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Experimental evidence that oxidative stress influences reproductive decisions

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Summary

1. There is considerable interest of evolutionary ecologists in the proximate mechanisms that constrain life-history variation. It is increasingly recognised that oxidative stress may be a prime physiological constraint on reproduction, but to the best of our knowledge this has never been tested experimentally.
2. To fill in this gap, we examined whether a specific and short-term experimental increase of pre-reproductive oxidative stress in females of a songbird (canary, *Serinus canaria*) would influence reproductive decisions (i.e., when and how many eggs to lay), and reproductive success (hatching and fledging success, number of hatchlings and of fledglings produced by each female), as compared to females whose oxidative stress levels were not manipulated.
3. Our experimental reduction of glutathione, a key antioxidant, increased oxidative stress and affected reproductive decisions: treated females significantly delayed the start of egg laying and laid significantly smaller clutches. However, both hatching and fledging success and the number of hatchlings and of fledglings produced by each female were similar between control and treated females.
4. Our results support the hypothesis that oxidative stress may be one proximate mechanism modulating key life history traits (such as the timing of laying and clutch size in birds) and therefore may act as a link between prevailing environmental conditions and fitness traits.

Key-words: Antioxidants, clutch size, constraint, glutathione, life history, oxidative damage, reproduction, *Serinus canaria*

Introduction

Life history research has been a topic of interest to evolutionary ecologists for several decades. It seeks to describe the diversity among organisms in their rate of growth, age at maturity, longevity or reproductive success (e.g., hatching and fledging success in birds; litter size in mammals) and to determine what causes differences in evolutionary fitness among life-history variants within and among species (Roff 1992; Stearns 1992). Current theory proposes that life history traits cannot be simultaneously maximised because there are costs paid in the currency of fitness when a change in one trait, for example increased reproduction, results in a detrimental change in another, for example decreased survival (Roff 1992; Stearns 1992). The underlying reasoning is that resources (e.g., nutrients, energy, time) are finite and this gives rise to trade-offs in their allocation among traits, activities or processes that compete for the same resource. Therefore, allocation of a resource to one trait means that less can be invested to other traits. This simple concept, referred to as a trade-off, has had a prominent role in our understanding of how life history variation arises.

There are also constraints that limit trait expression, implying that the ability of the phenotype to evolve is inhibited or canalised along certain paths (Schwenk 2002). It is recognised that elucidating the physiological mechanisms that work as constraints is essential to our understanding of what causes negative associations among life history traits and diversification of life histories (Zera & Harshman 2001; Ricklefs & Wikelski 2002). Work on physiological constraints of reproduction has had a prominent role in life history research. For example, nutrient and energy limitation in females lead to reduced allocation of nutrients to their offspring (Derrickson & Lowas 2007; Speakman 2008; Surai 2002), reduced reproductive success (Koskela *et al.* 1998; Ruffino *et al.* 2014) or reduced capacity to

withstand the metabolic demands of offspring rearing (Fletcher *et al.* 2013; Giordano, Costantini & Tschirren 2015).

Constraints may also be manifested at the cellular level. One mechanism thought to be particularly important in this regard is oxidative stress (OS), a complex multifaceted biochemical condition of the organism that occurs when generation of oxidative molecular damage increases. In the last decade, there has been increasing interest of evolutionary ecologists in OS as a prime physiological constraint driving the outcome of many life history tradeoffs because the resultant tissue degradation may influence reproductive success, growth patterns, senescence and survival (Costantini 2008, 2014; Metcalfe & Alonso-Alvarez 2010; Isaksson, Sheldon & Uller 2011; Blount *et al.* 2015; Beaulieu *et al.* 2015). Much work has been done on whether reproduction results in OS. This work showed that oxidative damage is often positively associated with offspring rearing effort across females of various species, but also that oxidative damage is lower in reproducing females than in non-reproducing females (reviewed in Blount *et al.* 2015). These studies suggested that females might need to avoid OS prior to reproduction because OS may constrain their reproductive decisions (e.g., when and how many eggs to lay) and success or be costly to developing young. However, few studies have addressed whether pre-reproduction OS influences reproductive decisions and success. Moreover, to the best of our knowledge, experimental studies manipulating OS specifically are lacking. In humans, high OS is increasingly recognised as a cause of decreased female fertility or increased health issues for new-borns (Peter Stein *et al.* 2008; Ruder, Hartman & Goldman 2009). In other organisms, correlative evidence showed that female oxidative damage measured prior to reproduction was negatively associated with litter size at birth (Stier *et al.* 2012). Similarly, female oxidative damage measured while incubating was negatively associated with reproductive success (Costantini 2014). Although

