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RESEARCH ARTICLE

Does reproduction protect against oxidative stress?

David Costantini[1,2,*], Giulia Casasole[3] and Marcel Eens[1]

ABSTRACT

A central principle of life-history theory is that parents trade investment in reproduction against that in body maintenance. One physiological cost thought to be important as a modulator of such trade-off is oxidative stress. Experimental support for this hypothesis has, however, proved to be contradictory. In this study, we manipulated the nestling rearing effort of captive canaries (*Serinus canaria*) soon after the hatching of their nestlings using a brood-size manipulation to test whether an increase in nestling rearing effort translates into an increase in oxidative damage, an increase in ceruloplasmin (which is upregulated in response to oxidative damage) and a decrease in thiol antioxidants. We also compared the blood oxidative stress level of reproducing birds with that of non-reproducing birds, a crucial aspect that most studies have invariably failed to include in tests of the oxidative cost of reproduction. Compared with non-breeding canaries and pre-manipulation values, plasma oxidative damage (reactive oxygen metabolites and protein carbonyls) decreased in breeding canaries irrespective of sex and brood size. In contrast, oxidative damage did not change in non-breeding birds over the experiment. Ceruloplasmin activity in plasma and both non-protein and protein thiols in red blood cells did not change throughout the experiment in both treatment groups. Our results suggest that reproduction may result in decreased rather than increased blood oxidative stress. Our results may explain some of the inconsistencies that have so far been reported in experimental tests of the oxidative cost of reproduction hypothesis.

KEY WORDS: Antioxidants, Life history, Oxidative damage, Parental care, Thiols, Vertebrates

INTRODUCTION

Reproduction is a critical and demanding phase of an animal's life. From mate choice to offspring rearing, individuals are being continuously faced with multiple trade-offs in the allocation of resources and time between self-maintenance and reproductive activity (Stearns, 1992). The basic concept of a trade-off is that resource acquisition or time budget is limited and so increased allocation to one phenotypic trait is at the expense of other traits requiring the same resource (Stearns, 1992). That investment of resources into reproduction has pervasive effects on the individual is well documented by numerous studies showing dramatic increases in energy expenditure and mortality rate (Daan et al., 1996; Angilletta and Sears, 2000; Boonekamp et al., 2014) or decreased future reproductive perspectives (Law, 1979; Reed et al., 2008). However, very little is currently known about the physiological mechanisms underlying the reproductive costs.

One putative mechanism that has received considerable recent attention is the role of oxidative stress induced by an increase in oxidative molecular damage and oxidation of non-protein and protein thiols that regulate the cell oxidative balance (Jones, 2006; Halliwell and Gutteridge, 2007; Sohal and Orr, 2012). The major assumption underlying the oxidative cost of reproduction hypothesis is that high investment in reproduction would result in faster somatic deterioration and reduced life expectancy as resources allocated to reproduction are no longer available to protect the animal against oxidative stress. Experimental support for this hypothesis has, however, proved to be highly equivocal (Stier et al., 2012; Metcalfe and Monaghan, 2013; Speakman and Garratt, 2014; Costantini, 2014). This might be because of flaws in many experimental designs, such as lack of manipulation of reproductive effort, quantification of oxidative damage or comparison between reproducing and non-reproducing individuals (Metcalfe and Monaghan, 2013). Evidence in favour of an oxidative cost of reproduction, however, is equivocal even when flaws are limited (e.g. Garratt et al., 2013; Yang et al., 2013; Costantini et al., 2014a).

One reason for this might be that the intensity of trade-offs varies across environmental contexts. Hence, reproduction might not be very costly when the environmental conditions are favourable. Fluctuations in the quality of the habitat in which an animal lives can have major consequences for its reproductive activity. In a poor-quality habitat with low food availability, breeding individuals are faced with metabolically intensive foraging activity and scarcity of nutrients, which are likely to result in increased generation of oxidative stress (van de Crommenacker et al., 2011; Fletcher et al., 2013) and induction of inflammation-inducible proteins (e.g. ceruloplasmin, haptoglobin) that limit the spread of oxidative damage across tissues (Pacht and Davis, 1988; Jelena et al., 2013). In contrast, reproducing under good environmental conditions may mitigate the intensity of such trade-offs (van de Crommenacker et al., 2011; Fletcher et al., 2013). One consequence might be that the reproductive activity results in the stimulation of physiological self-maintenance mechanisms when it is mildly demanding for the organism [e.g. through hormetic mechanisms (Costantini, 2014)]. This would explain why several experimental reports found no change (e.g. Costantini et al., 2014a) or even decreases in oxidative damage (e.g. Garratt et al., 2011) or increases in antioxidant protection (e.g. Salomons, 2009) during reproduction.

In this study, we manipulated the nestling rearing effort in captive canaries, *Serinus canaria* (Linnaeus 1758) under favourable environmental conditions [mimicking reproductive conditions that occur in good quality environments (e.g. van de Crommenacker et al., 2011; Fletcher et al., 2013)] and investigated whether higher effort induces an increase in blood oxidative damage, an increase in ceruloplasmin activity in plasma and a reduction in the non-protein and protein thiols. To this end, we enlarged or reduced the brood size, which is a relatively easy way of altering parental effort in

[1]Department of Biology, Ethology Group, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium. [2]Institute for Biodiversity, Animal Health and Comparative Medicine, University of Glasgow, Glasgow G12 8QQ, UK. [3]Institute of Environmental Sciences, Jagiellonian University, Ulica Gronostajowa 7, 30-387 Kraków, Poland.

