

## RESEARCH ARTICLE

# Evolved changes in maternal care in high-altitude native deer mice

Cayleigh E. Robertson\* and Grant B. McClelland

## ABSTRACT

At high altitude (HA), unremitting low oxygen and persistent cold push small mammals close to their metabolic ceilings, leaving limited scope for aerobically demanding activities. However, HA breeding seasons are relatively short and endemic rodents compensate with larger litters than low altitude (LA) conspecifics. Rodent mothers are the sole source of heat and nutrition for altricial offspring and lactation is energetically costly. Thus, it is unclear how HA females balance energy allocation during the nursing period. We hypothesized that HA female rodents invest heavily in each litter to ensure postnatal survival. We measured maternal energetic output and behaviour in nursing deer mice (*Peromyscus maniculatus*) native to LA (400 m a.s.l.) and HA (4350 m a.s.l.) under control (24°C, 760 mmHg) and cold hypoxia conditions, simulating HA (5°C, 430 mmHg). Strikingly, resting metabolic rates of lactating HA and LA females under cold hypoxia were 70–85% of their maximum aerobic capacity. In cold hypoxia, LA mothers increased both nursing time and milk fat content, however their pups were leaner and severely growth restricted at weaning. HA mothers also increased nursing in cold hypoxia but for far less time than LA mothers. Despite receiving less care, HA pups in cold hypoxia only experienced small growth restrictions at weaning and maintained body composition. As adults, HA mice raised in cold hypoxia had increased aerobic capacity compared to controls. These data suggest that HA mothers prioritize their own maintenance costs over investing heavily in their offspring. Pups compensate for this lack of care, likely by reducing their own metabolic costs during development.

**KEY WORDS:** Maternal behaviour, Lactation, Developmental plasticity, Maternal-offspring conflict, *Peromyscus*

## INTRODUCTION

High altitude (HA) regions are characterized by low ambient temperatures and hypobaric hypoxia. As a result of these combined environmental stressors, the daily energy expenditure of small, obligate endotherms native to HA is much higher than conspecific lowlanders. In fact, measurements of field metabolic rate in deer mice (*Peromyscus maniculatus*) living at HA suggest they routinely operate close to their maximum aerobic capacity ( $\dot{V}_{O_{2,max}}$ ; Hayes, 1989). This high cost of living limits aerobic reserve ( $\dot{V}_{O_{2,max}}$ –field metabolic rate), leaving little scope for additional aerobically demanding activities. Despite these significant metabolic challenges, small rodents and lagomorphs have the highest altitudinal ranges of any mammals across numerous mountain ranges (Hock, 1964; Kramer et al., 1999; Ci et al., 2009; Storz et al.,

2020). These species also complete their entire reproductive cycle at HA, under the persistent cold and unremitting hypoxic conditions of this hostile environment.

The physiological challenges at HA are further exacerbated when animals are required to engage in energetically costly activities. Of note, for a female mammal lactation is the most energetically demanding period of her adult life (Miller, 1979; Speakman, 2008). Indeed, energy requirements in all lactating mammals increase 2- to 3-fold (Prentice and Prentice, 1988), most of this energy goes directly to milk production. For example, milk production in lactating laboratory mice accounts for ~50% of energy intake (Johnson et al., 2001). During the early postnatal period, young eutherian mammals rely exclusively on maternal milk for nourishment (Langer, 2008). Thus, this period after birth is when nursing mammalian mothers and their offspring are in the most direct conflict for resources (Trivers, 1974; Rogowitz, 1996). As such, maternal investment during the early nursing period varies considerably in mammals and numerous distinct life-history strategies have evolved (Stearns, 1989; Langer, 2008). However, it is unclear how maternal investment strategies have evolved under the extreme conditions at HA.

Environmental conditions at low altitude (LA) have been shown to affect the quantity and quality of maternal investment between reproductive bouts within an individual's lifespan, particularly during the lactation period (e.g. Stewart and McAdam, 2014). Factors that may vary between environments include care behaviour (e.g. time spent nursing), milk output and milk nutrient content. The physiological limits to food intake (Johnson et al., 2001) result in females often prioritizing their own maintenance over that of their offspring, particularly during periods of high energetic stress (Rogowitz, 1996). For example, wild rodent mothers spend less time caring for or nursing pups when temperatures are colder, or when population density is high and competition for food increases (e.g. Hill, 1972; Stewart and McAdam, 2014). At its extreme, decreases in maternal investment when rodents are energetically stressed can take the form of infanticide (e.g. Sikes, 1996). Ultimately, by decreasing investment in one litter when conditions are poor, resources can be preserved for future litters and can help optimize a female's lifetime reproductive fitness (Stearns, 1989).

At high elevations, fewer frost-free days likely result in a relatively short breeding season compared with LA environments of the same latitude (McLean et al., 2019). In rodents with broad altitudinal distributions, females from populations at higher elevations tend to give birth to larger litters than their low altitude counterparts, likely to compensate for this shorter breeding season (e.g. *Phyllotis xanthopygus*, Sassi et al., 2018; *P. maniculatus*, Dunmire, 1960; Halfpenny, 1980; Robertson et al., 2019; McLean et al., 2019). However, litter size is one of the primary drivers of energy requirement during lactation, with more pups requiring greater milk production (Miller, 1979). Thus, HA mothers would have a greater energetic burden to raise these larger litters.

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The overall cost of care may be even higher for altricial species where mothers act not only as a source of food, but also as an external source of heat until offspring are able to independently thermoregulate (Hill, 1972). We have previously shown that thermoregulatory mechanisms mature more slowly postnatally in HA native deer mice compared with LA conspecifics (Robertson et al., 2019; Robertson and McClelland, 2019). Even at 10 days after birth, HA pups are unable to maintain body temperature in the face of a very mild cold stress (10 min at 24°C), whereas by this age, LA pups can successfully thermoregulate. This developmental delay extends in highlanders until weaning at 21 days of age, after which they quickly surpass lowlanders in overall thermogenic capacity (Robertson and McClelland, 2019). This suggests that HA pups may rely more heavily on external maternal heat during the nursing period, and that energetic costs during lactation are exacerbated at HA by a need for increased maternal care.

Studies on laboratory rats suggest LA native rodents are unable to adequately provision their offspring at HA (Weihe, 1965). In the wild, this species is found exclusively at LA (Jochams-Lemoine et al., 2015), and when laboratory rats were mated at HA, their offspring were of poor condition compared to those born at LA (Weihe, 1965). Milk production is inhibited for several LA mammals living at HA or exposed to simulated altitudes greater than 3000 m above sea level (a.s.l.), and reduced milk production directly results in severe growth restrictions of their offspring (Moore and Price, 1948; Weihe, 1965; Bruder et al., 2008). When exposed to combined hypoxia and cold experienced at HA, rats are unable to produce enough milk to provision their pups, even when supplemented with a high fat diet. As a result, offspring mortality is extremely high (Weihe, 1965).

