



OXIDATIVE DAMAGE INCREASES WITH REPRODUCTIVE ENERGY EXPENDITURE AND IS REDUCED BY FOOD-SUPPLEMENTATION

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A central principle in life-history theory is that reproductive effort negatively affects survival. Costs of reproduction are thought to be physiologically based, but the underlying mechanisms remain poorly understood. Using female North American red squirrels (*Tamiasciurus hudsonicus*), we test the hypothesis that energetic investment in reproduction overwhelms investment in antioxidant protection, leading to oxidative damage. In support of this hypothesis we found that the highest levels of plasma protein oxidative damage in squirrels occurred during the energetically demanding period of lactation. Moreover, plasma protein oxidative damage was also elevated in squirrels that expended the most energy and had the lowest antioxidant protection. Finally, we found that squirrels that were food-supplemented during lactation and winter had increased antioxidant protection and reduced plasma protein oxidative damage providing the first experimental evidence in the wild that access to abundant resources can reduce this physiological cost.

KEY WORDS: Antioxidant protection, daily energy expenditure, doubly-labeled water, energetics, food-supplementation, life-history theory.

Life-history theory assumes that individuals maximize fitness by balancing the allocation of limited energy or resources to the competing demands of growth, reproduction, and somatic maintenance (Roff 1992; Stearns 1992). One of the best-studied trade-offs is how allocation toward reproduction compromises survival

(Reznick 1992; Daan et al. 1996). In line with the theory that costs of reproduction are mediated by allocation trade-offs, theoreticians have hypothesized (van Noordwijk and de Jong 1986; McNamara and Houston 1996; Reznick et al. 2000) and empiricists have demonstrated (Tuomi et al. 1983; Reznick et al. 2000;

Bonnet et al. 2002) that the negative effects of reproduction on survival are reduced when animals have access to abundant energy/resources. The reduction of survival costs of reproduction in these circumstances is thought to result because animals with abundant resources can allocate more energy/resources to somatic maintenance (Reznick et al. 2000). However, the physiological mechanisms that underlie how costs of reproduction are incurred, or can be reduced, remain a mystery (Zera and Harshman 2001; Speakman 2008).

Much recent interest has focused on oxidative damage as an important physiological mechanism responsible for costs of reproductive effort in free-ranging animals (Costantini 2008; Dowling and Simmons 2009; Monaghan et al. 2009; Selman et al. 2012). Lactation in mammals and chick rearing in birds are associated with high levels of daily energy expenditure (DEE) that may approach physiological limits (reviewed in McNab 2002). Experimental increases in the number of offspring supported leads to increases in parental DEE in free-ranging birds (e.g., Daan et al. 1996) and mammals (Humphries and Boutin 2000) suggesting a mechanistic link between DEE and reproductive effort. Previous research examining reproductive effort has used a variety of indicator variables (e.g., litter size, offspring mass and growth; e.g., Clutton-Brock 1984), but a more integrative measure of reproductive effort, such as DEE, might be more reflective of parental care costs than traditional indicators. Reactive oxygen species (ROS) are primarily by-products of aerobic energy expenditure that can cause oxidative damage to proteins, lipids, and nucleic acids (Beckman and Ames 1998; Finkel and Holbrook 2000). A suite of enzymatic and nonenzymatic antioxidants function to neutralize ROS before they cause damage (Beckman and Ames 1998); however, if there is an imbalance between the production of ROS from energy expenditure on one hand, and antioxidant protection and repair mechanisms on the other, oxidative damage occurs (Selman et al. 2012).

Comprehensive examinations of the oxidative damage hypothesis in free-living animals are currently required. Most tests of the oxidative damage hypothesis on wild-captured animals have been conducted in captivity (Alonso-Alvarez et al. 2004; Wiersma et al. 2004; Alonso-Alvarez et al. 2006; Bertrand et al. 2006; Selman et al. 2008a,b; Garratt et al. 2011; Garratt et al. 2012; Oldakowski et al. 2012), with studies on free-ranging animals appearing only more recently (Bize et al. 2008; Nussey et al. 2009; Bergeron et al. 2011; Christe et al. 2011; Markó et al. 2011; Losdat et al. 2011; Heiss and Schoech 2012). These studies have produced equivocal results potentially because they have not obtained measures of DEE, antioxidant protection, and oxidative damage within the same individual (Selman et al. 2012). Moreover, experimental studies examining the effect of energy/resource availability on oxidative damage are rare in wild animals (Losdat et al. 2011; for captive studies see Bertrand et al. 2006; Costantini

2010). Experimentally increased energy/resource availability is expected to reduce oxidative damage if the balance between the production of ROS from energy expenditure on one hand, and antioxidant protection and repair mechanisms on the other, is in favor of the latter. However, how this balance may be resolved in response to increased energy/resource availability is complicated for two reasons. First, the direction of the relationship between energy/resource availability and DEE is currently unresolved (Tinbergen and Verhulst 2000; Thomas et al. 2001; Speakman et al. 2003; Welcker et al. 2010); thus, it is unknown how energy/resource availability influences ROS production. Second, it is generally unknown whether levels of antioxidant protection are influenced by energy/resource availability in the wild (Losdat et al. 2011).

