

Daily energy expenditure during lactation is strongly selected in a free-living mammal

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Summary

1. Energy expenditure is a trait of central importance in ecological and evolutionary theory. We examined the correlates of, the strength of selection on, and the heritability of, daily energy expenditure (DEE; kJ day⁻¹) during lactation in free-ranging North American red squirrels (*Tamiasciurus hudsonicus*).
2. Over 7 years, lactating squirrels with greater DEE had higher annual reproductive success (ARS; standardized selection gradient: $\beta' = 0.47$; top 12% of published estimates). Surprisingly, positive fecundity selection on lactation DEE for increased ARS did not result because lactation DEE was correlated with typical measures of reproductive performance and/or investment.
3. We found no evidence of costs of elevated lactation DEE acting through female survival, subsequent year lactation DEE or subsequent year reproduction.
4. Lactation DEE was not significantly repeatable, and heritability was not significantly different from zero.
5. Elevated lactation DEE enhances ARS through a link between DEE and an unidentified measure of maternal or environmental quality, but there is limited evolutionary potential for lactation DEE to respond to our documented selection.

Key-words: costs of reproduction, energetics, heritability, reproductive success

Introduction

Energy expenditure is a trait of central importance in ecological and evolutionary theory. The rate at which animals expend energy is an important determinant of the resource needs, demographics and distributions of populations (Damuth 1981; Yodzis & Innes 1992; Carbone & Gittleman 2002; Brown *et al.* 2004; Kearney & Porter 2009; Humphries & McCann 2013). The fitness-promoting activities of growth (Wieser 1994), reproduction (Drent & Daan 1980; Gittleman & Thompson 1988) and maintenance

(Delahay, Speakman & Moss 1995; Martin, Scheuerlein & Wikelski 2003; Scantlebury *et al.* 2007; Careau, Thomas & Humphries 2010 but see Speakman & Król 2010) are all energetically demanding. As a result, a large body of literature has examined the importance of energy expenditure as a determinant of allocation trade-offs (e.g. Metcalfe & Monaghan 2001; Zera & Harshman 2001) and fitness itself (see Table 1).

At first glance, a positive relationship between energy expenditure and reproductive success is expected because reproduction is energetically costly (McNab 2002; Angilletta 2009). Thus, if animals can expend more energy during reproduction, they should have increased reproductive success. However, high rates of energy expenditure may not be correlated with reproductive investment or success because energy expenditure is also influenced by environmental factors. For example, blue tits (*Parus caeruleus*) expended the most energy in poor-food years when they

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Table 1. Observational studies examining the relationship between energetic traits and fitness (survival, reproductive success or surrogates thereof). These studies were conducted on animals in captivity, in field enclosures, as well as on free-ranging animals

Study type/species	Energetic trait(s)	Number of individuals	Survival	Reproductive success	Ref.
Captivity					
Cotton rat (<i>Sigmodon hispidus</i>)	BMR	115		Energy investment into reproduction	1
Deer mouse (<i>Peromyscus maniculatus</i>)	BMR	47		Litter size, litter mass, interlitter interval	2
House mice (<i>Mus domesticus</i>)	BMR, MMR	74		Litter size, litter mass, mean offspring mass	3
Large-eared tenrecs (<i>Geogale aurita</i>)	DEE, RMR	10		Litter size	4
Shrew tenrecs (<i>Microgale dobsoni</i>)	RMR	4		Litter size, neonate mass, litter mass	5
Laboratory mice (<i>Mus musculus</i>)	RMR	108		Litter size, litter mass, mean offspring mass	6
Zebra Finches (<i>Taeniopygia guttata</i>)	DEE, RMR	24		Clutch size, clutch mass, brood mass	7
C57BL/6J mice	BMR	304		Litter mass, litter size, pup mass at birth and weaning, weaning success, pup loses prior to weaning	8
MF1 mice (<i>M. musculus</i>)	RMR	271		Litter size, litter mass at weaning	9
Speckled cockroach (<i>Nauphoeta cinerea</i>)	SMR	174		Gestation duration, number of offspring	10
Speckled cockroach (<i>N. cinerea</i>)	SMR	96	Survival during food and water restriction		11
Swiss Webster mice	BMR	30		Offspring growth rate	12
Field enclosures					
Common garden snail (<i>Helix aspersa</i>)	SMR	285	Mark-recapture study		13
Bank voles (<i>Myodes glareolus</i>)	BMR	136	Overwinter survival		14
Free-ranging					
Golden-mantled ground squirrels (<i>Spermophilus saturatus</i>)	DEE	20		Litter size	15
Pied Flycatchers (<i>Ficedula hypoleuca</i>)	DEE	41		Chick growth rate	16
Deer mouse (<i>P. maniculatus</i>)	MMR	278	Mark-recapture study		17
Short-tailed field vole (<i>Microtus agrestis</i>)	RMR	173	Overwinter survival		18
Brown trout (<i>Salmo trutta</i>)	SMR	347	Mark-recapture study		19
Leach's storm-petrels (<i>Oceanodroma leucorhoa</i>)	BMR	95		Egg volume, hatching date, hatching success, chick growth rates	20
Bank vole (<i>M. glareolus</i>)*	BMR, MMR	337	Mark-recapture study		21
Bank vole (<i>M. glareolus</i>)*	BMR, MMR	153		Mating success and offspring recruitment based on reconstructed parentage analyses	22
Little Auk (<i>Alle alle</i>)	DEE	43	Survival until next breeding season		23
N. American red squirrels (<i>Tamiasciurus hudsonicus</i>)	RMR, DEE	53	Overwinter survival		24
Eastern chipmunk (<i>Tamias striatus</i>)	DEE	57		Litter size	25
Eastern chipmunk (<i>T. striatus</i>)	DEE, RMR	68	Juvenile overwinter survival		26

The total number of individuals with energetic trait measures is given in the table regardless of the number of energetic traits that were quantified on these individuals. Resting metabolic rate (RMR) is the minimal rate of metabolism necessary to maintain basic physiological processes in a thermoneutral animal. Basal metabolic rate (BMR) is a similar measure to RMR, but it also requires that the animal is non-growing, non-reproductive and in a post-absorptive state. Standard metabolic rate (SMR) is the minimum cost of maintenance in ectotherm organisms exposed to a specified temperature. Daily energy expenditure (DEE) equals the total amount of energy expended

over the period of a day, or over the period of multiple days. Maximum metabolic rate (MMR) quantifies the maximum metabolic capacity of an animal. Animals can be pushed to their MMR using different tests (treadmill, cold or forced swim).

