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RESEARCH ARTICLE

Oxidative stress during courtship affects male and female reproductive effort differentially in a wild bird with biparental care

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ABSTRACT

Oxidative stress has been suggested as one of the physiological mechanisms modulating reproductive effort, including investment in mate choice. Here, we evaluated whether oxidative stress influences breeding decisions by acting as a cost of or constraint on reproduction in the brown booby (*Sula leucogaster*), a long-lived seabird with prolonged biparental care. We found that during courtship, levels of lipid peroxidation (LP) of males and females were positively associated with gular skin color, a trait presumably used in mate choice, while levels of reactive oxygen species (ROS) were higher as laying approached and in early breeding pairs. Evidence of a constraining effect of oxidative stress for females was suggested by the fact that females with higher ROS during courtship laid smaller first eggs and had chicks with lower rates of body mass gain, and higher female LP was associated with lower offspring attendance time. No evidence of an oxidative cost of parental effort was found; from courtship to parental care, levels of ROS in males and females decreased, and changes in LP levels were non-significant. Finally, using a cross-fostering experiment we found that offspring ROS was unrelated to rearing and genetic parents' ROS. Interestingly, offspring LP was positively associated with the LP during courtship of both the rearing parents and the genetic father, suggesting that offspring LP might have both a genetic and an environmental component. Hence, in the brown booby, oxidative stress may be a cost of investment in reproductive traits before egg laying and constrain females' investment in eggs and parental care.

KEY WORDS: Color, Constraint, Cost of reproduction, Life-history trade-off, Parental care

INTRODUCTION

The existence of an energetic trade-off based on limited resources has been traditionally proposed to explain the cost of reproduction, as resources invested in reproductive activities are no longer available for other life functions (Stearns, 1992; Reznick et al., 2000). However, other types of trade-off may arise when reproduction and its associated processes directly inflict somatic damage and affect other life-history components through their physiological by-products (Harshman and Zera, 2007). Oxidative stress could underlie this type of trade-off. Oxidative stress occurs when the normal cellular redox homeostasis is disrupted, leading to harmful cellular damage (Jones, 2006). While a small amount of reactive oxygen species (ROS) plays an important cellular signaling role (Hurd and Murphy, 2009; Dickinson and Chang, 2011), un-neutralized ROS are involved in further reactions that can damage lipids, proteins and DNA (Beckman and Ames, 1998; Finkel and Holbrook, 2000). Increased susceptibility to oxidative stress as by-product of an increase in energy used during reproduction (Angilletta and Sears, 2000; Nilsson, 2002; but also see Williams and Vézina, 2001) has been suggested as a potential short- and long-term physiological cost of reproduction, independent of energy or resource allocation trade-offs (von Schantz et al., 1999; Salmon et al., 2001; Wang et al., 2001; Alonso-Alvarez et al., 2004; Dowling and Simmons, 2009; Monaghan et al., 2009; Costantini, 2008).

Evidence of an oxidative cost of reproductive effort remains unclear (Metcalfe and Monaghan, 2013; Speakman and Garratt, 2014). Various studies in wild animals have found the predicted positive associations between reproduction, daily energy consumption and oxidative damage in lipids and proteins (Bergeron et al., 2011; Heiss and Schoech, 2012; Fletcher et al., 2013; Costantini et al., 2014). Furthermore, studies in wild and captive animals have found evidence of a potential oxidative imbalance resulting from reproduction, including higher production of reactive oxygen metabolites (Casagrande et al., 2012; Stier et al., 2012; Guindre-Parker et al., 2013), decreased antioxidant capacity (Wiersma et al., 2004; Costantini et al., 2010; van de Crommenacker et al., 2011) and diminished resistance to rapid temporary ROS overproduction (Alonso-Alvarez et al., 2004; Bertrand et al., 2006; Losdat et al., 2011; Christe et al., 2012). Nevertheless, other studies have failed to find a relationship between reproductive effort and oxidative damage (Nussey et al., 2009; Isaksson et al., 2011a,b; Aloise-King et al., 2013; Wegmann et al., 2015), have found increased antioxidant response in females' liver cells during parental care (Yang et al., 2013; Xu et al., 2014), or have found less oxidative damage in serum, liver, kidney and muscle cells of reproductive compared with non-reproductive individuals (Garratt et al., 2011, 2013; Ołdakowski et al., 2012). Recently, it has been proposed that the transition from non-reproductive status to reproduction may activate additional protective mechanisms that may shield individuals – especially mothers, and by maternal effects, their offspring – from excessive oxidative damage due to parental investment (Blount et al., 2015).

An alternative, non-exclusive, explanation that may account for the inconsistent results suggests a confounding influence of a potential constraining effect of oxidative stress on reproductive investment (Metcalfe and Alonso-Alvarez, 2010). This proposal states that oxidative stress may prevent individuals from increasing their reproductive effort in the first place when they are in an oxidative imbalance, thereby avoiding potential oxidative damage to bio-molecules during reproduction (Metcalfe and Alonso-Alvarez, 2010; Stier et al., 2012). Accordingly, studies evaluating the influence of oxidative stress on colorful sexual traits, an important

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component of reproductive effort, suggest that higher levels of oxidative stress can constrain investment in such ornaments, particularly when they are carotenoid-dependent (Torres and Velando, 2007; Mougeot et al., 2010; Alonso-Alvarez and Galván, 2011; Vergara et al., 2012; Hill and Johnson, 2012; but see Isaksson and Andersson, 2008). Also, lower red blood cell resistance to oxidative burst and higher levels of oxidative damage in lipids and proteins before pair formation or during courtship or incubation have been associated with smaller litter size at birth in laboratory mice (Stier et al., 2012), decreased male parental effort in Florida scrub jays (Heiss and Schoech, 2012), smaller clutch size, lower female hatching probability and lower male survival probability to the next reproductive event in alpine swifts (Bize et al., 2008), and a decline in lifetime total breeding events and greater aging rate in captive zebra finches (Kim et al., 2009). However, no association between oxidative markers upon arrival to the courting site and territory quality or number of fledglings produced by snow buntings was found (Guindre-Parker et al., 2013).

If oxidative stress is a general cost of reproduction and acts as an evolutionary force shaping phenotypic traits, selection for high ability to resist oxidative stress should be expected (Costantini, 2014). Oxidative stress is a complex, multivariable physiological trait; hence, to understand whether selection directly shapes resistance to oxidative stress, we need to understand how genetic, maternal and common environment effects influence different components of the ability to limit oxidative damage (Metcalfe and Alonso-Alvarez, 2010; Losdat et al., 2014). There is evidence that some components of oxidative balance have a genetic basis (e.g. antioxidant enzymes, Ito et al., 2004; resistance of red blood cells to haemolysis, Kim et al., 2010). Also, cross-fostering studies suggest that variation in some components of oxidative stress has a genetic basis (e.g. serum oxidative damage of nestling kestrels, Costantini and Dell'Omo, 2006; ROS production in painted dragon lizards, Olsson et al., 2009), while other components are principally influenced by environmental effects (e.g. serum antioxidant barrier, Costantini and Dell'Omo, 2006; glutathione peroxidase in great tits, Norte et al., 2009). However, we still know little about the potential relative influences and synergies of the genetic makeup and the environmental effects on offspring resistance to oxidative stress (Metcalfe and Alonso-Alvarez, 2010; Stier et al., 2015).

