Intergenerational Costs of Oxidative Stress: Reduced Fitness in Daughters of Mothers That Experienced High Levels of Oxidative Damage during Reproduction

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ABSTRACT

Parental condition transfer effects occur when the parents' physiological state during reproduction affects offspring performance. Oxidative damage may mediate such effects, yet evidence that oxidative damage experienced by parents during reproduction negatively affects offspring fitness is scarce and limited to early life stages. We show in Japanese quail (*Coturnix japonica*) that maternal levels of oxidative damage, measured during reproduction, negatively predict the number of offspring produced by daughters. This maternal effect on daughters' reproductive success was mediated by an effect on hatching success rather than on the number of eggs laid by daughters. We also observed a negative association between fathers' oxidative damage levels and the number of eggs laid by daughters but a positive association between fathers' oxidative damage levels and the hatching success of those eggs. These opposing paternal effects canceled each other out, resulting in no overall effect on the number of offspring produced by daughters. No significant association between a female's own level of oxidative damage during reproduction and her reproductive success was observed. Our results suggest that oxidative damage experienced by parents is a better predictor of an individual's reproductive performance than oxidative damage experienced by the individual itself. Although the mechanisms underlying these parental condition transfer effects are currently unknown, changes in egg composition or (epi)genetic alterations of gametes may play a role. These findings highlight the importance of an intergenerational perspective when quantifying costs of physiological stress.

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Introduction

The phenotype of the parents can shape the phenotype of the offspring (Mousseau and Fox 1998; Qvarnström and Price 2001; Crean and Bonduriansky 2014). Such parental effects can be exerted during the gamete stage or during embryo or juvenile development and can often have long-lasting consequences for offspring fitness (Mousseau and Fox 1998). Parental effects can enhance offspring's ability to cope with environmental conditions and thus can be adaptive (Mousseau and Fox 1998; predictive adaptive response [Gluckman et al. 2005]). Alternatively, parental effects may merely be a (nonadaptive) consequence of the parents' condition during reproduction, often referred to as "parental transmissive effects" or "parental condition transfer effects" (Qvarnström and Price 2001; Marshall and Uller 2007; Crean and Bonduriansky 2014). As a consequence, offspring of parents with an enhanced physiological state display a better performance than those of parents with a poor physiological state (silver spoon effect; Grafen 1988; Monaghan 2008). Oxidative stress is a key physiological driver of biological processes (Metcalfe and Alonso-Alvarez 2010) and may mediate such parental condition transfer effects.

Oxidative stress is a dynamic process where the rate of reactive oxygen species (ROS) production exceeds the antioxidant defense capacity of an organism (Halliwell and Gutteridge 2007; Monaghan et al. 2009). Oxidative damage to proteins, lipids, or DNA is a direct consequence of nonalleviated oxidative stress and can impair physiological processes and individual performance (Halliwell and Gutteridge 2007; Monaghan et al. 2009). Oxidative stress may also disrupt the redox signaling pathways, but with poorly understood consequences (Ayala et al. 2014; Schieber and Chandel 2014). Cell metabolism, and cellular respiration in particular, contribute substantially to ROS production (Halliwell and Gutteridge 2007). However, extrinsic factors, such as radiation, changes in temperature, exposure to xenobiotics, and pollution, can also increase ROS levels in an organism (Araujo et al. 2008; Isaksson 2010), and indeed this additional burden may often tip the balance and result in oxidative damage (Isaksson 2010). High levels of oxidative damage

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are associated with the occurrence of certain diseases, such as cancer (Valko et al. 2007; Ayala et al. 2014), and they have been related to the ageing process (Martinez de Toda et al. 2020) and shorter life span (Noguera et al. 2012; Vitikainen et al. 2016), as well as impaired reproductive success (Stier et al. 2012). However, the causal role of oxidative damage in mediating these processes is still debated (e.g., Metcalfe and Alonso-Alvarez 2010; Speakman and Selman 2011; Blount et al. 2015; Alonso-Alvarez et al. 2017).

Importantly, the negative consequences of oxidative damage experienced by an individual might not be limited to the individual itself but may expand to the next generation (Blount et al. 2015). A parent experiencing oxidative damage may, for example, be constrained in its ability to optimally provision or care for the developing offspring (e.g., because of impaired uterus and placental function or reduced milk yield; Al-Gubory et al. 2010; Burton and Jauniaux 2011; Napierala et al. 2016, 2019). Alternatively, oxidized molecules may be directly transferred from the mother to the offspring (e.g., via the egg [Mohiti-Asli et al. 2008; Surai et al. 2016], placenta [Rossner et al. 2009], or milk [Napierala et al. 2019]) and influence offspring development. Furthermore, oxidative damage may induce (epi)genetic changes in the parents that may be transferred to the next generation (Velando et al. 2008; Aitken et al. 2016). For example, oxidative damage might shorten telomeres (Kalmbach et al. 2013; Heidinger and Young 2020) or affect DNA methylation patterns (Tunc and Tremellen 2009; Menezo et al. 2016) in gametes. As a result, oxidative damage experienced by a parent during reproduction may negatively affect offspring development and, ultimately, offspring fitness. Because of such potential parental condition transfer effects, it has been proposed that parents may actively decrease oxidative damage levels before reproduction to shield the offspring from harmful effects (oxidative shielding hypothesis; Blount et al. 2015). Yet to date, evidence for negative fitness consequences of oxidative damage experienced by the parents for the next generation is scarce and limited to early life stages (i.e., low birth weight and illness in infants [Al-Gubory et al. 2010; Napierala et al. 2016, 2019; Viblanc et al. 2018] and reduced early-life survival [Bize et al. 2008; Vitikainen et al. 2016; Dupoué et al. 2020]) or indirectly inferred from parental exposure to oxidative stress–related factors, such as xenobiotics (Hamlin and Guillette 2011). However, the consequences of oxidative damage experienced by parents for the long-term performance of the offspring are currently unknown. A long-term perspective is required, however, to fully understand the fitness consequences of oxidative damage experienced by parents for the next generation and to quantify the relative importance of oxidative damage–mediated parental condition transfer effects versus effects of oxidative damage experienced by the adult individual itself. Such an intergenerational view is essential not only to gain insights into the role of oxidative damage in life history evolution but also to assess the consequences of environmental stressors on the resilience and stability of natural populations.

Here, using Japanese quail (*Coturnix japonica*) as a study system, we quantified whether maternal and/or paternal levels of oxidative damage to lipids (quantified as plasma levels of malondialdehyde [MDA]) when the offspring were conceived predict key fitness components—namely, reproductive success and life span—in daughters. Furthermore, we quantified the relative importance of oxidative damage–mediated parental condition transfer effects versus effects of oxidative damage experienced by the focal individual itself on these fitness components. Given the short-term negative fitness consequences (i.e., reduced early-life survival) of parental oxidative damage observed in previous studies (Bize et al. 2008; Vitikainen et al. 2016; Dupoué et al. 2020), we predict that levels of parental oxidative damage will be negatively associated with daughters' fitness. Furthermore, on the basis of previous findings where high within-individual levels of oxidative damage were related to reduced fitness (Noguera et al. 2012; Stier et al. 2012; Costantini et al. 2016; Vitikainen et al. 2016), we predict a negative association between a focal female's oxidative damage levels and her reproductive success and life span.

