RESEARCH ARTICLE

Effects of reproduction on immuno-suppression and oxidative damage, and hence support or otherwise for their roles as mechanisms underpinning life history trade-offs, are tissue and assay dependent

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SUMMARY

Life history parameters appear to be traded off against each other, but the physiological mechanisms involved remain unclear. One hypothesis is that potentially energetically costly processes such as immune function and protection from oxidative stress may be compromised during reproductive attempts because of selective resource allocation. Lower temperatures also impose energy costs, and hence allocation decisions might be more pronounced when animals are forced to reproduce in the cold. Here, we experimentally tested whether reproduction at different ambient temperatures was associated with elevated oxidative stress and suppressed immune function in Mongolian gerbils (*Meriones unguiculatus*). Using a variety of different markers for both immune function and oxidative stress, we found that some measures of immune function (serum bactericidal capacity and size of the thymus) were significantly suppressed, while some measures of oxidative protection [serum superoxide dismutase (SOD) activity and glutathione peroxidase (GPx) activity] were also reduced, and a marker of oxidative damage (protein carbonyls in serum) was increased in lactating compared with non-reproductive gerbils. These changes were in line with the selective resource allocation predictions. However, the phytohaemagglutinin response and serum total immunoglobulin (IgG) were not suppressed, and other markers of oxidative damage [malondialdehyde (MDA) (TBARS) and protein carbonyls in the liver] were actually lower in lactating compared with non-reproductive gerbils, consistent with increased levels of SOD activity and total antioxidant capacity in the liver. These latter changes were opposite of the expectations based on resource allocation. Furthermore, other measures of protection (GPx levels in the liver and protein thiols in both serum and liver) and damage [MDA (TBARS) in serum] were unrelated to reproductive status. Ambient temperature differences did not impact on these patterns. Collectively, our results indicated that the inferred effects of reproduction on immunosuppression and oxidative damage, and hence support or otherwise for particular physiological mechanisms that underpin life history trade-offs, are critically dependent on the exact markers and tissues used. This may be because during reproduction individuals selectively allocate protection to some key tissues, but sacrifice protection of others.

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INTRODUCTION

Life-history theory assumes that trade-offs exist among various physiological functions (Stearns, 1992) because of the need to selectively allocate limited resources (Zera and Harshman, 2001; Monaghan et al., 2009). Reproduction, particularly lactation, is the most energetically demanding period of a mammal's life (Wade and Schneider, 1992; Bronson, 1998; Johnson et al., 2001a; Speakman, 2008; Bergeron et al., 2011). The high energy demands of reproduction, in a situation where total available resources are limited, is therefore predicted to lead to trade-offs with other costly processes. It has been suggested that two such processes that might be compromised during reproduction are defense against oxidative damage and maintenance of the immune system.

Oxidative stress is defined as an imbalance arising when the rate of production of reactive oxygen species (ROS) exceeds the capacity of the antioxidant defense and repair mechanisms (Monaghan et al., 2009; Metcalfe and Alonso-Álvarez, 2010). A variety of ROS, produced as a by-product of normal metabolism, can cause oxidative damage to biomolecules (e.g. DNA, proteins and lipids) unless quenched by antioxidants (Balaban et al., 2005; Dowling and Simmons, 2009). Several studies of birds and *Drosophila melanogaster* have found that an increase in reproductive effort is associated with a decrease in activity of antioxidants or resistance to oxidative stress (Salmon et al., 2001; Wang et al., 2001; Alonso-Álvarez et al., 2004; Wiersma et al., 2004).

Oxidative damage increases during lactation in some domesticated and laboratory animals (Upreti et al., 2002; Castillo et al., 2005; Stier et al., 2012). Positive correlations between litter size and oxidative damage in chipmunks (*Tamias striatus*) (Bergeron et al., 2011) and red squirrels (*Tamiasciurus hudsonicus*) (Fletcher et al., 2013) in the

wild have also been observed. However, several measures of oxidative stress were actually reduced during lactation in captive house mice (*Mus musculus*) (Garratt et al., 2011; Garratt et al., 2013) and bank voles (*Myodes glaeolus*) (Ołdakowski et al., 2012).

A growing body of evidence suggests that raising an immune response is also energetically costly, and may play a key role in physiological trade-offs (Lochmiller and Deerenberg, 2000; Martin et al., 2008) in both mammals (Christe et al., 2000; Drazen et al., 2003) and birds (Nordling et al., 1998; Knowles et al., 2009; Christe et al., 2012). Immunosuppression caused by reproduction could be the result of an energetic trade-off between reproductive demands and immune function, which is in line with the standard predictions of life history theory (Stearns, 1992). However, an alternative explanation that might explain the diversity of responses in oxidative damage is that immunosuppression may be an adaptive response to minimize oxidative stress. Although a previous study has evaluated the effects of reproductive effort on both immune function and oxidative stress in a bird (Christe et al., 2012), to date no such studies have been conducted in mammals. Here we provide results from such a study that simultaneously evaluated the effects of reproduction on both immune function and oxidative stress using a variety of markers for both these traits.

