Density Triggers Maternal Hormones That Increase Adaptive Offspring Growth in a Wild Mammal

Ben Dantzer *et al.*
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Density Triggers Maternal Hormones That Increase Adaptive Offspring Growth in a Wild Mammal

Ben Dantzer,[1]*† Amy E. M. Newman,[2] Rudy Boonstra,[3] Rupert Palme,[4] Stan Boutin,[5] Murray M. Humphries,[6] Andrew G. McAdam[1,2]

In fluctuating environments, mothers may enhance the fitness of their offspring by adjusting offspring phenotypes to match the environment they will experience at independence. In free-ranging red squirrels, natural selection on offspring postnatal growth rates varies according to population density, with selection favoring faster-growing offspring under high-density conditions. We show that exposing mothers to high-density cues, accomplished via playbacks of territorial vocalizations, led to increased offspring growth rates in the absence of additional food resources. Experimental elevation of actual and perceived density induced higher maternal glucocorticoid levels, and females with naturally or experimentally increased glucocorticoids produced offspring that grew faster than controls. Therefore, social cues reflecting population density were sufficient to elicit increased offspring growth through an adaptive hormone-mediated maternal effect.

Fluctuations in food availability and the resultant changes in the population density of consumers are thought to be important ecological agents of natural selection in many animal populations (*1, 2*). Temporal variation in natural selection characterized by recurrent pulses in food or density can favor the evolution of adaptive phenotypic plasticity when there are reliable cues that predict the direction or the magnitude of these agents of selection (*3, 4*). Phenotypic plasticity is beneficial in such changing environments because it enables individuals to track fluctuating fitness optima (*5, 6*). Similarly, if the parental environment or phenotype provides reliable cues of the conditions that offspring will experience, parents may induce adaptive changes in offspring that increase both parental and offspring fitness [adaptive parental effects (*7, 8*)].

The role of parental effects in the adaptation of offspring to changing environments is intriguing, but little is known about their importance in free-living animals. Not only do the agents of natural selection on offspring phenotype need to be identified, but the cues parents use to predict changes in the agent of selection, and the mechanism that mediates the parental effect, also need to be known. This is further complicated when considering population density as a cue because it is often confounded with food availability, which might also relieve resource constraints and cause resource-mediated or permissive parental effects (*7*). Identification and experimental manipulation of the mechanisms that mediate parental effects requires a combination of field physiology, experimental ecology, and longitudinal studies of natural selection that have not been achieved to date. We identified and experimentally manipulated the social density cues and stress hormones responsible for an adaptive maternal effect in a natural population of North American red squirrels (*Tamiasciurus hudsonicus*).

Individual male and female red squirrels defend exclusive territories around a central midden (*9, 10*) containing cached white spruce cones [*Picea glauca* (*11, 12*)], and juveniles that fail to acquire a territory before their first winter do not survive (*13*). Red squirrels experience recurrent fluctuations in population density because of pronounced episodic fluctuations in the availability of white spruce seeds (Fig. 1A) (*11, 12*). Increased autumn spruce cone production is associated with increased squirrel density in the following spring (Fig. 1B) (*14*). In our 23-year study in the Yukon, Canada, we found that these changes in density have notable effects on red squirrels because we documented density-dependent selection on offspring postnatal growth rates. In years when spring density was high, females that produced faster-growing offspring had more offspring survive their first winter and recruit into the adult population, whereas when density was low there was no benefit to producing faster-growing offspring [$n = 463$ females, offspring growth × density, $t_{726} = 2.15$, $P = 0.016$ (table S1)].

In such variable environments, the evolution of adaptive maternal effects may be favored, but this requires the presence of reliable cues that enable an accurate prediction of natural selection on offspring (*3, 4*). Therefore, cues of population density in red squirrels might induce adaptive increases in offspring growth when density is high. Red squirrels emit territorial vocalizations called rattles to defend their territories, and the

[1]Department of Zoology, Michigan State University, East Lansing, MI 48824, USA. [2]Department of Integrative Biology, University of Guelph, Guelph, ON N1G 2W1, Canada. [3]Centre for the Neurobiology of Stress, University of Toronto Scarborough, Toronto, ON M1C 1A4, Canada. [4]Department of Biomedical Sciences/Biochemistry, University of Veterinary Medicine, A-1210 Vienna, Austria. [5]Department of Biological Sciences, University of Alberta, Edmonton, AB T6G 2E9, Canada. [6]Natural Resource Sciences, Macdonald Campus, McGill University, Ste-Anne-de-Bellevue, QC H9X 3V9, Canada.

*Corresponding author. E-mail: bendantzer@gmail.com
†Present address: Department of Zoology, University of Cambridge, Cambridge CB2 3EJ, UK.
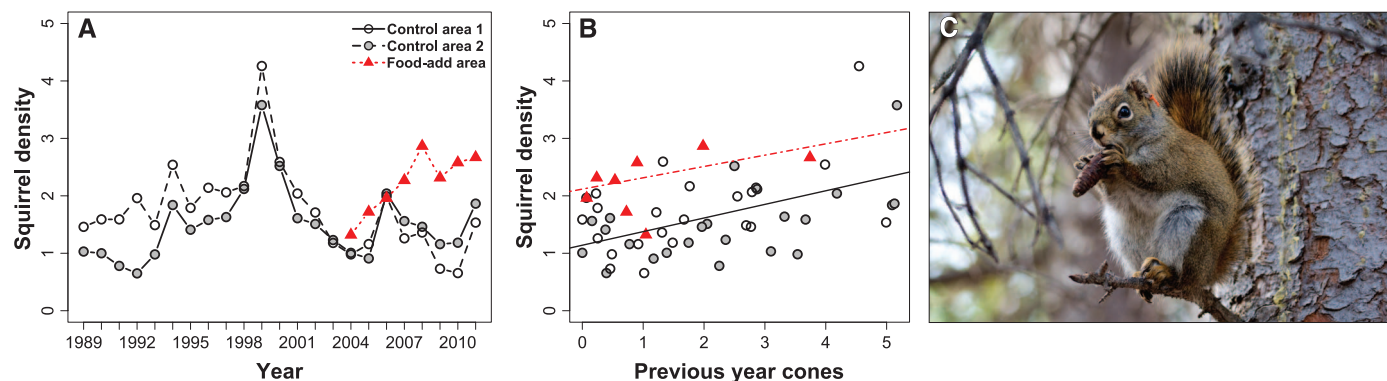
Fig. 1. Population density of North American red squirrels in the Yukon, Canada, fluctuates annually in response to the availability of spruce cones. (**A**) Yukon red squirrels experience recurrent fluctuations in population density (squirrels/ha) because of interannual variation in white spruce cone abundance (*11, 12*). (**B**) Spruce cone production in the previous autumn is associated with increased spring population density in two control study areas ($b = 0.24 \pm 0.05$, $t_{53} = 4.3$, $P < 0.0001$) and one study area (Food-add) where squirrels have been provided with supplemental food since autumn 2004 ($b = 0.20 \pm 0.19$, $t_{53} = -0.22$, $P > 0.5$). Autumn spruce cone production is an index on a ln scale (*11*). Regression lines from a linear mixed-effects model. (**C**) Red squirrel extracting seeds from a white spruce cone. [Photo credit: R. W. Taylor]

frequency with which they hear rattles in their neighborhood accurately predicts density (10). We hypothesized that territorial vocalizations provide a cue of density that allows females to adaptively adjust offspring growth in anticipation of the density-dependent selection that they will experience. We tested this hypothesis by simulating high-density conditions using audio playbacks of red squirrel rattles (9, 10). This corresponded to a perceived density of 4.92 squirrels/ha, which was sixfold higher than the perceived density of females exposed to a control stimulus (bird vocalizations, 0.81 squirrel/ha) and similar to the maximum historical density (Fig. 1A) (10). Such a high-density environment would typically be associated with a strong positive relationship between offspring growth and fitness (table S1), whereas offspring growth does not affect fitness in the low-density control environment.