Taken together, these data suggest that at HA neonates require substantial energetic investment from their mothers to survive until weaning. Along with the many energetic challenges, females living at HA likely have limited reproductive opportunities during a compressed breeding season (McLean et al., 2019). Females who have evolved at HA probably concentrate their reproductive efforts across a smaller number of more costly litters. Thus, they may not be able to prioritize their own maintenance costs over the energetic demands of their pups. The goal of this study was to understand how maternal investment has evolved at HA. Therefore, we tested the hypothesis that HA adapted rodents, such as deer mice, employ an 'all or nothing' approach to reproduction, prioritizing heavy investment into their offspring during the postnatal period.

To test this hypothesis, we used a common garden experimental design to examine maternal energy output and behaviour in LA and HA populations of deer mice (*P. maniculatus*) under both simulated LA (warm normoxia) and HA (cold hypoxia) conditions. This design allows us to isolate the effects of HA ancestry from those of the cold hypoxic environment. We allowed females to raise their natural litter sizes and we predicted that under control LA conditions litter size would be the major factor distinguishing difference in maternal investment. However, under conditions of cold hypoxia we predicted that LA females would decrease maternal investment and prioritize their own maintenance, while HA females would not. Any differences in maternal care should be reflected in offspring phenotype during nursing and subsequently their phenotypes at adulthood.

## MATERIALS AND METHODS

### Animals

Breeding pairs used in this study were the first generation (G<sub>1</sub>) laboratory-born progeny of wild caught HA and LA native deer

mice [*Peromyscus maniculatus* (Wagner 1845)]. Wild HA mice (*P.m. rufinus*) and LA mice (*P.m. nebracensis*) were trapped at the summit of Mount Evans, CO (4350 m a.s.l.) and from Nine Mile Prairie, NE (430 m a.s.l.; Cheviron et al., 2012), respectively. Since latitude of origin strongly influences breeding sensitivity to photoperiod in *Peromyscus* (Dark et al., 1983), this LA subspecies was chosen as they are the geographically closest (~750 km), while genetically distinct population, found at a similar latitude (~1.2 deg) as the HA deer mice. These two subspecies are approximately 200,000 generations diverged but are interfertile and hybrid intermediates exist all along the altitudinal cline that separates them (Natarajan et al., 2015). Wild mice were transported to McMaster University, Canada (~90 m a.s.l.) and bred within their respective populations under common garden conditions (24°C, 760 mmHg, 14 h:10 h light:dark cycle, with rodent chow and water *ad libitum*). These conditions mimic the LA breeding season. Once they reached adulthood (90 days old), 10 virgin G<sub>1</sub> females from each population were mated to unrelated G<sub>1</sub> males of the same altitude ancestry. Once pregnancy was confirmed, males were removed until the litter was weaned at postpartum day (P) 21. After weaning, G<sub>2</sub> pups were housed with their same-sex littermates until adulthood (P90) with a maximum of 5 individuals per cage. Breeding success in captivity is highly variable, so we allowed breeding pairs to have 3 litters for females to establish their own maternal care phenotype before starting the experiment at litter number 4. Breeder males were removed from the cages once pregnancy was confirmed. Females were re-paired with their original breeder male for litter 5. As such, all offspring from a given female are full siblings. All procedures were approved by the McMaster University Animal Research Ethics Board.

### Experimental design

Once the females gave birth to their fourth litter, they were given 24 h with their pups undisturbed. On P1, mothers and pups were weighed and placed in a clean cage. Food was weighed and the cage was moved to an observation room with the same light cycle, temperature and oxygen level as the breeding room described above. All measures were repeated on the next litter but raised under cold hypoxic conditions (CH), simulating the native HA environment (5°C, 430 mmHg). Mothers and pups were moved into the cold room only once the litter was born. To isolate the effects of CH on postnatal care strategy we deliberately chose not to pre-expose females to cold hypoxia during pregnancy as gestational hypoxia can cause both fetal intrauterine growth restriction and impact maternal fat accumulation and remodelling of neural circuitry that are important for care/lactation (Napso et al., 2018). The next day (P1) mothers, pups and food were weighed and placed in a clean cage inside hypobaric chambers at 430 mmHg. These conditions mimic the summit of Mount Evans, CO during the summer months (United States National Weather Service; <https://www.weather.gov/>). We quantified maternal behaviour between P2 and P8, leaving mothers and pups undisturbed. After a final maternal care observation on P8, we performed a maternal provisioning test (see below). At P12, maternal resting metabolic rate (RMR) was measured. At P21 mothers and all pups were weighed, and pup body composition was determined for 1 male and 1 female from each litter (see below). After weaning at P21, remaining pups were returned to common control holding conditions and raised until adulthood (P90), at which time thermogenic capacity was measured (detailed below). We started with 10 females from each population. Once the experiment began, females who did not successfully raise their control or cold hypoxic litter to weaning were given 1 additional breeding attempt in that

experimental treatment. 7 HA and 6 LA females completed the control trials. Under cold hypoxia, 3 HA females repeatedly committed infanticide of their entire litters. By contrast all LA females attempted to raise their entire litters to weaning under cold hypoxia. There was 1 LA female mortality during cold hypoxia.

### Maternal behaviour

We performed maternal behaviour observations starting at 12:00 h on P2 until 08:00 h on P8 using comprehensive protocols for scoring maternal behaviour previously developed for laboratory rodents (Champagne et al., 2003) and validated in wild *P. maniculatus* (Stewart and McAdam, 2014). Five observation periods of 1 h each were performed during a 24 h cycle, 3 equally spaced during the light phase (08:00 h, 12:00 h and 17:00 h) and 2 during the dark phase under red light (22:30 h, 02:30 h). During each 1 h observation period, 21 observations were made. Every 3 min, maternal behaviour was scored, first as nursing (either attached pups visible or incubating pups in nest) or not nursing (not in contact with pups). Other behaviours were noted that were not mutually exclusive (eating, drinking, grooming, active, nest building, carrying pups). Each mother's scores for each behaviour were averaged for every timepoint across the 7 day observation period.

### Provisioning test

On P8, while pups remained in the home cage mothers were moved to a new cage and separated from their pups for 4 h. During that time, mothers were fed *ad libitum*, but pups were not fed. At the end of this separation, mothers and pups were individually weighed. Each pup was given a distinctive mark using a (nontoxic) marker. Mothers were then returned to the home cage with pups and filmed for the next 3 h to determine the time spent nursing. At the end of the 3 h, pups and mothers were again individually weighed. Provisioning rate was calculated by multiplying litter weight gain by hour nursing (Hager and Johnstone, 2006, 2007; Stewart and McAdam, 2014). We used the quotient of litter weight gain and maternal weight loss during the time spent nursing as a measure of provisioning efficiency.