One suggested reason why studies of animals in the wild diverge from those in captivity, in terms of the reported effects of reproduction on oxidative damage, is because in captivity animals have access to unlimited food supplies, while in the wild resources may be limited (Selman et al., 2012; Metcalfe and Monaghan, 2013). Such free access to resources in the laboratory may negate the need to selectively allocate between contrasting functions. This interpretation, however, assumes that at peak reproduction animals are limited by external resources rather than by intrinsic physiological capacities. Such physiological limitations have been demonstrated to apply in at least some animals such as laboratory mice (e.g. Hammond and Diamond, 1992; Johnson and Speakman, 2001; Speakman and Król, 2011) and may be more generally important both in captivity and in the wild (Drent and Daan, 1980; Peterson et al., 1990; Hammond and Diamond, 1997; Speakman and Król, 2010; Piersma and Van Gils, 2011). Given that such physiological limitations may be routinely more important than extrinsic supplies of energy, we might expect that if animals are challenged during reproduction, by additional energy-demanding tasks (or these physiological capacities are manipulated), then the need to trade-off functions against each other will be magnified (Speakman and Garratt, 2014). Changes in environmental temperatures directly impose energy demands on animals and may also cause oxidative stress (Selman et al., 2000; Metcalfe and Alonso-Álvarez, 2010; Venditti et al., 2010). Animals may respond to temperature challenges by upregulating antioxidant enzymes in tissues (Selman et al., 2000) and mobilizing dietary antioxidants (Eraud et al., 2007). However, this capacity may be compromised when animals are reproducing because of the extra energy demands required for reproduction. Hence cold temperatures may exacerbate the requirement to trade off protective functions such as the immune system and protection from oxidative damage against reproduction.

However, an alternative interpretation is that reproductive investment is constrained by the physiological capacity to dissipate heat (Król et al., 2007; Speakman and Król, 2011). Because capacity to dissipate heat is greater at lower ambient temperatures, placing reproducing animals in the cold may actually diminish the requirement to trade-off costly functions because the total capacity for intake is increased, while conversely in hot conditions the trade-off may be magnified. In addition, animals may respond to cold

temperatures by increasing expression of uncoupling proteins that reduce ROS production and increase survival (Speakman et al., 2004; Selman et al., 2008; Caldeira da Silva et al., 2008; Keipert et al., 2011). Hence, there may be complex relationships between reproduction, immune function and oxidative status depending on the ambient temperature.

In the present study, we tested whether oxidative stress and immunosuppression are consequences of reproduction in lactating gerbils, and whether the relationship between these traits and reproduction is affected by ambient temperature. Specifically, we simultaneously measured a number of markers of oxidative stress (including oxidative damage and antioxidants in the liver, mammary gland and serum) and immune responses [phytohaemagglutinin (PHA) response, serum total immunoglobulin (IgG) and bactericidal capacity] in lactating and non-reproductive gerbils acclimated to three ambient temperatures (10, 21 and 30°C). We predicted, based on life history theory, that lactation would be associated with immunosuppression and elevated oxidative stress, unless immunosuppression serves to protect animals from oxidative stress. Further, we predicted that if lowered ambient temperatures impose additional energetic burdens on animals, then lowering ambient temperature would strengthen the requirement to trade off immune function and oxidative protection against reproduction. However, if animals are limited by the capacity to dissipate heat, we predicted that the requirement to trade off these functions would be increased under hot conditions.

MATERIALS AND METHODS
Animals and housing conditions
Mongolian gerbils [*Meriones unguiculatus* (Milne-Edwards 1867)] are small, seasonally breeding, non-hibernating, granivorous rodents that are distributed in the desert and semi-arid regions of Mongolia and Northern China (Walker, 1968). They experience marked seasonal fluctuations in environmental temperature. The average temperature in the short summer is 18.8°C, and the average temperature is −22.3°C in the winter, which lasts approximately 6–7 months (Chen, 1988).

Ninety-six virgin female Mongolian gerbils (range 6–7 months old and weighing 59–88 g) from our breeding colony were maintained at the Institute of Zoology, Chinese Academy of Sciences for these experiments. Our breeding colony has been maintained since captured from wild in 1999. Since this time the colony was rejuvenated several times using animals captured from the field, most recently in 2010. Gerbils were individually housed in plastic cages (30×15×20 cm) with sawdust as bedding for 2 weeks prior to the start of the experiment, and maintained at the room temperature of 21±1°C, under a 16 h:8 h light:dark cycle (lights on at 04:00 h). Commercial standard rat pellet chow (Beijing KeAo Feed Co., Beijing, China) and water were provided *ad libitum*. All animal procedures were approved by the Animal Care and Use Committee of Institute of Zoology, Chinese Academy of Sciences.

Experimental procedures
Thirty randomly selected females were used as non-reproductive control animals. The remaining 66 females were paired with males for 15 days at 21±1°C, after which the males were removed; of these animals, only 36 became pregnant. The 30 animals that did not become pregnant took no further part in this study. The pregnant gerbils were checked twice a day to determine the day of parturition (day 0 of lactation). On day 1 of lactation, all the lactating females were randomly allocated to three temperatures: 10±1°C [cold temperature and lactation (CL), *N*=13], 21±1°C [warm temperature

and lactation (WL), N=11] and 30±1°C [high temperature and lactation (HL), N=12]. These three groups were balanced for body mass and litter size. The non-reproductive gerbils were also allocated to the three temperatures for 17 days [cold temperature and non-reproduction (CN), N=10; warm temperature and non-reproduction (WN), N=10; and high temperature and non-reproduction (HN), N=10], and these three groups were also balanced for body mass. Initial (day of parturition) and final (day 18 of lactation) body mass, litter size and litter mass were recorded.