The brown booby [*Sula leucogaster* (Boddaert 1783)] is a long-lived species with an extended period of obligate biparental care. Modal clutch size is two eggs and both the male and female incubate the clutch on average for 42 days (Nelson, 1978). Brown boobies show obligate siblicide: during the first days of life, the older chick typically eliminates the younger one, even when enough food is available for both chicks (Drummond et al., 2003; Osorno and Drummond, 2003), resulting in only one surviving offspring per breeding event. The offspring is attended and fed by both parents for approximately 3 months (Tershy and Croll, 2000). Parents feed their chicks by transferring small portions of semi-digested fish directly into the chick's mouth (Nelson, 1978). Eggs and young chicks are vulnerable to predation and chicks are unable to thermoregulate (and therefore cannot be left unattended by the parents) until they reach approximately 4 weeks of age. During courtship, males display green–bluish coloration on bare skin under the bill (hereafter gular color), and females exhibit yellow–greenish color in this tegument. Gular skin color in both sexes has been found to be carotenoid-dependent (the presence of carotenoids was determined by high-performance liquid chromatography; B.M., C. Flores and R.T., unpublished data). Males with greener gular color attend their offspring longer, feed them more and have chicks with higher body mass gain, while chicks of genetic fathers with greener gulars show higher structural growth, suggesting that gular color is an honest sexual signal that indicates both direct and indirect benefits to females (Montoya and Torres, 2015).

The aims of this study were to evaluate whether oxidative stress (1) modulates reproductive investment by parents, either as a cost of or a constraint on reproductive effort, and (2) is associated with environmental and/or genetic variation in the offspring. To estimate oxidative stress, we measured levels of ROS production and lipid peroxidation (LP), which is a marker of oxidative damage to lipids. To distinguish environmental from genetic effects on offspring measurements, we performed a cross-fostering experiment. First, if gular color in the brown booby is related to oxidative status, we expected a negative association of gular color during courtship with ROS and LP levels. Second, if oxidative stress constrains reproduction, we predicted that males and females with higher levels of ROS or LP during courtship would have later laying dates, smaller clutch volume, lower offspring attendance, lower food provisioning and chicks with lower body mass and size. Third, if oxidative stress arises as a cost of reproduction, we expected ROS and LP levels to increase in males and females during parental care compared with their own courtship levels, and for this increase to be positively associated with their breeding effort (i.e. offspring attendance, food provisioning and chick growth). Finally, if offspring oxidative stress is largely determined by environment, we expected offspring ROS and LP to be correlated with rearing parents' ROS and LP, while if oxidative stress is associated with genetic variation, we expected a correlation between chick ROS and LP and their genetic parents' ROS and LP levels.

MATERIALS AND METHODS

The study was carried out at the brown booby breeding colony of Isla Larga, Parque Nacional Islas Marietas, Nayarit, Mexico (20°41′ N 105°36′W), from June to September 2011. During the courtship period, we captured 52 pairs and individually marked both members of each pair with a white numbered polymethylmethacrylate leg band (PMMA leg bands; Interrex, Poland). For each captured bird, body mass (±20 g), ulna length (±1 mm) and gular color were measured. Individuals' ages were unknown. Gular color was quantified as the mean reflectance curve of three sequential measurements of non-overlapping patches from the gular skin using a portable spectrophotometer that determines the reflectance from 360 to 740 nm (±10 nm) (MINOLTA CM 2600d, Osaka, Japan). Morphometric measurements, color measurements and blood sampling (see below) of each pair were completed in roughly 10 min, after which birds were returned to their courting site. Pairs' courting sites were marked with a numbered flag and the study area was monitored daily between 18:00 and 20:00 h to determine laying dates of focal pairs. On the day of laying, egg volume in mm^3 (length×$width^2$×0.51/1000; Hoyt, 1979) was measured.

Permission was granted by Parque Nacional Islas Marietas and SEMARNAT (02083/11). This research complies with current laws of Mexico and Animal Behavior Guidelines.

Cross-fostering

Fifteen days after the first egg of the clutch was laid, clutches were randomly assigned to either the cross-fostered or the control group. Control and cross-fostered clutches did not differ in laying date ($F_{1,41}$=0.37, P=0.55) or clutch size ($\chi^2_{1,41}$=3.40, P=0.07). In the cross-fostered group (n=34, 28 two-egg and 6 one-egg clutches), complete clutches were swapped between pairs with similar laying date (±3 days) and equal clutch size (one or two eggs). Additionally,

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to address another research question for a different study, chicks were cross-fostered by males that had the greatest difference from their genetic fathers in gular reflectance at 540 nm (i.e. the peak wavelength in the green range of male gular color; relationship of male gular color within swapped clutches r_p=−0.50 P=0.008; see Montoya and Torres, 2015). In the control group (n=18, 12 two-egg and 6 one-egg clutches), the swapping procedure was simulated, but clutches were returned to their original nests (Montoya and Torres, 2015). Nests were monitored every 5 days during incubation, and daily after hatching until the chicks were 15 days old. Chick body mass (±1 g) and, as a proxy of skeletal growth, ulna length (±1 mm) were measured at 1, 5, 10 and 15 days of age.

During the experiment, 13 nests were lost: no hatching occurred in five nests (three cross-fostered and two control), and in eight nests the surviving chick died before reaching 15 days of age (three cross-fostered and five control). Hence, sample size for the analyses was 39 nests with a single chick that survived up to the age of 15 days (36 first chicks and three second chicks).

Blood sampling

To determine LP and ROS levels, blood samples of parents were taken during courtship (mean±s.d.; 12.56±1.33 days before laying) and during parental care (15 days after the first chick hatched), and chicks were blood sampled 15 days after hatching. Blood samples of adults (2 ml) and chicks (1 ml) were taken from the brachial vein, kept on ice until they were centrifuged at 10,000 ***g*** for 10 min, stored in the field for approximately 2 months in liquid nitrogen within roughly 2 h of collection, and maintained at −80°C until analyses were performed. The sex of the chicks was determined according to Griffiths et al. (1998).

Behavioral records

Parental behavior and chick begging were recorded when the chicks were 1, 5, 10 and 15 (+3 days) days old. Observations were performed from 07:00 to 09:00 h and from 17:00 to 21:00 h, the time periods when most diurnal parental care activity occurs in this breeding colony (B.M., unpublished data). Behavioral observations were carried out by four trained observers located at a distance of 3–6 m from the focal nest. Inter-observer reliability was high (≥90%) and observers were uninformed about whether the observed nest belonged to the control or the cross-fostered group. Behaviors recorded were: (1) number of provisioning events by the male and the female (when a parent places the bill over the chick's head and the chick places its head into the parent's bill to be fed), (2) the occurrence at 5 min intervals (1–0 records) of chick begging (when the chick raises the head and vocalizes with a 'tac-tac' sound) to the male or the female, and (3) the duration of male and female attendance of offspring (the minute and second of arrivals to and departures from the nest by each parent). For each chick, behaviors recorded during the four days of observation were pooled together for the analyses.