Here, we provide a comprehensive test of the oxidative damage hypothesis in a free-ranging population of North American red squirrels (*Tamiasciurus hudsonicus*; hereafter red squirrels). We quantified the DEE, antioxidant protection, and oxidative damage of female squirrels during three seasonal and reproductive stages: (1) lactation, (2) nonbreeding during the summer, and (3) winter. To investigate the role of energy/resource availability in mediating oxidative damage, squirrels were examined on study areas where individuals only had access to natural food, as well as on experimental food-supplementation study areas where individuals were provided with supplemental peanut butter. To our knowledge, our study is the first in the wild to combine measures of DEE, antioxidant protection, and oxidative damage into a single study.

Materials and Methods

STUDY REGION AND SQUIRREL LIFE-STAGES

Female squirrels were studied during the spring/summer of 2006 and the winter of 2006 to 2007 in the boreal forest of Yukon, Canada (61°N, 138°W). The average temperature in the coldest and warmest months ranges between -22°C and 12.8°C (Environment Canada: 1967–2006; Burwash, Yukon; ~ 70 km from study area). Squirrels were examined on six study areas. The minimum and maximum distances between study areas were approximately 200 m and 8 km, respectively. Study areas were dominated by white spruce (*Picea glauca*) with an understory of willow (*Salix* spp.).

Following long-term data collection protocols, we tracked the reproductive output of all adult females living on our study areas during the spring/summer (for further details, see Boutin et al. 2006; McAdam et al. 2007). All adult females living on our study areas were ear-tagged and pregnancy and parturition was tracked by palpating their abdomens and attempting to express milk from their nipples following repeated live-trapping. When a female was determined to have given birth, her pups were

Table 1. Sample sizes of oxidative damage and antioxidant protection samples collected from female squirrels within the three life-stages (lactation, nonbreeding, and winter), on natural-food and food-supplementation study areas. Females within the lactation and nonbreeding life-stages living on the natural-food study areas either experienced high or low natural-food levels. A proportion of the squirrels with oxidative damage/antioxidant protection samples were associated with and without paired DEE estimates.

	Lactation			Nonbreeding		Winter	
	Natural-Food		Food-Supplementation	Natural-Food		Natural-Food	Food-Supplementation
	High	Low		High	Low		
With DEE measurement	7	5	4	7	2	11	2
Without DEE measurement	1	2	1	0	1	6	5

temporarily removed from the nest to be counted and weighed. The parturition date of each female was estimated based on her live-trapping records and the masses of her pups when they were removed from the nest. Pups were removed from the nest a second time at approximately 25 days postpartum to be ear-tagged. We ascertained that some females had lost their litters during lactation when we could no longer express milk from their nipples prior to the estimated date of weaning (approximately 70 days postpartum; McAdam et al. 2007; S. Boutin, A. G. McAdam, M. M. Humphries, unpubl. data).

Oxidative damage and antioxidant protection were sampled in female squirrels during three seasonal and reproductive stages (hereafter, life-stages): (1) lactation, (2) nonbreeding during summer (henceforth, nonbreeding), and (3) winter. Lactating females were sampled between 2 May 2006 and 30 June 2006 during a consistent period of lactation (90% of females were sampled when they were 35–42 days postpartum; range = 35 to 49). Energetic demands are likely high during this period of lactation because this is just prior to when pups emerge from the nest at approximately 42 days postpartum and start transitioning to natural food items (pups are weaned at approximately 70 days postpartum; McAdam et al. 2007; S. Boutin, A. G. McAdam, M. M. Humphries, unpubl. data). All lactating females were attempting to raise their first litter of the year. Nonbreeding females were sampled over a calendar period that approximately overlapped when lactating females were sampled (14 June 2006 to 4 July 2006). Two types of females were included in the nonbreeding life-stage: females that lost their litter at least 29 days previously ($n = 3$), and females that did not attempt to breed in 2006 ($n = 7$). There was no significant difference in either the DEE ($P = 0.32$), oxidative damage ($P = 0.13$), or antioxidant protection ($P = 0.43$) between the two types of nonbreeding females. Females during the winter were sampled between 17 January 2007 and 1 April 2007. None of the females in winter life-stage that we sampled were pregnant. We knew this based on four types of information: (1) autopsy after sacrifice ($n = 11$; S. B. Woods, unpubl. data); (2) known parturition dates ($n = 8$); (3) trapping records when females were known not to be preg-