### Maternal resting metabolic rate

We used flow-through respirometry (Robertson et al., 2019) to determine maternal resting metabolic rate (RMR) during lactation. Trials were performed when pups were at P12, which corresponds to the period of peak lactation (Król and Speakman, 2003). To determine metabolic rates, mothers were placed in 475 ml plexiglass chambers placed inside a Peltier temperature control cabinet (Sable Systems, NV) maintained at either 28°C or 5°C. Incurrent air was either outside air (normoxic) or 12% O<sub>2</sub> (88% N<sub>2</sub>, hypoxic), dried, stripped of CO<sub>2</sub> and pushed through chambers at 1000 ml min<sup>-1</sup> using a mass flow controller (Sable Systems). Excurrent air was subsampled at 150 ml min<sup>-1</sup>, dried using magnesium perchlorate and passed through O<sub>2</sub> and CO<sub>2</sub> analyzers (Sable Systems). Each trial lasted 2 h, with the first 45 min as an adjustment period. Resting metabolic rate was determined as the average of the 3 lowest stable 5 min of  $\dot{V}_{O_2}$  in this last 75 min. Data acquisition and analysis were performed using Expedata software (Sable Systems).

### Milk collection

After the RMR trial, mothers were placed in a clean cage and fed *ad libitum* for an additional 2 h to allow milk to accumulate. Mothers then received 2 IU kg<sup>-1</sup> oxytocin via intraperitoneal injection to promote milk let down (Willingham et al., 2014). After oxytocin

injection, mothers were anesthetized using isoflurane (2 litres min<sup>-1</sup>), each teat was manually palpated, and droplets of milk were collected using a 20 µl pipette. Milk samples were frozen and stored at -80°C for further analysis (Görs et al., 2009).

### Milk creatinocrit

Milk samples were thawed and collected in 100 µl glass capillary tubes. Tubes were centrifuged at 10,000 g for 15 min. Creatinocrit was determined as the ratio of the height of cream layer to the total height of milk (×100) and is directly proportional to fat content (Fleet and Linzell, 1964; Lucas et al., 1978).

### Pup body composition

We used a quantitative magnetic resonance imager (EchoMRI 4-in1, EchoMRI, Houston, Texas) to non-invasively determine lean mass (g) and fat mass (g) of G<sub>2</sub> pups at P21 and adulthood. Body composition was determined as: 100×fat mass/body mass.

### Offspring thermogenic capacity

To assess the long-term effects of maternal care phenotype on offspring adult (P90) metabolic phenotype, we measured thermogenic capacity (defined as cold-induced  $\dot{V}_{O_2,max}$ ) under normoxic (21% O<sub>2</sub>) and hypoxic (12% O<sub>2</sub>) conditions (Robertson and McClelland, 2019). Briefly, adult mice were placed in sealed glass respirometry chambers (475 ml) at -5°C. Heliox (21% or 12% O<sub>2</sub> balanced with He) was flowed through the chambers at 1000 ml min<sup>-1</sup>. Subsampled excurrent air was dried before being passed through O<sub>2</sub> and CO<sub>2</sub> analysers (FoxBox Respiratory System, Sable Systems). Data acquisition was performed using LabChart software (ADI instruments) and cold-induced  $\dot{V}_{O_2,max}$  was calculated from 10 s of the highest stable  $\dot{V}_{O_2}$  (Tate et al., 2017). Rectal body temperature was taken before and after each trial to ensure that the trial had induced hypothermia.

### Statistical analysis

We used linear mixed effects models fit with restricted maximum likelihood (REML), using the lmer package (<https://github.com/lme4/lme4/>) for R (<https://www.r-project.org/>), to test the effects of population (i.e. altitude ancestry) and treatment (warm normoxia versus cold hypoxia) on data for litter size, weekly maternal energy intake, change in litter mass per nursing bout, provisioning rate, provisioning efficiency, creatinocrit, maternal behavioural trait, and pup body composition at weaning. All data were nested within family (for pups) or individual (for dams) to avoid pseudo-replication and to account for the fact that not all females completed both treatment conditions (Bolker et al., 2009).

We included the effect of time for measurements taken repeatedly over the nursing period (daily energy intake, maternal body mass, pup mass). We included sex as a fixed factor when testing measurements of offspring adult phenotype (adult body mass, adult body composition, adult thermogenic capacity). Additionally, we included body mass as a covariate for measures of metabolic rate (maternal RMR, offspring cold-induced  $\dot{V}_{O_2,max}$ ). A chi-squared test was used to compare the proportions of pups in a litter who lost weight during a nursing bout. A linear regression was used to test the effects of litter size on change in pup mass per nursing bout. We used an alpha of 0.05 to determine statistical significance.

## RESULTS

### Maternal energy intake

To understand how mothers adjusted energy intake while nursing, we tracked food consumption over the initial lactation period

(P2–P8). All mothers increased food consumption over this period (significant effect of postnatal day on energy intake,  $P < 0.001$ ) but HA mothers consumed more food than LA mothers (significant effect of population,  $P = 0.033$ ) (Fig. 1A). This increased food consumption in HA mothers could be accounted for principally by increased litter size, as food intake per pup was equivalent between the populations ( $P = 0.416$ ) (Fig. 1B). In the cold hypoxic (CH) conditions, we tracked weekly food intake to avoid disturbing the hypobaric chambers but found there was no increase in food consumption in CH compared with warm normoxic control conditions for either population (no significant effect of treatment conditions,  $P = 0.598$ ) (Fig. 1C). In CH conditions, HA mothers also ate more than LA mothers ( $P = 0.046$ ), but these mothers consumed the same amount of food per individual pup ( $P = 0.637$ ) (Fig. 1D).

### Maternal energy output

HA mothers were heavier throughout the lactation period than were LA mothers, regardless of environmental conditions ( $P = 0.020$ ). HA mothers also gave birth to larger litters, with a mean of 6.5 pups while LA mothers had a mean litter size of 4.3 pups ( $P = 0.049$ ), consistent with previous reports on the same populations (Robertson et al., 2019; Robertson and McClelland, 2019). However, in CH conditions, litter size was generally smaller ( $P = 0.029$ , Fig. 2B).

