Inter-generational 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 the daughter's reproductive success was mediated by an effect on hatching success, rather than the number of eggs laid by daughters. We also observed a negative association between the father's oxidative damage levels and the number of eggs laid by daughters, but a positive association between the father's oxidative damage levels and the hatching success of these eggs. These opposing paternal effects cancelled 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 are 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 inter-generational perspective when quantifying costs of physiological stress.

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 the offspring's ability to cope with environmental conditions, and thus be adaptive (Mousseau and Fox 1998; 'predictive adaptive response' Gluckman et al 2005). Alternatively, parental effects may merely be a (non-adaptive) 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 enhanced physiological state display a better performance than those of parents with 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 defence capacity of an organism (Halliwell and Gutteridge 2007; Monaghan et al. 2009). Oxidative damage to proteins, lipids or DNA is a direct consequence of non-alleviated 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 signalling pathways, but with poorly understood consequences (Schieber and Chander 2014; Ayala et al. 2014). Cell metabolism, and cellular respiration in particular, contribute substantially to ROS production (Halliwell and Gutteridge 2007). However, also

extrinsic factors, such as radiation, changes in temperature, exposure to xenobiotics or pollution can 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 are associated with the occurrence of certain diseases, like cancer (Valko et al. 2007; Ayala et al. 2014) and they have been related to the ageing process (Martinez de Toda et al 2020), shorter lifespan (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. Speakman and Selman 2011; Metcalfe and Alonso-Alvarez 2010; 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 the 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 in order 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 are 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 vs. effects of oxidative damage experienced by the adult individual itself. Such an inter-generational 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, we quantified if 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 in their daughters, namely reproductive success and lifespan, using Japanese quail (*Coturnix japonica*) as a study system. Furthermore, we quantified the relative importance of oxidative damage-mediated parental condition transfer effects vs. 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 the daughters' fitness. Furthermore, based on previous findings where high within-individual levels of oxidative damage were related to reduced fitness (Stier et al. 2012; Costantini et al. 2016; Noguera et al. 2012; Vitikainen et al. 2016), we predict a negative association between a focal female's oxidative damage levels and her reproductive success and lifespan.

Methods

Breeding conditions

The study was conducted in a captive population of Japanese quail (*Coturnix japonica*) maintained in large outdoor aviaries (7m × 5.5 m each) at the University of Zurich, Switzerland. Females were maintained in a single sex aviary, and males in a mixed sex aviary together with non-experimental females (see Pick et al. 2016 for a detailed description of the breeding and husbandry conditions). For this study, adult males and females (age: 189-295 days old), were randomly selected from the population and housed during three weeks in pairs (N = 22 randomly assigned breeding pairs) in breeding cages (122 x 50 x 50 cm) in the animal facility on a 16:8 light:dark cycle at approximately 20 °C. Eggs were collected on the day they were laid, weighed (to the nearest 0.01g), and artificially incubated (Favorit, HEKA Brutgeräte). During the first 14 days, 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 × 57× 25 cm, Kükenaufzuchtbox Nr 4002/C, HEKA Brutgeräte) for two weeks after hatching. The first five days the temperature was kept at 35–38 °C, then slowly lowered to 25 °C over the next 9 days. After two weeks, chicks were transferred to rearing cages within the breeding facility. At the age of four weeks, the birds were released into the outdoor aviaries (see Pick et al. 2016 for details). Thus, all offspring were reared in mixed-family groups under standardised conditions. Body mass was measured at hatching (to the nearest 0.01g) and at adulthood (i.e. when six months old; to the nearest 1g). We focused on daughters because males show a low variation in reproductive performance (Pick et al. 2017).

At the age of six months, 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 during three weeks to determine their reproductive success during the reproductive event. To avoid potential effects of incubation conditions, the eggs were removed on the day they were laid and incubated under standardised conditions as described above to determine hatching success and the number of offspring produced. After breeding, the focal females were moved back into the outdoor aviaries where they were kept for their entire life to record their lifespan. All focal females thus experienced the same standardised conditions from incubation to death. Death of the focal females occurred either naturally (N = 17) or they were euthanized because they had reached a pre-defined humane endpoint (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 (approx. 100µl) were taken from the brachial

vein using heparinised capillary tubes. Samples were stored at 4ºC until centrifugation (5 min at 20 °C and 2000 *g*) within 4 h. Plasma was then separated and frozen at −80 °C until analysis.

Quantification of lipid oxidative damage

Although oxidative stress not always results in oxidative damage, oxidative damage is generated under non-alleviated 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 malondialdehyde acid (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; Vagasi et al. 2019) and plasma levels of MDA have been reported to reflect those in other tissues, thus being a proxy of 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 (reviews in Ayala et al. 2014), as well as impaired fitness in non-human animals (Noguera et al. 2012; Vitikainen et al. 2016; Vagasi et al. 2019).