nant ($n = 4$); and (4) the female disappeared from the study area, and thus was presumed dead (Descamps et al. 2009) prior to becoming being pregnant ($n = 1$). The females in this study were at least 1-year old (S. Boutin, A. G. McAdam, M. M. Humphries, unpubl. data); however, we did not investigate the effect of age in this study because we only knew the birth year of 50% of the females.

NATURAL-FOOD AND FOOD-SUPPLEMENTATION STUDY AREAS

We examined squirrels on four study areas where individuals only had access to natural food (approximately 40 ha each). Two of the natural-food study areas have been the subject of consistent long-term research on completely enumerated ear-tagged squirrels since 1987 (Boutin et al. 2006; McAdam et al. 2007). Red squirrels at this site hoard white spruce cones while they are mature during the autumn (Fletcher et al. 2010; Archibald et al. 2012) to help meet the energetic demands of winter and reproduction the following spring (Boutin et al. 2006). Past research at this site demonstrates that the production of white spruce cones in the previous year is the key factor shaping the ecology of squirrels. Large cone crops in the previous year are correlated with increased juvenile survival (Humphries and Boutin 2000; McAdam and Boutin 2003), increased adult overwinter survival (LaMontagne 2007), earlier parturition dates, and faster pup growth rates (Boutin et al. 2006). Lactating and nonbreeding squirrels on three of the natural-food study areas experienced high levels of spruce cones, whereas lactating and nonbreeding squirrels on the fourth natural-food study area experienced low levels of spruce cones (see Table 1). Spruce cone levels were low on the natural-food study area during winter. For further details on the availability of cones in this study and the effect of spruce cone abundance on squirrel ecology, see Supplementary Material S1.

Squirrels were also examined on two food-supplementation study areas where every individual was provided with supplemental natural peanut butter. We are confident that target squirrels from these food-supplemented populations had additional

food resources (i.e. peanut butter) because: (1) peanut butter was placed in closed plastic feeders near the center of each squirrel's territory and the feeders were actively defended, (2) squirrels were frequently observed inside the feeders, and (3) the peanut butter was depleted. Continued ownership of the peanut butter feeders by the target squirrels was confirmed with live-trapping. It is possible that some peanut butter was eaten by nontarget individuals or species but based on our field observations, it seems likely that the vast majority of the peanut butter was eaten by the intended recipients. The food-supplementation study area where squirrels were sampled during lactation (approximately 45 ha) was different from the food-supplementation study area where squirrels were sampled during winter (approximately 3.2 ha). On the food-supplementation study area where lactating squirrels were sampled, approximately 1 kg of natural peanut butter was provided to every resident squirrel (all squirrels on the study area were marked with ear-tags) every 6 weeks starting in October and continuing until the pups were weaned the following year (May to June). One kilogram of peanut butter has the approximate nutritional composition of the seed contained in 5000 spruce cones (Health Canada 1990; LaMontagne 2007), which is sufficient to meet the resting metabolic rate requirements of female red squirrels in this population for approximately 70 days (M. M. Humphries, unpubl. data). This food-supplementation started in 2004, and the reproductive output of females on this study area was also tracked following the same long-term data collection protocols as on the natural-food study areas. The squirrels that were food-supplemented during winter were provided with 1 kg of peanut butter at least every 6 weeks (Woods 2009). This study area was only provided with peanut butter during the winter of 2007 for winter energetics research (Woods 2009). Study animals on the winter food-supplementation study area were sacrificed at the end of winter (Woods 2009); therefore, it was not possible to conduct this research on the study area where lactating squirrels were sampled because destructive sampling would have interfered with the long-term data collection protocols.

We chose to spatially separate the natural-food and food-supplementation study areas instead of interspersing the food-supplemented squirrels amongst individuals that were not food-supplemented (i.e., natural-food squirrels). This design presumably minimized peanut butter pilfering and intruder pressure for ownership of the peanut butter feeders from the squirrels that were not food-supplemented. Moreover, we do not think that the food-supplementation results were confounded by location because of the similarity of squirrel habitat and densities on the study areas (Boonstra et al. 2001; S. Boutin and C. J. Krebs, unpubl. data).