Measurement of the PHA response

To determine the PHA response on the last day of temperature exposure, we measured the footpad thickness of their left hind foot with a micrometer (Tesa Shopcal, Renens, Switzerland) to ±0.01 mm at 09:00 h. Immediately thereafter, we injected subcutaneously 0.1 mg of PHA (PHA-P, Sigma L-8754, Sigma-Aldrich, St Louis, MO, USA) dissolved in 0.03 ml of sterile PBS (pH 7.4) in the middle of the footpad. Six hours after the injection, we measured the footpad thickness again. The PHA response was calculated as the difference between pre- and post-injection measurements divided by initial footpad thickness [PHA response=(post-PHA–pre-PHA)/pre-PHA]. Each measurement of footpad thickness was replicated six times (Goüy de Bellocq et al., 2006; Yang et al., 2012). The maximum PHA response occurs after 6 h of PHA injection in this species (Xu and Wang, 2010). Swelling in the footpad is roughly equal to the strength of the cell-mediated immune response, and therefore more swelling is equivalent to a greater infiltration and proliferation of macrophages and lymphocytes (Demas et al., 2011). Following the measurement of the PHA response, each gerbil was euthanized by CO_2 asphyxiation. The spleen and thymus were collected and weighed to the nearest 0.001 g. The serum, liver and mammary gland (only in lactating gerbils) were also collected and then stored at −80°C until assayed for immune parameters or markers of oxidative stress. Two studies have previously evaluated whether the PHA response test affects oxidative stress markers, with mixed results. Costantini and Dell'Omo (Costantini and Dell'Omo, 2006) found that PHA challenge (0.05 mg/0.05 ml) was correlated with an increase in levels of reactive oxygen metabolites and decreased total antioxidants in wild nestlings of the Eurasian kestrel. However, another study showed that PHA challenge (0.5 mg/0.1 ml) did not appear to be associated with oxidative stress (i.e. no change for total plasma antioxidant status and lipid oxidative damage) in adult red-legged partridges (Perez-Rodriguez et al., 2008).

Measurements of serum total IgG and bactericidal capacity

IgG is the most abundant immunoglobulin in circulation and a non-specific component of innate immunity, and may represent a state of immunological 'readiness' (Greives et al., 2006). Serum total IgG levels were determined using a rat enzyme-linked immunosorbant assay (ELISA) kit (RapidBio Lab, Calabasas, CA, USA). The sensitivity by this assay is 1.0 μg ml^{-1}, and 10 μl serum samples were used. Inter- and intra-assay variations were both <15%.

A serum bactericidal assay was performed in a sterile laminar flow cabinet to assess the functional response by the animal's innate immune system against a relevant pathogen, *Escherichia coli* (Tieleman et al., 2005; French et al., 2009). This assay allows characterization of a relevant immune response that involves that action of phagocytes, opsonizing proteins and natural antibodies (predominantly IgM and IgA) (Demas et al., 2011). Briefly, serum samples were diluted 1:20 in CO_2-independent medium (Gibco no. 18045, Carlsbad, GA, USA). A standard number of colony-forming units (CFUs) of *E. coli* (ATCC no. 8739, Microbial Culture

Collection Center of Guangdong Institute of Microbiology, China) was added to each sample in a ratio of 1:10, and the mixture was allowed to incubate at 37°C for 30 min to induce bacterial killing. After incubation, 50 μl of each sample was added to tryptic soy agar plates in duplicate. All plates were covered and left to incubate upside down at 37°C for 24 h, and then total CFUs were counted and bactericidal capacity was calculated as 100% minus the mean number of CFUs for each sample divided by the mean number of CFUs for the positive controls (containing only medium and standard bacterial solution), i.e. the percentage of bacteria killed relative to the positive control.

Measurements of oxidative damage

Oxidative damage was estimated as lipid peroxidation and protein oxidation. Lipid peroxidation was assessed by quantifying malondialdehyde (MDA) (Del Rio et al., 2005) using a thiobarbituric acid reactive substances (TBARS) assay kit (Nanjing Jiancheng, Nanjing, China) following the manufacturer's instructions. The absorbance of the eluent was monitored spectrophotometrically at 532 nm (Synergy 4 Hybrid Microplate Reader, BioTek, Winooski, VT, USA). Both within- and among-sample variations for this assay were <1.5%. Lipid peroxidation was expressed as nmol MDA mg^{-1} protein or nmol MDA ml^{-1} serum. It should be noted that this method has low specificity as a measurement of MDA levels (Moore and Roberts, 1998). However, because MDA is the main aldehyde that reacts with TBA, previous researchers have often used the term 'MDA' when measuring TBARS. For clarification we use the term 'MDA (TBARS)' in this article.

Protein oxidation was assessed by the determination of levels of protein carbonyls (Mateos and Bravo, 2007) using a kit (Nanjing Jiancheng) according to the manufacturer's instructions. Briefly, proteins in liver and serum were reacted with 2,4-dinitrophenylhydrazine (DNPH) in hydrochloric acid for 30 min at 37°C, precipitated with trichloroacetic acid and washed four times by resuspension in ethanol/ethyl acetate (1:1 v/v). Proteins were solubilized in guanidine hydrochloride and centrifuged to remove insoluble material. Carbonyl groups were monitored spectrophotometrically at 370 nm (DU 800 UV/Vis spectrophotometer, Beckman Coulter, Brea, CA, USA). Carbonyls were expressed as nmol mg^{-1} protein.

Antioxidant assays

Superoxide dismutase (SOD) activity, glutathione peroxidase (GPx) activity, total antioxidant capacity (T-AOC) and protein thiol content were measured using kits (Nanjing Jiancheng) according to the manufacturer's instructions. One unit of SOD was defined as the amount of enzyme that causes 50% inhibition of superoxide radical produced by the reaction between xanthine and xanthine oxidase at 37°C. One unit of GPx was the enzyme in 1 mg protein or in 0.1 ml serum that oxidizes 1 μmol l^{-1} glutathione (GSH) per minute at 37°C. Blanks were run to correct for spontaneous reactions in the absence of GPx.

One unit of T-AOC was defined as the extent to which optical density is increased by 0.01 per milligram protein or per millilitre serum per minute. Protein thiol content was measured using an ELISA kit (Nanjing Jiancheng). The sensitivity by this assay is 0.01 mol l^{-1}, and 10 μl samples were used. Protein thiol content was expressed as μmol g^{-1} protein in tissues or μmol l^{-1} in serum.

