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Tissue-dependent changes in oxidative damage with male reproductive effort in house mice

Michael Garratt[1], Francis McArdle[2], Paula Stockley[1], Aphrodite Vasilaki[2], Robert J. Beynon[3], Malcolm J. Jackson[2] and Jane L. Hurst*[,1]

[1]*Mammalian Behaviour and Evolution Group, Institute of Integrative Biology, University of Liverpool, Leahurst Campus, Neston CH64 7TE, UK;* [2]*Pathophysiology Research Group, Institute of Ageing and Chronic Disease, University of Liverpool, Liverpool L69 3GA, UK; and* [3]*Protein Function Group, Institute of Integrative Biology, University of Liverpool, Liverpool, L69 7ZB, UK*

Summary

1. Investment in reproduction is anticipated to be costly and can decrease survival or future reproductive success. For males, substantial reproductive costs may be accrued when competing for mates, particularly when individuals need to invest heavily in the production of sexual signals to attract females. On a proximate level, increased male signalling effort can cause somatic damage because of oxidative stress, although this has been demonstrated only in species with visual sexual signals.

2. We tested whether reproductive effort (comprising reproduction, aggression and scent signalling) is associated with increased oxidative stress in male house mice (*Mus musculus domesticus*). Sexual signalling in this species involves the production and deposition of scent signals containing a high concentration of protein around a defended territory. Male reproductive investment was manipulated by housing males alone, with a female or with a female and in the vicinity of competitors.

3. Males breeding in the vicinity of competitors invested the most in olfactory signalling as well as having regular aggressive interactions with other males. These males tended to show greater oxidative damage to lipids in the gastrocnemius muscle but no other indication of increased oxidative stress. Instead, lipid oxidation was lower in the serum and liver of reproductive males compared with those housed alone.

4. Our results highlight that oxidative stress does not always occur simply as a function of increasing reproductive effort. The lack of a consistent increase in oxidative damage could be due to adaptive regulation of antioxidants and/or a consequence of the scent signalling system of house mice, which differs considerably from the visual signalling of birds previously examined in this context.

Key-words: life history, major urinary proteins, mammals, oxidative stress, reproduction, scent signalling, sexual selection

Introduction

Investment in reproduction is expected to be costly and can decrease survival or future reproductive success (Stearns 1992). Reproductive costs may be generated by numerous processes and behaviours relating to reproduction, such as the growth of sex organs, the act of copulation and investment in parental care (Rose 2005). Competition for mates is likely to be a particularly costly component of reproduction for males. Territory defence, male-male combat and the production of morphological weapons such as antlers and horns can consume energy and increase risk of mortality (reviewed in Andersson 1994). Further costs may be entailed when investing in the production and maintenance of sexual signals used to attract females. Costs associated with such signals may help to maintain honesty (Grafen 1990) but can have negative impacts on other life history components (Mappes *et al*. 1996; Hunt *et al*. 2004).

Oxidative stress has been highlighted as a possible physiological consequence of reproduction that could limit investment in other life history components (Costantini 2008; Dowling & Simmons 2009; Monaghan, Metcalfe & Torres 2009). Normal metabolic processes produce a variety of reactive oxygen species (ROS) that can damage biomolecules

*Correspondence author. E-mail: jane.hurst@liv.ac.uk

unless regulated by enzymatic and non-enzymatic 'antioxidant' systems (Balaban, Nemoto & Finkel 2005). Oxidative stress results when there is a serious imbalance between the production of ROS and the capacity to control their damaging effects (Monaghan, Metcalfe & Torres 2009). It causes impaired redox regulation and altered cellular signalling (Jones 2006), is implicated in the onset of hundreds of diseases (Halliwell & Gutteridge 1999) and may play a major role in the ageing process (Harman 1956; Beckman & Ames 1998). As the majority of ROS are produced from oxidative phosphorylation during energy metabolism, investment in energetically demanding components of reproduction could increase ROS production and potentially cause oxidative stress (Alonso-Alvarez *et al.* 2004; Speakman 2008). Males may be particularly susceptible to this when engaging in aggressive behaviours and sexual signalling (Alonso-Alvarez *et al.* 2007; Metcalfe & Alonso-Alvarez 2010), as investment in both of these activities can increase metabolic rate (Haller 1995; Buchanan *et al.* 2001; Basolo & Alcaraz 2003; Decarvalho, Watson & Field 2004; Radwan *et al.* 2006; Briffa & Sneddon 2007; Hasselquist & Bensch 2008). However, increased metabolism does not produce a proportional increase in ROS production (Barja 2007) and increased ROS production does not necessarily cause long-term oxidative stress. Healthy individuals have a variety of defence mechanisms that are up-regulated in response to increased ROS production, repairing oxidative damage and limiting its subsequent occurrence (Droge 2002). Therefore, even when investing in components of reproduction that have substantial energy requirements, animals may be able to limit the occurrence of oxidative stress (Garratt *et al.* 2011).

To date, the relationship between oxidative stress and male reproductive investment has been examined almost entirely in relation to the production of visual signals involved in attraction of mates. In species with these traits, the allocation or synthesis of several pigments may heighten the trade-off between sexual signalling and oxidative stress. This may facilitate honest signalling of male quality, as individuals that can cope with increased susceptibility to oxidative stress while signalling are likely to be healthy and have good resistance to parasites and pathogens (Von Schantz *et al.* 1999; Kurtz *et al.* 2006). Many yellow-red signals are composed of carotenoids that are obtained through the diet and may be a limited resource. These molecules have other roles as immunostimulants and antioxidants, creating the potential for a trade-off in their allocation between sexual signalling and antioxidant defence (Monaghan, Metcalfe & Torres 2009). It has also been suggested that individuals may be required to sacrifice some aspects of antioxidant protection when investing maximally in melanin-based visual traits (Galvan & Alonso-Alvarez 2009). Several empirical studies have now linked investment in both types of visual sexual signals with oxidative damage. For example, in red grouse (*Lagopus lagopus scoticus*), experimental elevation of testosterone levels increased the size of a carotenoid-based trait but caused oxidative damage to lipids through lipid peroxidation (Mougeot *et al.* 2009). In greenfinches (*Carduelis chloris*), lipid peroxidation occurred when investment in a melanin-based trait was increased through administration of buthionine sulfoximine (BSO), a selective inhibitor of the antioxidant glutathione (Horak *et al.* 2010). However, it is largely unknown whether increased investment in non-visual sexual signals causes similar oxidative damage. In this study, we explore the impact of investment in olfactory sexual signals, reproduction and territorial aggression on oxidative damage in male house mice.