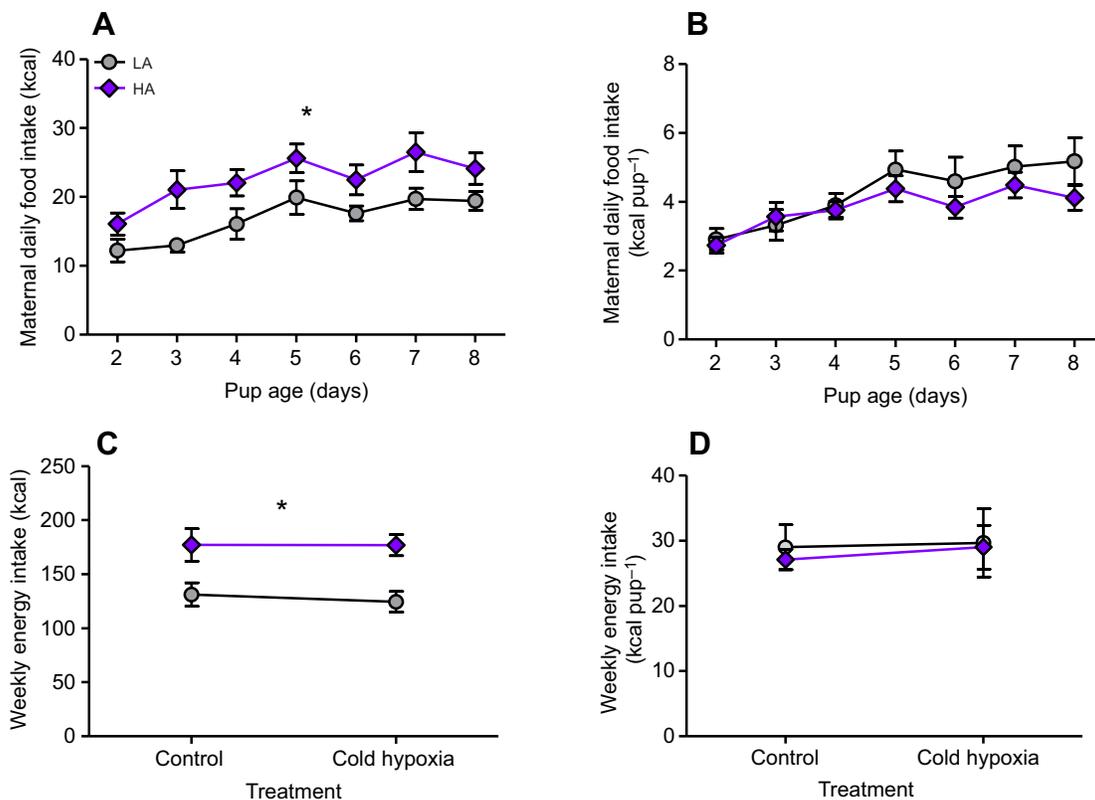
The RMR of lactating females was similar between the populations ( $P = 0.330$ ), regardless of exposure conditions and was 1.5–1.8-fold higher than virgin females under control conditions. RMR of lactating females increased by ~36–52% under CH

environmental conditions compared with control conditions ( $P < 0.001$ ) (Fig. 2C).

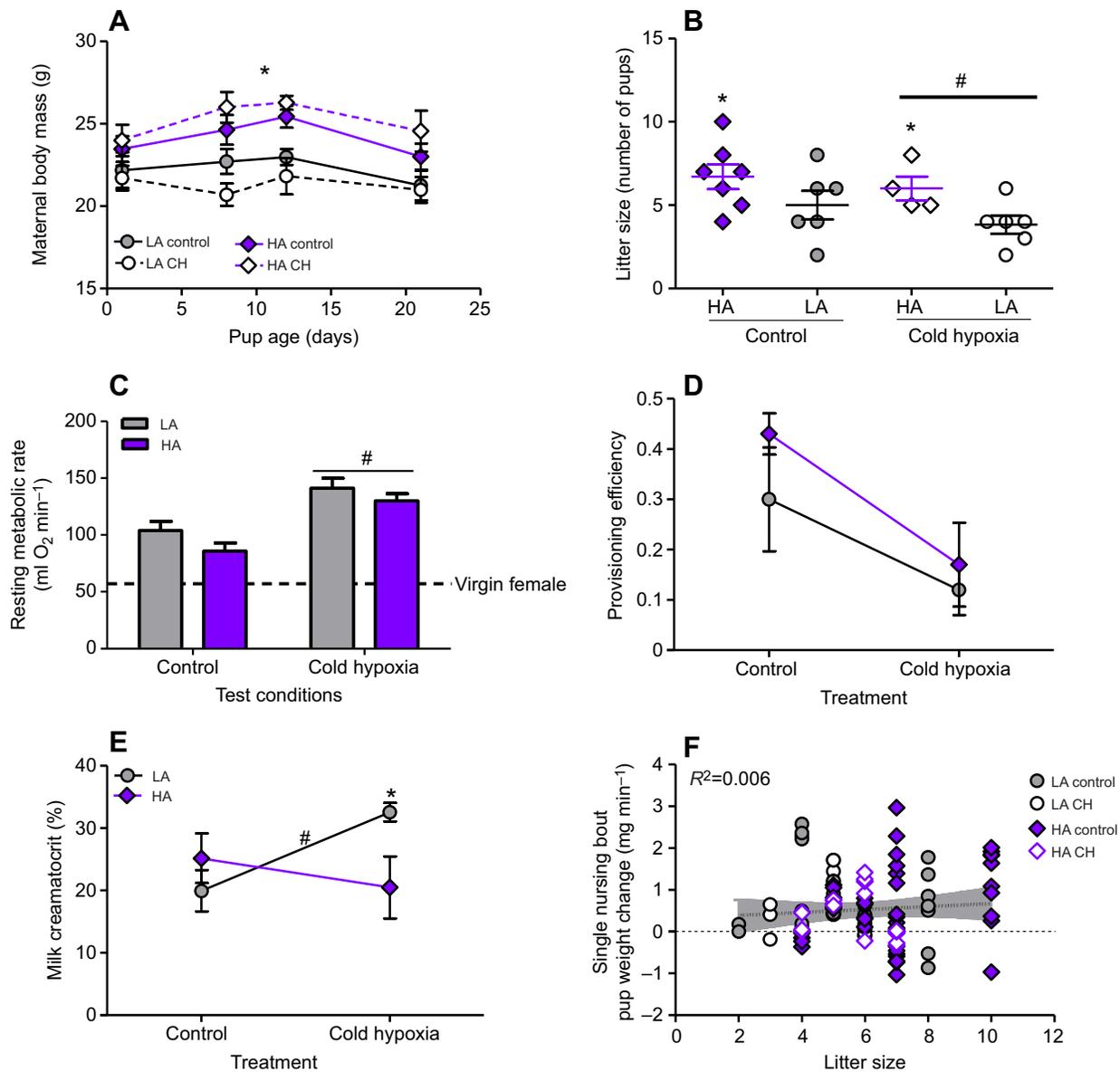
Maternal provisioning rate, defined as pup weight gain after a single nursing bout, was similar between populations ( $P = 0.522$ ). We also compared litter weight gain to maternal weight loss over the same nursing bout to determine provisioning efficiency. We saw no effect of population ( $P = 0.667$ ) nor of experimental treatment ( $P = 0.260$ ) on provisioning efficiency (Fig. 2D). Creamatocrit was used as an index of milk energy and fat content. This measure was also similar between populations under control conditions. However, LA mothers showed an increase in milk fat when caring for pups in CH (a significant Population  $\times$  Treatment interaction,  $P = 0.008$ ) (Fig. 2E). We found that during a single nursing bout, ~20–30% of pups per litter lost at least some weight (Fig. 2F). However, the proportion of pups losing weight did not significantly change between any of the groups tested ( $\chi^2 = 1.089$ ,  $P = 0.780$ ). When assessing weight changes over a single nursing bout and taking into account the individual pups who lost weight, there was no significant effect of either population ( $P = 0.611$ ) or environment ( $P = 0.207$ ) on the mean pup weight change. There was also no correlation between this factor with litter size ( $R^2 = 0.006$ ,  $P = 0.653$ ).

