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Maternal oxidative stress and reproduction: testing the constraint, cost and shielding hypotheses in a wild mammal

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Abstract :

1.Oxidative stress has been proposed as a central causal mechanism underlying the life history trade-off between current and future reproduction and survival in wild animals.

2.Whereas mixed evidence suggests that maternal oxidative stress may act both as a constraint and a cost to reproduction, some studies have reported a lack of association between reproduction and maternal oxidative stress.

3.The oxidative shielding hypothesis offers an alternative explanation, suggesting that mothers may pre-emptively mitigate the oxidative costs of reproduction by increasing antioxidant defences prior to reproduction.

4.We tested the oxidative constraint, cost, and shielding hypotheses using a longitudinal field study of oxidative stress levels in a species that breeds using daily energy income, the Columbian ground squirrel (*Urocitellus columbianus*).

5.Elevated maternal oxidative damage prior to reproduction was associated with higher maternal investment in litter mass at birth, but not at weaning.

6.Breeding females increased their antioxidant capacity and decreased their oxidative damage from birth to lactation compared to non-breeding females measured at the same time periods. However, lower maternal oxidative stress during lactation was not associated with higher offspring survival or mass growth over this period.

7.Our results provide little evidence for maternal oxidative stress acting as a constraint on, or cost to, reproduction in Columbian ground squirrels, but partially support the idea that oxidative shielding

occurred to buffer potential oxidative costs of reproduction.

Keywords : antioxidant, body condition, cost of reproduction, life history, mammal, mitochondria, trade-off

Introduction

Central to life-history theory, the "cost of reproduction" hypothesis predicts that breeding individuals should trade-off high investments into current reproduction with future survival or reproductive prospects (Williams 1966; Hirshfield & Tinkle 1975; Reznick 1985; Stearns 1992). While fitness costs to reproduction have often been highlighted (Linden & Møller 1989; Nager, Monaghan & Houston 2001; Nur 2002; Koivula *et al.* 2003; Penn & Smith 2007; Descamps *et al.* 2009; Flatt 2011; Lehto Hurlimann *et al.* 2014), the mechanisms responsible for such costs remain poorly understood, despite an increasing effort to integrate physiological approaches in understanding this compromise (Zera & Harshman 2001).

Recently, oxidative stress has been suggested as a proximate mechanism that underlies variation in fitness related to breeding effort (Alonso-Alvarez *et al.* 2004, 2007; Bize *et al.* 2008; Monaghan, Metcalfe & Torres 2009; Stier *et al.* 2012; Fletcher *et al.* 2012; Noguera 2017). This proposition stems from the notion that when adults are reproducing, increased metabolism should lead to increased oxidative stress as a consequence of enhanced mitochondrial activity during ATP synthesis (Raha & Robinson 2000). Specifically, mitochondrial processes involve the obligate by-production of reactive oxygen species (ROS), unstable molecules known to cause damage to lipids, proteins, and DNA. This may lead to reduced life expectancy, i.e. *the Disposomable Soma Theory* (Kirkwood 1977; Kirkwood & Holliday 1979; Metcalfe & Alonso-Alvarez 2010). Under this theory, the strength of oxidative stress suffered during reproduction depends on the organism's ability to

shield itself from ROS through exogenous and endogenous antioxidant detoxifying systems (Beckman & Ames 1998), and may therefore constitute a proximate cost to reproduction.

Whereas several studies have tested for an oxidative cost to reproduction, evidence has been mixed (see Metcalfe & Monaghan 2013; Speakman & Garratt 2014 for reviews). The main issue comes from the dogma that ROS production strictly follows the rate of mitochondrial ATP production and hence metabolic rate, but this is not always the case (Murphy 2009; Speakman & Selman 2011). Several studies conducted either in the field or under controlled laboratory conditions have led to positively weak, negative or no associations between oxidative stress and reproductive investment (Nussey et al. 2009; Garratt et al. 2011, 2013; Bergeron et al. 2011; Oldakowski et al. 2012). Specifically, studies comparing breeding vs. non-breeding individuals have often failed to note the expected increase in oxidative damage associated with reproduction (e.g. Garratt et al. 2011), possibly also because of high inter-individual variability in oxidative stress related to other important intrinsic (e.g. age, experience) or extrinsic (e.g. pollutants) factors (Bonisoli-Alquati *et al.* 2010; Isaksson 2010; Costantini *et al.* 2014b). This discrepancy has led authors to call beyond a simplistic trade-off model and to reconsider how the energy demands of reproduction may fit with a stressful metabolic situation (Metcalfe & Monaghan 2013; Speakman & Garratt 2014).

More recently, it has been proposed that maternal oxidative stress *prior* to reproduction may act as a constraint (Dowling & Simmons 2009; Stier *et al.* 2012), limiting parental investment into reproduction (Bize *et al.* 2008; Dowling & Simmons 2009). Here also, evidence is mixed. In wandering albatross, *Diomedea exulans*, maternal oxidative stress levels prior to reproduction did not negatively affect future breeding probability or outcome (Costantini *et al.* 2015). In laboratory mice, *Mus musculus*, females with higher oxidative damage levels prior to reproduction produced overall smaller litters (Stier *et al.* 2012). In canaries, *Serinus canaria*, experimentally reducing maternal antioxidant defences prior to reproduction delayed the onset of egg-laying and had a negative effect on clutch size (Costantini *et al.* 2016). It is important to note that the constraint and cost hypotheses are not mutually exclusive, as both may occur simultaneously during reproduction (Stier *et al.* 2012).

