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Is oxidative stress a physiological cost of reproduction? An experimental test in house mice

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Investment in reproduction is costly and frequently decreases survival or future reproductive success. However, the proximate underlying causes for this are largely unknown. Oxidative stress has been suggested as a cost of reproduction and several studies have demonstrated changes in antioxidants with reproductive investment. Here, we test whether oxidative stress is a consequence of reproduction in female house mice (Mus musculus domesticus), which have extremely high energetic demands during reproduction, particularly through lactation. Assessing oxidative damage after a long period of reproductive investment, there was no evidence of increased oxidative stress, even when females were required to defend their breeding territory. Instead, in the liver, markers of oxidative damage (malonaldehyde, protein thiols and the proportion of glutathione in the oxidized form) indicated lower oxidative stress in reproducing females when compared with non-reproductive controls. Even during peak lactation, none of the markers of oxidative damage indicated higher oxidative stress than among non-reproductive females, although a positive correlation between protein oxidation and litter mass suggested that oxidative stress may increase with fecundity. Our results indicate that changes in redox status occur during reproduction in house mice, but suggest that females use mechanisms to cope with the consequences of increased energetic demands and limit oxidative stress.

**Keywords:** oxidative stress; reproduction; lactation; Mus musculus domesticus; life history

1. INTRODUCTION

Investment in reproduction has costs to other life-history components, such as decreasing survival or future reproductive success [1–4]. This cost of reproduction is central to evolutionary theory and is a pivotal trade-off around which life histories are thought to evolve [2,5]. Traditional views for the cause of this cost centre on limits in the allocation of finite resources, such that investment in reproduction limits resource allocation to other areas [6]. However, reproduction, and the processes that enable it, may also directly inflict somatic damage [5,7], with direct negative effects on other life-history components.

One physiological consequence of reproduction, with possible negative impacts on other life-history components, may be oxidative stress [5,8–11]. Normal metabolic processes produce a variety of reactive oxygen species (ROS) that have the potential to damage biomolecules unless quenched by antioxidants [12]. Oxidative stress occurs when there is a serious imbalance between ROS production and the capacity to control their damaging effects [11]; this can cause extensive oxidative damage to proteins, lipids and DNA [13]. The potential negative consequences of oxidative stress are widespread, for example, oxidative stress may be a major determinant of the ageing process [14,15], is implicated in hundreds of diseases [16] and may constrain investment in reproduction [17]. Oxidative stress has therefore been highlighted as a possible proximate mediator of life-history trade-offs [11].

During reproduction, individuals typically have much higher rates of metabolism, which, potentially, could cause increased ROS production and result in oxidative stress [9,18]. However, greater metabolism does not necessarily mean greater ROS production [19] and further, animals often upregulate a host of antioxidant defences in response to increased ROS, repairing damage and limiting its subsequent occurrence [20,21]. Previous experimental studies examining oxidative stress as a cost of reproduction have investigated changes in antioxidant status with reproductive investment. In zebra finches, Taeniopygia guttata, experimentally increased brood size [18,22] and investment in a greater number of breeding events [23] leads to decreased antioxidant capacity. However, changes in antioxidant status may serve to maintain oxidant–antioxidant balance and do not necessarily indicate oxidative stress [11]. To establish whether oxidative stress is a consequence of reproduction, oxidative damage to cellular targets with biological functions needs to be assessed.

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In mammals, reproduction, and particularly lactation, is the most energetically demanding period of a female’s life. In the wild, investment in reproduction is associated with a decrease in survival in both large [24] and small [25–27] mammals. Energy intake typically increases by 66–188% [28], but in some animals such as laboratory mice, intake can increase by up to 400 per cent [29]. As a consequence, daily energy expenditure [30] and basal metabolic rate [31] can be increased in lactating females when compared with non-reproductive. This has led to the suggestion that oxidative stress may be elevated during lactation, unless antioxidant defences are sufficiently upregulated [9]. There is evidence that oxidative damage increases during lactation in some domesticated and laboratory animals [32,33]; however, these animals have been subject to strong artificial selection for increased reproductive output during domestication, which is likely to be at the expense of other life-history components. As yet, there has been very limited assessment of whether investment in reproduction elevates oxidative stress in mammals that have naturally selected levels of reproductive investment.

Here, we test whether oxidative stress is a consequence of reproduction in outbred female house mice (*Mus musculus domesticus*) that have been recently derived from wild populations. We used an approach that allowed females to freely invest in reproduction and tested for increased oxidative stress compared with non-reproductive females. Investment in reproduction and lactation in wild derived mice requires substantial increases in energetic intake [34,35]; therefore oxidative stress might result unless females adjust protective mechanisms. First, we examined whether oxidative stress increased after a long period of reproductive investment (four months) under semi-natural conditions. Our second experiment then explored whether oxidative stress increased specifically during lactation, when increases in metabolism are highest for females and oxidative stress might result from increased ROS production. We measured a number of markers of oxidative damage in the liver, gastrocnemius muscle and serum. We measured malonaldehyde (MDA), a secondary product of lipid peroxidation [11]; protein thiol groups, which are essential for stability and function of proteins but susceptible to oxidation [16]; glutathione, a ubiquitous antioxidant with an established antioxidant role *in vivo* [16]; and the proportion of glutathione that was oxidized, which is a common marker of oxidative stress and redox status [36].