References: 1. Derting & McClure (1989), 2. Earle & Lavigne (1990), 3. Hayes, Garland & Dohm (1992), 4. Stephenson & Racey (1993a), 5. Stephenson & Racey (1993b), 6. Johnson, Thomson & Speakman (2001a), 7. Vézina, Speakman & Williams (2006), 8. Johnston *et al.* (2007), 9. Duarte *et al.* (2010), 10. Schimpf, Matthews & White (2012a), 11. Schimpf, Matthews & White (2012b), 12. Sadowska, Gębczyński & Konarzewski (2013), 13. Artacho & Nespolo (2009), 14. Boratyński *et al.* (2010), 15. Kenagy *et al.* (1990), 16. Moreno, Potti & Merino (1997), 17. Hayes & O'Connor (1999), 18. Jackson, Trayhurn & Speakman (2001), 19. Álvarez & Nieceza (2005), 20. Blackmer *et al.* (2005), 21. Boratyński & Koteja (2009), 22. Boratyński & Koteja (2010), 23. Welcker *et al.* (2010), 24. Larivée *et al.* (2010), 25. Bergeron *et al.* (2011), 26. Careau *et al.* (2013).

*Paired studies on the same population of bank voles.

raised the slowest growing chicks (Thomas *et al.* 2001 but see Verhulst & Tinbergen 2001).

When the relationship between energy expenditure and survival is considered, a negative relationship is often assumed because high rates of energy expenditure may be associated with greater physiological costs (reviewed in Zera & Harshman 2001). Experimental increases in reproductive investment (e.g. clutch size manipulations), which are presumably associated with increased energetic costs, are generally associated with increased mortality rates (Dijkstra *et al.* 1990). However, there are very few studies that quantify levels of energy expenditure, and those that do, find equivocal evidence for a negative effect of energy expenditure on survival (Drent & Daan 1980; Daan, Deerenberg & Dijkstra 1996; Humphries & Boutin 2000; Skibieli, Speakman & Hood 2013). Moreover, high rates of energy expenditure may in fact be associated with improved survival because they are indicative of animals in better physiological condition (e.g. increased cold tolerance – Swanson 2001).

Because energy expenditure is considered an important determinant of fitness (Table 1), researchers have repeatedly quantified the heritability of energy expenditure to predict how it would respond to selection. In order for energy expenditure to respond to selection, it must have an underlying genetic component (i.e. additive genetic variance, V_A ; Falconer & Mackay 1996; but see Bonduriansky, Crean & Day 2012 for a review of non-genetic inheritance). Currently, we have a poor understanding of the heritability of energy expenditure in free-ranging animals because the heritability of energetic traits has primarily been estimated in captivity (Lacy & Lynch 1979; Lynch & Sulzbach 1984; Garland & Bennett 1990; Dohm, Hayes & Garland 1996, 2001; Lerman *et al.* 2002; Nespolo, Bacigalupe & Bozinovic 2003; Bacigalupe *et al.* 2004; Khazaeli, Van Voorhies & Curtsinger 2005; Konarzewski, Książek & apo 2005; Nespolo *et al.* 2005; Sadowska *et al.* 2005; Nespolo, Castañeda & Roff 2007; Rønning *et al.* 2007; Ketola & Kotiaho 2009; Sadowska *et al.* 2009; Tieleman *et al.* 2009; Wone *et al.* 2009; Careau *et al.* 2011; Mathot *et al.* 2013; Schimpf, Matthews & White 2013; for reviews see – Konarzewski & Książek 2013; White & Kearney 2013). The majority of the information regarding the heritability of energetic traits in free-ranging animals comes from estimates of repeatability, which sets an upper limit to heritability (Falconer & Mackay 1996; but see Dohm 2002).

These studies generally find that energetic traits are repeatable in free-ranging animals (Chappell, Bachman & Odell 1995; Chappell, Zuk & Johnsen 1996; Bech, Langseth & Gabrielsen 1999; Hayes & O'Connor 1999; Nespolo & Franco 2007; Szafrńska, Zub & Konarzewski 2007; Boratyński & Koteja 2009; Broggi *et al.* 2009; Larivée *et al.* 2010; Bouwhuis, Sheldon & Verhulst 2011; but see Bozinovic 2007). The few studies that have quantified the heritability of energy expenditure in free-ranging animals have demonstrated that it is significantly heritable (*Cyanistes caeruleus*, Nilsson, Akesson & Nilsson 2009; *Mustela nivalis*, Zub *et al.* 2012; *Ficedula hypoleuca*, Bushuev *et al.* 2012).

Development of the doubly labelled water (DLW) technique (Lifson & McClintock 1966; Nagy 1983; Speakman 1997; Butler *et al.* 2004) represented a major advance in animal and human energetics research because it allowed energy expenditure to be quantified on free-living animals over multiday periods (Drent & Daan 1980; Bryant 1997; Nagy, Girard & Brown 1999; Speakman 2000; Westerterp & Speakman 2008). The estimate of daily energy expenditure (DEE; kJ day^{-1}) includes the energy expended on maintaining baseline physiological processes, behaviour, thermoregulation, as well as the costs required to process and allocate energy. The vast majority of research examining selection on, and the heritability of, energy expenditure has focused on minimum (i.e. resting and basal metabolic rate, RMR and BMR respectively; henceforth RMR) and maximum (MMR) metabolic rates (Table 1). These measures of energy expenditure are quantified in the laboratory under highly standardized conditions, over a period of minutes or hours. Research examining the fitness consequences and heritability of DEE has been forced to use smaller sample sizes and has generally lagged behind research that has quantified RMR and MMR, due to the difficulty (i.e. animals must be captured twice over a period of days preferably at a specific time of day) and cost associated with quantifying DEE in a sufficient number of animals to be able to reasonably estimate natural selection.

In a previous study, we examined the correlates of female energy expenditure throughout the year. We found that lactation is an energetically demanding time of the year for free-ranging red squirrels (Fletcher *et al.* 2012). We also found that lactation DEE was positively correlated with resource levels, unrelated to body mass, and positively correlated with ambient temperatures over a

range of ambient temperatures that would have been below the lower critical temperature of its thermal neutral zone (Pauls 1981). Here, we examined the correlates of, the strength of selection on, and the heritability of, DEE during lactation in free-ranging North American red squirrels (*Tamiasciurus hudsonicus*; henceforth, squirrels). In our study, the DEE of lactating squirrels was estimated in 7 years at a consistent point during lactation. Detailed monitoring of the reproductive behaviour of females allowed us to examine how a suite of typical correlates of reproductive performance (maternal mass and age) and measures of reproductive investment (parturition date, litter size and nestling growth rate; e.g. Clutton-Brock [ed.] 1988) were correlated with lactation DEE. We examined both fecundity and viability selection on lactation DEE to assess potential trade-offs between these fitness components (Arnold & Wade 1984). Finally, we used an established pedigree from our study population to estimate the heritability of DEE. Combining these pieces of information allowed us to examine the fitness consequences of individual variation in lactation DEE, as well as the genetic influences on lactation DEE with the most comprehensive and the largest data set of DEE measures used to address these questions to date (Table 1).