The repeated observation that, in mammals, oxidative stress may actually be lower in breeding compared to non-breeding females, especially during lactation when energy investment into reproduction is highest (Garratt *et al.* 2011, 2013; Oldakowski *et al.* 2012; Schmidt, Blount & Bennett 2014), has led to a third hypothesis: the idea that animals may be selected to pre-emptively mitigate the obligate costs of ROS production during reproduction (see Blount *et al.* 2016). Oxidative shielding may be particularly important if maternal oxidative stress levels during reproduction has consequences on the development and survival of offspring (Mutinati *et al.* 2013; Herrera *et al.* 2014). The onset of reproduction is then expected to trigger a pre-emptive reduction in the levels of oxidative damage in mothers' tissues (Blount *et al.* 2016; Vitikainen *et al.* 2016). In particular, damaged gametes or transfer of damaged molecules through maternal milk (e.g. Rizzo *et al.* 2013) during lactation (i.e. oxidized fatty acids) could have deleterious effects on embryonic/juvenile growth and future health state, and such maternal tissues should be particularly preserved (Blount *et al.* 2016). Thus, considering potential trans-generational consequences of oxidative stress is likely to improve our understanding of oxidative stress in the "cost of reproduction" trade-off (Blount *et al.* 2016). However, the oxidative shielding hypothesis requires empirical evidence gathered in different species using longitudinal monitoring (e.g. Vitikainen *et al.* 2016) to test how its predictions fit with variable life histories (Blount *et al.* 2016). To date, only one study tested whether mothers actually mount an oxidative shield during reproduction. In a cross-sectional study of wild banded mongoose over pre-breeding, breeding and post-breeding periods, Vitikainen *et al.* (2016) found that plasma malondialdehyde (MDA, oxidized lipids) were reduced in pregnant vs. non-breeding females. MDA levels were also negatively related to reproductive output, higher MDA levels during pregnancy being correlated to lower offspring survival. However, the authors did not find increased antioxidant defences (vitamin E or superoxide dismutase activity) in reproducing females, and the mechanisms responsible for an oxidative shield remained unclear.

Here, we evaluated the oxidative constraint, cost and shielding hypotheses using a longitudinal study of free-living breeding (successful and non-successful) *vs.* non-breeding females Columbian ground squirrels (*Urocitellus columbianus*). We considered (1) how maternal antioxidant defences and

oxidative damage varied over the course of reproduction and (2) how maternal oxidative status was related to offspring production at birth and at weaning. Columbian ground squirrels are hibernating sciurid rodents with a short 3-4 month annual active season during which reproduction takes place (Murie & Harris 1982; Dobson, Badry & Geddes 1992; Neuhaus 2000a). Female ground squirrels are predominantly income breeders (diet consisting mostly of monocotyledonous grasses and dicotyledonous forbs before and during reproduction; Ritchie 1988; Bennett 1999) whose reproduction nonetheless benefits from the availability of stored energy capital at the start of reproduction (Broussard, Dobson & Murie 2005). Females are sexually mature at 2 years of age (Dobson & Murie 1987; Neuhaus *et al.* 2004), and can live up to 14 (oldest living female in our 24 year long-term data set). In a given year, females produce a single litter of 3 (1-7) pups on average and may occasionally skip reproduction to recoup body condition for subsequent breeding (Rubach *et al.* 2016). Gestation lasts for 24 days followed by 27 days of lactation, during which parental care is restricted to the mother and does not extend past a few days after weaning (Murie & Harris 1982). Several studies on litter size manipulations or comparisons of breeding vs. non-breeding females have found little evidence for a substantial cost of reproduction in terms of female survival (Murie & Dobson 1987; Hare & Murie 1992; Skibiel, Speakman & Hood 2013; Rubach *et al.* 2016; but see Neuhaus 2000). However, the costs of reproduction in this species may manifest in terms of future reproduction. For instance, resource allocation strongly differs between breeding and non-breeding females (Rubach *et al.* 2016). Not breeding in one year provides females with the possibility of accumulating extra body mass that can be carried-over into the subsequent season, increasing propensity for future reproduction (Risch, Dobson & Murie 1995; Broussard *et al.* 2005; Rubach *et al.* 2016). In addition, females in better condition during reproduction wean larger litters (Risch et al. 1995) of larger, faster growing pups (Skibiel, Dobson & Murie 2009), reflecting increasing returns (Dobson *et al.* 1999). Female ground squirrel condition has not been considered within the framework of oxidative stress, nor has maternal oxidative condition during the breeding season been tested for possible effects on the growth and survival of offspring.

We used plasmatic measures of antioxidant capacity (OXY adsorbent test) and oxidative damage (d-ROMs test) as global indices of the oxidative balance (Costantini 2011, 2016). The oxidative constraint, cost and shielding hypotheses, allow drawing of specific predictions that can be tested in our model system (see Fig. 1). First, following the oxidative constraint hypothesis (Dowling & Simmons 2009; Stier *et al.* 2012), we predicted that females with high levels of oxidative stress at emergence from hibernation should be constrained from investing in reproduction. Hence, we expected a negative relationship between female oxidative stress levels prior to reproduction (i.e. at emergence from hibernation) and the total offspring mass produced at birth and at weaning. Second, following the oxidative cost hypothesis (Alonso-Alvarez *et al.* 2004), we predicted that females investing heavily into reproduction (offspring production and lactation) would suffer from greater oxidative stress. Thus, we expected a positive relationship between a female's oxidative stress levels at weaning (and variation from emergence to weaning) and the total offspring mass she produced and reared. Third, following the oxidative shielding hypothesis (Blount *et al.* 2016), we predicted that breeding females should increase antioxidant defences compared to non-breeding females, resulting in low levels of oxidative damage around critical periods of offspring development (i.e. pregnancy, and post-parturition through to the end of lactation). In addition, maternal oxidative stress should be a reliable indicator of offspring mass growth and survival rate from birth to weaning. We expected high maternal oxidative damage after birth and during lactation to negatively affect offspring growth and survival to weaning. In addition, we tested whether maternal oxidative stress related to female age or female condition at the start of the season, given the importance of these factors in determining female annual fitness (Dobson *et al.* 1999; Broussard *et al.* 2003; Skibiel *et al.* 2009; Rubach et al. 2016).

Materials and Methods

General methods

Columbian ground squirrels were studied in two neighbouring subalpine colonies located *ca.* 1.5 km apart in the Sheep River Wildlife Provincial Park, Alberta, Canada (50°64N, 114°66W and 50°65N,

114°66W, elevation 1550m). These populations have been monitored since 1992 and 2002 respectively, and the age of all individuals was known from birth. In 2016, we followed a total of 76 females throughout the breeding season, classified as (1) successful breeders if they successfully raised a litter through to weaning (N = 37); (2) unsuccessful breeders if they produced a litter, but failed to wean any pups (N = 7); and (3) non-breeders if they did not mate and engage in breeding (N = 32). Females were trapped on the day of emergence from hibernation or the day after (from April 12 to May 14) using live traps (13 x 13 x 40 cm) baited with a small amount of peanut butter. Upon trapping, we collected a blood sample (see below) and each female was weighed to the nearest 5 g using a Pesola spring scale (Pesola Ag, Baar, Switzerland). Each female was identified by a unique ear tag number (#1-Monel metal; National Band and Tag Company, Newport, KY) and was given a unique mark of black hair dye (Clairol, Stamford, CT) for visual identification in the field.