Methods

Breeding Conditions

The study was conducted in a captive population of Japanese quail (*Coturnix japonica*) maintained in large outdoor aviaries (7 m × 5.5 m each) at the University of Zurich, Switzerland. Females were maintained in a single-sex aviary, and males were maintained in a mixed-sex aviary together with nonexperimental females (for a detailed description of the breeding and husbandry conditions, see Pick et al. 2016). For this study, adult males and females (age: 189–295 d old) were randomly selected from the population and housed for 3 wk in pairs ($N = 22$ randomly assigned breeding pairs) in breeding cages (122 cm × 50 cm × 50 cm) in the animal facility on a 16L : 8D cycle at approximately 20°C. Eggs were collected on the day that they were laid, weighed (to the nearest 0.01 g), and artificially incubated (Favorit, HEKA Brutgeräte). During the first 14 d, eggs were maintained at 37.8°C and 55% humidity. They were then transferred to a hatcher (Favorit, HEKA Brutgeräte) and kept at 37.6°C and 80% humidity until hatching. Chicks were kept in a heated cage (109 cm × 57 cm × 25 cm, Kükenaufzuchtbox Nr 4002/C, HEKA Brutgeräte) for 2 wk after hatching. For the first 5 d the temperature was kept at 35°C–38°C, then slowly lowered to 25°C over the next 9 d. After 2 wk, chicks were transferred to rearing cages within the breeding facility. At the age of 4 wk, the birds were released into the outdoor aviaries (for details, see Pick et al. 2016). Thus, all offspring were reared in mixed-family groups under standardized conditions. Body mass was measured at hatching (to the nearest 0.01 g) and at adulthood (i.e., when 6 mo old; to the nearest 1 g). We focused on daughters because males show a low variation in reproductive performance (Pick et al. 2017).

At the age of 6 mo, one randomly chosen virgin daughter per breeding pair (hereafter referred to as "focal females"; $N = 22$) was brought into the breeding facility and housed in a breeding cage with a random male from the population for 3 wk to determine their reproductive success during the reproductive event. To avoid potential effects of incubation conditions, the eggs were removed on the day that they were laid and incubated under standardized conditions, as described above, to determine hatching success and the number of offspring produced. After breeding, the focal females were moved back to the outdoor aviaries, where they were kept for their entire life to record their life span. All focal females thus

experienced the same standardized conditions from incubation to death. Death of the focal females occurred either naturally ($N = 17$) or by euthanization because they had reached a predefined humane end point ($N = 5$).

To quantify levels of oxidative damage, we took a blood sample from the parents of the focal females when they were moved into the breeding cages (i.e., when the focal female was conceived). We also blood sampled the focal females at the beginning of their reproductive event. Blood samples (approximately 100 μL) were taken from the brachial vein using heparinized capillary tubes. Samples were stored at 4°C until centrifugation (5 min at 20°C and 2,000 *g*) within 4 h. Plasma was then separated and frozen at −80°C until analysis.

Quantification of Lipid Oxidative Damage

Although oxidative stress does not always result in oxidative damage, oxidative damage is generated under nonalleviated oxidative stress conditions (Halliwell and Gutteridge 2007; Monaghan et al. 2009). Oxidative damage is thus a direct consequence of oxidative stress (Monaghan et al. 2009), and lipids are particularly susceptible to such damage (Del Rio et al. 2005; Hulbert et al. 2007). To quantify lipid oxidative damage in focal females and their parents, we measured plasma levels of MDA, one of the end point molecules in the lipid peroxidation cascade (Halliwell and Gutteridge 2007; Mateos and Bravo 2007). MDA is a commonly used marker of oxidative damage (Del Rio et al. 2005; Noguera et al. 2012; Vitikainen et al. 2016; Vágási et al. 2019), and plasma levels of MDA have been reported to reflect those in other tissues, thus being a proxy for whole-body oxidative damage (Argüelles et al. 2004; Margaritelis et al. 2015). MDA has also been found to be an extremely toxic and mutagenic molecule with high reactivity, interacting with DNA and proteins (Del Rio et al. 2005; Nair et al. 2007). High MDA concentrations have been related to numerous illnesses in humans (reviewed in Ayala et al. 2014), as well as impaired fitness in nonhuman animals (Noguera et al. 2012; Vitikainen et al. 2016; Vágási et al. 2019).

MDA quantification was performed using high-performance liquid chromatography (HPLC) following the protocol of Agarwal and Chase (2002) with modifications by Nussey et al. (2009). In short, a standard curve for calibration was prepared using a 1,1,3,3-tetraethoxypropane stock solution (5 μM in 40% ethanol) serially diluted using 40% ethanol. We added 50 μL of a butylated hydroxytoluene solution (0.05% w/v in 95% ethanol), 400 μL of a phosphoric acid solution (0.44 M), and 100 μL of a thiobarbituric acid (TBA) solution (42 mM) to 20 μL of plasma and 30 μL of Milli-Q water or to 50 μL of standard, vortexed and heated at 100°C for 1 h to allow for the formation of MDA-TBA adducts. The reaction was stopped by placing samples and standards on ice. We then added 250 μL of *n*-butanol to extract the MDA-TBA complex. Tubes were subsequently vortexed and centrifuged at 18,000 *g* for 3 min at 4°C. We then moved 100 μL of the upper (*n*-butanol) phase to HPLC vials, which were immediately saturated with N_2 to avoid oxidation (see also Romero-Haro and Alonso-Alvarez 2014). Samples were injected into an Agilent 1100 series HPLC system (Agilent, Waldbronn, Germany) fitted with a fluorescence detector set and a 5-μm ODS-2 C18 (4.0 × 250-mm) column maintained at 37°C. The mobile phase was a ratio of MeOH : KH_2PO_4 (50 mM; 40 : 60 v/v), running isocratically for 10 min at a flow rate of 1 mL/min. Chromatograms were collected at 515 nm (excitation) and 553 nm (emission). Some samples were measured in duplicate both within and across laboratory sessions to quantify repeatabilities (intrasession: $r = 0.95, N = 28, P < 0.001$; intersession: $r = 0.76, N = 12, P = 0.001$). Quantification of MDA could not be done in two fathers and four focal females because samples were lost during handling, resulting in lower sample sizes for some comparisons. MDA concentrations were log transformed for the statistical analyses.

Statistical Analyses

First, we ran linear models to test whether a focal female's own oxidative damage (i.e., plasma MDA levels) or the oxidative damage experienced by her parents when she was conceived predicted the number of offspring (i.e., number of hatchlings) produced by the focal female during the reproductive event. Second, we further explored variation in focal female reproductive success by running generalized linear models with a quasibinomial error structure to test whether a focal female's own oxidative damage or the oxidative damage experienced by her parents when she was conceived predicted the number of eggs she laid during the reproductive event or the hatching success of those eggs. Quasibinomial models were used instead of binomial models because of overdispersion. Third, we ran linear models to test whether a focal female's own oxidative damage or the oxidative damage experienced by her parents when she was conceived predicted focal female life span while accounting for the cause of death (natural/euthanized). Focal female body mass at adulthood was included as an additional covariate in the models described above. Fourth, we used linear models to test whether parental oxidative damage levels during reproduction were associated with the size of the egg the focal female developed in, her hatching mass, or her adult body mass. Finally, we used linear models to test for parent-offspring resemblance in oxidative damage during the reproductive period. Standardized MDA values were used for parent-offspring regressions.

