



Oxidative physiology of reproduction in a passerine bird: a field experiment

Péter L. Pap^{1,2} · Orsolya Vincze^{1,2} · Attila Fülöp^{1,2} · Orsolya Székely-Béres¹ · Laura Pătraș^{3,4} · Janka Péntes¹ · Csongor I. Vágási^{1,2}

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Abstract

Organisms face resource trade-offs to support their parental effort and survival. The life-history oxidative stress hypothesis predicts that an individual's redox state modulates the trade-off between current and residual fitness, but this has seldom been tested experimentally in non-captive organisms. In this study, we manipulated the brood size in breeding pairs of barn swallows (*Hirundo rustica*) and found that females tending enlarged broods had increased levels of plasma oxidative damage (malondialdehyde concentration). This effect, however, was not accompanied by either a depletion, or defensive upregulation in antioxidants (glutathione, total antioxidant capacity, and uric acid) that may explain the increase in oxidative damage. Brood size manipulation and the level of plasma oxidative damage during brood rearing are not translated into decreased annual return rate, which does not support the oxidative stress hypothesis of life-history trade-offs. On the contrary, we found that female's oxidative damage and total glutathione levels, an important intracellular non-enzymatic antioxidant measured at hatching decreased and correlated positively, respectively with annual return rate, suggesting that oxidative condition at hatching might be a more important contributor to fitness than the oxidative physiology measured during chick rearing. We also show that individual traits and ecological factors, such as the timing of breeding and the abundance of blood-sucking nest mites, correlated with the redox state of males and females during brood care.

Significance statement

Oxidative stress is one of the most important physiological costs of reproduction and thus a key modulator of life-history trade-offs. In this study, we manipulated reproductive effort in breeding pairs of barn swallows and found that females tending enlarged broods had increased levels of plasma oxidative damage. This effect, however, was not accompanied by either a depletion or upregulation in

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✉ Péter L. Pap
peterlpap@gmail.com

¹ Evolutionary Ecology Group, Hungarian Department of Biology and Ecology, Babeş-Bolyai University, Clinicilor Street 5–7, 400006 Cluj Napoca, Romania

² MTA-DE Behavioural Ecology Research Group, Department of Evolutionary Zoology and Human Biology, University of Debrecen, Egyetem square 1, Debrecen 4032, Hungary

³ Department of Molecular Biology and Biotechnology, Babeş-Bolyai University, Clinicilor Street 5–7, 400006 Cluj Napoca, Romania

⁴ Molecular Biology Centre, Institute for Interdisciplinary Research in Bio-Nano-Sciences, Babeş-Bolyai University, Treboniu Laurean Street 42, 400271 Cluj Napoca, Romania

antioxidants that may explain the increase in oxidative damage. We found that female's oxidative damage and total glutathione levels measured at hatching decreased and correlated positively, respectively with annual return rate, suggesting that oxidative condition at hatching might be an important contributor to fitness. Brood size manipulation and the increased levels of plasma oxidative damage are not translated into decreased annual return rate; thus, our results support the hypothesis that reproductive effort has a transient effect on oxidative physiology.

Keywords Antioxidants · Barn swallows · Life-history trade-offs · Lipid peroxidation · Oxidative stress · Parasitism

Introduction

A central paradigm in life-history evolution is that reproductive effort is traded-off against survival and/or future

reproductive prospects (Stearns 1989). Recently, ecophysiologists have proposed that oxidative stress is one of the most important physiological costs of reproduction and thus a key modulator of life-history trade-offs (Costantini 2008 2014; Monaghan et al. 2009; Stier et al. 2012; Speakman and Garratt 2014; Speakman et al. 2015; Blount et al. 2016). Oxidative stress is defined as an imbalance between reactive oxygen species (ROS) production and the capacity of the antioxidant defenses to neutralize them, which will result in the formation of oxidative damage to important cell components such as DNA, lipids, or proteins (Halliwell and Gutteridge 2007). Thus, because the oxidation hypothesis for life-history trade-offs argues that reproduction is highly demanding and redirects investment from self-maintenance into progeny, it is arguable to expect oxidative stress in breeding animals. In agreement with this hypothesis, experimental studies that altered reproductive effort suggest that organisms sacrifice their oxidative homeostasis for elevated reproductive performance (Alonso-Alvarez et al. 2004; Wiersma et al. 2004; Costantini et al. 2014a), or at least that oxidative damage is correlated with reproductive success and survival (Bize et al. 2008; Freeman-Gallant et al. 2011; Markó et al. 2011; Noguera et al. 2012; Costantini and Dell’Omo 2015; Rey et al. 2015). However, a recent meta-analysis found little support for this hypothesis (Blount et al. 2016), and a further case-study showed that redox state is altered only under conditions when reproduction and self-maintenance are traded-off with direction of alteration depending on whether the studied organism prioritizes reproduction over survival, or vice versa (Beaulieu et al. 2015). Thus, the extent to which the oxidation hypothesis of life-history trade-offs is relevant remains an unresolved issue in evolutionary biology and ecophysiology (Selman et al. 2012; Stier et al. 2012; Metcalfe and Monaghan 2013; Speakman et al. 2015; Blount et al. 2016).

Whether relevant or not, the oxidative stress hypothesis of life-history trade-offs formulates clear predictions about the possible relationships and effects of an increased reproductive effort on oxidative physiology, and about how oxidative damage is carried over to impact performance during subsequent life-history stages (Metcalfe and Monaghan 2013). In conflict with this theory, however, studies conducted on captive and wild-living animals have produced ambiguous results (Wiersma et al. 2004; Losdat et al. 2011; Christe et al. 2012; Fletcher et al. 2012; Stier et al. 2012; Aloise King et al. 2013; Garratt et al. 2013; Yang et al. 2013; Costantini et al. 2014a, b; Cram et al. 2015; Emaresi et al. 2016; review in Blount et al. 2016). One conflict may be due to methodological issues, the most important of which are related to the housing condition of study animals, a lack of experimental manipulation of breeding effort, measures used to assess oxidative status (Metcalfe and Monaghan 2013), and a shortage of good alternative hypotheses (Speakman et al. 2015; but see the “oxidation shielding” hypothesis of Blount et al. 2016). For

instance, it is highly desirable to assess multiple components of oxidative physiological state (i.e., not just antioxidants; Costantini and Verhulst 2009) and important that studies are conducted where ever possible under natural conditions and on wild-living organisms, because captive conditions with unlimited food supply may quench any oxidative costs of reproduction that emerge only in harsher nutritional and energetic circumstances (Metcalfe and Monaghan 2013; Beaulieu et al. 2015). Finally, the cost of breeding can be translated into oxidative stress over either short- or long-timescales (Losdat et al. 2011), calling for studies addressing the impact of oxidative stress on survival. Yet, since the review of Metcalfe and Monaghan (2013), few studies have fulfilled these expectations. As Blount et al. (2016) pointed out, studies to date on the oxidative cost of reproduction are in line with this theory, but experiments on wild animals where both antioxidants and oxidative damage are measured are lacking (but see Costantini et al. 2014a; Cram et al. 2015 for discussion of the limited exceptions).

In addition to parental effort a number of other aspects of reproduction, such as the timing of egg-laying and the extent of nest infestation with parasites, might also be integral to redox state. Indeed, the oxidative condition of breeding birds may impact their laying dates, which may subsequently affect breeding success (Costantini et al. 2016). This is especially relevant for long-distance migrants like the barn swallow (*Hirundo rustica*) because only those birds that can endure the antioxidant cost of early arrival (Ninni et al. 2004) can breed early and raise multiple broods per season (Møller 1994). However, determination of breeding time using oxidative physiology is unclear and both positive and negative effects can be expected. The change in antioxidants and oxidative damage during breeding can be related to the change in food supply because proteins and specifically amino acids serve as precursors for some of the antioxidants (Halliwell and Gutteridge 2007). Although the potential limitation of breeding by oxidative physiology has received some attention (Stier et al. 2012; Speakman and Garratt 2014; Blount et al. 2016; Costantini et al. 2016), no clear predictions have been formulated for the direction of the relationship between timing, antioxidant level, and oxidative damage. If early breeding is limited by the depletion of antioxidants, and an increase in oxidative damage due to the energetic cost of fast spring migration (see Jenni-Eiermann et al. 2014), then one might predict a positive correlation between reproductive onset and antioxidants, as well as a negative association with oxidative damage. On the contrary, if early breeders are superior in their physiological capacity to control oxidative stress due to faster returns to breeding grounds, then any association between timing of breeding, antioxidants, and oxidative damage is expected to reverse. However, change in the oxidative physiology of breeding birds over time can also be related to haematophagous parasite load (Sepp et al. 2012; López-

Arrabé et al. 2015). Parasites can either directly impact on redox state (e.g., by triggering ROS-based immune response; Costantini and Møller 2009; Sorci and Faivre 2009), or the temporal dynamics of parasite abundance may have evolved to exploit deterioration in the oxidative state of their hosts during the breeding season. However, whether parasites deplete, or elevate, levels of different antioxidants, and whether they can induce oxidative damage is still poorly understood (Sepp et al. 2012; van de Crommenacker et al. 2012; López-Arrabé et al. 2015; Pap et al. 2015).