For rodents, olfaction is one of the dominant senses and most sexual signalling involves chemical scent signals (Blaustein 1981). Male-specific olfactory signals are testosterone dependent (Kimura & Hagiwara 1985; Ferkin & Johnston 1993; Ferkin *et al.* 1994), and their expression has been associated with metabolic costs (Gosling *et al.* 2000; Radwan *et al.* 2006; Zala, Potts & Penn 2008). In some species, such as house mice (*Mus musculus domesticus*; Fig. 1), males are highly territorial. In addition to directly attacking intruders (Mackintosh 1981), males deposit urine scent marks throughout their territory (Hurst & Beynon 2004), which influence their attractiveness to females (Rich & Hurst 1998, 1999). Investment in scent marking may be a costly component of reproductive effort for male mice, due to the energy that is required to scent mark a territory and to synthesize the molecules that convey information in scent. Major urinary proteins (MUPs), the main involatile scent components in mouse urine, are produced in the liver and excreted in urine at extremely high concentrations, sometimes reaching 70 mg mL^{-1} (Beynon & Hurst 2003). These proteins bind volatile components and release these slowly from scent marks (Hurst *et al.* 1998). The MUP patterns expressed are highly polymorphic and facilitate individual recognition through scent (Hurst *et al.* 2001; Cheetham *et al.* 2007). One particular male-specific MUP, darcin, acts as a sex pheromone that attracts females to spend time near a male's scent and stimulates females to remember and become attracted to

Fig. 1. A wild house mouse (*Mus musculus domesticus*) in a chicken farm near Sydney, Australia. Photograph by Michael Garratt.

the airborne urinary odour of that particular male (Roberts *et al*. 2010). In addition to involatile urine components, males produce a number of androgen-dependent volatile signals that attract females or stimulate reproductive physiology (Hurst & Beynon 2004). These include E,E-α-farnesene and E-β farnesene ('farnesenes'), which are produced in the preputial glands with greater investment among dominant males (Harvey, Jemiolo & Novotny 1989).

To examine whether oxidative stress is a cost of overall reproductive effort among male house mice, in this study we manipulated a male's opportunity to reproduce and the need to defend a territory over a 16-week period. As males should vary investment in costly sexual signals in relation to the requirements of their ecological or social environment (Badyaev & Qvarnstrom 2002), we expected that isolated males (which could not reproduce and had no aggressive interactions) would signal at a low rate and have the lowest oxidative stress. By contrast, we expected that males housed with a female while defending a territory would show the highest investment in reproductive effort and experience the greatest oxidative stress. A third group of males were housed with a female without the need to defend a territory to investigate the costs of reproduction alone. Markers of oxidative stress were measured at the end of the 16-week period. We also measured changes in urinary testosterone levels, as this hormone may mediate the trade-off between reproductive investment, including sexual signalling, and protection from oxidative stress (Alonso-Alvarez *et al*. 2007). Testosterone is required for male-specific expression of particular sexual signals in mice (Maruniak, Desjardins & Bronson 1977; Clissold, Hainey & Bishop 1984), so we expected testosterone levels to be highest in the group of males investing the most in sexual signalling.

Materials and methods

SUBJECTS

Subjects were adult captive-bred male house mice from a colony of mice originally derived from several populations in the North West of England, UK, and outbred in captivity for up to six generations. Subject males (n = 28) were the offspring of 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 28 days after birth. They were then housed singly in cages (48 × 11·5 × 12 cm) to prevent intermale aggression until the beginning of the experiment (range, 31–49 weeks old). Females (n = 20) that were paired with unrelated males in the two reproduction groups were bred in enclosures in an identical manner to males but stayed in the enclosures with their dam and female siblings until the start of the experiment (range, 31–49 weeks old). Stimulus males (n = 10) used in intruder trials were bred in cages (45 × 28 × 13 cm) and housed singly (48 × 11·5 × 12 cm) from 28 days old. All mice had paper-wool nest 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 amount 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}). Cages were lined with Corn Cob Absorb 10/14 substrate. Mice were maintained on a 12 : 12 h reversed light cycle with lights off at 09:00. Experimental procedures were carried out in the dark phase under dim red light.

EXPERIMENTAL PROTOCOL

The social environment of subject males was manipulated in one of three ways over a period of 16 weeks. Some males were housed alone and had no direct contact with other animals (isolated control, C, n = 8). A second set of males were each housed with a female and were able to mate but had no further contact with other animals (reproduction only, R, n = 10). A third set were each housed with a female in contact with a neighbour pair and experienced territory intrusions to stimulate investment in territory defence (reproduction plus territory defence, RT, n = 10). Subjects were randomly allocated to experimental groups, although siblings were spread across treatments, and replicates were run in two successive batches.

To create a semi-natural environment, subjects 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. To increase the 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 *ad libitum* olfactory and visual contact with a neighbouring pair; those in reproduction only and control pairs had solid walled enclosures. Seven Perspex tiles (15 × 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 neighbouring 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 10 s 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 isolated males were also confined for an identical duration. To further increase the perception of territorial intrusions, three times per week a cage containing an intruder male (one of five cage-housed stimulus males) was introduced to the enclosures of reproduction plus territory defence pairs, permitting visual and olfactory contact for a 2-h period (with an equivalent empty cage added to enclosures of control and reproduction females).