Absolute MDA levels were used in the models described above. In addition we ran the same models with MDA levels corrected for circulating triglyceride concentrations (see app. A). Furthermore, analyses of associations between mothers' and fathers' oxidative damage levels are presented in appendix B.

For quasibinomial models, significance of predictors was determined by comparing two nested models, with and without the factor of interest, using likelihood ratio tests. All statistical analyses were performed in R version 3.6.2 (R Development Core Team 2014). Terms were removed from the final models if $P > 0.05$. Normality of the residuals of linear models was confirmed by visual inspection and Shapiro-Wilk tests. Means ± SEs are presented.

Results

Focal Female Reproductive Success

A focal female's own levels of oxidative damage, measured during the reproductive event, did not predict the number of

offspring she produced ($t_{1, 15} = 0.895$, $P = 0.385$; fig. 1*a*). In contrast, levels of oxidative damage in the mother, measured when the focal female was conceived, were negatively associated with the number of offspring produced by the focal female ($\beta \pm \text{SE} = -13.208 \pm 5.816$, $t_{1, 20} = -2.271$, $P = 0.034$; fig. 1*b*). No association between the father's oxidative damage, measured when the focal female was conceived, and the number of offspring produced by the focal female was found ($t_{1, 12} = -0.107$, $P = 0.916$; fig. 1*c*).

We further dissected variation in focal female reproductive success by separately analyzing the number of eggs the focal female laid during the reproductive event and the hatching success of those eggs. The number of eggs laid by a focal female was predicted neither by her own oxidative damage levels ($\chi^2 = 0.440$, $P = 0.715$; fig. 2*a*) nor by those of her mother, measured when the focal female was conceived ($\chi^2 = 3.257$, $P = 0.315$; fig. 2*b*). In contrast, the number of eggs laid by a focal female was negatively associated with the father's levels of oxidative damage, measured when the focal female was conceived ($\beta \pm \text{SE} = -7.724 \pm 2.316$, $\chi^2 = 46.155$, $P < 0.001$; fig. 2*c*). Hatching success was not predicted by the focal female's own levels of oxidative damage during reproduction ($\chi^2 = 0.951$, $P = 0.354$; fig. 3*a*), but the mother's oxidative damage levels, measured when the focal female was conceived, negatively predicted the hatching success of eggs laid by the focal female ($\beta \pm \text{SE} = -3.554 \pm 1.715$, $\chi^2 = 4.355$, $P = 0.038$; fig. 3*b*). Furthermore, there was a positive association between the father's oxidative damage levels, measured when the focal female was conceived, and hatching success ($\beta \pm \text{SE} = 2.545 \pm 1.267$, $\chi^2 = 4.127$, $P = 0.043$; fig. 3*c*).

These results did not change when including the focal female's body mass at adulthood as an additional factor in the models. Focal female body mass was not significantly associated with the focal female's reproductive success (all $P > 0.285$).

Female Life Span

A focal female's life span was associated neither with her own levels of oxidative damage, measured during reproduction ($t_{1, 12} = 0.250$, $P = 0.807$), nor with oxidative damage levels in the mother ($t_{1, 20} = -1.272$, $P = 0.218$) or the father ($t_{1, 17} = 0.646$, $P = 0.527$), measured when the focal female was conceived. Including the cause of death (natural or euthanized) in the analysis did not change the results, and there was no association between the parents' or focal female's levels of oxidative damage and the cause of death (all $P > 0.176$). These results did not change when including the focal female's body mass at adulthood as an additional factor in the model. Focal female body mass was not significantly associated with life span ($t_{1, 16} = 0.493$, $P = 0.628$).

Egg Size and Female Body Mass

There was no significant association between the mother's or the father's level of oxidative damage when the focal female was conceived and the size of the egg the focal female developed in (mother MDA: $t_{1, 17} = -0.720$, $P = 0.482$; father MDA: $t_{1, 17} = 0.794$, $P = 0.438$), the focal female's body mass at hatching (mother MDA: $t_{1, 17} = -0.501$, $P = 0.623$; father MDA: $t_{1, 17} = 0.449$, $P = 0.659$), or the focal female's body mass at adulthood (mother

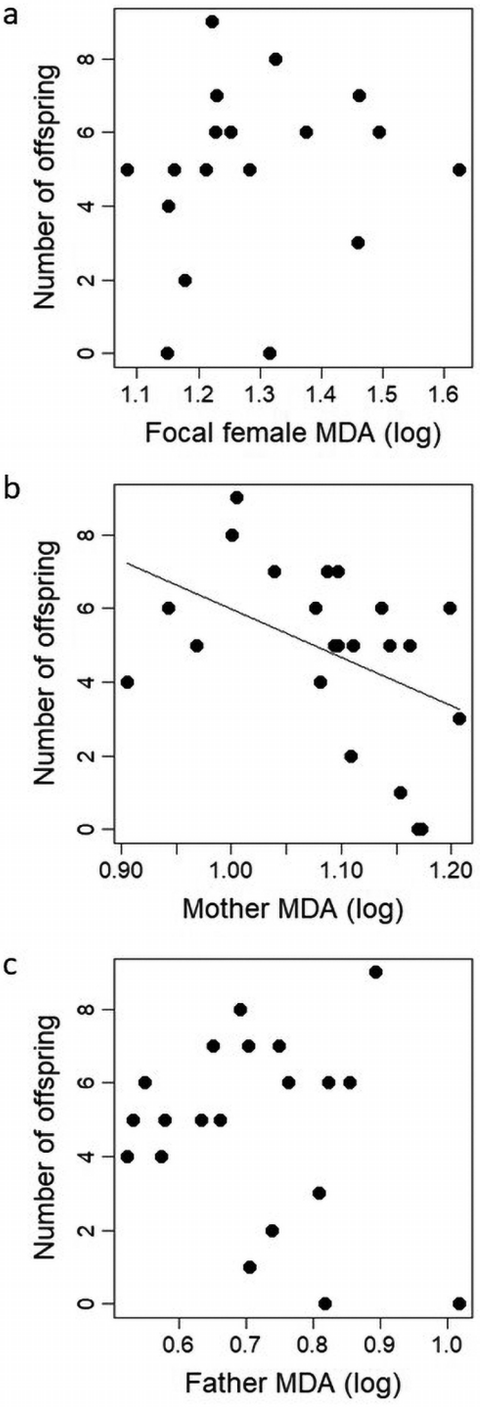

Figure 1. Association between the number of offspring produced by the focal female during a reproductive event and her own plasma malondialdehyde (MDA) levels during reproduction (*a*), her mother's plasma MDA levels when the focal female was conceived (*b*), and her father's plasma MDA levels when the focal female was conceived (*c*). A regression line is presented for significant associations.