*Author for correspondence (davidcostantini@libero.it)

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altricial avian species (Dijkstra et al., 1990; Santos and Nakagawa, 2012). We also compared the blood oxidative stress level of reproducing birds with that of non-reproducing birds. Using a non-breeding group offered us the opportunity to have a control group for the reproductive activity, which is a crucial aspect that most studies have invariably failed to include in tests of the oxidative cost of reproduction (Metcalfe and Monaghan, 2013). If reproductive activity results in oxidative stress, we would predict higher oxidative damage, higher ceruloplasmin and lower thiols (i) in reproducing than in non-reproducing birds and (ii) in parents rearing enlarged broods than in those rearing reduced broods. In contrast, if reproducing under our favourable experimental conditions is stimulatory rather than challenging for the individual physiological state, we would predict that (i) reproducing birds would have lower oxidative damage than non-reproducing birds, (ii) the activity of ceruloplasmin and concentration of thiol groups would not differ between reproducing and non-reproducing birds because antioxidant defences are stable in reproducing birds, and (iii) reproducing birds would have similar levels of oxidative damage, ceruloplasmin and thiols irrespective of brood size.

RESULTS

Compared with levels in non-breeding canaries and pre-manipulation values, reactive oxygen metabolites decreased in breeding canaries irrespective of sex and brood size (Table 1, Fig. 1). Similarly, compared with control (non-breeding) canaries and pre-manipulation values, protein carbonyls (expressed per mg of protein or total) decreased in breeding canaries irrespective of sex and brood size (Table 1, Fig. 1). Both reactive oxygen metabolites and protein carbonyls were unchanged in non-breeding birds over the experiment. For both measurements of protein carbonyls, there was a significant interaction between sex and treatment group: while control males and females had similar levels of both protein carbonyl measurements, males of both reduced and enlarged broods had lower levels than their respective female mates (Tables 1, 2). Values of both reactive oxygen metabolites and protein carbonyls

Table 1. Linear mixed models of factors affecting plasma oxidative damage (reactive oxygen metabolites and protein carbonyls), thiol status of red blood cells (non-protein and protein thiols) and plasma ceruloplasmin of canaries

		Full model				Final model			
Variable	Effect	Num d.f.	Den DF	*F*	*P*	Num d.f.	Den d.f.	*F*	*P*
ROMs	Sex	1	30	2.48	0.1255	1	1.33	2.04	0.3431
	Treatment group	2	30	1.41	0.2597	2	26.3	1.21	0.3157
	Period	1	60	28.36	<0.0001	1	63	27.83	<0.0001
	Period × treatment group	2	60	8.27	0.0007	2	63	8.12	0.0007
	Sex × period	1	60	3.71	0.0588				
	Sex × treatment group	2	30	2.77	0.0786				
	Sex × treatment group × period	2	60	0.24	0.7862				
Protein carbonyls	Sex	1	3.54	0.78	0.4320	1	3.54	0.78	0.4320
	Treatment group	2	25.8	1.29	0.2935	2	25.8	1.29	0.2935
	Period	1	60	21.76	<0.0001	1	63	21.85	<0.0001
	Period × treatment group	2	60	3.44	0.0385	2	63	3.46	0.0377
	Sex × period	1	60	2.66	0.1082				
	Sex × treatment group	2	26.5	6.01	0.0070	2	26.5	6.01	0.0070
	Sex × treatment group × period	2	60	0.04	0.9561				
Total protein carbonyls	Sex	1	3.19	1.26	0.3385	1	3.19	1.26	0.3385
	Treatment group	2	23	1.63	0.2176	2	23	1.63	0.2176
	Period	1	60	42.61	<0.0001	1	63	42.82	<0.0001
	Period × treatment group	2	60	9.58	0.0002	2	63	9.63	0.0002
	Sex × period	1	60	2.34	0.1316				
	Sex × treatment group	2	25.2	4.99	0.0149	2	25.2	4.99	0.0149
	Sex × treatment group × period	2	60	0.18	0.8392				
Non-protein thiols	Sex	1	60	19.48	<0.0001	1	62	19.91	<0.0001
	Treatment group	2	60	0.11	0.9007	2	62	0.11	0.8986
	Period	1	60	0.64	0.4271	1	64	0.63	0.4295
	Period × treatment group	2	60	0.15	0.8576				
	Sex × period	1	60	14.01	0.0004	1	64	13.86	<0.0001
	Sex × treatment group	2	60	0.32	0.7245				
	Sex × treatment group × period	2	60	2.21	0.1191				
Protein thiols	Sex	1	30	1.32	0.2588	1	32	1.37	0.2513
	Treatment group	2	30	0.11	0.8938	2	30	0.11	0.8938
	Period	1	60	3.49	0.0665	1	64	3.53	0.0649
	Period × treatment group	2	60	0.32	0.7245				
	Sex × period	1	60	4.30	0.0424	1	64	4.34	0.0411
	Sex × treatment group	2	30	0.53	0.5958				
	Sex × treatment group × period	2	60	1.35	0.2667				
Ceruloplasmin	Sex	1	30.2	3.41	0.0745	1	31.9	2.71	0.1096
	Treatment group	2	31.1	0.60	0.5537	2	29.9	0.79	0.4613
	Period	1	60	0.53	0.4714	1	65	0.54	0.4652
	Period × treatment group	2	60	0.54	0.5861				
	Sex × period	1	60	2.05	0.1574				
	Sex × treatment group	2	30.2	0.43	0.6524				
	Sex × treatment group × period	2	60	0.08	0.9238				

ROMs, reactive oxygen metabolites.

