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RESEARCH ARTICLE

Experimental evidence that litter size imposes an oxidative challenge to offspring

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ABSTRACT

The post-natal environment in which young develop can substantially impact development, adult phenotype and fitness. In wild mice, competition among litter-mates affects development rate and adult behaviour. We manipulated post-natal litter size in a cross-fostering design to investigate the effects of enlarged and reduced litter sizes on sexual signalling, oxidative stress and the links between them. Oxidative stress causes somatic damage that can limit reproductive success and lifespan, and is predicted to mediate investment in life-history traits, including sexual signals. We predicted that litter enlargement would cause an increase in potential oxidative stress, inhibit growth and reduce sexual signalling in male mice. Males reared in enlarged litters were smaller at weaning and, despite rapid growth immediately after weaning, remained smaller at 10 weeks of age than those reared in smaller litters. Females from enlarged litters were consistently smaller throughout post-weaning development and showed no increase in growth rate compared with females from reduced litters. In enlarged litters, protein thiol concentration was lower at weaning in the liver and kidneys, with this trend continuing at 10 weeks of age in the kidneys only. Aconitase enzyme activity was also lower in mice from enlarged litters at weaning and 10 weeks of age in the kidneys. Male mice from enlarged litters scent marked more frequently and had larger preputial glands than those from reduced litters, indicating greater sexual signalling investment irrespective of this increased oxidative challenge. The results of this study are the first to reveal oxidative costs of developmental stress in small mammals.

KEY WORDS: Oxidative stress, Sexual signalling, Rearing environment, Growth rate, Development

INTRODUCTION

Life-history theory predicts physiological trade-offs associated with investment in life-history traits such as growth pattern, age of maturity, reproduction (number, size and sex ratio of offspring) and lifespan (Harman, 1956). Allocating resources to one trait prevents those resources being invested in another (Stearns, 1992). For example, females that invest heavily in reproduction often incur higher energetic costs (Bergeron et al., 2011), which can reduce survival in natural conditions (Koivula et al., 2003).

Oxidative stress is an important physiological cost that has long been suggested to mediate investment in life-history traits (Harman, 1956). Oxidative stress occurs when reactive oxygen species (ROS) production outweighs the capacity of antioxidant defences to protect against damage (Monaghan et al., 2009). Increased ROS production does not always lead to oxidative stress as excess ROS can be regulated with an increased investment of anti-oxidant defences and repair mechanisms (Monaghan et al., 2009). However, when surplus ROS are not controlled, they can oxidise susceptible biomolecules and cause damage (Monaghan et al., 2009). This oxidative damage can contribute to increased ageing and decreased reproductive success, sometimes generating more ROS (Monaghan et al., 2009).

Evidence that investment in reproduction results in an increase in oxidative stress is currently equivocal (see reviews in Stier et al., 2012; Metcalfe and Monaghan, 2013; Speakman and Garratt, 2014). Bergeron et al. (2011) found that wild chipmunk (*Tamias striatus*) females with higher reproductive output incur slightly higher levels of serum oxidative stress than those that invest less in reproduction. Garratt et al. (2013) found the opposite; female mice (*Mus musculus domesticus*) housed in laboratory conditions with experimentally enlarged litters incurred lower levels of one marker of oxidative damage and showed no difference in other markers when compared with females with reduced litters and with non-reproductive females. Xu et al. (2014) similarly failed to observe an increase in oxidative damage when litters were experimentally enlarged in Brandt's voles (*Lasiopodomys brandtii*). Aloise King et al. (2013) also reported no increase in oxidative stress with a manipulated increase in litter size or when female house mice were simultaneously lactating for one litter and gestating a second litter.

With little evidence that elevated reproductive effort causes oxidative stress in females, we predicted that oxidative costs of large litter sizes might be more detectable in the females' offspring. Both among and within species, there is a trade-off between offspring number and quality (McNamara and Houston, 1992; Stearns, 1992). Females may produce many, lower quality offspring instead of fewer offspring of higher quality, investing the same amount into each litter regardless of the number of young. Offspring from larger broods or litters are often smaller and less developed at weaning (Machin and Page, 1973; Koskela, 1998; Burness et al., 2000), with negative effects that persist into adulthood, including later first reproduction (Alonso-Alvarez et al., 2006) and lower survival probability (Humphries and Boutin, 2000). Skibiel et al. (2013) found that in a wild population of Columbian ground squirrels (*Urocitellus columbianus*), offspring reared in experimentally enlarged litters had a lower survival probability than those from reduced litters.