As predicted, offspring produced by females experiencing experimentally heightened density grew significantly faster than those produced by control females (Fig. 2). Consistent with life-history theory (15), the growth rates of offspring produced by control females declined significantly as litter size increased, but this effect was attenuated by 67% in females exposed to playbacks of territorial vocalizations [playback × litter size, $t_{186} = 1.98$, $P = 0.024$ (table S2 and Fig. 2)]. In fact, the trade-off between litter size and growth rate in females exposed to playbacks of territorial vocalizations was greatly reduced ($r = -0.12$, $t_{66} = -1.57$, $P = 0.06$) compared with that in control females ($r = -0.37$, $t_{64} = -4.43$, $P < 0.0001$). Female red squirrels, therefore, increase offspring growth in response to conspecific density because of the fitness benefits of doing so in high-density years. These growth-enhancing maternal effects in high-density years are adaptive for mothers and offspring by increasing the probability that their offspring will survive their first winter (16), which is a major component of their lifetime fitness (17). However, faster offspring growth rates are not favored under low-density conditions [≤1 squirrel/ha (table S1)], and in some years there is significant negative selection on offspring growth (16). Increased reproductive effort does not appear to incur a survival cost to mothers (18, 19). However, offspring born in high-density years have a reduced adult life span (20), suggesting that faster offspring growth, which enhances recruitment when density is high, might incur a cost to offspring later in life. Such conditions will promote the evolution of plasticity in maternal effects, whereby increased offspring growth coincides with the high-density conditions under which it enhances fitness.

These adaptive maternal effects on offspring were mediated by the physiological stress responses of females experiencing heightened population density. Across 6 years (2006 to 2011), we found a positive relationship between local density and concentrations of fecal cortisol metabolites [FCM; $t_{155} = 3.63$, $P = 0.0002$ (table

Fig. 2. Female red squirrels experiencing increased perceived or actual density produced faster-growing offspring than controls. Female red squirrels experiencing experimentally increased perceived population density (rattle playbacks, $n = 19$ females, 67 pups) produced offspring that grew significantly faster than those produced by controls ($n = 19$ females, 65 pups) but similar to those produced by food-supplemented females ($n = 16$ females, 55 pups) experiencing increased actual density. Values on the y axis represent residuals from a linear mixed-effects model (table S2).
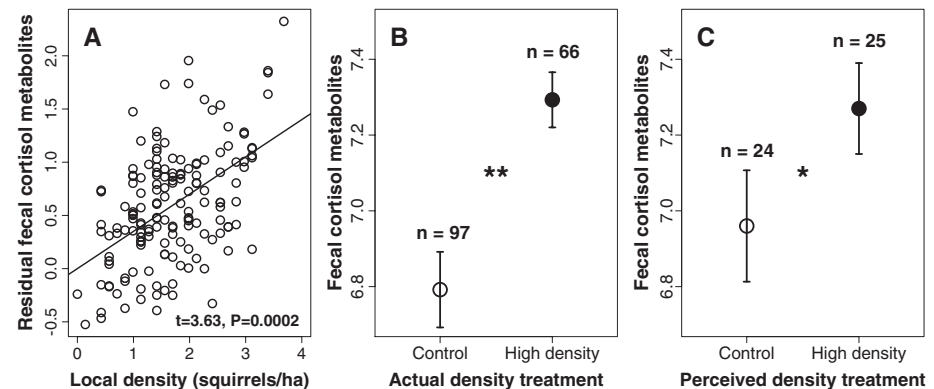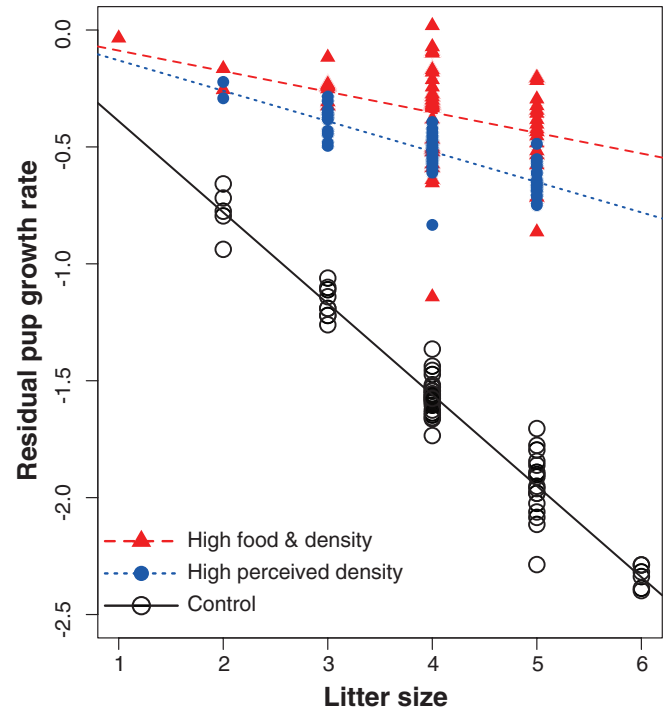
Fig. 3. Female red squirrels experiencing higher population density had higher glucocorticoid levels. (**A**) Female red squirrels living under high-density conditions had higher concentrations of FCM. Squirrels experiencing experimentally increased (**B**) actual density resulting from long-term food supplementation or (**C**) perceived density (rattle playbacks) had significantly higher concentrations of FCM than controls. Values on the y axis represent either (A) residuals from a linear mixed-effects model (table S4) or [(B) and (C)] raw FCM (ln ng/g of dry feces). Sample sizes refer to the number of fecal samples analyzed. **$P < 0.01$ and *$P < 0.05$ (table S4). Error bars indicate ± SE.

S4 and Fig. 3A)]. Females from a study area with experimentally increased density resulting from food supplementation [75% higher density than control study areas (Fig. 1)] had concentrations of FCM that were 49% higher [$t_{162} = 3.82$, $P < 0.0001$ (table S4 and Fig. 3B)] than those of females in control study areas. Females experiencing increased perceived density through the playback experiment had concentrations of FCM that were 30% higher than those of control females [$t_{48} = 2.24$, $P = 0.015$ (table S4 and Fig. 3C)]. These results confirm that increases in concentrations of FCM were driven by perceived density rather than by food abundance (21).