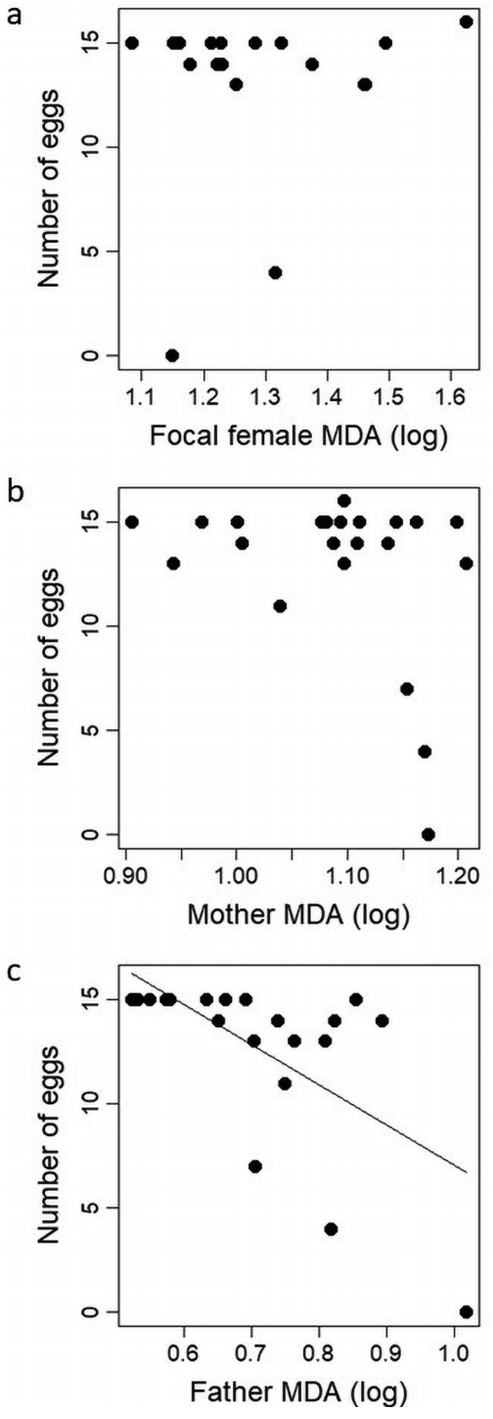

Figure 2. Association between the number of eggs laid by the focal female during a reproductive event and her own plasma malondialdehyde (MDA) levels during reproduction (*a*), her mother's plasma MDA levels when the focal female was conceived (*b*), and her father's plasma MDA levels when the focal female was conceived (*c*). A regression line is presented for significant associations.

MDA: $t_{1,17} = -0.917$, $P = 0.372$; father MDA: $t_{1,17} = 0.979$, $P = 0.341$).

Parent-Daughter Resemblance in Oxidative Damage

There was no significant mother-daughter ($\beta \pm \text{SE} = 0.097 \pm 0.230$, $t_{1,16} = 0.422$, $P = 0.678$) resemblance in the levels of oxidative damage measured during reproduction. There was also no significant father-daughter ($\beta \pm \text{SE} = -0.028 \pm 0.208$, $t_{1,14} = -0.133$, $P = 0.896$) resemblance in the levels of oxidative damage measured during reproduction.

Discussion

Our study provides evidence for an intergenerational link between parental oxidative damage at offspring conception and key components of offspring reproductive success. We observed associations between offspring reproductive performance and the oxidative damage of both parents, but different fitness components were affected depending on parental sex. The mother's level of oxidative damage at offspring conception was significantly negatively associated with the number of offspring that a focal female (i.e., her daughter) produced, and this effect was mainly mediated through an effect on the hatching success of eggs laid by a focal female rather than on the number of eggs she laid. In contrast, the father's level of oxidative damage was negatively associated with the number of eggs laid by the focal female but positively associated with the hatching success of those eggs. These opposing paternal effects canceled each other out, resulting in no significant association between the father's level of oxidative damage and the number of offspring produced by a focal female. No significant association between a focal female's own oxidative damage during reproduction and her reproductive success was observed. Furthermore, no direct or parental effect of oxidative damage on focal female life span was observed. These results show that parental physiological states (i.e., levels of oxidative damage) can have long-term fitness consequences for the next generation and suggest that intergenerational effects may be a stronger predictor of offspring performance than levels of oxidative damage experienced by the individual itself during reproduction.

Three previous studies have reported a negative association between maternal oxidative stress–related measurements during reproduction and offspring survival early in life. In Alpine swifts (*Apus melba*), females with erythrocytes less resistant to an oxidative attack (KRL bioassay) laid eggs that were less likely to hatch (Bize et al. 2008). In banded mongoose (*Mungos mungo*), pups of females with high plasma MDA levels had a lower survival probability until emergence from the den (Vitikainen et al. 2016). And in common lizards (*Zootoca vivipara*), maternal plasma levels of oxidative damage (dROMs test, hydroperoxides) were negatively related to offspring survival early in life (Dupoué et al. 2020). We did not find paternal effects on offspring life span. To our knowledge, our study is the first, however, to demonstrate long-term consequences of parental oxidative damage on key components of offspring reproductive success.

Currently, we can only speculate about the mechanisms underlying the observed parental effects on offspring reproductive performance. The environment an individual encounters during the first stages of life can have long-lasting consequences (Lindström 1999). The observed effect of oxidative damage experienced by

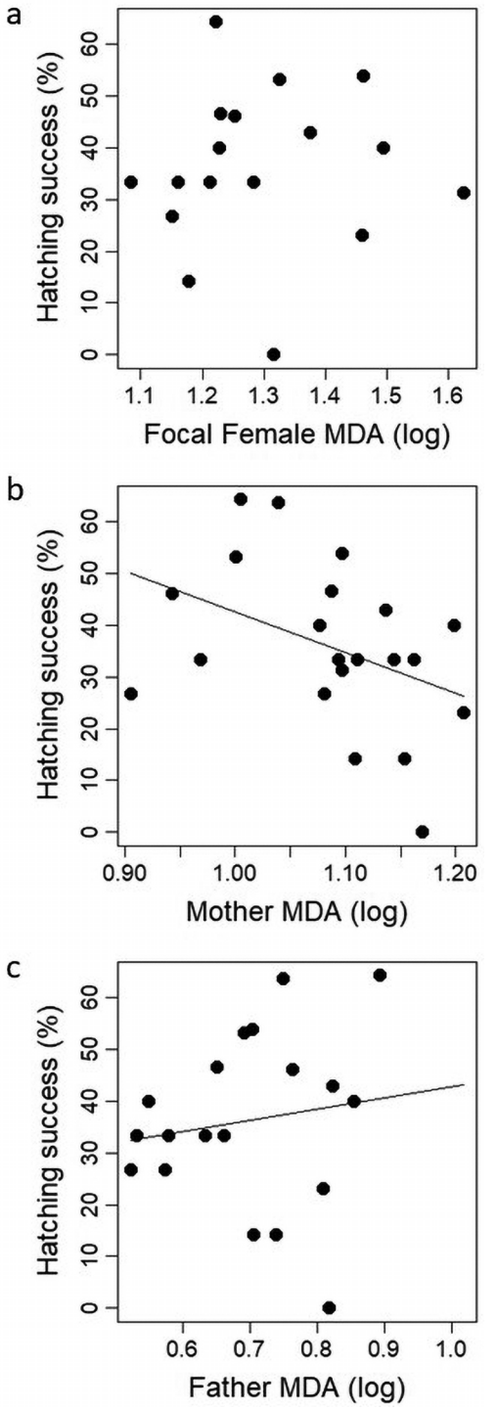

Figure 3. Association between the hatching success of the eggs laid by the focal female and her plasma malondialdehyde (MDA) levels during reproduction (*a*), her mother's plasma MDA levels when the focal female was conceived (*b*), and her father's plasma MDA levels when the focal female was conceived (*c*). A regression line is presented for significant associations.