### 2. MATERIAL AND METHODS

**(a) Subjects**

Subjects were adult captive-bred female house mice from a colony of mice originally derived from several populations in the northwest of England, UK, and outbred in captivity for up to six generations. Females in our first experiment (n = 28), which examined long-term investment in reproduction, were derived from eight pairs of mice each housed in separate enclosures (122 × 60 × 76 cm); they remained in these enclosures with their dam and female siblings until the beginning of the experiment (range 31–49 weeks old). Females in our second experiment (n = 28), examining lactation, were either bred in enclosures (116 × 58 × 80 cm) with their siblings, dam and sire until weaning at 28 days of age (n = 18), or were bred in cages (45 × 28 × 13 cm; n = 10); females from different pre-weaning environments were balanced between experimental groups. After weaning, all females were housed in cages (45 × 28 × 13 cm) in single sex groups with their siblings (2–5 individuals per cage) until the start of the second experiment when females were 37–47 weeks old. Males used in each experiment (n = 38 and 28, experiments 1 and 2, respectively) were unrelated to their mates and were either bred in cages or enclosures in an identical manner to females, but at 28 days males were weaned and housed singly in cages (48 × 11.5 × 12 cm) to prevent intrasexual aggression. Cages were lined with Corn cob Absorb 10/14 substrate. All mice had paper-wood bedding material, and ad libitum access to water and to a homogeneous diet that contained no antioxidants for food preservation, no added vitamin C or carotene, and only a conservative supplementation of vitamin E to meet basic nutritional requirements (Lab diet 5002, International Product Supplies Limited, London, UK: Vitamin E = 65 IU kg⁻¹, carotene = 1.6 mg kg⁻¹). This diet should thus be equivalent to a natural grain-based diet. Mice were maintained on a 12 L: 12 D reversed light cycle with lights off at 09.00 h. Experimental procedures were carried out in the dark phase under dim red light.

**(b) Experiment 1: oxidative stress and long-term investment in reproduction**

In the first experiment, we compared markers of oxidative damage in females that were allowed to reproduce freely (housed with a male) or not (housed with another female) in semi-natural enclosures over a 16 week period. This represents a substantial period of reproduction for a mouse given that extrinsic mortality is extremely high and in many wild populations most mice die within their first year [37,38]. We further manipulated social conditions, such that some male–female pairs were housed without contact with other animals (reproduction-only; n = 10), while others were housed in contact with a neighbour pair and experienced territorial intrusions to stimulate investment in territory defence (reproduction plus territory defence; n = 10). Although this was primarily to examine territorial expenditure in the males (data to be presented elsewhere), which is associated with metabolic costs [39–41], females also invest in territory defence [42,43]. This group therefore allowed us to examine whether female investment in other potential energetically demanding activities during reproduction influences the extent of oxidative damage. The production and excretion of a large quantity of urinary signalling proteins, major urinary proteins (MUPs), plays an important role in territory defence among male mice [44] and is likely to be metabolically costly [39]. As female house mice can also make quite substantial investment in these urinary proteins [45], we measured the concentration of MUP in urine samples taken at two and 16 weeks using the method of Cheetham et al. [46]. Non-reproductive females were housed in same-sex pairs without contact with other mice (control: n = 8). One reproduction-only female died prior to the end of the experiment and was therefore removed from all analysis. Females in different social environments did not differ in body mass at the start of the experiment (mean ± s.e. for control: 18.40 ± 1.10 g; reproduction only: 19.13 ± 0.86 g; reproduction plus territory defence: 18.74 ± 1.11 g; F2,26 = 0.12, p = 0.89).
To create a semi-natural environment, pairs were each housed in melamine enclosures (116 × 58 × 80 cm) that contained a nest-box, food and water hopper, four small bricks and a Perspex sheet for cover. Offspring were removed from the enclosures when aged 28–31 days. Male–female pairs in a reproduction plus territory defence environment received a number of manipulations to increase their investment in territory defence. These pairs had two mesh gates (40 mm diameter) in one wall of their enclosure that provided continuous olfactory and visual contact with a neighbouring pair. Once every week neighbouring pairs were also allowed to interact aggressively for 30 min or until three persistent aggressive interactions had occurred. An additional three times per week, a caged intruder male (one of five cage-housed stimulus males) was added to their enclosures for a 2 h period, allowing visual and olfactory contact. Further investment in territory defence was manipulated by swapping scents between enclosures of reproduction plus territory defence pairs and their neighbours, as investment in territorial behaviour can increase in response to invested in territory defence. These pairs had two mesh gates (40 mm diameter) in one wall of their enclosure that permitted olfactory and visual contact with their prospective partner but did not allow direct contact. Control females were housed with offspring that were at peak lactation, and primiparous females that had recently finished lactating. Adult females were randomly allocated to one of three groups: control (n = 8), peak lactation (n = 10) and post-lactation (n = 10). Females assigned to these three groups did not differ in body mass at the start of the experiment (mean ± s.e. for control: 19.58 ± 0.89 g; peak lactation: 19.57 ± 0.81 g; post-lactation: 19.77 ± 1.02 g; F_{2,24} = 0.02, p = 0.98). Females in the peak and post-lactation groups were housed with males for 18 days to breed, following a 7 day familiarity period in cages (45 × 28 × 13 cm) that permitted olfactory and visual contact with their prospective partner but did not allow direct contact. Control females were housed with another unrelated and unfamiliar female after an identical familiarization period with a male. Females in peak and post-lactation groups were culled either 14 days after the birth of their pups, corresponding to peak lactation in house mice [47], or 28 days after birth, when offspring were fully weaned [34,48]. In a sample of mice from our wild-derived colony that had been randomly allocated to reproduce or not, reproductive females doubled their food intake 14 days after the birth of their offspring when compared with parturition (day of parturition: 5.01 ± 0.92; 14 days after parturition: 11.32 ± 0.77), and ate approximately three to four times more food than the non-reproductive control group sampled at the same time points (control non-parturition: 3.02 ± 0.13; control 14 days later: 3.16 ± 0.25; repeated-measures ANOVA, reproductive versus control group P_{1,10} = 36.61, p < 0.0001; time × female group P_{1,10} = 20.26, p = 0.001). To ensure that there was no bias in sample storage duration, mice were paired such that females in all groups were culled over the same week. Four control females were paired at the same time as the post-lactation group and four were paired to match the peak lactation group. Two females from the post-lactation group and one female from the peak lactation group did not breed, and so were excluded from the experiment.