In this study, we experimentally manipulated the breeding effort of barn swallows and measured the effect on non-enzymatic antioxidants (i.e., glutathione, total antioxidant capacity, and uric acid) and plasma oxidative damage in male and female parents. We followed the effects of manipulations on the birds over the breeding season and noted differences in haematophagous nest mite infestation intensities. We also assessed the long-term effect of oxidative condition on survival probability during the subsequent year (both with, and without, experimental manipulation).

Materials and methods

Field data collection

Our study was carried out in Cojocna village in central Transylvania (46°45'N, 23°50'E), Romania, during the breeding seasons 2013–2015. At this study site, barn swallows usually breed in stall buildings in natural nests most often solitarily, but in some cases several pairs aggregate into small loose colonies (for details see Fülöp et al. 2017). Adult barn swallows were captured with nest traps on day one (females only) and on day 13 post-hatching (both parents) (hatching day = day 0), usually between 10 am and 6 pm for biometric measurements and blood sampling. In a few cases, adults were captured some days later than day 13 for practical reasons (i.e., weather conditions); these delays did not cause a difference in sampling date between sexes and experimental groups (linear mixed-effects model with year and individual as random factors, sex: $F = 0.59$, $df = 1$, $P = 0.444$; group: $F = 1.18$, $df = 2$, $P = 0.310$). Upon first capture (day one), we marked all females with a unique numbered aluminum ring and took a blood sample (ca. 100 μ L) from the ulnar vein within 5 min following capture. Blood samples were stored in a cool box for up to 10 h until being centrifuged for 5 min at 6200 g to separate the plasma and packed cell fractions. The samples were stored at -20°C for a maximum of 6 months for subsequent laboratory analyses. Sex was determined by visual examination of the presence of a brood patch (only females develop a brood patch) and for each nest, we

determined the laying date (appearance of the first egg) by examination of the nests at every 4–5 days, clutch size and brood size at days 1 and 12 post-hatching.

In 2013 and 2014, we performed a brood size manipulation experiment using synchronous broods, increasing or decreasing the original brood size by one nestling on the day after hatching (day one). Broods were considered synchronous when hatching of the first nestling in both nests occurred on the same day while those that could not be paired served as controls. The nestlings to be fostered were randomly selected from the experimental nests. We randomized the broods within experimental groups eliminating any bias, which could be created by the preferential choice of nests in terms of location, or the size of the brood or nestlings. In a few cases, however, when more than two broods hatched on the same day, broods were paired in function of their size, i.e., those who were more similar in size were paired and the rest were used as controls. Control nests were inspected as the experimental nests, including the counting and handling of all one-day-old nestlings, except that no chicks were fostered. Therefore, the disturbance of birds was similar between groups, except switching the nestlings between experimental groups. We used the data from 103 broods, and the sample size between experimental groups was balanced (see Table 1). The sample size of males and females may slightly differ between groups because in a few cases birds could not be captured, or because of the limited blood sample some of the oxidative stress assays could not be measured. The laying date and the brood size before manipulation did not differ between experimental groups (laying date: $F = 0.53$, $df = 2$, $P = 0.587$; brood size: $F = 2.70$, $df = 2$, $P = 0.072$). We recorded provisioning rate of males and females once in the period of 7–12 days after hatching for 1 hour (between 9.00 and 19.00) either by direct observations or by using video cameras from a hide. One-hour observation of feeding rate is the optimum sampling duration, i.e., the shortest observation time that can explain the most variation in total daily visits per unit time (Lendvai et al. 2015). We recorded feeding rate only on sunny and/or warm days and whenever possible on the same day for the paired nests. The feeding rate of barn swallows drops dramatically during rainy and/or cold days because the flying insects on which they rely are inactive, and this makes it impossible to accurately measure the parental effort. Within 1 week following fledging, we estimated the abundance of the haematophagous tropical fowl mite (*Ornithonyssus bursa*) in each nest by placing one hand on the rim of the nest with fingers inside the nest for 10 s (see Møller 1990). The number of mites on the hand were then estimated with increments of ten until 100 mites, and increments of 50 above 100 mites. Repeatability of the number of nest mites measured on subsequent days was very high (intraclass correlation coefficient [ICC] = 0.94, 95% confidence interval [CI] = 0.82–0.99, $F_{10,11} = 35.0$, $P < 0.0001$).

Table 1 Difference in clutch size and post manipulation brood size at day 1 and day 12, as well as feeding rates by males and females across the three experimental groups (mean $\pm$ SE). Letters denote significant differences at $P < 0.05$, as indicated by the general linear mixed-effects models. The feeding rate of females in the enlarged group tended to be larger than in the reduced and control groups at $P = 0.07$ and 0.09 , respectively (§)

	Group			df	χ^2	P	Year		
	Reduced	Control	Enlarged				df	χ^2	P
<i>N</i>	31	42	30						
Clutch size	4.79 $\pm$ 0.28	4.79 $\pm$ 0.28	4.91 $\pm$ 0.28	2	0.74	0.691	1	11.64	0.001
Brood size at 1 day old	3.44 $\pm$ 0.29 ^a	3.99 $\pm$ 0.27 ^a	5.57 $\pm$ 0.28 ^b	2	72.66	<0.0001	1	2.46	0.117
Brood size at 12 days old	3.47 $\pm$ 0.37 ^a	3.73 $\pm$ 0.36 ^a	4.89 $\pm$ 0.37 ^b	2	33.82	<0.0001	1	6.65	0.010
Feeding rate of females	13.12 $\pm$ 1.48 ^a	13.48 $\pm$ 1.33 ^a	17.59 $\pm$ 1.43 [§]	2	7.05	0.030	1	0.00	1.000
Feeding rate of males	14.09 $\pm$ 1.48 ^a	14.50 $\pm$ 0.83 ^a	17.98 $\pm$ 1.41 ^a	2	5.03	0.081	1	0.00	1.000

The barn swallow population from Cojocna has been carefully monitored in a series of population studies since 2011, with more than 99% of all breeding birds captured annually. Previous observations on barn swallows (Schaub and von Hirschheydt 2009), and our own capture–recapture data, indicate high breeding philopatry (Pap et al. 2005). Indeed, as the capture rate of the marked birds was high, and constant, over the study period, survival of birds between years can be accurately assessed; annual return rate of birds breeding in 2013 and 2014 was measured using the recapture data from the following years 2014 and 2015, respectively. All birds breeding in 2013 and recaptured in 2015 were caught in 2014 as well, indicating that those birds that returned in multiple years were always recaptured in the following season after the treatment took place. Therefore, the estimation of the annual return rate was similarly precise both for birds treated in 2013 and in 2014 (i.e., recapture in 2016 was not necessary). However, because aging can affect oxidative measures (i.e., Stier et al. 2015), we controlled for the age effect in our analyses. Note however, that monitoring in this population started in 2011, therefore, we could only accurately determine age to a maximum of 4 years. Thus, in order to minimize measurement error, age was used in the models as a two level factor, representing 1 year or older birds. A further argument in favor of this categorization is that the change in breeding performance with age in barn swallows is significant between the first and second years of life (Møller and de Lope 1999). To minimize observer bias, all laboratory measurements were conducted blind. Nonetheless, feeding rate of adult birds was not possible to record blind because our study involved focal animals in the field.

Oxidative physiology assays

Total antioxidant capacity

Non-enzymatic antioxidant capacity is an indication of an organism's ability to defend itself against free radicals. Thus,

we measured total antioxidant capacity (TAC) following the method described by Erel (2004), with slight modifications following Sepp et al. (2010). This assay relies on the ability of non-enzymatic antioxidants (i.e., uric acid, vitamins, sulfhydryl groups of proteins, glutathione) to decolorize blue-green ABTS⁺ (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate)) to a degree proportionate to concentrations that can be measured spectrophotometrically at 660 nm. Briefly, the first absorbance was measured after mixing 5 μ L of plasma with 200 μ L of 0.4 M acetate buffer (pH 5.8) as a sample blank, while the last absorbance was measured 20 min after incubating the previous mixture with 20 μ L of 10 mM ABTS⁺ (Sigma A1888) in 30 mM acetate buffer (pH 3.6). An antioxidant of known concentration (Trolox, Sigma 23,881-3) was then used as a standard for the calculation of antioxidant levels, and assay results were expressed as mM Trolox equivalents (for details, and repeatability, see Pap et al. 2015); since UA is a component of TAC as well as a product of amino acid catabolism, we controlled TAC for UA levels by ordinary least squares regression, and calculated residual TAC as suggested by Cohen et al. (2007). Results for residual TAC were very similar to those for raw TAC and so are omitted throughout. Repeatability of duplicate measures was very high (ICC = 0.94, 95% CI = 0.89–1.00, $F_{13,28} = 52.0$, $P < 0.0001$).

Uric acid

Uric acid (UA) is an endogenously produced micromolecular antioxidant, by far the most abundant circulating antioxidant (Surai 2002). In birds this is also the end product of amino acid metabolism and the main way that nitrogen is excreted (Klasing and Austic 1984). Plasma UA concentration was thus determined from 5 μ L of plasma by using a spectrophotometrical uricase/peroxidase method (Uric Acid liquicolour kit, Human, Wiesbaden, Germany), and results are given as mg/dL plasma. Repeatability of duplicate measures was very high (ICC = 0.99, 95% CI = 0.96–1.00, $F_{9,10} = 131.7$, $P < 0.001$).