We followed females throughout their single day of mating, and determined mating dates from the occurrence of above-ground copulations and below-ground consortships with males (Raveh *et al.* 2010, 2011). When matings were not detected, we inspected female's vulvar condition and fur around the genital area for the presence of copulatory plug material and dried sperm, as indicators of successful mating (Murie & Harris 1982). We established the location of single-entrance natal nest-burrows by daily observations of females' morning emergence from these burrows, and from females frequently stocking them with loads of dry grass material collected from the meadow. We marked natal nest burrows with coloured flags. We trapped females about 22 days after mating and 2-3 days before expected parturition date (May 5 – June 4). Females were blood sampled and weighed (nearest 5 g) a second time, brought to an on-site laboratory, and temporarily housed in polycarbonate microvent cages (267 x 483 x 200 mm; Allentown Caging Equipment Company, Allentown, NJ) (see details in Hare & Murie (1992)).

After parturition, mothers (nearest 5g) and pups (nearest 0.01g) were weighed. Mothers and their neonates were released the day after birth into their nest burrows. Mothers were caught, weighed, and blood was sampled at mid-lactation (around day 16 post-partum; from May 20 to June 20), and a final time when young first emerged from the natal burrow at weaning (June 1st to July 2nd). All pups

were caught when they first emerged from natal nest burrows, weighed to the nearest 1g and given unique ear tags and dye markings.

Blood sampling

Upon capture (at emergence from hibernation, pre-parturition, mid-lactation and weaning), we collected 0.5 mL of blood from the saphenous vein using a 27G needle fitted to a 1 mL heparinised syringe. Syringes were kept on ice packs in a cooler box while in the field. After centrifugation (3000g for 10 min) within 1-2 hours of collection, plasma was separated and kept frozen at -20°C until the end of the field season, before transportation on dry ice and subsequent frozen storage at -80°C until analyses.

Oxidative stress measurements

Female oxidative status was assessed using global measures of oxidative damage and antioxidant defences in plasma: (1) Oxidative damage was assessed using the d-ROMs test (8 µl of plasma) (Diacron International, Grosseto, Italy), that measures reactive oxygen metabolites (ROMs) in plasma (Stier *et al.* 2012; Costantini 2016). This test measures hydroperoxides, the main compounds contributing to plasma oxidant activity, expressed as mg H_2O_2 equivalent.dl^{-1}. (2) Oxidative defences were evaluated using the OXY adsorbent test (5 µl of 1:100 diluted plasma) (Diacron International) that measures plasma's total antioxidant capacity (OXY) (Costantini 2011; Stier *et al.* 2012), i.e. the ability of plasma to a buffer massive oxidation through hydroperoxide acid expressed in µmolHCL.mL^{-1}. All sample measurements were duplicates. Intra-plate variation was 3.71% and 4.74% for ROMs and OXY, respectively. Inter-plate variation based on a standard sample repeated over plates was 13.86% and 7.06% for ROMs and OXY, respectively.

Statistics

All statistical analyses were run in JMP 9.0.1 (© 2010 SAS Institute Inc) and R 3.0.2 (© 2013 The R Foundation for Statistical Computing). For all analyses, continuous independent variables were standardized so that model estimates (β coefficients) are comparable. We inspected Variation Inflation Factors (VIFs) for independent variables to ensure there was no substantial bias from collinearity in the analyses (Zuur *et al.* 2009). Results are reported as means ± SE. Sample sizes vary across analyses due to varying success in data collection (capture or blood sampling success) and laboratory analyses. In total, we were able to acquire oxidative stress measures from blood samples for 72 females at emergence from hibernation, 67 females at parturition, 55 females at mid-lactation and 61 females at weaning.

Oxidative stress dynamics over the breeding season

We tested for differences in oxidative stress kinetics between female ground squirrels that were either (1) successful breeders, (2) failed breeders and (3) non-breeders females over the course of the 2016 breeding season using linear mixed models (LMMs). ROM (or OXY) levels were set as independent variables in the models, and breeding status (successful breeders, failed breeders, non-breeders) and stage (emergence, birth, lactation, weaning) were set as independent variables. We considered the *breeding status x stage* interaction to test for different dynamics in oxidative stress metrics between stages. Female age and mass at capture were entered as separate covariates to test for effects on oxidative status. Female ID was entered as a random factor to account for repeated measures on the same individuals.

Testing the oxidative constraint hypothesis

We tested whether ROM and OXY levels at emergence from hibernation acted as a constraint on the upcoming reproductive event (Stier *et al.* 2012) affecting total litter mass at birth and weaning. We

performed a linear model (LM) with total litter mass at birth (mean ± SD = 38.02 ± 10.02g, range = 22.13 – 64.20g) or at weaning (mean ± SD = 309 ± 79g, range = 73 – 437g) as the dependent variable and mother's ROM and OXY at emergence from hibernation levels as the independent variable. Mother's age and mass at the start of the breeding season were included as covariates in the analyses to account for potential effects on reproduction.

Testing the oxidative cost hypothesis

We tested whether maternal ROM and OXY levels measured at weaning were related to the total litter mass a female produced and reared through lactation, i.e. a cost to reproduction. Maternal ROM or OXY levels at weaning were specified as dependent variables in separate models and litter mass at weaning as the independent variable, controlling for mother's age and mass at the start of the breeding season. In addition, we tested whether changes in maternal ROM and OXY levels over the entire reproductive season, i.e. weaning – emergence levels, were related to litter mass at weaning.

Testing the oxidative shielding hypothesis

We tested whether changes in maternal ROM and OXY levels from (1) birth to mid-lactation, and (2) mid-lactation to weaning, affected the survival of dependent individual offspring from birth to weaning, and individual offspring mass growth during lactation. We used a Generalized Linear Mixed model with a binomial distribution (0/1: death/survival) to test for effects of changes in ROM and OXY levels (independent variable) on offspring survival (dependent), while controlling for mother age and mass at the start of the breeding season as covariates. For offspring mass growth, we used a LMM with the same variables, further accounting for litter size at birth, because of the known trade-off between litter size and mean offspring mass. For all models, mother ID was specified as a random factor in the analyses to account for the non-independence of offspring born from the same mother.

Ethics

Animal care conformed to Auburn University IACUC protocol # 2015-2626 also approved by the University of Calgary. Authorization to conduct research and collect samples in the Sheep River provincial park was obtained from the Department of Alberta Tourism, Parks and Recreation, permit # 16-017; and from Alberta Environment and Parks, permits # 56989 and 56990.