(d) Measurements of oxidative stress
At the end of both experiments, females were culled humanely and a blood sample taken immediately via cardiac puncture; the liver and gastrocnemius muscle were removed quickly, snap-frozen in liquid nitrogen and stored at −80°C. Owing to limitations in the quantity of sample available for analysis, sample sizes for each oxidative damage assay differed slightly (see electronic supplementary material, table S1 for sample sizes of each assay). Total and oxidized glutathione content were measured using the automated glutathione recycling assay [49] modified for use on a 96-well plate reader (Benchmark, Bio-Rad, UK) [50]. Protein thiol content was measured as described by Di Monte et al. [51] modified for use on a 96-well plate reader [50]. MDA in tissues was measured using the high-performance liquid chromatography method of Fukunaga et al. [52].

(c) Experiment 2: oxidative stress during lactation
In experiment 2, we tested whether oxidative stress increases over the period when females make maximum lactational investment in a particular litter by comparing markers of oxidative damage between virgin females, primiparous females that were at peak lactation, and primiparous females that had recently finished lactating. Adult females were randomly allocated to one of three groups: control (n = 8), peak lactation (n = 10) and post-lactation (n = 10). Females assigned to these three groups did not differ in body mass at the start of the experiment (mean ± s.e. for control: 19.58 ± 0.89 g; peak lactation: 19.57 ± 0.81 g; post-lactation: 19.77 ± 1.02 g; F_{2,24} = 0.02, p = 0.98). Females in the peak and post-lactation groups were housed with males for 18 days to breed, following a 7 day familiarity period in cages (45 × 28 × 13 cm) that permitted olfactory and visual contact with their prospective partner but did not allow direct contact. Control females were housed with another unrelated and unfamiliar female after an identical familiarization period with a male. Females in peak and post-lactation groups were culled either 14 days after the birth of their pups, corresponding to peak lactation in house mice [47], or 28 days after birth, when offspring were fully weaned [34,48]. In a sample of mice from our wild-derived colony that had been randomly allocated to reproduce or not, reproductive females doubled their food intake 14 days after the birth of their offspring when compared with parturition (day of parturition: 5.01 ± 0.92; 14 days after parturition: 11.32 ± 0.77), and ate approximately three to four times more food than the non-reproductive control group sampled at the same time points (control non-parturition: 3.02 ± 0.13; control 14 days later: 3.16 ± 0.25; repeated-measures ANOVA, reproductive versus control group P_{1,10} = 36.61, p < 0.0001; time × female group P_{1,10} = 20.26, p = 0.001). To ensure that there was no bias in sample storage duration, mice were paired such that females in all groups were culled over the same week. Four control females were paired at the same time as the post-lactation group and four were paired to match the peak lactation group. Two females from the post-lactation group and one female from the peak lactation group did not breed, and so were excluded from the experiment.