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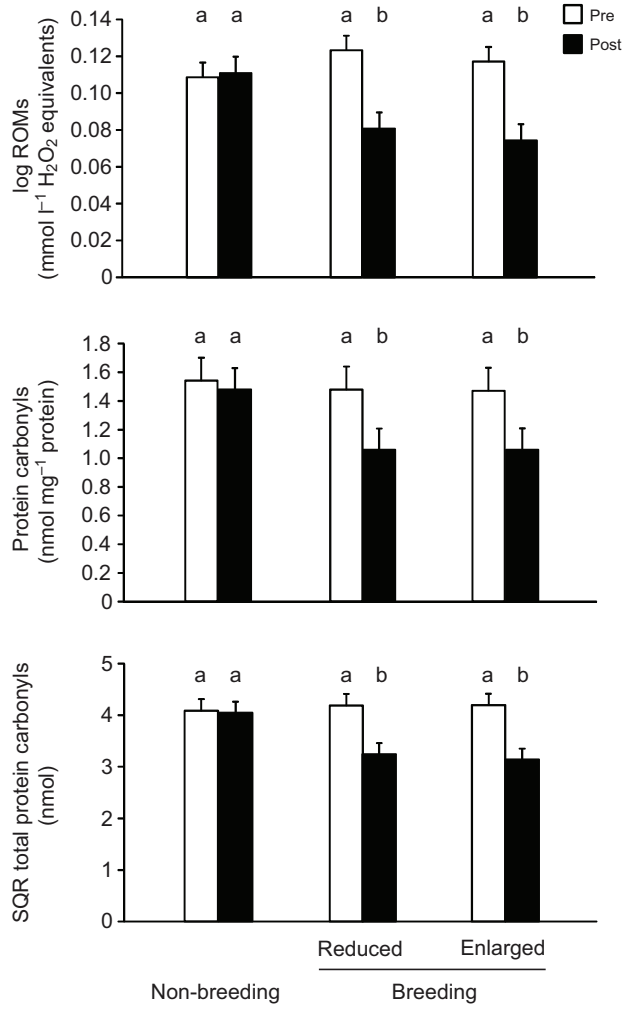

Fig. 1. Pre- and post-manipulation levels of plasma oxidative damage of canaries in relation to treatment. Canaries were either non-breeding or breeding with reduced or enlarged broods. Plasma oxidative damage (reactive oxygen metabolites and protein carbonyls) levels were obtained before pair formation (Pre) and at day 12 post-hatching (Post). Reactive oxygen metabolites (ROMs) were log(x+1) transformed; total protein carbonyls were square-root (SQR) transformed. Means that do not share the same letter are significantly different from each other (Tukey, P<0.05). Values are shown as least square means + s.e.

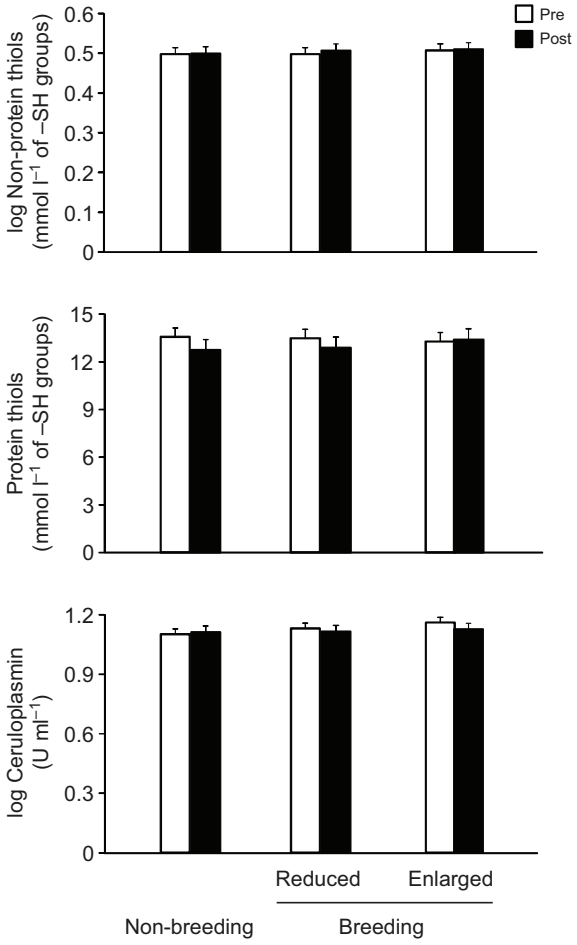

Fig. 2. Pre- and post-manipulation levels of red blood cell non-protein and protein thiols and of plasma ceruloplasmin of canaries in relation to treatment. Canaries were either non-breeding or breeding with reduced or enlarged broods. Non-protein thiols and ceruloplasmin were log(x+1) and log(x) transformed, respectively. Treatment groups did not differ in any molecular biomarker. Values are shown as least square means + s.e.

were significantly repeatable within individuals across time (P≤0.025).

Non-protein thiols were similar among experimental groups (Table 1, Fig. 2) and significantly increased in males and decreased in females, resulting in a significant interaction between period and sex (Tables 1, 2). Protein thiols were also similar among experimental groups (Table 1, Fig. 2); they also significantly decreased in females, while they were stable in males over the experimental period, which resulted in a significant interaction between period and sex (Tables 1, 2). Females also had a significantly higher concentration of non-protein thiols than did males (estimate ± s.e., −0.059±0.019; P=0.003; Table 2). Non-protein thiols were significantly repeatable within individuals across time (P<0.001), while intra-individual repeatability of protein thiols was close to significance (P=0.07).