Protein quantification

Because redox reactions depend on the amount of substrate available for oxidation, and the amount of substrate may vary between samples of the same volume (e.g. owing to differences in diet), estimations per concentration of substrate, instead of per sample volume, are more appropriate (Barja de Quiroga et al., 1991). Lipids, because of their hydrophobic nature, are transported in the bloodstream within lipoproteins (Vance and Vance, 2002), and lipoproteins may be attacked by ROS, resulting in LP (Halliwell and Gutteridge, 1999). Therefore, we quantified in each sample the amount of blood proteins as an estimate of substrate for redox reactions using the bicinchoninic acid (BCA) method for protein quantification. The protocol of the BCA protein assay kit (Pierce, Rockford, IL, USA) was followed adjusting the samples from 20 to 10 µl to be able to run all the assays in triplicate. In this assay, the chelation of two molecules of BCA with one cuprous ion produces a purple colored reaction, which increases with protein concentration (Smith et al., 1985). The absorbance of the resulting complex was measured at 562 nm of absorbance using an ELISA spectrophotometer (Model 550, Bio-Rad Laboratories, Hercules, CA, USA). The results were calibrated with a standard curve of bovine albumin (catalog no. 23209, Thermo Fisher Scientific, Waltham, MA, USA). LP and ROS assays require a sample of approximately 50 µg of protein, thus protein concentrations in our plasma samples were diluted 1:100 before subsequent analysis to achieve the suggested concentration.

Lipid peroxidation

The thiobarbituric acid reactive substances (TBARS) assay estimates peroxidative damage to lipids through the formation of the pink chromogen $[TBA]_2$–malondialdehyde adduct (Halliwell and Chirico, 1993). Briefly, a 100 µl aliquot of the diluted plasma was added to 100 µl of trichloroacetic acid (10% v/v) and centrifuged at 3000 ***g*** for 10 min. The supernatant was added to 1 ml of thiobarbituric acid reagent (0.375% TBA and 2% acetic acid), and the mixture was incubated at 92°C for 45 min. After reaction, samples were placed on ice for 10 min. The absorbance of the thiobarbituric acid–MDA complex was measured at 532 nm using an ELISA spectrophotometer (Bio-Rad Model 550). Estimates of lipid peroxidation were calculated as MDA equivalents interpolating into a concentration curve of 1,1,3,3-tetraethoxypropane (Fluka Chemie Co., USA) ranging from 0 to 5 nmol l^{-1}. Throughout the document, lipid peroxidation is expressed as nmol MDA mg^{-1} protein. The average coefficient of variation (CV) between replicates (measured as the ratio of the standard deviation to the mean and expressed as a percentage) was lower than 5.11% within plates and 11.83% among plates.

Reactive oxygen species

The technique we used to estimate ROS is based on the oxidation of the non-fluorescent molecule dihydrorhodamine-123 to the fluorescent rhodamine-123 by hydrogen peroxide in the presence of peroxidases (Henderson and Chappell, 1993). Rhodamine-123 results from the action of peroxynitrite and hydrogen peroxide, two biological oxidants produced by the radicals superoxide anion and nitric oxide. Peroxynitrite and hydrogen peroxide can damage lipids, DNA and proteins, and alter the modulation of various cell signal transduction pathways (Pacher et al., 2007). Therefore, the amount of rhodamine-123 has been used as an estimate of ROS/RNS production (Jallali et al., 2005). Briefly, 180 µl buffer A (140 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 0.8 mmol l^{-1} $MgSO_4{\cdot}7H_2O$, 1.8 mmol l^{-1} $CaCl_2$, 5 mmol l^{-1} glucose, 15 mmol l^{-1} Hepes) and 20 µl dihydrorhodamine-123 (1 mmol l^{-1}, Aldrich Chemical Co., Milwaukee, WI, USA) were added to 20 µl aliquots of the diluted plasma samples. The mixture was placed in a 96-well plate and read at 505 nm in an ELISA spectrophotometer (Bio-Rad Model 550). The results were interpolated from a standard curve of rhodamine-123 (Aldrich Chemical Co., Milwaukee, WI, USA) in buffer A ranging from 0 to 10 µmol l^{-1}. ROS quantity is expressed as µmol rhodamine-123 mg^{-1} protein throughout. The average CV between replicates was lower than 4.54% within plates and 8.18% among plates.

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Statistical analysis

Male gular color was synthesized by performing a principal component analysis (PCA) that included UV (sum of reflectance from 360 to 400 nm/sum of total reflectance from 360 to 740 nm), blue (sum of reflectance from 430 to 470 nm/total reflectance) and green chroma (sum of reflectance from 480 to 550 nm/total reflectance). Three PCs were extracted, explaining 59.86%, 34.38% and 5.74% of the variation (eigenvalues PC1=1.79, PC2=1.03 and PC3=0.17). For statistical analyses, we used PC1 (hereafter, male gular color) with factor loadings of 0.71 for UV chroma, 0.34 for blue chroma and –0.60 for green chroma. From females' gular spectral curves, we calculated the maximum reflectance value within 360–740 nm as an indicator of female gular color. Although gular color measurements during courtship were unavailable for six females, female gular color did not differ between courtship and chick rearing (paired *t*-test=−0.87, P=0.38, n=29 females for which we were able to measured gular color during both courtship and chick rearing period), so color measurements from chick rearing were used to represent color during courtship for four of these females.

Prior to statistical analyses, ROS and LP estimates were ln transformed. Independent models were fitted to explore the effect of ROS and LP on each response variable. General linear models with a normal error distribution were fitted to evaluate the effects of ROS and LP on all the response variables except for parental provisioning. To analyze parental provisioning, generalized linear models with a negative binomial distribution to correct for over-dispersion were fitted. Of the 39 total focal families, ROS estimates of four males, 14 females and four chicks, and LP estimates of nine males, 10 females and three chicks could not be obtained because of missing samples. Sample sizes thus vary among analyses, and the number of covariates in statistical models was minimized to deal with the unbalanced dataset. The sex of the chicks was included in the initial models of parental effort but was never significant, hence it is not included in the models reported. Minimal adequate models were obtained by backward elimination of non-significant terms (α=0.05). Effect size for principal results was calculated through η^2 using the 'lsr' package (Navarro, 2015). Residuals from all final models were normally distributed. Analyses were carried out using R 3.0.2 (R Foundation for Statistical Computing, http://www.R-project.org/) or SAS software 9.0 (SAS Institute, Cary, NC, USA). Assumptions underlying statistical tests were verified.

To analyze the effects of oxidative markers on gular color, models included ROS or LP of males or females, days to laying, and the interaction of days to laying with ROS and LP. In the model of male gular color, one outlier was identified and dropped (ID 21, Bonferroni outlier test, P=0.01). Models evaluating the potential constraining effect of ROS and LP on laying date, clutch volume and first egg volume included LP or ROS of both parents as the main variable, as well as the interaction between the male's and female's oxidative markers. As ROS levels were significantly correlated with days to laying, residuals of this correlation were used in further analyses where ROS levels were included. For analyses during chick rearing, hatching date was included as a covariate in the initial models, but was dropped because it was never significant (P>0.07).