Linear mixed effects (LMEs) models were used to test differences between groups in experiment 1, fitted by maximum likelihood using the lme4 package in R and controlling for non-independence between non-reproducing pairs and territorial neighbours by adding experimental enclosure as a random effect. Significance was assessed by comparison of models with and without the group term included. General linear models (conducted in SPSS v. 16) assessed differences between groups in experiment 2, where females were independent. Data were transformed logarithmically where necessary to meet parametric assumptions (liver MDA and total glutathione). The reproductive groups were each compared with the respective control group using planned contrasts. In the second experiment, serum MDA content could not be normalized to meet parametric assumptions, so groups were compared using a Kruskal–Wallis test. Spearman’s rank correlations on untransformed data tested for relationships between individual reproductive investment (number of offspring, number of litters) and markers of oxidative damage in each experiment. Data displayed in figures are untransformed.

3. RESULTS
(a) Experiment 1: oxidative stress and long-term investment in reproduction
If oxidative stress is a consequence of investment in reproduction, increased oxidative damage should be evident after a long period of reproductive investment. All females in the two reproduction groups produced and weaned at least one litter over the 16 week experiment. However, markers of oxidative damage in the liver indicated lower (not higher) oxidative stress among females in the two reproduction groups when compared with control non-reproductive females. MDA content of females in the reproduction and the reproduction plus territory defence groups was 61 per cent that of control females (not breed, and so were excluded from the experiment).
groups ($\chi^2 = 5.65, p = 0.059$; figure 1c). However, in this case, planned contrasts revealed that females in the reproduction-only group had lower levels than controls ($p = 0.008$), but those with additional territory defence did not ($p = 0.95$; electronic supplementary material, table S1). There was also evidence that liver antioxidants were upregulated among reproducing females in both groups; their total content of the antioxidant glutathione was higher when compared with controls ($\chi^2 = 8.27, p = 0.016$; figure 1d; electronic supplementary material, table S1). While there was strong evidence that oxidative stress was decreased in the livers of reproductive females, similar differences were generally not evident in the gastrocnemius muscle. Only MDA content showed a significant difference between groups ($\chi^2 = 6.29, p = 0.043$), again reflecting a lower level of lipid peroxidation among reproductive females when compared with non-reproductive controls (electronic supplementary material, table S1). MDA content in serum did not differ between groups (electronic supplementary material, table S1).

There was no difference between females in the reproduction-only or reproduction plus territory defence groups either for the total number of offspring per female (mean $\pm$ s.e. for reproduction only: $8.8 \pm 1.68$; reproduction plus territory defence: $11.9 \pm 2.16$; LME $\chi^2 = 4.65, p = 0.051$) or for the days since their last litter at the end of the experiment, when measures were taken (reproduction-only: $20.11 \pm 4.65$ days; reproduction plus territory defence: $18.11 \pm 4.39$; $\chi^2 = 0.43, p = 0.74$). There was, however, evidence that females differed in their investment of resources to scent signalling, which is involved in territory defence, because groups excreted different concentrations of MUP in their urine when sampled at two weeks (control, $3.6 \pm 0.3$ mg mg$^{-1}$ creatinine; reproduction-only, $3.9 \pm 0.5$; reproduction plus territory defence, $6.2 \pm 0.7$; $\chi^2 = 7.62, p = 0.022$) and at 16 weeks (control, $5.8 \pm 1.0$; reproduction-only, $10.5 \pm 1.7$; reproduction plus territory defence, $15.3 \pm 2.1$; $\chi^2 = 9.99, p = 0.007$). Planned contrasts showed that MUP output was consistently greater among reproduction plus territory defence females than control females at both time points (two weeks, $p = 0.011$; 16 weeks, $p = 0.001$), while output was not significantly greater among reproduction-only females than among controls (two weeks, $p = 0.77$; 16 weeks, $p = 0.063$).

Because individual females varied greatly in the number of offspring and litters they produced (number of litters: 1–3; total number of offspring: 3–22), we also tested for a relationship between reproductive investment and oxidative stress. However, there were no significant correlations between individual female investment in reproduction (number of offspring or number of litters) and any measured marker of oxidative damage (all $p > 0.05$, data not shown).

(b) Experiment 2: oxidative damage during lactation

Metabolic rate is at its highest during peak lactation in mice, which potentially could cause increased oxidative stress specifically during this period. However, when we compared virgin females, primiparous females at peak lactation, and primiparous females after their first litter was fully weaned, we found no evidence of increased oxidative stress at peak lactation. Indeed, liver MDA was much lower in females at peak lactation when compared...
with virgin females ($F_{2,22} = 31.07, p < 0.0001$; figure 2a), indicating less (not more) oxidative damage to lipids. In contrast to experiment 1, we found no evidence of an upregulation of the antioxidant glutathione ($F_{2,21} = 0.48, p = 0.63$; figure 2b), or a change in the amount of glutathione that was oxidized ($F_{2,21} = 0.19, p = 0.83$; figure 2c), which can also indicate increased oxidative stress and changes in redox potential. Protein thiol content also did not differ significantly between groups ($F_{2,22} = 2.55, p = 0.10$; figure 2d), although levels tended to be higher in peak and post-lactation females consistent with slightly lower (not higher) levels of protein oxidation. None of the markers of oxidative damage in the gastrocnemius muscle differed between groups (electronic supplementary material, table S1), although protein thiol tended to be higher at post-lactation similar to the liver; serum MDA concentration did not differ significantly between groups ($n = 20, \chi^2 = 1.74, p = 0.42$).