Although smaller at weaning, young from enlarged litters can increase their post-weaning growth rate to match a projected growth rate, had they not been born smaller, compared with others within the population (Alonso-Alvarez et al., 2007). Such compensatory growth can deliver the ecological benefits of not being small (Arendt, 1997; Metcalfe and Monaghan, 2003), timely sexual

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Journal of Experimental Biology (2015) 218, 3911-3918 doi:10.1242/jeb.116327

maturity and early reproduction (Monteiro and Falconer, 1966). However, compensatory growth can be associated with costs such as impaired cognitive performance (Fisher et al., 2006) and increased susceptibility to oxidation of red blood cells (Alonso-Alvarez et al., 2007) that can persist into adulthood and even decrease lifespan (Lee et al., 2013).

Factors associated with the rearing environment, such as maternal investment, can have subsequent effects on an offspring's adult physiology and reproductive success (Machin and Page, 1973; Mousseau and Fox, 1998; Burness et al., 2000; Criscuolo et al., 2008). Sexual signalling is an important contributor to lifetime reproductive success, signalling information about an individual's health and status to competitors and potential mates (Hurst, 1990; Rich and Hurst, 1998; Maynard Smith and Harper, 2003; Malone et al., 2005). For example, male wild house mice use urine deposits as scent marks to convey information about their sex, quality, territory ownership and dominance status to conspecifics (Hurst and Beynon, 2004). Scent marking is important in the advertisement of successful territory defence. Males regularly re-apply scent marks to their territories and mark over competitor male scents in order to maintain attractiveness to females (Rich and Hurst, 1998, 1999). Thus, for wild mice, the ability to invest in scent marking is an important life-history fitness component.

In this study, we explored the effects of rearing environment and compensatory growth on wild-derived house mice (*Mus musculus domesticus* Linnaeus) offspring oxidative stress and subsequent investment in sexual signalling. We manipulated litter size by cross-fostering newborn pups into (reduced) litters of three pups or (enlarged) litters of eight pups. Oxidative stress was analysed in sub-samples of pups culled at weaning age or at 10 weeks of age. Growth rate and sexual signalling (in males) were measured between weaning and 10 weeks of age. We predicted that pups from enlarged litters would be smaller at weaning than pups from reduced litters and they would increase their growth rate such that the variation of body mass between treatments would disappear by 10 weeks of age. We also predicted that pups from enlarged litters would incur higher oxidative stress at weaning and 10 weeks of age. With this imposed oxidative challenge, we predicted that males from enlarged litters would have a reduced capacity for reproductive allocation and, therefore, scent mark less frequently and have smaller preputial glands.

MATERIALS AND METHODS

Subjects and animal husbandry

Experimental animals were second or third generation house mice (*Mus musculus domesticus*) derived from individuals that were wild-caught at a chicken farm in northwest Sydney, Australia. Animals were kept in polypropylene cages (514×213×130 mm) lined with cobb bedding (Techniplast Australia Pty Ltd, Lane Cove, NSW, Australia) and supplied with toilet rolls, shredded paper, and tissues for nesting. Each cage was supplied with rodent pellets (Gordon's Specialty Stockfeeds, Yanderra, NSW, Australia) and water *ad libitum*. Mice were kept on a 12 h:12 h reverse light cycle with white lights on at 20:00 h and off at 08:00 h. All experimental measures were performed during the dark period using a red light. This experiment was approved by the University of New South Wales Animal Care and Ethics Committee (approval: 12/89B). As a result of time constraints, this experiment was conducted in three blocks over the course of 5 months to ensure statistical power. All statistics and means presented are for all blocks inclusive.

Cross-fostering

Non-related males and females (dam age ranged from 125 to 447 days of age) were introduced by placing them side-by-side in independent cages for 5 days. Soiled bedding was then swapped between mating pairs' cages to prime females for oestrus (Jemiolo et al., 1985). After 3 days, the mating pairs were weighed and caged together (block 1, N=15 pairs; block 2, N=17 pairs; block 3, N=8 pairs). When pregnancy was established (mass gain of ≥3 g from pre-pregnancy mass), mating pairs were split to prevent post-partum pregnancy, and pregnant females were placed in clean cages. Females were randomly assigned to either the enlarged (8 pups) or reduced (3 pups) litter treatment. There were no differences in the foster dams' body mass ($F_{1,38}$=0.74, P=0.396) or age ($F_{1,38}$=1.27, P=0.267) between treatments at the commencement of each block. The females' reproductive history was also as evenly spread across treatments as possible depending on the availability of female stocks (enlarged litter treatment: virgin N=6, previously mated N=6; reduced litter treatment: virgin N=12, previously mated N=16). There were also no significant differences in the number of pups in the dam's natural litter ($F_{1,38}$=0.42, P=0.519) or the mass of the natural pups ($F_{1,38}$=0.51, P=0.479) between treatments.