Materials and methods

STUDY SITE AND FEMALE REPRODUCTION MONITORING

We studied a natural population of red squirrels in the Kluane region of south-western Yukon, Canada (60°57'N, 138°2'W), from 2002 to 2008. Squirrels were monitored on two adjacent study areas (40 ha each) that were bisected by the Alaska Highway. Our study areas were in a glacial valley composed of boreal forest dominated by white spruce (*Picea glauca*), with an understorey of willow (*Salix* spp.), interspersed with stands of trembling aspen (*Populus tremuloides*) and some open meadows (Krebs & Boonstra 2001).

The reproductive activity of every female living on the two study areas was tracked following standardized protocols of live-trapping and natal nest entries (Berteaux & Boutin 2000; Humphries & Boutin 2000; Boon, Réale & Boutin 2007; McAdam *et al.* 2007; Lane *et al.* 2010). Overall, these protocols allowed us to determine the age of each breeding female, in addition to the parturition date, litter size and average nestling growth rate of each litter. The majority of females examined in this study (90/117) were ear-tagged (Monel #1) within their natal nest, thus their ages were known with certainty. The birth year of immigrant females to the population was assigned to be the previous year if the colour of her nipples suggested that she had never bred (i.e. pink nipples); otherwise, her birth year was assigned to be 2 years previous, and thus represents a minimum estimate (see also Becker, Boutin & Larsen 1998). All records from females 5 years of age and older ($n = 18$) were pooled into a single age class. From March to August, we live-trapped females and determined whether they were pregnant or lactating by palpating their abdomens and attempting to express milk from their nipples. Females were radio-collared as soon as they were determined to have given birth and were tracked to their nest. Nestlings were temporarily removed from the nest, individually marked and weighed. The female's litter size was the number of nestlings present at this time.

Parturition date (days since 1 January) was estimated based on trapping records (sudden weight loss or the expression of milk from teats), the weight of the nestlings the first time they were removed from the nest or behavioral observations indicating parturition (i.e. spending more time in their nest, or the presence of large nipples visible through binoculars; see also Becker 1993; Boutin & Larsen 1993; Dantzer *et al.* 2013). Nestlings were temporarily removed from their nest a second time when they were ~25 days old to be weighed and ear-tagged. The average nestling growth rate (g day^{-1}) between the first and second time that the nestlings were removed from the nest was used in all analyses. For consistency with previous research at this site, only individuals attempting their first litter of the season were analysed (McAdam & Boutin 2003; Réale *et al.* 2003). All animals were captured and handled in compliance with the guidelines of the Canadian Council on Animal Care.

LACTATION DAILY ENERGY EXPENDITURE

We obtained 147 DEE estimates from 117 lactating females using the DLW technique (Nagy 1983; Speakman 1997). We obtained repeated DEE measures on 24% of individuals ($n = 28$) with the median time difference between measures being *c.* 1 year (median = 356 days, 1st–3rd quartiles: 332–404 days). We attempted to estimate the DEE of every female present on the two study areas during lactation when females were between 35 and 40 days post-partum (89% of samples were collected during this time range; the date range of the additional samples was between 33 and 49 days post-partum). This time window reflects a period when females likely experience peak energetic demands of lactation because nestlings have grown to their largest body size prior to first emergence from the nest when they start supplementing their diet with solid food (~42 days; S. Boutin unpublished data). Nestlings are fully weaned at ~70 days post-partum (S. Boutin, unpublished data).

The methodology of the DLW technique is presented in Fletcher *et al.* (2012). Briefly, CO₂ production was calculated based on the differential washout of hydrogen (²H) and oxygen (¹⁸O) isotopes over a period of 2–5 days. Ninety-seven per cent of final blood samples were obtained within 2 h of a 24-h interval from the initial blood sample (141/146; for one individual, the times of the initial and final blood samples were not recorded), which controlled for circadian rhythms of activity (Speakman & Racey 1988; Speakman *et al.* 1994; Berteaux *et al.* 1996). CO₂ production over the DLW interval was converted to an estimate of DEE in kJ day^{-1} (Speakman 1997). We determined the mass of individuals at the beginning and end of the DLW technique. The average of these two masses was used in all analyses. One hundred and seventeen lactation DEE values were originally presented in Fletcher *et al.* (2012).

STATISTICAL ANALYSES

Data screening and sample sizes

Observations were omitted from the analyses for three possible reasons: (i) the number of nestlings the second time the nest was entered was less than the first time it was entered due to partial litter loss (omitted from the correlates of lactation DEE and natural selection on lactation DEE analyses), (ii) nests were entered for the first time when the nestlings were too old to estimate parturition dates, litter size and average nestling growth rates (omitted from the correlates of lactation DEE and natural selection on lactation DEE analyses), and (iii) an unnatural death prevented the offspring or mother from surviving to 1 May the following year (omitted from the natural selection on lactation DEE

analyses). Overall, 116 DEE estimates were included in the correlates of lactation DEE analysis, 136 DEE estimates were included in the selection analysis examining annual reproductive success (ARS), and 137 DEE estimates were included in the selection analysis examining maternal survival. All 147 lactation DEE measures were included in the quantitative genetics of lactation DEE analysis.

Correlates of lactation DEE

We examined the correlates of lactation DEE using a linear mixed model analysis (lme4; Bates & Maechler 2009). Year and squirrel identity (henceforth, ID) were included as random effects in this analysis. The significance of fixed effects was assessed using stepwise backward elimination, with the fixed effects with the largest P values removed first, retaining all effects with $P \leq 0.05$. P -values were determined using likelihood ratio tests (-2 times the difference in log-likelihoods between hierarchical models estimated using maximum likelihood, tested against a chi-square distribution with the number of degrees of freedom equal to the difference in the number of terms estimated in the hierarchical models). Unless stated otherwise, significance was based on $\alpha < 0.05$, and all analyses were performed using R (R Development Core Team 2011).