Female energy intake during peak lactation can increase with litter size and mass [28,53–55], which could influence the rate of free radical production and oxidative stress. Since females in both reproduction groups showed a large variation in litter sizes and masses at the end of the experiment (peak lactation litter size, 5–8; litter mass, 34–55 g; post-lactation litter size, 2–8; litter mass, 28–115 g), we also tested for a relationship between litter mass and maternal oxidative damage. There was no relationship between any marker of oxidative damage and litter mass among females post-lactation (electronic supplementary material, table S2). However, among peak lactation females, there were negative correlations between protein thiols and total litter mass in both the liver ($n = 8, r_s = -0.86, p = 0.007$; figure 3a) and the gastrocnemius muscle ($n = 7, r_s = -0.86, p = 0.014$; figure 3b), indicating greater protein oxidation at peak lactation among females that were rearing a heavier mass of offspring.

4. DISCUSSION
Reproduction is often the most energetically demanding period of an animal’s life. However, contrary to predictions, we found no evidence of increased oxidative damage to a number of key biomarkers among reproductive female house mice when compared with non-reproductive controls. Indeed, markers in the liver indicated lower oxidative damage in reproductive females. However, during peak lactation when metabolism is particularly high, there was a positive relationship between female litter mass and protein oxidation, suggesting a possible link between fecundity and oxidative stress among breeding females, which is discussed further below. It is most notable though that peak lactation females did not have higher protein oxidation than virgin females and lipid peroxidation was much lower.

The energetic investment made by female house mice during reproduction is very high; therefore, it is unlikely that the lack of oxidative damage with reproduction was owing to an insufficient increase in metabolism in comparison to other animals. Although some energy is exported directly to offspring in milk [47], a large proportion is metabolized by females, increasing female daily energy expenditure and resting metabolic rate. Further, it has been demonstrated in laboratory rats that physiological changes occur to aid fat and carbohydrate metabolism [56] and metabolic activity per unit mass increases in specific tissues, including the liver [57], where we observed substantial decreases in markers...
of oxidative damage. While laboratory strains have been subject to selection for larger litter sizes than those of wild mice [58], recently derived mice from the wild still double their food intake during lactation ([34]; present study), and have increased resting metabolic rates [59], suggesting that their increase in metabolism is also substantial. Our results suggest that females alter aspects of their physiology during lactation to limit oxidative damage occurring as a consequence of these metabolic changes.

Organisms possess a variety of defence mechanisms that can protect against oxidative stress [60] and regulation of antioxidant defence systems occurs to maintain the correct oxidant–antioxidant balance [16]. Glutathione, an important low-molecular weight non-enzymatic antioxidant, was more concentrated in the livers of females that had been investing in reproduction over four months, suggesting that upregulation of this antioxidant by reproducing females was one mechanism used to defend against oxidative stress. Moreover, since glutathione maintains ascorbate and α-tocopherol as their functional forms and is a coenzyme for two important antioxidant families, glutathione peroxidases and glutathione S-transferases [61], the increase in glutathione suggests that other antioxidants may also have been upregulated. Glutathione is synthesized through activation of the enzymes γ-glutamylcysteine synthetase and GSH synthetase, whose activity is controlled by a number of factors, including changes in redox status [36]. Increases in glutathione may have resulted in a decrease in oxidative damage and may be especially relevant to the increase in protein thiol content, because glutathione can cause significant dethiolation of oxidized proteins and subsequent reactivation of enzymes inactivated by thiolation [62]. It must also be noted, however, that proteins vary in both their abundance of thiol groups and the susceptibility of these groups to oxidation [63,64]. Therefore, any substantial changes in the liver proteome with reproduction could influence total protein thiol content and contribute to differences between reproductive and non-reproductive females. Although less protein oxidation is consistent with the general decrease in oxidative damage in reproducing females’ livers, further research examining other markers of protein oxidation, such as protein carbonyls, may confirm these changes.

When looking at oxidative stress during and after a single lactation (experiment 2), MDA content was much lower at peak lactation without any significant increase in glutathione. This suggests that changes in markers of oxidative damage are not entirely related to increased glutathione and additional mechanisms are involved. Under temporary conditions of increased ROS, redox-sensitive transcription factors are expressed, which increase the expression of a number of antioxidants that protect against oxidative stress [21]. If ROS production was increased transiently in reproductive females during this experiment, transcription factors such as AP-1 and NF-kB may have been activated, leading to increased production of a variety of defence systems against oxidative stress. Further research is required to understand the cause of the decrease in oxidative damage at peak lactation and the extent to which this occurs in different tissues.