Average parturition dates for lactating females in this study were earliest on the high natural-food study area (9 April) and were later on the low natural-food study area (4 May) and the

food-supplementation study area (7 May). We did not sample all females attempting to raise their first litter of the year on these study areas; however, the lactating females we sampled on the high natural, low natural, and food-supplementation study areas ranged from having early to late parturition dates with respect to the other squirrels on their respective study areas (percentile range of parturition dates from sampled females: high natural = 31 to 100, low natural = 5 to 93, food-supplementation = 15 to 83). Parturition dates on the food-supplementation study area are generally earlier than on control study areas, which is consistent with the intent of the food-supplement to mimic naturally high levels of spruce cones (Boutin et al. 2006; S. Boutin, A. G. McAdam, M. M. Humphries, unpubl. data). However, parturition dates on the food-supplementation study area were later than on high natural-food study areas in this study presumably because in the previous year (2005) there was a large cone crop on the high natural-food study areas, whereas there was a small cone crop on the food supplementation study area (S. Boutin, A. G. McAdam, M. M. Humphries, unpubl. data; see Boutin et al. 2006 for the correlation between earlier parturition dates and abundant cone production in the previous year).

DOUBLY-LABELED WATER (DLW) TECHNIQUE

We quantified DEE using the DLW technique (Nagy 1983; Speakman 1997) using the methodology presented in Fletcher et al. (2012). Eleven lactation, seven nonbreeding, and nine female winter DEE values were initially presented in Fletcher et al. (2012). Briefly, CO₂ production was calculated based on the differential washout of the hydrogen (²H) and oxygen (¹⁸O) isotopes in the DLW over a period of 72 to 120 h, and this value was converted to an estimate of DEE in kJ/day. For individuals with DEE measures, the average body mass of squirrels at the initial and final blood samples of the DLW technique was used in all analyses. See Supplementary Material S2 for further details on DLW methodology.

OXIDATIVE DAMAGE AND ANTIOXIDANT PROTECTION

All oxidative damage samples were paired with antioxidant protection samples. Blood samples used to quantify oxidative damage and antioxidant protection were collected in live-trapped animals not exposed the DLW technique, at the same time as the initial or final samples of the DLW technique, or from animals following sacrifice. Supplementary Material S3 provides more details on how blood samples were collected in the three life-stages and demonstrates that our major conclusions are robust to the influence of these different types of blood collection. All squirrel oxidative damage/antioxidant protection blood samples were collected into heparanized glass capillary tubes, and kept on ice for

≤ 4 h before spinning. Samples were spun for 10 min at 5000 r.p.m., stored at -20°C , and then subsequently at -80°C until they were analyzed. The number of oxidative damage/antioxidant protection samples collected within the three life-stages, on the natural-food and food-supplementation study areas, with and without paired DEE estimates are presented in Table 1.

Plasma protein oxidative damage was determined by measuring protein carbonyls using the 2,4-dinitrophenylhydrazine (DNPH) method (nmol protein carbonyls/mg protein; BIOCELL Corporation Ltd., New Zealand). This marker reflects the oxidation of plasma proteins by ROS (Stadtman 1992; Berlett and Stadtman 1997) and it has been used as a marker of oxidative damage in a diversity of species (e.g., Selman et al. 2002; Salmon et al. 2009; Oldakowski et al. 2012; Heiss and Schoech 2012; Archer et al. 2012). Protein levels were determined using the Bradford assay prior to the oxidative damage assay to determine the amount of sample required. We quantified total antioxidant capacity using a commercially available kit (mM Trolox; Cayman Chemical Company, Ann Arbor, MI). This technique provides an integrated measure of nonenzymatic antioxidant protection that takes into account the important interactions among antioxidants that determine protection against ROS (Cohen et al. 2007; Somogyi et al. 2007; Archer et al. 2012).

STATISTICAL ANALYSES

DEE, oxidative damage, antioxidant protection, and body mass values were \log_{10} transformed in all analyses. Raw average values are presented in the text $\pm SE$. Data are displayed in the figures as raw values on a \log_{10} -transformed scale. All analyses were performed using R (R Development Core Team 2011) with $\alpha = 0.05$. Our analysis proceeded in three steps. First, we used three linear models to compare DEE, oxidative damage, and antioxidant protection between the three life-stages (lactation, nonbreeding, and winter) for individuals living on natural-food study areas (the effect of mass and the mass by life-stage interaction were also included in these two models). Second, we used three linear models to examine the effect of food treatment (natural-food vs. food-supplementation), life-stage (lactation and winter), and their interaction, on DEE, antioxidant protection, and oxidative damage (the antioxidant protection and oxidative damage analyses incorporated females with and without DEE measurements; Table 1). Third, we used seven linear models to examine how DEE and antioxidant protection influenced oxidative damage. Life-stage and food treatment were included in a subset of these models and a subset of models were restricted to single life-stages. These models allowed us to determine whether the effects of both DEE and antioxidant protection on oxidative damage were confounded by life-stage and food treatment effects. Because the seven linear models involved the testing of multiple hypotheses, we performed a false discovery rate (FDR)

correction to control the expected proportion of falsely rejected null hypotheses (Benjamini and Hochberg 1995; for a review see García 2004).