Total glutathione

Glutathione (GSH) is the most important intracellular, endogenous, non-enzymatic antioxidant (Halliwell and Gutteridge 2007). In this study, total GSH (tGSH) concentration was assayed using a commercial kit (Sigma-Aldrich, St Louis, MO) following the methods in Galván and Alonso-Álvarez (2008) and Hörak et al. (2010) but with a number of modifications. After thawing on ice, each erythrocyte pellet was washed three times with phosphate-buffered saline and spun at 600 *g* for 10 min at 4 °C. Next, we weighed the pellets ($\pm$ 0.1 mg) and deproteinized them with 5% 5-sulfosalicylic acid (SSA; 1:1 *w/v*; i.e., a 100 mg pellet was diluted in 100 μ L of 5% SSA). The resultant solution was vigorously vortexed, kept on ice for 10 min, and then centrifuged at 10,000 *g* for 10 min at 4 °C to remove precipitated proteins. The supernatant (5 μ L) was transferred to another test tube, diluted tenfold and used subsequently for tGSH detection, according to the manufacturer's instructions. Total GSH determination is based on a kinetic assay in which nanomoles of GSH cause a continuous reduction of 5,5'-dithiobis(2-nitrobenzoic acid) to 5-thio-2-nitrobenzoic acid (TNB), and GSH oxidized to GSSG is subsequently recycled by glutathione reductase and reduced nicotinamide adenine dinucleotide phosphate (NADPH). The yellow color of the TNB product is proportional to GSH concentration; its absorbance was measured spectrophotometrically at 412 nm at intervals of 1 min for 5 min. Changes in absorbance were compared to a standard curve ($R^2 = 0.99$) generated by serial dilution of reduced GSH, and results are given in nM of tGSH/mg of erythrocytes. Repeatability of the subsample measured twice was moderate but significant (ICC = 0.64, 95% CI = 0.06–1.00, $F_{6,7} = 4.5$, $P = 0.035$).

Malondialdehyde

Malondialdehyde (MDA) is a carbonyl compound that is produced by the peroxidative degeneration of membrane polyunsaturated fatty acids by ROS, a widely used marker of oxidative stress (Del Rio et al. 2005). MDA concentration was determined from 10 μ L of plasma by High Performance Liquid Chromatography (HPLC) on a HPLC SUPELCOSIL™ LC-18 column (5 μ m particle size; Sigma-Aldrich) with UV detection at 254 nm (Jasco, UV-2075 Plus, Japan) (Bókonyi et al. 2014). The mobile phase was 30 mM monopotassium phosphate (KH₂PO₄)–methanol (65:35, *v/v* %) and the flow rate 0.5 mL/min. The retention time of MDA recorded was around 6 min. MDA concentration in the sample was determined using a calibration curve ($R^2 = 0.99$) of a series of standards generated by acidic hydrolysis of 1,1,3,3-tetraethoxypropane (TEP; Sigma-Aldrich). Results are given as μ g/mL plasma and are not corrected for the dilution factor. Repeatability when the subsample was measured twice was very high (ICC = 0.91, 95% CI = 0.77–1.00, $F_{9,10} = 20.2$, $P < 0.0001$).

Triglyceride

Triglycerides constitute the main chemical form by which vertebrates store and transport lipids in the body. Because the circulating triglyceride level may covary with plasma MDA and therefore may confound the results on lipid peroxidation (Pérez-Rodríguez et al. 2015), we measured the concentration of these markers on a subsample of plasma collected in 2015 from breeding adult birds captured on day 13 post-hatching. Plasma level of triglycerides was measured using a commercial kit (11,528 from Biosystems, Barcelona) based on the glycerol phosphate oxidase/peroxidase method in a direct colorimetric procedure (Bucolo and David 1973; Fossati and Prencipe 1982). Analyses were run in 96-well plates using a microplate reader. Two hundred fifty milliliter of the chromogen was added to 5 mL of plasma or the standard (a 200 mg/dL glycerol solution). Plates were incubated for 5 min at 37 °C, subsequently measuring absorbance at 500 nm. Results are given as mg/mL plasma. Repeatability of a subsample that was measured in duplicate was very high (ICC = 0.82, 95% CI = 0.65–0.90, $F_{18,19} = 10.2$, $P < 0.0001$).

Statistical analyses

First, we tested whether clutch size differed among experimental groups and whether manipulation had any effect on brood size at one and 12 days after hatching, on parental provisioning rate or on oxidative parameters of females measured prior the manipulation. To do this, we built separate linear mixed-effects models (LMEs) for each of these parameters separately, including the identity of the parents (only in the case of feeding rate and oxidative parameters), age (only in the case of oxidative parameters), and year as random factors. Group was included as a fixed factor. Second, we tested the effect of manipulation on body mass and oxidative traits of females and males measured 13 days post-hatching. To do this, we built LMEs for each sex and oxidative parameter separately, using the latter as response variables and including bird identity and year as random factors, group and age as fixed factors, hatching date and the number of nest mites as covariates. Third, given that the body mass and oxidative physiology of females was measured both prior the manipulation and 13 day post-hatching, we tested the difference in post-treatment values, for each response variable separately after controlling for pre-treatment values. To do this, we constructed ANCOVA models, using post-treatment measurements as response variables and including pre-treatment measurements as covariates. The latter procedure is often preferred over repeated measure ANOVA, due to a usually more robust and powerful treatment effect assessment (e.g., Senn 2008). All other explanatory variables and random factors included in these models were identical to models in the second model set. Fourth, to test the sex differences in the effect of manipulation

on oxidative markers we built LMEs using the oxidative parameters of both sexes combined measured at day 13 as response variables, where the non-independence of pairs was controlled by including nest identity as a random factor in the models. All explanatory variables and random factors were identical to the LMEs for females and males (second model set).

To test how feeding rate correlates with oxidative traits measured on day 13, we included feeding rate as covariates to models of oxidative physiology of the two sexes (second model set), while all other explanatory variables were retained. Moreover, to test the possible confounding effect of triglycerides on MDA, we analyzed the association between these two physiological parameters measured on day 13 post-hatching using samples taken in 2015. We constructed models for females and males separately, using MDA as dependent variable, and we included hatching date and triglyceride concentration as covariates. The results of the latter two models showed that the MDA significantly and positively correlated with the concentration of triglycerides in males ($F = 6.67$, $df = 1,43$, $P = 0.013$), but not in females ($F = 2.04$, $df = 1,46$, $P = 0.160$), suggesting a limited effect of triglycerides on MDA in females (see “Discussion”).

In an additional set of analyses, we used generalized linear mixed models (GLMMs) to test the effect of brood size manipulation, hatching date, and the number of nest mites on the annual return rate of male and female barn swallows. Annual return rate served as a binomial dependent variable (i.e., 1 = survived, 0 = died), while year and bird identity were included as random factors. Group, hatching date, and the number of nest mites were included as covariates in the models, and analyses were then re-run with adding oxidative parameters measured at day one (females only) and day 13 (females and males) post-hatching in separate models. This approach allowed us to test the effect of oxidative physiology parameters on annual return rate while controlling for the potentially confounding effects of manipulation, hatching date, and nest mite abundance. Binomial models were tested for overdispersion using the function `overdisp_fun` (specified at <http://glmm.wikidot.com/faq>) from the package “lme4” for in R (Bolker et al. 2009; Bates et al. 2014); analyses show that none of the survival models were overdispersed. P values were not adjusted for multiple comparison in order to avoid inflation of the type II error probability (Rothman 1990, 2014). During the second year of the study, adult birds that returned from the previous breeding season experienced different breeding effort due to brood size manipulation, which may affect their responses. In total 46 birds were recaptured in 2014 and included again in one of the experimental groups. Therefore, we re-run all analyses where we included the experimental group from the previous year as random factor. Birds that were not included in the study during the previous year were listed in the control group. However, because the

results remained unchanged after controlling for this effect, we decided to present the original models without experimental groups from the previous year included as random factor in the models. Prior to statistical analyses, tGSH, TAC, and UA were log-transformed to achieve residual normality in the models. We report minimal models, in which all main effects were retained; these were all obtained using stepwise backward selection based on the largest non-significant (> 0.05) P value. Second-order interactions were not included because of the high number of parameters estimated and the limited sample size. All statistical analyses were conducted in the R statistical environment, version 3.2.2 (R Core Team 2016). Specifically, mixed models were constructed using the “lme4” package (Bates et al. 2014) and analysis-of-variance statistics were obtained using Wald test as implemented in the R package “car” (Fox and Weisberg 2011).

Data availability The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Results

Brood size, parental feeding rate, and body mass

Results show that although clutch size did not differ between experimental groups (Table 1), brood size manipulation on the first day after hatching resulted in significant differences in brood size among experimental groups, and these group differences persisted until day 12 post-hatching (Table 1). Note, however, that brood sizes both at day one and day 12 post-hatching were statistically similar in the control and reduced groups. The feeding rate of females from the enlarged group was higher by 23.4 and 25.4% than in the control and reduced groups, respectively, while no significant difference was observed between the latter two groups (Table 1). Brood size manipulation did not affect the feeding rate of males (Table 1), although it was slightly higher in the enlarged group in comparison to the other two groups. Group differences were similar in males and females in a model where the feeding rate of females and males were included simultaneously, as indicated by the non-significant group $\times$ sex interaction ($\chi^2 = 0.07$, $df = 2$, $P = 0.964$). Nonetheless, when sexes were analyzed separately, the increase in feeding rate in the enlarged group was only apparent in females, but not males (Table 1).