MDA quantification was performed using 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

196 (5 µM in 40% ethanol), serially diluted using 40% ethanol. 50 µL of a butylated
197 hydroxytoluene (BHT) solution (0.05% w/v in 95% ethanol), 400 µL of a phosphoric
198 acid solution (0.44 M), and 100 µL of a thiobarbituric acid (TBA) solution (42 mM)
199 were added to 20 µL of plasma and 30µL of Milli-Q water or to 50 µL of standard,
200 vortexed and heated at 100ºC for 1 h to allow for the formation of MDA-TBA
201 adducts. The reaction was stopped by placing samples and standards on ice. 250 µL of
202 *n*-butanol was then added to extract the MDA-TBA complex. Tubes were
203 subsequently vortexed and centrifuged at 18 000 *g* for 3 min at 4ºC. 100 µL of the
204 upper (*n*-butanol) phase were then moved to HPLC vials, which were immediately
205 saturated with N_2 to avoid oxidation (see also Romero-Haro and Alonso-Alvarez
206 2014). Samples were injected into an Agilent 1100 series HPLC system (Agilent,
207 Waldbronn, Germany) fitted with a fluorescence detector set and a 5-µm ODS-2 C-18
208 4.0 x 250-mm column maintained at 37ºC. The mobile phase was MeOH : KH_2PO_4
209 (50 mM; 40 : 60 v/v), running isocratically for 10 min at a flow rate of 1 mL/min.
210 Chromatograms were collected at 515 nm (excitation) and 553 nm (emission). Some
211 samples were measured in duplicate both within and across laboratory sessions to
212 quantify repeatabilities (intrasession: $r = 0.95$, $n = 28$, $P < 0.001$, intersession: $r =$
213 0.76, $n = 12$, $P = 0.001$). Quantification of MDA could not be done in two fathers and
214 four focal females because samples were lost during handling, resulting in lower
215 sample sizes for some comparisons. MDA concentrations were log transformed for
216 the statistical analyses.
217
218 *Statistical analyses*
219 First, we ran linear models to test if a focal female's own oxidative damage (i.e.
220 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 generalised linear models with a quasibinomial error structure to test if 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 these eggs. Quasibinomial models were used instead of binomial models because of overdispersion. Third, we ran linear models to test if a focal female's own oxidative damage, or the oxidative damage experienced by her parents when she was conceived, predicted focal female lifespan 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 if 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 Supplementary material S1). Furthermore, analyses of associations between mother and father oxidative damage levels are presented in Supplementary material S2.

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 ± SE 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. 1a). 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 (β ± SE: -13.208 ± 5.816; $t_{1, 20} = -2.271$, $P = 0.034$, Fig. 1b). 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. 1c).

In a second step, 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 these eggs. The number of eggs laid by a focal female was neither predicted by her own oxidative damage levels ($\chi^2 = 0.440$, $P = 0.715$; Fig. 2a) nor by those of her mother, measured when the focal female was conceived ($\chi^2 = 3.257$, $P = 0.315$, Fig. 2b). 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 (β ± SE = -7.724 ± 2.316; $\chi^2 = 46.155$, $P < 0.001$, Fig. 2c). 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. 3a) 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 ($\beta \pm$ SE = -3.554 ± 1.715, $\chi^2 = 4.355$, $P = 0.038$, Fig. 3b). Furthermore, there was a positive association between the father' oxidative damage levels, measured when the focal female was conceived, and hatching success ($\beta \pm$ SE = 2.545 ± 1.267, $\chi^2 = 4.127$, $P = 0.043$, Fig. 3c).

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

Female lifespan

A focal female's lifespan was neither associated with her own levels of oxidative damage measured during reproduction ($t_{1, 12} = 0.250$, $P = 0.807$) nor by 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 into the model. A focal female's body mass was not significantly associated with lifespan ($t_{1, 16} = 0.493$, $P = 0.628$).

Egg size and female body mass

There was no significant association between the mother's or the father's levels of oxidative damage when the focal female was conceived and the size of the egg the

296 focal female developed in (mother MDA: $t_{1, 17}$ = -0.720, P = 0.482, father MDA: $t_{1, 17}$
297 = 0.794, P = 0.438), the focal female's body mass at hatching (mother MDA: $t_{1, 17}$ = -
298 0.501, P = 0.623, father MDA: $t_{1, 17}$ = 0.449, P = 0.659) or the focal female's body
299 mass at adulthood (mother MDA: $t_{1, 17}$ = -0.917, P = 0.372, father MDA: $t_{1, 17}$ = 0.979,
300 P = 0.341).
301
302 *Parent-daughter resemblance in oxidative damage*
303 There was no significant mother-daughter (β ± SE = 0.097 ± 0.230, $t_{1, 16}$ = 0.422, P =
304 0.678) or father-daughter resemblance (β ± SE = -0.028 ± 0.208, $t_{1, 14}$ = -0.133, P =
305 0.896) in the levels of oxidative damage measured during reproduction.
306
307 **Discussion**
308 Our study provides evidence for an inter-generational link between parental oxidative
309 damage at offspring conception and key components of offspring reproductive
310 success. We observed associations between offspring reproductive performance and
311 the oxidative damage of both parents, but different fitness components were affected
312 depending on parental sex. The mother's level of oxidative damage at offspring
313 conception was significantly negatively associated with the number of offspring a
314 focal female (i.e. her daughter) produced, and this effect was mainly mediated
315 through an effect on the hatching success of eggs laid by a focal female, rather than
316 the number of eggs she laid. In contrast, the father's level of oxidative damage was
317 negatively associated with the number of eggs laid by the focal female, but positively
318 associated with the hatching success of these eggs. These opposing paternal effects
319 cancelled each other out, resulting in no significant association between the father's
320 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 lifespan 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 inter-generational 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 early life offspring survival (Dupoué et al. 2020). We did not find paternal effects on offspring lifespan. 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 mothers during reproduction on the reproductive success of their daughters could thus be mediated by an inability of physiologically stressed females