SCENT-MARKING TRIALS

The scent-marking rate of each subject male was assessed at two, six and sixteen weeks after introduction to enclosures. Scent marking was recorded on two Perspex tiles (15 × 15 cm) wrapped in Benchkote, placed against each 116-cm wall of the enclosure for four hours on three consecutive days. Females and any offspring were confined with the nest box under the up-ended base of a cage that the

male could not enter. Marking patterns were visualized using a Bio-Rad Fluor-S MultiImager (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) and QUANTITYONE software (BioRad) with parameters adjusted for mouse urine (12-s exposure duration, 530DF60 filter, UV light source Epi illumination, high-resolution mode). The number of scent marks was counted automatically using the 'Analyze Particles' tool in IMAGEJ version 1.38× (http://rsb.info.nih.gov/ij/). This counts the number of physically separated scent marks so slightly underestimates the total number of scent marks deposited. The mean number of scent marks deposited over the 4 h period was calculated for each male and time point.

URINE COLLECTION

The urine used to measure protein concentration was collected from males via bladder massage at six and sixteen weeks; this method allowed samples to be collected and stored immediately at −20 °C. Each male was held by the scruff of the neck over a clean Eppendorf tube and their bladder massaged gently using the tip of the index finger and thumb until they urinated. To collect a greater volume of urine for the testosterone assay, urine was collected by confining males above a cage on a mesh grid for a maximum of 150 min. Urine was collected in the week prior to the experiment and at sixteen weeks. Cages under males were checked for urine every 30 min, and any urine present was collected and stored at −20 °C.

MEASUREMENT OF URINARY PROTEIN CONCENTRATION

Urinary protein concentration was assessed using the Coomassie plus® protein assay reagent kit from Perbio Science UK Ltd (Cramlington, Northumberland, UK). as described by Cheetham *et al.* (2009). To correct for urinary dilution, we measured urinary creatinine (Beynon & Hurst 2004) using the method of Cheetham *et al.* (2009). MUP concentration is expressed as mg per mg creatinine. To confirm that urinary protein consisted of MUPs rather than other proteins that had leaked through the glomerular filter, urine samples were analysed by SDS-PAGE gels (Laemmli 1970). Samples were diluted to apply an equal amount of urinary creatinine (0·25 μg) to each lane.

URINARY TESTOSTERONE CONCENTRATION

Testosterone concentration was measured using enzyme immunoassay methods previously validated for mouse urine (Munro *et al.* 1991; Muir *et al.* 2001). Testosterone was obtained from Sigma chemicals, UK, and antibodies to testosterone and corresponding horseradish peroxidise conjugates from the Department of Population Health and Reproduction at the University of California, USA. NUNC Maxisorb plates were coated in 50 μL of antibody stock diluted 1 : 10 000 in a coating buffer (50 mM bicarbonate buffer, pH 9·6) then stored for 12–14 h at 4 °C. Wash solution (0·15 M NaCl solution containing Tween 20) then rinsed away any unbound antibody. 25 μL of phosphate buffer (0·1 M sodium phosphate buffer, pH 7·0 containing 8·7 g NaCl and 1 g BSA), 50 μL of standard or sample (urine samples were diluted 1 : 11 in phosphate buffer) and then 50 μL of testosterone horseradish peroxidase (diluted 1 : 25 000) were added to wells. Plates were incubated at room temperature for 2 h before rewashing. 100 μL of substrate solution (citrate buffer, H_2O_2 and 2,2′-azino-bis) was added and left to incubate at room temperature until the density of blank wells reached one. Plates were read with a single filter at 405 nm and urinary creatinine corrected for the dilution of each sample.

MEASUREMENTS OF OXIDATIVE STRESS

At the end of the experiment, males were culled humanely by cervical dislocation, a blood sample taken immediately via cardiac puncture, the liver and gastrocnemius muscle quickly removed, the liver weighed, and then both were snap frozen in liquid nitrogen and stored at −80 °C. The preputial glands were removed, trimmed of excess fat and weighed. Total and oxidized glutathione content were measured in the liver using the automated glutathione recycling assay (Anderson 1996) modified for use on a 96-well plate reader (Benchmark; Bio-Rad) (Vasilaki *et al.* 2006); glutathione is a ubiquitous antioxidant with an established antioxidant role *in vivo* (Halliwell & Gutteridge 1999), and the proportion of glutathione in the oxidized form is a common marker of oxidative stress and redox status (Griffith 1999). Protein thiols, which are groups on proteins essential for stability but susceptible to oxidation (Halliwell & Gutteridge 1999), were measured in liver as described by Dimonte *et al.* (1984), modified for use on a 96-well plate reader (Vasilaki *et al.* 2006). Malonaldehyde (MDA), a secondary product of lipid peroxidation (Monaghan, Metcalfe & Torres 2009), was measured in serum, liver and gastrocnemius muscle using the high performance liquid chromatography (HPLC) method of Fukunaga, Suzuki & Takama (1993). With the exception of the proportion of glutathione in the oxidized form, markers of oxidative damage in tissues are reported as a concentration per gram of protein. The amount of MDA in serum is displayed per litre. Owing to limitations in the quantity of sample available for analysis, sample sizes differed slightly between oxidative damage assays.