mothers during reproduction on the reproductive success of their daughters could thus be mediated by an inability of physiologically stressed females to optimally provision or care for their offspring early in life, with long-term consequences for their reproductive performance. Given that in our study eggs were artificially incubated and chicks were reared under standardized conditions, such maternal provisioning effects would have to occur before egg laying through a change in egg size or quality. We did not observe a relationship between maternal oxidative damage and egg size. However, mothers experiencing high levels of oxidative damage could be constrained in the amount of antioxidants they transfer to the egg because of a direct trade-off between the use of antioxidants for self-maintenance or reproduction. Indeed, in great tits (*Parus major*), plasma levels of oxidative damage in females were negatively associated with the levels of yolk antioxidants in their eggs (Giordano et al. 2015). Similarly, dietary antioxidant supplementation reduces the levels of oxidative damage and increases the levels of antioxidants in females and in the eggs and chicks they produce (Surai et al. 2016). Also, the allocation of different maternal resources to the eggs is nonindependent (Royle et al. 2001; Blount et al. 2002; Boulinier and Staszewski 2008). Thus, maternal oxidative damage may not only affect the allocation of antioxidants to eggs but also the allocation of other egg components, such as hormones (Groothuis and Schwabl 2008) or antibodies (Boulinier and Staszewski 2008), with downstream consequences for offspring development and performance (Groothuis et al. 2005; Surai et al. 2016), potentially affecting reproductive performance.

Alternatively, oxidized molecules in the mother's circulation may be directly incorporated into the eggs (Grune et al. 2001; Mohiti-Asli et al. 2008). Both a reduced allocation of antioxidants to eggs and the direct transfer of oxidized molecules to eggs as a consequence of high levels of oxidative damage in the mother may increase oxidative stress experienced by focal females during prenatal development. The developing organism is particularly sensitive to oxidative stress (Monaghan et al. 2009; Metcalfe and Alonso-Alvarez 2010), which may negatively affect the development of the reproductive system and exert negative, long-lasting effects on reproductive success of the individual. For example, in zebra fish (*Dario rerio*), levels of oxidative stress experienced during development reduced the odds of breeding and the rate of fertilized eggs (Newman et al. 2015). Furthermore, in laboratory rats, adult females that developed under gestational hypoxia, a condition commonly used to generate placental oxidative stress in biomedical studies (Silvestro et al. 2020), showed lower ovarian reserve than females that developed under normoxia (Aiken et al. 2019*a*, 2019*b*). Interestingly, we observed no significant association between maternal oxidative damage at offspring conception and offspring body mass at hatching or at adulthood. This makes it unlikely that impaired growth and/or catch-up growth (Metcalfe and Monaghan 2001) mediated the observed maternal effects on daughters' reproductive success.

In addition to the significant negative associations between maternal oxidative damage at offspring conception and a focal female's reproductive success, we observed a significant negative association between paternal oxidative damage and the number of eggs laid by a focal female. Furthermore, and unexpectedly, we found a significant positive association between paternal oxidative damage and the hatching success of those eggs. First, paternal effects on offspring reproductive performance could arise if females

change the investment in eggs in response to partner quality (Burley 1986; Gowaty 2008). Thus, the observed paternal effects on aspects of offspring reproductive success may indeed be maternal effects. Females may perceive a male's oxidative status through condition-dependent signals, such as color ornaments (Galván and Alonso-Alvarez 2009), vocalizations (Messina et al. 2017), and behavioral displays (Metcalfe and Alonso-Alvarez 2010), and change egg provisioning accordingly. We did not observe a relationship between the father's oxidative damage level and the size of the eggs his partner laid. Yet changes in egg composition may occur in response to partner quality. Indeed, females have been found to differentially deposit hormones and antioxidants in eggs in relation to partner quality (Gil et al. 1999; Saino et al. 2002; Williamson et al. 2006; Remes 2011), which may affect offspring reproductive performance later in life (Müller et al. 2009). Second, the negative link between the father's oxidative status and the number of eggs laid by daughters could arise because of direct mutagenic effects of oxidative damage on the male's germ line, with negative consequences for the offspring (Aitken and Krausz 2001; Gavriliouk and Aitken 2015). Spermatozoa are known to be particularly sensitive to oxidative stress, and indeed oxidative stress is considered to be one of the main causes of male infertility (Tremellen 2008) and increased offspring morbidity (Aitken and Krausz 2001; Aitken et al. 2014). Furthermore, the observed relationships between paternal oxidative damage and aspects of offspring reproductive performance could be caused by the transfer of nongenetic information from the father to the zygote via sperm. Only relatively recently has it been recognized that sperm contains more than just paternal DNA and that nongenetic sperm components, such as DNA methylation, chromatin modifications, RNAs, and proteins, can have long-term consequences for offspring development and performance (reviewed in Krawetz 2005; Immler 2018). The specific mechanisms underlying the connection between paternal sperm characteristics and offspring performance are still poorly understood, although changes in gene expression patterns during the embryonic period have been pointed out (Chen et al. 2016). Importantly, previous studies suggest that oxidative damage experienced by a male may affect these nongenetic sperm components and thus offspring development. For example, in laboratory mice, adult females conceived by sperm with experimentally increased levels of oxidative damage were smaller and showed increased adiposity and reduced glucose tolerance compared with control females (Lane et al. 2014). Furthermore, environmental oxidative stress–related factors, such as exposure to toxins and pollutants, have also been shown to modify nongenetic sperm components and affect the phenotype and health of descendants (reviewed in Jiménez-Chillarón et al. 2015; Xavier et al. 2019), highlighting the role of oxidative stress and the resulting oxidative damage in mediating environmentally induced epigenetic remodeling. Importantly, direct DNA damage in gametes caused by oxidative damage and the transfer of nongenetic information (e.g., epigenetic states) to the offspring through the gamete could also contribute to the maternal oxidative damage effect on daughters' reproductive success.

Our study is correlational, and experimental manipulations of parental oxidative status are needed to further understand why and how maternal and paternal physiological states affect different components of daughters' reproductive performance. In addition, analyses of oxidative stress in gametes, eggs, and embryos may help to identify the relative importance of the different, but not mutually exclusive, mechanisms discussed above.