Cross-fostering was performed with newborn pups up to 48 h of age with sibling pups randomly distributed between treatments where possible. Each dam received either a reduced litter of 3 pups or an enlarged litter of 8 pups from two or more dams; no dam received any of her biological pups during cross-fostering. The sex ratio of cross-fostered litters was not manipulated in this study. As litter size manipulations were performed within 48 h of birth, we were sometimes limited by available pup numbers at the time of treatment application and were able to apply only the reduced litter treatment on two occasions and only the enlarged litter treatment on one occasion. Cross-fostered pups did not vary in average body mass ($F_{1,38}$=0.05, P=0.823) between treatments and the number of males and females in each litter was independent of treatment (χ^2_1=1.484, P=0.223). It is common in wild mice that fatalities occur within litters in the first few days after birth. Although cross-fostering of 3 pups and 8 pups per litter was performed, because of subsequent deaths, the final litter sizes varied. Cross-fostered litters remained in the experiment if reduced treatment litters consisted of 1–3 pups (28 litters, N=72 pups) and enlarged treatment litters contained 6–8 pups (12 litters, N=83 pups) at weaning age. All other variations of litter size were removed from the study. For each block, cross-fostering of litters was performed over a period of 3–9 days.

Weaning

At weaning age (28 days old), pups were removed from the dam and sex was determined (enlarged litter treatment: N=37 males, N=45 females; reduced litter treatment: N=38 males, N=31 females). Depending on the number of each sex within the litter, a subsample of mice of each sex from each litter were culled and assessed for oxidative stress in both treatments (early group; enlarged litter treatment: N=16 males, N=19 females; reduced litter treatment: N=11 males, N=10 females). For example, if there were 5 females and 3 males within a litter, 2 females and 1 male would be culled. The remaining pups were weighed and continued in the experiment until 10 weeks of age (late group; see below). Female pups were housed with 1–2 non-related females to avoid stress from isolation, and this housing condition was evenly applied across treatments. Males were housed individually to prevent aggression and fatal fights. Animals in the early group were culled by cervical dislocation and organ samples were taken. The liver and kidneys were removed, weighed and then snap-frozen in liquid nitrogen. Organs were subsequently placed in a −80°C freezer until oxidative stress assays were performed. Preputial glands were removed from male subjects, compressed to remove as much preputial fluid as possible and weighed as a measure of preputial gland size.

Late treatment

Mice assigned to be culled at 10 weeks of age were weighed weekly (enlarged litter treatment: N=21 males, N=26 females; reduced litter treatment: N=27 males, N=21 females). We also tested males for scent marking weekly from 6 to 10 weeks old. Scent mark frequency was measured by placing a male in a clean cage lined with absorbent paper (Benchkote; Sigma-Aldrich, St Louis, MO, USA) (Humphries et al., 1999), with no food or water for 1 h. Scent marking frequency was determined by placing the Benchkote samples under ultraviolet light and counting the

Journal of Experimental Biology (2015) 218, 3911-3918 doi:10.1242/jeb.116327

number of marks deposited (Desjardins et al., 1973). One researcher counted the scent marks, applying the same counting techniques to all samples obtained. The researcher was blinded to which sample was in each treatment by the numbering system used, as treatment was not recorded on the scent marking collection paper. At 10 weeks old (±1 day), all late treatment animals were culled and organ samples were obtained using the same measurements as in the subsample of animals analysed at weaning age.

Oxidative stress analysis

To assess oxidative stress, we examined two oxidation markers in two tissues (liver and kidney) of 4 and 10 week old pups of both sexes. Protein thiol concentrations were used as a biomarker of oxidative stress as they are susceptible to oxidation by free radicals (Vasilaki et al., 2006). Organ samples were macerated into a fine powder in liquid nitrogen and homogenised in 1% 5-sulfosalicylic acid dihydrate buffer. Samples were then centrifuged for 10 min at 10,000 ***g*** at 4°C to separate the pellet. The lower molecular weight material (supernatant containing glutathione) was separated from the pellet and discarded. Protein thiols were measured as described by Di Monte et al. (1984; see protein sulfhydryl groups measure description under Methods section 'Measurement of protein mixed disulfides, protein sulfhydryl groups and protein binding' in their study), altered for use on a plate reader by transferring sample and glutathione standards (for calibration curve) to a 96-well microplate and measuring the absorbance at 405 nm in an automatic microplate reader (Biotek Instruments, Winooski, VT, USA). This adaptation has been used previously (Garratt and Brooks, 2012; Garratt et al., 2013, 2014).

As an increase in ROS production can lead to oxidative stress, aconitase enzyme activity, which is susceptible to deactivation by certain ROS (in particular, a superoxide radical) and is thus is used as a marker of ROS production and subsequently potential oxidative stress (Hausladen and Fridovich, 1994, 1996; Gardner et al., 1995; Gardner, 1997), was also measured. Aconitase plays an important role in the tricarboxylic acid cycle and is principally located within mitochondria (Wiegand and Remington, 1986; Gardner et al., 1995); therefore, citrate synthase, a marker of mitochondrial density, was also measured (Pichaud et al., 2010). For both aconitase and citrate synthase activity assays, tissue samples were homogenised by macerating tissue into a fine powder in liquid nitrogen and suspended in 100 mmol l^{-1} potassium phosphate buffer. For full method of both the aconitase and citrate synthase activity assays, see Garratt et al. (2013) (see also Pichaud et al., 2010).