Statistical analysis

Data were analyzed using SPSS 13.0 software (IBM, Armonk, NY, USA). Prior to all statistical analysis, data were examined for normality

Table 1. Effects of different ambient temperatures on litter mass and oxidative stress markers in mammary gland in lactating gerbils

Parameter	10°C (CL)	21°C (WL)	30°C (HL)	P
Initial litter mass (g)	19.1±1.1	20.1±0.8	19.1±1.1	NS
Final litter mass (g)	55.9±4.0^b	75.3±1.4^a	69.8±3.1^a	<0.001
MDA (nmol mg^{-1} protein)	0.303±0.018	0.305±0.019	0.338±0.036	NS
Protein thiol (µmol g^{-1} protein)	61.1±3.1	67.7±5.5	68.9±3.2	NS
SOD activity (U mg^{-1} protein)	12.4±0.4	12.4±0.6	12.1±0.3	NS
GPx activity (U mg^{-1} protein)	13.4±1.1	11.0±0.8	12.7±0.6	NS

Different letters indicate statistically significant differences among group means ($P<0.05$). Values are means ± s.e.m. MDA, malondialdehyde; SOD, superoxide dismutase; GPx, glutathione peroxidase; NS, no significant difference.

and homogeneity of variance using Kolmogorov–Smirnov and Levene's tests, respectively. Differences in liver and serum markers of oxidative stress [MDA (TBARS), protein carbonyls, protein thiols, SOD activity, GPx activity, T-AOC] and immunity (PHA response, serum total IgG levels, bactericidal activity, spleen and thymus) were analyzed by two-way ANOVA (temperature and reproductive status as factors) followed by Tukey's *post hoc* comparisons. Litter mass and markers of oxidative stress [MDA (TBARS), protein thiols, total-SOD activity and GPx activity] in the mammary gland were analyzed by a one-way ANOVA (temperature as the factor) followed by Tukey's *post hoc* comparisons. Differences between group means were considered statistically significant at $P<0.05$.

RESULTS
Initial and final litter size and mass
Initial litter sizes at birth (day 0) in lactating gerbils in cold (CL), warm (WL) and hot (HL) conditions were 6.0±0.4, 6.5±0.3 and 6.1±0.4, respectively, and final litter sizes at weaning (day 18) in these three groups were 5.6±0.4, 6.3±0.3 and 6.0±0.4, respectively. Litter size and litter mass on day 0 of lactation did not differ significantly among the groups (all $F<0.44$, $P>0.65$; Table 1). At the end of lactation when the pups were weaned (day 18), litter size also did not differ significantly between the temperature groups ($F_{2,33}=1.07$, $P>0.05$); however, final litter mass was significantly lower in the CL group when compared with both the WL and HL groups ($F_{2,33}=11.70$, $P<0.001$).

Immunity
Lactating gerbils had significantly lower thymus masses and serum bactericidal capacity and higher mass of the spleen and serum total IgG levels than non-reproductive gerbils (Table 2). However, the PHA response was not significantly affected by reproductive status (Fig. 1, Table 2; see Table 3 for statistical details).

Serum total IgG levels in lactating gerbils were significantly increased by high temperature, but the masses of the spleen and thymus and the bactericidal capacity and PHA response were not

significantly related to ambient temperature. The bactericidal capacity was significantly reduced during reproduction at all temperatures.

Oxidative damage and antioxidant activity
Liver MDA (TBARS), liver protein carbonyls, serum SOD activity and serum GPx activity were all significantly lower in lactating compared with non-reproductive gerbils (Table 4). Serum protein carbonyls, liver SOD and liver T-AOC activity were significantly higher in lactating compared with non-reproductive gerbils (Table 4). In contrast, serum MDA (TBARS), liver GPx activity, liver protein thiol and protein thiols in serum were unaffected by reproductive status (Figs 2, 3, Tables 2, 4).

MDA, protein thiols and GPx activity in liver, serum and mammary gland (Table 1), protein carbonyls in liver and serum, and SOD activity in serum and mammary gland were not related to ambient temperature. However, T-AOC and SOD in liver were significantly increased in animals at reduced ambient temperature (Table 4). With respect to protective antioxidants, there was a significant interaction effect of reproduction and temperature. In particular, SOD activity in the liver was increased during reproduction to a greater extent under either cold or hot conditions than at warm temperature (Fig. 3A).

Correlations between reproductive investment and markers of oxidative stress
Litter mass was negatively correlated with liver GPx activity ($r=-0.488$, $P=0.003$), liver SOD activity ($r=-0.401$, $P=0.015$) and GPx activity in mammary gland ($r=-0.580$, $P<0.001$), and the same significant correlations existed between litter size and serum SOD activity ($r=-0.399$, $P=0.016$; supplementary material Figs S1–S4). Serum protein carbonyl concentrations were also positively correlated with litter size ($r=0.468$, $P=0.003$; supplementary material Fig. S5).