In contrast to the associations between parental oxidative damage and focal female reproductive success, we found no link between a focal female's own levels of oxidative damage and her fitness, which is in disagreement with some previous studies (Bize at al. 2008; Noguera et al. 2012; Stier et al. 2012; Costantini et al. 2016; Vitikainen et al. 2016; but see Losdat et al. 2012; van de Crommenacker et al. 2017; Fowler et al. 2018). The potential importance of the mechanisms triggering intergenerational effects discussed above, the enhanced sensitivity to oxidative stress early in life, and the fact that an individual's levels of oxidative damage during development and at adulthood appear to be unrelated (Romero-Haro and Alonso-Alvarez 2014) might explain this unexpected result.

In conclusion, our study provides evidence that both the mother's and the father's oxidative states at offspring conception have long-term consequences for key aspects of offspring reproductive performance. Such intergenerational oxidative damage effects may promote the evolution of oxidative shielding mechanisms in parents during reproduction to protect the descendants and, in turn, increase fitness return via an enhanced reproductive success of daughters (Blount et al. 2015). Importantly, parental levels of oxidative damage were stronger predictors of offspring fitness than levels of oxidative damage experienced by the adult individual itself. This finding highlights the importance of an inter- and transgenerational perspective in the study of oxidative stress and life history evolution, and it suggests that natural or human-induced environmental stressors may have delayed transgenerational effects on natural populations, leading to an underestimation of their effect on population health, resilience, and stability.

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Data have been deposited in the Dryad Digital Repository (https://doi.org/10.5061/dryad.1ns1rn8v6; Romero-Haro et al. 2021).

APPENDIX A

Analyses of Focal Female Fitness Using Malondialdehyde (MDA) Controlled for Circulating Levels of Triglycerides as Explanatory Variables

Plasma MDA levels have been shown to positively correlate with plasma triglyceride levels across bird species (Pérez-Rodríguez et al. 2015). This is expected, since triglycerides are the main form of storage and transport of polyunsaturated fatty acids, which are the main target of lipid peroxidation (Mateos and Bravo 2007). So it has been recommended to report both absolute and relative (controlled for circulating triglyceride levels) MDA levels (Romero-Haro and Alonso-Alvarez 2014; Pérez-Rodríguez et al. 2015).

In addition, differences in absolute MDA levels between sexes and generations (see the *x*-axis in figs. 1–3) were found. Since all individuals in this study—males and females as well as parents and daughters—were reared under the same standardized conditions, such differences could potentially be explained by variation in circulating plasma triglycerides levels. To confirm the patterns presented in the main text and test whether differences in triglyceride levels explain the sex and generational differences in MDA, we repeated the analyses presented in the main text using triglyceride-controlled MDA levels as explanatory variables instead of absolute MDA levels.

To this end, we quantified triglyceride concentrations in all plasma samples using the glycerol phosphate oxidase/peroxidase method. We used a commercial kit (BioSystems, Barcelona) and followed the manufacturer's instructions. Repeatability of triglyceride measures was high ($r = 0.98, N = 20, P < 0.001$). Because of limited plasma samples, triglyceride levels were not measured in two samples of fathers, resulting in lower sample sizes for some comparisons.

To check whether triglyceride levels predict MDA levels across all of the samples, we ran a linear model including triglyceride levels as a fixed factor. We found that plasma triglyceride levels were positively related to plasma MDA levels ($\beta \pm \text{SE} = 0.0003 \pm 0.00002$, $t_{1, 56} = 11.18, P < 0.0001$). We then obtained triglyceride-controlled MDA levels separately for each group (i.e., focal females, mothers, and fathers). Triglyceride-controlled MDA was defined as the residuals of a linear model with MDA levels as the response variable and triglyceride level the as predictor.

To explore whether the differences in MDA levels between focal females, mothers, and fathers shown in figures 1–3 were caused by differences in triglyceride levels, we ran three linear models including MDA, triglycerides, or triglyceride-controlled MDA levels as response variables and the group of analysis (a three-level factor representing focal females, mothers, or fathers) as a predictor. The post hoc comparisons were performed with Tukey tests. We found that MDA and triglyceride levels differed between groups (MDA: $F_{2, 55} = 113.29, P < 0.001$; fig. A1*a*; triglycerides: $F_{2, 55} = 248.92$, $P < 0.001$; fig. A1*b*). MDA and triglyceride levels in focal females were higher than those in mothers and fathers, and levels in mothers were higher than in fathers (all $P < 0.001$). In contrast, triglyceride-controlled MDA levels did not differ among groups ($F_{2, 55} = 0.021$, $P = 0.979$; fig. A1*c*; for post hoc comparisons, all $P > 0.853$). This suggests that differences in MDA levels observed across groups are caused by differences in triglyceride levels (fig. A1). We show the mean, SE, and range of the variables in table A1.

What could cause differences in triglyceride levels across groups? Differences between sexes could have originated because of sex differences in the metabolism, use, or storage of lipids for reproduction (Lawrence and Riddle 1916; Riddle and Burns 1927). In other words, females may need higher circulating levels of triglycerides to produce eggs, which means higher levels of easily oxidizable substrate (Romero-Haro and Alonso-Alvarez 2014; Pérez-Rodríguez et al. 2015; Alonso-Alvarez et al. 2017). Indeed, males showing lower

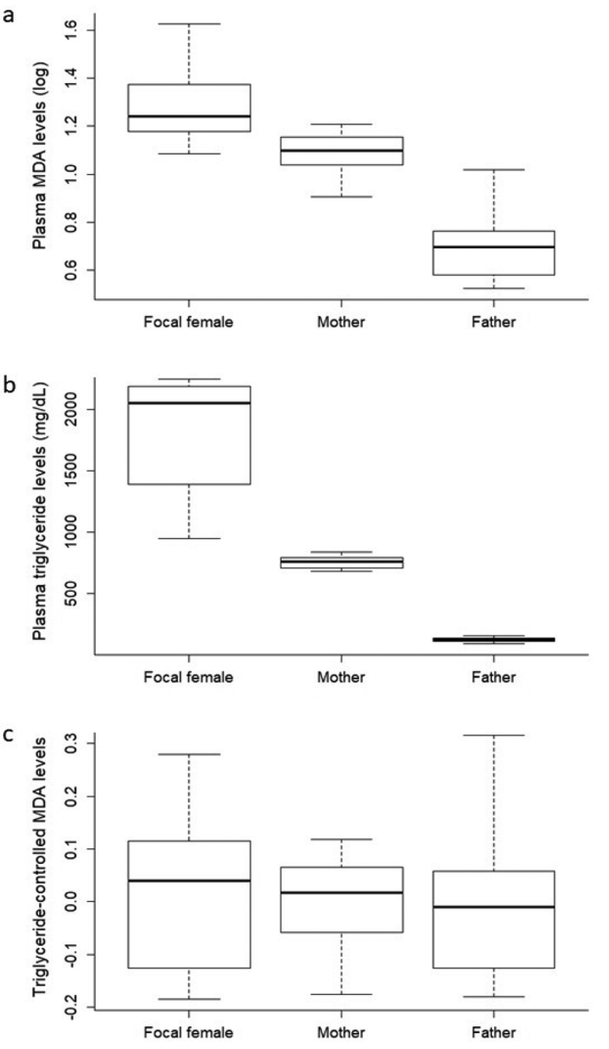

Figure A1. Focal female, mother, and father plasma levels of malondialdehyde (MDA; i.e., oxidative damage in lipids; *a*), triglycerides (*b*), and triglyceride-controlled MDA (*c*). The midline of the boxes indicates median values, and the lower and upper edges of the boxes indicate the first and third quartiles, respectively. Whiskers indicate greatest and lowest values.