these studies suggest that OS might work as a constraint on reproduction, it is now urgently needed to address this OS-constraint hypothesis experimentally.

In this study, we tested for the first time whether an experimental increase of pre-reproductive OS in females of a songbird (canary, *Serinus canaria*) would influence reproductive decisions and reproductive success as compared to females whose OS levels were not manipulated. To this end, we reduced specifically the body concentration of a key antioxidant (glutathione) in females before the start of reproduction in order to induce OS. Then we examined the effects of the treatment on multiple key reproductive traits, including laying date, clutch size, hatching success, fledging success, and number of hatchlings and of fledglings produced by each female.

Material and methods

HOUSING CONDITIONS AND EXPERIMENTAL SETUP

This study was conducted on a captive colony of canaries. Canaries are considered as seasonal opportunistic breeders because they do not rely on photoperiod only, but also react to changes in environmental conditions (Leitner, Van't Hof & Gahr 2003). On the 28th of March 2015, both male and female canaries were moved from our single-sex outdoor to single-sex indoor aviaries. Birds were kept on a long-day photoperiod (14:10 h light to dark) to stimulate their reproductive activity. On the 8th of April 2015, females were moved from aviaries to single breeding cages (50×61×40 cm; GEHU cages, the Netherlands) maintained in a room on a 14:10 light to dark photoperiod and at room temperature of 19–23 °C. The room temperature was within the thermoneutral zone of canaries (Weathers 1985). One week later, the manipulation of OS was started. Half of the females (N=23) were randomly allocated to the experimental group whose OS level was increased by decreasing the glutathione concentration, while the second half of females (N=23) were allocated to the

control group. All females were three years old and had previous breeding experience, but they did not have any previous breeding experience with their partners in this experiment.

To decrease the glutathione concentration, treated females were injected intramuscularly with 100 µl of a solution containing 50 mg of DL-buthionine-(S,R)-sulfoximine (Sigma-Aldrich B2640) per ml of saline solution (PBS). Sulfoximine reduces cellular levels of glutathione by inhibiting its synthesis (Griffith & Meister 1979; Bailey 1998). The injection was done every second day for a total of four times, resulting in an amount of 20 mg of sulfoximine given to each treated female. This amount of sulfoximine was chosen according to previous work on other songbird species (Galván & Alonso-Alvarez 2008; Romero-Haro & Alonso-Alvarez 2015). This resulted in variable doses among females (range of body mass of treated females from 16.5 to 19.6 grams), but the body mass did not differ between treatment groups nor was it affected by the treatment (see Table 1). The females in the control group were subjected to the same regime, but injected with PBS only. A sample of venous blood was taken from the wing vein the day before the first injection and the day after the last injection. The blood was collected by venipuncture using a heparinized microvette (Sarstedt, Nümbrecht, Germany). Blood samples were maintained cool and were then centrifuged to separate plasma from red blood cells. Samples were stored at -80°C. The body mass of each female was taken using an electronic balance (0.01 grams) the day before the first injection and again the day after the last injection.

The day after the post-treatment sample of blood was taken, each female was randomly mated with a non-related sexual partner randomly taken from our single-sex indoor aviaries containing males of the same age. In order to avoid any influence of variation in diet quality, all birds were provided with canary seed mixture (van Camp, Belgium), water, shell

grit, and cuttlefish bone. Birds were also provided with egg food (van Camp, Belgium) daily after the nestlings had hatched. Cages were equipped with nest boxes and nesting material.