### Maternal behaviour

Daily maternal behaviour was quantified to assess any impacts of altitude ancestry on maternal care. Females of both populations spent ~95% of their time during the day in their nests, nursing their pups (significant effect of time of day,  $P < 0.001$ ), consistent for a



**Fig. 1. Energy intake of lactating mothers from low- and high-altitude native *Peromyscus maniculatus* in warm normoxic control and simulated high altitude conditions (cold hypoxia).** Daily maternal food intake (A) and energy intake per pup (B) under control conditions during postnatal (P) days 2–8. Weekly maternal food intake (C) and energy intake per pup (D) under control [low altitude (LA),  $N = 6$ , high altitude (HA),  $N = 7$ ] and cold hypoxic (CH) (LA,  $N = 5$ , HA,  $N = 4$ ) conditions. \* indicates significant main effect of population (altitude ancestry) as determined in linear mixed models. Data are means  $\pm$  s.e.m.



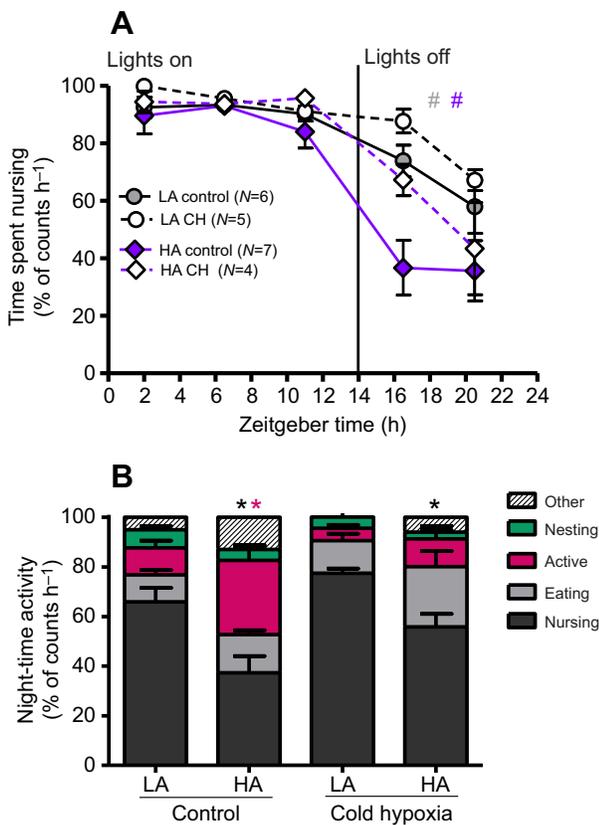
**Fig. 2. Energy output of nursing mothers from low- and high-altitude native *P. maniculatus*, under warm normoxic control and simulated high altitude conditions.** (A) Maternal body mass throughout the nursing period. (B) Litter size. (C) Resting metabolic rate (RMR) of lactating females measured at peak lactation (postnatal day 12–14). Dashed line represents mean RMR of virgin LA and HA females (unpublished data). (D) Provisioning efficiency, the ratio of provisioning rate (litter mass gain in  $\text{g h}^{-1}$ ) to rate of maternal weight loss ( $\text{g h}^{-1}$ ). (E) Milk creatocrit (% cream), an indicator of fat content. (F) Change in individual pup mass per minute of nursing bout. Dashed line at  $0 \text{ mg min}^{-1}$  represents no net change in pup mass. Solid line represents the line of best fit by linear regression, including 95% confidence intervals (shaded grey). \* indicates a significant effect of population (altitude ancestry); # indicates effect of treatment within a population as determined by liner mixed model. Control (LA,  $N=6$ ; HA,  $N=7$ ) and cold hypoxia (CH) (LA,  $N=5$ ; HA,  $N=4$ ) data (A–E) are means  $\pm$  s.e.m.

nocturnal species (Fig. 3A). We, therefore, carried out a more in-depth analysis of night-time behaviour and found that during this period, HA mothers spent 30% less time nursing or in contact with pups ( $P=0.006$ ) than LA mothers (Fig. 3A,B). Overall, CH exposure caused mothers from both populations to increase time spent in the nest nursing their pups ( $P=0.026$ ). However, there was no effect of CH treatment on the population difference in time spent nursing at night (Population  $\times$  Treatment interaction,  $P=0.546$ ). Interestingly, HA mothers spent a similar time nursing pups under CH conditions as LA mothers under warm normoxic, control conditions. In the control conditions, HA mothers spent 20% more time being active away from their pups (running, climbing etc.), but under CH conditions, HA mothers decreased their out-of-nest activity while

LA mothers did not (significant Treatment  $\times$  Population interaction,  $P=0.006$ ). However, there was no difference between populations in nest building activity ( $P=0.411$ ) or under either experimental treatment ( $P=0.182$ ). In addition to these behaviours, we found that in CH conditions, 3 of the 7 HA mothers repeatedly abandoned their litters or committed infanticide resulting in 100% pup mortality. In contrast, for LA mothers, barring one mortality, raised all their pups to weaning without abandonment under CH conditions.

#### Offspring postnatal phenotype

Overall, LA pups were smaller than HA pups throughout the nursing period (from P1 to P21,  $P=0.029$ ). The CH treatment led to growth restrictions in both populations ( $P=0.036$ ). However, for



**Fig. 3. Effect of simulated high altitude on maternal behaviour of low- and high-altitude deer mice (*P. maniculatus*) during days 2 to 8 of lactation.** (A) Daily pattern of time spent nursing. Zeitgeber time (ZT) 0 is 06:00 h. The solid vertical line indicates ZT hour 14, when lights were turned off. # indicates a main effect of treatment on behaviour within a population. (B) Proportional time spent of nighttime activities. \* indicates a significant population difference within treatment ( $P < 0.05$ ). All data are presented as means  $\pm$  s.e.m. A sample size of  $N=1$  represents all data from a single mother over 7 repeated day/night cycles.

LA pups this environmental effect was much more severe, and by P21 these pups only achieved 65% of the mass of their control siblings. In comparison, by weaning, HA pups in CH conditions grew to 88% of the mass of pups in control conditions (Fig. 4A). For LA pups, this reduction in mass occurred in conjunction with a substantial decrease in % body fat at P21, which was reduced from ~23% body fat in control conditions to ~18.5% in CH conditions (Population  $\times$  Treatment;  $P=0.006$ ). This effect was not observed in HA pups who maintained a constant ~23% body fat in either control or CH conditions (Fig. 4B).

### Offspring phenotype at adulthood

To understand the potential persistent effects of rearing environment on metabolic phenotype later in life we assessed offspring phenotypes of LA and HA deer mice at adulthood. We only included data from families that successfully completed both the control and CH treatment. In pups of LA and HA families that were exposed to both control and CH conditions we found significant effects of sex for measures of body composition and body size. Therefore, data from males and females were considered separately.

There was no difference in adult body mass of LA and HA male mice regardless of developmental treatment (Population,  $P=0.518$ ; Treatment,  $P=0.652$ ). However, LA females were smaller than HA females ( $P=0.001$ ) (Fig. 5A).

LA mice were generally leaner than HA mice ( $P=0.030$ ). However, LA males raised in CH conditions until weaning showed increased fat mass as adults, resulting in a similar body composition to HA males (Population  $\times$  Treatment,  $P=0.026$ ) (Fig. 5B). There was no effect of rearing environment on body composition of female mice (Population,  $P=0.1471$ ; Treatment,  $P=0.5741$ ).