Table A1: Triglyceride and malondialdehyde (MDA) plasma levels of focal females, mothers, and fathers included in this study

	Mean	SE	Range
Focal females:			
Triglycerides (mg/dL)	1,873	100.2	947.4–2,246.7
MDA (μM)	20.6	1.82	12.1–42.1
Log-transformed MDA	1.29	.03	1.08–1.62
Triglyceride-controlled MDA	0	.03	−.017 to .029
Mothers:			
Triglycerides (mg/dL)	758	10.2	680.9–839.5
MDA (μM)	12.5	.48	8.05–16.1
Log-transformed MDA	1.09	.02	.91–1.21
Triglyceride-controlled MDA	0	.02	−.16 to .11
Fathers:			
Triglycerides (mg/dL)	123.6	4.83	90.8–160.7
MDA (μM)	5.41	.39	3.34–10.4
Log-transformed MDA	.71	.03	.52–1.02
Triglyceride-controlled MDA	0	.03	−.18 to .031

levels of circulating lipids than females have been reported previously in birds (Riddle and Burns 1927), and we have found the same result in another population of quail during reproduction (A. A. Romero-Haro, L. Pérez-Rodríguez, and B. Tschirren, unpublished data). Regarding the differences between generations—focal females showing higher triglyceride levels than mothers—this may be caused, for example, by slight differences between food batches generated by the manufacturer (note that samples from mothers and daughters were collected at different time points).

Considering the results above, to confirm the patterns presented in the main text (where absolute MDA levels were used in the analyses), we repeated the same models reported in the main text but using triglyceride-controlled MDA levels instead of absolute MDA levels as explanatory variables. In agreement with the results presented in the main text, the number of offspring produced by a focal female was explained neither by her own triglyceride-controlled MDA levels ($t_{1, 15} = 1.182, P = 0.256$; fig. A2*a*) nor by the father's triglyceride-controlled MDA levels ($t_{1, 10} = -0.370$, $P = 0.719$; fig. A2*c*). However, we again found a negative association with triglyceride-controlled MDA of the mother during reproduction ($\beta \pm \text{SE} = -13.307 \pm 6.188, t_{1, 20} = -2.150, P = 0.044$; fig. A2*b*).

Regarding the number of eggs laid by a focal female, the results are again the same as those presented in the main text: the number of eggs was predicted neither by the focal female's triglyceride-controlled MDA levels ($\chi^2 = 3.657, P = 0.144$; fig. A3*a*) nor by her mother's triglyceride-controlled MDA levels ($\chi^2 = 3.743$, $P = 0.201$; fig. A3*b*). However, we again found a negative association with the triglyceride-controlled MDA levels of the father ($\beta \pm \text{SE} = -8.372 \pm 2.242, \chi^2 = 45.072, P < 0.001$; fig. A3*c*).

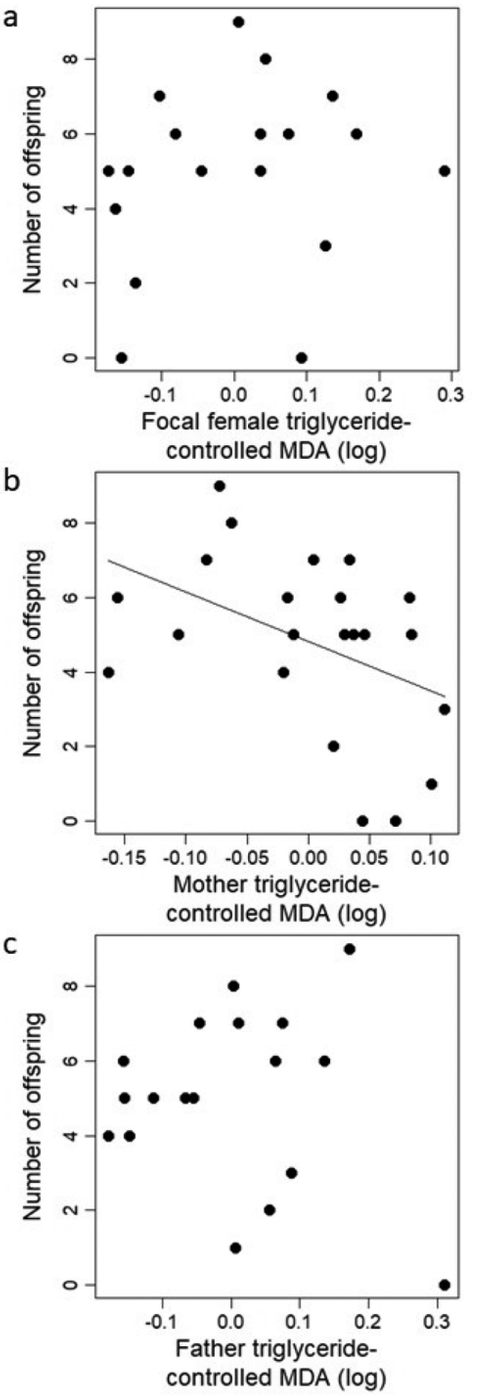

Figure A2. Association between the number of offspring produced during a reproductive event by the focal female and her plasma triglyceride-controlled malondialdehyde (MDA) levels during reproduction (*a*), her mother's plasma triglyceride-controlled MDA levels when the focal female was conceived (*b*), and her father's plasma triglyceride-controlled MDA levels when the focal female was conceived (*c*). A regression line is presented for significant associations.

Slight differences were observed when analyzing hatching success of eggs laid by a focal female during the reproductive event.

As in the main text, the hatching success was not predicted by the focal female's levels of triglyceride-controlled MDA ($\chi^2 = 0.855$, $P = 0.376$; fig. A4*a*). Unlike in the analyses presented in the main text, the mother's and father's levels of triglyceride-controlled MDA also did not significantly predict hatching success (mother: $\chi^2 = -2.698$, $P = 0.112$; father: $\chi^2 = 1.369$, $P = 0.246$; fig. A4*b*, A4*c*). This absence of association between the father's triglyceride-controlled MDA levels and the hatching success of the daughter's eggs could be explained by the lower statistical power of this analysis, as we were not able to obtain triglyceride