346 to optimally provision or care for their offspring early in life, with long-term
347 consequences for their reproductive performance. Given that in our study eggs were
348 artificially incubated and chicks reared under standardized conditions, such maternal
349 provisioning effects would have to occur before egg laying, through a change in egg
350 size or quality. We did not observe a relationship between maternal oxidative damage
351 and egg size. However, mothers experiencing high levels of oxidative damage could
352 be constrained in the amount of antioxidants they transfer to the egg because of a
353 direct trade-off between the use of antioxidants for self-maintenance or reproduction.
354 Indeed, in great tits (*Parus major*), plasma levels of oxidative damage in females were
355 negatively associated with the levels of yolk antioxidants in their eggs (Giordano et al.
356 2015). Similarly, dietary antioxidant supplementation reduces the levels of oxidative
357 damage and increases the levels of antioxidants in females, and in the eggs and chicks
358 they produce (Surai et al. 2016). Also, the allocation of different maternal resources to
359 the eggs is non-independent (Royle et al 2001; Blount et al. 2002; Boulinier and
360 Staszewski 2008). Thus, maternal oxidative damage may not only affect the allocation
361 of antioxidants to eggs, but also the allocation of other egg components, such as
362 hormones (Groothuis and Schwabl 2008) or antibodies (Boulinier and Staszewski
363 2008), with downstream consequences for offspring development and performance
364 (Groothuis et al. 2005; Surai et al. 2016), potentially affecting reproductive
365 performance.
366 Alternatively, oxidized molecules in the mother's circulation may be directly
367 incorporated into the eggs (Grune et al. 2001; Mohiti-Asli et al. 2008). Both, a
368 reduced allocation of antioxidants to eggs or the direct transfer of oxidized molecules
369 to eggs as a consequence of high levels of oxidative damage in the mother, may
370 increase oxidative stress experienced by focal females during prenatal development.

371 The developing organism is particularly sensitive to oxidative stress (Monaghan et al.
372 2009; Metcalfe and Alonso-Alvarez 2010), which may negatively affect the
373 development of the reproductive system and exert negative, long-lasting effects on
374 reproductive success of the individual. For example, in zebrafish (*Dario rerio*), levels
375 of oxidative stress experienced during development reduced the odds of breeding and
376 the rate of fertilized eggs (Newman et al 2015). Furthermore, in laboratory rats, adult
377 females that developed under gestational hypoxia, a condition commonly used to
378 generate placental oxidative stress in biomedical studies (Silvestro et al 2020),
379 showed lower ovarian reserve than females that developed under normoxia (Aiken et
380 al. 2019a, b). Interestingly, we observed no significant association between maternal
381 oxidative damage at offspring conception and offspring body mass at hatching or at
382 adulthood. This makes it unlikely that impaired growth and / or catch-up growth
383 (Metcalfe and Monaghan 2001) mediated the observed maternal effects on the
384 daughters' reproductive success.
385 In addition to the significant negative associations between maternal oxidative
386 damage at offspring conception and a focal female's reproductive success, we also
387 observed a significant negative association between paternal oxidative damage and
388 the number of eggs laid by a focal female. Furthermore, and unexpectedly, we found a
389 significant positive association between paternal oxidative damage and the hatching
390 success of these eggs. Paternal effects on offspring reproductive performance could
391 arise if females change the investment in eggs in response to partner quality (Burley
392 1986; Gowaty 2008). Thus, the observed paternal effects on aspects of offspring
393 reproductive success may indeed be maternal effects. Females may perceive a male's
394 oxidative status through condition-dependent signals, such as colour ornaments
395 (Galván and Alonso-Alvarez 2009), vocalisations (Messina et al. 2017), or

behavioural displays (Metcalfe and Alonso-Alvarez 2010) and change egg provisioning accordingly. We did not observe a relationship between the father's oxidative damage levels 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 germline, 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 non-genetic information from the father to the zygote via sperm. Only relatively recently it has been recognized that sperm contains more than just paternal DNA and that non-genetic sperm components, such as DNA methylation, chromatin modifications, RNAs or 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 non-genetic 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 to 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 non-genetic 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 non-genetic information (e.g. epigenetic states) to the offspring through the gamete could also contribute to the maternal oxidative damage effect on daughter's 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 the 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 a 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; Stier et al. 2012; Noguera et al. 2012; Costantini et al. 2016; Vitikainen et al. 2016, but see Losdat et al. 2012; Fowler et al. 2018; van de Crommenacker et al. 2017). The potential importance of the mechanisms triggering

446 inter-generational effects discussed above, the enhanced sensitivity to oxidative stress
447 early in life, and the fact that an individual's levels of oxidative damage during
448 development and at adulthood appear to be unrelated (Romero-Haro and Alonso-
449 Alvarez 2014) might explain this unexpected result.
450 In conclusion, our study provides evidence that both the mother's and the father's
451 oxidative state at offspring conception have long-term consequences for key aspects
452 of offspring reproductive performance. Such inter-generational oxidative damage
453 effects may promote the evolution of oxidative shielding mechanisms in parents
454 during reproduction to protect the descendants and, in turn, increase fitness return via
455 an enhanced reproductive success of daughters (Blount et al. 2015). Importantly,
456 parental levels of oxidative damage were stronger predictors of offspring fitness than
457 levels of oxidative damage experienced by the adult individual itself. This finding
458 highlights the importance of an inter-and transgenerational perspective in the study of
459 oxidative stress and life history evolution, and it suggests that natural or human-
460 induced environmental stressors may have delayed, transgenerational effects on
461 natural populations, leading to an underestimation of their effect on population health,
462 resilience and stability.
463
464 Data accessibility
465 Data available from the Dryad Digital Repository:
466 https://doi.org/10.5061/dryad.1ns1rn8v6
467
468 Ethics

All procedures were conducted under licenses provided by the Veterinary Office of the Canton of Zurich, Switzerland (permit numbers 195/2010; 14/2014; 156) and the ethical committee of the University of Exeter (permit eCORN002475).

Authors' contributions

AAR-H and BT designed and performed the research, analysed the data and wrote the manuscript. LP-R enabled the lab analyses. All authors discussed the results and commented on the manuscript.