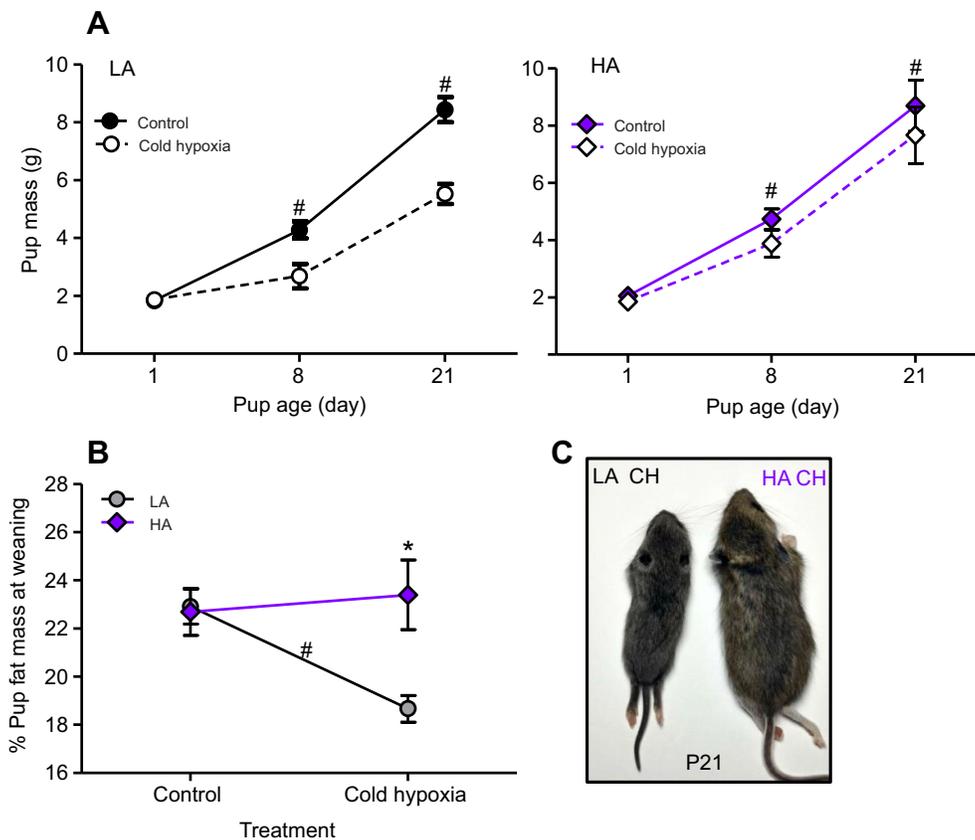
Rearing environment had a significant effect on adult thermogenic capacity, depending on population both when determined in normoxia (Population  $\times$  Treatment,  $P=0.001$ ) and hypoxia (Population  $\times$  Treatment,  $P=0.003$ ). Early life CH exposure increased adult cold-induced  $\dot{V}_{O_{2,max}}$  in HA mice when tested under both normoxia ( $P < 0.001$ ) and hypoxia ( $P=0.003$ ) (Fig. 5C,D). There was no influence of sex, independent of body mass on cold-induced  $\dot{V}_{O_{2,max}}$  determined in hypoxia ( $P=0.329$ ) or in normoxia ( $P=0.207$ ). In contrast, for LA mice, there was no effect of developmental exposure on cold-induced  $\dot{V}_{O_{2,max}}$ . This was true of both cold-induced  $\dot{V}_{O_{2,max}}$  in normoxic ( $P=0.780$ ) and hypoxic ( $P=0.614$ ).

### DISCUSSION

In this study we tested the hypothesis that in HA deer mice, mothers have evolved to maximize investment in their offspring during lactation compared with LA conspecifics. However, our results show that female HA deer mice consistently invest less energy in their pups relative to LA females. Although the cost of lactation was a remarkably high proportion of aerobic capacity, especially under CH, both LA and HA mothers showed substantial capacity for flexibility in maternal care when rearing pups in these conditions. However, only HA mothers were able to adequately rear pups in cold hypoxic conditions. HA pups maintained body composition at weaning and only experienced mild growth restrictions in CH conditions. This occurred despite HA mothers providing significantly less maternal care than LA mothers, whose pups, by contrast, were much smaller and leaner at weaning than were controls. In fact, as adults, HA mice that were reared in these high-altitude simulating conditions vastly outperform their control siblings and LA conspecifics, with respect to cold-induced  $\dot{V}_{O_{2,max}}$ . This important metabolic trait has been directly linked to survival at high altitude. These findings suggest that a shift in energy allocation between mothers and their offspring may have evolved at high altitude.

### Altitude ancestry alters maternal care

Contrary to our initial predictions, we found that HA and LA females allocate energy differently during the lactation period even under warm normoxic control conditions. Mothers had similar proportional energy intake as they consumed the same amount of food per pup in a litter. However, HA mothers typically had two additional pups and therefore required greater food intake commensurate with provisioning their larger litters. Indeed, increasing food consumption to accommodate greater litter size is a common response in many rodent species, including in *P. maniculatus* (Miller, 1975; Glazier, 1985). However, HA mothers also spent significantly less time nursing their pups, and more time active outside the nest (running, climbing, jumping, etc.) during the nocturnal phase than did LA mothers. They also maintained a heavier body mass throughout lactation. This suggests that, in warm normoxia, lactating HA mothers were expending less of their total energy intake on feeding their offspring. Females from both populations also had similar metabolic rates during lactation, provisioning rates, provisioning efficiencies and milk creatinocrit. Taken together, these data suggest that milk output (a reflection of maternal mass loss during a nursing bout; Derting and Austin, 1998) and cost of milk production were also similar in LA and HA mice



**Fig. 4. Effect of simulated high altitude on postnatal growth during nursing of low- and high-altitude native *P. maniculatus* pups.** (A) Pup mass in control and cold hypoxia (CH) conditions until weaning (P21). Each sample represents an average of all pups within a control (LA,  $N=6$ ; HA,  $N=7$ ) and CH (LA,  $N=5$ ; HA,  $N=4$ ) litter. (B) Body composition (% fat mass) at weaning. (C) Representative images of LA and HA pups maintained in CH conditions until weaning. # indicates significant effect of treatment within a population; \* indicates significant effect of population within a treatment. All data are means  $\pm$  s.e.m.