DATA ANALYSIS

Linear mixed effect models (LMEs) were constructed to test differences between treatment groups for continuous response variables, fitted by maximum likelihood using the lme4 package in R. Litter of origin, batch (first, second) and enclosure (to control for non-independence between neighbours in the territory defence group) were included in each model as simple, scalar random effects and treatment group as a fixed effect. For longitudinal data (repeated measures through time), time was included as an additional fixed effect with subject ID as a random effect using models with random slopes and intercepts (Schielzeth & Forstmeier 2009). To assess the effect of treatment group on organ weights, total body mass was included as a covariate. Significance of a treatment group or time effect (or interaction between group and time) was assessed by comparison of the model with and without the group or time term (or interaction) included, using likelihood ratio tests. Where a significant interaction between group and time was revealed, separate models were constructed to assess differences between treatment groups at each time point or to assess change through time for each treatment group. Where a significant treatment group effect was detected, models were constructed for each pair of groups to assess which group comparisons accounted for the difference. Inclusion of litter, batch and enclosure had no significant effects except for a significant effect of litter on total body weight ($P = 0{\cdot}0004$). To assess whether groups differed in the relationship between individual investment in scent marking and measures of oxidative stress, we constructed LME models to test for an interaction between treatment group and scent marking at week 16 on measures of oxidative stress. LME models also confirmed that groups did not differ before the experiment in age ($\chi^2_2 = 0{\cdot}16$,

$P = 0{\cdot}92$), body mass ($\chi^2_2 = 1{\cdot}86$, $P = 0{\cdot}39$), urinary protein concentration ($\chi^2_2 = 0{\cdot}09$, $P = 0{\cdot}96$) or testosterone levels ($\chi^2_2 = 2{\cdot}37$, $P = 0{\cdot}31$). A generalized linear mixed model (GLMM) tested the fixed effect of treatment group on the number of offspring per male, with enclosure, litter of origin and batch as random effects, using the Poisson distribution and logarithm link function to analyse count data. Data transformed logarithmically were necessary to meet parametric assumptions (scent mark frequency, preputial gland mass, liver MDA, testosterone). Where this was not possible (serum MDA), groups were compared using a non-parametric Kruskal–Wallis test, using the mean response of two neighbouring males in the territory defence group to control for non-independence, and differences between pairs of groups checked using Mann–Whitney tests. Spearman's rank correlations with untransformed data tested for a relationship between olfactory signalling and oxidative stress.

Results

INVESTMENT IN REPRODUCTION AND OLFACTORY SEXUAL SIGNALLING

All males that were housed with a female sired at least one litter over the experiment, with reproduction only and reproduction plus territory defence males siring a similar number of offspring (mean ± SE for reproduction only: 8·9 ± 1·5; reproduction plus territory defence: 11·9 ± 2·2; $\chi^2_1 = 0{\cdot}86$, $P = 0{\cdot}35$). Males in different reproductive and competitive environments differed substantially in their investment in olfactory signalling, which was generally attributable to greatly increased investment in signalling by reproduction plus territory defence males. First, males in different environments varied in the number of scent marks deposited on test tiles throughout the experiment (treatment group: $\chi^2_2 = 22{\cdot}0$, $P < 0{\cdot}0001$; interaction between group and time: $\chi^2_4 = 3{\cdot}63$, $P = 0{\cdot}46$; Fig. 2a). Reproduction plus territory defence males scent marked at a higher rate than both isolated ($\chi^2_1 = 16{\cdot}35$, $P < 0{\cdot}0001$) and reproduction only males ($\chi^2_1 = 13{\cdot}6$, $P = 0{\cdot}0002$), while the difference between isolated and reproduction only males was not significant ($\chi^2_1 = 2{\cdot}73$, $P = 0{\cdot}098$). Secondly, the concentration of urinary protein also differed between groups (Fig. 2b; treatment group: $\chi^2_2 = 23{\cdot}4$, $P < 0{\cdot}0001$; interaction between group and time: $\chi^2_2 = 4{\cdot}87$, $P = 0{\cdot}09$) and consisted almost entirely of MUP (Fig. 2c). Reproduction plus territory defence males greatly increased urinary protein concentration compared with both isolated ($\chi^2_1 = 19{\cdot}2$, $P < 0{\cdot}0001$) and reproduction only males ($\chi^2_1 = 13{\cdot}8$, $P = 0{\cdot}0002$), while reproduction only males had slightly higher urinary protein than isolated males ($\chi^2_1 = 4{\cdot}86$, $P = 0{\cdot}03$).

To examine morphological changes in males that resided in different reproductive and competitive environments, we assessed changes in total body weight during the experiment and weights of the liver [the main site of urinary MUP production (Finlayson *et al.* 1965)], and the preputial glands [which produce farnesenes involved in male sexual signalling (Caroom & Bronson 1971; Jemiolo, Xie & Novotny 1991)] at the end of the experiment. While there was no difference in body weight between groups immediately before the experiment ($\chi^2_2 = 1{\cdot}86$, $P = 0{\cdot}39$), changes in body weight over the experiment differed between groups (interaction between group and time: $\chi^2_4 = 13{\cdot}0$, $P = 0{\cdot}011$). Separate models revealed no difference in body weight between groups after two ($\chi^2_2 = 2{\cdot}86$, $P = 0{\cdot}24$) and 6 weeks ($\chi^2_2 = 0{\cdot}58$,