Results

Oxidative stress dynamics over the breeding season

Over the breeding season, ROM and OXY levels showed markedly different dynamics in successful breeders, failed breeders and non-breeding females (see significant interaction terms; ESM 1). Overall, a female's mass was negatively related to her ROM levels (*estimate* = -14.49 ± 6.55; t = -2.21, P = 0.03; ESM1) and positively related to her OXY levels (17.12 ± 6.47; t = 2.65, P = 0.01; ESM1). Age was not significantly related to either ROM or OXY levels (all P > 0.85; ESM1).

In successful and failed breeders, ROM and OXY levels followed similar patterns (Fig. 2A and 2B). ROM levels remained similar from emergence of hibernation to birth, before decreasing substantially from birth to mid-lactation (significantly by 35% for successful breeders, and non-significantly by 27% for failed breeders; Fig. 2A; Table 1). ROM levels then increased again from mid-lactation to weaning (significantly by 31% for successful breeders, and non-significantly by 18% for failed breeders; Fig. 2A; Table 1). OXY levels decreased non-significantly from emergence of hibernation to birth (by 12% for successful breeders and by 21% for failed breeders), before increasing again significantly by 17% for successful breeders and by 40% for failed breeders (Fig. 2B; Table 2). OXY levels remained similar from mid-lactation to weaning (Fig. 2B; Table 2)

ROM and OXY dynamics were markedly different in non-breeding females: ROM were lower (non-significantly) than in successful and failed breeders at emergence from hibernation and around the period of births (significantly), but showed a progressive 45% significant increase between the

period of births and the period of weaning (Fig. 2A; Table 1). In contrast, OXY levels remained relatively stable over the course of the study (Fig 2B; Table 2).

Relationships between oxidative balance and fitness

Oxidative constraint hypothesis: Controlling for mother's age and mass at the start of the breeding season, maternal oxidative damage (ROM) and antioxidant capacity (OXY) at emergence of hibernation were both related to total litter mass at birth, but not at weaning (Table 3). The relationship was positive for ROM (β = 3.16 ± 1.36; Fig. 3A), and negative for OXY (β = -2.28 ± 1.14, P = 0.053; Fig. 3B). Further, maternal mass at emergence was positively related to litter mass at birth (β = 8.57 ± 2.08; Fig. 3C). Variance inflation factors indicated no substantial bias from collinearity among independent variables (1.01< VIFs < 1.09).

Oxidative cost hypothesis: Controlling for mother's age and mass at the start of the breeding season, maternal ROM and OXY levels at weaning were not significantly associated with the total mass of the litter weaned (Table 4). Similarly, the differences in ROM and OXY levels from the start (emergence) to the end (weaning) of reproduction were not significantly associated with litter mass at weaning. Variance inflation factors indicated no substantial bias from collinearity (1.00 < VIFs < 1.01).

Oxidative shielding hypothesis: Controlling for mother's age and mass at the start of the breeding season, changes in maternal ROM and OXY levels from birth to mid-lactation or from mid-lactation to weaning were not related to offspring survival (GLMM, Table 5) or offspring mass gain (LMM, Table 5). As expected, controlling for litter size at weaning in the model revealed a negative trade-off between litter size and growth in offspring mass (see Table 5). Variance inflation factors indicated no substantial bias from collinearity (1.04 < VIFs < 1.60).

Discussion

In recent years, oxidative stress has been suggested as an important proximate mechanism mediating life-history trade-offs (Monaghan *et al.* 2009; Speakman *et al.* 2015). In this study, we considered variation in maternal oxidative stress over the course of reproduction in an income breeder, focusing on maternal oxidative stress effects on dependent young. We considered three alternative hypotheses: (1) oxidative stress at the onset of reproduction constrains maternal investment into reproduction (Dowling & Simmons 2009; Stier *et al.* 2012), (2) higher maternal oxidative stress results from higher investments into reproduction (Alonso-Alvarez *et al.* 2004; Metcalfe & Alonso-Alvarez 2010), and (3) females actively and pre-emptively mitigate oxidative stress by oxidative shielding (Blount *et al.* 2016; Vitikainen *et al.* 2016).

Maternal oxidative stress as a constraint on reproduction

Our results provide relatively little evidence for maternal oxidative stress acting as a constraint on reproduction in Columbian ground squirrels. Indeed, not only did breeding females (whether successful or not) have marginally higher oxidative stress levels than non-breeding females prior to reproduction (when emerging from hibernation), higher oxidative stress levels prior to reproduction in breeding females were actually associated with larger litter sizes produced at birth (contrary to our prediction). The relatively few studies having tested for the relations between pre-breeding oxidative stress levels and reproductive output have suggested that oxidative stress may act as a constraint on reproduction (see Metcalfe & Alonso-Alvarez 2010; Stier *et al.* 2012, for reviews). For example, in laboratory mice, oxidative damage prior to reproduction was negatively related to litter size at birth, but not at weaning (Stier *et al.* 2012). In wild Alpine swifts, *Apus melba*, female resistance to oxidative stress is positively related to clutch size and hatching success, whereas hatching failure is

associated with low resistance to oxidative stress (Bize *et al.* 2008), though in this case maternal oxidative status was measured after egg-laying. In canaries, *Serinus canaria*, experimentally reducing maternal antioxidant defences prior to reproduction delayed the onset of egg-laying and had a negative effect on clutch size (Costantini *et al.* 2016).

Our observation that breeders (successful or not) experienced slightly higher oxidative damage (ROM levels) than non-breeders at emergence from hibernation could be due to differences in metabolic torpor and arousal patterns. Whereas we consider emergence from hibernation as the moment an individual is first seen above ground, breeding individuals in hibernating species actually become euthermic below ground earlier, preparing the reproductive system for the upcoming breeding season (gonadal growth and maturation) (Sheriff *et al.* 2011; Siutz, Franceschini & Millesi 2016). Early arousal from hibernation and hypoxia at a time where ambient temperatures are still low is likely to increase the potential for oxidative stress associated with tissue re-oxygenation and thermogenesis (Orr *et al.* 2009; Metcalfe & Alonso-Alvarez 2010).

In Columbian ground squirrels, most non-breeding females are young animals, which typically emerge later from hibernation (Young 1990; Neuhaus 2000a). Indeed in our study, non-breeding animals were on average 3.2 years younger than breeders (1.2 ± (SD) 0.5; range: 1-3 *vs.* 4.4 ± 1.8; range: 2-10 years old; $F_{1,74}$ = 96.83, $P < 0.001$), and emerged on average 7.1 days later (108.3 ± 4.9; range: 101-118 *vs.* 115.4 ± 6.5; range: 105-129 Julian days; $F_{1,74}$ = 29.85, $P < 0.001$). Age *per se* might be an important factor influencing oxidative stress levels, since young growing individuals might be in a different physiological state. Yet, in our models, age was not particularly associated with ROM and OXY levels.