In mammalian species, increases in maternal glucocorticoid levels can cause profound changes in offspring phenotype (22) and may provide offspring with reliable hormonal cues about their future environment. Three lines of evidence indicate that increases in maternal glucocorticoid levels are responsible for the adaptive increase in offspring growth under high-density conditions. First, females exposed to heightened perceived density had increased concentrations of FCM during pregnancy (Fig. 3C) and also produced faster-growing offspring than controls (Fig. 2). Second, increased maternal FCM concentrations were positively associated with offspring growth in females measured over a 6-year
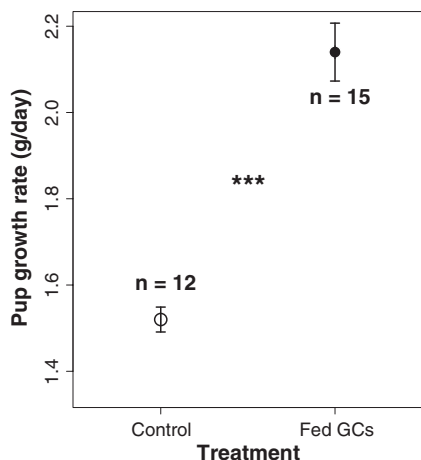
Fig. 4. Offspring produced by female red squirrels provisioned with cortisol grew significantly faster than those from controls. Raw offspring growth rates (mean ± SE) are shown on y axis. Sample sizes denote number of pups. Fed GCs corresponds to provisioning with three different cortisol concentrations (fig. S2). ***$P < 0.0001$ (table S6).

period [$t_{98} = 1.94$, $P = 0.028$ (table S5)]. Last, offspring born to females with experimentally increased glucocorticoid levels during pregnancy [fed cortisol (fig. S1)] grew 41% faster than those produced by control females [$t_{26} = 4.98$, $P < 0.0001$ (table S6 and Fig. 4)].

Our results suggest that elevated maternal glucocorticoid levels in response to heightened population density induced an adaptive hormone-mediated maternal effect on offspring growth. In contrast to the widespread assumption that heightened maternal glucocorticoid levels are detrimental to offspring (22), our results emphasize that in free-living animals they can instead lead to adaptive adjustments in offspring (23, 24). Under high-density conditions, squirrels spend less time feeding and in the nest (10), suggesting that increased offspring growth is not a simple outcome of increased maternal care or milk provisioning. Alternatively, elevated exposure to glucocorticoids early in life (22, 25) could increase offspring growth by directly influencing offspring physiology or behavior (22, 26) and subsequent changes in growth hormone secretion in offspring (27).

For nearly 100 years, food availability has been considered to be a universal variable affecting population dynamics and life-history traits (28). Increased food availability also increases the population density of consumers, which has made it difficult to distinguish whether the plasticity in life-history traits after periods of high food availability is due to relaxation of food limitation or to adaptive reproductive adjustments to changes in density-mediated selection. Our results provide evidence that female red squirrels can produce faster-growing offspring in the absence of additional resources but only do so when the fitness prospects warrant this increased invest-

ment. In fact, offspring produced by females exposed to high-density cues but with no access to additional food grew as fast as those produced by food-supplemented females that were also experiencing increased density [1.79 ± 0.09 squirrels/ha (Fig. 2 and table S2)]. Therefore, some of the plasticity in female life history traits is due to the expected fitness benefits of producing faster-growing offspring under high-density conditions rather than only reflecting a relaxation of food limitation.

Experimental increases in food resources that result in increased reproductive output are typically interpreted as evidence for resource limitations on reproduction (29). However, if animals use food abundance as a cue of upcoming density-mediated selection, then reproductive responses to food supplementation might reflect not only relaxation of food limitation but also an adaptive adjustment to an anticipated change in natural selection resulting from an impending increase in density. Cues of population density may be a general signal that animals use to make adaptive reproductive adjustments in anticipation of density-dependent natural selection on offspring phenotypes.

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The Cross-Bridge Spring: Can Cool Muscles Store Elastic Energy?

N. T. George,[1] T. C. Irving,[2] C. D. Williams,[1,3] T. L. Daniel[1]*

Muscles not only generate force. They may act as springs, providing energy storage to drive locomotion. Although extensible myofilaments are implicated as sites of energy storage, we show that intramuscular temperature gradients may enable molecular motors (cross-bridges) to store elastic strain energy. By using time-resolved small-angle x-ray diffraction paired with in situ measurements of mechanical energy exchange in flight muscles of *Manduca sexta*, we produced high-speed movies of x-ray equatorial reflections, indicating cross-bridge association with myofilaments. A temperature gradient within the flight muscle leads to lower cross-bridge cycling in the cooler regions. Those cross-bridges could elastically return energy at the extrema of muscle lengthening and shortening, helping drive cyclic wing motions. These results suggest that cross-bridges can perform functions other than contraction, acting as molecular links for elastic energy storage.

Elastic energy storage is heralded as a critical design characteristic of animal movement, because it promotes efficient locomotion. Canonical examples of elastic energy-storage sites include tendons of mammals and resilin, the rub-

berlike protein in insect cuticle (1, 2). Elastic energy storage is particularly important to flying insects, reducing the otherwise prohibitive inertial power costs of accelerating and decelerating the wings (3, 4). Two main sites of elastic

Supplementary Materials for

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*Corresponding author. E-mail: bendantzer@gmail.com

This PDF file includes:

Materials and Methods

Red squirrels

Male and female red squirrels defend separate, year-round territories around a central larder hoard ("midden") containing cached white spruce cones (*10*, *30*). White spruce is the only conifer tree in our study area and spruce seed is the major food item of red squirrels (*11*, *12*). The larder hoard of spruce cones that is cached in the autumn is necessary for overwinter survival and fuels breeding in the following spring (*11*, *12*). Squirrels advertise their presence on the territory and defend their territory using territorial vocalizations called rattles (*10*, *30*).

White spruce is a masting tree species that produces a super-abundance of cones in some years (mast years) with fewer to no cones produced in other years (*11*, *12*). Increased spruce cone availability in the autumn is associated with an increase in spring breeding density (Fig. 1B, *10*, *14*). In high-density years, which follow pulses of cone availability, many juveniles compete for each territory while a smaller number compete for each vacant territory in lower density years. An important predictor of whether a juvenile red squirrel acquires a vacant territory before its first winter is its rate of postnatal growth (the rate of linear growth from ~0-25 d post-parturition: *16*). In years of high population density, following years of elevated spruce cone availability, natural selection favors females that produce faster growing offspring (Table S1). In years of low density, there is relaxed selection on offspring postnatal growth rates (Table S1) and in some years slower growth rates are favored by natural selection (*16*).

Estimating population density

We monitored a natural population of red squirrels in the southwest Yukon (61° N 138° W) on two unmanipulated control study areas (40 hectares each) from 1989-2011 using a combination of live-trapping and behavioral observations. In the spring (May) and autumn (August) of each year of study, we completely enumerated all squirrels defending territories on each of our study areas. Territory ownership was confirmed using live-trapping and behavioral observations (*10*, *30*) from ~ March to ~1 September of each year. Any changes in territory ownership that occurred from the spring to autumn were recorded as we followed all individual squirrels over this time period. We estimated study area spring breeding population density (number of unique squirrels defending a territory/total area of study area) and also local spring breeding population density for each squirrel as the number of unique squirrels defending a territory within 150 m of each female's midden (a 7.07-ha area: *10*).