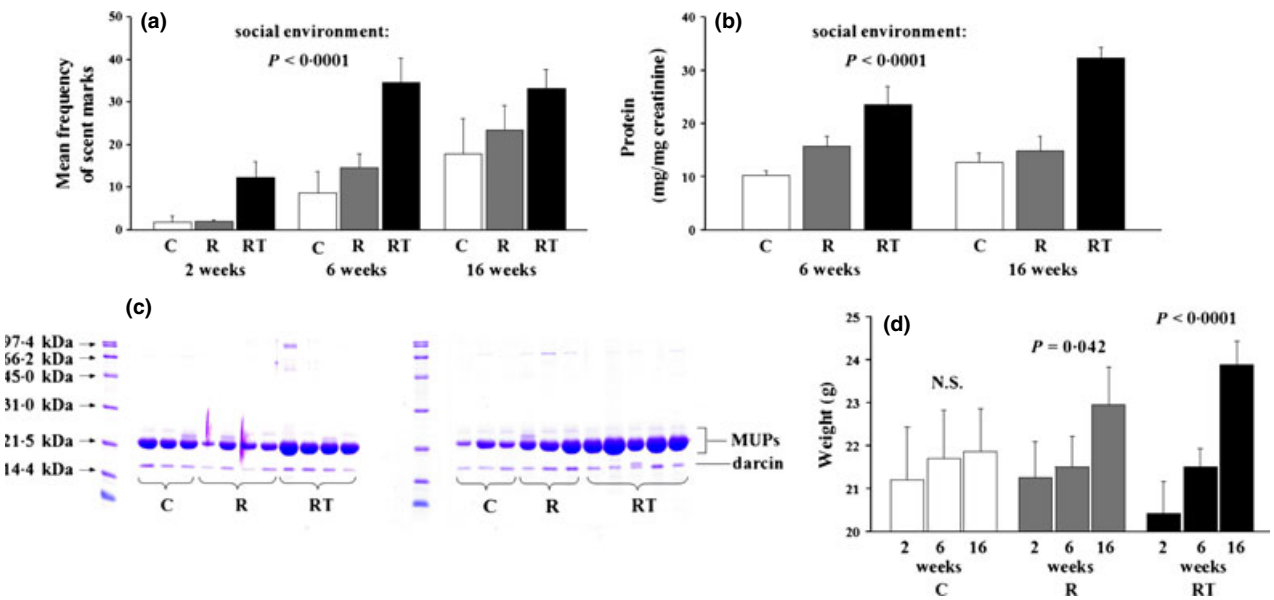

Fig. 2. Investment in olfactory signalling by males that were isolated (C, open bars), reproducing (R, grey bars) or reproducing while defending their territory from intruders (RT, black). Males in different reproductive and competitive environments differed greatly in their mean four hour scent-marking frequency over the experiment (a) and in their urinary protein output at 6 weeks and at the end of the experiment (b). *P* values indicate the difference between social treatment groups. Samples run on SDS-PAGE (diluted to add 0·25 μg urinary creatinine to each lane) revealed that almost all urinary protein was major urinary proteins (c). Males in both reproductive environments showed a significant increase in mass over the experiment while isolated males did not (d). *P* values indicate the effect of time within each treatment group.

$P = 0{\cdot}75$), but a difference after 16 weeks ($\chi^2_2 = 9{\cdot}15$, $P = 0{\cdot}01$). This was attributable to an increase in body weight among reproduction only (effect of time: $\chi^2_2 = 6{\cdot}32$, $P = 0{\cdot}042$) and reproduction plus territory defence males ($\chi^2_2 = 27{\cdot}8$, $P < 0{\cdot}0001$), which occurred mostly between weeks 6 and 16, but there was no weight increase apparent among isolated males ($\chi^2_2 = 1{\cdot}32$, $P = 0{\cdot}52$; Fig. 2d). This resulted in lower final body weight among isolated males compared with reproduction only ($\chi^2_1 = 4{\cdot}92$, $P = 0{\cdot}027$) and reproduction plus territory defence males ($\chi^2_1 = 6{\cdot}83$, $P = 0{\cdot}009$), while there was no difference between the two reproduction groups ($\chi^2_1 = 0{\cdot}72$, $P = 0{\cdot}40$). Males in different reproductive and competitive environments differed significantly in liver mass (mean ± SE for isolated males: 0·76 ± 0·05 g; reproduction only: 0·84 ± 0·05 g; reproduction plus territory defence: 1·03 ± 0·04 g; effect of group with total body weight included as a covariate: $\chi^2_2 = 14{\cdot}3$, $P = 0{\cdot}0008$) and preputial gland mass (mean ± SE for isolated males: 0·040 ± 0·004 g; reproduction only: 0·048 ± 0·010 g; reproduction plus territory defence: 0·087 ± 0·011 g; $\chi^2_2 = 8{\cdot}72$, $P = 0{\cdot}013$). This was a consequence of much heavier livers and preputial glands among reproduction plus territory defence males (comparison of liver to isolated males, $\chi^2_1 = 11{\cdot}0$, $P = 0{\cdot}0009$; to reproduction only males, $\chi^2_1 = 10{\cdot}1$, $P = 0{\cdot}001$; comparison of preputial glands to isolated males, $\chi^2_1 = 6{\cdot}60$, $P = 0{\cdot}010$; to reproduction only males, $\chi^2_1 = 5{\cdot}61$, $P = 0{\cdot}018$). Reproduction only and isolated males had similar liver and preputial gland masses (liver: $\chi^2_1 = 0{\cdot}43$, $P = 0{\cdot}51$; preputial glands: $\chi^2_1 = 0{\cdot}12$, $P = 0{\cdot}73$). Not surprisingly, total body weight also influenced both liver mass ($\chi^2_1 = 18{\cdot}5$, $P < 0{\cdot}0001$) and preputial gland mass ($\chi^2_1 = 12{\cdot}3$, $P = 0{\cdot}0005$).