Within breeding females, our result that higher oxidative stress levels prior to breeding are associated with larger litter sizes at birth is contrary to predictions from the oxidative constraint hypothesis. One explanation is that those females producing larger litters at birth are generally higher quality females that invest more in a suite of correlated phenotypic traits (*e.g.* metabolic rate,

territoriality, boldness, dominance, home range size) (Careau *et al.* 2008; Biro & Stamps 2010; Careau & Garland 2012), such that the net fitness gains in terms of offspring survival after birth offset the potential oxidative stress seen as a constraint on reproduction.

Oxidative stress as a cost of reproduction

Because of increased metabolic demands during reproduction (e.g. Speakman 2008), it has been suggested that oxidative stress may be a proximate cost of reproduction, arising as a by-product of natural cell metabolism. Whereas several studies have highlighted positive links between some form of reproductive investment and oxidative stress (Reichert *et al.* 2014; Costantini *et al.* 2014b), others have failed to detect such associations (Garratt *et al.* 2011, 2013). More importantly, recent criticism has questioned the idea that oxidative stress may actually be a proximate cost of reproduction (Speakman & Garratt 2014), based on the growing evidence that increased metabolism does not necessarily scale 1:1 with increased oxidative stress (Criscuolo *et al.* 2005; Speakman & Selman 2011).

It has been suggested that the comparison of breeding vs. non-breeding animals may not be a valid one, since breeding animals control their own level of investment into reproduction thereby possibly avoiding the costs of oxidative stress (Metcalfe & Monaghan 2013). However, as pointed out by Speakman and Garratt (2014), regardless of their level of investment into reproduction, breeding animals cannot avoid the increase in metabolic rate compared to non-breeding animals. Thus, longitudinal approaches where breeding and non-breeding individuals are monitored repeatedly before and over the course of a breeding season are likely to provide powerful assessments of the oxidative cost of reproduction theory (Alonso-Alvarez *et al.* 2004; Stier *et al.* 2012).

Our longitudinal exploration of the links between female reproductive investment (total litter mass weaned) and oxidative stress levels at weaning provided little evidence of substantial oxidative stress resulting from reproduction in Columbian ground squirrels. Those results are in line with

previous studies in the same species failing to detect survival costs to reproduction either via experimental manipulations of female reproductive effort (Hare & Murie 1992; Skibiel & Hood 2013; but see Neuhaus 2000b) or long-term life history approaches (Murie & Dobson 1987; Rubach *et al.* 2016). As mentioned above, non-reproducing females in our study were typically younger females for which energy is primarily allocated to somatic demands (Broussard *et al.* 2003). Taken together, our results suggest that when compared to non-breeders, breeding females seem to suffer little from increased oxidative stress during reproduction.

Oxidative shielding in breeding females

The oxidative shielding hypothesis was first advanced to reconcile the conundrum that levels of oxidative damage were sometimes observed to actually be lower in breeders compared to non-breeders (Blount *et al.* 2016). The rationale behind this theory is that oxidative stress may be viewed as a type of negative maternal effect having detrimental trans-generational impacts on offspring, e.g. via impaired placental function, and decreased maternal milk yield (Gupta *et al.* 2007; Al-Gubory, Fowler & Garrel 2010; Mutinati *et al.* 2013; Herrera *et al.* 2014). Thus, breeding mothers should be selected to pre-emptively decrease their levels of oxidative stress in order to shield their developing offspring from detrimental effects (Blount *et al.* 2016; Vitikainen *et al.* 2016; see Costantini, Casasole & Eens 2014a for some interesting results in birds).

Our results provide mixed-evidence for the oxidative shielding hypothesis. Maternal oxidative stress levels during lactation were not related to offspring survival or mass growth over this period. Further, oxidative shielding before birth (i.e. during pregnancy) did not seem to occur, although this is a sensitive period for developing embryos and miscarriages associated with high levels of oxidized lipids in placenta are known to occur (*e.g.* in humans; Hempstock *et al.* 2003). How maternal plasma oxidative stress measurements correlate with placental levels in ground squirrels remains to be determined. However, one possibility is that rather than relying mostly on maternal

transfer of antioxidants (Debier & Larondelle 2005), ground squirrel embryos are able to produce sufficient self-protection via early-stage expression of anti-oxidant enzymes (Frank & Groseclose 1984; Guerin 2001). From a life history perspective, ground squirrels are hibernating rodents that have to cope repeatedly with oxidative bursts during annual ischemia-reperfusion following hibernation (Ma *et al.* 2005), including in their first year of life. Those are similar to the challenge faced by the new-born organism during the post-hypoxic re-oxygenation period of tissues (Saugstad 2005; Chouchani *et al.* 2016), and it is likely that in the ground squirrels efficient antioxidant mechanisms are set very early in life in the developing embryo.

Despite a lack of relationship between maternal oxidative stress levels and offspring survival and mass growth, our results clearly show contrasting dynamics between breeding and non-breeding females in terms of the dynamics of oxidative stress. Whereas non-breeding females generally exhibited no change in oxidative stress (or slight increase in ROM and concurrent decrease in OXY plasma levels) over the course of the breeding season, breeding females exhibited a clear decrease in oxidative stress levels from birth to weaning that increased afterwards. Failed breeders (*viz.* females that gave birth but failed to wean) exhibited remarkably similar dynamics to breeding females, except for the fact that oxidative stress levels did not increase as much from mid-lactation to weaning. Those results support the idea that breeding females pre-emptively mitigate the oxidative increase associated with reproduction by mounting higher antioxidant capacity prior to lactation (*i.e.* the peak metabolic period for breeding mammals) (Blount *et al.* 2016). Such mitigation might be directed to females themselves, rather than for shielding offspring.

Alternatively, in our study, maternal oxidative stress on offspring may have had more subtle effects than affecting survival directly. For instance, one interesting idea is that maternal oxidative stress may have affected offspring biological ageing during early development by acting on telomere loss, since telomere sequences are highly susceptible to oxidative stress (von Zglinicki 2002) and mostly lost during early growth (Salomons *et al.* 2009; Heidinger *et al.* 2012; Boonekamp *et al.* 2014).