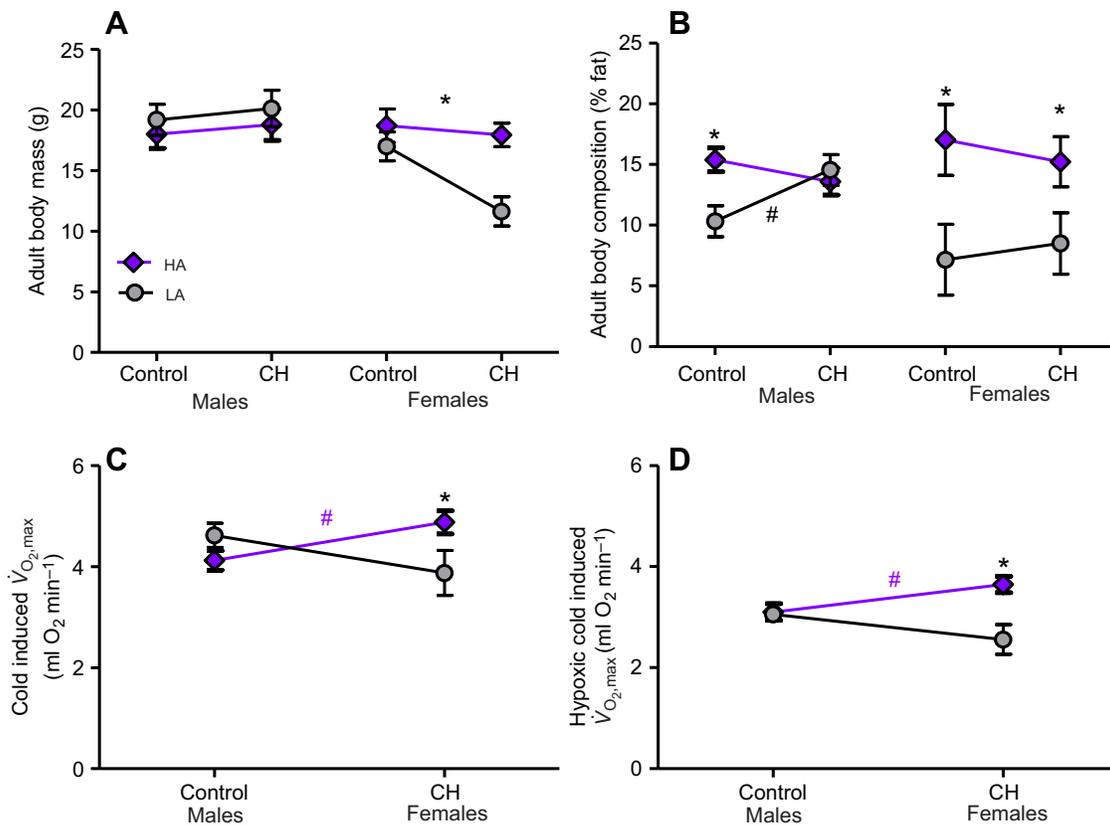
despite the HA mothers provisioning more pups (Hager and Johnstone, 2006, 2007). To our knowledge, this is the first measure of milk quality in a HA-adapted population of a small species. Milk quality also does not appear to change with adaptation to HA in larger mammals as there was no association between milk energy content and native elevation in humans, yaks or dairy cows (Bartl et al., 2009; Qiao et al., 2013; Barsila et al., 2014; Quinn et al., 2016). We also found that during a single nursing bout early in lactation (P8), litters gained between 0.20 and 0.31 g h<sup>-1</sup> regardless of altitude ancestry. It is possible that variation in evaporative water loss of mothers or pups may have confounded this result. However, the litter weight gain reported here is equivalent to previous reports on wild LA native *P. maniculatus gracilis*, where litter gain was 0.315 g h<sup>-1</sup> (Stewart and McAdam, 2014), further supporting the conclusion that, under control conditions, nursing HA mothers are producing similar quantities and quality of milk as their LA counterparts, despite having a greater number of pups to feed. Altogether, these results show a distinct difference in allocation of energy when deer mouse mothers of different altitude ancestry are rearing pups in warm normoxic conditions, and that HA mothers allocate less energy to each individual pup. That these differences are evident under control common garden conditions suggests that they have evolved at high altitude. Remarkably, lower maternal investment did not negatively affect growth of HA pups, who were larger overall than their LA conspecifics.

#### Flexibility of maternal care in response to simulated high altitude

When mothers were lactating in CH conditions, maternal metabolic rate increased substantially from warm normoxia (by 36–52%) but remained similar between the two populations. These metabolic rates represent a remarkable 85% of hypoxic cold-induced  $\dot{V}_{O_{2,max}}$

in LA mothers, but only 70% of  $\dot{V}_{O_{2,max}}$  for HA mothers because HA deer mice have evolved a higher aerobic capacity in hypoxia (Hayes and O'Connor, 1999; Cheviron et al., 2013). Curiously, these high resting metabolic rates are sustained without a corresponding increase in food intake. This result was surprising as others have found that cold exposure alone increases food intake in both lactating (Hammond and Kristan, 2000) and non-lactating (Hammond et al., 2001) *P. maniculatus*. In fact, it has been suggested that food intake during lactation is limited by a mother's ability to dissipate metabolically produced heat (the heat dissipation limit hypothesis, see Speakman and Król, 2010, for review). Thus, in cold environments, lactating females are able to consume more food than would be possible at warmer temperatures (Johnson and Speakman, 2001). However, hypoxia is a known anorexic agent, and has been found to decrease food intake in lactating rats (Bruder et al., 2008). Non-lactating *P. maniculatus* housed at 3800 m also do not show the same cold-induced increase in food intake as those at lower elevations. So, it is possible that hypoxia at HA mitigates the stimulatory effects of cold on appetite (Hammond et al., 2001). It is unclear how lactating females can compensate for the increased energetic demands of offspring care without increasing energy intake and despite a reduced aerobic reserve in hypoxia ( $\dot{V}_{O_{2,max}}$ –field metabolic rate; Hayes, 1989).

In many species, simulated or natural hypobaric hypoxia limits milk output (e.g. Walton and Uruski, 1946; Moore and Price, 1948; Weihe, 1965; Bruder et al., 2008). However, we observed that during a single nursing bout in cold hypoxia there was no change in litter mass gained compared with levels in warm normoxia, suggesting that milk output is maintained. These data suggest that mothers in both populations are not limiting milk output in CH conditions as a means to conserve energy. Instead, LA mothers actually increased milk creatinocrit, likely increasing the energetic



**Fig. 5. Effect of developmental environment on adult phenotype of low- and high-altitude native *P. maniculatus* raised in warm normoxic control or cold hypoxia conditions.** (A) Adult body mass, (B) body composition (% fat mass), (C) thermogenic capacity (cold-induced  $\dot{V}_{O_{2,max}}$ ) in normoxia. (D) Cold-induced  $\dot{V}_{O_{2,max}}$  in hypoxia. # indicates a significant effect of developmental treatment within a population (altitude ancestry). \* indicates a significant effect of population within a treatment as determined in linear mixed models. Control (LA,  $N=8$ ; HA,  $N=8$ ) and CH (LA,  $N=7$ ; HA,  $N=8$ ) data are presented as mean body mass and body composition data or estimated marginal mean of  $\dot{V}_{O_{2,max}}$  corrected for body size  $\pm$  s.e.m.

cost of milk production. A similar response is seen in cold-adapted rodents that tend to increase milk fat content (Barnett and Dickson, 1984). The increased cost of lactation provides evidence for an increased energetic conflict between mothers and offspring in these CH conditions (Trivers, 1974). This energetic conflict is particularly notable because both populations increased the amount of time spent nursing in cold hypoxia. As such, mothers must allocate more overall energy to their pups at a cost to themselves. How do they compensate for this additional energetic burden without increasing food intake and how are HA mothers able to maintain heavier body mass?