The protein concentration of all samples for each assay was measured using the method developed and outlined by Lowry et al. (1951). Organ samples were analysed at the completion of all three experimental blocks. Assays were performed separately by organ, with the samples from all blocks and ages (weaning or 10 weeks old) randomly placed on the microplate for reading.

Statistical analysis

All statistical analyses were performed using SPSS version 2.1 (IBM Corp, Armonk, NY, USA). In all analyses, we fitted foster dam as a random effect to account for non-independence of individuals originating from the same litter in all models unless otherwise described. Experimental block was also fitted as a random factor to all analyses to control for the time differences of each group of experimental mice. Growth rate and scent marking data were analysed using general linear mixed models (GLMMs) with mouse ID as a subject nested within foster dam to account for the repeat measure nature of the data. GLMMs were also used to test for effects on male organ size and for oxidative stress measures. Preputial gland mass was transformed to the fourth root and oxidative stress variables were log transformed if needed to ensure a normal data distribution. Scent marking frequency was also transformed to $\log_e(x+1)$ to account for measures of zero deposits and normalise the data. A backward stepwise procedure was followed for all models and significance was determined at $P \leq 0.05$.

RESULTS

Growth

At weaning (week 4 in Fig. 1), pups from enlarged litters were lighter than individuals from reduced litters (effect of litter size: $F_{1,35}=17.6$, $P<0.001$) and females were lighter than males (effect of sex: $F_{1,127}=67.0$, $P<0.001$; marginal means±s.e.m. of reduced litter: males 10.83±0.26 g, females 9.22±0.27 g; marginal means± s.e.m. of enlarged litter: males 8.85±0.34 g, females 7.85±0.34 g). The sex by treatment interaction was marginally significant (interaction between treatment and sex: $F_{1,127}=3.7$, $P=0.057$) probably due to the large early treatment effect within males (Fig. 1).

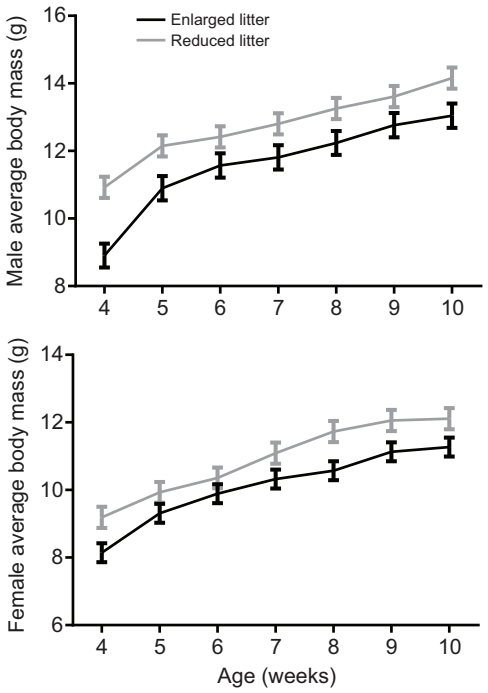

Fig. 1. Growth rate in the enlarged and reduced litter treatments. Growth rate was calculated from mean body mass from weaning age (4 weeks) to 10 weeks of age for males (reduced litter *N*=27, enlarged litter *N*=21) and females (reduced litter *N*=21, enlarged litter *N*=26) in each treatment. Males from enlarged litters grew slower than those from reduced litters ($P<0.001$). Females from enlarged litters were consistently smaller than those from reduced litters throughout development ($P=0.032$). Data are presented as estimated marginal means±1 s.e.m. for each measure from general linear mixed models for growth rate.

Overall, the GLMM revealed a significant interaction between sex, age and treatment ($F_{6,546}=2.2$, $P=0.044$; Fig. 1) for weekly growth analysis. We therefore prepared partial datasets to present GLMMs for males and females separately. Female pups from enlarged litters were consistently smaller than those from reduced litters (effect of treatment: $F_{1,45}=4.9$, $P=0.032$). There was no difference in the growth rate between treatments (interaction between treatment and age for females: $F_{6,270}=1.4$, $P=0.217$). For males, there was a significant interaction between age and treatment; males from enlarged litters grew faster (interaction between weekly mass and litter size: $F_{6,276}=5.8$, $P<0.001$).