DISCUSSION
In this study, we found that serum bactericidal capacity was reduced and oxidative damage to proteins in serum (protein carbonyls) was

Table 2. Effects of reproductive status and different ambient temperatures on immune organs and some liver or serum oxidative stress markers

Parameter	Lactation			Non-reproduction			P		
	10°C (CL)	21°C (WL)	30°C (HL)	10°C (CN)	21°C (WN)	30°C (HN)	La	Ta	La × Ta
Spleen (mg)	85±18^a	146±57^a	82±23^a	57±6^b	52±3^b	42±4^b	0.039	NS	NS
Thymus (mg)	21±2^b	20±2^b	25±5^b	87±51^{a,b}	59±10^a	43±5^a	0.013	NS	NS
Liver T-AOC (U mg^{-1} protein)	0.39±0.02^a	0.33±0.02^{a,c}	0.33±0.03^{a,c}	0.34±0.02^{a,b}	0.26±0.02^c	0.30±0.02^{b,c}	0.01	0.02	NS
Liver thiol (µmol g^{-1} protein)	75.3±9.2	65.6±4.1	75.9±7.7	59.4±4.6	59.4±3.8	69.1±6.9	NS	NS	NS
Serum protein thiol (µmol l^{-1})	812 ±60	863±101	685±19	790±23	778±44	747±43	NS	NS	NS

Different superscripted letters indicate statistically significant differences among group means ($P<0.05$). Values are means ± s.e.m.
T-AOC, total antioxidant capacity; La, lactation; Ta, ambient temperature; La × Ta, interaction of lactation and ambient temperature; NS, no significant difference.
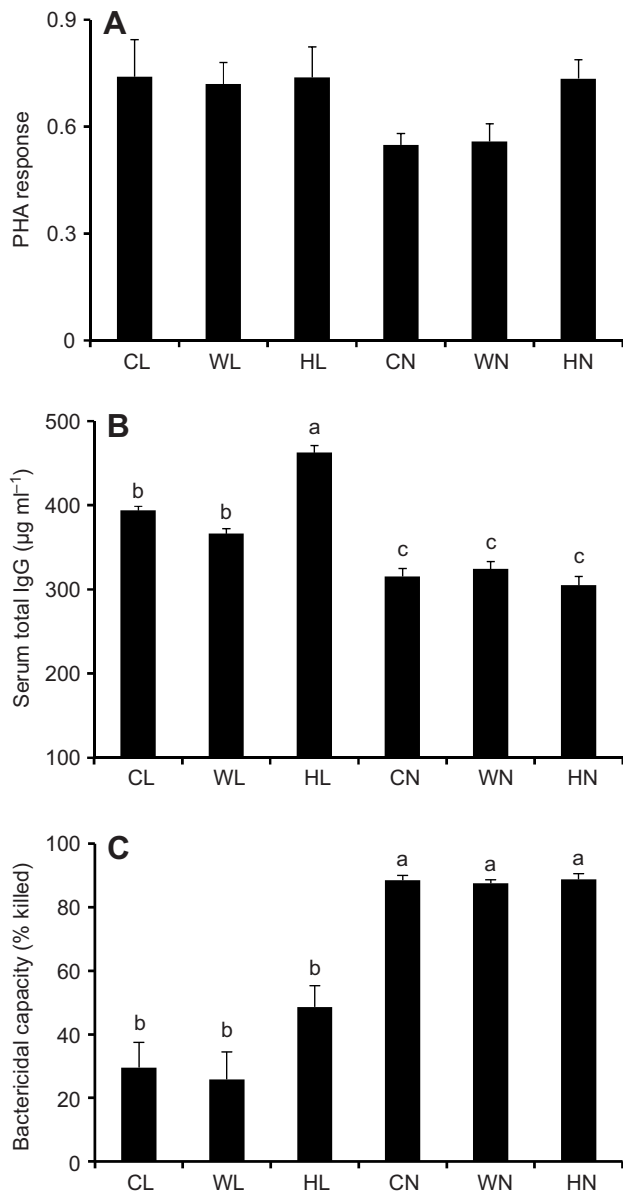
Fig. 1. Phytohaemagglutinin (PHA) response (A), serum total immunoglobulin (IgG) levels (B) and bactericidal capacity (C) in lactating and non-reproductive Mongolian gerbils acclimated to cold (10±1°C), warm (21±1°C) or high (30±1°C) ambient temperature. C, W and H represent cold, warm and high ambient temperature, respectively. L and N represent lactation and non-reproduction, respectively. Significant differences are indicated by different letters if $P<0.05$. Values are means ± s.e.m.

antioxidants can be altered in response to altered ROS production (Isaksson et al., 2011). The observed elevation in levels of liver antioxidants was consistent with the reduced oxidative damage observed in the liver. These changes are directly opposite to the expectations based on the resource allocation model, which predicts that investment in antioxidant defenses should decline during reproduction, leading to elevated oxidative damage. This reduction in oxidative damage, however, was consistent with the observation that oxidative damage was reduced during lactation in house mice [measured by MDA and protein oxidation in the liver – protein thiols and carbonyls (Garratt et al., 2011; Garratt et al., 2013)] and bank voles [measured by MDA and protein carbonyls in the kidneys (Ołdakowski et al., 2012)].

In contrast to the observations in the liver, we found that serum SOD activity and serum GPx activity were significantly lower, and damage correspondingly greater, in lactating compared with non-reproductive gerbils. Field tests of the oxidative stress hypothesis in small mammals, based on serum sampling, have also suggested that oxidative damage increases during reproduction (Bergeron et al., 2011; Fletcher et al., 2013) [see also Stier et al. (Stier et al., 2012) for a laboratory study of mice based on serum samples that showed the same positive effect of reproductive investment on oxidative damage]. Previous studies have also found that an increase in reproductive effort in zebra finches (*Taeniopygia guttata*) is associated with a decrease in the overall antioxidant capacity of the blood (Alonso-Álvarez et al., 2004). Although it has been previously observed that oxidative stress may differ between tissues (Sohal et al., 1995; Costantini, 2008), the reasons for the diametrically opposite responses in the liver and serum remain unclear. One potential explanation for the contrasting trends is that any elevation in damage in the liver might be diluted by hypertrophy of the liver during lactation (Kennedy et al., 1958; Speakman and McQueenie, 1996; Johnson et al., 2001b; Król et al., 2003). Although there is also a large increase in blood volume during lactation, this appears to be mostly added water, rather than greater numbers of cells (Suzuki et al., 1993). However, differential hypertrophy seems an unlikely explanation because the reduction in damage was not observed in all markers (protein thiols, for example, were unchanged). Moreover, the activity of some of the protective enzymes was increased in the liver, the opposite of what might be expected by a simple dilution effect.