Natural selection on lactation DEE

Following previous research at this study site, the fitness components of reproduction and survival were based on recapture data assuming that squirrels that disappeared from our study areas had died (McAdam & Boutin 2003; Réale *et al.* 2003; Boon, Réale & Boutin 2007). The fitness of a female resulting from her ARS (W_{ARS}) was defined as the number of her offspring surviving overwinter until 1 May the following year (McAdam & Boutin 2003). Survival to this age is a major episode of selection for juveniles in this population (McAdam *et al.* 2007). Our study considers the early survival of offspring as a component of the mother's fitness, rather than her offspring's fitness (see also Réale *et al.* 2003). The fitness of lactating females associated with her survival (W_S) was defined as whether or not she survived to 1 May the following year (1 = survived, 0 = died). Our ability to estimate ARS and maternal survival is enabled by the high visibility and fidelity of squirrels to their territories. Our estimates of ARS do not appear biased by dispersal from the study area because the recruitment of juveniles born in the core 20 ha of each study area is not different from the recruitment of juveniles born in the surrounding 20-ha periphery of the study areas (McAdam *et al.* 2007). In addition, capture-mark-recapture models performed on this population suggest that our probability of detecting a squirrel given that it is alive is one (Descamps *et al.* 2009). Our ability to determine the survival of squirrels likely results because our study areas are large in size (40 ha each) relative to the dispersal distance of juvenile squirrels (mean dispersal distance = 96 ± 94 m from their natal area; Berteaux & Boutin 2000). Moreover, adult squirrels tend to occupy the same territory for their entire lives (S. Boutin, A. G. McAdam and M. M. Humphries unpublished data).

Fitness components for each female were converted to relative fitness (w_{ARS} and w_S) by dividing the absolute fitness measures (W_{ARS} and W_S) by population average fitness measures (\bar{W}_{ARS} and \bar{W}_S). Average fitness measures were calculated each year including all females in this study, pooling the two study areas. Average fitness for ARS (\bar{W}_{ARS}) was the mean number of offspring surviving to the following year, whereas average fitness for maternal survival (\bar{W}_S) was the proportion of females surviving to the following year.

To examine the relationship between lactation DEE and both ARS and maternal survival, we estimated standardized selection

gradients following Lande & Arnold (1983). Maternal traits were standardized (zero mean, unit standard deviation) within each year to avoid confounding selection gradients with among-year covariances between environmental conditions, trait values and fitness (see McAdam & Boutin 2003; Garant *et al.* 2007). The standardized selection gradients (β') of the maternal traits were the coefficients generated from a linear model. Using a separate linear model, we also estimated standardized quadratic selection gradients for the traits (γ_{ii} ; two times the regression coefficient, following Stinchcombe *et al.* 2008) and the correlational selection gradients (γ_{ij}) between all standardized maternal traits. Standard errors (SE) for all selection analyses were determined using a jackknifing procedure in which all records for each individual were removed during each iteration. The significance of the differentials and gradients in the separate analyses were determined using t -tests (coefficient/jackknifed SE). To control for repeated measurements of individual females, the degrees of freedom in these jackknife analyses reflected the number of individuals in the analysis minus one.

Quantitative genetics of lactation DEE

Prior to running our quantitative genetics analysis of lactation DEE, we selected fixed effects to include in the linear mixed-effects animal model (Wilson 2008). To determine the fixed effects that influenced lactation DEE, we examined potential environmental variables (food availability, squirrel density and ambient temperature) in addition to the typical correlates of reproductive performance and investment described above. The seeds of white spruce trees are an important food source of squirrels (Fletcher *et al.* 2013a). In late-summer of each year, we quantified the amount of white spruce cones available to red squirrels on each study area using a ln-scaled cone index (LaMontagne & Boutin 2007, 2009). We included both the previous year's and current year's cone index (average index of both study areas; the indices on the two study areas were highly correlated: $r = 0.97$) in our set of potential fixed effects because they both can influence life-history parameters (Boutin *et al.* 2006). The other two environmental variables that we examined as potential fixed effects were the average ambient temperature over the course of the DLW interval (T_a – hourly T_a readings; Fletcher *et al.* 2012) and the average spring breeding population density on the two study areas (see Dantzer *et al.* 2013). Our candidate model set included all possible combinations of models involving at least one of the potential fixed effects (previous year's cone index, current year's cone index, T_a , density, maternal mass, maternal age, parturition date, litter size and nestling growth rate; 511 models), with no interactions and two random effects (year and squirrel ID). The fixed effects that we included in our animal model were those in the model with the lowest AIC_c value (Burnham & Anderson 2002).

Variance components and the heritability of lactation DEE were estimated using restricted maximum likelihood animal models (Lynch & Walsh 1998) in ASREML 3.0 (Gilmour *et al.* 2009). See Réale *et al.* (2003), McFarlane *et al.* (2011) and Taylor *et al.* (2012) for previous quantitative genetics analyses on this population. We included the current year's cone index, squirrel density, maternal mass, parturition date (days since 1 January) and nestling growth rate as fixed effects, which were identified by the AIC_c model selection analysis (see paragraph above; Table S1 presents the top 25 models, Supporting information). Year was included as a random factor in all models because multiple lactation DEE values were obtained in each year. The null model included only the random effect of year. We first fitted an individual variance term (V_1) that incorporated all sources of individual variance: permanent environment variance (V_{PE}), additive genetic variance (V_A) and maternal effect variance (V_M ; Table 4 – row 2). In a subsequent model, we partitioned the V_1 term into the constituent V_{PE}

and V_A components (Table 4 – row 3). Permanent environment effects arise due to repeatable non-genetic differences between individuals (Kruuk & Hadfield 2007), which may result due to maternal effects. Thus, in the model presented in row 3 of Table 4, the V_{PE} term incorporated V_M (Kruuk & Hadfield 2007). Permanent environment effects can be estimated from repeated measures of a trait on the same individual and were fitted to prevent this source of variation from artificially inflating our estimate of additive genetic variance (Lynch & Walsh 1998; Kruuk & Hadfield 2007). Finally, we ran a model that explicitly included terms reflecting the V_M and V_{PE} . The repeatability (r), narrow-sense heritability (h^2), maternal effects (m^2) and permanent environment effects (pe) were calculated as $r = V_I/V_P$, $h^2 = V_A/V_P$, $m^2 = V_M/V_P$ and $pe = V_{PE}/V_P$, respectively, where V_P is the total phenotypic variance (Table 4 – row 4). All models also included a V_R term, reflecting the residual variance.

The pruned pedigree used in the animal model analysis included 233 individuals. This pedigree included all known dams, sires and ancestors of individuals with lactation DEE measures. Dams were assigned for individuals that were ear-tagged prior to emergence from their natal nest. Sires within this pedigree were assigned using previously established protocols (Gunn *et al.* 2005; Lane *et al.* 2008; 95% confidence using CERVUS 3.0, Kalinowski, Taper & Marshall 2007). The descriptive statistics of the pruned pedigree are provided in Table S2 (Supporting information) (R library: Pedantics; Morrissey 2012).