To analyze the potential constraining effect of parental oxidative stress on chick size and growth, we constructed separate models of chick mass at fledging, size at fledging, mass gain rate and ulna growth up to 15 days of age as a function of the rearing parents' ROS or LP and swapping treatment (swapped or control) and their interaction, including egg volume as a covariate. The swapping treatment was included in the analyses because cross-fostering disrupted the correlation between the genetic mother's provisioning rate and offspring begging (B.M. and R.T., unpublished data), and this disruption might influence parental care behavior, offspring growth, and oxidative stress markers of parents and chicks. In the analyses of hatchling mass and chick mass gain rate, ulna length at hatching and ulna growth rate, respectively, were included as covariates to control for structural size. Models analyzing the effects of ROS and LP levels at courtship on parental feeding and offspring attendance included the swapping treatment as a factor, chick body mass at hatching as a covariate, and the interaction between the swapping treatment and parental oxidative markers. In the analyses of parental

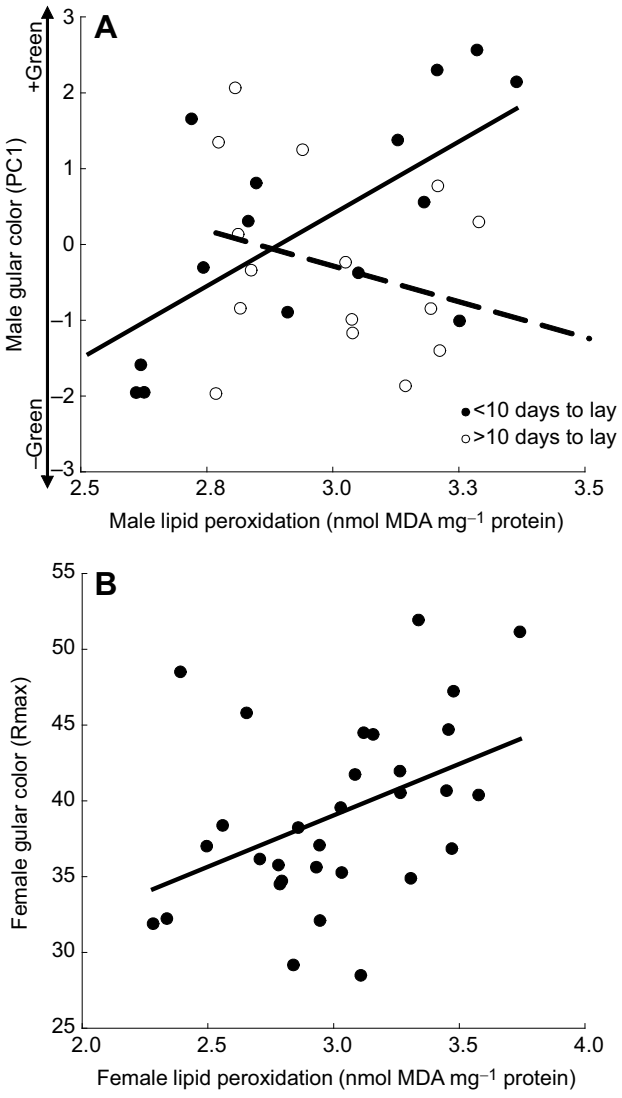

Fig. 1. Gular color in the brown booby during courtship is positively associated with lipid peroxidation. (A) Male gular color (PC1) versus lipid peroxidation during courtship as a function of laying date (≤10 days, closed circles and solid line, R^2=0.43; >10 days, open circles and dashed line, R^2=0.12). For clarity, PC1 was multiplied by –1 (in the plot only, not for analyses), such that positive values indicate higher green chroma and negative values higher UV chroma [general linear model (GLM), LP: $F_{1,27}$=8.05, P=0.009; days to laying: $F_{1,27}$=6.41, P=0.02; LP×days to laying: $F_{1,27}$=6.73, P=0.02, n=30, η^2=0.19]. (B) Female gular color (maximum reflectance) versus lipid peroxidation at courtship (GLM, $F_{1,29}$=6.43, P=0.02, n=31, η^2=0.18).

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provisioning, chick begging rate and its interaction with parental oxidative markers were also included.

To evaluate the potential occurrence of an oxidative cost of reproduction, we fitted repeated-measures general linear models to analyze the change in rearing parents' ROS and LP levels between courtship and parental care. Models included nest identity as the subject term, reproductive stage (courtship or parental care) as the repeated measure, swapping treatment as a fixed factor, chick body mass at 15 days, parental provisioning and nest attendance as covariates, and the interaction of swapping treatment with parental provisioning and swapping treatment with offspring attendance. Intra-individual repeatability of parents' ROS and LP levels between courtship and parental care was calculated using the R package rptR (Nakagawa and Schielzeth, 2010). Parametric bootstrapping was used to obtain confidence intervals for the mixed-model-based repeatability, and statistical significance of the repeatability estimates was tested by restricted maximum likelihood (Nakagawa and Schielzeth, 2010). Significant repeatability indicates that an individual's measurements are more similar than measurements of different individuals.

Finally, the effect of genetic and environmental variation on offspring ROS and LP levels was not evaluated within the same model because of a lack of statistical power associated with missing values. Therefore, to explore this question, we fitted independent models including either the genetic mother and father ROS and LP levels, or the rearing mother and father ROS and LP levels as main variables. In the cross-fostered group, oxidative markers during courtship of rearing and genetic fathers or mothers were not correlated (ROS levels of fathers, r_p=−0.30, P=0.20; mothers, r_p=−0.22, P=0.73; LP levels of fathers, r_p=0.29, P=0.31; mothers, r_p=−0.39, P=0.34). All models included swapping treatment as a factor, egg volume, rate of body mass gain and chick weighted begging (sum of chick begging rate to mother and father plus one divided by sum of provisioning of father and mother plus one; one was added to avoid zeros in the numerator or denominator) as covariates, and the interactions between the parents' oxidative markers and the swapping treatment.

RESULTS

During courtship, ROS levels of males and females increased as laying approached (males, $F_{1,37}$=8.16, P=0.007, β=−0.02±0.008, η^2=0.18; females, $F_{1,26}$=4.24, P=0.050, β=−0.02±0.01, η^2=0.14), but LP levels were unrelated to the timing of laying (males, $F_{1,30}$=0.07, P=0.79; females, $F_{1,30}$=0.55, P=0.46). After controlling for variation related to the timing of laying, ROS production and LP levels tended to be positively correlated during courtship in males (ROS: $F_{1,29}$=4.07, P=0.053; days to laying: $F_{1,29}$=1.23, P=0.27), but not females (ROS: $F_{1,25}$=0.01, P=0.91; days to laying: $F_{1,25}$=0.12, P=0.73). There was no correlation between pair members in ROS or LP at courtship (LP: $F_{1,24}$=0.38, P=0.5; ROS: $F_{1,25}$=2.29, P=0.14). Hence, preparing for the establishment of a clutch increased ROS production by both males and females, and in males only, greater ROS was marginally related to greater LP levels.

Are sexual ornaments related to oxidative stress level?

During courtship, gular color was not associated with ROS levels of males ($F_{1,37}$=0.009, P=0.92; days to laying: $F_{1,36}$=0.52, P=0.47; ROS×days to laying: $F_{1,35}$=0.21, P=0.65) or females ($F_{1,25}$=2.02, P=0.17; days to laying: $F_{1,24}$=1.46, P=0.24; ROS×days to laying: $F_{1,23}$=3.94, P=0.06). However, gular color was positively related to the level of LP in males and females. Males with greener gular and less UV reflectance had greater LP levels as laying date approached (LP: $F_{1,27}$=8.05, P=0.009; days to laying: $F_{1,27}$=6.41, P=0.02; LP×days to laying: $F_{1,27}$=6.73, P=0.02; Fig. 1A). Females with brighter gular color had greater LP levels ($F_{1,29}$=6.43, P=0.02; Fig. 1B), regardless of the timing of laying (days to laying: $F_{1,28}$=0.43, P=0.52; LP×days to laying: $F_{1,27}$=0.32, P=0.58). Thus, displaying bright gular color during courtship was related to greater levels of LP, but in males this was only as laying approached.