Monitoring female reproduction

All squirrels on our study areas were individually marked with uniquely numbered metal ear tags (National Band and Tag, Newport, KY, USA) as well as a unique combination of colored wires or pipe cleaners threaded through their ear tags that enabled individual identification from afar. Squirrels were live-trapped on their middens every 3-14 days using Tomahawk live traps. Females were classified as non-breeding, pregnant (fetus palpable in abdomen), or lactating (milk expressed from teats). Pregnant females nearing parturition were trapped more frequently (every 3 days) and

2

radiocollared immediately prior to parturition. Parturition was typically confirmed by frequent live-trapping where we documented a sudden weight loss or the expression of milk from teats. We also used frequent (nearly daily) behavioral observations of pregnant females known to be nearing parturition to note behavioral changes indicating parturition (i.e., spending more time in their nest) or the presence of large nipples (visible through binoculars). In some cases we assigned ages to neonates based upon their mass when accessed in their natal nest within 1 to 3 days of birth (*31*, *32*), which is a highly reliable method of estimating parturition dates (*31*) and a period of time where the variation in pup body mass is lowest (*32*). Conception dates were estimated by subtracting 35 days from known dates of parturition (*33*).

Female red squirrels typically raise one successful litter per year and only produce a second litter successfully in anticipation of an upcoming spruce cone mast (*14*). Additionally, if the offspring from their first litter in that year do not survive to first emergence (~35 days of age), females will attempt another litter. We focus all of our analyses on the first litter produced by each female in a year. We documented parturition dates, litter sizes, and offspring growth rates for each female's first litter of the year from 1989-2011. To measure offspring postnatal growth rates, pups were temporarily removed from their natal nests once at ~0 d post-parturition and a second time at ~25 d post-parturition. During each nest entry, pups were weighed (using a calibrated electronic portable balance to 0.1 g), sexed, and individually marked (ear notches at first entry and metal ear tags at second entry). We used the rate of change in mass from birth to 25 d post-parturition as our estimate of offspring growth rate. This is a linear period of growth (*16*, *32*, *34*) during which offspring are entirely dependent upon milk from their mothers for nutrition, as it is before their first emergence from the natal nest (*35*) and has been used in previous analyses (*16*, *17*, *34*, *36*). Red squirrels are relatively sexually monomorphic in adult body mass (*32*) and juvenile males and females do not differ in their size or rate of growth during this pre-weaning period (*32*, *34*). We were able to document the total number of offspring produced by a female that survived to the following spring by completely enumerating all squirrels living on our study years in the spring and autumn of each year using live-trapping and behavioral observations (see above, *17*). There is no sex-bias in natal dispersal and successful juvenile dispersal distances from their natal territory are often <100 m, which is < 1-2 territory widths away from their natal territory (*13*, *37*) and well within the scale of our study areas (40 hectares each). This allows accurate assessments of juvenile survival and recruitment (discussed in *17*). We used the number offspring produced that survived to the following spring as our measure of female reproductive success (see below, *17*).

Manipulation of actual population density using food-supplementation

From 2006-2011, we recorded the litter characteristics of females on a nearby study area (~6 km from other study areas) whose population density was experimentally increased using a long-term food supplementation (Fig. 1A). Starting in the autumn of 2004, we provided supplemental peanut butter (no sugar or salt added: *10*) to 100-120 middens on this study area (45 hectares). Squirrels were provided with one kg of peanut butter in a bucket that was hung between two trees in the center of their midden. Peanut butter was replenished approximately every 6 weeks from approximately October to May of each year. Squirrels cannot hoard peanut butter and it does not appear to influence

female body mass (*10*). The average population density (study area wide estimate) on the food-addition study area from 2006-2011 (2.44 ± 0.13 squirrels/ha) was significantly higher (75%) than on the control study areas from 2006-2011 (1.39 ± 0.13 squirrels/ha; general linear model, $t_{15} = 5.1$, $P < 0.0001$, Fig. 1A).

Diet can influence fecal hormone metabolite concentrations (*38*). We have previously found that feeding red squirrels peanut butter biases concentrations of FCM downwardly (*39*). Consequently, our test of the prediction that concentrations of FCM would be higher on the food-supplemented study area (because actual density was experimentally increased) is made more conservative by these effects of diet on FCM concentrations. Because feeding squirrels peanut butter reduces their FCM concentrations relative to squirrels fed spruce seed (*39*), any direct effects of peanut butter on FCM concentrations would have been in the opposite direction of our predicted responses to elevated population density in the food-supplemented population.

Manipulation of perceived population density using playbacks

In one year (2010), we experimentally increased perceived population density using audio playbacks of territorial vocalizations (rattle playbacks), which we have previously used to simulate high-density conditions effectively (*10*). We exposed females to playbacks of recorded rattles to simulate high-density conditions and compared their endocrine and reproductive responses to those of a separate group of control females exposed to the vocalizations of boreal chickadees (*Poecile hudsonicus*), which is a non-predatory resident species in our study area. Squirrels were exposed to the playbacks near their middens as soon as pregnancy was confirmed (mid-gestation) and until 5 days post-parturition (~34 continuous days) for approximately 12 hrs/day (800-2000 h). This portion of the reproductive cycle was chosen for exposure to this form of social stress based upon previous laboratory studies in rodents documenting that this is important period of time for brain and physiological development in rodents (*40*, *41*) and that maternal stress during mid- and late-pregnancy can increase the postnatal growth rates of their offspring early in life (*42*, *43*).

During the active part of their day, red squirrels emit ~1 rattle every 7-min (*10*). Rattles contain individually distinct signature signals (*44*), which enabled us to manipulate the number of unique rattles that females heard during reproduction. We simulated an increase of 4 additional neighboring squirrels around each females territory by exposing them to audio playbacks of rattles recorded from 4 different squirrels. Similarly, control females were exposed to 4 different chickadee vocalizations every 7-min. Each female in the rattle playback treatment was exposed to a unique combination of rattles (2 rattles recorded from different males and 2 recorded from different females). We used 7-min behavioral observation sessions (*10*) to quantify the number of rattles that rattle-playback (n = 184 sessions) and chickadee-playback (n = 90) females heard while they were being exposed to the playbacks. We used the number of rattles heard during these behavioral observation sessions to estimate the perceived population density rattle playback females were experiencing (*10*).

We performed these manipulations of perceived density on four different study areas all located within 1-10 km of the control and food-addition study areas. Two of these study areas had both treatments with the restriction that females exposed to chickadee

playbacks had to be >150 m away from a female exposed to rattle playbacks (i.e., out of hearing distance: *30*, *45*).