In addition, we used global (non-specific) indices of oxidative damage and defence, i.e. ROM and OXY tests (Costantini 2011, 2016). However, multiplying markers of oxidative damage (e.g. lipid peroxidation, protein carbonyls) or defences (e.g. catalase, superoxydismutase, glutathione peroxydase) in different tissues (brown adipose tissue, liver, etc. – with the great disadvantage of being terminal measures) is likely to provide a more comprehensive assessment of female oxidative status (Selman *et al.* 2012).

Notably, measures focusing on oxidative damage in lipids (e.g. MDA) and endogenous enzymatic defences may be particularly relevant for this long-term hibernating species, for which body fat is crucial to overwinter survival. Finally, one caveat to our results is the frequency at which we sampled blood. Indeed, our sample at mid-lactation may have been too late to actually measure the total oxidative shield set-up by breeding females. Given that oxidative stress is expected to increase with lactating effort (Blount *et al.* 2016), measures of oxidative damage and antioxidant defences earlier during lactation (shortly after birth) may have revealed even lower levels of oxidative stress in breeding females.

Conclusions

Taken together, our results provide little evidence that oxidative stress acts either as a constraint on, or a cost of, reproduction in Columbian ground squirrels. Breeding females appeared to mitigate the expected increase in oxidative stress during lactation by mounting higher antioxidant defences prior to lactation, but with no consequence on offspring development or survival as predicted by the oxidative shielding hypothesis. Further studies are needed to determine whether this increase may occur to buffer potential long-term oxidative costs of repeated reproductions in adults and/or precocial ageing in offspring.

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Data accessibility

The data related to this manuscript is available from the Dryad Digital Repository. doi:10.5061/dryad.s8045 (Viblanc et al, 2017)

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Figure captions

Fig 1. Schematic representation of the oxidative constraint, oxidative cost and oxidative shielding hypotheses.

Fig 2. Marginal means for plasma (A) reactive oxygen metabolite (ROM) levels and (B) antioxidant capacity (OXY) for female Columbian ground squirrels over the course of a breeding season. For clarity, only marginal means are presented in the figure. Full statistical details including SEs, CIs and Tukey's HSD tests can be found in the tables (see also ESM). Marginal means were estimated from separate LMMs with ROM or OXY as the dependent variables, breeding status (breeder, failed-breeder and non-breeder), stage (emergence, birth, lactation and weaning) and breeding status x stage specified as independent variables. Female age and mass were included as covariates in the analyses to test for potential effects on the oxidative stress metrics. Female ID was specified as a random factor. Models were run on n = 255 observations for N = 76 females for ROM and n = 254 observations for N = 75 females for OXY.

Fig 3. Relationships between (A) reactive oxygen metabolite (ROM) levels, (B) antioxidant capacity (OXY), and (C) female mass at emergence from hibernation (pre-breeding) and total litter mass-

produced at birth in Columbian ground squirrels. Effect leverage plots show the unique effect of given terms in the model. The dashed horizontal line shows the constrained model without the term of interest. The model prediction (full line) and 95% CI (dashed lines) are given. The linear model included litter mass at birth as the dependent variable, and ROM and OXY levels as the independent variables. Female mass at emergence and age were added as covariates in the analyses to account for age/mass potential effects on reproduction.

Tables

Table 1. Observed and model marginal means (± SE) for plasma oxidative damage (ROM; in mg H_2O_2.dl^{-1}) in female Columbian ground squirrels (*Urocitellus columbianus*) over the course of a reproductive season. Sample sizes for observed means vary due to varying success in data collection (capture or blood sampling success) and laboratory analyses. Marginal means were estimated from a LMM with ROM as the dependent variable, breeding status (breeder, failed-breeder and non-breeder), stage (emergence, birth, lactation and weaning) and *breeding status x stage* specified as independent variables. Female age and mass were included as covariates in the analyses to test for potential effects on the ROM levels. Female ID was specified as a random factor. Overall model was run on n = 255 observations for N = 76 females. Marginal means with different letters are significantly different for $P < 0.05$ (Tukey HSD). Full model statistics are given as Electronic Supplemental Materials.

Status	Stage	Mean ± SE	N	Marginal mean ± SE	95% CI	Tukey HSD
Successful breeders	Emergence	228.77 ± 8.36	34	227.88 ± 10.42	207.33 – 248.43	a
	Birth	218.69 ± 9.41	35	234.73 ± 11.86	211.32 – 258.14	a
	Lactation	141.13 ± 8.67	29	153.38 ± 11.06	131.58 – 175.18	b,d
	Weaning	189.82 ± 9.83	37	200.86 ± 10.56	180.02 – 221.70	a,c
Failed breeders	Emergence	232.14 ± 16.29	7	225.17 ± 20.19	185.37 – 264.97	a,b
	Birth	198.32 ± 14.51	6	205.36 ± 21.79	162.44 – 248.29	a,b,c,d
	Lactation	144.57 ± 14.39	4	149.92 ± 26.38	97.96 – 201.89	a,b,c,d
	Weaning	166.50 ± 10.52	4	177.37 ± 26.68	124.81 – 229.92	a,b,c,d
Nonbreeders	Emergence	202.09 ± 9.17	31	176.80 ± 14.67	147.82 – 205.79	a,b,c,d

	Birth	153.57 ± 8.27	26	142.33 ± 12.58	117.51 – 167.15	c,d
	Lactation	175.55 ± 12.36	23	172.07 ± 12.51	147.39 – 196.74	a,b,c,d
	Weaning	204.12 ± 15.52	20	206.52 ± 13.26	180.37 – 232.67	a,b

Table 2. Observed and model marginal means (± SE) for plasma antioxidant capacity (OXY in µmolHCL.mL^{-1}) in female Columbian ground squirrels (*Urocitellus columbianus*) over the course of a reproductive season. Sample sizes for observed means vary due to varying success in data collection (capture or blood sampling success) and laboratory analyses. Marginal means were estimated from a LMM with OXY as the dependent variable, breeding status (breeder, failed-breeder and non-breeder), stage (emergence, birth, lactation and weaning) and *breeding status x stage* specified as independent variables. Female age and mass were included as covariates in the analyses to test for effects on OXY levels. Female ID was specified as a random factor. Overall model was run on n = 254 observations for N = 76 females. Marginal means with different letters are significantly different for $P < 0.05$ (Tukey HSD). Full model statistics are given as Electronic Supplemental Materials.