OXIDATIVE STRESS

If oxidative stress is a cost of reproductive effort for male house mice, we would expect the highest oxidative damage in reproduction plus territory defence males. These individuals made the greatest investment in olfactory signalling, in addition to engaging in regular aggressive interactions with conspecifics and mating with a female. However, oxidative damage only followed this prediction in the gastrocnemius muscle, where a marginally non-significant difference between groups in the oxidation of lipids ($\chi^2_2 = 5{\cdot}63$, $P = 0{\cdot}06$; Fig. 3a) was a result of higher lipid peroxidation in reproduction plus territory defence males compared with reproduction only ($\chi^2_1 = 6{\cdot}61$, $P = 0{\cdot}01$) and isolate males ($\chi^2_1 = 3{\cdot}07$, $P = 0{\cdot}08$), while there was no difference between reproduction only and isolated males ($\chi^2_1 = 1{\cdot}97$, $P = 0{\cdot}16$). Lipid peroxidation differed between groups in serum (Fig. 3b; Kruskal–Wallis test, $n = 21$, $\chi^2_2 = 6{\cdot}63$, $P = 0{\cdot}036$), but this was attributable to lower (not higher) lipid peroxidation within both reproductive groups when compared to controls (Mann–Whitney test for reproduction only: $P = 0{\cdot}022$; reproduction plus territory defence: $P = 0{\cdot}045$). There was a marginally significant difference between groups in lipid peroxidation in the liver (Fig. 3c; $\chi^2_2 = 5{\cdot}94$, $P = 0{\cdot}05$), similarly reflecting lower lipid peroxidation in reproduction plus territory defence males compared with isolated males ($\chi^2_1 = 6{\cdot}29$, $P = 0{\cdot}012$), but intermediate levels among reproduction only males did not differ significantly from isolated ($\chi^2_1 = 2{\cdot}35$, $P = 0{\cdot}12$) or reproduction plus territory defence males ($\chi^2_1 = 0{\cdot}99$, $P = 0{\cdot}32$). Analysis of other markers of oxidative damage in the liver failed to reveal an increase in oxidative stress with reproductive effort. There was no difference between males in the concentration of protein thiols (mean ± SE for isolated males: 57·5 ± 6·8 μmols g^{-1} protein; reproduction only: 55·2 ± 4·9 μmols g^{-1} protein; reproduction plus territory defence: 55·8 ± 9·0 μmols g^{-1} protein; $\chi^2_2 = 0{\cdot}06$, $P = 0{\cdot}97$), an indicator of protein oxidation. There was also no difference in the proportion of glutathione in the oxidized form (isolated: 0·050 ± 0·003; reproduction only: 0·046 ± 0·002; reproduction plus territory defence: 0·054 ± 0·004; $\chi^2_2 = 4{\cdot}55$,

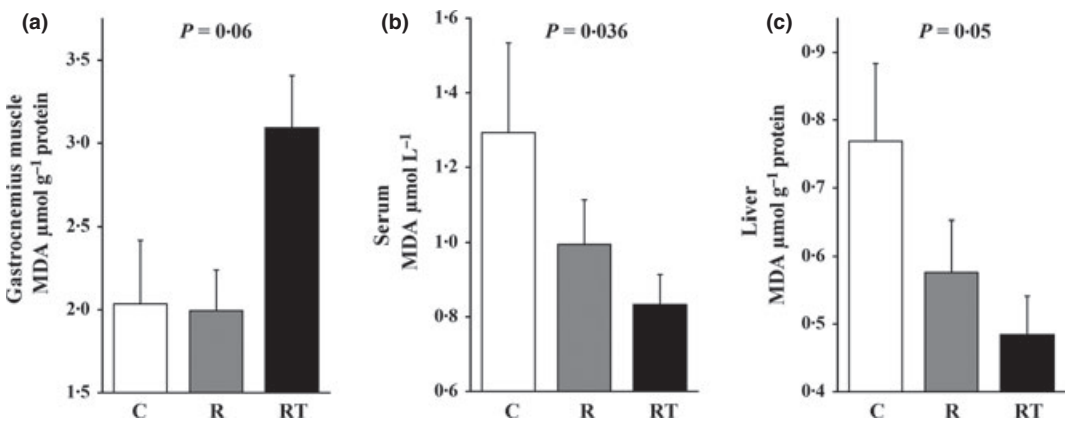

Fig. 3. The effect of reproductive and competitive environment on malonaldehyde content in different tissues of male mice. In gastrocnemius muscle (a) lipid peroxidation tended to be the highest in those males that were reproducing and defending their territory from intruders (RT, black). However, in serum (b) lipid peroxidation was higher in isolated males (C, open bars) than in those males that were reproducing (R, grey bars) and RT males. In the liver (c) the difference between groups was weaker but consistent with higher lipid peroxidation in isolated males. *P* values indicate the difference between groups.

$P = 0{\cdot}10$), or in the total amount of glutathione in the liver (isolated: $60{\cdot}6 \pm 7{\cdot}4$ μmols g^{-1} protein; reproduction only: $56{\cdot}8 \pm 3{\cdot}8$ μmols g^{-1} protein; reproduction plus territory defence: $57{\cdot}7 \pm 4{\cdot}6$ μmols g^{-1} protein; $\chi^2_2 = 0{\cdot}23$, $P = 0{\cdot}89$).

Although differences in lipid peroxidation between groups suggest redox changes with social environment, an overall increase in oxidative damage with reproductive effort was not evident. Notably, all males in the reproduction plus territory defence group invested heavily in sexual signalling (in addition to regular aggressive interaction with other males). However, those in the reproduction only and isolated groups showed a much greater range of individual investment in sexual signalling. To assess whether the relationship between individual scent mark investment and oxidative stress differed between groups, we examined whether there was an interaction between group and scent mark investment at week 16 on measures of oxidative stress. In the liver, there was a significant interaction between group and scent mark investment for the concentration of total glutathione ($\chi^2_2 = 7{\cdot}94$, $P = 0{\cdot}019$), the proportion of glutathione in the oxidized form ($\chi^2_2 = 6{\cdot}15$, $P = 0{\cdot}046$) and the oxidation of protein thiols ($\chi^2_2 = 9{\cdot}00$, $P = 0{\cdot}01$). Isolated males with the greatest scent-marking rates had a lower concentration of total liver glutathione ($n = 7$, $r_s = -0{\cdot}96$, $P = 0{\cdot}001$; Fig. 4a), a higher proportion of glutathione in the oxidized form ($n = 7$, $r_s = -0{\cdot}85$, $P = 0{\cdot}016$; Fig. 4b) and greater oxidation of protein thiols ($n = 7$, $r_s = -0{\cdot}78$, $P = 0{\cdot}041$; Fig. 4c). Isolated males with the highest scent-marking rates also had the highest lipid peroxidation in serum ($n = 7$, $r_s = 0{\cdot}87$, $P = 0{\cdot}024$; Fig. 4d). By contrast, there were no consistent correlations between markers of oxidative stress and sexual signalling in either reproductive group (see Table S1, Supporting Information), although there was a strong positive correlation between liver glutathione and urinary MUP concentrations among reproduction plus territory defence males ($n = 9$, $r_s = 0{\cdot}85$, $P = 0{\cdot}004$).