ANALYSES OF BLOOD OXIDATIVE STATUS METRICS

In order to validate the effect of treatment on glutathione concentration and oxidative damage, two methods commonly applied to vertebrates were used (e.g., Costantini *et al.* 2006; van de Crommenacker *et al.* 2010; Sinha *et al.* 2014). Firstly, high-performance liquid chromatography with electrochemical detection was applied for determination of reduced glutathione (GSH) in red blood cells by a Reversed-Phase HPLC of Shimadzu (Hai Zhonglu, Shanghai). The protocol as described in Sinha et al. (2014) was applied. Concentrations of GSH were expressed as μmol g^{-1} fresh weight of red blood cells. Secondly, the d-ROMs assay (Reactive Oxygen Metabolites; Diacron International, Grosseto, Italy) was used to measure plasma oxidative damage metabolites (mostly organic hydroperoxides) that are generated early in the oxidative cascade. Our work relied on this metric of oxidative damage because GSH is used to detoxify the organism from organic hydroperoxides (Halliwell & Gutterdige 2007). Hence, a decrease of GSH is expected to result in an increase of organic hydroperoxides (Halliwell & Gutterdige 2007). Analyses of reactive oxygen metabolites were done according to manufacturer's instructions as in previous studies. Quality controls (Diacron International) were also assessed in each assay. Values of reactive oxygen metabolites have been expressed as mM of H_2O_2 equivalents.

STATISTICAL ANALYSES

Analyses were run using R (version 3.1.1; R Core Team, 2013). Two control and two treated females were excluded from the analyses because they did not breed during the experimental period. Linear mixed models (lmer in package 'lme4') with a repeated measures design were

used to assess the effect of treatment on glutathione concentration in red blood cells, on plasma oxidative damage and on body mass (see Table S1 in Supporting Information). In each model, treatment group, sampling period (pre- and post-treatment values) and their interaction were included as fixed factors; individual female was entered as a random factor. Welch two-sample t-test was used to assess the effect of treatment on laying date (square-root transformed) and clutch size (see Table S2 in Supporting Information). General linear models with a binomial error distribution were used to test the effect of treatment on hatching success and fledging success. To do this, two variables were bound together using 'cbind' into a single one; these two variables comprised the number of hatched and unhatched eggs for hatching success and of nestlings that fledged or died for fledging success, respectively. This approach was used because the use of percentages as response variable raises a few concerns: the errors are not normally distributed; the variance is not constant; the response is bounded; information of the sample size from which the percentage is estimated is lost (chapter 16 in Crawley 2007). The number of hatchlings and of fledglings produced by each female (see Table S2 in Supporting Information) was compared between groups using a Welch two-sample t-test. For each significant outcome, the effect size was calculated as Pearson's r (plus confidence interval) using the package 'compute.es'.

Results

The experimental treatment induced blood oxidative stress, but it did not affect body mass. Compared with levels in control females and pre-manipulation values, the concentration of GSH in red blood cells decreased significantly in treated females (Table 1, Fig. 1). The effect size (expressed as r Pearson) was 0.68 (95% confidence interval: 0.46 to 0.82). As expected, a decrease of GSH resulted in an increase of our metric of oxidative damage. Compared with levels in control females and pre-manipulation values, plasma oxidative damage increased

significantly in treated females (Table 1, Fig. 1). The effect size (expressed as r Pearson) was 0.60 (95% confidence interval: 0.35 to 0.77). Control and stressed females did not differ in body mass at the beginning (mean±SE: control 18.6±0.3 grams, stressed 17.6±0.2 grams) and at the end (mean±SE: control 18.6±0.4 grams, stressed 17.6±0.2 grams) of the treatment (Table 1).