The activity of plasma ceruloplasmin did not change over the experimental period (Table 1) and did not differ among groups (Table 1, Fig. 2), or between males and females (Table 1).

Significant correlations were found between: reactive oxygen metabolites and total protein carbonyls; protein carbonyls and both total protein carbonyls and non-protein thiols; total protein carbonyls and non-protein thiols; and non-protein thiols and protein thiols (Table 3).

DISCUSSION

The results of our study show that reproduction can result in decreased rather than increased blood oxidative damage. In fact, compared with non-breeding canaries, breeding birds showed a reduction in plasma oxidative damage. Moreover, breeding and non-breeding birds had similar and constant levels of plasma ceruloplasmin and of both non-protein and protein thiols in red blood cells throughout the experimental period, showing that reproducing birds were capable of maintaining their redox state and did not need to increase the synthesis of thiols and ceruloplasmin.

Our canaries were housed under good husbandry conditions, with an unlimited supply of food, limited foraging effort and thermoneutral conditions. Therefore, the conclusions gathered by this experiment cannot be generalized, but should be contextualized to those individuals that reproduce in good territories, where the costs of reproduction may be mitigated. Indeed, under favourable

Table 2. Protein carbonyl and non-protein and protein thiol measurements in control males and females and those with reduced and enlarged broods

		Males	Females
Protein carbonyls	Controls	1.62±0.65	1.40±0.62
	Enlarged	1.08±0.44	1.46±0.82
	Reduced	1.05±0.48	1.49±0.64
Total protein carbonyls	Controls	18.51±7.71	16.23±7.62
	Enlarged	12.67±6.00	16.37±9.40
	Reduced	12.76±6.04	16.73±7.95
Non-protein thiols	Pre	1.86±0.51	2.62±0.60
	Post	2.05±0.56	2.48±0.59
Protein thiols	Pre	12.79±2.43	14.09±2.48
	Post	13.00±3.01	13.00±2.82

Control males and females had similar levels of both protein carbonyl measurements (P≥0.31), while males of both reduced (estimate ± s.e., protein carbonyls, −0.472±0.173, P=0.01; total protein carbonyls, −0.681±0.233, P=0.007) and enlarged (estimate ± s.e., protein carbonyls, −0.419±0.173, P=0.021; total protein carbonyls, −0.620±0.233, P=0.013) broods had lower levels than their respective female mates.
Non-protein thiols significantly increased in males (estimate ± s.e., −0.026±0.008, P=0.002) and decreased in females (estimate ± s.e., 0.017±0.008, P=0.043). Protein thiols also significantly decreased in females (estimate ± s.e., 20.106±7.176, P=0.007), while they were stable in males (estimate ± s.e., −1.046±7.176, P=0.88). Pre- and post-manipulation data are shown as least square means and s.d.

conditions, experimental brood enlargements in wild birds may result in parents being able to raise all the nestlings without incurring any costs in terms of reduced survival or future breeding performance (Dijkstra et al., 1990; Santos and Nakagawa, 2012). Studies on wild birds or mammals have also found that individuals reproducing in high quality environments had lower oxidative stress than those reproducing under poorer environmental conditions. For example, plasma oxidative damage (reactive oxygen metabolites) in Seychelles warbler (*Acrocephalus sechellensis*) was higher in those individuals breeding in low quality territories compared with those breeding in high quality territories. Moreover, the individual oxidative balance was sensitive to relatively short-term changes in territory quality, meaning that an increase in territory quality while breeding would translate to reproduction not increasing oxidative damage (van de Crommenacker et al., 2011). Similarly, in a study on wild American red squirrels (*Tamiasciurus hudsonicus*), Fletcher et al. (Fletcher et al., 2013) found that plasma oxidative damage (protein carbonyls) increased with reproductive energy expenditure, but it was reduced by food supplementation. Hence, it is possible that under favourable environmental conditions, reproduction not only does not result in oxidative stress but also might stimulate self-maintenance mechanisms at no cost for reproductive activity (see also Garratt et al., 2011; Ołdakowski et al., 2012). It will be important to replicate this experiment, maintaining animals under less favourable environmental conditions than those of the present study in order to identify which conditions give rise to the oxidative costs of reproduction.

Another explanation for our results might lie with individual quality. Many pairs allocated to the breeding group failed to successfully reproduce; hence, those birds that successfully bred (and were consequently included in the experiment) might have been those that were best prepared to breed (i.e. high quality individuals). Previous studies on birds or mammals showed that the cost of reproduction may be evident only in low quality individuals (Cichoń et al., 1998; Hamel et al., 2009). In our case, any effect of individual quality does not, however, appear strong because breeding and failed-breeding pairs did not differ in clutch size (t-test, P=0.11) or in body mass (GLM, group P=0.89, group×sex P=0.76).

Overall these results may reconcile some of the seemingly contrasting findings that have been so far reported. Various studies on both captive and free-living birds have, for example, demonstrated that an increase in workload may decrease antioxidant defences (Alonso-Alvarez et al., 2004; Wiersma et al., 2004; Costantini et al., 2014a) and increase oxidative damage (Casagrande et al., 2011) or that engaging in reproduction may increase plasma oxidative damage compared with birds that skipped the breeding season (Costantini et al., 2014b). Other studies, however, found no such effects (Salomons, 2009; Beaulieu et al., 2011) or found that any effects on oxidative balance are transient (Losdat et al., 2011) or contingent on the territory quality (van de Crommenacker et al., 2011). As with birds, there is little direct experimental evidence that investment in reproduction elevates oxidative stress in mammals. In captive house mice (*Mus musculus*), lipid peroxidation and protein damage in liver were even lower in females that were allowed to reproduce than in non-reproducing females (Garratt et al., 2011). Similarly, oxidative status in the heart and gastrocnemius muscle did not differ between house mouse females rearing large or small litters (Garratt et al., 2013). Surprisingly, oxidative protein damage was lower in the livers of females with a litter size of eight than in females with two pups or non-reproductive control females (Garratt et al., 2013).