Rattles used in the playbacks were recorded from the same general area, but were recorded at least 500 m away from the study areas used in this experiment such that females likely only experienced rattles from unrelated or at least unfamiliar squirrels (successful dispersal from natal territory is generally <100 m: *13*, *37*). We used publicly available recordings of boreal chickadee vocalizations from various commercial sources (www.xenocanto.org, The Cornell Lab of Ornithlogy Macauley Library) to develop 17 unique combinations of 4 chickadee vocalizations. Methods for recording rattles and handling the sound files are discussed elsewhere (*10*, *45*).

Squirrel or chickadee playbacks were broadcast from an MP3 player (Coby MP-300, Lake Success, NY, USA) through two separate speakers (two different alternating vocalizations were broadcast through each speaker: Altec Lansing Orbit, San Diego, CA, USA). We placed each speaker in a random cardinal direction ~15 m away from the center of the midden so that the vocalizations did not simulate a territory intrusion. One rattle or chickadee playback was broadcast from each speaker every 3.5 min in an alternating fashion, which ensured that a squirrel was exposed to 4 different rattle or chickadee playbacks every 7-min. Sound pressure levels of the chickadee and rattle vocalizations from each speaker were standardized to 100 dB from 1 m from the speaker using a digital sound pressure meter (Radio Shack Digital Sound Level Meter #33-2055).

Experimental elevation of female glucocorticoid levels

We determined whether the elevation of maternal glucocorticoid levels associated with high-density conditions caused the change in offspring growth rates by experimentally increasing maternal glucocorticoid levels during pregnancy and early lactation and comparing their life history responses with control females. In one year (2012), we fed females either 3, 6, or 12 mg of cortisol (Sigma Chemical, #H4001, hydrocortisone). We used these doses because previous studies in similar sized rodents have shown that a dose of 12 mg/day of cortisol induces chronic mild stress (*46-48*). We dissolved the cortisol into 1 ml of 100% ethanol and then into 3 ml of peanut oil. After adding the peanut oil, the entire vial containing this solution was sonicated for 5 min and then left at room temperature overnight with the lid off so that the ethanol evaporated. We then sonicated this mixture again for 5 min and thoroughly mixed it with a specific amount of peanut butter and wheat germ (to achieve a tractable consistency). These peanut butter doses were placed individually into separate containers and frozen until use. Control doses of peanut butter were produced in the same manner except cortisol was not included. We provided females with these peanut butter doses approximately every day when pregnancy was first noted (~20 days into a ~35 day pregnancy) until 5 days after parturition, which is the same period of time that we experimentally increased perceived population density using audio playbacks (see above, *40*, *41*). We placed the peanut butter doses in buckets that were hung on the midden of the specific squirrel (described above). We confirmed that females were consuming these peanut butter doses by checking the buckets each day.

This supplementation procedure achieved our desired result of experimentally increasing female glucocorticoid levels around parturition. Compared with control females not fed any peanut butter or fed the same amount of peanut butter without

dissolved cortisol (hereafter, control females), females treated with cortisol had significantly higher maternal FCM concentrations during pregnancy ($t_{29} = 1.99$, $P = 0.028$, Fig. S1).

Measuring concentrations of fecal cortisol metabolites

We measured the effects of our density and glucocorticoid experimental manipulations on maternal glucocorticoid levels by measuring concentrations of fecal cortisol metabolites, which accurately reflect plasma glucocorticoid concentrations (*49*). We collected fecal samples from underneath the live traps during live-trapping, placed them individually into 1.5 mL vials using forceps, placed them on wet ice in an insulated container, and then placed them in a -20 C freezer within 5 h of collection. Squirrels were in traps for less than 2 h before collection of fecal samples, which is not long enough for FCM concentrations to be affected by trap-induced stress from the current capture event (*39*, *50*). We only analyzed fecal samples from squirrels that had not been live-trapped and handled within the previous 72 h. We determined the effects of population density and the density manipulations on maternal FCM concentrations from samples collected from pregnant females around parturition (31-40 days post-conception). We chose this time period for analysis because maternal FCM concentrations peak around parturition (*50*).

Fecal samples were stored, prepared, and FCM were extracted using previously validated methods (*33*, *39*, *50*). Samples were run in duplicate and samples from different years and treatments were randomly allocated to different assays (n = 71 plates of 35 samples/plate). The intra- and inter-assay coefficients of variation for the FCM enzyme-immunoassays were 7.6 and 14.9%. The repeatability of our measures of concentrations of FCM was very high (85% total variance in measurement attributable to sample itself) and the total amount of variance in the concentrations of FCM that could be attributed to assay variability was very low (4% of total variance due to duplicate within plate or within-assay measurements; <1% of total variance due to among-assay measurements).

Statistical analyses

We used a linear mixed-effects model (LMM) to determine how the relative reproductive success of female red squirrels (n = 463) over 22 years of study was affected by three of their life history characteristics (parturition date, litter size, offspring growth rate) that are known to influence fitness (*16*, *17*, *51*). We calculated relative female reproductive success as the number of offspring produced in that year that survived to the following spring divided by the average number of offspring produced in that year that survived to the following spring for that study area-year combination (*16*, *17*). We assigned offspring survival to maternal fitness because the period during which we measured offspring growth rates occurs when offspring are entirely dependent upon their mother for nutrition (milk), have never emerged from their natal nest on their own (*35*), and most of the variation (81%) in offspring growth rates during this time period is due to the nursing mother (*34*). We included a fixed effect for spring breeding density on that study area (number of squirrels/hectare on that study area in that year) experienced by the female during reproduction and a 2-way interaction between population density and each of the three life history traits to determine how female relative reproductive success was affected by these three life history traits under low and high density conditions. All of the

life history characteristics were standardized (mean of 0 and unit variance) within each study area-year combination.

We used LMMs to determine how neonate mass, litter size, and offspring growth rates varied among control females (females exposed to chickadee or no playbacks, n = 29 litters from 28 females), those living on the food-addition study area (n = 33 litters from 33 females), and those exposed to the rattle playbacks (n = 35 litters from 35 females). We combined females exposed to chickadee playbacks and those exposed to no playbacks from two control study areas because the chickadee playbacks did not affect FCM concentrations (LMM containing treatment, days post-conception, and days post-conception^2 as fixed effect terms: $t_{144} = -1.03$, $P = 0.15$). In each LMM, we included a fixed effect term for treatment (3 level categorical variable: control, rattle playbacks, or food-addition), litter size, and their interaction. In the LMM for the treatment effects on neonate mass, we also included a fixed effect for age of pups (see above: *32*, *33*). We used a generalized linear mixed-effects model (GLMM; binomial errors, logit link) to determine if litter sex ratio varied among the three treatments (dispersion parameter from a generalized linear model without random intercept term – see below - was 1.12). We determined the effects of experimentally increased perceived or actual density as well as increased local population density on maternal FCM concentrations using LMMs that contained a fixed effect term for either local population density (squirrels/hectare: *10*) or a 2-level fixed effect term for actual density treatment (food-addition or control study area) or perceived density treatment (rattle playbacks or control). We also included a fixed effect term for days post-conception to control for the effects of reproductive condition when the samples were obtained (*33*, *50*). We confirmed that there were no differences in concentrations of FCM in females in both treatment groups prior to being exposed to any playbacks (Fig. S3) using LMMs containing treatment (rattle or chickadee playbacks), days post-conception, and an interaction term between treatment and days post-conception as fixed effects.