In CH conditions, mothers of both populations altered their nighttime behaviour to increase the time spent nursing pups. However, the high levels of activity HA mothers displayed outside the nest in warm normoxic conditions provided a greater scope for behavioural changes to reduce energy expenditure in CH conditions, than was available to LA mothers. HA mothers reduced nocturnal activity levels substantially in CH conditions compared with LA mothers (3-fold versus 2-fold, respectively). It is the greater scope for behavioural change that is likely to allow HA mothers to reduce activity and conserve energy in CH, maintaining high body mass. It appears that HA mothers have reserve energy available in control conditions for either activity or maintenance costs that is then shifted to nursing in HA conditions. What is the source of this energy reserve and how are HA mothers able to provision larger litters of pups without increasing milk output or energy content? The answer may lie in the physiology and behaviour of the pups themselves.

### Pup energetics

Deer mice have 6 teats for nursing and HA mothers routinely have litters with greater than 6 pups, thus exceeding the number of teats (Robertson et al., 2019). In this study the average litter size of HA females was 6.5 (ranging from 2 to 8 pups). Thus, they often cannot feasibly provision all offspring simultaneously, whereas LA mothers have smaller litters and avoid this limitation. Moreover, for rodents, it is well known that larger litter sizes increase intra-litter competition for milk (Seitz, 1954), and that pups from these larger litters tend to be smaller in size (Kaufman and Kaufman, 1987; Rogowitz, 1996). Surprisingly, we found that HA pups were larger than their LA counterparts, especially when raised under CH conditions. This was despite having more siblings and receiving less milk from their mothers. During a single nursing bout, we did not find any evidence that a greater number of pups from HA litters were failing to gain weight, suggesting that their own metabolic costs are lower than were those of LA pups. It is unclear how the HA pups are reducing their metabolic costs. One possibility is that HA pups are not actively thermoregulating when they experience bouts of cold in the absence of their mothers (Hill, 1972; Robertson et al., 2019; Velotta et al., 2020). This developmental delay of homeothermic endothermy in HA pups may not fully explain why their metabolic costs appear lower during nursing bouts when their mothers are present, but it would allow substantially more energy to be allotted to maintaining growth and preserving valuable fat stores.

The ability to thermoregulate develops sometime during the postnatal period in deer mice, and we have previously shown that

thermogenesis matures more slowly in HA pups (Robertson et al., 2019). During the same period where maternal care was monitored in this study (P2–P8), LA pups can activate their brown adipose tissue (BAT) and elevate their metabolic rate in response to acute cold exposure. In contrast, HA pups show a significant reduction in body temperature and do not activate BAT or increase metabolic rate at these ages (Robertson et al., 2019). In fact, HA pups have a consistently blunted metabolic response to cold throughout the nursing period (Robertson and McClelland, 2019). It has been suggested that this is an adaptive cost saving strategy that evolved to allow HA pups to allocate energy towards growth rather than thermogenesis (Velotta et al., 2020). At least one other small, altricial mammalian species, the desert hamster (*Phodopus roborovskii*) has been shown to use a similar ‘precocial torpor’ strategy early in postnatal development to save energy in the cold (Geiser et al., 2019). HA mothers may be able to successfully provision their pups, while providing less milk and care than lowlanders because of the lowered metabolic needs of their offspring. What influence do these differing strategies and environments have on offspring and resulting adult phenotype?

### Influence of maternal care and rearing environment on offspring and adult phenotypes

Rearing environment can have profound effects on offspring physiology through developmental plasticity (West-Eberhard, 2005). In species with substantial maternal care, rearing environment is the result of both the abiotic stressors of the surrounding environment and the mother’s ability to buffer her offspring against them (Wells, 2019). We have found that when raised under CH conditions, HA and LA deer mice show evidence of developmental plasticity that can be considered examples of adaptive and maladaptive responses, respectively. These distinct responses likely reflect evolved genetic variation in the capacity for plasticity (West-Eberhard, 2005). Despite increasing maternal care, LA mothers were unable to buffer their pups against adverse effects of experiencing CH during postnatal development. In contrast, raising HA mice in CH conditions better prepares them to face the challenges of that environment as adults. These mice have higher cold-induced  $\dot{V}_{O_{2,max}}$  as adults when tested under either normoxic or hypoxic conditions than their control siblings. An elevated cold-induced  $\dot{V}_{O_{2,max}}$  confers a fitness benefit at HA, improving survival (Hayes and O’Connor, 1999). For LA mice, it is unlikely that the small lean pups raised in CH conditions would survive after weaning in cold conditions at HA. For LA females who did manage to survive to adulthood, the lack of developmental plasticity in  $\dot{V}_{O_{2,max}}$  would put them at an even greater disadvantage relative to HA females raised in the same conditions. For female highlanders, an increase in  $\dot{V}_{O_{2,max}}$  would allow for a greater aerobic reserve to cope with the high energetic costs of nursing.

### Conclusions

We have shown that the metabolic costs of lactation in cold and hypoxia are exceptionally high and require lactating mothers to adjust their energy allocation dramatically. It is clear that females native to LA are unable to effectively reallocate resources in HA conditions, resulting in negative short- and long-term consequences for the growth of their offspring. In contrast, HA mothers compensate for the increased energetic demands of HA by decreasing activity, which allows them to increase time nursing while maintaining stable body condition. This is likely aided by their pups, which suppress energetically costly thermoregulation during the nursing period (Robertson et al., 2019; Robertson and McClelland, 2019). This may be an example of maternal–offspring coadaptation (Wolf and Brodie,

1998), which occurs when selection favours the coevolution of a combination of parental and offspring traits that ultimately improve fitness of both parties. Indeed, the developmental delays in thermoregulation observed in HA deer mouse pups are known to be adaptive (Velotta et al., 2020). This delay may have evolved in response to decreased maternal investment at HA relative to LA. It is important to note that mice in our study only experienced CH conditions during lactation and not during pregnancy, which may have impacted our results. Mammalian mothers extensively remodel their physiology during the last trimester of pregnancy in preparation for lactation. Hypoxia exposure can impair this process in LA native rodents (reviewed in Robertson and Wilsterman, 2020). It is unclear how HA adaptation has impacted pregnancy-induced remodelling, though it likely compounds the influences reported here. Regardless of mechanism, given the incredibly high costs of lactation at HA, overcoming maternal–offspring conflict for resources during the early nursing period would have been critical for deer mice and other species to colonize HA ecosystems.

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

Conceptualization: C.E.R., G.B.M.; Methodology: C.E.R.; Formal analysis: C.E.R.; Data curation: C.E.R.; Writing - original draft: C.E.R.; Writing - review & editing: C.E.R., G.B.M.; Supervision: G.B.M.; Funding acquisition: G.B.M.

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### Data availability

All data are available in Figshare at:  
[https://figshare.com/articles/Maternal\\_Care/12202418](https://figshare.com/articles/Maternal_Care/12202418)

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