We also found differences in oxidative balance between males and females. Although their response to the experimental treatment was similar, females tended to have more protein damage and a higher concentration of non-protein thiols than males. Sexual differentiation in oxidative balance has been reported in many vertebrate species (Alonso-Alvarez et al., 2004; Wiersma et al., 2004; Costantini, 2014). The reasons for this sexual differentiation in oxidative balance have not been elucidated yet.

Finally, bivariate correlations among biomarkers of blood oxidative status presented in Table 3 highlight the importance of using multiple assays and determining both sides of the oxidative balance to estimate the oxidative stress level.

Table 3. Bivariate correlations among blood oxidative status parameters

	ROMs	Protein carbonyls	Total protein carbonyls	Non-protein thiols	Protein thiols
Protein carbonyls	0.08	–			
Total protein carbonyls	**0.24**	**0.94**	–		
Non-protein thiols	–0.02	**0.22**	**0.16**	–	
Protein thiols	0.12	–0.14	–0.09	**0.18**	–
Ceruloplasmin	–0.03	0.09	0.10	–0.11	0.08

ROMs, reactive oxygen metabolites.
Significant correlations are shown in bold type. The total sample size is 132.

In conclusion, the results of our study suggest that the hypothesis that reproduction should incur an oxidative cost is not ubiquitous. Rather, the extent to which reproduction leads to an increased oxidative stress is likely to be contingent on several features, such as conditions of the breeding environment or individual quality. We certainly need more studies in order to elucidate when reproduction is stimulating for the self-maintenance mechanisms or is costly. It might be that reproduction activates behavioural or physiological mechanisms that protect against oxidative stress in order to improve immediate reproductive success and, possibly, to not compromise future reproduction. This may be especially important in those species, like the canary, with multiple reproductive cycles over the course of a lifetime (iteroparous species).

It is also not clear to what extent the results we observed are dependent upon the number of breeding events that birds experience with a reduced or an enlarged brood. It is possible that the results would have been different if the birds had been at their first breeding experience when the experiment took place or if the birds experienced repeated enlargement or reduction of their brood size during their lifetime. It is also unclear how the overall oxidative cost of reproduction comes up. In fact, various factors (e.g. hormones, foraging effort, food intake, food quality) might influence the oxidative balance over the transition from a non-reproducing to a reproducing stage. For example, reproducing birds might consume more food than non-reproducing birds in order to have more resources to invest in self-maintenance mechanisms. Therefore, future challenges will also include determining how the conflictual or synergistic interactions of these factors determine under which conditions reproduction incurs an oxidative cost that can impact on immediate, as well as on future, individual evolutionary fitness.

MATERIALS AND METHODS

This study was carried out in agreement with Belgian and Flemish legislation and was approved by the ethical committee.

Housing conditions and experimental setup

This study was conducted on a captive colony of canaries. In December 2013, birds were moved from our outdoor to single-sex indoor aviaries. We kept birds on a long-day photoperiod (14 h:10 h light:dark) and room temperature (19–23°C) to stimulate their reproductive activity. In mid-January 2014, we randomly mated the birds with a non-related sexual partner. Each couple allocated to the breeding group was housed in a single breeding cage (50×61×40 cm; GEHU cages, The Netherlands). The male and the female of a non-breeding couple were also housed in a single cage, but they were kept separated from each other using a mesh panel to prevent them from breeding. All the breeding and non-breeding birds were maintained in a same room on a 14 h:10 h light:dark photoperiod and room temperature of 19–23°C. The room temperature was within the thermoneutral zone of canaries (Weathers, 1985); note that at ambient temperatures outside this zone both metabolic and oxidative costs increase (Weathers, 1985; Beaulieu et al., 2014). We initially had 46 pairs allocated to the breeding group and 23 pairs allocated to the non-breeding group. Data included in this study were, however, collected from 22 breeding pairs (11 reduced and 11 enlarged broods) and from 11 non-breeding pairs randomly chosen because some breeding pairs did not breed, bred asynchronously, had eggs (the whole or part of the clutch) that failed to hatch or had nestlings that died in the first days of life. Therefore, these pairs were not suitable for answering the questions of our experiment.

Groups did not differ in body mass (overall mean ± s.e., 19.97±0.27 g; P=0.91), tarsus length (overall mean ± s.e., 20.28±0.09 mm; P=0.17) and age (overall mean ± s.e., 2.70±0.06 years; P=0.45). Between-sex variation in body mass, tarsus length and age was similar across experimental groups (GLM, group×sex, P≥0.58). There were also no differences in initial values of all parameters of oxidative status (GLM, group×sex, P≥0.15).

In order to avoid any influence of variation in diet quality, both breeding and non-breeding birds were provided with canary seed mixture (van Camp, Belgium), water, shell grit and cuttlefish bone *ad libitum*. We also provided both the breeding and non-breeding birds with egg food (van Camp, Belgium) twice a week and daily after the nestlings had hatched. Cages of breeding pairs were equipped with nest boxes and nesting material.

A first sample of blood was collected from the wing vein on the day birds were moved from the indoor aviaries to the breeding cages (i.e. mid-January). A second sample of blood was taken when nestlings in a brood were 12 days old because this age falls within the maximal (i.e. linear) phase of growth (Müller et al., 2010). The same number of non-breeding males and females was bled concomitantly with breeding birds to avoid any influence of temporal variation in bleeding period.

