive interactions during the nest-building period could also have led to an increase in glucocorticoid hormones (i.e., through an activation of the hypothalamic-pituitary-adrenal axis) to help an individual recover and survive an immediate challenge (Sapolsky, Romero, & Munck, 2000). An increase of glucocorticoids in the blood stream after aggressive interactions has been reported both in seasonally territorial (e.g., Charlier *et al.*, 2009) and year-round territorial vertebrates (Landys *et al.*, 2010, Ros *et al.*, 2014). Further, a direct link between glucocorticoids and oxidative stress has previously been reported (Haussmann & Marchetto 2010; Costantini, Marasco & Møller 2011; Haussmann *et al.* 2012; Majer *et al.* 2019), and females seem to be more susceptible to the effects of glucocorticoids on oxidative stress than males (reviewed by Costantini *et al.* 2011, but see Ouyang *et al.*

2016). Thus, the interaction between glucocorticoids and oxidative status could provide an alternative explanation of our results that we cannot resolve at the moment: aggression may lower OXY concentrations directly as a consequence of an increased metabolism or indirectly via an increase in glucocorticoid concentration, or both.

5. Conclusions

Until now, the effect of aggression on oxidative stress was investigated in the framework of personality traits (Costantini et al. 2008; Isaksson et al. 2011). Our study demonstrates, for the first time, that acute increases in aggression can increase oxidative stress, and suggests that the mechanisms mediating oxidative stress during agonistic encounters differ between the sexes. Altogether, the three studies suggest that aggressive behaviors performed over both short- and long-term have direct consequences on the redox balance of an individual. Remarkably, by generating a change in non-enzymatic antioxidants and not in the concentration of pro-oxidants as previously suggested. Given that oxidative stress can shape central life history traits such as longevity, the oxidative status of an individual might be a key physiological mechanism underlying different life-history strategies in individuals that show different levels of aggression.

Ethical statement

The experimental procedures were approved by the Ethics Committee of Animal Experimentation (CEUA) of the Facultad de Ciencias, Universidad de la República, Uruguay (University of the Republic of Uruguay). Protocol number 186, file 2400-11000090-16.

Competing interests

We declare no competing interests.

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