Status	Stage	Mean ± SE	N	Marginal mean ± SE	95% CI	Tukey HSD
Successful breeders	Emergence	353.26 ± 10.12	34	356.22 ± 10.19	336.11 – 376.32	a,b,c,d
	Birth	330.08 ± 8.27	35	313.08 ± 11.64	290.10 – 336.05	b,d
	Lactation	376.99 ± 8.82	29	365.75 ± 10.78	344.49 – 387.02	a
	Weaning	365.93 ± 8.98	37	354.64 ± 10.36	334.18 – 375.10	a,c
Failed breeders	Emergence	352.75 ± 12.97	7	360.68 ± 19.56	322.11 – 399.25	a,b,c,d
	Birth	291.69 ± 19.04	6	283.60 ± 21.03	242.14 – 325.05	c,d
	Lactation	405.25 ± 18.91	4	396.88 ± 25.31	347.01 – 446.75	a,b
	Weaning	412.13 ± 22.75	4	397.23 ± 25.61	346.77 – 447.70	a,b
Nonbreeders	Emergence	343.84 ± 6.84	31	370.51 ± 14.47	341.93 – 399.08	a,b,c,d
	Birth	346.78 ± 10.85	26	359.30 ± 12.34	334.95 – 383.65	a,b,c,d
	Lactation	357.37 ± 12.86	22	361.99 ± 12.43	337.47 – 386.52	a,b,c,d
	Weaning	359.48 ± 11.48	20	357.40 ± 12.94	331.87 – 382.92	a,b,c,d

Table 3. Linear model estimates (± SE) for independent variables tested in the oxidative constraint hypothesis (see Methods). Variation in sample size is due to varying success in data collection (capture or blood sampling success) and laboratory analyses. Sample sizes are given as number of mothers (N) for which we had information on all parameters.

Hypothesis	Dependent	Independent (standardized)	*β coefficient* ± SE	*t* value	*P* >|*t*|	*N*
(1) Oxidative constraint	Litter mass at birth	Intercept	31.70 ± 1.96	16.16	<.0001*	40
		ROM	3.16 ± 1.36	2.32	0.026*	
		OXY	-2.28 ± 1.14	-2.00	0.053	
		Mass	8.57 ± 2.08	4.11	0.000*	
		Age	1.13 ± 1.46	0.78	0.442	
	Litter mass at weaning	Intercept	309.31 ± 25.61	12.08	<.0001*	34
		ROM	12.27 ± 15.60	0.79	0.438	
		OXY	-10.61 ± 12.39	-0.86	0.399	
		Mass	-0.49 ± 24.37	-0.02	0.984	
		Age	-5.12 ± 17.11	-0.30	0.767	

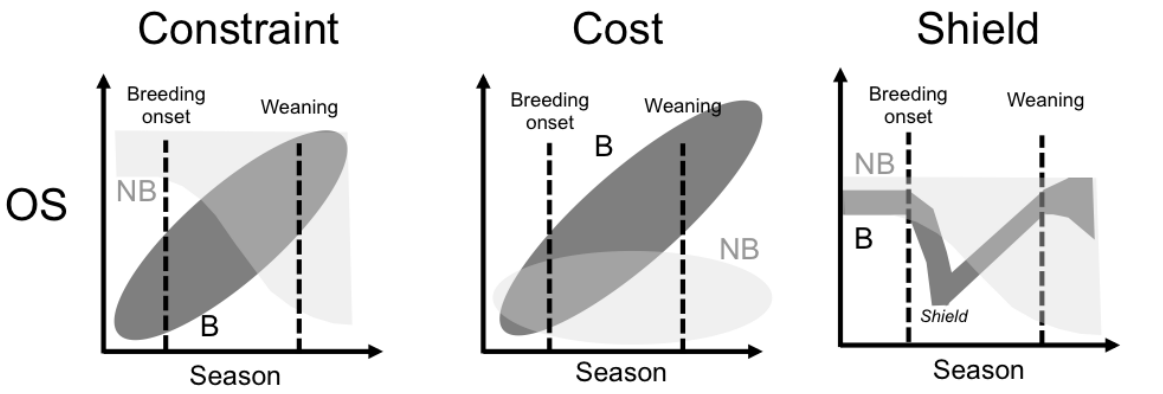
Table 4. Linear model estimates (± SE) for independent variables tested in the oxidative cost hypothesis (see Methods). Variation in sample size is due to varying success in data collection (capture or blood sampling success) and laboratory analyses. Sample sizes are given as number of mothers (N) for which we had information on all parameters.

Hypothesis	Dependent	Independent (standardized)	β ± SE	*t* value	*P* >|*t*|	*N*
(2) Oxidative cost	ROM	Intercept	189.27 ± 18.47	10.25	<.0001*	37
		Litter mass	11.13 ± 10.17	1.09	0.282	
		Mass	7.65 ± 16.70	0.46	0.650	
		Age	-6.05 ± 12.40	-0.49	0.629	
	OXY	Intercept	355.36 ± 17.13	20.75	<.0001*	37
		Litter mass	-1.13 ± 9.43	-0.12	0.906	
		Mass	14.14 ± 15.48	0.91	0.368	
		Age	1.10 ± 11.50	0.10	0.924	
	Δ ROM	Intercept	-36.53 ± 23.44	-1.56	0.130	34
		Litter mass	0.69 ± 13.17	0.05	0.958	
		Mass	-1.87 ± 22.28	-0.08	0.934	
		Age	-2.95 ± 15.78	-0.19	0.853	
	Δ OXY	Intercept	8.63 ± 21.32	0.40	0.689	34
		Litter mass	9.94 ± 11.98	0.83	0.413	
		Mass	2.37 ± 20.27	0.12	0.908	
		Age	-0.78 ± 14.36	-0.05	0.957	

Table 5. Generalized Linear Mixed Model estimates (± SE) for independent variables tested in the oxidative shielding hypothesis (see Methods). Variation in sample size is due to varying success in data collection (capture or blood sampling success) and laboratory analyses. Sample sizes are given as number of offspring (n) and number of mothers (N) for which we had information on all parameters.