Scent marking and preputial glands

Preputial glands produce pheromones that attract females (Bronson and Caroom, 1971). Preputial gland size is thought to be an important component of reproductive investment; glands are heavier in dominant males than in subordinates (Bronson and Marsden, 1973). Males from enlarged litters had significantly larger preputial glands than those from reduced litters, and treatment affected weaning age and 10 week old mice similarly (Table 1, Fig. 2). Preputial glands were also smaller in weaning age males

Journal of Experimental Biology (2015) 218, 3911-3918 doi:10.1242/jeb.116327

Table 1. Preputial gland mass and scent marking frequency analysed with body mass as a covariate

	Preputial gland mass					Weekly scent marking frequency				
	F	d.f.	*P*	Coefficient	s.e.m.	*F*	d.f.	*P*	Coefficient	s.e.m.
Treatment	5.90	1, 70	0.018	0.024	0.010	5.00	1, 47	0.030	0.900	0.402
Age	11.31	1, 71	0.001	−0.045	0.013	11.14	1, 190	0.001	2.460	0.737
Age^2	–	–	–	–	–	9.77	1, 190	0.002	−0.144	0.046
Treatment×age	0.09*	1, 69	0.771	–	–	1.34*	1, 189	0.249	–	–
Treatment×age^2	–	–	–	–	–	0.32*	1, 188	0.575	–	–
Body mass	45.77	1, 69	>0.001	0.019	0.003	3.50	1, 86	0.065	0.199	0.106

Preputial gland mass (g) was transformed to the fourth root. Scent marking frequency was $\log_e(x+1)$ transformed. There are no age^2 results for preputial gland mass because it was not measured as a trajectory.
*Not included in the final model.

than in 10 week old males (Table 1, Fig. 2). These analyses all included preputial body mass as a covariate (Table 1).

Scent marking frequency was recorded between 6 and 10 weeks of age. Scent marking frequency reached a peak and then slowed, and so we tested the fit of a quadratic term for age. Scent marking frequency increased with age (Table 1), but this effect slowed, resulting in a significant negative quadratic term (Table 1) for both treatments, indicating a non-linear rate of change over time; therefore, age^2 remained in the model before further analysis. This component of olfactory signalling was also marginally related to body mass (Table 1). Males from enlarged litters scent marked more frequently than those from reduced litters regardless of age (Table 1). This trend became marginal when body mass was not fitted as a covariate (effect of litter size: $F_{1,46}$=3.28, P=0.077).

Oxidative stress

For all oxidative stress marker analyses, analyses are presented without body mass as a covariate, as the difference in body mass was a direct effect of the applied treatment. The plate number on which each sample was run in the plate reader was fitted as a random factor to control for between-plate variability. For assays in which repeat samples were run (all except protein thiol concentration because of insufficient sample volume), repeatability tests were run with sample repeats no lower than R=0.928.

There was a significant interaction between litter size and age in protein thiol concentration in the liver (Table 2, Fig. 3A). Although this interaction was not present in the kidneys, pups from enlarged litters had lower protein thiol concentrations than those from reduced litters overall (Table 2, Fig. 3B). In the kidneys, pups at weaning also had lower protein thiol concentrations than 10 week old individuals. Females also had lower protein thiol levels in the kidneys than males, but there were no sex differences in liver thiols (Table 2).

GLMMs revealed that pups from enlarged litters had significantly lower aconitase enzyme activity to citrate synthase activity (mitochondrial density) ratio than those from reduced litters, indicating higher levels of ROS production in the kidneys (Table 2, Fig. 3D). This decrease in aconitase enzyme activity was not observed in the liver (Table 2, Fig. 3C). Females also had lower enzyme activity than males in the liver and kidney regardless of age or treatment (Table 2).

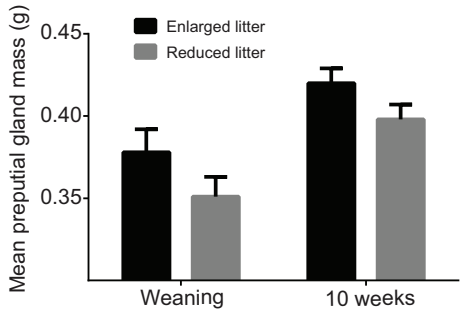

Fig. 2. Preputial gland mass in the enlarged and reduced litter treatments. Individual preputial gland mass (fourth root transformation) was obtained for males at weaning (reduced litter N=11, enlarged litter N=16) and 10 weeks of age (reduced litter N=27, enlarged litter N=21) for each treatment. Males from enlarged litters had larger preputial glands than those from reduced litters at both weaning and 10 weeks of age (P=0.018). Older males also had larger preputial glands than younger males (P=0.001). Data are presented as estimated marginal means±1 s.e.m. for each measure from a general linear mixed model for preputial gland mass.