Brood size manipulation significantly affected body mass of females measured on day 13 post-hatching, as indicated by ANCOVA models including body mass measured prior the experiment as a covariate ($\chi^2 = 12.02$, $df = 2$, $P = 0.0024$). The latter model indicated that body mass of control females was significantly higher than that of females from the enlarged

group ($t = 3.20$, $df = 98$, $P = 0.005$) or the reduced group ($t = 2.63$, $df = 96$, $P = 0.026$), but no difference was detected between the latter two groups ($t = 0.51$, $df = 92$, $P = 0.867$). Brood size manipulation significantly affected the body mass of males ($\chi^2 = 6.69$, $df = 2$, $P = 0.035$); however, none of the contrasts between groups from the LME were significant (all $P > 0.06$).

Oxidative physiology and breeding effort

Oxidative traits of females measured on the first day after hatching (i.e., prior the manipulation) did not differ between experimental groups (tGSH: $\chi^2 = 0.23$, $df = 2$, $P = 0.893$; TAC: $\chi^2 = 1.49$, $df = 2$, $P = 0.475$; UA: $\chi^2 = 0.47$, $df = 2$, $P = 0.793$; MDA: $\chi^2 = 2.22$, $df = 2$, $P = 0.330$; Table S1). Similarly, tGSH, TAC, and UA concentrations in females measured at 13 days post-hatching did not differ among the experimental groups, while MDA was significantly affected by brood size manipulation and was higher in females in the enlarged group compared with the control and reduced groups (Table 2; Table S1). No differences were observed between the latter two groups (Fig. 1a; Table 2), while the effect of

manipulation on MDA concentration remained significant when the values measured at day one were introduced into the models (Table 2). None of the oxidative traits in males measured 13 days post-hatching, except the MDA were statistically different among the experimental groups (Fig. 1b; Table 2; Table S1). Brood size manipulation slightly and significantly affected the MDA of males; however, none of the contrasts between groups from the LMEs were significant. Group differences in all oxidative markers were similar in males and females, since the group $\times$ sex interactions were non-significant in models where the data of females and males were included simultaneously (tGSH: $\chi^2 = 1.53$, $df = 2$, $P = 0.465$; TAC: $\chi^2 = 6.21$, $df = 2$, $P = 0.051$; UA: $\chi^2 = 1.50$, $df = 2$, $P = 0.473$; MDA: $\chi^2 = 2.15$, $df = 2$, $P = 0.341$). Nonetheless, when sexes were analysed separately, the increase in MDA in the enlarged group was only apparent in females, but not males (Figs. 1a, b). In none of the models did feeding rate significantly explain oxidative parameters of females or males (female, tGSH: β (SE) = 0.01 (0.01), $\chi^2 = 2.77$, $P = 0.096$; TAC: β (SE) = -0.00 (0.01), $\chi^2 = 0.13$, $P = 0.713$; UA: β (SE) = -0.01 (0.01), $\chi^2 = 1.60$, $P = 0.206$; MDA: $\chi^2 = 1.21$, $P = 0.271$; male, tGSH: β (SE) = 0.01

Table 2 The effect of the brood size manipulation on the redox state of adult female and male barn swallows at day 13 post-hatching (tGSH total glutathione, TAC total antioxidant capacity, UA uric acid, MDA malondialdehyde). Random effects are italicized. The number of observations (no. obs.) give the total number of data included in the model, and the number of individuals (individ.) are data without repeated measures of the same birds sampled in both years

		tGSH		TAC		UA		MDA	
	df	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>
Female at day 13 post-hatching									
Group	2	2.29	0.318	1.65	0.438	0.75	0.687	8.43	0.015
Age	1	1.99	0.158	0.02	0.877	0.33	0.569	0.61	0.434
Hatching date	1	8.78	0.003	0.30	0.581	1.08	0.300	2.56	0.110
Nest mite	1	0.00	0.957	2.03	0.154	1.00	0.317	3.85	0.050
<i>Year</i>	1	4.99	0.026	5.86	0.016	55.56	<0.0001	3.07	0.080
		No. obs 96, individ. 73		No. obs 93, individ. 72		No. obs 92, individ. 72		No. obs 96, individ. 73	
Female at day 13 post-hatching with values at day one included in the models									
Group	2	2.63	0.268	1.19	0.551	1.91	0.385	10.70	0.012
Age	1	3.13	0.077	0.20	0.653	1.21	0.271	0.53	0.465
Hatching date	1	12.96	<0.001	0.00	0.971	0.68	0.410	2.33	0.127
Nest mite	1	0.02	0.903	1.29	0.256	1.02	0.314	3.84	0.050
Parameter at day 1	1	6.26	0.012	1.72	0.190	1.88	0.171	0.44	0.508
<i>Year</i>	1	11.02	<0.001	2.57	0.109	34.64	<0.0001	3.44	0.064
		No. obs 92, individ. 72		No. obs 85, individ. 67		No. obs 81, individ. 67		No. obs 93, individ. 71	
Male at day 13 post-hatching									
Group	2	0.41	0.814	4.94	0.085	5.78	0.056	6.07	0.048
Age	1	5.63	0.018	0.21	0.648	1.11	0.293	3.62	0.057
Hatching date	1	25.02	<0.0001	0.15	0.695	0.54	0.463	0.03	0.873
Nest mite	1	5.63	0.018	2.60	0.107	3.00	0.083	5.13	0.024
<i>Year</i>	1	16.41	<0.0001	10.84	0.001	41.13	<0.0001	4.54	0.033
		No. obs 95, individ. 75		No. obs 94, individ. 74		No. obs 93, individ. 74		No. obs 96, individ. 76	

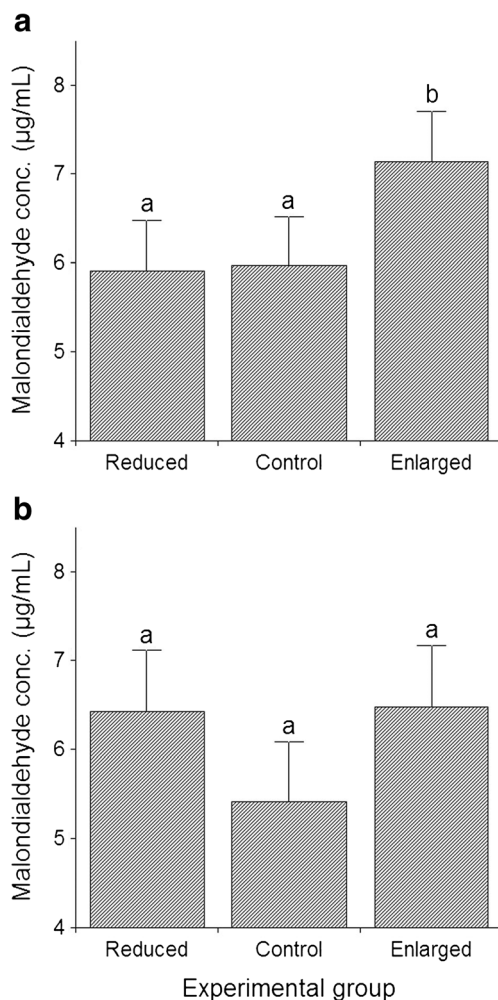


Fig. 1 Malondialdehyde concentration (Mean + SE) of female (**a**) and male (**b**) barn swallows. The values are measured at day 13 post-hatching in the three experimental groups. Different letters denote significant differences at $P < 0.05$, as indicated by the general linear mixed-effects models. The number of observations (no. obs.) give the total number of data included in the model, and the number of individuals (individ.) are data without repeated measures of the same birds sampled in both years (female: no. obs. = 96, individ. = 73; male: no. obs. = 95, individ. = 75)

(0.01), $\chi^2 = 2.13$, $P = 0.144$; TAC: β (SE) = -0.00 (0.01), $\chi^2 = 0.06$, $P = 0.799$; UA: β (SE) = -0.01 (0.01), $\chi^2 = 3.22$, $P = 0.078$; MDA: β (SE) = -0.09 (0.43), $\chi^2 = 0.05$, $P = 0.833$).

Oxidative physiology, breeding time, and parasitism

None of the female oxidative traits we measured, with the exception of tGSH, differed between day 1 and day 13 post-hatching as indicated by the effect of the sampling time in a repeated measure ANOVA (TAC: $\chi^2 = 0.00$, $df = 1$, $P = 0.988$; UA: $\chi^2 = 1.18$, $df = 1$, $P = 0.276$; MDA: $\chi^2 = 0.72$, $df = 1$, $P = 0.396$). In contrast, the tGSH of females significantly increased by day 13 post-hatching ($\chi^2 = 27.01$, $df = 1$, $P < 0.001$). None of the oxidative traits measured at day 13

post-hatching differed between females and males (tGSH: $\chi^2 = 0.61$, $df = 1$, $P = 0.434$; TAC: $\chi^2 = 0.06$, $df = 1$, $P = 0.806$; UA: $\chi^2 = 0.89$, $df = 1$, $P = 0.346$; MDA: $\chi^2 = 0.24$, $df = 1$, $P = 0.625$) when the confounding effects of brood size manipulation, hatching date, the number of mites, the identity of the nest and individuals, and year were controlled.