Table 1. Linear models evaluating the effects of rearing parents reactive oxygen species (ROS) and lipid peroxidation (LP) levels at courtship on offspring size at hatching and growth

	Hatching				Growth rate			
	Body mass		Ulna		Body mass		Ulna	
Variable	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Rearing parents ROS								
Swapping treatment	1.12	0.30	0.13	0.72	1.88	0.18	0.22	0.64
Egg volume	**5.79**	**0.02**	0.001	0.97	**5.69**	**0.03**	0.26	0.61
Hatchling ulna	**19.97**	**<0.001**	–	–	–	–	–	–
Ulna growth rate	–	–	–	–	**22.32**	**<0.001**	–	–
Father ROS	0.04	0.85	0.04	0.85	0.15	0.70	0.28	0.60
Mother ROS	0.28	0.60	0.46	0.51	**4.64**	**0.04**	0.27	0.60
Father ROS×swapping	1.83	0.20	1.26	0.28	1.22	0.28	0.04	0.84
Mother ROS×swapping	1.71	0.21	0.06	0.81	0.06	0.80	0.01	0.90
Error d.f. final model	34		37		19		35	
Rearing parents LP								
Swapping treatment	1.12	0.29	0.11	0.75	2.28	0.14	0.003	0.95
Egg volume	**5.79**	**0.02**	0.20	0.66	2.74	0.12	0.17	0.68
Hatchling ulna	**19.97**	**<0.001**	–	–	–	–	–	–
Ulna growth rate	–	–	–	–	**24.99**	**<0.001**	–	–
Father LP	0.17	0.68	2.68	0.12	0.07	0.79	2.25	0.14
Mother LP	0.16	0.69	0.41	0.53	0.04	0.84	1.07	0.21
Father LP×swapping	0.04	0.85	0.01	0.92	0.001	0.97	0.03	0.87
Mother LP×swapping	2.11	0.16	0.45	0.51	0.04	0.84	0.91	0.35
Error d.f. final model	34		37		35		35	

The residuals of the regression of ROS on days to laying were used for analyses. Growth rate=daily body mass (g day^{-1}) and ulna length (mm day^{-1}) growth to 15 days post-hatching. Table shows *F* and *P* values from variables in the initial model at the moment of their exclusion; variables in the final model are in bold.

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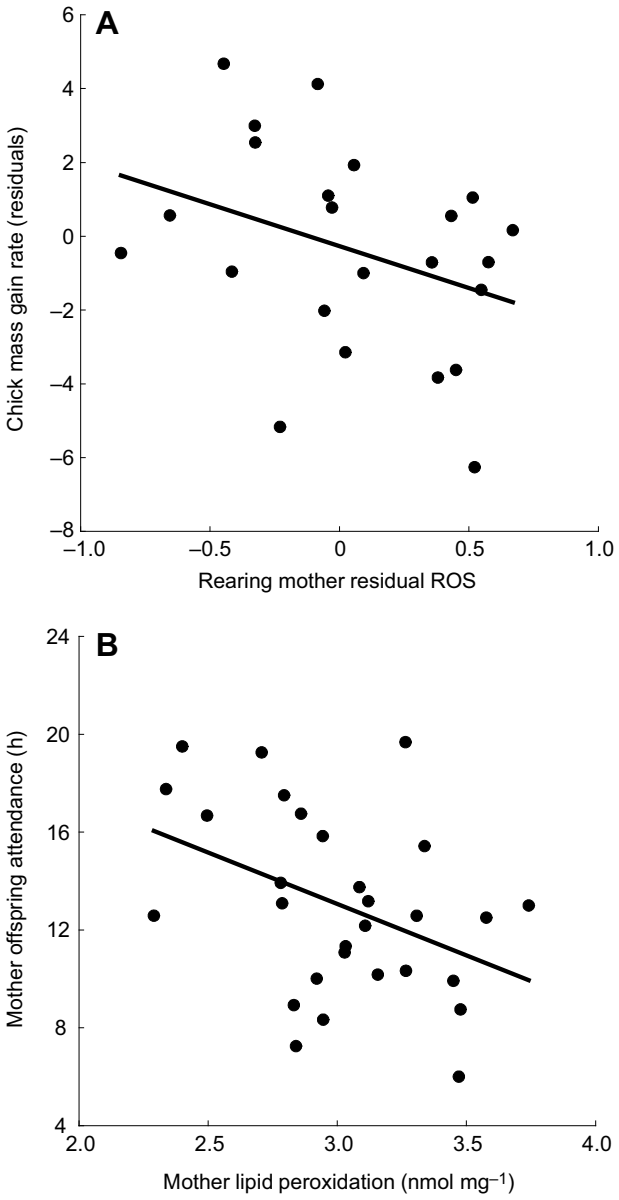

Fig. 2. Higher levels of oxidative markers during courtship are negatively related to maternal effort. (A) Chick body mass gain rate from hatching to 15 days old versus rearing mother residual reactive oxygen species (ROS) at courtship. Residual ROS was calculated as the residuals of the regression of mother ROS μmol mg^{-1} at courtship on days to laying. Residuals of the final model dropping mother residual ROS are plotted on the *y*-axes (GLM, $F_{1,19}$=4.64, P=0.04, n=23, η^2=0.16). (B) Mother offspring attendance versus levels of lipid peroxidation during courtship (GLM, $F_{1,27}$=5.63, P=0.03, n=29, η^2=0.17).

Does oxidative stress during courtship constrain reproductive effort?

Laying date and egg size

Pairs with higher ROS levels during courtship established earlier clutches (male ROS: $F_{1,24}$=6.32, P=0.02; female ROS: $F_{1,24}$=5.62, P=0.03; interaction: $F_{1,23}$=0.19, P=0.66). Total clutch volume was not associated with male or female ROS (male ROS: $F_{1,21}$=0.41, P=0.53; female ROS: $F_{1,22}$=1.37, P=0.25; interaction: $F_{1,20}$=0.58, P=0.45). However, females with higher ROS laid smaller first eggs ($F_{1,24}$=6.94, P=0.01), while male ROS was not associated with first egg volume (male: $F_{1,23}$=0.03, P=0.86; male×female: $F_{1,22}$=0.07, P=0.79). Second egg volume was not related to the male's or female's ROS levels at courtship (male: $F_{1,26}$=0.60, P=0.44; female: $F_{1,17}$=0.17, P=0.87).

Within pairs, LP levels were unrelated to laying date (male LP: $F_{1,30}$=2.30, P=0.14; female LP: $F_{1,23}$=0.16, P=0.69; interaction: $F_{1,22}$=0.38, P=0.54), clutch volume (male LP: $F_{1,26}$=0.79, P=0.38; female LP: $F_{1,20}$=0.001, P=0.97; interaction: $F_{1,19}$=0.01, P=0.91), first egg volume (male LP: $F_{1,29}$=0.51, P=0.48; female LP: $F_{1,22}$=0.03, P=0.86; interaction: $F_{1,21}$=0.58, P=0.45) or second egg volume (male LP: $F_{1,16}$=0.002, P=0.96; female LP: $F_{1,20}$=0.49, P=0.49; interaction: $F_{1,15}$=1.82, P=0.20).