None of the markers of oxidative damage were higher in reproducing females than in non-reproductive controls during the rearing of a single litter, although reproducing females with the heaviest litters had the lowest protein thiol concentration in both liver and skeletal muscle. Assuming that this equates to greater protein oxidation, it suggests a link between fecundity and oxidative stress. Either females with the lowest concentration of protein thiols produced larger litters, or producing a larger litter increased protein oxidation. Theoretically, either explanation is plausible. Individuals with high metabolic rates prior to reproduction could, according to the ‘free radical’ theory of ageing [14,15], have higher levels of oxidative damage. If resting metabolic rate is related to sustained maximal metabolic rate [65], individuals with a high metabolic rate could sustain lactation for a greater litter mass. However, this hypothesis relies on increased ROS production among individuals with high metabolic rates and a positive correlation between resting and maximal metabolic rate, assumptions which have been criticized [19,66]. Alternatively, and also in accordance with the ‘free radical’ hypothesis, individuals with the heaviest litters are likely to require the highest rates of metabolism during lactation, so damage could occur specifically during this time. The latter hypothesis would be consistent with previous studies that observed decreased antioxidant capacity in birds with increased brood sizes or investment in a greater number of breeding events [18,22,23].

Our experiment was designed to test the hypothesis that oxidative stress occurs as a by-product of
reproductive investment, and thus other challenges to females were limited to allow us to test this specific physiological trade-off. Females were fed a diet that, while only conservatively supplemented with vitamins to prevent deficiencies, still provided for the nutritional requirements of females. When females increase food intake during lactation, they may also increase the gross quantity of vitamins consumed, and it is possible that this could help limit oxidative damage. It is possible that if the diet available to females is deficient in certain nutrients/vitamins, they may be less able to adapt to metabolic changes. Furthermore, increased metabolic investment in more challenging conditions may also influence redox status during reproduction. When breeding females experienced the additional stress of territory intrusions, the proportion of oxidized glutathione in the liver increased relative to females in a reproduction-only environment. While territory defence is principally undertaken by territorial males [67], females also engage in aggressive territorial behaviour [42,43]. We further show that territorial female mice increased their investment in the concentration of MUP signalling proteins in their urinary scent marks. This territorial behaviour might divert investment of resources away from reproduction and also increase stress hormones that can cause oxidative damage [68]. Females generally become much more aggressive in defence of their litters during lactation [69,70], so increased social pressure when reproducing at high population densities might contribute to increased oxidative damage. Other factors, such as suboptimal temperatures, might also increase stress associated with reproduction. Further studies need to explore the extent to which oxidative damage occurs when females reproduce under more challenging conditions, which may often be experienced in the wild. One recent cross-sectional study tested for a correlation between reproductive investment and plasma MDA in Soay sheep living in semi-wild conditions [71]. No significant relationship was detected; however, the authors suggest this may be owing to the cross-sectional nature of the study and also highlighted the need for long-term, individual-based studies examining oxidative stress and life histories in the wild.

A cost to reproduction is central to life-history theory, but the proximate physiological mechanisms for this are still largely unknown. Although it has been suggested that oxidative stress may be a consequence of reproduction, with the potential to constrain investment in other life-history traits, our results indicate that for female house mice, living in good conditions and allowed to invest freely in reproduction, this does not occur. A correlative study examining trade-offs between reproduction and lifespan in captive zoo populations found that investment in reproduction had little effect on lifespan [72]. It is therefore feasible that animals living in optimum conditions adjust their reproductive effort to reduce the negative consequences of reproduction. Manipulation of reproductive investment, through litter size manipulation, may reveal whether oxidative stress is a consequence of reproduction when females are less able to tailor their investment. This may be especially revealing in house mice as both larger litter masses and investment in defence of the breeding territory influence redox status. Manipulation of female investment in reproduction may also give a greater insight into the ability of individuals to upregulate defences in more demanding conditions. In a novel paper, Spekman & Krol [73] recently suggested that increased reproductive activity will not reduce lifespan because somatic maintenance mechanisms are not likely to trade-off against reproduction; instead they suggested that reproduction is constrained by the ability to dissipate heat. Our results are in accord with their hypothesis, which predicts that somatic damage will not arise from reproduction as protective mechanisms can be maintained in spite of reproductive effort. However, measurement of oxidative stress after manipulation of litter size or with additional energetic challenges will be a more powerful test of this hypothesis, as a lack of somatic damage (e.g. oxidative damage) would still be predicted, in addition to a limit in the amount that females can increase investment owing to the constraint of heat dissipation.

This research adhered to the Association for the Study of Animal Behaviour/Animal Behaviour Society Guidelines for the Use of Animals in Research, the legal requirements of the country in which the work was carried out and all institutional guidelines.