Competing interests

The authors declare no competing interests.

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738

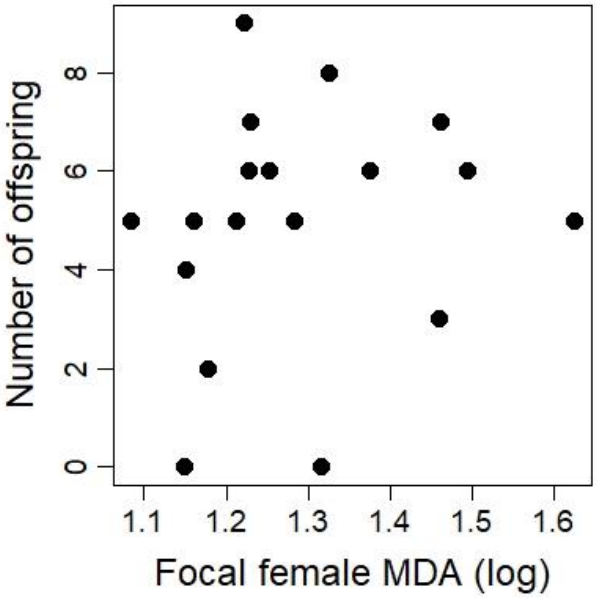
Figure legends

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

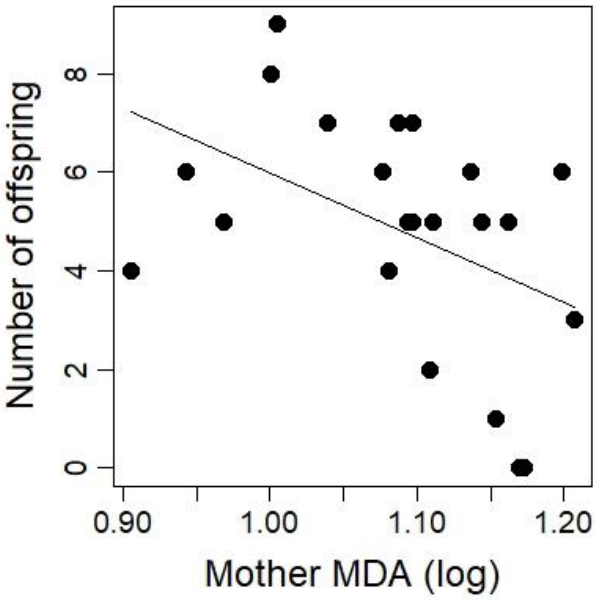
Figure 2. Association between the number of eggs laid by the focal female during a reproductive event and a) her own plasma MDA levels during reproduction, b) her mother's plasma MDA levels when the focal female was conceived, and c) her father's plasma MDA levels when the focal female was conceived.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

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

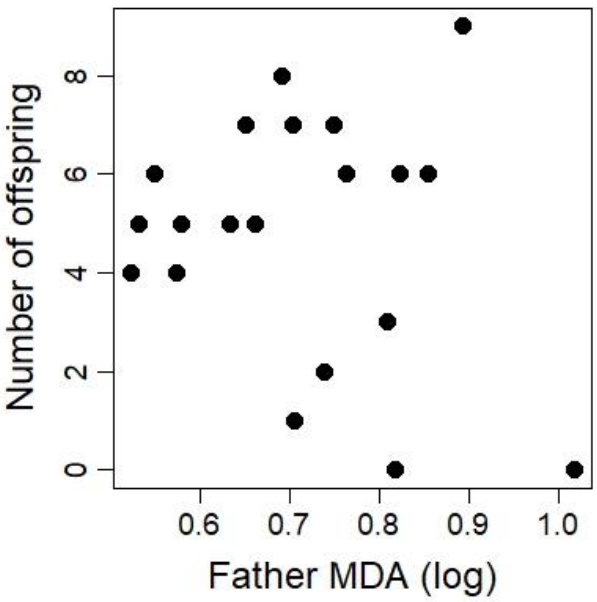
a


Number of offspring
0
2
4
6
8
1.1
1.2
1.3
1.4
1.5
1.6
Focal female MDA (log)

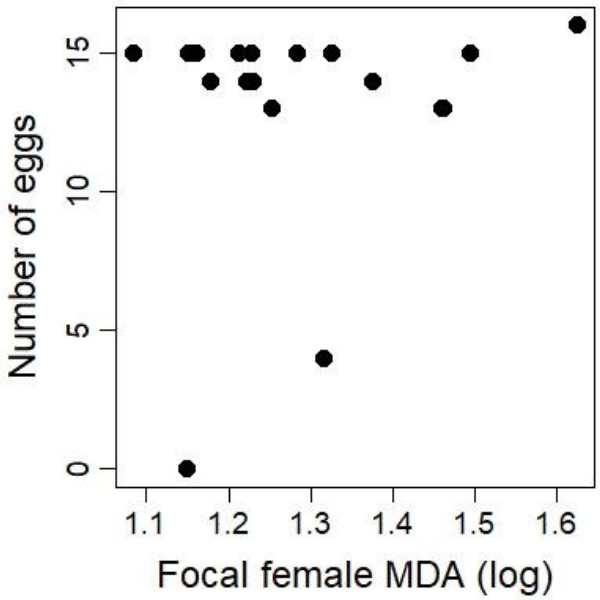
b


Number of offspring
0
2
4
6
8
0.90
1.00
1.10
1.20
Mother MDA (log)