DISCUSSION

Offspring from enlarged litters produce more ROS and may suffer more oxidative stress than those from reduced litters at both weaning and 10 weeks of age. One marker of ROS production used in this study, aconitase enzyme, plays a key role in the tricarboxylic acid cycle within mitochondria that generates energy from derivatives of carbohydrates, fats and proteins in aerobic organisms. A decrease in this enzyme could significantly alter the cycle's efficiency and aconitase has been shown to decline with age in the kidneys of laboratory mice (Yarian et al., 2006). In the current study, mice originating in enlarged litters had decreased aconitase activity at both weaning and 10 weeks of age in the kidneys compared with reduced litter offspring, indicating a decreased turnover rate of the tricarboxylic acid cycle and potential increased ageing, possibly leading to oxidative stress. As oxidative stress has been suggested to be associated with increased ageing and shorter lifespan (Harman, 1956; Beckman and Ames, 1998; Finkel and Holbrook, 2000), our results indicate that originating in a larger litter can potentially impose considerable physiological and fitness costs in later adulthood. The exact pathways by which being reared in a larger litter leads to oxidative stress, and the ways in which female optimisation of litter size is related to offspring oxidative stress and potential damage remain to be resolved. The extent to which the oxidative costs arise due to constraints on maternal investment into each offspring, the stress of competing within large litters, or the challenges of compensatory growth also remain unknown. Our cross-fostered experimental manipulation of litter size, however, illustrates that oxidative costs to offspring may be an important element of female reproductive optimisation.

In the current study, there was a large difference in body mass between young that originated from larger litters and those from litters with fewer pups at 4 weeks of age (see also Monteiro and

Journal of Experimental Biology (2015) 218, 3911-3918 doi:10.1242/jeb.116327

Table 2. Oxidative stress results for protein thiol concentration and aconitase enzyme activity in the liver and the kidneys

	Liver						Kidneys					
	Protein thiol concentration			Aconitase enzyme activity			Protein thiol concentration			Aconitase enzyme activity		
	F	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*
Treatment	5.93	1, 33	0.020	2.50	1, 27	0.125	13.80	1, 33	0.001	3.92	1, 140	0.050
Age	2.35	1, 113	0.128	0.00	1, 111	0.978	23.33	1, 123	>0.001	2.69	1, 139	0.103
Sex	0.94	1, 128	0.335	11.57	1, 130	0.001	38.69	1, 138	>0.001	10.66	1, 139	0.001
Treatment×age	8.89	1, 114	0.004	0.33*	1, 111	0.567	2.51*	1, 121	0.116	0.61*	1, 139	0.435
Sex×treatment	2.82*	1, 129	0.096	1.54*	1, 133	0.216	0.34*	1, 137	0.559	0.32*	1, 137	0.573
Sex×treatment×age	0.07*	2, 115	0.933	0.08*	2, 114	0.920	0.97*	2, 125	0.381	1.89*	2, 135	0.154

Protein thiol concentration was measured as μmol g^{-1} protein; aconitase enzyme activity was obtained as a ratio to citrate synthase activity (U mg^{-1} protein). Kidney protein thiol concentration, and liver and kidney aconitase enzyme activity/citrate synthase were log transformed. Models were also fitted with plate number as a random factor to control for variation between assay plates.
*Not included in the final model.

Falconer, 1966; Machin and Page, 1973). This was expected as maternal investment determines weaning body mass and so larger litters result in smaller pups (Cox et al., 1959). Within the few weeks post-weaning, this difference in body mass decreased (predominately in males), indicating that mice from large litters grew faster than those from small litters. This could reflect a release from within-litter competition for milk, as mouse pellets were provided *ad libitum*. Although there was a clear increase in growth rate for males, young of both sexes from larger litters were still smaller at 10 weeks of age than those from small litters (see also Monteiro and Falconer, 1966), indicating a long-term trade-off between offspring number and quality. The difference in mean body mass of males between treatments was more pronounced at weaning than at 10 weeks of age, as indicated by a statistically significant treatment by age interaction, suggesting that compensatory growth had occurred (see also Monteiro and Falconer, 1966). This trend was not observed in females.

There is conflicting evidence regarding the effect of rearing environment quality on subsequent adult survival in mice and other small rodents. Sun et al. (2009) found that laboratory mice from enlarged litters had a higher median and maximum lifespan than those from smaller litters. Consistent with this, Kappeler et al. (2009) reported reduced IGF-1 signalling in mice from enlarged litters. Reduced IGF-1 signalling is linked with longer lifespan and increased oxidative stress resistance (Holzenberger et al., 2003). These results could be seen as contradictory to those obtained in the current study. In congruence with the current study, Skibiel et al. (2013) found that young from experimentally enlarged litters had a decreased survival probability in wild populations of ground squirrels, and Hürlimann et al. (2014) reported the same result in