None of the oxidative traits we measured on the first day after hatching in females correlated significantly with hatching date (tGSH: β (SE) = 0.01 (0.01), $\chi^2 = 2.03$, $P = 0.154$; TAC: β (SE) = 0.01 (0.01), $\chi^2 = 0.82$, $P = 0.365$; UA: β (SE) = 0.00 (0.01), $\chi^2 = 0.83$, $P = 0.361$; MDA: β (SE) = -0.09 (0.31), $\chi^2 = 0.09$, $P = 0.763$). At day 13 post-hatching; however, tGSH concentration in females was significantly positively correlated with hatching date (β (SE) = 0.02 (0.01); Fig. 2a; Table 2). Moreover, MDA at day 13 was positively correlated with the number of nest mites in females (β (SE) = 2.69 (1.37); Fig. 3a; Table 2). In males, tGSH concentration was significantly positively correlated with hatching date at day 13 (β

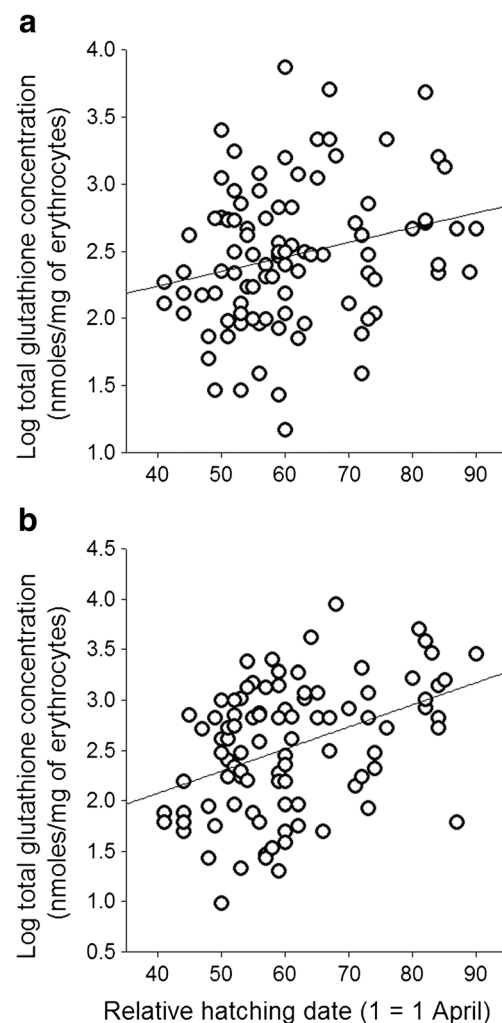


Fig. 2 Relationship between total glutathione concentration and hatching date in female (**a**) and male (**b**) barn swallows. Oxidative parameters were measured at day 13 post-hatching (female: no. obs. = 96, individ. = 73; male: no. obs. = 95, individ. = 75)

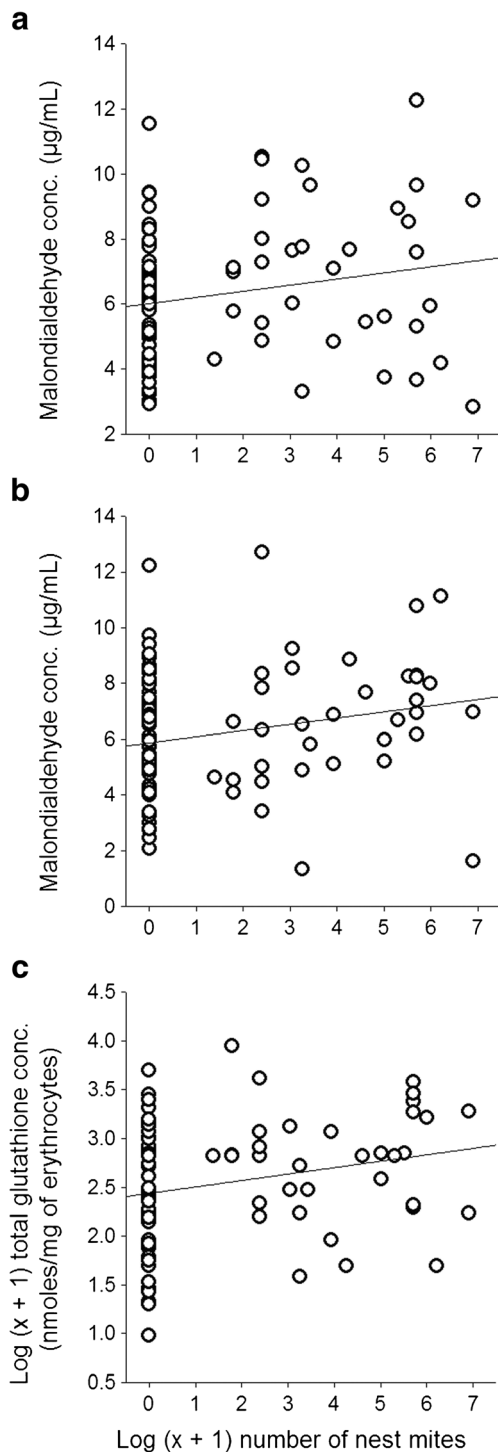


Fig. 3 Relationship between malondialdehyde concentration, number of nest mites, and total glutathione concentration. The correlation between malondialdehyde concentration and the number of nest mites are for female (a) and male (b) barn swallows, and the correlation between total glutathione concentration and the number of nest mites in males (c). Oxidative parameters were measured at day 13 post-hatching (MDA vs. nest mite; female: no. obs. = 96, individ. = 73; male: no. obs. = 95, individ. = 75; tGSH vs. nest mite; male: no. obs. = 96, individ. = 76)

(SE) = 0.03 (0.01); Fig. 2b; Table 2). The number of nest mites in males was also significantly positively correlated with

tGSH concentration (β (SE) = 0.06 (0.03)) and MDA (β (SE) = 3.29 (1.45) measured on day 13 (Fig. 3a, b; Table 2).

Oxidative physiology and annual return rate

In 2013, 47.2% ($N = 53$) of females and 44.2% of males ($N = 52$) returned, while in 2014, these values were 40.0% ($N = 50$) and 44.9% ($N = 49$), respectively (Table S2). Brood size manipulation, hatching date, and the number of nest mites did not affect the annual return rate of females and males (Table 3), and including all oxidative state markers measured at day 13 post-hatching in the models did not alter the previous results or predict the annual return rate of females and males (Table 3). Among the oxidative traits of females measured on the first day after hatching, MDA and tGSH significantly predict individual return rate (Fig. 4a, b; Table 3); birds with high plasma oxidative damage and low tGSH concentrations were less likely to return to the breeding ground the following year.

Discussion

Oxidative physiology, breeding effort, and annual return rate

Our study shows that female barn swallows tending enlarged broods increase their feeding frequency compared with birds from the control and reduced groups, and that this increased maternal workload was associated with higher oxidative damage. Interestingly, this effect was not accompanied by either a depletion of non-enzymatic antioxidants or an upregulation of antioxidants as indicators of activated defense mechanisms. Our results thus seem to contradict Costantini et al.'s (2014a) study on the same species because similar brood size manipulation did not impact on oxidative damage while plasma non-enzymatic antioxidant levels decreased in females rearing enlarged broods, albeit the measures used in these two studies were different, which may partly explain the divergent results. Nevertheless, both studies do suggest that environmental and/or life-history trait differences among populations of the same species may govern the relationship between oxidative state and parental effort. In our study, however, none of the antioxidants changed following brood size manipulations, which may explain the increase in oxidative damage at least in females in the enlarged group. If the amount of ROS increases due to higher workload (see Emaresi et al. 2016), and antioxidants are not upregulated to neutralize ROS, our results suggest organisms enter an oxidative stress state (see Costantini and Verhulst 2009). With this in mind, it is interesting to note that a study by Pérez-Rodríguez et al.

Table 3 Result of the generalized linear models showing the effect of brood size manipulation, hatching date, the number of nest mites, and oxidative parameters measured at days 1 and 13 post-hatching on the annual return rate of male and female barn swallows. Random effects are italicized. The number of observations (no. obs.) give the total number of data included in the model, and the number of individuals (individ.) gives the data without repeated measures of the same birds sampled in both years

	Female at day 1 post-hatching			Female at day 13 post-hatching		Male at day 13 post-hatching	
	df	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>
Models without oxidative parameters included							
Group	2	3.20	0.202	3.20	0.202	5.22	0.074
Age	1	0.17	0.680	0.17	0.680	0.22	0.639
Hatching date	1	0.03	0.865	0.03	0.865	0.01	0.928
Nest mite	1	0.40	0.529	0.40	0.529	1.56	0.212
Year	1	0.00	1.000	0.00	1.000	0.00	1.000
		No. obs 100, individ. 77		No. obs 100, individ. 77		No. obs 98, individ. 78	
Models with oxidative parameters included							
Group	2	4.29	0.117	2.80	0.247	5.27	0.072
tGSH	1	5.31	0.021	0.48	0.487	3.04	0.081
TAC	1	0.03	0.872	0.48	0.488	0.24	0.626
UA	1	2.27	0.132	0.10	0.751	0.02	0.883
MDA	1	9.02	0.003	0.28	0.597	0.02	0.892
		No. obs 84, individ. 69		No. obs 89, individ. 70		No. obs 95, individ. 75	