Chick growth

Rearing mothers with higher ROS levels during courtship had chicks with a lower rate of body mass gain (β=−3.46±1.61; Table 1, Fig. 2A). Rearing father ROS level was not associated with chick body mass gain rate (Table 1). Rearing mother and father ROS levels were not associated with hatchling body mass, ulna length or chick structural growth (Table 1). Rearing parents' LP levels were unrelated to hatchling body mass and size, or the rates of chick body mass gain and structural growth (Table 1). Swapping treatment or its interaction with parental ROS and LP were not related to chick growth (Table 1).

Parental investment

Rearing parents' ROS levels during courtship were not associated with their provisioning effort or time attending offspring (Table 2). Mothers with higher LP levels during courtship spent less time attending their chicks (β=−4.02±1.78; Table 2, Fig. 2B), but did not affect their provisioning effort (Table 2). Father's LP during courtship was unrelated to attendance or provisioning of chicks (Table 2). Swapping treatment or its interaction with parental ROS and LP during courtship were not related to mothers' or fathers' parental investment (Table 2).

Hence, pairs with higher ROS production during courtship established clutches early in the season, but higher ROS levels in females were associated with the production of smaller first eggs and chicks with lower mass gain. No associations were found between courtship LP levels of males and females and the timing of breeding, egg/clutch size, chick growth or male parental effort, but females with higher LP during courtship spent less time with their chicks.

Is oxidative stress a cost of reproductive effort?

Both males and females had higher ROS levels during courtship than during chick rearing (Table 3, Fig. 3). Chick mass at 15 days, parental provisioning, chick attendance, swapping treatment and its interaction with parental provisioning or attendance were all unrelated to this decline in parental ROS from courtship to chick rearing (Table 3). Male and female LP levels did not significantly vary from courtship to chick rearing and were not related to any of the covariates tested (Table 4). Thus, no evidence of an oxidative cost of parental effort was found.

Intra-individual variation from courtship to parental care was not repeatable for ROS levels (males, P=0.55; females, P=0.44); however, repeatability of LP levels was 33% for males (95% CI=0.0–62.8%, P=0.05) and 61.1% for females (95% CI=30.4–81.2%, P=0.001).

Sources of individual variation in chick oxidative stress

Chick ROS levels were unrelated to rearing or genetic parents' ROS levels during courtship, or to swapping treatment, its interaction with rearing parents ROS, or the covariates included in the analyses

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Table 2. Linear models evaluating the effects of rearing parents' reactive oxygen species (ROS) and lipid peroxidation (LP) levels at courtship on parental effort

	Paternal effort				Maternal effort			
	Provisioning		Attendance		Provisioning		Attendance	
Variable	χ^2	*P*	*F*	*P*	χ^2	*P*	*F*	*P*
Parental levels of ROS								
Swapping treatment	0.001	0.98	1.68	0.20	0.14	0.71	0.003	0.98
Hatchling mass	0.93	0.33	2.09	0.16	**12.63**	**<0.001**	0.002	0.98
Begging rate[a]	**6.88**	**0.009**	–	–	**15.06**	**<0.001**	–	–
Father ROS	0.15	0.69	0.77	0.39	0.93	0.33	1.89	0.19
Mother ROS	0.001	0.97	0.02	0.89	0.98	0.32	0.05	0.82
Parent ROS×swapping	0.10	0.76	0.58	0.45	0.19	0.66	0.002	0.96
Parent ROS×begging rate[a]	0.86	0.35	–	–	0.78	0.38	–	–
Error d.f. final model	36		38		34		38	
Parental levels of LP								
Swapping treatment	3.15	0.08	1.68	0.20	0.15	0.70	0.03	0.87
Hatchling mass	0.31	0.57	2.09	0.16	**12.63**	**<0.001**	0.25	0.62
Begging rate[a]	**6.88**	**0.009**	–	–	**15.06**	**<0.001**	–	–
LP father	0.73	0.39	0.48	0.50	0.04	0.85	2.08	0.16
LP mother	3.20	0.07	0.12	0.74	0.14	0.71	**5.63**	**0.03**
LP parent×swapping	3.19	0.07	0.01	0.92	0.001	0.97	0.05	0.83
LP parent×begging rate[a]	2.09	0.15	–	–	0.001	0.98	–	–
Error d.f. final model	36		38		34		27	

The residuals of the regression of ROS on days to laying were used for analyses. General linear models with normal error distribution were use to analyze attendance time, while generalized linear models with Poisson error distribution were used to analyze provisioning. Table shows *F* and *P* values from variables in the initial model at the moment of their exclusion; variables in the final model are in bold.

[a]Begging rate to the father was included in the model of paternal provisioning while begging rate to the mother was included in the model of maternal provisioning.

(Table 4). However, chick LP levels were positively related to rearing mother (β=0.30±0.15; Fig. 4A) and rearing father LP levels during courtship (β=0.78±0.23; Table 4, Fig. 4B). Chicks from the control group had higher LP levels than chicks from swapped nests, but the interaction between swapping treatment and rearing parents' LP was not significant (Table 4). Additionally, chicks from larger eggs showed higher LP levels (β=0.02±0.01; Table 4). Interestingly, chick LP was associated with genetic father LP levels (β=0.86±0.28; Table 4, Fig. 4C), but not with genetic mother LP levels at courtship (Table 4). Chicks' body mass gain and weighted begging were not related to their LP levels (Table 4). Thus, chick LP was related to the LP levels of rearing parents and to the LP level of the genetic father at courtship, while chick ROS production was unrelated to either the rearing or genetic parents' ROS.

DISCUSSION

We evaluated whether oxidative stress constrains reproductive effort or is a proximate cost of reproductive investment, and whether oxidative status in offspring is due to genetic and/or environmental variation. We found that oxidative stress (1) may be a cost of displaying elaborate gular coloration and preparing for early laying, but not of increased parental effort, (2) may constrain female, but not male reproductive investment in eggs and offspring, and (3) in chicks, might result from genetic and environmental variation.

In males, ROS levels during courtship were higher when laying date approached, and lipid damage was higher when males displayed greener and less UV-reflective gular coloration as laying approached. These results suggest that developing colorful ornaments, particularly during the period just before egg laying, might entail oxidative costs. In the brown booby, male skin color is associated with direct and indirect benefits for females (Montoya and Torres, 2015); hence, male gular color likely functions as a sexual signal. Additionally, the negative relationship between green and UV chromas agrees with the idea that skin color may result from the combined effect of a structural color (UV) and the allocation of

Table 3. Repeated-measures models to evaluate the change in rearing parental reactive oxygen species (ROS) and lipid peroxidation (LP) levels from courtship to parental care

	ROS change				LP change			
	Father		Mother		Father		Mother	
Variable	*F*	*P*	*P*	*F*	*F*	*P*	*F*	*P*
Within-subjects effects								
Stage	**18.19**	**<0.001**	**11.54**	**0.002**	0.20	0.66	0.001	0.98
Stage×swapping treatment	2.03	0.17	0.04	0.85	0.01	0.93	0.10	0.76
Stage×chick mass at 15 days	0.17	0.69	0.24	0.63	0.03	0.88	0.67	0.42
Stage×provisioning	0.11	0.74	0.001	0.96	0.62	0.44	0.19	0.67
Stage×attendance	0.02	0.88	0.13	0.73	2.97	0.10	0.60	0.45
Stage×provisioning×swapping	0.03	0.86	0.10	0.76	0.09	0.76	1.23	0.29
Stage×attendance×swapping	0.30	0.59	0.51	0.49	0.50	0.49	1.90	0.19
Error d.f. final model	28		20		26		22	

Table shows *F* and *P* values from variables in the initial model at the moment of their exclusion; variables in the final model are in bold.