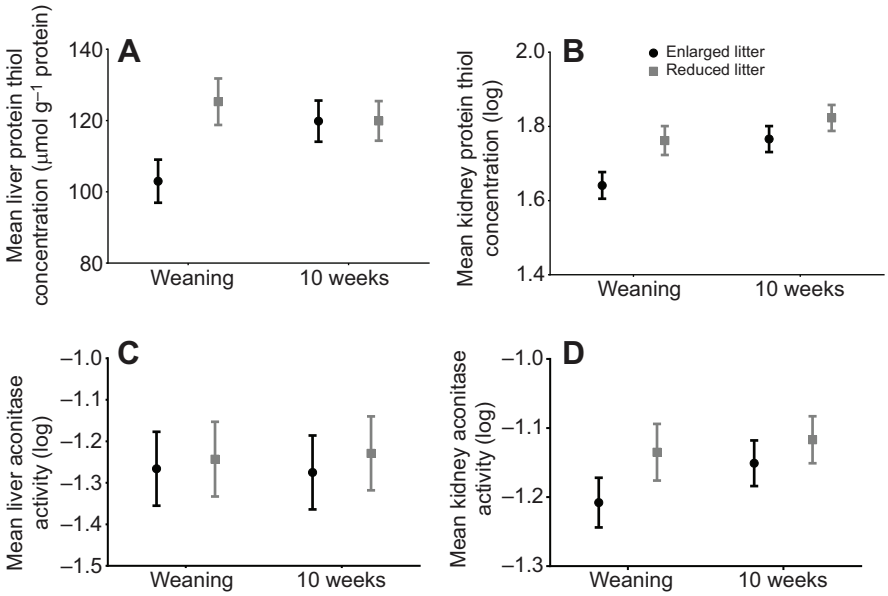

Fig. 3. Mean protein thiol concentration and aconitase enzyme activity for the enlarged and reduced litter treatments. (A,B) Mean protein thiol concentration in the liver (A; 4 weeks: enlarged litter *N*=35, reduced litter *N*=21; 10 weeks: enlarged litter *N*=46, reduced litter *N*=43) and kidney [B; 4 weeks: enlarged litter *N*=35, reduced litter *N*=21; 10 weeks: enlarged litter *N*=47, reduced litter *N*=46; kidney data (μmol g^{-1} protein) were log transformed] for each treatment. (C,D) The ratio of mean aconitase enzyme activity to citrate synthase activity in the liver (C; 4 weeks: enlarged litter *N*=35, reduced litter *N*=21; 10 weeks: enlarged litter *N*=46, reduced litter *N*=45) and kidney [D; 4 weeks: enlarged litter *N*=33, reduced litter *N*=21; 10 weeks: enlarged litter *N*=46, reduced litter *N*=45; both measures (U mg^{-1} protein) were log transformed] for each treatment. Mice from enlarged litters had lower protein thiol concentrations in the liver than those from reduced litters at 4 weeks of age, but not at 10 weeks of age (*P*=0.004). Protein thiol levels in the kidneys were lower for mice from enlarged litters at both weaning and 10 weeks of age (*P*=0.001). There was no difference in aconitase activity in the liver (*P*=0.125); however, aconitase activity was lower for mice from enlarged litters in the kidneys (*P*=0.050). Data are presented as estimated marginal means±1 s.e.m. for each measure from general linear mixed models for each tissue sample.

a laboratory study on wild-derived bank voles (*Microtus arvalis*). The studies by Sun et al. (2009) and Kappeler et al. (2009) used laboratory strains of mice, whereas Skibiel et al. (2013) and Hürlimann et al. (2014) used wild and wild-derived animals, suggesting that these different strains might be employing different life-history strategies for managing stress associated with a poor quality rearing environment. There could also be a hormesis effect occurring, where early life exposure to a stressor increases oxidative challenges, as reported in the current study, but later in life this assists with stress resistance, subsequently increasing lifespan (Ristow and Schmeisser, 2011) as a result of using a captive population.

Contrary to our *a priori* prediction, males reared in enlarged litters had larger preputial glands and scent-marked more frequently despite consistently maintaining a smaller body mass than males from reduced litters. Preputial glands, which are responsible for the production of certain volatile signalling molecules released in male urine (Bronson, 1966; Bronson and Caroom, 1971), are larger in dominant males (Bronson and Marsden, 1973; Collins et al., 1997) and dominant males experience higher reproductive success. The measure of scent marking frequency at 10 weeks of age probably captures variation in both the degree of sexual maturation and in the allocation of resources to sexual signalling. This indicates that males from enlarged litters in the current study might be reaching sexual maturity at a younger age and contributing more resources toward reproductive investment than those from reduced litters.

Gosling et al. (2000) suggested that smaller male lab mice can maintain dominance over larger males by investing more in olfactory signalling and increased preputial gland mass. This investment appeared to come at a cost, however, because these originally small males had a slower growth rate. The large preputial glands and high level of signalling displayed by males in our enlarged litter treatment might, therefore, have contributed to the smaller size of these males. Gosling et al. (2000) also report an eventual dominance reversal in which larger males eventually became dominant over the small males, an effect also reported by Ryan and Wehmer (1975). Whilst in the current study smaller males scent marked more frequently up to the end of the experiment at 10 weeks of age, this behaviour may have reversed later on in adulthood.