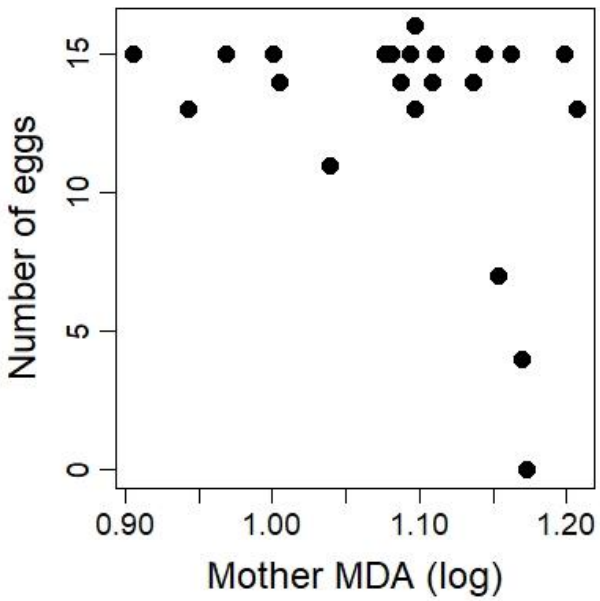
c


Number of offspring
0
2
4
6
8
0.6
0.7
0.8
0.9
1.0
Father MDA (log)

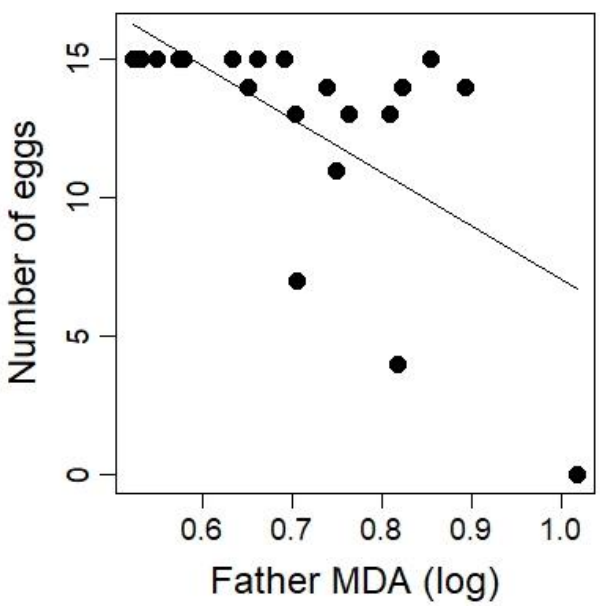
a


Number of eggs
0
5
10
15
1.1
1.2
1.3
1.4
1.5
1.6
Focal female MDA (log)

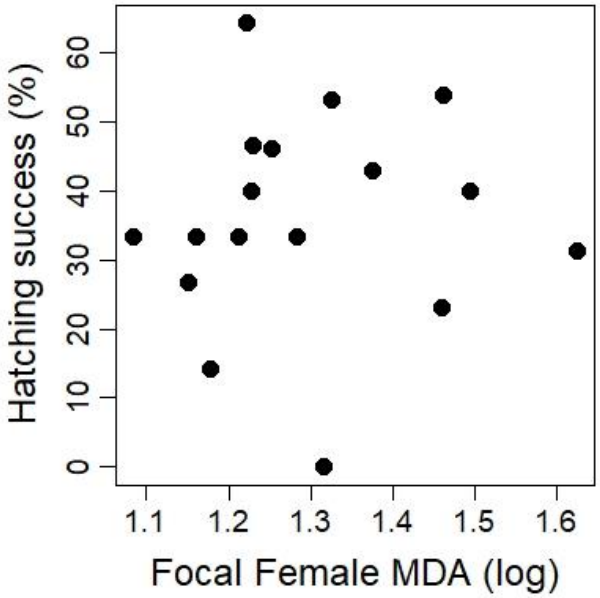
b


Number of eggs
0
5
10
15
0.90
1.00
1.10
1.20
Mother MDA (log)

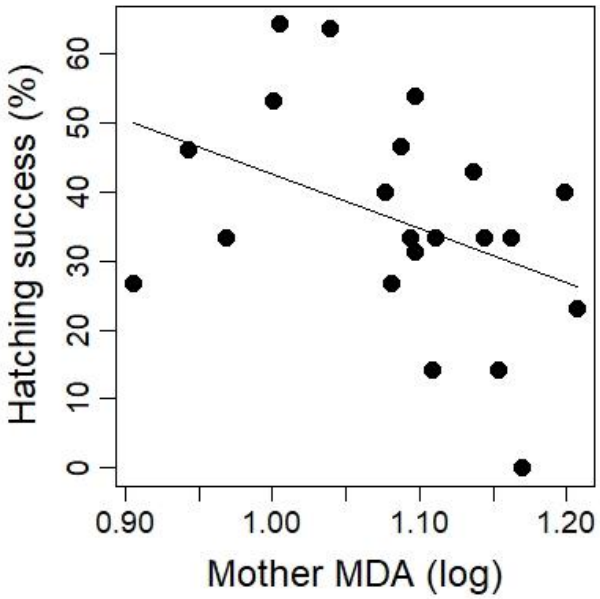
c


Number of eggs
0
5
10
15
0.6
0.7
0.8
0.9
1.0
Father MDA (log)