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REFERENCES


Supplementary method of territory defence manipulation

To increase perception of territory intrusions for those in reproduction plus territory defence pairs, two mesh gates (40 mm diameter) in one wall of their enclosure provided continuous olfactory and visual contact with a neighbouring pair; those in reproduction only and control pairs had solid walled enclosures. Seven Perspex tiles (15 x 15 cm) were placed on the floor of each enclosure to become scent marked. In reproduction plus territory defence pairs, these tiles were swapped with those of their neighbouring pair three times per week to simulate scent marking intrusions of neighbours. To control for this manipulation, tiles in the reproduction only and control enclosures were swapped with a replicate set of tiles from their own enclosure (i.e. own scent marks) and stored outside of the enclosure in an area free from odours. Reproduction plus territory defence pairs were also allowed to interact physically with their neighbour pair once per week for up to 30 min or until three persistent aggressive interactions had occurred. During these interactions, the nest box and any offspring from each pair were confined under the up-ended base of an empty cage; interactions were observed continuously and any prolonged aggressive attacks were broken up after 10s to ensure the mice did not suffer any injuries. Both males and females took part in aggressive behaviour. While neighbour interactions took place between reproduction plus territory defence pairs, the nest boxes and any offspring of reproduction only and control females were also confined for an identical duration. To further increase the perception of territorial intrusions, three times per week a caged intruder male (one of five cage housed stimulus males) was added to the enclosures of reproduction plus territory defence pairs permitting visual and olfactory contact for a two hour period. Equivalent empty cages were added to enclosures of control and reproduction females over the same time period.
Electronic supplementary material

Supplementary table 1. Markers of oxidative damage in the liver, gastrocnemius muscle and serum of females after long term investment in reproduction or during a single reproductive event (mean ± s.e.m.). Differences between groups in experiment 1 (long term investment in reproduction) were examined using linear mixed effects (LMEs) models; differences between groups in experiment 2 (during a single breeding event) were tested for using general linear models (GLMs).

<table>
<thead>
<tr>
<th></th>
<th>(a) Long term investment (experiment 1)</th>
<th>non-R*</th>
<th>R†</th>
<th>R + TD‡</th>
<th>n</th>
<th>χ²</th>
<th>p-value</th>
<th>planned contrast to control</th>
<th>R</th>
<th>R + TD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MDA (μmol/g protein)</strong></td>
<td><strong>Liver</strong></td>
<td>0.89±0.07</td>
<td>0.51±0.09</td>
<td>0.47±0.06</td>
<td>8.9±0.10</td>
<td>10.41</td>
<td>0.006</td>
<td>0.009</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>gastrocnemius muscle</strong></td>
<td>3.39±0.32</td>
<td>1.71±0.35</td>
<td>1.77±0.39</td>
<td>5.09</td>
<td>2.59</td>
<td>0.043</td>
<td>0.022</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>serum (μmol/l)</strong></td>
<td>0.98±0.18</td>
<td>0.73±0.05</td>
<td>0.78±0.05</td>
<td>6.8±0.10</td>
<td>1.98</td>
<td>0.375</td>
<td>0.344</td>
<td>0.81</td>
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<tr>
<td><strong>Protein thiol (μmol/g protein)</strong></td>
<td><strong>Liver</strong></td>
<td>24.1±3.4</td>
<td>16.0±4.2</td>
<td>44.9±3.7</td>
<td>8.9±0.10</td>
<td>12.89</td>
<td>0.002</td>
<td>0.019</td>
<td>0.001</td>
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<tr>
<td></td>
<td><strong>gastrocnemius muscle</strong></td>
<td>67.4±6.7</td>
<td>75.3±8.2</td>
<td>77.3±3.2</td>
<td>7.7±0.10</td>
<td>1.80</td>
<td>0.395</td>
<td>0.360</td>
<td>0.192</td>
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<tr>
<td><strong>Total glutathione (μmol/g protein)</strong></td>
<td><strong>Liver</strong></td>
<td>23.6±2.5</td>
<td>22.0±3.4</td>
<td>22.5±2.4</td>
<td>8.9±0.10</td>
<td>8.27</td>
<td>0.016</td>
<td>0.003</td>
<td>0.019</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>gastrocnemius muscle</strong></td>
<td>0.03±0.003</td>
<td>0.03±0.002</td>
<td>0.03±0.003</td>
<td>0.03±0.001</td>
<td>0.03±0.001</td>
<td>0.03±0.001</td>
<td>0.03±0.001</td>
<td>0.019</td>
<td></td>
</tr>
<tr>
<td><strong>Proportion of glutathione oxidised</strong></td>
<td><strong>Liver</strong></td>
<td>0.03±0.003</td>
<td>0.03±0.002</td>
<td>0.03±0.003</td>
<td>0.03±0.001</td>
<td>0.03±0.001</td>
<td>0.03±0.001</td>
<td>0.03±0.001</td>
<td>0.019</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>gastrocnemius muscle</strong></td>
<td>0.03±0.004</td>
<td>0.03±0.003</td>
<td>0.03±0.003</td>
<td>0.03±0.001</td>
<td>0.03±0.001</td>
<td>0.03±0.001</td>
<td>0.03±0.001</td>
<td>0.019</td>
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<table>
<thead>
<tr>
<th></th>
<th>(b) Single litter (experiment 2)</th>
<th>control</th>
<th>peak lactation</th>
<th>post lactation</th>
<th>n</th>
<th>F</th>
<th>p-value</th>
<th>planned contrast to control</th>
<th>peak lactation</th>
<th>post lactation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MDA (μmol/g protein)</strong></td>
<td><strong>Liver</strong></td>
<td>1.26±0.09</td>
<td>0.54±0.03</td>
<td>0.54±0.02</td>
<td>8.7</td>
<td>31.07</td>
<td>&gt;0.0001</td>
<td>&gt;0.0001</td>
<td>0.001</td>
<td></td>
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<tr>
<td></td>
<td><strong>gastrocnemius muscle</strong></td>
<td>0.45±0.06</td>
<td>0.36±0.05</td>
<td>0.34±0.04</td>
<td>8.7</td>
<td>1.38</td>
<td>0.276</td>
<td>0.206</td>
<td>0.147</td>
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<tr>
<td><strong>Protein thiol (μmol/g protein)</strong></td>
<td><strong>Liver</strong></td>
<td>78.4±7.3</td>
<td>89.6±3.4</td>
<td>94.9±3.8</td>
<td>8.7</td>
<td>2.55</td>
<td>0.103</td>
<td>0.138</td>
<td>0.04</td>
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<tr>
<td></td>
<td><strong>gastrocnemius muscle</strong></td>
<td>40.0±2.3</td>
<td>41.1±0.0</td>
<td>48.0±5.5</td>
<td>8.7</td>
<td>2.33</td>
<td>0.134</td>
<td>0.317</td>
<td>0.044</td>
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<tr>
<td><strong>Total glutathione (μmol/g protein)</strong></td>
<td><strong>Liver</strong></td>
<td>61.7±9.1</td>
<td>51.8±5.3</td>
<td>52.7±9.1</td>
<td>8.7</td>
<td>0.48</td>
<td>0.608</td>
<td>0.379</td>
<td>0.44</td>
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<tr>
<td></td>
<td><strong>gastrocnemius muscle</strong></td>
<td>7.3±0.4</td>
<td>7.3±0.3</td>
<td>7.3±0.6</td>
<td>8.7</td>
<td>0.34</td>
<td>0.718</td>
<td>0.455</td>
<td>0.933</td>
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<tr>
<td><strong>Proportion of glutathione oxidised</strong></td>
<td><strong>Liver</strong></td>
<td>0.05±0.017</td>
<td>0.03±0.013</td>
<td>0.06±0.027</td>
<td>8.7</td>
<td>0.19</td>
<td>0.835</td>
<td>0.563</td>
<td>0.658</td>
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<tr>
<td></td>
<td><strong>gastrocnemius muscle</strong></td>
<td>0.02±0.005</td>
<td>0.02±0.005</td>
<td>0.03±0.007</td>
<td>8.7</td>
<td>0.44</td>
<td>0.651</td>
<td>0.977</td>
<td>0.425</td>
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</table>