Results

DEE was greatest during lactation on the natural-food study areas (470 ± 44 kJ/day), exceeding nonbreeding DEE by 48% (318 ± 40 kJ/day), and DEE during winter by 90% (247 ± 14 kJ/day; $F_{2,29} = 12.6$, $P = 0.0001$; Tukey's HSD: $P < 0.01$). Plasma protein oxidative damage was also greatest during lactation on the natural-food study areas (0.71 ± 0.08 nmol/mg), exceeding nonbreeding oxidative damage by 1.9 times (0.38 ± 0.05 nmol/mg), and oxidative damage during winter by 4.1 times (0.17 ± 0.02 nmol/mg; $F_{2,39} = 30.4$, $P < 0.0001$; Tukey's HSD: $P < 0.01$). Antioxidant protection on the natural-food study areas during lactation (1.44 ± 0.03 mM Trolox) was greater than antioxidant protection during winter by 18% (1.22 ± 0.10 mM Trolox; $F_{2,39} = 3.4$, $P = 0.04$; Tukey's HSD: $P = 0.04$); however, there was no difference in antioxidant protection between lactating and nonbreeding females (1.33 ± 0.03 mM Trolox; Tukey's HSD: $P = 0.73$). In all three of these analyses, the effects of mass, and the mass by life-stage interactions, were not significant ($P > 0.13$) and were removed from the models.

When squirrels exposed to high and low natural-food were compared, squirrels on high natural-food study areas had greater DEE ($t_{10} = 2.9$, $P = 0.02$; Fig. 1A) and antioxidant protection ($t_{13} = 3.1$, $P = 0.008$; Fig. 1B) compared to squirrels on low natural-food study areas. Plasma protein oxidative damage did not differ between the squirrels on high and low natural-food study areas ($t_{13} = 0.8$, $P = 0.44$; Fig. 1C).

There was a trend for squirrels on food-supplementation study areas to have lower DEE than squirrels on the natural-food study areas (Fig. 1A; $F_{1,26} = 2.8$, $P = 0.11$). Squirrels on food-supplementation study areas had greater antioxidant protection than squirrels on natural-food study areas (Fig. 1B; $F_{1,41} = 9.1$, $P = 0.004$). Food-supplemented squirrels had lower plasma protein oxidative damage than squirrels on natural-food study areas by 2.9 times during lactation and 5.9 times during winter (Fig. 1C; $F_{1,41} = 65.8$, $P < 0.0001$).

Oxidative damage was positively related with DEE (Fig. 2, solid line) and negatively related with antioxidant protection when the effect of life-stage was controlled for (Table 2, Model number 1). However, the effects of DEE and antioxidant protection on plasma protein oxidative damage were confounded by food treatment because the effects of DEE and antioxidant protection on oxidative damage were reduced to trends ($P \leq 0.13$) when the effect of food treatment was added to the model (Table 2, Model number 2). During lactation, plasma protein oxidative damage was positively related with DEE and negatively related