Nests were checked daily. Laying activity started around 3 weeks after the first blood sample was taken. All pairs included in this experiment laid eggs synchronously. In order to limit hatching asynchrony, the first two eggs laid by a female were swapped with dummy eggs and were then put back in the nest soon after the female laid her third egg. When nestlings were 1 day old, the brood size was manipulated so as to create two groups: (i) pairs raising a reduced brood (2–3 nestlings); (ii) pairs raising an enlarged brood (5–6 nestlings). The manipulation resulted in the following brood sizes (means ± s.e.): reduced brood group, 2.45±0.52; enlarged brood, 5.45±0.69 (Z=–4.1, P<0.001). At the end of the experiment (i.e. when nestlings were 12 days old), brood sizes were as follows: reduced brood group, 2.0±0.0; enlarged brood, 4.5±0.82; rank test (Z=–4.3, P<0.001). Cumulative brood mass at the end of the experiment was significantly higher in enlarged than in reduced broods (Z=–3.3, P=0.001).

Analyses of blood oxidative status

We assessed blood oxidative status using assays commonly applied to birds, as well as to other vertebrates (e.g. Costantini and Dell'Omo, 2006; Beaulieu et al., 2011; van de Crommenacker et al., 2010; Garratt et al., 2011; Garratt et al., 2013; Montgomery et al., 2011; Costantini et al., 2013).

The d-ROMs assay (Diacron International, Grosseto, Italy) was used to measure plasma oxidative damage compounds (mostly hydroperoxides) that are generated early in the oxidative cascade. The small interference of the enzyme ceruloplasmin that was found in humans (Alberti et al., 2000) did not occur in canary plasma. Inhibition of ceruloplasmin activity with 50 µmol l^{-1} or 1 mmol l^{-1} of sodium azide (inhibitor of ceruloplasmin activity; Sigma-Aldrich, code 08591) did not cause any decrease in absorbance [paired t-test, P≥0.18; mean ± s.d. of readings of absorbance values: no addition of sodium azide, 0.132±0.015; addition of 50 µmol l^{-1} sodium azide, 0.134±0.017; addition of 1 mmol l^{-1} sodium azide, 0.136±0.017; coefficient of variation (CV) of measures: 3.03±1.75%]. Moreover, the reaction of a dilution series of cumene hydroperoxide with the d-ROMs reagents was highly linear (range 0–4.5 µmol l^{-1}, R^2=0.9996; physiological values in vertebrates) at the temperature incubation of 37°C required by the manufacturer's instructions. Incubation at lower temperatures (4 and 24°C) reduced the efficiency of the Fenton reaction (i.e. the chemical reaction of the d-ROMs assay), as testified by a strong and similar reduction in absorbance of both plasma samples and cumene hydroperoxide. Analyses of reactive oxygen metabolites were therefore performed according to the manufacturer's instructions as in previous studies. Quality controls (Diacron International) were also assessed in each assay. Values of reactive oxygen metabolites are expressed as mmol l^{-1} of H_2O_2 equivalents. Analyses were done in duplicate and the mean intra- and inter-assay CV was 2.5% and 9.6%, respectively.

The Protein Carbonyl Colorimetric assay (Cayman Chemical Company, Ann Arbor, MI, USA) was used to measure the plasma concentration of protein carbonyls. The assay is based on the protocol of Levine et al. (Levine et al., 1990). Protein carbonyls indicate oxidative damage to proteins caused by lipid peroxidation (free radical) products [malondialdehyde and hydroxynonenal (Halliwell and Gutteridge, 2007)]. All plasma samples were first diluted with distilled water in order to have a concentration of 2 mg protein ml^{-1}, as measured using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA) using albumin as a reference standard. Nucleic acids were removed by adding 1 volume of a 10% solution of

streptomycin sulphonate (Sigma-Aldrich, code S6501) to 9 volumes of sample. Then, analyses were done according to the protocol of Levine et al. (Levine et al., 1990). A control plasma sample was also assessed in each assay. The concentration of protein carbonyls is expressed as nmol mg^{-1} protein or as total by multiplying the concentration per mg of protein by the total concentration of proteins in the plasma. Analyses were done in duplicate and the mean intra- and inter-assay CV was 13.6% and 19.1%, respectively.

The –SHp test (Diacron International) was used to quantify the concentration of both non-protein and protein thiols in haemolysate. First, red blood cells were diluted 1:100 with distilled water. Then, an aliquot of each haemolysate was further diluted 1:1 with cold distilled water or with cold trichloroacetic acid (30%) to precipitate proteins. Samples were then centrifuged at 6000 rpm for 2 min. The supernatant of samples diluted in only distilled water was used to measure the total concentration of thiols, while the haemolysates diluted with trichloroacetic acid were used to measure the concentration of non-protein thiols. The concentration of protein thiols was then calculated as the difference between total thiols and non-protein thiols. Analyses were done according to manufacturer's instructions. Quality controls (Diacron International) were also assessed in each assay. Concentrations of thiol groups are expressed as mmol l^{-1} of –SH groups. Analyses were done in duplicate and the mean intra- and inter-assay CV was 8.3% and 7.4%, respectively.