* non-reproducing controls
† reproduction only
‡ reproduction plus territory defence
Supplementary table 2. Relationship between individual litter mass and markers of oxidative stress among primiparous females at peak and post lactation (Spearman’s rank correlations).

<table>
<thead>
<tr>
<th></th>
<th>peak lactation</th>
<th></th>
<th>post lactation</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>( n )</td>
<td>( r_z )</td>
<td>( p )-value</td>
<td>( n )</td>
</tr>
<tr>
<td>MDA (µmol/g protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>liver</td>
<td>8</td>
<td>-0.10</td>
<td>0.82</td>
<td>7</td>
</tr>
<tr>
<td>gastrocnemius muscle</td>
<td>7</td>
<td>0.54</td>
<td>0.22</td>
<td>7</td>
</tr>
<tr>
<td>serum</td>
<td>8</td>
<td>0.10</td>
<td>0.82</td>
<td>7</td>
</tr>
<tr>
<td>protein thiol (µmol/g protein)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>liver</td>
<td>8</td>
<td>-0.86</td>
<td>0.007**</td>
<td>7</td>
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<tr>
<td>gastrocnemius muscle</td>
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<td>-0.86</td>
<td>0.014*</td>
<td>7</td>
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<tr>
<td>GSH (µmol/g protein)</td>
<td></td>
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<tr>
<td>liver</td>
<td>8</td>
<td>0.14</td>
<td>0.74</td>
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<td>7</td>
<td>0.57</td>
<td>0.18</td>
<td>7</td>
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<tr>
<td>proportion of glutathione oxidised</td>
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<td></td>
<td></td>
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<tr>
<td>liver</td>
<td>7</td>
<td>0.61</td>
<td>0.15</td>
<td>7</td>
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<tr>
<td>gastrocnemius muscle</td>
<td>7</td>
<td>0.00</td>
<td>1.00</td>
<td>7</td>
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