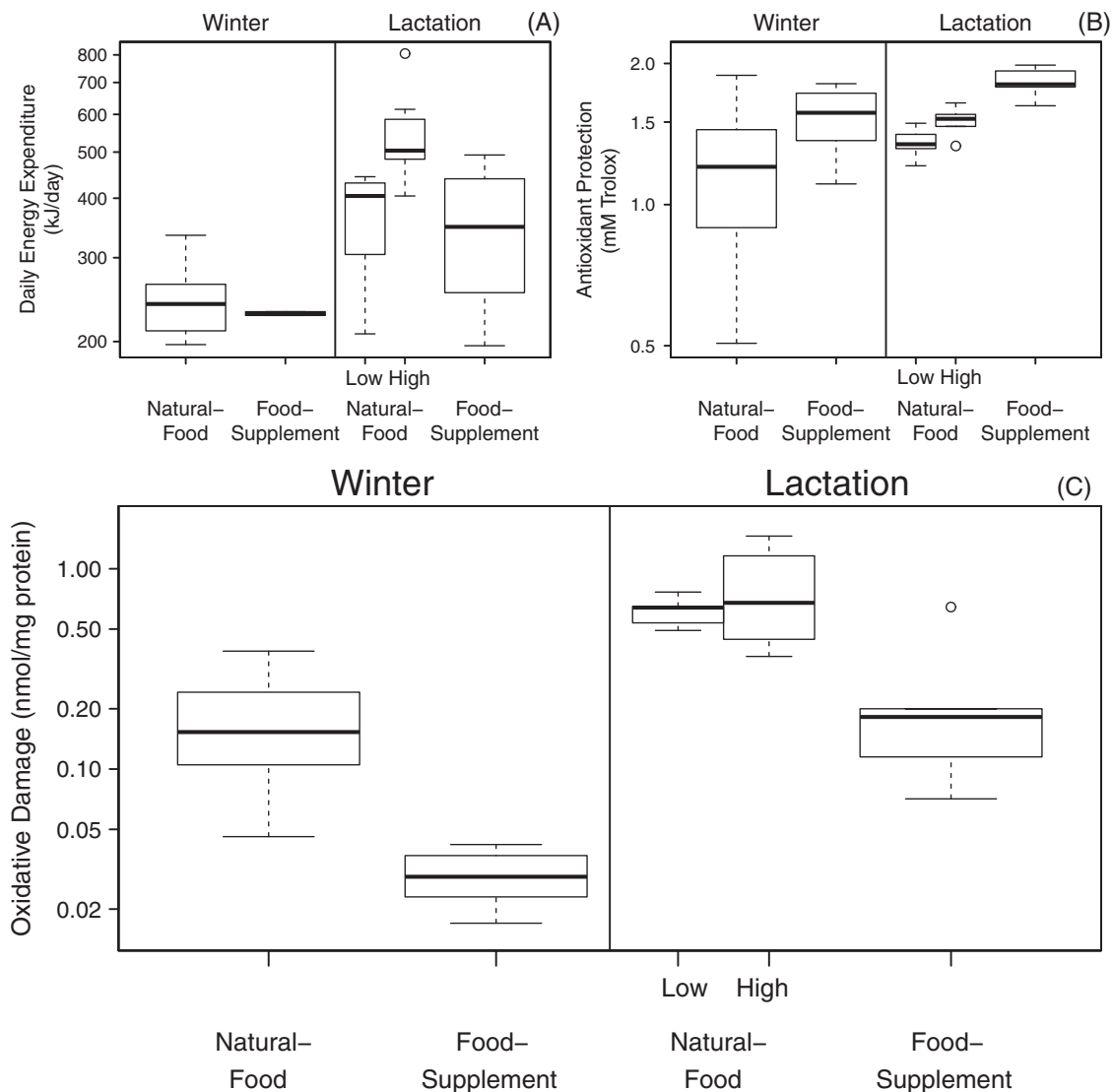


Figure 1. Effect of food-supplementation on (A) daily energy expenditure (DEE), (B) antioxidant protection, and (C) plasma protein oxidative damage during the life-stages of lactation and winter. Squirrels were examined on study areas where they only had access to only natural food sources ("Natural-Food") and on study areas where all individuals had access to supplemental peanut butter ("Food-Supplement"). The natural food levels experienced by lactating females were either high or low depending on which natural-food study area the squirrels inhabited. Whiskers represent 95% confidence intervals and boxes bound the interquartile range including a median line. Life-stage influenced DEE ($F_{1,26} = 27.5$, $P < 0.0001$), antioxidant protection ($F_{1,41} = 8.0$, $P = 0.007$), and oxidative damage ($F_{1,41} = 93.4$, $P < 0.0001$); however, food treatment did not interact with life-stage ($P > 0.10$), and was thus removed from these models.

with antioxidant protection (Table 2, Model number 3; note that the antioxidant protection effect was nonsignificant after the FDR correction); however, these effects were similarly confounded by food treatment (Table 2, Model number 4). Antioxidants were negatively related with oxidative damage during winter (Table 2, Model number 5; note that the antioxidant protection effect was nonsignificant after the FDR correction); however, this effect was also confounded by food treatment (Table 2, Model number 6). Plasma protein oxidative damage during the nonbreeding life-stage was not related to either DEE or antioxidant protection (Table 2, Model number 7).