As compared to control females, treated females started egg laying significantly later ($t_{39.9}$ = -2.28, n = 21 pairs each, P = 0.028; Fig. 2) and laid significantly smaller clutches ($t_{39.0}$ = 2.09, n = 21 pairs each, P = 0.044; Fig. 2). The effect size was 0.34 (95% confidence interval: 0.03 to 0.59) and 0.31 (95% confidence interval: 0 to 0.57) for laying date and clutch size, respectively. Both hatching success (z = 0.09, n = 21 pairs each, P = 0.93; Fig. 2) and fledging success (z = 0.84, n = 17 pairs each, P = 0.40; Fig. 2) did not differ between the two experimental groups. Control and experimental groups did not differ in the number of hatchlings ($t_{38.9}$ = 0.58, P = 0.57) and of fledglings ($t_{29.0}$ = -0.39, P = 0.70) produced by each female (Fig. 2).

Discussion

The results of our study support the hypothesis that OS may be one proximate mechanism modulating important aspects of reproduction. Using Cohen's benchmarks for interpreting effect sizes (Cohen 1988), we found that a short-term, large and significant increase of OS, resulted in a medium and significant delay of egg laying and reduction of clutch size, respectively. In contrast, hatching and fledging success, as well as the number hatchlings and of fledglings produced by each mother did not differ between control and stressed females. Although the fledging success was in the range of previous work on canaries (Hinde 1959; Beguin *et al.* 2006), it should be considered as low. The increase of damage and decrease of

glutathione were both within the physiological range; the effect size for both metrics of OS was actually similar to that of previous studies that tested the effect of various endogenous and exogenous stressors on the oxidative balance in both wild and captive vertebrates (e.g., immune activation and inflammation, Schneeberg, Czirják & Voigt 2013; thermal stress, Costantini, Monaghan & Metcalfe 2012; exposure to contaminants, Isaksson 2010; reproductive effort, Blount *et al.* 2015). This result suggests that OS may be universally involved as one mechanism contributing to the effects of environmental stressors on individual fitness.

When to start reproducing and how many offspring to have are critical decisions for animals. In vertebrates, females may optimise reproduction by adjusting their start of reproduction and the number of eggs laid or offspring produced to the prevailing environmental conditions. Yet despite considerable interest across a range of disciplines, the physiological mechanisms involved in the determination of both reproductive decisions and success remain poorly understood (Williams 2012). Our data suggest that OS might be one candidate proximate mechanism underlying the link between prevailing environmental conditions and reproductive decisions. In other words, animals might balance their reproductive decisions against OS in order to maximise their lifetime reproductive success. The risk of OS is, however, not constant, rather it likely varies in time and space during an individual's lifetime. Individuals should therefore have an ability to assess short-term changes in OS and adjust their reproductive decisions accordingly. One way to do so might be through hormonal regulation of reproductive decisions (Schwabl 1996; Sockman and Schwabl 1999) because OS may reduce production of reproductive hormones that regulate the transition from a non-reproductive to a reproductive status (Agarwal and Allamaneni 2004). The question then is why it is important for females to balance reproduction against

OS. One answer may lie with the detrimental effects of OS on fertility, and embryo development and survival (Costantini 2014). For example, it has been shown that high OS in oocytes reduces their probability to mature or to be fertilised (Knapen *et al.* 1999; Agarwal and Allamaneni 2004). In mammals, either high placental or systemic OS have been associated with defective embryogenesis and pregnancy loss (Gupta et al. 2007; Al-Gubory, Fowler & Garrel 2010). Moreover, generation of eggs may itself increase OS across females of various species (Travers *et al.* 2010; Olsson *et al.* 2012), potentially exacerbating any detrimental effects of OS on female condition and reproductive success. Importantly, oxidative damage products may also result in degeneration of yolk nutrients (Grune *et al.* 2001), potentially affecting embryo development and hatching probability. Our data give some support to recent suggestions that females need to reduce or to avoid increased OS before reproduction (Costantini, Casasole & Eens 2014; Blount *et al.* 2015). Recently, Blount *et al.* (2015) hypothesised that in vertebrates the cost of reproduction is mediated by dual impacts of maternally-derived oxidative damage on mothers and offspring, and that mothers may be adapted to diminish such damage. For example, pre-breeding reductions in oxidative damage in reproducing females may serve to shield gametes and developing offspring (Blount *et al.* 2015). Given the transient nature of OS (Losdat *et al.* 2011; Costantini 2014), delaying the start of reproduction may enable the organism to reduce oxidative damage back to baseline levels. If so, this strategy might explain why treated females had a reproductive success (hatching and fledging success, number of young produced) similar to that of control females. We do not know why this has occurred. It might be that the natural variation in hatching success was enough to offset the moderate effect on clutch size caused by treatment. Given the normal but low reproductive success, we suggest that further studies will be needed to examine how OS before reproduction may affect reproductive success.