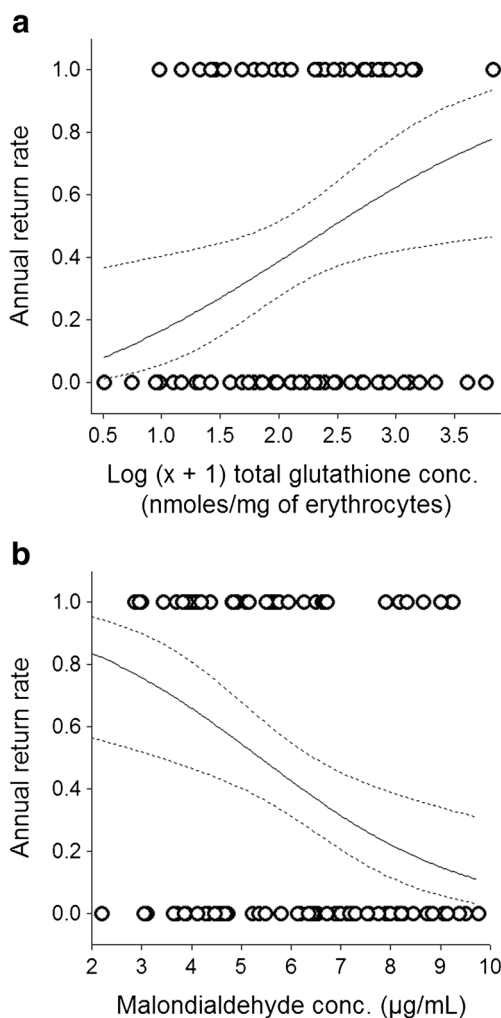


Fig. 4 The relationship between malondialdehyde (a) and total glutathione (b) concentration and annual return rate. The data are for female barn swallows measured at day one post-hatching and the return rate was measured following breeding season as predicted by the generalized linear mixed-effects model. The fitted (continuous) line shows the predicted return rate from generalized linear mixed model. Dashed lines show the upper and lower confidence intervals of the predicted return rate (no. obs. = 84, individ. = 69)

(2015) suggested a possible regulatory effect of triglycerides on oxidative damage as measured by MDA, because a high concentration of this lipid compound provides a substrate for lipid peroxidation. Triglyceride concentrations reflect an individual's state of fattening by indicating the amount of lipids absorbed a few hours before blood sampling. If so, then we expect that birds attending enlarged broods will have lower triglyceride concentrations in their blood because they deplete their reserves more than birds from the reduced group and with low parental feeding rates. In this case, MDA concentration is expected to be lower in the enlarged group because of lower substrate availability. Our results show that MDA concentration increased in the enlarged group relative to the control and reduced groups, suggesting that this possible confounding effect of triglycerides on oxidative damage was probably minor (see López-Arrabé et al. 2015, 2016). The weak effect of triglycerides on MDA concentration at least in females is supported by the absence of correlation between these two parameters. Note however, that brood size manipulation significantly affected body mass in females and it had a marginally non-significant effect on body mass in males. In case we assume a correlation between mass and the concentration of fatty acids (Jenni-Eirmann et al. 2002), the latter results might indeed point at the possible regulatory effect of triglycerides on MDA, but further insights and measurements of plasma MDA and triglyceride levels are needed for a better understanding of the possible interplay between body mass, triglycerides and MDA. The oxidative damage in females during brood rearing has only a short-term effect because this damage had no apparent carry over effect and did not impact survival during the non-breeding season. Thus, our results support the hypothesis that reproductive effort has a transient effect on oxidative physiology (see also Losdat et al. 2011), and contradict the idea that redox state modulates a trade-off between current and residual fitness

(Monaghan et al. 2009; Speakman et al. 2015). Indeed, oxidative damage to females and tGSH concentration on the first day post-hatching predicts annual return rates and thus suggests that oxidative condition at hatching is more important in fitness determination than a transient change in oxidative physiology triggered by elevated investment in offspring production (Bize et al. 2008; Freeman-Gallant et al. 2011; Noguera et al. 2012; Costantini and Dell’Omo 2015). A possible explanation for the difference in MDA and tGSH measured on day 1 and on day 13, respectively, on the survival is that egg production and incubation, which is performed by females alone, impose higher costs to females than rearing the nestlings, an activity that is shared with the male. MDA concentration did not change between hatching and brood rearing, suggesting that the effect of oxidative damage on survival was probably masked by confounding factors, such as nest mites, which increase in number over brood rearing (Møller 1994). Our results also support Saino et al.’s (2011) study that plasma non-enzymatic antioxidant level positively predicts the annual survival in the barn swallow. The work of Saino et al. (2011) and Costantini et al. (2014a), on the same Italian barn swallow population, proved that females rearing enlarged broods show a decrease in their plasma non-enzymatic antioxidants during the nestling rearing period and that the same antioxidant measure predicts annual return rate. These studies suggest that in this particular Italian population antioxidants, rather than oxidative damage, mediate the trade-off between breeding and survival. The absence of any effect on annual return rate from increased MDA of females in the enlarged group may be related to high physiological tolerance to lipid peroxidation in birds because of the higher availability of substrates (see Pérez-Rodríguez et al. 2015), although this effect has so far not been demonstrated. All previous studies on barn swallows thus suggest that environmental and/or life-history trait differences among populations of the same species may govern different relationships between oxidative state and survival.

Our study also shows that parents responded differently to manipulation; only females moderately increased their feeding rate when faced with an enlarged brood, compared to the control and reduced groups. This sex difference is also reflected in plasma oxidative damage, as MDA increased in females in the enlarged group, not in males. Nonetheless, group differences were similar in males and females when the data of sexes were analyzed together, since the group by sex interactions were non-significant. These results indicate that the difference between males and females in responses to manipulation was slight. Our results suggest that the sexes may differ in their susceptibility to oxidative stress when experimentally loaded with extra parental work (see Emaresi et al.

2016), which is already unequal in barn swallows; incubation is carried out by females alone, while all other parental activities are shared between sexes (Møller 1994). Our results on the sex-specific oxidative responses of parents to modified parental effort agree with Costantini et al. (2014a) who worked on barn swallow, and with Emaresi et al. (2016) who worked on the tawny owl (*Strix aluco*). In all these studies, the sex that made the higher contribution to parental activity was affected in oxidative state by increased parental effort.

Oxidative physiology and breeding time

On the first day post-hatching, none of our oxidative measures correlated with the hatching date of females. We show, however, that tGSH concentrations in females and males measured at day 13 post-hatching are higher in late breeders than in early breeders. Our results thus suggest that individuals that started breeding earlier experienced increased depletions of a key intracellular antioxidant. Since GSH is synthesized by the cell from cysteine and other amino acids such as methionine (Wu et al. 2004; Isaksson 2013; Romero-Haro and Alonso-Alvarez 2015), its synthesis depends on the availability of proteins in the diet (Romero-Haro and Alonso-Alvarez 2015), and poor nutrition will lead to depleted levels (e.g., Partadiredja et al. 2009). Because barn swallows are aerial insectivores, their food supply is highly temperature dependent; feeding conditions thus improve as the season progresses and hence low tGSH concentrations in early breeders can be caused by limited protein intake (see Saino et al. 2004). Nevertheless, our results tend to support the hypothesis that there is an oxidative cost to early breeding in this species, at least during brood rearing, when the food supply is in high demand due to food provisioning. This argument is supported by the significant observed increase of tGSH in females between day one and day 13 post-hatching revealed by our results.

Oxidative physiology and parasitism

Data also show that the number of haematophagous nest mites was positively related to tGSH levels in males, and to oxidative damage in both sexes, in agreement with previous studies on the oxidative cost of parasitism (e.g., Sepp et al. 2012; van de Crommenacker et al. 2012; López-Arrabé et al. 2015). Oxidative stress is higher in infested compared to uninfested birds (e.g., van de Crommenacker et al. 2012) although the responses of antioxidants to infection can vary. For example, depleted GSH has been measured in pied flycatchers (*Ficedula hypoleuca*) infested with a nest-dwelling ectoparasite (López-Arrabé et al. 2015), an opposite response to that seen in our study. Clearly, infestation can deplete the pool

of antioxidants as an increase in ROS-mediated inflammatory immune response is required (Costantini and Møller 2009; Sorci and Faivre 2009). Animals may also upregulate their antioxidant system to protect cells from damage, or to stimulate and regulate their immune systems (Costantini 2014).

Conclusions

The results of this long-term field study support the hypothesis of an oxidative cost to reproduction while at the same time not strengthening the hypothesis that oxidative stress plays a role in life-history trade-offs between reproduction and survival in wild barn swallows. The increase in oxidative damage seen in females exposed to a higher workload can be related to the absence of antioxidant variation with altered parental effort; increased oxidative damage in females with a high workload had a short-term effect because our manipulation does not translate this effect into a decreased likelihood of return rate. On the contrary, natural levels of oxidative damage and the concentration of a key intracellular antioxidant in females measured at hatching predict annual return rate, suggesting that the oxidative condition at hatching may be more important in fitness than a transient increase in oxidative status during brood rearing. Our results clearly show that the timing of breeding and infection by blood-sucking nest parasites can also be integral to the redox state during brood care, both areas for research in the future.