Results

CORRELATES OF LACTATION DEE

None of the potential correlates of reproductive performance or measures of reproductive investment were strong predictors of lactation DEE (Table 2). There was a weak trend for positive relationships between lactation DEE and both maternal mass ($P = 0.11$; Table 2; Fig. 1) and parturition date (greater lactation DEE with later parturition

Table 2. Correlates of lactation daily energy expenditure (DEE; linear mixed-effects model incorporating repeated measures on individuals over 7 years). Lactation DEE was quantified on free-ranging squirrels using the doubly labelled water technique between 35 and 40 days after a female had given birth to her litter

	Full model coefficient \pm SE	χ^2	P
Maternal body mass (g)	1.1 \pm 0.7	2.55	0.11
Parturition date (d. since 1 January)	1.5 \pm 1.0	2.54	0.11
Maternal age (years)	7.6 \pm 11.2	0.56	0.46
Average nestling growth (g day ⁻¹)	-13.7 \pm 39.6	0.11	0.74
Litter size	-1.6 \pm 18.1	7.9 ^{e-3}	0.93
Year (random)		11.3	0.0008
ID (random)		0	1

All fixed effect predictors were non-significant and thus were removed from the model using a backward stepwise elimination procedure working from the bottom of the table upwards. Chi-square and P values are those that were used to eliminate terms from the linear mixed model. The coefficients of the fixed effects from the full model are plotted in Fig. 1. The significance of the random effects of year and ID were tested in a model that excluded the fixed effects.

dates, $P = 0.11$; Table 2; Fig. 1). None of the other potential correlates of reproductive performance (maternal age) or measures of reproductive investment (litter sizes and average nestling growth rate) were correlated with lactation DEE ($P \geq 0.46$; Table 2; Fig. 1). Because Fig. 1 presents partial residual lactation DEE, we have included scatter plots relating raw lactation DEE with all potential correlates of reproductive performance and measures of reproductive investment in Fig. S1 (Supporting information).

NATURAL SELECTION ON LACTATION DEE

We detected positive linear selection on lactation DEE for increased ARS ($\beta' = 0.49$, $t_{108} = 3.1$, $P = 0.002$; Table 3; Fig. 2). Linear selection gradients for potential correlates of reproductive performance (maternal mass and age) and measures of reproductive investment (parturition date, litter size and average nestling growth rate) were all non-significant (Table 3). Lactation DEE was also positively correlated with ARS in a model that excluded the potential correlates of reproductive performance and measures of reproductive investment [standardized selection differential: $s' = 0.49 \pm 0.17$ (SE), $t_{108} = 2.8$, $P = 0.005$]. Moreover, if lactation DEE is excluded from the selection analysis, the linear selection gradients of the remaining variables remained non-significant (results not shown). Overall, these results suggest that the positive selection on lactation DEE for increased reproductive success was not mediated by correlations between lactation DEE and measures of reproductive performance and investment.

All quadratic and correlation selection gradients for ARS were non-significant (Table S3, Supporting information). In this model, the linear selection gradient on lactation DEE remained significant ($\beta' = 0.42 \pm 0.16$, $t_{108} = 2.6$, $P = 0.01$).

The linear selection gradient of lactation DEE for maternal survival was low and non-significant ($\beta' = 0.04$; Table 3). We detected marginally significant negative viability selection on maternal age (Table 3), but the linear viability selection gradients of maternal body mass, parturition date, litter size and average nestling growth rate were all non-significant (Table 3). All quadratic viability selection gradients were non-significant (Table S3). None of the correlational selection gradients involving lactation DEE were significant (Table S3). However, there was a significant positive correlational selection gradient between maternal mass and average nestling growth rate ($\gamma_{ij} = 0.39 \pm 0.17$, $t_{108} = 2.3$, $P = 0.02$).

OTHER POSSIBLE FITNESS COSTS OF ELEVATED DEE

Because elevated lactation DEE was not associated with reduced survival, we tested whether elevated DEE was associated with other potential fitness costs that could be realized during the following year. Specifically, we examined whether elevated DEE was associated with (i) reduced

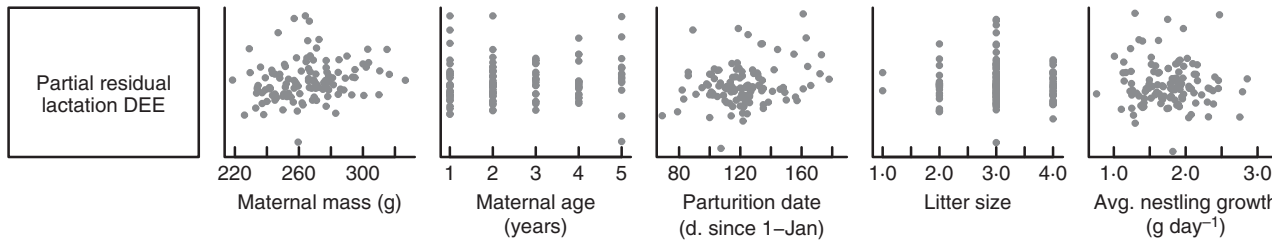


Fig. 1. Correlations between partial residual lactation daily energy expenditure (DEE) and potential measures of reproductive performance (maternal mass and age) and investment (parturition date, litter size and average nestling growth rate). Partial residual lactation DEE values hold constant the influence of all other correlates listed in the bottom row (coefficients presented in Table 1; model includes random effects of year and ID). The scales of the y-axes are not given because these values are uninformative.

Table 3. Standardized linear selection gradients ($\beta' \pm SE$) for six maternal traits in two separate analyses. Annual reproductive success (ARS) and maternal survival were the dependent variables in these two analyses

	ARS $\beta' \pm SE$	Maternal survival $\beta' \pm SE$
Lactation daily energy expenditure	0.49 \pm 0.16	0.04 \pm 0.09
Maternal body mass	0.09 \pm 0.18	0.04 \pm 0.09
Maternal age	-0.23 \pm 0.12*	-0.17 \pm 0.09*
Parturition date	-0.21 \pm 0.15	-0.1 \pm 0.09
Average nestling growth	0.06 \pm 0.16	0.03 \pm 0.09
Litter size	0.08 \pm 0.12	-0.11 \pm 0.08

Bold estimates are significant at $P < 0.05$. Significance was assessed by jackknifing. * P values between 0.05 and 0.10.