We determined whether concentrations of FCM were associated with offspring growth rates using a LMM that contained a fixed effect term for concentrations of FCM. Concentrations of FCM were first corrected for when they were collected during the reproductive period by performing a separate general linear model (GLM) containing days post-conception and days post-conception^2 with FCM as the response variable (*50*). We used residual FCM from this GLM as the corrected values to determine how they affected offspring growth rates. We also included covariates for white spruce cone production in the current and previous year, Julian parturition date, and litter size at our first nest entry (~0 d post-parturition).

We grouped females fed the three doses of cortisol together into one group (n = 14 litters from 14 females) because of low sample sizes within each treatment group and compared their life history responses and concentrations of FCM to control females (n = 16 litters from 16 females). However, there was a dose-dependent effect of the three different doses of hydrocortisone on offspring growth rates where females fed higher doses of hydrocortisone produced faster growing offspring (Fig. S2). We determined how provisioning females with hydrocortisone affected concentrations of FCM during pregnancy using a LMM that contained a 2-level categorical variable for treatment (fed GCs or control). We used general linear models or LMMs to determine how birth weight, litter size, and offspring growth rates varied among control females and those fed

hydrocortisone by including a 2-level categorical variable for treatment (fed GCs or control)**.** We also included a covariate for estimated age of the offspring for the birth weight model and a covariate for litter size for the growth rate model. We used a generalized linear model (binomial errors, logit link, dispersion parameter from entirely fixed-effects model = 1.57) containing a 2-level categorical variable for treatment (fed GCs or control) to determine if litter sex ratio varied among the two treatment groups (Table S7).