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Author contributions PLP and CIV conceived and designed the study. PLP, OV, AF, OSB, LP, and CIV performed the study. OV and PLP analyzed the data. PLP, CIV, OV, and AF wrote the manuscript. All authors commented, read, and approved the final manuscript.

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Compliance with ethical standards

Ethical approval Birds were handled in strict accordance with good animal welfare and ethical prescriptions.

The authors declare that this publication complies with the current laws of the country in which the experiment was performed (Romania).

Conflict of interest The authors declare that they have no conflict of interest.

References

- Aloise King ED, Garratt M, Brooks R (2013) Manipulating reproductive effort leads to changes in female reproductive scheduling but not oxidative stress. *Ecol Evol* 3(12):4161–4171. <https://doi.org/10.1002/ece3.786>
- Alonso-Alvarez C, Bertrand S, Devevey G, Prost J, Faivre B, Sorci G (2004) Increased susceptibility to oxidative stress as a proximate cost of reproduction. *Ecol Lett* 7(5):363–368. <https://doi.org/10.1111/j.1461-0248.2004.00594.x>
- Bates D, Maechler M, Bolker B, Walker S (2014) lme4: linear mixed-effects models using Eigen and S4. R package version 1:1–7 <https://github.com/lme4/lme4/> <http://lme4.r-forge.r-project.org/>
- Beaulieu M, Geiger RE, Reim E, Zielke L, Fischer K (2015) Reproduction alters oxidative status when it is traded-off against longevity. *Evolution* 69(7):1786–1796. <https://doi.org/10.1111/evo.12697>
- Bize P, Devevey G, Monaghan P, Doligez B, Christe P (2008) Fecundity and survival in relation to resistance to oxidative stress in a free living bird. *Ecology* 89(9):2584–2593. <https://doi.org/10.1890/07-1135.1>
- Bolker BM, Brooks ME, Clark CJ, Geange SW, Poulsen JR, Stevens MHH, White JSS (2009) Generalized linear mixed models: a practical guide for ecology and evolution. *Trends Ecol Evol* 24(3):127–135. <https://doi.org/10.1016/j.tree.2008.10.008>
- Blount JD, Vitikainen EIK, Stott I, Cant MA (2016) Oxidative shielding and the cost of reproduction. *Biol Rev* 91(2):483–497. <https://doi.org/10.1111/brev.12179>
- Bókonyi V, Lendvai ÁZ, Vágási CI et al (2014) Necessity or capacity? Physiological state predicts problem-solving performance in house sparrows. *Behav Ecol* 25(1):124–135. <https://doi.org/10.1093/beheco/art094>
- Bucolo G, David H (1973) Quantitative determination of serum triglycerides by the use of enzymes. *Clin Chem* 19(5):476–482
- Christe P, Glaizot O, Strepparava N, Devevey G, Fumagalli L (2012) Twofold cost of reproduction: an increase in parental effort leads to higher malarial parasitaemia and to a decrease in resistance to oxidative stress. *Proc R Soc Lond B* 279(1731):1142–1149. <https://doi.org/10.1098/rspb.2011.1546>
- Cohen A, Klasing K, Ricklefs R (2007) Measuring circulating antioxidants in wild birds. *Comp Biochem Physiol B* 147(1):110–121. <https://doi.org/10.1016/j.cbpb.2006.12.015>
- Costantini D (2008) Oxidative stress in ecology and evolution: lessons from avian studies. *Ecol Lett* 11(11):1238–1251. <https://doi.org/10.1111/j.1461-0248.2008.01246.x>
- Costantini D (2014) Oxidative stress and hormesis in evolutionary ecology and physiology. A marriage between mechanistic and evolutionary approaches. Springer, Berlin

- Costantini D, Bonisoli-Alquati A, Rubolini D, Caprioli M, Ambrosini R, Romano M, Saino N (2014a) Nestling rearing is antioxidant demanding in female barn swallows (*Hirundo rustica*). *Naturwissenschaften* 101(7):541–548. <https://doi.org/10.1007/s00114-014-1190-2>
- Costantini D, Casasole G, Eens M (2014b) Does reproduction protect against oxidative stress? *J Exp Biol* 217(23):4237–4243. <https://doi.org/10.1242/jeb.114116>
- Costantini D, Casasole G, Abdelgawad H, Asard H, Eens M (2016) Experimental evidence that oxidative stress influences reproductive decisions. *Funct Ecol* 30(7):1169–1174. <https://doi.org/10.1111/1365-2435.12608>
- Costantini D, Dell’Omo G (2015) Oxidative stress predicts long-term resight probability and reproductive success in Scopoli’s shearwater (*Calonectris diomedea*). *Conserv Physiol* 3:cov024
- Costantini D, Møller AP (2009) Does immune response cause oxidative stress in birds? A meta-analysis. *Comp Biochem Physiol A* 153(3):339–344. <https://doi.org/10.1016/j.cbpa.2009.03.010>
- Costantini D, Verhulst S (2009) Does high antioxidant capacity indicate low oxidative stress? *Funct Ecol* 23(3):506–509. <https://doi.org/10.1111/j.1365-2435.2009.01546.x>
- Cram DL, Blount JD, Young AJ (2015) The oxidative costs of reproduction are group-size dependent in a wild cooperative breeder. *Proc R Soc B* 282(1819):20152031. <https://doi.org/10.1098/rspb.2015.2031>
- Del Rio D, Stewart AJ, Pellegrini N (2005) A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutr Metab Cardiovasc Dis* 15(4):316–328. <https://doi.org/10.1016/j.numecd.2005.05.003>
- Emaresi G, Henry I, Gonzalez E, Roulin A, Bize P (2016) Sex- and melanin-specific variations in the oxidative status of adult tawny owls in response to manipulated reproductive effort. *J Exp Biol* 219(1):73–79. <https://doi.org/10.1242/jeb.128959>
- Erel O (2004) A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clin Biochem* 37(4):277–285. <https://doi.org/10.1016/j.clinbiochem.2003.11.015>
- Fletcher QE, Selman C, Boutin S, McAdam AG, Woods SB, Seo AY, Leeuwenburgh C, Speakman JR, Humphries MM (2012) Oxidative damage increases with reproductive energy expenditure and is reduced by food-supplementation. *Evolution* 67:1527–1536
- Fossati P, Prencipe L (1982) Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. *Clin Chem* 28(10):2077–2080
- Fox J, Weisberg S (2011) An {R} companion to applied regression, 2nd edn. Sage, Thousand Oaks, CA
- Freeman-Gallant CR, Amidon J, Berdy B, Wein S, Taff CC, Haussmann MF (2011) Oxidative damage to DNA related to survivorship and carotenoid-based sexual ornamentation in the common yellow-throat. *Biol Lett* 11:429–432
- Fülöp A, Vágási CI, Pap PL (2017) Cohabitation with farm animals rather than breeding effort increases the infection with feather-associated bacteria in the barn swallow *Hirundo rustica*. *J Avian Biol* 48(7):1005–1014. <https://doi.org/10.1111/jav.01262>
- Galván I, Alonso-Alvarez C (2008) An intracellular antioxidant determines the expression of a melanin-based signal in a bird. *PLoS One* 3(10):e3335. <https://doi.org/10.1371/journal.pone.0003335>
- Garratt M, Pichaud N, Aloise King ED, Brooks RC (2013) Physiological adaptations to reproduction. I. Experimentally increasing litter size enhances aspects of antioxidant defence but does not cause oxidative damage in mice. *J Exp Biol* 216(15):2879–2888. <https://doi.org/10.1242/jeb.082669>
- Halliwell B, Gutteridge J (2007) Free radicals in biology and medicine. Oxford University Press, Oxford
- Hörak P, Sild E, Soomets U, Sepp T, Kilk K (2010) Oxidative stress and information content of black and yellow plumage coloration: an experiment with greenfinches. *J Exp Biol* 213(13):2225–2233. <https://doi.org/10.1242/jeb.042085>
- Isaksson C (2013) Opposing effects on glutathione and reactive oxygen metabolites of sex, habitat, and spring date, but no effect of increased breeding density in great tits (*Parus major*). *Ecol Evol* 3(8):2730–2738. <https://doi.org/10.1002/ece3.663>
- Jenni-Eiermann S, Jenni L, Kvist A, Lindström Å, Piersma T, Visser GH (2002) Fuel use and metabolic response to endurance exercise: a wind tunnel study of a long-distance migrant shorebird. *J Exp Biol* 205(Pt 16):2453–2460
- Jenni-Eiermann S, Jenni L, Smith S, Costantini D (2014) Oxidative stress in endurance flight: an unconsidered factor in bird migration. *PLoS One* 9(5):e97650. <https://doi.org/10.1371/journal.pone.0097650>
- Klasing KC, Austic RE (1984) Changes in plasma tissue and urinary nitrogen metabolites due to an inflammatory challenge. *Proc Soc Exp Biol Med* 176(3):276–284. <https://doi.org/10.3181/00379727-176-41871>
- Lendvai AZ, Akçay Ç, Ouyang JQ, Dakin R, Domalik AD, St John PS, Stanback M, Moore IT, Bonier F (2015) Analysis of the optimal duration of behavioral observations based on an automated continuous monitoring system in tree swallows (*Tachycineta bicolor*): is one hour good enough? *PLoS One* 10(11):e0141194. <https://doi.org/10.1371/journal.pone.0141194>
- López-Arrabé J, Cantarero A, Pérez-Rodríguez L, Palma A, Alonso-Alvarez C, González-Braojos S, Moreno J (2015) Nest-dwelling ectoparasites reduce antioxidant defences in females and nestlings of a passerine: a field experiment. *Oecologia* 179(1):29–41. <https://doi.org/10.1007/s00442-015-3321-7>
- Losdat S, Helfenstein F, Gaude B, Richner H (2011) Reproductive effort transiently reduces antioxidant capacity in a wild bird. *Behav Ecol* 22(6):1218–1226. <https://doi.org/10.1093/beheco/arr116>
- Markó G, Costantini D, Michl G, Török J (2011) Oxidative damage and plasma antioxidant capacity in relation to body size, age, male sexual traits and female reproductive performance in the collared flycatcher (*Ficedula albicollis*). *J Comp Physiol B* 181(1):73–81. <https://doi.org/10.1007/s00360-010-0502-x>
- Metcalfe NB, Monaghan P (2013) Does reproduction cause oxidative stress? An open question. *Trends Ecol Evol* 28(6):347–350. <https://doi.org/10.1016/j.tree.2013.01.015>
- Møller AP (1990) Effects of parasitism by the haematophagous mite *Ornithonyssus bursa* on reproduction in the barn swallow *Hirundo rustica*. *Ecology* 71(6):2345–2357. <https://doi.org/10.2307/1938645>
- Møller AP (1994) Sexual selection and the barn swallow. Oxford University Press, Oxford
- Møller AP, de Lope F (1999) Senescence in a short-lived migratory bird: age-dependent morphology, migration, reproduction and parasitism. *J Anim Ecol* 68(1):163–171. <https://doi.org/10.1046/j.1365-2656.1999.00274.x>
- Monaghan P, Metcalfe NB, Torres R (2009) Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. *Ecol Lett* 12(1):75–92. <https://doi.org/10.1111/j.1461-0248.2008.01258.x>
- Ninni P, de Lope F, Saino N, Haussy C, Møller AP (2004) Antioxidants and condition-dependence of arrival date in a migratory passerine. *Oikos* 105(1):55–64. <https://doi.org/10.1111/j.0030-1299.2004.12516.x>
- Noguera JC, Kim S-Y, Velando A (2012) Pre-fledgling oxidative damage predicts recruitment in a long-lived bird. *Biol Lett* 8(1):61–63. <https://doi.org/10.1098/rsbl.2011.0756>
- Pap PL, Pătraș L, Osváth G, Buehler DM, Versteegh MA, Sesarman A, Banciu M, Vágási CI (2015) Seasonal patterns and relationships among coccidian infestations, measures of oxidative physiology, and immune function in free-living house sparrows over an annual cycle. *Physiol Biochem Zool* 88(4):395–405. <https://doi.org/10.1086/681243>