ARS, (ii) reduced lactation DEE, (iii) later parturition dates, (iv) smaller litter sizes or (v) slower average nestling growth rates in the following year. To test for these potential fitness costs, we performed a linear regression between standardized levels of DEE and standardized levels of all the potential fitness costs in the following year (values were standardized to zero mean, unit standard deviation). In all five analyses, elevated DEE was not associated with a potential fitness cost the following year (Fig. 3).

QUANTITATIVE GENETICS OF LACTATION DEE

Quantitative genetic analyses using our multigenerational pedigree revealed that the repeatability and heritability of lactation DEE were low and not significantly different from zero (Table 4). Based on log-likelihood values, the best-supported model included only the random effect of year (i.e. the null model, Table 4, Table S4, Supporting information). In a model that included fixed effects, the V_A of lactation DEE was fixed to be a very small value by ASREML to prevent this variance component from becoming negative in the iterative process of the animal model (Table 4). As a result, the heritability of lactation DEE in the model that included fixed effects was essentially zero. In a model that excluded fixed effects, the V_A was estimated (551.4 ± 1983.9), but the standard error of this esti-

mate was very large (Table S4). Moreover, the V_A of lactation DEE yielded by this model was small in comparison with the large V_P of lactation DEE ($V_P = 22827.7$; Table S4). Overall, these two analyses suggest that heritability of lactation DEE is low and not significantly different from zero.

Discussion

Females that expended more energy during mid-lactation recruited more offspring into the population the following year (i.e. had higher ARS; Fig. 2). The positive linear selection gradient of lactation DEE for ARS ($\beta' = 0.49$) ranked in the top 12% of selection gradients compiled in a comprehensive meta-analysis (including longitudinal and cohort studies of reproductive success; Kingsolver *et al.* 2001a,b).

Although positive selection on DEE for increased ARS was relatively strong, there was a considerable amount of residual variation associated with the correlation between lactation DEE and ARS (Fig. 2). A high amount of residual variation is typical of selection analyses on wild populations, because the survival of juveniles (and thus the ARS of the mother) is influenced by many factors (e.g. nutrition, territory quality, behaviour) and circumstances (e.g. predation, starvation and other hazards). It is possible that the selection on DEE was in fact on an unmeasured character, but the inclusion of an extensive suite of other potential fitness correlates makes this interpretation unlikely (*sensu* Brodie 1992).

Elevated lactation DEE neither reduced survival until the next year's breeding season (Table 3), nor negatively affected ARS, lactation DEE and reproductive investment in the following year (Fig. 3). Our results are consistent with recent avian studies that have found no relationship between DEE during the breeding season and annual survival (Welcker *et al.* 2009, 2010). Given that our measure of maternal survival was whether or not females survived to the following year, it remains possible that the maternal survival costs of elevated lactation DEE are realized at later ages. However, a lack of repeatability of lactation DEE complicates any analysis examining the relationship between lactation DEE and lifelong survival.

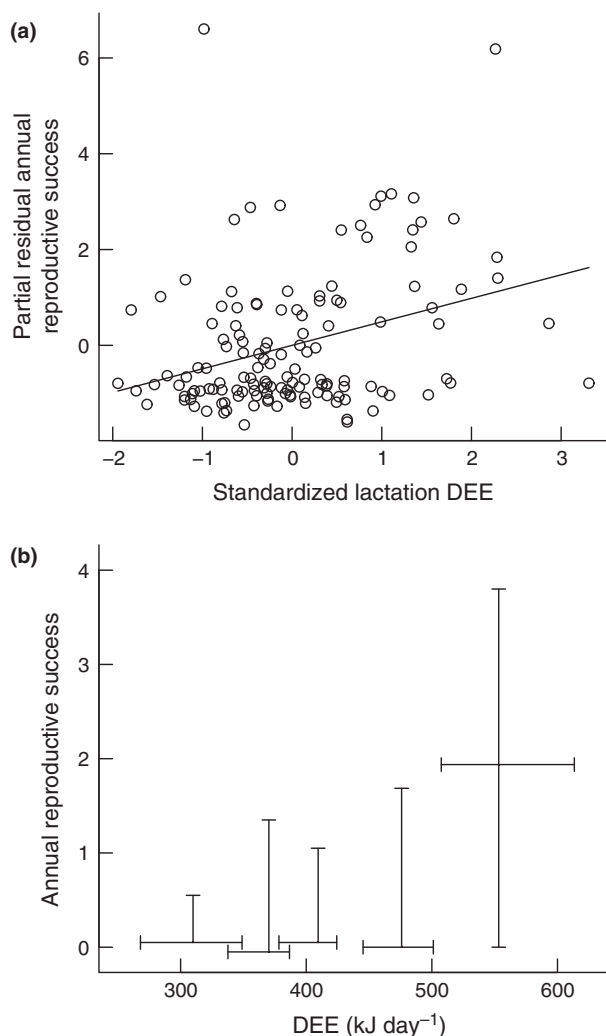


Fig. 2. Positive selection on lactation daily energy expenditure (DEE) for increased annual reproductive success (ARS). (a) The relationship between partial residual ARS and lactation DEE. Partial residual ARS holds constant the effects of the other modelled traits in the selection gradient analysis (Table 3). ARS reflects relative fitness, which is calculated by dividing the number of each females' offspring that survived overwinter by the population average number of offspring from each female that survived overwinter. The standardized selection gradient for lactation DEE (β' , Table 3) is represented by the slope of the line. The standardized selection gradient for lactation DEE is significant after removing the two potential outlier points ($\beta' = 0.46 \pm 0.12$, $P = 0.002$) with residual fitness values >6 . (b) To reflect raw values of ARS and lactation DEE, lactation DEE values were ranked and then grouped into five groups that each encompassed 20% of the lactation DEE values. Within each group, the 25th percentiles, medians (intersection) and 75th percentiles of relative ARS and raw DEE are plotted. The 25th percentile and median ARS values for the first three groups are zero, but they have been staggered around zero to show the 25th and 75th percentiles of DEE.

Considerable attention has been given to hypothesis that survival costs of reproduction are mediated by elevated levels of oxidative stress, which are elevated due to high levels of energy expenditure during reproduction (for reviews see: Costantini 2008; Dowling & Simmons 2009; Monaghan, Metcalfe & Torres 2009; Metcalfe & Alonso-Alvarez 2010;

Selman *et al.* 2012; Metcalfe & Monaghan 2013). In squirrels at this site, levels of oxidative damage to plasma proteins are elevated during lactation, especially in lactating females with high DEE (Fletcher *et al.* 2013b). These results suggest that lactating squirrels face short-term oxidative costs during reproduction; however, the results of the current study suggest that the extent and/or permanency of these costs do not appear to be great enough to compromise survival.