All of our statistical analyses were conducted in R (version 2.14.1: *52*) and we used lme4 (version 0.999375-42: *53*) to conduct our LMMs and GLMMs. We included random intercept terms for squirrel identification (*54*) to account for any lack of independence among repeated observations for a single female (e.g., multiple offspring growth rates or fecal samples per female). When we had repeated measures within a year, we also included a random intercept term for year or study area-year combination. We visually inspected the residuals from our models for normal distribution, homoscedasticity, and if there were outlying observations with high leverage. We also used Markov Chain Monte Carlo methods (mcmcsamp function in lme4) to estimate the 95% credible (confidence) intervals around the parameter estimates in the general linear models and linear mixed-effects models for statistical inference. Continuous fixed effects were standardized (mean of 0 and unit variance) prior to statistical analysis and reported in Tables S1-S7.
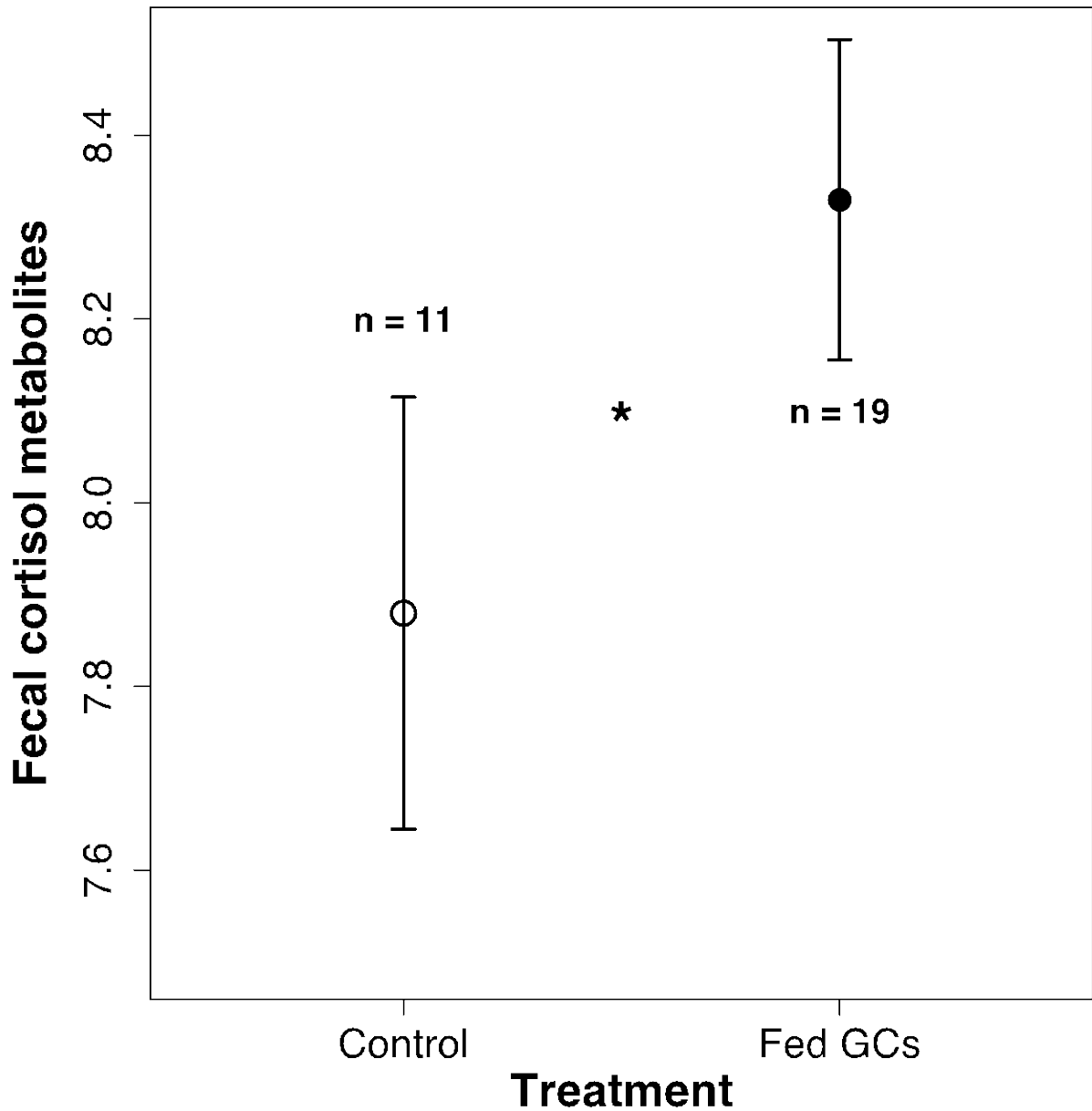
Fig. S1. Pregnant female red squirrels fed cortisol (3, 6, or 12 mg/day) had significantly higher concentrations of fecal cortisol metabolites (FCM) than control females (t_{29} = 1.99, P = 0.028). Raw concentrations of FCM (ng/g dry feces) on a ln-scale are shown on y-axes. Sample sizes represent the number of fecal samples analyzed. Significant differences are noted by "*" (P < 0.05).
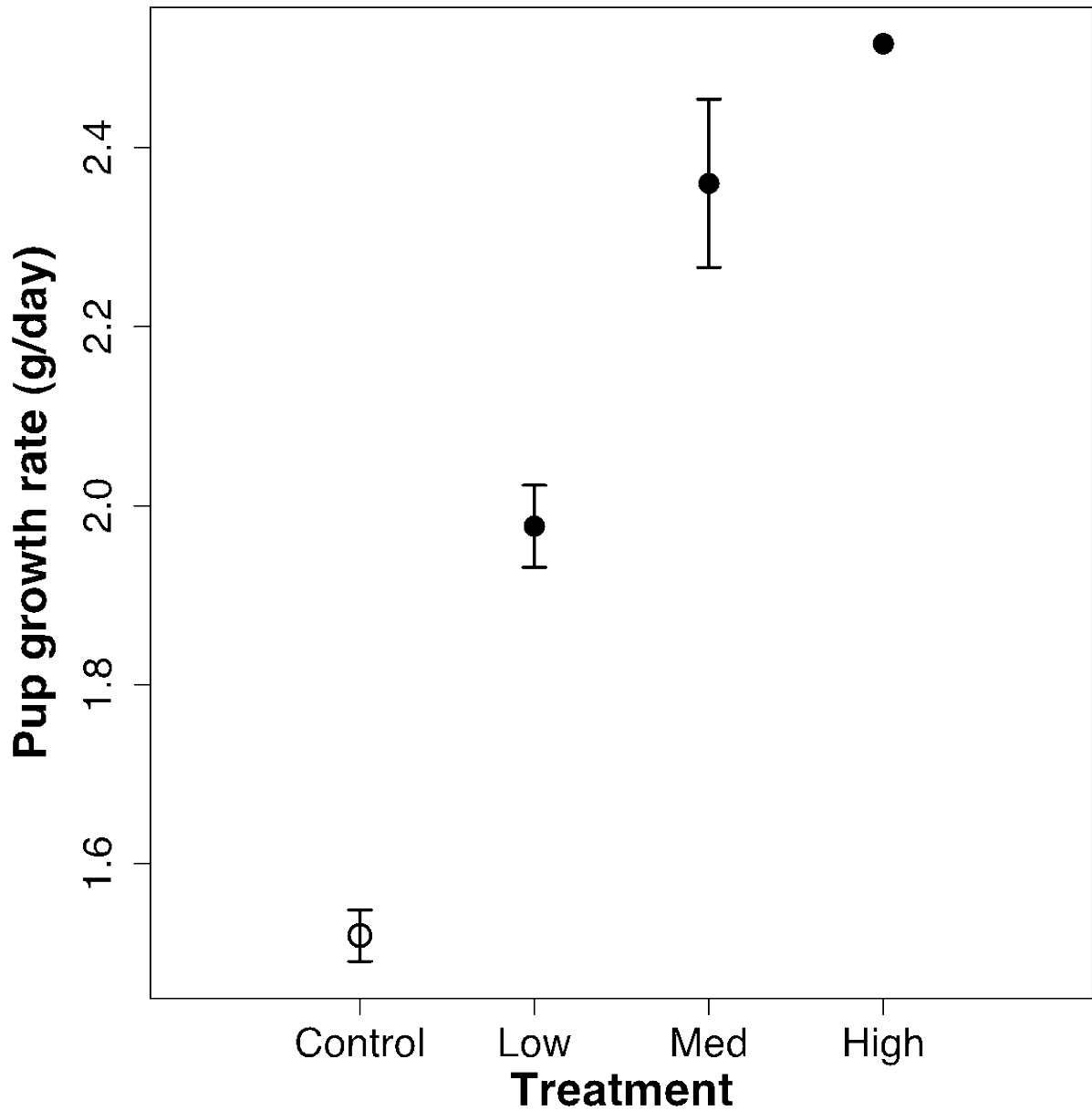
Fig. S2. The relationship between offspring growth rates and maternal glucocorticoid treatment exhibited a dose-response relationship with offspring produced by female red squirrels fed low (3 mg/day, n = 9 pups from 2 females), medium (6 mg/day, n = 5 pups from 2 females), and high (12 mg/day, n = 1 pup from 1 female) doses of cortisol growing faster than control females (n = 12 pups from 3 females).
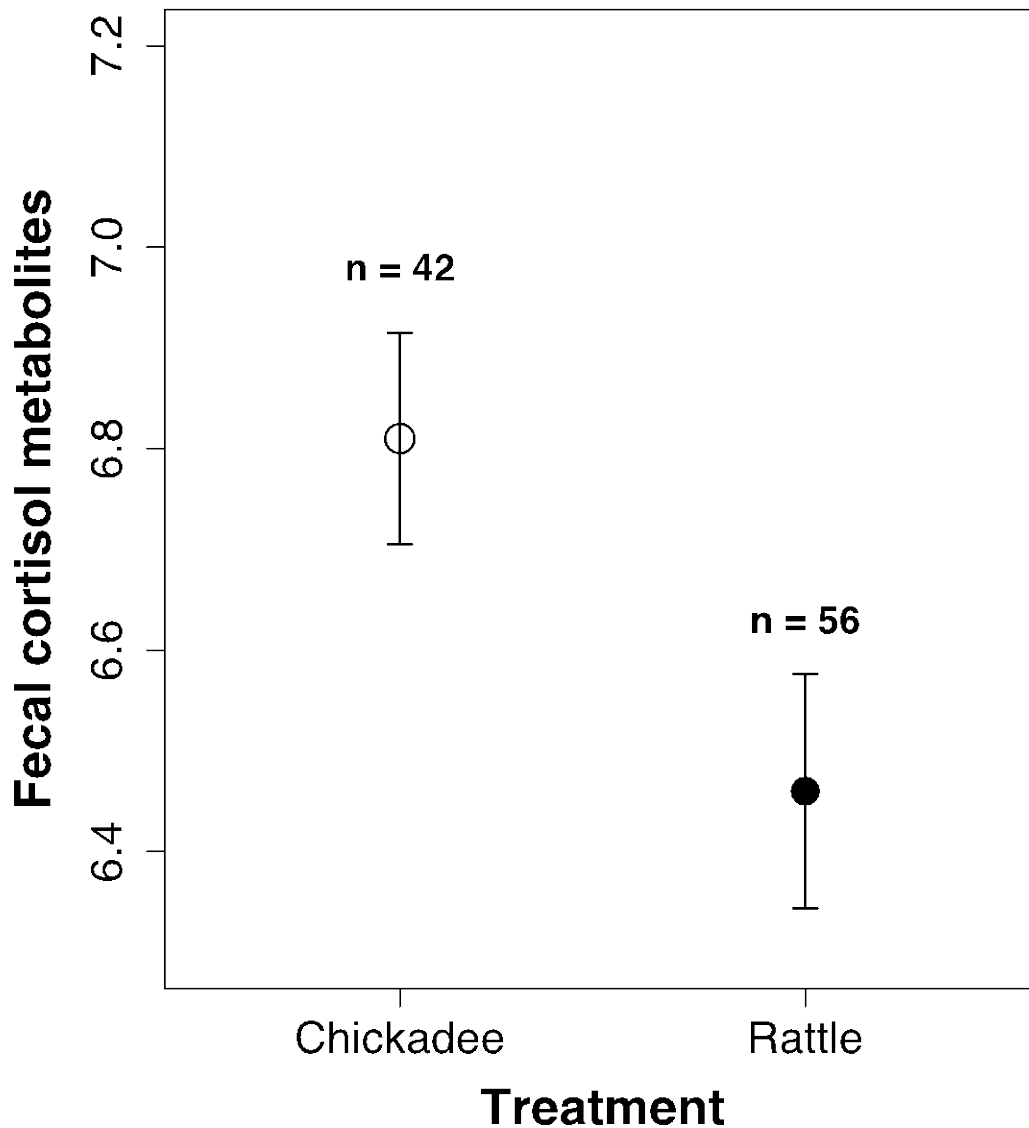
Fig. S3. After we controlled for the effects of reproductive condition when the samples were collected (*50*), females in the rattle and chickadee playback treatment groups had statistically similar concentrations of fecal cortisol metabolites (FCM) prior to being exposed to any playbacks on their own midden or those of neighboring squirrels (t_{97} = -0.93, *P* = 0.17). Sample sizes represent the number of fecal samples analyzed. Values on y-axis are raw concentrations of FCM (ln ng/g dry feces).