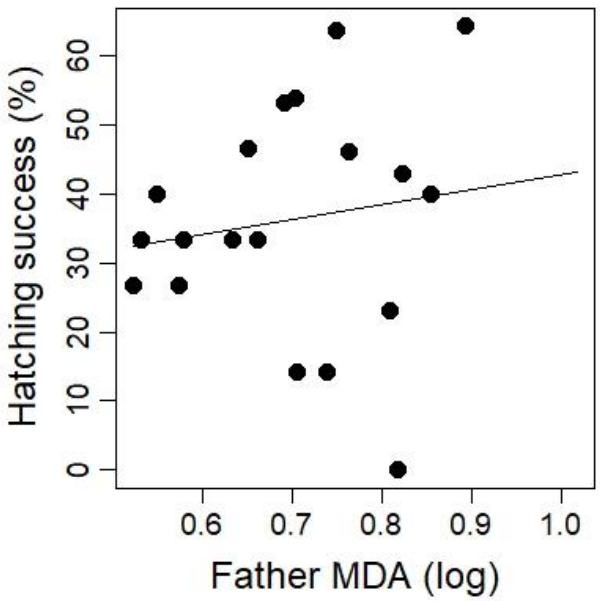
a


Hatching success (%)
0
10
20
30
40
50
60
1.1
1.2
1.3
1.4
1.5
1.6
Focal Female MDA (log)

b


Hatching success (%)
0
10
20
30
40
50
60
0.90
1.00
1.10
1.20
Mother MDA (log)

c


Hatching success (%)
0
10
20
30
40
50
60
0.6
0.7
0.8
0.9
1.0
Father MDA (log)

Supplementary material for:

Inter-generational costs of oxidative stress: reduced fitness in daughters of mothers that experienced high levels of oxidative damage during reproduction

Ana Ángela Romero-Haro[1*], Lorenzo Pérez-Rodríguez[2] & Barbara Tschirren[1]

Supplementary analysis S1. Analyses of focal female fitness using 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 x-axis of the main text figures 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 if 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, $P < 0.001$). Due to limited plasma samples, triglyceride levels were not measured in two samples of fathers, resulting in lower sample sizes for some comparisons.

In order to check if triglyceride levels predict MDA levels across all 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 (β ± SE: 0.0003 ± 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 response variable and triglyceride level as predictor.

To explore if the differences in MDA levels between focal females, mothers and fathers observed in the figures of the main text 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 by Tukey tests. We found that MDA and triglyceride levels differed between groups (MDA: $F_{2, 55}$ = 113.29, $P < 0.001$, fig. S1a; triglycerides: $F_{2, 55}$ = 248.92, $P < 0.001$, fig. S1b). 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-values < 0.001). In contrast, triglyceride-controlled MDA levels did not differ among groups ($F_{2, 55}$ = 0.021, P = 0.979, fig. S1c, all P values of post-hoc comparisons > 0.853). This suggests that differences in MDA levels observed across groups are caused by differences in triglyceride levels (fig. S1). We show the mean, SE and range of the variables in the table S1.

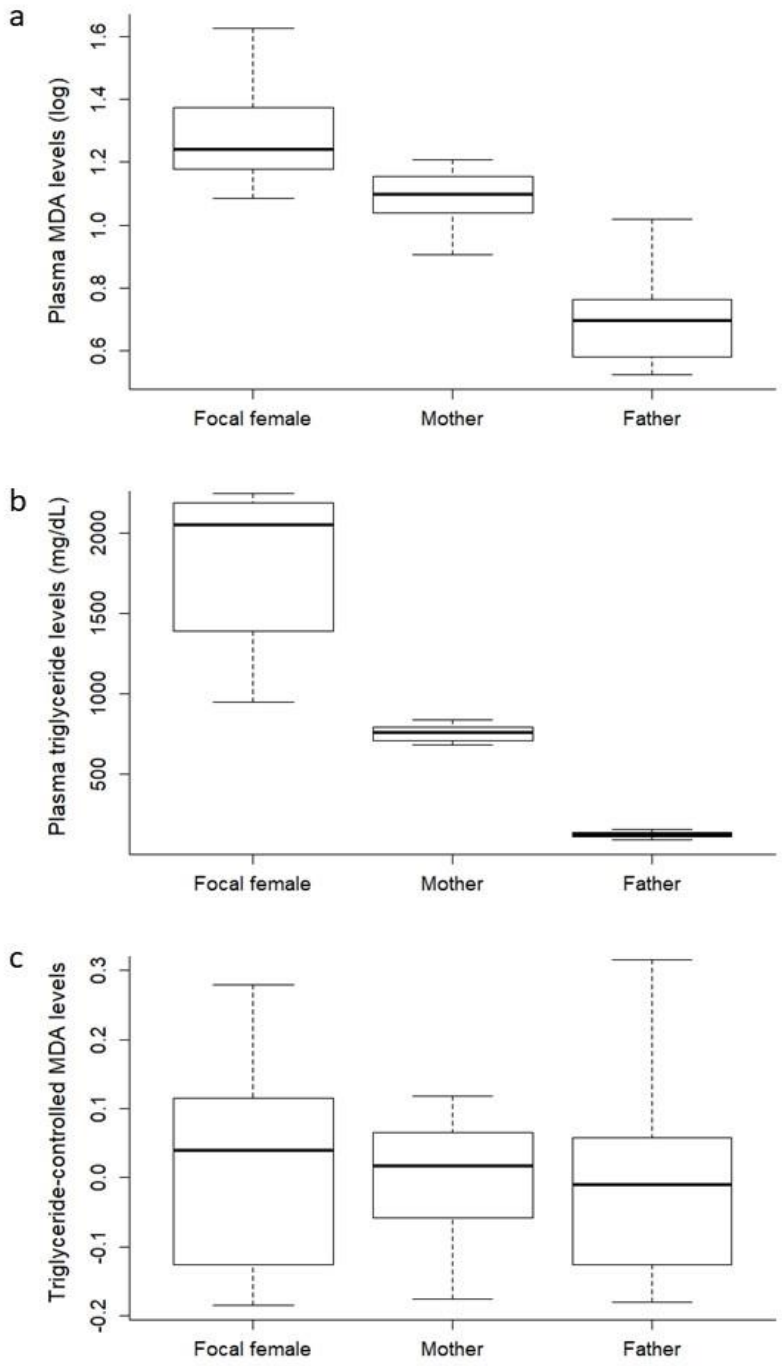


Figure S1: Focal female, mother and father plasma levels of: a) MDA (i.e. oxidative damage in lipids), b) triglycerides and c) triglyceride-controlled MDA. The midline of the boxes represents median values, and the lower and upper edges of the boxes represent the first and third quartiles, respectively. Whiskers represent greatest and lowest values.