The DetectX Ceruloplasmin Activity Kit (Arbor Assays, Ann Arbor, MI, USA) was used to measure the activity of ceruloplasmin in plasma. Ceruloplasmin is an inflammation-inducible protein that limits the spread of oxidative damage across tissues (Pacht and Davis, 1988). For example, increased physical effort (e.g. during the nestling rearing period) may raise ceruloplasmin because of inflammatory processes that occur in the muscles (Liesen et al., 1977; Kenyon et al., 2011). Plasma samples were diluted 1:30 and were then analysed according to manufacturer's instructions. The activity of ceruloplasmin is expressed as U ml^{-1}. Analyses were done in duplicate and the mean intra- and inter-assay CV was 3.6% and 6.2%, respectively.

Statistical analyses

Statistical analyses were carried out using SAS Version 9.3 (Cary, NC, USA). Linear mixed models with a repeated measures design were used to test the effects of our treatment on blood oxidative status parameters. Response variables were as follows: reactive oxygen metabolites, protein carbonyls (expressed as per unit of protein or total), non-protein thiols, protein thiols and ceruloplasmin. In each model, we included treatment group (non-breeding, reduced brood, enlarged brood), sex (male and female) and sampling period (pre- and post-manipulation) as fixed factors; individual (nested within pair) and pair were included as random factors. The number of the laboratory assay was included as random factor to control for the inter-assay random variation only when its inclusion reduced the Akaike information criterion (AIC) ≥2. However, its inclusion or exclusion did not alter the output of models. We also included two- and three-way interactions among fixed factors. We used a backward elimination process to exclude independent variables with $P>0.05$, starting from the three-way interaction. *Post hoc* comparisons were performed using the Tukey test when we found a statistically significant interaction effect. Dependent variables were transformed where needed to respect assumptions of normality of residuals and homoscedasticity.

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Competing interests

The authors declare no competing financial interests.

Author contributions

D.C. and M.E. designed the study; D.C. and G.C. collected samples; D.C. and G.C. analysed samples and data; D.C. wrote the manuscript with contributions from G.C. and M.E.

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References

Alberti, A., Bolognini, L., Macciantelli, D. and Caratelli, M. (2000). The radical cation of N,N-diethyl-para-phenylendiamine: a possible indicator of oxidative stress in biological samples. *Research on Chemical Intermediates* **26**, 253-267.

Alonso-Alvarez, C., Bertrand, S., Devevey, G., Prost, J., Faivre, B. and Sorci, G. (2004). Increased susceptibility to oxidative stress as a proximate cost of reproduction. *Ecol. Lett.* **7**, 363-368.

Angilletta, M. J., Jr and Sears, M. W. (2000). The metabolic cost of reproduction in an oviparous lizard. *Funct. Ecol.* **14**, 39-45.

Beaulieu, M., Reichert, S., Le Maho, Y., Ancel, A. and Criscuolo, F. (2011). Oxidative status and telomere length in a long-lived bird facing a costly reproductive event. *Funct. Ecol.* **25**, 577-585.

Beaulieu, M., Haas, A. and Schaefer, H. M. (2014). Self-supplementation and effects of dietary antioxidants during acute thermal stress. *J. Exp. Biol.* **217**, 370-375.

Boonekamp, J. J., Salomons, M., Bouwhuis, S., Dijkstra, C. and Verhulst, S. (2014). Reproductive effort accelerates actuarial senescence in wild birds: an experimental study. *Ecol. Lett.* **17**, 599-605.

Casagrande, S., Dell'omo, G., Costantini, D., Tagliavini, J. and Groothuis, T. (2011). Variation of a carotenoid-based trait in relation to oxidative stress and endocrine status during the breeding season in the Eurasian kestrel: a multi-factorial study. *Comp. Biochem. Physiol.* **160A**, 16-26.

Cichoń, M., Olejniczak, P. and Gustafsson, L. (1998). The effect of body condition on the cost of reproduction in female Collared Flycatchers *Ficedula albicollis*. *Ibis* **140**, 128-130.

Costantini, D. (2014). *Oxidative Stress and Hormesis in Evolutionary Ecology and Physiology*. Berlin; Heidelberg: Springer-Verlag.

Costantini, D. and Dell'Omo, G. (2006). Effects of T-cell-mediated immune response on avian oxidative stress. *Comp. Biochem. Physiol.* **145A**, 137-142.

Costantini, D., Monaghan, P. and Metcalfe, N. B. (2013). Loss of integration is associated with reduced resistance to oxidative stress. *J. Exp. Biol.* **216**, 2213-2220.

Costantini, D., Bonisoli-Alquati, A., Rubolini, D., Caprioli, M., Ambrosini, R., Romano, M. and Saino, N. (2014a). Nestling rearing is antioxidant demanding in female barn swallows (*Hirundo rustica*). *Naturwissenschaften* **101**, 541-548.

Costantini, D., Meillère, A., Carravieri, A., Lecomte, V., Sorci, G., Faivre, B., Weimerskirch, H., Bustamante, P., Labadie, P., Budzinski, H. et al. (2014b). Oxidative stress in relation to reproduction, contaminants, gender and age in a long-lived seabird. *Oecologia* **175**, 1107-1116.

Daan, S., Deerenberg, C. and Dijkstra, C. (1996). Increased daily work precipitates natural death in the kestrel. *J. Anim. Ecol.* **65**, 539-544.

Dijkstra, C., Bult, A., Bijlsma, S., Daan, S., Meijer, T. and Zijlstra, M. (1990). Brood size manipulations in the kestrel (*Falco tinnunculus*): effects on offspring and parent survival. *J. Anim. Ecol.* **59**, 269-285.

Fletcher, Q. E., Selman, C., Boutin, S., McAdam, A. G., Woods, S. B., Seo, A. Y., Leeuwenburgh, C., Speakman, J. R. and Humphries, M. M. (2013). Oxidative damage increases with reproductive energy expenditure and is reduced by food-supplementation. *Evolution* **67**, 1527-1536.