Another possibility is that different tissues have different priorities for protection depending on the rate at which they generate oxygen radicals during reproduction and the consequences of failing to protect against such radicals. In other words, the animals may trade off the allocation of protection. Finally, an effect may occur because of chemical differences in the proteins found in serum and in the liver and their propensity to be oxidized in a manner leading to carbonyl formation. Carbonyl groups are produced primarily on protein side chains (especially of Pro, Arg, Lys and Thr) when they are oxidized (Dalle-Donne et al., 2003). Thus greater levels of these amino acid residues might lead to a greater level of protein carbonyl formation. Whatever the cause of these differences, it is evident that if we had only measured one tissue we would have reached opposite conclusions regarding the predictions of life history theory depending on the tissue we had chosen for analysis. Therefore, we should be extremely cautious in interpreting data from studies where only single tissues and/or single assays are utilized to characterize immune function or oxidative stress status. A complete picture of what is happening will likely only emerge using comprehensive surveys of tissues, utilizing multiple assays of protection and damage (see also Selman et al., 2012).

increased in lactating compared with non-reproductive gerbils. These effects were independent of ambient temperature and potentially indicated a twofold cost of reproduction in gerbils: reduced immune function and elevated oxidative stress. Such effects were consistent with the hypothesis that oxidative stress and immune function are physiological factors underpinning the life history trade-off between reproduction and survival.

However, lactating gerbils had significantly lower MDA (TBARS) and protein carbonyls in their livers compared with non-reproductive gerbils, indicating oxidative damage in the liver was decreased during lactation. In addition, MDA (TBARS) in serum and protein thiols in both the liver and serum were unaffected by reproductive status. The production or deployment of various

Table 3. Statistical summary for immune assays

Immune and stress assays	Reproduction status			Temperature			Interaction	
	F	P	Change	F	P	Change	F	P
PHA response	2.75	0.10	No	0.79	0.46	No	0.66	0.52
Serum IgG	193.86	<0.001	Up	12.26	<0.001	Up	26.50	<0.001
Bactericidal capacity	93.31	<0.001	Down	1.81	0.17	No	1.53	0.23
Spleen	4.48	0.04	Up	0.81	0.45	No	0.66	0.52
Thymus	6.49	0.013	Down	0.56	0.58	No	0.76	0.47

PHA, phytohaemagglutinin; IgG, immunoglobulin.
Up or down, increase or decrease in lactating compared with non-reproductive gerbils or gerbils at 10 or 30°C compared with those at 21°C; no, no change.

Although the levels of oxidative damage in the liver during reproduction were decreased relative to non-reproducing gerbils, there was a significant positive relationship between litter size and protein carbonyls in serum, suggesting a possible link between reproductive effort and oxidative damage. Similar associations have also been found in other species (Upreti et al., 2002; Castillo et al., 2005; Bergeron et al., 2011; Fletcher et al., 2013; Stier et al., 2012). However, no relationship was found between reproductive status and oxidative damage in wild Soay sheep (*Ovis aries*) based on the assay of MDA in serum after reproduction was completed (Nussey et al., 2009). Similar data to ours were also observed in wild derived house mice, where there was increased damage (reflected by reduced thiol levels) in relation to reproductive status, despite the overall damage levels (lipid and protein damage in liver) in reproducing individuals being lower than in non-reproductive mice (Garratt et al., 2011). There is a potential issue with all these studies, in that the level of reproductive investment was not experimentally manipulated (Metcalfe and Monaghan, 2013). A recent manipulation of litter size in wild derived house mice revealed no effect of reproductive level on protein oxidation in the heart and gastrocnemius muscle and decreased damage in the livers of mice with increased levels of reproductive effort that had been experimentally manipulated (Garratt et al., 2013).

The levels of markers of oxidative stress and protection have been previously shown to depend on environmental conditions (Selman et al., 2000; Monaghan et al., 2009; but see Selman et al., 2008). We found that only T-AOC and total SOD activity in the liver were affected by ambient temperatures. All the markers of oxidative stress in the mammary glands were unaffected by ambient temperature. We did not find any evidence to support the idea that there were elevated or diminished trade-offs between reproduction and the extent of immunosuppression or oxidative stress at lower or higher temperatures.

The absence of any impact of ambient temperature was surprising because we anticipated *a priori* that low temperatures might exacerbate the trade-off between energy use for reproduction and oxidative protection or immune function by challenging the gerbils with an additional cost. Alternatively, high temperatures might compromise the mice because of heat dissipation constraints that cap the total available energy for allocation (Król et al., 2007; Speakman and Król, 2011). These observations therefore call into question our simplistic interpretations from life history theory that these features are being traded off against each other with energy as the primary currency mediating that trade-off. Consistent with this view is the fact that two of the main antioxidant enzymes (SOD and catalase) do not require energy to perform their catalytic functions (Gems and Partridge, 2013), although this is not true of some other antioxidant protection processes – such as the recycling of reduced glutathione (Speakman and Garratt, 2014) or immune function (Martin et al., 2008). At present this absence of a temperature effect remains unexplained.