Previous work at this site suggests that females raising natural litter sizes may limit their lactation DEE at a rate below that which would negatively impact their survival and lifetime reproductive success (Humphries & Boutin 2000; McAdam *et al.* 2007). Humphries & Boutin (2000) experimentally augmented the litters of female squirrels and found that females had increased rates of lactation DEE compared to females raising natural litter sizes. These results suggest that females raising natural litter sizes are able to have higher rates of lactation DEE, and thus, they are operating below their maximal capacity. Moreover, based on limited data, females raising augmented litters seemed to prioritize their survival over the survival of their offspring (Humphries & Boutin 2000). However, one key assumption of this hypothesis that needs to be tested is that lactating females raising natural litter sizes are able to increase their total energy intake as well as their DEE. One limitation of the DLW technique when it is performed on lactating mammals is that it does not quantify total energy intake. This technique quantifies the energy demands associated with additional foraging and milk synthesis, but it does not quantify the energy exported in milk (Król & Speakman 2003). Thus, females raising natural litter sizes may have capacity to increase their lactation DEE, but their total energy intake may be limited at a fixed ceiling.

Positive selection on lactation DEE for increased ARS was not mediated by a correlation between lactation DEE and maternal mass. Lactation DEE was neither strongly correlated with mass (Fig. 1; Table 2; Fig. S1), nor was the correlation between lactation DEE and ARS driven by a correlation between lactation DEE and maternal mass. This is counter to studies on laboratory rodents, which have generally found positive correlations between lactation energy expenditure and reproductive investment that are explained by positive correlations between lactation energy expenditure and maternal mass (Derting & McClure 1989; Earle & Lavigne 1990; Hayes, Garland & Dohm 1992; Johnson, Thomson & Speakman 2001a).

The weak relationship between lactation DEE and maternal body mass was unexpected (see also Fletcher *et al.* 2012). This result was surprising because body mass is strongly correlated with RMR (McNab 2002; Speakman, Król & Johnson 2004). Moreover, the DLW technique generates whole-animal estimates of CO₂ production, and therefore, all else being equal, larger animals should have higher DEE. The largest lactating female in our study was 49% larger than the smallest (327 g vs. 219 g; Fig. 1),

suggesting that it is unlikely that the lack of a relationship between maternal body mass and lactation DEE resulted because of low variation in the mass of adult red squirrels. One possible explanation for the lack of a relationship is that smaller squirrels were more active, either physiologically or behaviourally, than larger squirrels during lactation. Along similar lines, it is also possible that interindividual variation in activity during lactation swamps any influence of interindividual variation in body mass as a predictor of lactation DEE. This claim is consistent with previous work on red squirrels, suggesting that maternal mass and DEE are only correlated during stages when individuals are relatively inactive (winter: Humphries *et al.* 2005 Fletcher *et al.* 2012).

Positive selection on lactation DEE for increased ARS was also not mediated by correlations between lactation DEE and measures of reproductive investment (parturition date, litter size and average nestling growth rate). As stated above, lactation DEE does not quantify the energy exported in milk (Król & Speakman 2003). Consequently, our results suggest that there is no correlation between lactation DEE and milk energy export because if this correlation existed we would have observed positive correlations between lactation DEE and both litter size and nestling growth rates (Johnson, Thomson & Speakman 2001b). It is surprising that positive selection on lactation DEE for increased ARS was not mediated through measures of reproductive investment. Lactating females with greater DEE neither had earlier parturition dates (there was a trend for greater lactation DEE to be associated with later parturition dates; Table 2), larger litter sizes, nor faster-growing nestlings. Future research is required to determine the mechanistic link underlying positive selection on lactation DEE for increased ARS. Possible areas of study include those associated with early-life maternal care and/or resource allocation (e.g. Boulinier & Staszewski 2008; Sheriff & Love 2013).

Previous research at this site including 13 years of data (1989–2001) suggested that juveniles were more likely to recruit into the population if they were born earlier in the breeding season [all years $\beta' = -0.20 \pm 0.07$ (\pm SE by jack-knifing)] and had faster growth rates in the nest (all years $\beta' = 0.34 \pm 0.10$; McAdam & Boutin 2003). In our study, the linear selection gradients of parturition date ($\beta' = -0.21 \pm 0.15$) and average nestling growth rates

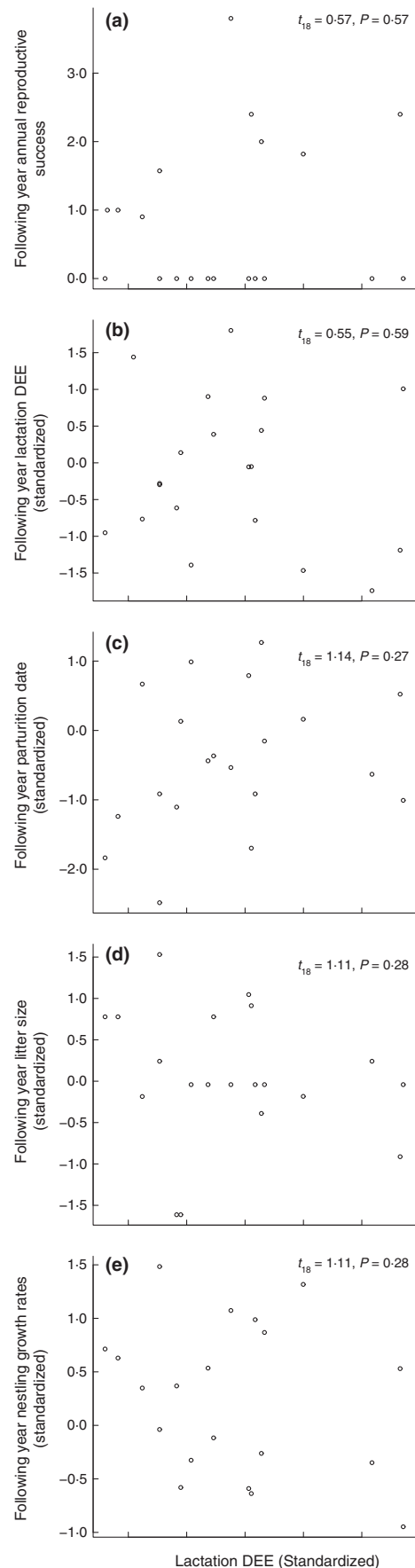


Fig. 3. Lactation daily energy expenditure (DEE) was not correlated with a suite of potential fitness costs that could be realized the following year. The five potential fitness costs we examined were (a) annual reproductive success (ARS), (b) lactation DEE, (c) parturition dates, (d) litter size and (e) average nestling growth rates. All maternal traits (DEE, parturition date, litter size and average nestling growth) were standardized (zero mean, unit standard deviation) within each year. ARS reflects relative fitness (see Fig. 2 legend or Materials and methods for details). t - and P -values from the linear regressions are presented in the top right corner of each panel.