Garratt, M., Vasilaki, A., Stockley, P., McArdle, F., Jackson, M. and Hurst, J. L. (2011). Is oxidative stress a physiological cost of reproduction? An experimental test in house mice. *Proc. Biol. Sci.* **278**, 1098-1106.

Garratt, M., Pichaud, N., King, E. D. A. and Brooks, R. C. (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**, 2879-2888.

Halliwell, B. H. and Gutteridge, J. M. C. (2007). *Free Radicals in Biology and Medicine*, 4th edn. Oxford: Oxford University Press.

Hamel, S., Côté, S. D., Gaillard, J. M. and Festa-Bianchet, M. (2009). Individual variation in reproductive costs of reproduction: high-quality females always do better. *J. Anim. Ecol.* **78**, 143-151.

Jelena, A., Mirjana, M., Desanka, B., Svetlana, I. M., Aleksandra, U., Goran, P. and Ilijana, G. (2013). Haptoglobin and the inflammatory and oxidative status in experimental diabetic rats: antioxidant role of haptoglobin. *J. Physiol. Biochem.* **69**, 45-58.

Jones, D. P. (2006). Redefining oxidative stress. *Antioxid. Redox Signal.* **8**, 1865-1879.

Kenyon, C. L., Basaraba, R. J. and Bohn, A. A. (2011). Influence of endurance exercise on serum concentrations of iron and acute phase proteins in racing sled dogs. *J. Am. Vet. Med. Assoc.* **239**, 1201-1210.

Law, R. (1979). The cost of reproduction in annual meadow grass. *Am. Nat.* **113**, 3-16.

Levine, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, I., Lenz, A. G., Ahn, B. W., Shaltiel, S. and Stadtman, E. R. (1990). Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol.* **186**, 464-478.

Liesen, H., Dufaux, B. and Hollmann, W. (1977). Modifications of serum glycoproteins the days following a prolonged physical exercise and the influence of physical training. *Eur. J. Appl. Physiol. Occup. Physiol.* **37**, 243-254.

Losdat, S., Helfenstein, F., Gaude, B. and Richner, H. (2011). Reproductive effort transiently reduces antioxidant capacity in a wild bird. *Behav. Ecol.* **22**, 1218-1226.

Metcalfe, N. B. and Monaghan, P. (2013). Does reproduction cause oxidative stress? An open question. *Trends Ecol. Evol.* **28**, 347-350.

Montgomery, M. K., Hulbert, A. J. and Buttemer, W. A. (2011). The long life of birds: the rat-pigeon comparison revisited. *PLoS ONE* **6**, e24138.
Müller, W., Vergauwen, J. and Eens, M. (2010). Testing the developmental stress hypothesis in canaries: consequences of nutritional stress on adult song phenotype and mate attractiveness. *Behav. Ecol. Sociobiol.* **64**, 1767-1777.
Ołdakowski, L., Piotrowska, Z., Chrząścik, K. M., Sadowska, E. T., Koteja, P. and Taylor, J. R. E. (2012). Is reproduction costly? No increase of oxidative damage in breeding bank voles. *J. Exp. Biol.* **215**, 1799-1805.
Pacht, E. R. and Davis, W. B. (1988). Role of transferrin and ceruloplasmin in antioxidant activity of lung epithelial lining fluid. *J. Appl. Physiol.* **64**, 2092-2099.
Reed, T. E., Kruuk, L. E. B., Wanless, S., Frederiksen, M., Cunningham, E. J. A. and Harris, M. P. (2008). Reproductive senescence in a long-lived seabird: rates of decline in late-life performance are associated with varying costs of early reproduction. *Am. Nat.* **171**, E89-E101.
Salomons, H. M. (2009). Fighting for fitness: telomeres, oxidative stress and life history trade-offs in a colonial corvid. PhD thesis, University of Groningen, Groningen, The Netherlands.
Santos, E. S. A. and Nakagawa, S. (2012). The costs of parental care: a meta-analysis of the trade-off between parental effort and survival in birds. *J. Evol. Biol.* **25**, 1911-1917.
Sohal, R. S. and Orr, W. C. (2012). The redox stress hypothesis of aging. *Free Radic. Biol. Med.* **52**, 539-555.
Speakman, J. R. and Garratt, M. (2014). Oxidative stress as a cost of reproduction: beyond the simplistic trade-off model. *BioEssays* **36**, 93-106.
Stearns, S. C. (1992). *The Evolution of Life Histories*. Oxford: Oxford University Press.
Stier, A., Reichert, S., Massemin, S., Bize, P. and Criscuolo, F. (2012). Constraint and cost of oxidative stress on reproduction: correlative evidence in laboratory mice and review of the literature. *Front. Zool.* **9**, 37.
van de Crommenacker, J., Komdeur, J., Burke, T. and Richardson, D. S. (2011). Spatio-temporal variation in territory quality and oxidative status: a natural experiment in the Seychelles warbler (*Acrocephalus sechellensis*). *J. Anim. Ecol.* **80**, 668-680.
Weathers, W. W. (1985). Energy cost of incubation in the canary. *Comp. Biochem. Physiol.* **81A**, 411-413.
Wiersma, P., Selman, C., Speakman, J. R. and Verhulst, S. (2004). Birds sacrifice oxidative protection for reproduction. *Proc. Biol. Sci.* **271**, S360-S363.
Yang, D.-B., Xu, Y.-C., Wang, D.-H. and Speakman, J. R. (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**, 4242-4250.