Hypothesis	Dependent	Period	Independent (standardized)	β ± SE	*z (t)* value	*P* >| *z (t)*|	*n(N)*
(3) Oxidative shielding	**GLMM (binomial)** **Offspring survival (0/1)**	Oxidative shield (lactation-birth)	Intercept	7.78 ± 2.72	2.86	0.004*	91 (30)
			Δ ROM	-0.07 ± 1.29	-0.06	0.955	
			Δ OXY	-1.11 ± 2.34	-0.48	0.633	
			Mass	0.33 ± 1.44	0.23	0.818	
			Age	0.95 ± 1.49	0.64	0.524	
		Oxidative shield (weaning-lactation)	Intercept	8.38 ± 2.24	3.74	<0.001*	102 (33)
			Δ ROM	-0.06 ± 1.42	-0.04	0.968	
			Δ OXY	0.98 ± 1.92	0.51	0.610	
			Mass	0.17 ± 1.28	0.13	0.897	
			Age	0.92 ± 1.66	0.55	0.579	
	LMM **Offspring mass growth**	Oxidative shield (lactation-birth)	Intercept	95.79 ± 4.16	23.02	<0.001*	73 (27)
			Δ ROM	2.68 ± 3.92	0.68	0.503	
			Δ OXY	-4.75 ± 3.98	-1.19	0.246	
			Mass	-1.64 ± 4.07	-0.40	0.691	
			Age	5.42 ± 4.30	1.26	0.222	
			Litter size at birth	-20.66 ± 4.95	-4.17	<0.001*	
		Oxidative shield (weaning-lactation)	Intercept	96.63 ± 4.28	22.57	<0.001*	76 (28)
			Δ ROM	-2.49 ± 4.24	-0.59	0.562	
			Δ OXY	0.72 ± 4.87	0.15	0.883	
			Mass	-3.22 ± 4.03	-0.80	0.433	
			Age	5.07 ± 4.87	1.04	0.309	
			Litter size at birth	-20.38 ± 5.03	-4.05	<0.001*	

Figures



Oxidative constraint hypothesis: Predicts that females with high levels of oxidative stress (OS) prior to breeding should be constrained from investing into reproduction. Non-breeding females (NB, light shading) are likely those starting the season with high OS, which may remain high (chronic stress) or subsequently decrease allowing reproduction in subsequent years. Breeding females (B, dark shading) increase their oxidative stress levels throughout reproduction. This relies on the assumption that increased metabolic demands are associated with increased production of reactive oxygen species and/or decreased investment in antioxidant defenses. For simplicity, OS is represented as increasing linearly with reproductive investment. However, this may not necessarily the case.

Cost of reproduction hypothesis: Predicts an increase in OS with increasing maternal reproductive investment within a breeding season. Breeding females (B, dark shading) are expected to display higher levels of OS at the end of the breeding season than non-breeders (NB, light shading). This relies on the assumption that increased metabolic demands are associated with increased production of reactive oxygen species and/or decreased investment in antioxidant defenses. For simplicity, OS is represented as increasing linearly with reproductive investment. However, this may not necessarily the case.

Oxidative shielding hypothesis: Predicts that breeding females (B, dark shading) should pre-emptively mitigate the oxidative cost of reproduction by mounting an antioxidant shield prior to the costly period of reproduction when compared to non-breeders (NB, light shading). Further predicts that mothers should shield their offspring from damaging effects of maternal oxidative stress by mountain an antioxidant shield around the sensitive periods of offspring development (in utero and during lactation). For simplicity, OS is represented as increasing linearly with reproductive investment. However, this may not necessarily the case.

Fig. 1

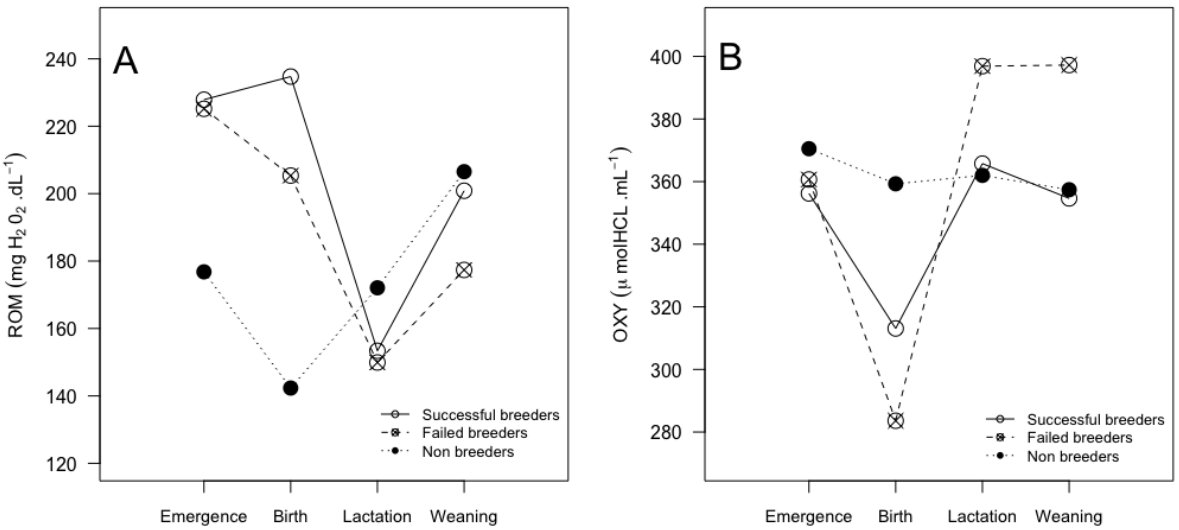


Fig. 2

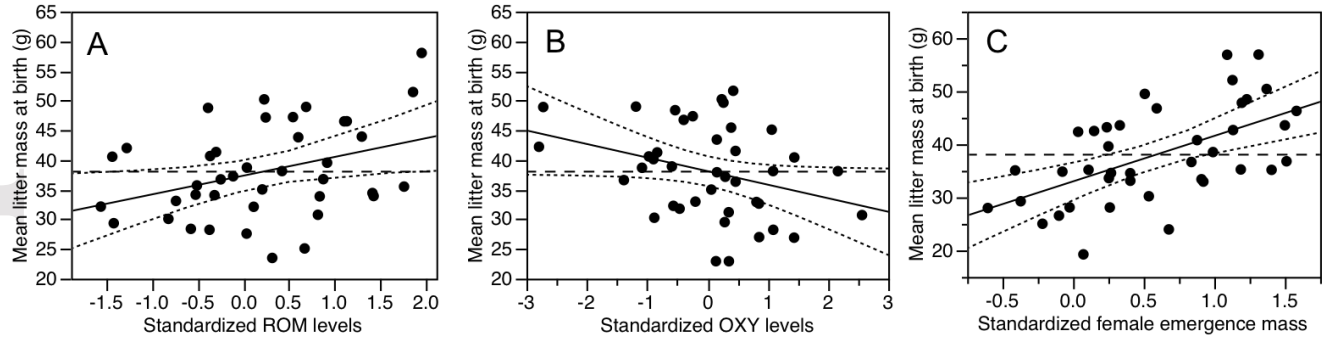


Fig. 3