Environmental conditions outside of captivity can vary in time and space, indicating that delaying reproduction as we observed in the treated females might become maladaptive if conditions get worse with time. For example, in seasonal environments, availability of food decreases with the progress of the reproductive season, resulting in lower chances of success for those individuals that reproduce late in the season (Verhulst & Nilsson 2008; Voigt *et al.* 2011). Similarly, delaying reproduction in order to limit negative effects of OS for reproductive success may be costly if on a strict reproductive schedule. Experimental testing of these hypotheses is an important area for future research.

In many iteroparous species individual fitness components, such as reproductive decisions and success, are also strongly affected by female age (Clutton-Brock 1984; Proaktor, Milner-Gulland & Coulson 2007). Moreover, resistance to oxidative stress may also change with age (reviewed in Costantini 2014). Our experiment included females of the same age (3 years old), hence we can safely exclude any bias due to variation in age. It would be interesting in future studies to assess the interplay among female age, resistance to OS and reproductive decisions and success.

In conclusion, our study showed that a short-term experimental increase of blood OS affected female reproductive decisions (i.e., start of laying and clutch size). In contrast, the treatment did not show any evident effect on reproductive success. Our results point to OS as a mechanism linking prevailing environmental conditions to female reproductive decisions. Future challenges include determining the way both males and females balance their reproductive decisions against OS under different circumstances, such as in environments that differ with regard to predation risk or stability of resources. Our work was limited to testing effects of a short-term increase of OS. It will be interesting to replicate this

experiment to test the effect of a prolonged state of OS on reproductive decisions and reproductive success.

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Data accessibility

Data are provided as supplementary material.

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Table captions

Table 1. Outcomes of linear mixed models with a repeated measures design used to assess the effect of treatment on glutathione concentration in red blood cells, plasma oxidative damage

and body mass. Sampling period refers to pre- and post-manipulation levels of plasma oxidative damage, reduced glutathione and body mass.

Figure captions

Figure 1. Pre- and post-manipulation levels of reduced glutathione and plasma oxidative damage of canaries in relation to treatment. The experimental treatment was able to decrease red blood cell concentration of reduced glutathione and to increase plasma oxidative damage (reactive oxygen metabolites). Means that do not share the same letter are significantly different from each other (Tukey, $P < 0.05$). Data are shown as mean ± standard error.

Figure 2. Reproductive traits in relation to treatment. As compared to controls, those females whose oxidative stress was experimentally increased significantly delayed the start of egg laying and laid significantly smaller clutches. Control and experimental groups did not differ in hatching success, fledging success, number of hatchlings and of fledglings produced by each female. Laying date is expressed as days after the second bleeding (i.e., day 0). For illustrative purposes, hatching success and fledging success are shown as percentages. * indicates $P < 0.05$. Data are shown as mean ± standard error.

Dependent variable	Effect	Estimate	df	t-value	P
Reactive oxygen metabolites (oxidative damage metric)	Treatment group	-0.092	76.9	-2.701	0.0085
	Sampling period	-0.097	40.0	-3.524	0.0011
	Treatment group × sampling period	0.082	40.0	4.698	< 0.001
Reduced glutathione (endogenous antioxidant)	Treatment group	5.202	64.8	4.078	0.0001
	Sampling period	4.320	40.0	3.728	0.0006
	Treatment group × sampling period	-4.244	40.0	-5.791	< 0.001
Body mass	Treatment group	-0.753	78.7	-1.440	0.154
	Sampling period	0.219	40.0	0.601	0.551
	Treatment group × sampling period	-0.142	40.0	-0.619	0.539

Table 1