Table S1: Triglyceride and MDA plasma levels of focal females, mothers and fathers included in this study.

	Mean	SE	Range
Focal females			
Triglycerides (mg/dL)	1873	100.2	947.4 – 2246.7
MDA (µM)	20.6	1.82	12.1 – 42.1
Log-transformed MDA	1.29	0.03	1.08 – 1.62
Triglyceride-controlled MDA	0	0.03	-0.017 – 0.029
Mothers			
Triglycerides (mg/dL)	758	10.2	680.9 – 839.5
MDA (µM)	12.5	0.48	8.05 – 16.1
Log-transformed MDA	1.09	0.02	0.91 – 1.21
Triglyceride-controlled MDA	0	0.02	-0.16 – 0.11
Fathers			
Triglycerides (mg/dL)	123.6	4.83	90.8 – 160.7
MDA (µM)	5.41	0.39	3.34 – 10.4
Log-transformed MDA	0.71	0.03	0.52 – 1.02
Triglyceride-controlled MDA	0	0.03	-0.18 – 0.031

What could cause differences in triglyceride levels across groups? Differences between sexes could have been 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; Perez-Rodriguez et al. 2015; Alonso-Alvarez et al. 2017). Indeed, males showing lower levels of circulating lipids than females has been reported previously in birds (Riddle and Burns 1927), and we have found the same result in another population of quail during reproduction (unpublished data). Regarding the differences between generations, focal females showing higher triglyceride levels than mothers, the difference may be caused, for example, by slight differences between food batches generated by the manufacturer(note that mother and daughter samples 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 were neither explained by her own triglyceride-controlled MDA levels ($t_{1, 15}$ = 1.182, P = 0.256, Fig. S2a) nor the father's triglyceride-controlled MDA levels ($t_{1, 10}$ = -0.370, P = 0.719, Fig. S2c). However, we again found a negative association with triglyceride-controlled MDA of the mother during reproduction (β ± 1SE: -13.307 ± 6.188; $t_{1, 20}$ = -2.150, P = 0.044, Fig. S2b).

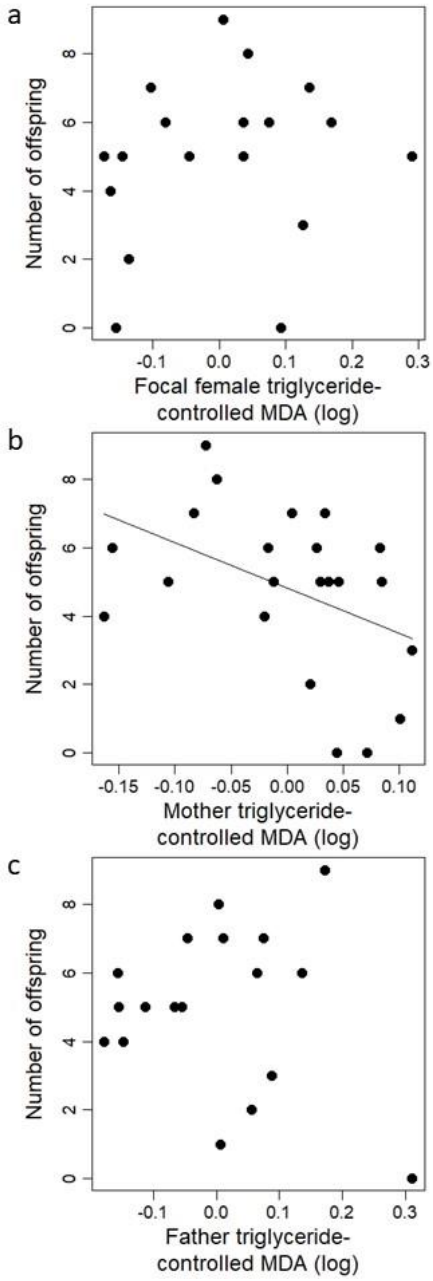


Figure S2. Association between the number of offspring produced during a reproductive event by the focal female and a) her plasma triglyceride-controlled MDA levels during reproduction, b) her mother's plasma triglyceride-controlled MDA levels when the focal female was conceived, and c) her father's plasma triglyceride-controlled MDA levels when the focal female was conceived. A regression line is presented for significant associations.

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 neither predicted by the focal female's nor by her mother's triglyceride-controlled MDA levels (χ^2 = 3.657, P = 0.144 and χ^2 = 3.743, P = 0.201, respectively; Figs. S3a, b). However, we again found a negative association with the triglyceride-controlled MDA levels of the father (β ± 1SE = -8.372 ± 2.242; χ^2 = 45.072, P < 0.001, Fig. S3c).