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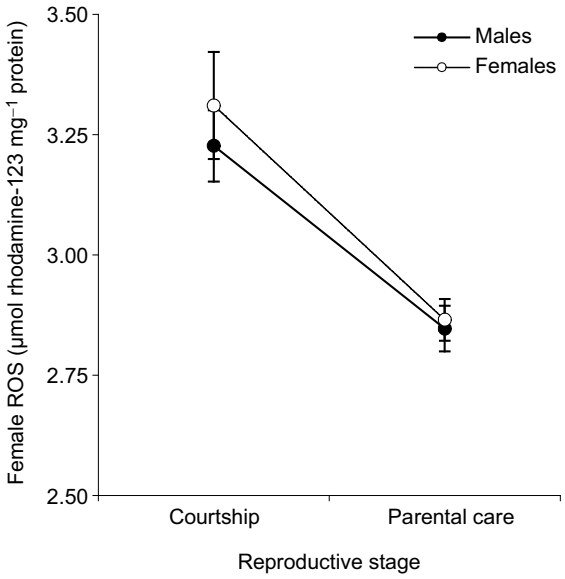

Fig. 3. Reactive oxygen species (ROS) are higher during courtship than during parental care. Measurements of ROS during parental care were obtained 15 days after the chick hatched and are expressed as means±s.e.m. (GLM, males: $F_{1,28}$=18.19, P<0.001, n=58 samples from 29 individuals; females: $F_{1,20}$=11.54, P=0.002, n=42 samples from 21 individuals). Males: closed circles and solid line; females: open circles and dotted line.

carotenoid pigments. The role of male skin color as an honest signal during courtship might be maintained through a handicap mechanism (Zahavi, 1975; Grafen, 1990; Iwasa et al., 1991; Alonso-Alvarez et al., 2007), where individuals that can afford higher levels of oxidative damage can also invest more heavily in colorful ornaments, because they are in better general condition. Individual condition is expected to be linked to higher biochemical efficiency of vital cellular processes (Hill, 2011; Hill and Johnson, 2012), and carotenoid-dependent coloration might signal such cellular efficiency when its production shares pathways with vital cellular processes (Hill and Johnson, 2012). Thus, brown booby male gular color may signal biochemical efficiency of cellular processes, which might explain why colorful males can afford higher LP levels, as suggested for other species with sexual colors dependent on keto-carotenoids (Mundy et al., 2016). Interestingly, in females, ROS levels during courtship were also higher as laying approached and brighter gular coloration was associated with higher levels of LP. In the brown booby, mutual mate choice and assortative mating is expected because of the long period of biparental care (Burley, 1977; Amundsen, 2000; Johnstone et al., 1996; Kokko and Johnstone, 2002). Accordingly, we recently found assortative mating by color in the brown booby (B.M., R.T., unpublished data). Thus, females might pay an oxidative cost for displaying brighter gular coloration to attract males that are better parents, as signaled by their more elaborate coloration (Montoya and Torres, 2015). At present, our results suggest that developing and maintaining colorful ornaments and preparing to establish a clutch are costly for both sexes.

Does oxidative stress constrain reproduction?

A constraining effect of oxidative stress on reproductive investment is expected when individuals with higher oxidative damage cannot pay the cost of high reproductive effort (Dowling and Simmons, 2009; Metcalfe and Alonso-Alvarez, 2010). As predicted when oxidative stress constrains reproductive investment, females with higher ROS at courtship laid smaller first eggs and their offspring had a lower rate of body mass gain, while females with higher LP at courtship spent less time attending the offspring. However, contrary to expectations, pairs with higher ROS levels during courtship established clutches earlier, not later. Therefore, increased ROS levels during courtship do not appear to delay breeding of males and females, and may rather arise as a consequence of breeding earlier. Early breeding may be favored, even at the potential costs of maintaining higher ROS levels, to avoid the breeding success decline suffered by late breeders (Verhulst and Nilsson, 2008).

Unlike females, ROS and LP levels during courtship did not influence male parental effort. Hence, oxidative stress during courtship may constrain female, but not male, reproductive investment during egg laying and chick rearing periods. In brown boobies, biparental care is obligate, but females likely invest more in reproduction than males. Besides investment in sexual ornaments and egg production, brown booby females attend the nestlings longer than males (Montoya and Torres, 2015). Therefore, it is possible that females' investment surpasses a threshold that males never reach (Velando and Alonso-Alvarez, 2003). The constraining effect observed in this study may therefore be part of a life-history strategy by which females buffer the impact of current reproductive effort on future reproduction (Blount et al., 2016). Our results suggest the existence of differences between sexes in the strategies

Table 4. Linear models evaluating the effects of rearing and genetic parental reactive oxygen species (ROS) and lipid peroxidation (LP) levels on offspring ROS and LP levels

	Rearing parents				Genetic parents			
	Chick ROS		Chick LP		Chick ROS		Chick LP	
Variable	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Swapping treatment	0.84	0.36	**8.25**	**0.01**	0.84	0.36	**7.09**	**0.01**
Egg volume	0.002	0.97	**5.50**	**0.03**	0.11	0.74	**5.37**	**0.03**
Body mass gain rate	0.001	0.97	0.03	0.87	0.15	0.70	0.13	0.72
Weighted begging rate	1.60	0.22	0.89	0.36	0.008	0.93	0.66	0.43
Father ROS/LP[a]	0.05	0.82	**11.94**	**0.003**	0.22	0.64	**9.49**	**0.005**
Mother ROS/LP[a]	0.04	0.85	**6.10**	**0.02**	0.35	0.56	0.48	0.50
Father ROS/LP[a]×swapping	0.50	0.49	0.77	0.40	0.43	0.52	0.39	0.54
Mother ROS/LP[a]×swapping	0.10	0.77	0.45	0.52	0.01	0.92	1.15	0.30
Error d.f. final model	34		17		33		23	

Chick weighted begging rate was calculated as the sum of chick begging rate to mother and father plus one divided by the sum of provisioning of father and mother plus one; one was added to avoid zeros in the numerator or denominator. Table shows *F* and *P* values from variables in the initial model at the moment of their exclusion; variables in the final model are in bold.

[a]Independent models for ROS and LP, and for rearing parents and genetic parents were fitted.