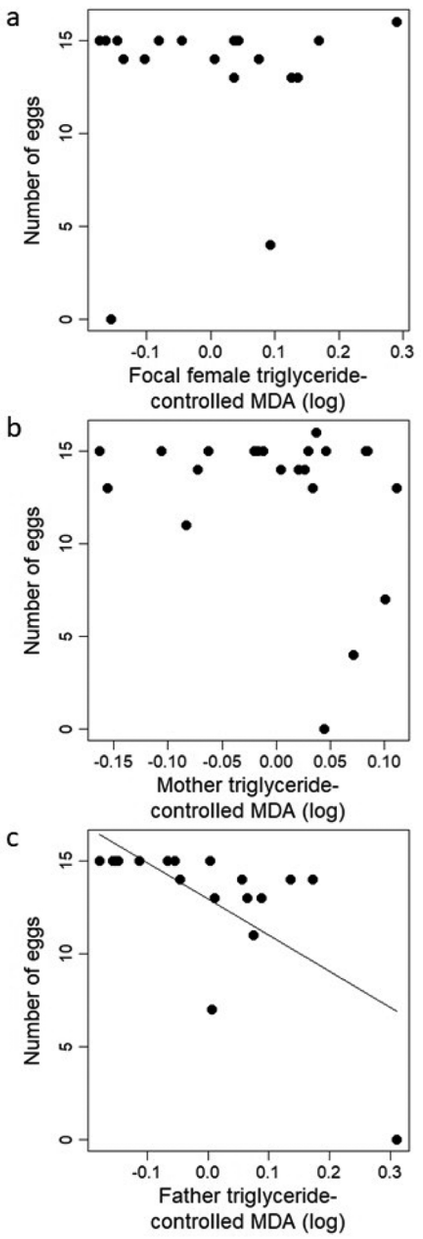

Figure A3. Association between the number of eggs laid during a reproductive event by the focal female and her plasma triglyceride-controlled malondialdehyde (MDA) levels during reproduction (*a*), her mother's plasma triglyceride-controlled MDA levels when the focal female was conceived (*b*), and her father's plasma triglyceride-controlled MDA levels when the focal female was conceived (*c*). A regression line is presented for significant associations.

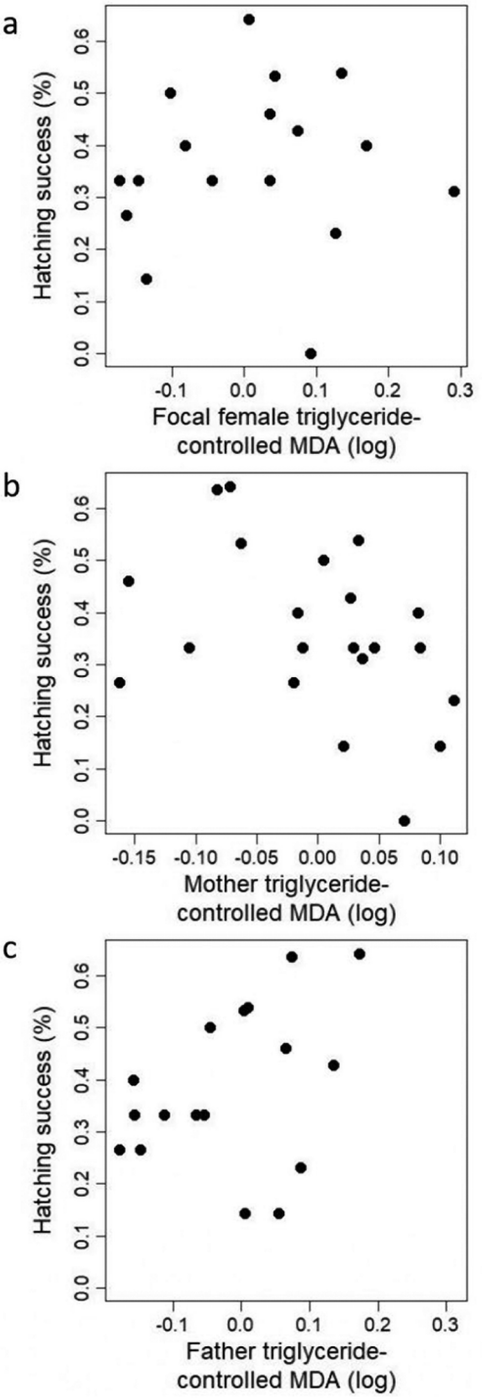

Figure A4. Association between the hatching success of the eggs laid by the focal female and her plasma triglyceride-controlled malondialdehyde (MDA) levels during reproduction (*a*), her mother's plasma triglyceride-controlled MDA levels when the focal female was conceived (*b*), and her father's plasma triglyceride-controlled MDA levels when the focal female was conceived (*c*).

levels (and, consequently, triglyceride-controlled MDA levels) from two males. However, this cannot explain the absence of a negative association between the mother's triglyceride-controlled MDA levels and the hatching success of the daughter's eggs, since the sample size is the same for absolute and triglyceride-controlled MDA. This result suggests that the association found in the main text could be led by maternal triglyceride levels instead of MDA levels. This highlights the modulating role of circulating triglyceride levels for the interpretation of lipid oxidative damage results (Romero-Haro and Alonso-Alvarez 2014; Pérez-Rodríguez et al. 2015; Alonso-Alvarez et al. 2017).

In agreement with the results presented in the main text, life span of focal females was explained neither by their own triglyceride-controlled MDA levels during reproduction ($t_{1, 10} = 0.075$, $P = 0.942$) nor by those of the mother or father when the focal female was conceived (mother: $t_{1, 20} = -0.921$, $P = 0.368$; father: $t_{1, 15} = 0.646$, $P = 0.733$). There was no significant association between the mother's or the father's levels of triglyceride-controlled MDA when the focal female was conceived and the size of the egg the focal female developed in (mother MDA: $t_{1, 15} = -0.186$, $P = 0.855$; father MDA: $t_{1, 16} = 1.467$, $P = 0.162$), the focal female's body mass at hatching (mother MDA: $t_{1, 15} = -0.016$, $P = 0.987$; father MDA: $t_{1, 16} = 0.644$, $P = 0.528$), or the focal female's body mass at adulthood (mother MDA: $t_{1, 17} = -0.402$, $P = 0.693$; father MDA: $t_{1, 16} = 0.644$, $P = 0.528$). There was neither a mother-daughter resemblance nor a father-daughter resemblance in the levels of triglyceride-controlled MDA measured during reproduction (mother-daughter resemblance: $t_{1, 16} = 0.909$, $P = 0.377$; father-daughter resemblance: $t_{1, 12} = 0.002$, $P = 0.999$).

Although the associations between hatching success and parental levels of triglyceride-controlled MDA became nonsignificant, overall, the results obtained from either absolute or triglyceride-controlled MDA levels are very similar. Thus, the proposed underlying mechanisms and, more importantly, the conclusions of the study do not change.

APPENDIX B

Correlation between Mother and Father Plasma MDA Levels

Although mothers and fathers were randomly selected and blood sampled just before being placed in cages for breeding (i.e., before meeting each other), their levels could—by chance—be correlated. We tested for an association between maternal and paternal absolute MDA, triglyceride, and triglyceride-controlled MDA levels using Pearson's correlation coefficient and standardized values. Unexpectedly, maternal and paternal levels of MDA were positively correlated ($r = 0.454$, $P = 0.044$, $N = 20$). However, neither triglyceride levels ($r = 0.153$, $P = 0.544$, $N = 18$) nor triglyceride-controlled MDA levels ($r = 0.247$, $P = 0.323$, $N = 18$) of both pair members were correlated. Since they were sampled before they were placed together in cages (i.e., before they had met), a direct influence on each other can be excluded.

Given the opposite effects of maternal and paternal MDA levels on the daughter's reproductive success, this correlation does not bias the results or conclusions presented in the main text. Specifically, the results presented in appendix A show that the results are robust when using triglyceride-controlled MDA levels, for which no correlation between mothers and fathers is observed.

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