Although temperature had no significant effect on the levels of most markers of oxidative stress and immune function, there were significant impacts of temperature on the levels of pup growth. These effects were similar to the more detailed impacts of temperature on reproductive performance reported previously in gerbils (Yang et al., 2013). This pattern is consistent with the heat dissipation limit theory for temperatures between 21 and 30°C, but suggest that at cooler temperatures, other factors, notably the growth capacity of

Table 4. Statistical summary for oxidative assays

Oxidative assays	Reproduction status			Temperature			Interaction	
	F	P	Change	F	P	Change	F	P
MDA in liver	61.72	<0.001	Down	0.85	0.43	No	0.67	0.51
MDA in serum	5.84	0.02	No	2.79	0.07	No	1.43	0.25
MDA in mammary gland				0.57	0.57	No		
Protein carbonyls in liver	42.27	<0.001	Down	0.05	0.95	No	2.08	0.13
Protein carbonyls in serum	146.94	<0.001	Up	0.85	0.43	No	2.43	0.10
Protein thiol in liver	3.34	0.07	No	1.22	0.30	No	0.35	0.71
Protein thiol in serum	0.11	0.75	No	1.82	0.17	No	0.81	0.45
Protein thiol in mammary gland				0.92	0.41	No		
T-AOC in liver	6.66	0.01	Up	4.09	0.02	Up	0.22	0.81
Total SOD in liver	95.41	<0.001	Up	3.12	0.05	No	4.01	0.02
Total SOD in serum	186.78	<0.001	Down	1.38	0.26	No	0.03	0.97
Total SOD in mammary gland				0.11	0.90	No		
GPx in liver	1.92	0.17	No	4.93	0.01	Up	0.36	0.70
GPx in serum	32.37	<0.001	Down	0.35	0.71	No	0.69	0.51
GPx in mammary gland				2.12	0.14	No		

Up or down, increase or decrease in lactating compared with non-reproductive gerbils or gerbils at 10 or 30°C compared with those at 21°C; no, no change.
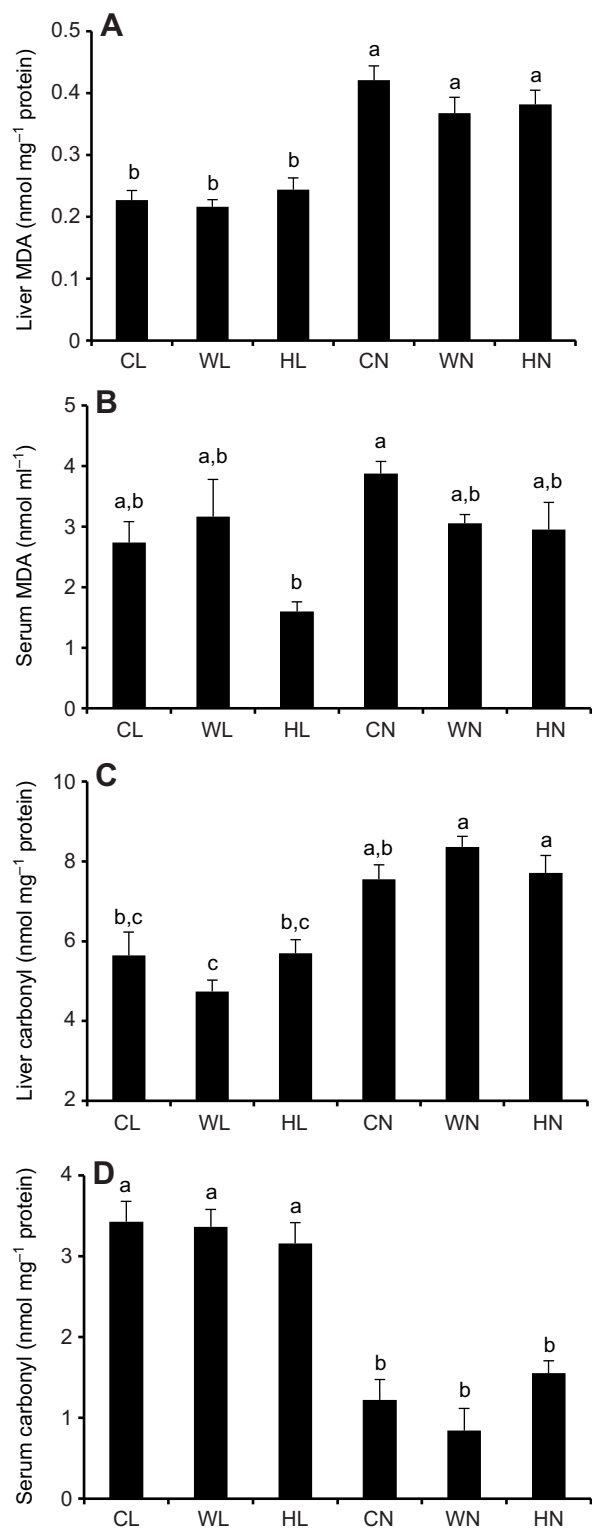
Fig. 2. Liver malondialdehyde (MDA) (TBARS) (A), serum MDA (TBARS) (B), liver protein carbonyls (C) and serum protein carbonyls (D) in lactating and non-reproductive Mongolian gerbils acclimated to cold (10±1°C), warm (21±1°C) or high (30±1°C) ambient temperature. Significant differences are indicated by different letters if $P<0.05$. Values are means ± s.e.m.
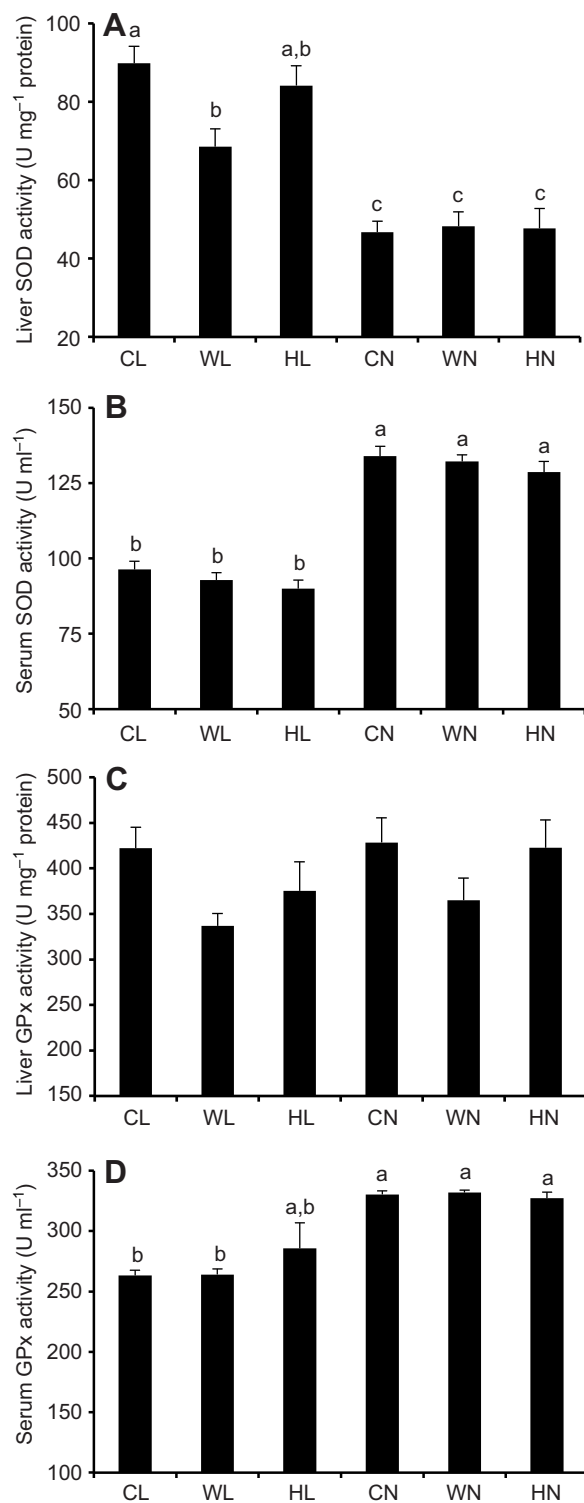