TESTOSTERONE

Isolated, reproduction only and reproduction plus territory defence males showed different changes in testosterone levels over the experiment (interaction between group and time: $\chi^2_2 = 12{\cdot}3$, $P = 0{\cdot}002$), but the difference between groups was not in the direction predicted. Males in both reproductive groups decreased urinary testosterone levels over the experiment (mean ± SE for reproduction only, pre-experiment: $30{\cdot}9 \pm 6{\cdot}0$ ng mg^{-1} creatinine; 16 weeks: $13{\cdot}5 \pm 2{\cdot}3$ ng mg^{-1} creatinine; reproduction plus territory defence, pre-experiment $33{\cdot}0 \pm 7{\cdot}3$ ng mg^{-1} creatinine; 16 weeks: $16{\cdot}9 \pm 3{\cdot}9$ ng mg^{-1} creatinine; effect of time, reproduction only: $\chi^2_1 = 8{\cdot}03$, $P = 0{\cdot}005$; reproduction plus territory defence: $\chi^2_1 = 6{\cdot}50$, $P = 0{\cdot}011$), whereas testosterone levels did not change significantly among isolated males (pre-experiment: $24{\cdot}8 \pm 5{\cdot}3$ ng mg^{-1} creatinine; 16 weeks: $42{\cdot}5 \pm 13{\cdot}3$ ng mg creatinine; effect of time: $\chi^2_1 = 2{\cdot}59$, $P = 0{\cdot}11$). There was no difference between the groups before the start of the experiment ($\chi^2_2 = 2{\cdot}37$, $P = 0{\cdot}31$), but a significant group effect at the end of the experiment ($\chi^2_2 = 9{\cdot}89$, $P = 0{\cdot}007$) was attributable to lower urinary testosterone in males of both reproductive groups compared with isolated males.

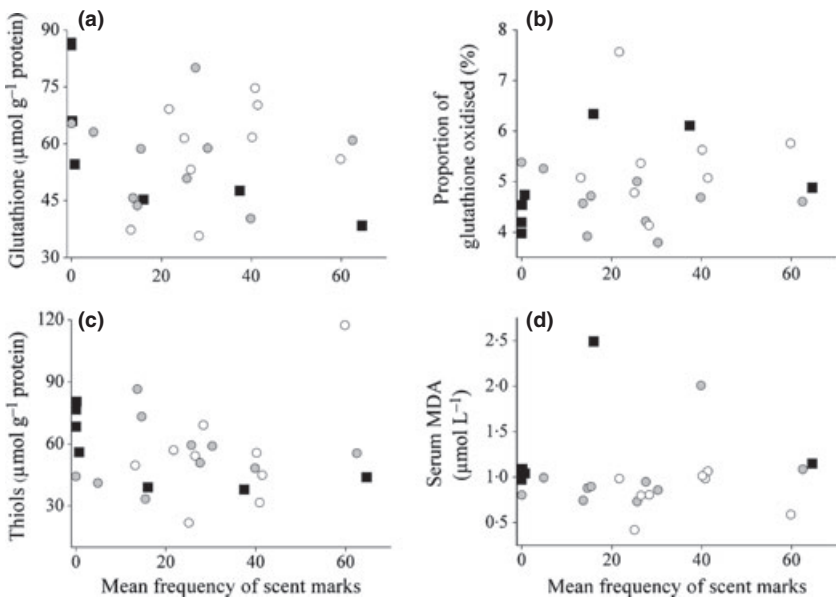

Fig. 4. Relationships between male scent-marking frequency and markers of oxidative stress within each treatment group. Isolated males with the highest scent-marking rates had the greatest oxidative stress in liver and serum. Black squares: isolated males; grey circles: reproductive males; white circles: reproductive males that were also defending their territory from intruders. In the liver, isolated males (■) with the highest scent-marking rates had the lowest concentration of total glutathione (a), a higher proportion of glutathione in the oxidized form (b) and greater oxidation of protein thiols (c). Isolated males with the highest scent-marking rates also had the greatest lipid peroxidation in serum (d).

Discussion

Oxidative stress has been suggested as a proximate cost of reproduction. For males, this could occur as a consequence of investment in a variety of traits involved in competing for mates and in particular from investment in sexual signalling (Alonso-Alvarez *et al.* 2007; Metcalfe & Alonso-Alvarez 2010). Here, we found little evidence that oxidative stress increases in line with reproductive effort in male house mice. Reproductive males that were defending a territory tended to have higher oxidative damage in the gastrocnemius muscle. However, lipid peroxidation in serum and liver instead was lower in reproduction plus territory defence males, even though these individuals were clearly investing the most in various aspects of reproduction. This highlights the benefits of examining markers of oxidative stress in multiple tissues as changes can be tissue dependent. A variety of factors could generate tissue-dependent changes in oxidative stress. Increased activity rates as a consequence of scent marking and aggression may have increased ROS production specifically in the muscles of reproduction and territory defence males (Davies *et al.* 1982; Jackson, Edwards & Symons 1985), causing localized oxidative stress. Particular tissues may differ also in their susceptibility to oxidative damage. Muscle is a post-mitotic tissue with a high energy demand and so may be more susceptible to oxidative damage than the liver (Wei, Kao & Lee 1996), a tissue with a much greater cell turnover. The increase in liver mass observed in reproduction plus territory defence males, which could be a consequence of increased metabolic demands and/or MUP production, could also have induced cellular changes that altered susceptibility to oxidative stress in this tissue. Another potentially important factor, particularly related to the attenuation of oxidative stress in liver and serum, is the adaptive modulation of antioxidants that can occur in relation to increased energy metabolism and associated ROS production. This has previously been associated with decreased lipid peroxidation (Schulz *et al.* 2010).