Discussion

Plasma protein oxidative damage was greatest during the energetically demanding life-stage of lactation as compared to the nonbreeding and winter life-stages. Our results suggest that the oxidative damage experienced by animals varies considerably through the year in response to large life-stages differences in energy expenditure. DEE during lactation was nearly twice as high as DEE during winter, whereas plasma protein oxidative damage during lactation was more than four times greater than plasma protein oxidative damage during winter. Previous studies

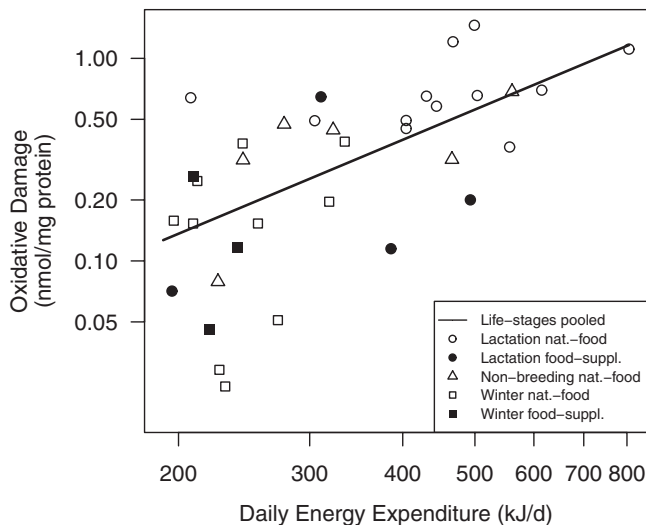


Figure 2. The positive relationship between daily energy expenditure and plasma protein oxidative damage pooling individuals on natural-food and food-supplementation study areas. The line of best fit for all life-stages pooled (solid line; $F_{1,36} = 18.8$, $P = 0.0001$; $R^2 = 0.34$) is displayed because this relationship was significant when the effects of life-stage and antioxidant protection were controlled for (Table 2; Model number 1).

examining the role of oxidative damage as a cost of reproduction in captivity or in the wild have generally conducted their studies during or around the reproductive season (Alonso-Alvarez et al. 2004, 2006; Wiersma et al. 2004; Bertrand et al. 2006; Bize et al. 2008; Garratt et al. 2011; Markó et al. 2011), when levels of energy expenditure are not likely to vary to the same extent as in our study that incorporated DEE measures of lactating, nonbreeding, and winter females. DEE has been quantified in one other study that examined oxidative damage (*Tamias striatus*; Bergeron et al. 2011). In Bergeron et al. (2011), DEE was less variable than in our study (P. Bergeron pers. comm.: coefficient of variation = 31%; c.v. in our study = 42%). Moreover, they found that litter size was positively correlated with both DEE and oxidative damage, which suggested that there was a positive link between DEE and oxidative damage. However, the correlation between DEE and oxidative damage could not be tested directly by Bergeron et al. (2011) because DEE and oxidative damage were quantified in different individuals.

In addition to differing in DEE, squirrels in different life-stages presumably differed in hormone levels that may have influenced oxidative damage. It has been demonstrated in laboratory mammal studies conducted *in vivo* and *in vitro* that estrogens can induce antioxidant effects (Behl et al. 1997; Viña et al. 2003; Razmara et al. 2007; Miller et al. 2007; Persky et al. 2000); although other laboratory studies (reviewed in Cavalieri et al. 2000) and a study on captive Eurasian kestrels (*Falco tinnunculus*; Casagrande et al. 2012) reported the opposite effect.

Squirrels on the food-supplementation study areas had elevated antioxidant protection and reduced plasma protein oxidative damage during winter and lactation relative to squirrels on natural-food study areas. One possible explanation for this result is that antioxidants present in peanut butter were responsible for the reduction in oxidative damage. Peanut butter contains the polyphenolic compound resveratrol (Sobolev and Cole 1999; Ibern-Gómez et al. 2000), which is a known antioxidant (Murcia and Martínez-Tomé 2001). Moreover, roasted peanuts, which are used to make peanut butter, also have antioxidant properties that primarily result from the presence of other polyphenolics (especially p-coumaric acid; Talcott et al. 2005; Duncan et al. 2006). Considerable research has also demonstrated beneficial effects of specific antioxidants on, for example, growth, immunocompetence, and sexual selection signals (reviewed extensively in Catoni et al. 2008). For example, supplementing breeding female zebra finches (*Taeniopygia guttata*) with antioxidants (i.e., carotenoids) reduces the negative effect of reproductive effort on the ability to resist oxidative stress (Bertrand et al. 2006). Alternatively, squirrels on the food-supplementation study areas may have been able to increase allocation to antioxidant protection relative to squirrels on the natural-food study areas as a result of being in a better energetic “state” (*sensu* McNamara and Houston 1996). The greater antioxidant protection in squirrels on high natural-food versus low natural-food study areas is also consistent with the hypothesis that increased food availability increased antioxidant protection as a result of an improved energetic state. This improved energetic state may involve improved body condition (fat and protein reserves), reduced activity requirements, and reduced thermoregulatory requirements resulting from favorable nutritional and energetic conditions created by access to more, higher quality food. Overall, our results comparing DEE, antioxidant protection, and oxidative damage between food treatments were based on small sample sizes (see Table 1). Thus, future research should follow up on our promising results by food-supplementing animals with natural diets and those rich in antioxidants.