Competition among littermates during development might encourage male investment in competitive traits such as preputial gland size and scent marking behaviour (Collins et al., 1997), and aggression (Mendl and Paul, 1991). With our litter size manipulation, males from the enlarged litter treatment were inevitably raised with a greater number of siblings than those from reduced litters, and so would have greater social experience. This may represent one pathway by which litter size manipulation affected scent marking frequency; it was as a consequence of the treatment we applied.

It is also possible that increased male reproductive allocation to sexual signalling is a response to greater oxidative challenges. Assuming that increased oxidative stress equates to reduced survival prospects, males from large litters may invest more in reproduction immediately after weaning because there is less motivation to withhold resources for allocation to reproduction or other areas later in life – because they may not survive to these ages. Alternatively, increased reproductive investment might contribute to the production of ROS, which, in turn, can result in higher levels of oxidative stress. Scent marking frequency was higher in males from enlarged litters, which could have contributed to increased oxidative challenges as opposed to being a result of it. This prediction is feasible as scent marking entails energetic costs that could increase ROS production (Gosling et al., 2000; Garratt et al., 2012).

So far, evidence linking oxidative stress to olfactory signalling is weak and contradictory. Garratt et al. (2012) reported oxidative damage in the gastrocnemius muscle as a result of increased reproductive effort (reproduction, aggression and sexual signalling) in wild-derived male mice. Garratt et al. (2014) also reported a decline in oxidative stress in laboratory mice that increased their investment in olfactory signalling over a 3 week period. We tested for a relationship between protein thiols and aconitase activity, and scent marking frequency and found no relationship ($P>0.1$ in all cases). Thus, if oxidative challenges are directly related to scent marking frequency, it is not through a simple linear relationship and is likely to have been compounded by the fact that these males increased investment prior to full sexual maturity, or because they were currently undergoing a period of fast growth. Females might also be accelerating the onset of sexual maturity, although we did not measure age of maturity in the current study. The ability to begin producing offspring at a younger age can optimise reproductive scheduling in response to a shorter lifespan, even if it potentially hastens the onset of death (Williams, 1966; Kokko, 1997).

Somatic compensatory growth has been suggested to impose oxidative stress. A study by Alonso-Alvarez et al. (2007) manipulated brood size of zebra finches and found that the increased growth rate (compensatory growth) negatively affected red blood cell resistance to free radical attacks, indicating a potential increased level of oxidative stress. In the current study, males from enlarged litters exhibited an increased growth rate. In females, the differences in body mass between treatments were slightly more pronounced at weaning age than at 10 weeks of age, indicating a possibly small increase in growth rate, although not significantly so. While compensatory growth could be contributing to the imposed oxidative challenges in pups from enlarged litters, treatment differences in protein thiols and aconitase activity were present in 4 week old individuals possibly before any compensatory growth would have occurred.

While compensatory growth may have contributed to a potential increase in oxidative stress, there are many other potential factors in this study such as maternal investment, sibling competition and litter size-dependent stress. In guinea pigs (*Cavia aperea* f. *porcellus*), during the period of development when nutrients are predominantly gained from lactating females, pups from large litters had higher levels of the stress hormone cortisol than those from small litters as a result of increased within-litter competition (Fey and Trillmich, 2008). An increase in glucocorticoids (the hormone group to which cortisol belongs) has been linked to increased oxidative stress in vertebrates (Costantini et al., 2011), so this early life stress should not be ruled out as a potential factor for the results of this study.

The theoretic prediction that oxidative damage might be one cost of pregnancy and lactation (Speakman, 2008; Dowling and Simmons, 2009), thus constraining female reproductive investment, has not been upheld (Garratt et al., 2011; Oldakowski et al., 2012; Yang et al., 2013; Speakman and Garratt, 2014; Xu et al., 2014), particularly in house mice (Aloise King et al., 2013; Garratt et al., 2013). Our results suggest that physiological costs of investment in greater numbers of offspring might arise as oxidative stress to the young as a trade-off between offspring number and quality.

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Conclusions

Offspring originating in an enlarged litter experienced an increased oxidative challenge compared with those from reduced litters, suggesting that oxidative costs may be mediating a trade-off between offspring number and quality. The increased growth rate, or compensatory growth, associated with small weaning body mass and post-weaning development in a large litter may contribute to this increase in oxidative challenge in males. However, the relationship between increased growth rate and potential oxidative stress is not clear, as oxidative costs were also found in offspring directly at weaning, so the stress of being in a crowded litter with limited food resources may also contribute to this oxidative cost. As a result, males from enlarged litters might be investing more resources into reproductive effort by increasing sexual signalling to employ a faster pace of life as an altered life-history strategy. The age of sexual maturity might also be contributing to the increased oxidative costs of offspring, although this was not examined in the current study and is a point for further research.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

All authors collaborated to conceive the study and interpret the findings; A.B.G. conducted the research and wrote the manuscript. All authors revised the final manuscript.

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