Fig. 3. Liver total superoxide dismutase (SOD) activity (A), serum total SOD activity (B), liver glutathione peroxidase (GPx) activity (C) and serum GPx activity (D) in lactating and non-reproductive Mongolian gerbils acclimated to cold (10±1°C), warm (21±1°C) or high (30±1°C) ambient temperature. Significant differences are indicated by different letters if $P<0.05$. Values are means ± s.e.m.

the offspring, may impose limits on the lactation performance (see also Simons et al., 2011; Zhao et al., 2014).

Serum bactericidal capacity represents an aspect of constitutive innate immunity (Demas et al., 2011), and the energetic cost of

maintenance of innate immunity is relatively high (Martin et al., 2008). Serum bactericidal capacity was suppressed in lactating compared with non-reproductive gerbils independent of ambient temperature, indicating a trade-off between reproduction and

immune function, which could potentially be interpreted as a consequence of a reallocation of resources between the two physiological functions (Sheldon and Verhulst, 1996; Norris and Evans, 2000; Martin et al., 2008). There has been a burgeoning of interest in the relationship between immune function and oxidative stress (Monaghan et al., 2009). Non-adaptive processes, such as damage to immune cells caused by increased production of free radicals during periods of high metabolic activity (Leeuwenburgh and Heinecke, 2001), may play a role in immunosuppressive consequences of increased reproductive effort. It has also been suggested that reproductive effort reduces the level of antioxidants, downregulating immune function and leading to increased parasitaemia (Sorci and Faivre, 2009). Serum SOD activity and GPx activity were reduced in lactating relative to non-reproductive gerbils. Indeed, we found that there were significantly positive correlations between these two antioxidants and bactericidal capacity (supplementary material Figs S6, S7), potentially suggesting a suppressive effect of reduced antioxidants on immune function in lactating gerbils.

However, the PHA response and serum total IgG concentrations were not significantly decreased in lactating compared with non-reproductive gerbils. Lactating gerbils had higher serum total IgG concentrations than non-reproductive gerbils. In vertebrates, IgG are the primary class of immunoglobulins that transfer *via* milk (Boulinier and Staszewski, 2008). A possible explanation for increased serum total IgG during lactation may be the need of maternal transfer of antibodies, which can protect the neonate offspring from infection (Hasselquist and Nilsson, 2009). As with the oxidative stress responses, had we only measured a single immune response type then we could have reached completely opposite conclusions regarding the trade-offs predicted by life history theory, depending on the exact marker we had chosen.

Our data show that the inferred effects of reproduction on immunosuppression and oxidative damage were critically dependent on the exact markers used, and the samples in which they were measured. The functional significance of these differences remains uncertain. Prior work suggests that animals are able to tolerate some forms of oxidative stress (Andziak and Buffenstein, 2006; Yang et al., 2007; Zhang et al., 2009) without effects on mortality and lifespan, at least in the protected conditions of the laboratory. At present it is unclear what the functional consequences are when oxidative damage increases in one tissue, but simultaneously decreases in another, or is altered when using one measure but unaltered when using a different measure. The implication is that protection (and hence damage) may be selectively allocated between tissues. Given the different patterns of immunosuppression and oxidative damage we obtained, future studies need to pay careful attention to the markers that are used to estimate immune function and oxidative stress. These data suggest that studies based on single measures, or those based on measures from single, or small numbers of, tissues such as blood samples, should be considered with extreme caution. A complete understanding of the relationships between reproduction, oxidative stress and immune function is likely to emerge only by expanding the scope of investigations to include diverse markers measured across multiple tissues (as suggested by Selman et al., 2012) in addition to functionally relevant end points such as survival (see also Metcalfe and Monaghan, 2013).

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