- Pap PL, Tökölyi J, Szép T (2005) Frequency and consequences of feather holes in barn swallows *Hirundo rustica*. *Ibis* 146:169–175
- Partadiredja G, Worrall S, Bedi KS (2009) Early life undernutrition alters the level of reduced glutathione but not the activity levels of reactive oxygen species enzymes or lipid peroxidation in the mouse fore-brain. *Brain Res* 1285:22–29. <https://doi.org/10.1016/j.brainres.2009.06.010>
- Pérez-Rodríguez L, Romero-Haro AA, Sternalski A, Muriel J, Mougeot F, Gil D, Alonso-Alvarez C (2015) Measuring oxidative stress: the confounding effect of lipid concentration in measures of lipid peroxidation. *Physiol Biochem Zool* 88(3):345–351. <https://doi.org/10.1086/680688>
- R Core Team (2016) A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna <http://www.R-project.org>
- Rey B, Péliisson PF, Bel-Venner MC, Voituren Y, Venner S (2015) Revisiting the link between breeding effort and oxidative balance through field evaluation of two sympatric sibling insect species. *Evolution* 69(3):815–822. <https://doi.org/10.1111/evo.12586>
- Romero-Haro AA, Alonso-Alvarez A (2015) The level of an intracellular antioxidant during development determines the adult phenotype in a bird species: a potential organizer role for glutathione. *Am Nat* 185(3):390–405. <https://doi.org/10.1086/679613>
- Rothman KJ (1990) No adjustments are needed for multiple comparisons. *Epidemiology* 1(1):43–46. <https://doi.org/10.1097/00001648-199001000-00010>
- Rothman KJ (2014) Six persistent research misconceptions. *J Gen Int Med* 29(7):1060–1064. <https://doi.org/10.1007/s11606-013-2755-z>
- Saino N, Caprioli M, Romano M, Boncoraglio G, Rubolini D, Ambrosini R, Bonisoli-Alquati A, Romano A (2011) Antioxidant defenses predict long-term survival in a passerine bird. *PLoS One* 6(5):e19593. <https://doi.org/10.1371/journal.pone.0019593>
- Saino N, Romano M, Ambrosini R, Ferrari RP, Møller AP (2004) Timing of reproduction and egg quality covary with temperature in the insectivorous barn swallow, *Hirundo rustica*. *Funct Ecol* 18(1):50–57. <https://doi.org/10.1046/j.0269-8463.2004.00808.x>
- Schaub M, von Hirschheydt J (2009) Effect of current reproduction on apparent survival, breeding dispersal, and future reproduction in barn swallows assessed by multistate capture–recapture models. *J Anim Ecol* 78(3):625–635. <https://doi.org/10.1111/j.1365-2656.2008.01508.x>
- Selman C, Blount JD, Nussey DH, Speakman JR (2012) Oxidative damage, ageing, and life-history evolution: where now? *Trends Ecol Evol* 27(10):570–577. <https://doi.org/10.1016/j.tree.2012.06.006>
- Senn SS (2008) Baselines and covariate information. In: Senn SS (2008) statistical issues in drug development, 2nd edn. John Wiley & Sons Ltd, Chichester, pp 95–112
- Sepp T, Karu U, Blount JD, Sild E, Männiste M, Hõrak P (2012) Coccidian infection causes oxidative damage in greenfinches. *PLoS One* 7(5):e36495. <https://doi.org/10.1371/journal.pone.0036495>
- Sepp T, Sild E, Hõrak P (2010) Hematological condition indexes in greenfinches: effects of captivity and diurnal variation. *Physiol Biochem Zool* 83(2):276–282. <https://doi.org/10.1086/648580>
- Sorci G, Faivre B (2009) Inflammation and oxidative stress in vertebrate host–parasite systems. *Philos T Roy Soc B* 364(1513):71–83. <https://doi.org/10.1098/rstb.2008.0151>
- Speakman JR, Blount JD, Bronikowski AM, Buffenstein R, Isaksson C, Kirkwood TBL, Monaghan P, Ozanne SE, Beaulieu M, Briga M, Carr SK, Christensen LL, Cochemé HM, Cram DL, Dantzer B, Harper JM, Jurk D, King A, Noguera JC, Salin K, Sild E, Simons MJP, Smith S, Stier A, Tobler M, Vitikainen E, Peaker M, Selman C (2015) Oxidative stress and life histories: unresolved issues and current needs. *Ecol Evol* 5(24):5745–5757. <https://doi.org/10.1002/ece3.1790>
- Speakman JR, Garratt M (2014) Oxidative stress as a cost of reproduction: beyond the simplistic trade-off model. *BioEssays* 36(1):93–106. <https://doi.org/10.1002/bies.201300108>
- Stearns SC (1989) Trade-offs in life-history evolution. *Funct Ecol* 3(3):259–268. <https://doi.org/10.2307/2389364>
- Stier A, Reichert S, Criscuolo F, Bize P (2015) Red blood cells open promising avenues for longitudinal studies of ageing in laboratory, non-model and wild animals. *Exp Gerontol* 71:118–134. <https://doi.org/10.1016/j.exger.2015.09.001>
- Stier A, Reichert S, Massemin S, Bize P, Criscuolo F (2012) Constraint and cost of oxidative stress on reproduction: correlative evidence in laboratory mice and review of the literature. *Front Zool* 9(1):37. <https://doi.org/10.1186/1742-9994-9-37>
- Surai PF (2002) Natural antioxidants in avian nutrition and reproduction. Nottingham University Press, Nottingham
- van de Crommenacker J, Richardson DS, Koltz AM, Hutchings K, Komdeur J (2012) Parasitic infection and oxidative status are associated and vary with breeding activity in the Seychelles warbler. *Proc R Soc Lond B* 279(1733):1466–1476. <https://doi.org/10.1098/rspb.2011.1865>
- Wiersma P, Selman C, Speakman JR, Verhulst S (2004) Birds sacrifice oxidative protection for reproduction. *Proc R Soc Lond B* 271(Suppl_5):S360–S363. <https://doi.org/10.1098/rsbl.2004.0171>
- Wu G, Fang YZ, Yang S, Lupton JR, Turner ND (2004) Glutathione metabolism and its implications for health. *J Nutr* 134(3):489–492
- Yang DB, Xu YC, Wang DH, Speakman JR (2013) Effects of reproduction on immuno-suppression and oxidative damage, and hence support or otherwise for their roles as mechanisms underpinning life history trade-offs, are tissue and assay dependent. *J Exp Biol* 216(22):4242–4250. <https://doi.org/10.1242/jeb.092049>