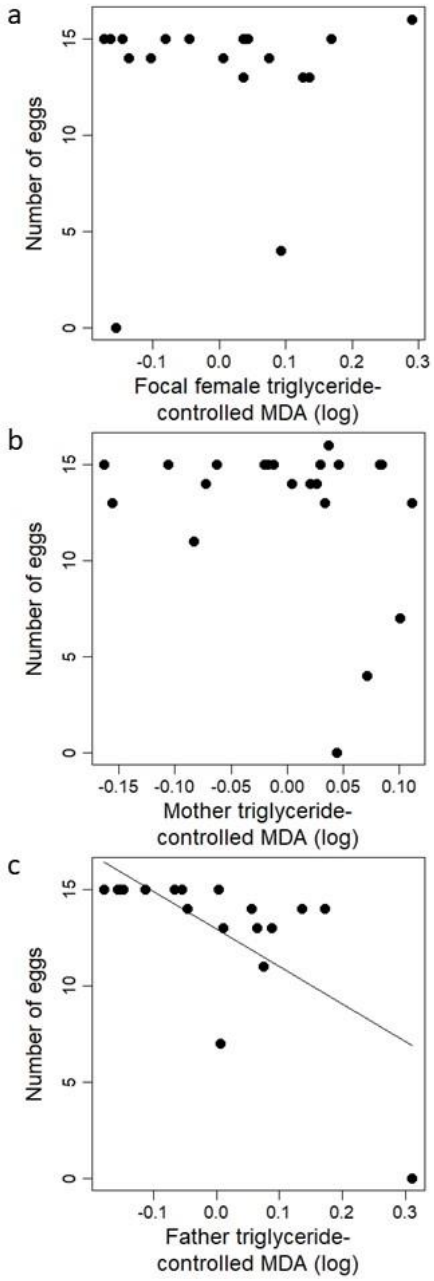


Figure S3. Association between the number of eggs laid during a reproductive event by the focal female and a) her plasma triglyceride-controlled MDA levels during reproduction, b) her mother's

plasma triglyceride-controlled MDA levels when the focal female was conceived, and c) her father's plasma triglyceride-controlled MDA levels when the focal female was conceived. 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 (χ^2 = 0.855, P = 0.376; Fig. S4a). Unlike in the analyses presented in the main text, also the mother's and father's levels of triglyceride-controlled MDA did not significantly predict hatching success (mother: χ^2 = -2.698, P = 0.112; father: χ^2 = 1.369, P = 0.246, respectively; Figs. S4b, c). This absence of association between father 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 levels (and, consequently, triglyceride-controlled MDA levels) from two males. However, this cannot explain the absence of a negative association between mother triglyceride-controlled MDA levels and 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. Anyway, this highlights the modulating role of circulating triglyceride levels for the interpretation of lipid oxidative damage results (Romero-Haro and Alonso-Alvarez 2014; Perez-Rodriguez et al. 2015; Alonso-Alvarez et al. 2017).

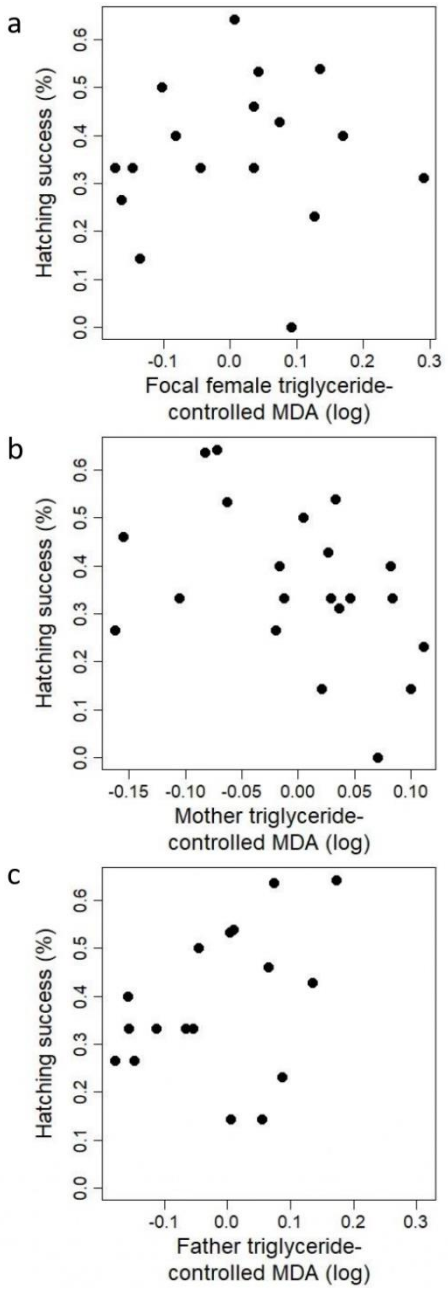


Figure S4. Association between the hatching success of the eggs laid by the focal female and a) her plasma triglyceride-controlled MDA levels during reproduction, b) her mother's plasma triglyceride-controlled MDA levels when the focal female was conceived, and c) her father's plasma triglyceride-controlled MDA levels when the focal female was conceived.

In agreement with the results presented in the main text, lifespan of focal females was neither explained by their own triglyceride-controlled MDA levels during reproduction ($t_{1, 10}$ = 0.075, P = 0.942) nor by those of their mother or father when they were conceived, ($t_{1, 20}$ = -0.921, P = 0.368 and $t_{1, 15}$ = 0.646, P = 0.733, respectively). 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 nor father-daughter resemblance in the levels of triglyceride-controlled MDA measured during reproduction ($t_{1, 16} = 0.909$, $P = 0.377$ and $t_{1, 12} = 0.002$, $P = 0.999$, respectively).

Although the associations between hatching success and parental levels of triglyceride-controlled MDA became non-significant, 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.

Supplementary analyses S2. 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 daughter reproductive success, this correlation does not bias the results or conclusions presented in the main text. Specifically, the results presented in Supplementary material S1 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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