Our results are consistent with the hypothesis that oxidative damage increases with DEE and decreases with antioxidant protection. However, the relationships between oxidative damage and both DEE and antioxidant protection primarily resulted because of the variation in DEE and antioxidant protection generated by the effect of food treatment (natural-food vs. food-supplementation). Specifically, food-supplementation decreased DEE ($P = 0.11$) and increased antioxidant protection ($P = 0.004$). As a result, the effects of DEE and antioxidant protection on oxidative damage were reduced to trends when the effect of food treatment was included in the model (compare Table 2, model numbers 1 and 2).

In conclusion, our results are consistent with the hypothesis that elevated oxidative damage results from energetic investment in reproduction that overwhelms antioxidant protection.

Table 2. Seven linear models examining the effects DEE, antioxidant protection, life-stage (winter, nonbreeding, and lactation), and food treatment (natural and food-supplementation) on plasma protein oxidative damage. Individuals with DEE measurements (see Table 1) were included in each analysis. Filled cells within each model show the terms that were included in each initial model. Nonsignificant terms were sequentially backward dropped from the models. Coefficients and *P*-values are either those that were used to eliminate the terms from the model, or were those in the final model. Significant variables remaining in the final models are bolded (coefficients \pm SE with *P*-values in brackets). Bolded *P*-values that are starred were nonsignificant after a false discovery rate correction (see Materials and Methods).

Model Number	Life-Stages Included	DEE	Antioxidant Protection	Life-Stage	Food Treatment
1	All	0.9 \pm 0.4 (0.03)	-1.9 \pm 0.6 (0.002)	(0.004)	
2	All	0.5 \pm 0.3 (0.13)	-0.9 \pm 0.8 (0.11)	(<0.0001)	(<0.0001)
3	Lactation	1.2 \pm 0.5 (0.02)	-3.0 \pm 1.3 (0.04*)		
4	Lactation	0.03 \pm 0.7 (0.96)	1.9 \pm 1.4 (0.19)		(0.0001)
5	Winter	-0.9 \pm 1.7 (0.62)	-1.9 \pm 0.7 (0.03*)		
6	Winter	-0.5 \pm 1.4 (0.71)	-1.2 \pm 0.6 (0.10)		(0.005)
7	Non breeding	0.7 \pm 0.6 (0.29)	-5.1 \pm 3.4 (0.19)		

This finding corroborates previous work on free-ranging animals suggesting that oxidative damage mediates life-history trade-offs (Bergeron et al. 2011; Heiss and Schoech 2012; Wilson et al. 2012). Ironically, support for the oxidative damage hypothesis is emerging from field studies at a time when laboratory animal and biomedical researchers are turning away from this hypothesis because of equivocal and contradictory evidence and emerging alternative mechanisms (Speakman and Selman 2011). This difference may simply reflect a stronger publication bias against negative results in the wild than in the laboratory where the oxidative damage hypothesis has been tested for a longer period of time. However, a possible biological interpretation of this conflicting support is that oxidative damage may play a more important role in defining the costs of reproduction in the wild than in the laboratory. In accord with our finding that favorable nutritional and energetic conditions can reduce oxidative damage, it is possible that life-history trade-offs are not mediated by oxidative damage in the laboratory because oxidative damage is mitigated by the reduced activity requirements, increased food availability, and balanced diets that characterize many laboratory studies.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Supplementary Material S1: White spruce cone abundance and squirrel ecology.

Figure S1. The average cone index on the two natural-food study areas that have been monitored consistently since 1987 (line: average of two study areas), as well as the cone indices in 2005 on the natural-food (open symbols) and food-supplementation (filled circle) study areas (two low cone index values in 2005 are staggered along the x-axis to aid visibility). The open symbol in 2006 represents the cone index that squirrels within the winter life-stage experienced in 2007 on both the natural-food and food-supplementation study areas.

Literature cited only in Supplementary Material S1.

Supplementary Material S2: Doubly-labeled water methodology.

Literature cited only in Supplementary Material S2.

Supplementary Material S3: Influence of the types of blood collection.