Table 4. Animal model analysis examining the sources of variation influencing lactation daily energy expenditure of free-ranging squirrels. A suite of fixed effects related to the environment (food availability, squirrel density) and typical correlates of reproductive performance and investment (maternal mass, parturition date and nestling growth rate) were included in the model

Model	V_P	V_R	V_{YEAR}	V_I	V_A	V_{PE}	V_M	r	h^2	pe	m^2	LogL
V_{YEAR}	14174.4 (1759.1)	13831.4 (1667.5)	343.0 (754.1)	–	–	–	–	–	–	–	–	761.94
$V_{YEAR} + V_I$	14174.5 (1759.0)	13831.5 (1667.5)	343.0 (754.0)	1.0^{e-2*}	–	–	–	0.0*	–	–	–	761.75
$V_{YEAR} + V_A + V_{PE}$	14174.5 (1759.0)	13831.5 (1667.5)	343.0 (754.0)	–	4.6^{e-3*}	1.7^{e-3*}	–	–	0.0*	0.0*	–	761.75
$V_{YEAR} + V_A + V_{PE} + V_M$	14174.5 (1759.0)	13831.5 (1667.5)	343.0 (754.0)	–	4.3^{e-3*}	3.9^{e-3*}	4.9^{e-2*}	–	0.0*	0.0*	0.0*	761.75

Variance components (\pm SE in brackets) are presented for four different random effect models. V_P , V_R and V_{YEAR} refer to the total phenotypic variance, the residual variance and the variance associated with year, respectively. In the second model, the individual variance (V_I) is not partitioned into V_A (additive genetic variance) and V_{PE} (permanent environment variance). In the third model, V_I is the sum of V_A and V_{PE} . V_M (maternal variance) is only examined in the fourth model. The repeatability (r), narrow-sense heritability (h^2), the permanent environment effects (pe) and the maternal effects (m^2) are presented where applicable. Log-likelihood values are provided for each model. The most supported model based on log-likelihoods is indicated in bold. Dashed cells were not fitted. *variance components and SEs that were fixed to a small positive value by ASREML – SEs are not shown in these cells.

($\beta' = 0.06 \pm 0.16$) for ARS were in the same direction; however, neither of these selection gradients was significant ($P > 0.16$). The lack of selection on nestling growth rates in our current study may be explained by the recent finding that selection on nestling growth rates is stronger in years of high squirrel density (Dantzer *et al.* 2013; study years: 1989–2011). Thus, McAdam & Boutin (2003) likely detected positive selection on nestling growth rates, whereas we did not because squirrel density was higher in the study years encompassed by McAdam & Boutin (2003; study years: 1989–2001) as compared to in our study (study years: 2002–2008; see Fig. 1A in Dantzer *et al.* 2013).

The lack of heritability in lactation DEE is in contrast with high heritability estimates for RMR in free-ranging birds and mammals (RMR $h^2 > 0.40$ in models including fixed effects – Nilsson, Akesson & Nilsson 2009; Bushuev *et al.* 2012; Zub *et al.* 2012). We recognize that animal models generally require large sample sizes (Wilson *et al.* 2010). Although the number of individuals with DEE data in our pedigree was comparable to other studies that have examined the heritability of RMR in free-ranging animals ($n = 413$ individuals, *F. hypoleuca*, Bushuev *et al.* 2012; $n = 128$ individuals, *M. nivalis*, Zub *et al.* 2012; $n = 122$, *C. caeruleus*, Nilsson, Akesson & Nilsson 2009), it was much smaller than what has been used in studies examining the heritability of morphological and phenological traits in the wild (Kruuk *et al.* 2002; Réale *et al.* 2003; Sheldon, Kruuk & Merilä 2003). It is recognized that the inclusion of fixed effects into animal model analyses influences heritability estimates (Wilson 2008). However, lactation DEE was not significantly heritable in animal model analyses that either included or excluded an extensive suite of fixed effects related to the environment and reproductive parameters (Table 4). Our animal model analysis indicated that lactation DEE was also not significantly repeatable (repeatability sets an upper limit to heritability – Falconer & Mackay 1996; but see Dohm 2002). The number of

individuals with repeated measures in our study ($n = 28$) ranked at the 41st percentile in an extensive meta-analysis examining the repeatability of energetic traits (using all sample sizes presented in White, Schimpf & Cassey 2013 – Table S1; see also Nespolo & Franco 2007).

Lactation DEE in squirrels likely has low levels of repeatability and heritability because in contrast to RMR, DEE is an integrative trait that also reflects the behaviour of animals and their responses to environmental conditions (McNab 2002). Moreover, it is also possible that the strong directional selection on lactation DEE has eroded the additive genetic variance underlying this trait by driving the high-fitness alleles to fixation (Fisher 1958). Regardless of the possible reason, the lack of heritability of lactation DEE suggests that this trait is not genetically correlated with other physiological, behavioral or life-history traits (Ricklefs & Wikelski 2002; Careau *et al.* 2008; Biro & Stamps 2010; Réale *et al.* 2010) and that there is limited evolutionary potential for lactation DEE to respond to our documented selection.

Research within this population of squirrels (McAdam & Boutin 2003; Réale *et al.* 2003; Descamps *et al.* 2008), and in many other study systems (e.g. Clutton-Brock [ed.] 1988), has focused on a suite of typical measures of reproductive performance (maternal mass and age) and measures of reproductive investment (parturition/laying date, litter/clutch size and juvenile mass/growth rates) as predictors of reproductive success. Here, we found positive linear selection on lactation DEE for ARS that was stronger than selection on these other traits in this population of red squirrels (McAdam & Boutin 2003; Réale *et al.* 2003) and in many other studies that have examined these traits (i.e. our DEE for ARS ranked in the top 12% of selection gradients for reproductive success; Kingsolver *et al.* 2001a, b). Overall, our results suggest that DEE is an important and under-quantified determinant of reproductive success in wild populations.

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DATA ACCESSIBILITY

Data deposited in the Dryad repository: <http://doi.org/10.5061/dryad.635md> (Fletcher *et al.* 2014).

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Supporting Information

Additional Supporting information may be found in the online version of this article:

Table S1. AIC_c analysis of the predictors of lactation DEE.

Table S2. Summary statistics for squirrel pedigree.

Table S3. Standardized quadratic selection gradients.

Table S4. Animal model analysis examining the sources of variation influencing lactation DEE.

Fig. S1. Scatter plots examining the predictors of lactation DEE.