In healthy individuals, a common response to an increase in ROS production is an up-regulation of antioxidant defences that repair oxidative damage and restore redox status (Droge 2002). The concentration of glutathione, an important non-enzymatic antioxidant, was not increased in reproductive males in the liver. However, mice have many other antioxidant defences, including a variety of antioxidant enzymes, which can be up-regulated dynamically to maintain the correct oxidant/antioxidant balance (Halliwell & Gutteridge 1999; Yoo, Chang & Rho 1999). In this study, we focused on oxidative damage, the outcome of oxidative stress, but measurement of antioxidant defences in future studies will determine whether these are indeed modulated with reproductive investment in male mice. While defences against oxidative stress can be effectively modulated in many situations, it must be noted that these defences are not always effective and large increases in ROS production can overwhelm defences, resulting in oxidative damage. Under more demanding conditions than those in this study, males that invest heavily in reproduction may be unable to effectively protect against ROS production. Further studies will be needed to determine the extent to which more demanding conditions limit the ability of the antioxidant defence system to protect against oxidative stress or whether males reduce investment in signalling and territory defence to protect against such effects.

Our study examined how markers of oxidative damage vary in response to male reproductive effort. However, oxidative stress may show strong links with one particular component of male reproductive investment, sexual signalling. Von Schantz *et al.* (1999) suggested that female choice should promote the evolution of signals in males that reliably reveal a lack of oxidative stress, as this could facilitate honest signalling of male quality. Because ROS can be produced from the highly polymorphic immune and detoxification systems (Von Schantz *et al.* 1999; Kurtz *et al.* 2006), males with good genetic resistance to parasites and pathogens may be less likely to suffer from oxidative stress. Oxidative stress is further linked to the onset and/or severity of hundreds of diseases (Halliwell & Gutteridge 1999) so could act as a proximate indicator of condition. For such signalling systems to evolve, mechanisms are required that ensure honesty. If investment in sexual signals increases susceptibility to oxidative stress, only those individuals with good antioxidant defences and pre-existing low levels of oxidative damage will be able to afford to invest heavily in such traits (Metcalfe & Alonso-Alvarez 2010).

The production of a number of visual signals in birds has been associated with oxidative stress (Mougeot *et al.* 2009; Horak *et al.* 2010), yet we found little evidence that mice that substantially increased investment in olfactory signalling suffered an overall increase in oxidative damage. Our results in relation to olfactory signalling should be interpreted cautiously because our manipulations also increased investment in other behaviours involved in reproductive investment, including aggressive competition. Nonetheless, strong correlations between scent mark investment and oxidative stress among isolated males suggest these components may be related in certain circumstances, as discussed further. However, one prominent feature of visual signalling is that investment in colourful traits can sometimes require males to reduce investment in specific components of the antioxidant defence system, for example, by redirecting carotenoids. In species that signal through other sensory modalities, such as mice, similar trade-offs have not been demonstrated, so individuals may be more able to adapt and protect against oxidative stress. Signalling of male quality does not necessarily require a relationship with oxidative stress. Moreover, sexual signals do not always have to entail condition-dependent physiological costs to remain honest (Maynard Smith & Harper 2003; Getty 2006). In house mice, there are additional mechanisms that help to ensure honest signalling of male quality through scent: scent marking itself provides spatial and temporal evidence of a male's ability to defend his territory, as only successful males can ensure that their scent marks predominate throughout the

defended area (Hurst & Beynon 2004). This does not mean that all individuals will have the same physiological capacity for olfactory signal investment. Investment in scent marking is generally regarded as metabolically costly (Gosling *et al*. 2000; Radwan *et al*. 2006; Zala, Potts & Penn 2008), so individuals that are in poor condition (and experiencing high oxidative stress) may be limited in their ability to invest in such traits. It is possible, though, that protection against oxidative stress may not limit investment in olfactory signalling among young adults that are in good condition.

Costs of male reproductive effort, in particular from investment in sexual signalling and aggression, may occur as a consequence of elevated testosterone levels (Folstad & Karter 1992; Alonso-Alvarez *et al*. 2007). In this experiment, reproductive males housed with females (whether actively defending a territory or not) showed a reduction in urinary testosterone levels, suggesting testosterone and male reproductive effort may not always be positively correlated in this species. Singly housed male mice have previously been reported to have higher testosterone than males caged with a resident female or in all-male groups (Macrides, Bartke & Dalterio 1975), although the reasons for this are not yet understood. By contrast, brief exposure to an unfamiliar female results in an immediate dramatic elevation in plasma testosterone whether males are housed singly or with a resident female (Macrides, Bartke & Dalterio 1975), suggesting that androgen levels may temporarily rise in response to an immediate opportunity for reproduction but decrease in more stable social situations. As oxidative stress can sometimes vary with testosterone (Alonso-Alvarez *et al*. 2007), a reduction in testosterone levels among reproductive males could have contributed to the changes in lipid peroxidation. If higher testosterone levels increase the likelihood of oxidative stress among males producing sexual signals (Alonso-Alvarez *et al*. 2008), this could account for the correlation between investment in scent marking and oxidative damage among isolated males, even though there was little evidence for such a relationship among males in reproductive groups. Manipulation of both testosterone levels and male investment in olfactory signalling (for example, by the manipulation of male social environment as outlined in this experiment) may help to tease apart the costs of sexual signalling from the costs of testosterone *per se*.

Our results suggest that oxidative stress does not always occur simply as a function of increasing reproductive effort among male mice. These results are concordant with our previous study, which demonstrated that oxidative damage was not a cost of reproduction for female mice under similar conditions (Garratt *et al*. 2011). However, under more demanding conditions, such as those experienced in the wild, it is possible that individuals may be unable to maintain redox balance during periods of high reproductive effort and oxidative stress could result. Controlled studies that examine the ability of males to invest in reproduction and sexual signalling in more naturalistic social and physical environments, including when temperature and food quality are suboptimal, may help to determine whether oxidative stress is a cost of male reproductive investment in the wild.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1. Relationship between oxidative stress and sexual signalling.

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