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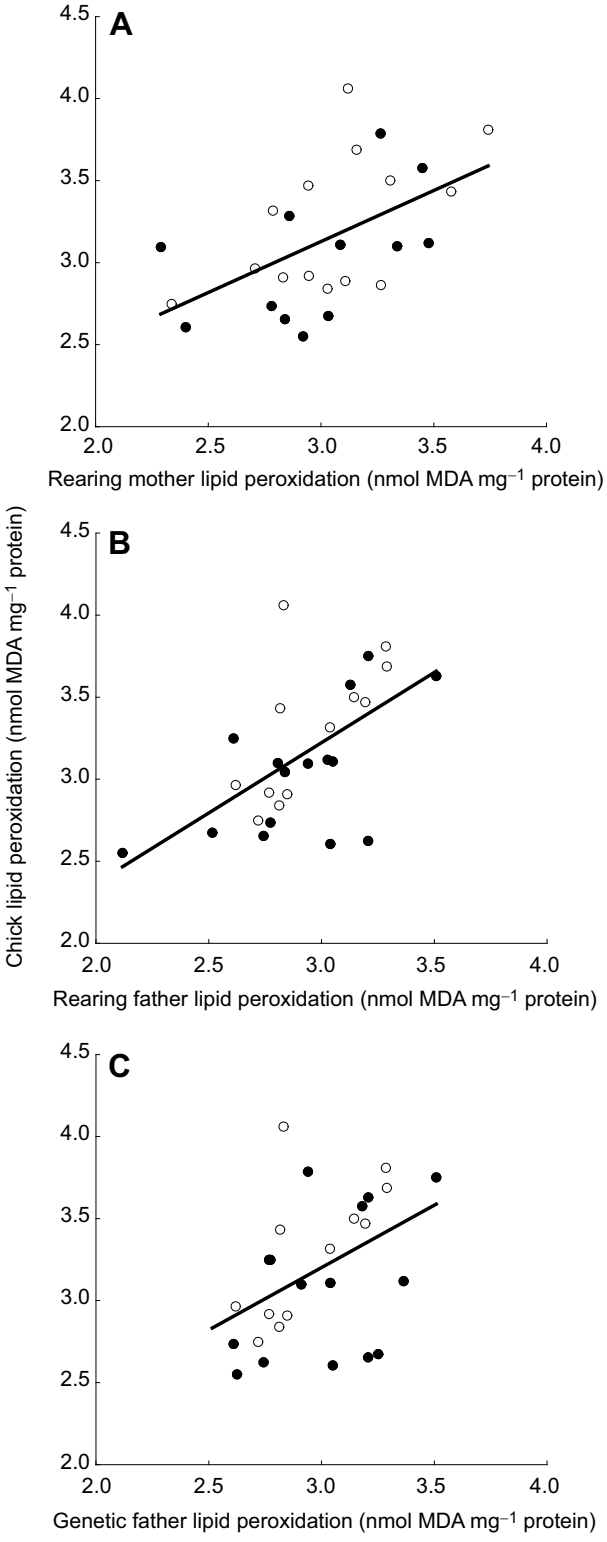

Fig. 4. Chick lipid peroxidation (LP) is associated with rearing parents' and genetic father's LP. Chick LP at 15 days post-hatching and LP during courtship of (A) rearing mother (GLM, $F_{1,17}$=6.10, P=0.02, n=26, η^2=0.10), (B) rearing father ($F_{1,17}$=11.94, P=0.003, η^2=0.19) and (C) genetic father ($F_{1,23}$=9.49, P=0.005, n=27, η^2=0.23). Control nests: open circles; cross-fostered nests: closed circles.

to cope with oxidative stress in particular, and reproductive demands in general (Wiersma et al., 2004; Bize et al., 2008). Future studies are needed to evaluate the fitness consequences for all family members, but particularly for offspring, of the apparent constraining effect on females of higher ROS and LP levels.

Is oxidative stress a cost of reproduction?

Basal metabolic rate increases during parental care (Nilsson, 2002; Steinhart et al., 2005). This increase in energy demand stimulates ATP production, and as a consequence, might increase the release of reactive species. Depending on the current oxidative balance of the individual, an increased release of reactive species may eventually lead to damage in bio-molecules (Halliwell and Gutteridge, 1999). However, contrary to expectations, in our study, males and females showed lower ROS production during chick rearing compared with their own ROS levels during courtship, and no significant change in the level of LP between stages. Furthermore, there was no association between the magnitude of the change in ROS or LP and our estimates of parental effort post-hatching. During courtship, brown booby males and females spent several hours every day during 4–6 weeks actively displaying and defending their territory (Nelson, 1978), while showing their carotenoid-dependent gular coloration (B.M. and R.T., unpublished data). Hence, breeding activities during courtship are likely to entail high oxidative costs. In long-lived species with limited breeding opportunities, such as the brown booby, individuals are expected to obtain more benefits from minimizing the physiological costs of current reproduction and favor survival and future reproduction (Kirkwood and Austad, 2000). Therefore, a constraining effect of oxidative stress on reproductive investment is more likely to occur than an oxidative cost of such investment in species with long survival expectancies (e.g. *Ovis aries*, Nussey et al., 2009; *Pygoscelis adeliae*, Beaulieu et al., 2011). At present, we have no evidence of increased levels of ROS and LP levels with parental effort as predicted if there was an oxidative cost of investment during the chick-rearing period. In the present study, antioxidant defences were not estimated. Hence, future research should evaluate whether an increase in antioxidant defences prior to reproduction could function as a mechanism to shield individuals from the oxidative stress of reproductive investment (Blount et al., 2016).

Sources of individual variation in chick oxidative stress

In our study, offspring ROS levels at 15 days of age were not associated with the ROS levels of either rearing or genetic parents. The lack of covariation between parental and offspring ROS levels might occur if the expression of genetic and non-genetic variation on ROS production depends on other environmental variables or the stage of development (Kim et al., 2010). For example, genetic variation in ROS production might be associated with changes in ROS in response to a challenge rather than with the baseline ROS levels (e.g. Olsson et al., 2009), or might increase as the chick develops (Kim et al., 2011). Interestingly, offspring LP was positively associated both with the rearing parents (mother LP, η^2= 0.10; father LP, η^2=0.19) and with the genetic father LP (η^2=0.23). In the brown booby it is possible that the relationship between genetic father LP and offspring LP resulted from maternal investment in eggs stimulated by the male phenotype (Rubolini et al., 2006; Losdat et al., 2014; Kahar et al., 2016); however, our experimental design does not allow us to separate maternal effects from genetic effects. Overall, our results may suggest that variation in the levels of serum LP of offspring has a genetic basis and might be further influenced by environmental conditions, including maternal effects, that the chicks experience during development. Offspring variation in the capacity to deal with oxidative stress can have important fitness consequences through increased survival (Bize et al., 2008; Noguera et al., 2012) or reproduction (Bize et al., 2008;

Costantini and Dell'Omo, 2015). Furthermore, in our study, male gular color and LP during courtship were positively related; if covariation between offspring and father LP levels have a positive impact on female fitness (through increased survival or reproduction of offspring), the evolution of sexual traits that indicate the ability to deal with oxidative stress, such as the gular color of male brown boobies, may be favored (von Schantz et al., 1999).

Conclusions

We found no evidence for an oxidative cost, estimated as an increment in ROS levels and oxidative damage to lipids, of parental effort. Interestingly, in the brown booby, elevated ROS production or lipid damage may arise from a higher investment in sexual ornaments, from the behavioral and physiological processes prior to the establishment of a clutch, and from early breeding. Moreover, oxidative stress might constrain female investment in first egg volume, offspring mass increase and chick attendance time, but such a constraining effect was not found in males, suggesting sexual differences in the susceptibility to oxidative stress, or in the strategies to cope with the demands of reproductive effort. Finally, our results suggest that offspring resistance to oxidative damage in serum lipids might depend on variation in their father's genetic contribution and on environmental conditions provided by the rearing parents during the chick's development. Hence, in the brown booby, oxidative stress may play a sex-specific role in shaping reproductive decisions at different stages of reproduction.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

B.M. and R.T. designed the experiment. B.M. collected behavioral data and blood samples. M.V. and E.R. advised and trained B.M. for the determination of oxidative stress markers and contributed during discussion of the results. B.M. and R.T. analyzed data and wrote the manuscript. All authors contributed to the preparation of later versions of the manuscript.

Data availability

Data are available from the Dryad Digital Repository http://dx.doi.org/10.5061/dryad.pn358 (Montoya et al., 2016).

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