Table S1. Across 23 years of study, female red squirrels that produced fast growing offspring under high-density conditions had significantly higher relative reproductive success (offspring growth x density).

Fixed effect	Parameter ± SE	95% CI	t	df	P
Intercept	1.47 ± 0.17	1.11 – 1.83	8.44	726	<0.0001
Density	**-0.19 ± 0.08**	**-0.37 – -0.02**	**-2.26**	**726**	**0.012**
Litter size	-0.19 ± 0.20	-0.58 – 0.20	-0.94	726	0.17
Offspring growth	-0.17 ± 0.19	-0.53 – 0.21	-0.88	726	0.19
Parturition date	-0.10 ± 0.23	-0.32 – 0.55	0.44	726	0.33
Litter size x Density	**0.20 ± 0.10**	**0.003 – 0.41**	**1.94**	**726**	**0.03**
Parturition date x Density	-0.15 ± 0.12	-0.38 – 0.08	-1.24	726	0.11
Offspring growth x Density	**0.20 ± 0.09**	**0.02 – 0.39**	**2.15**	**726**	**0.016**

Relative reproductive success calculated as the number of offspring that survived to the following spring over the average number of offspring produced for that study area-year combination. Density reflects the spring squirrel density (squirrels/hectare). Random intercept term for individual ($\sigma^2 = 0.16$) and study area-year ($\sigma^2 = 0$) were included. 95% credible intervals (CI) shown. Statistically significant results shown in bold.

Table S2. Offspring produced by female red squirrels experiencing experimentally increased perceived population density (rattle playbacks) grew significantly faster than those produced by control females but similar to those produced by food-supplemented females that were also experiencing increased actual density.

Response Variable	Fixed effect	Parameter ± SE	95% CI	t	df	P
Neonate mass	Intercept	15.1 ± 0.38	14.64 – 15.54	40.00	369	<0.0001
	Estimated age	**6.96 ± 0.20**	**6.70 – 7.21**	**34.64**	**369**	**<0.0001**
	Litter size	-0.11 ± 0.32	-0.52 – 0.29	-0.35	369	0.37
	Food-add	-0.23 ± 0.53	-0.86 – 0.41	-0.43	369	0.34
	Rattle playbacks	0.06 ± 0.53	-0.61 – 0.66	0.11	369	0.46
	Lit. size x Food-addition	-0.17 ± 0.46	-0.72 – 0.45	-0.37	369	0.35
	Lit. size x Rattle playbacks	-0.22 ± 0.51	-0.85 – 0.42	-0.43	369	0.33
Litter size	Intercept	4.07 ± 0.19	3.70 – 4.43	22.21	96	<0.0001
	Food-addition	-0.25 ± 0.25	-0.75 – 0.25	-0.99	96	0.16
	Rattle playbacks	-0.33 ± 0.25	-0.82 – 0.16	-1.32	96	0.09
Offspring growth	Intercept	1.51 ± 0.09	1.40 – 1.60	17.11	186	<0.0001
	Litter size	**-0.36 ± 0.07**	**-0.42 – -0.25**	**-4.93**	**186**	**<0.0001**
	Food-addition	0.21 ± 0.13	0.06 – 0.37	1.59	186	0.057
	Rattle playbacks	-0.04 ± 0.13	-0.17 – 0.11	-0.32	186	0.37
	Lit. size x Food-addition	**0.28 ± 0.11**	**0.13 – 0.39**	**2.54**	**186**	**0.006**
	Lit. size x Rattle playbacks	**0.24 ± 0.12**	**0.07 – 0.36**	**1.98**	**186**	**0.024**

Results are from a linear mixed-effects models (LMMs) that included a 2-level categorical variable for food-addition (on or off food-addition area) and playback treatment (rattle playbacks or control). A random intercept term for individual was included in the LMMs for neonate mass ($\sigma^2 = 3.5$) and offspring growth ($\sigma^2 = 0.13$).

Table S3. The effects of the experimental increase in perceived population density on litter sex ratio (proportion of males) depended upon litter size with larger litters produced by females experiencing increased perceived density (rattle playbacks) containing fewer males than did those from control females. Food-supplemented females experiencing heightened actual density produced litters with more males than control females.

Fixed effect	Parameter ± SE	z	P
Intercept	-0.26 ± 0.21	-1.29	0.09
Litter size	0.28 ± 0.18	1.51	0.07
Food-addition	**0.55 ± 0.26**	**2.09**	**0.02**
Rattle playbacks	0.37 ± 0.27	1.38	0.085
Litter Size x Food-addition	-0.20 ± 0.25	-0.79	0.21
Litter Size x Rattle playbacks	**-0.49 ± 0.29**	**-1.69**	**0.047**

Results are from a generalized linear mixed-effects model that included a random intercept term for individual (σ^2 = <0.01).

Table S4. Concentrations of fecal cortisol metabolites (FCM) were significantly higher in pregnant female red squirrels experiencing locally high population density or when actual population density was experimentally increased through long-term food addition, or when perceived density was experimentally increased with rattle playbacks compared with controls.

Density manipulation	Fixed effect	Parameter ± SE	95% CI	t	df	P
Local density	Intercept	6.45 ± 0.28	5.87 – 6.99	23.04	155	<0.0001
	Days post-conception	**-0.18 ± 0.05**	**-0.28 – -0.06**	**-3.55**	155	**0.0002**
	Population density	**0.35 ± 0.09**	**0.16 – 0.51**	**3.63**	**155**	**0.0002**
Actual density ⬆	Intercept	6.84 ± 0.19	6.37 – 7.30	34.77	162	<0.0001
	Days post-conception	**-0.18 ± 0.05**	**-0.28 – -0.06**	**-3.68**	162	**0.0001**
	Food-addition study area	**0.52 ± 0.14**	**0.27 – 0.73**	**3.82**	162	**<0.0001**
Perceived density ⬆	Intercept	6.90 ± 0.13	6.65 – 7.16	52.16	48	<0.0001
	Days post-conception	**-0.26 ± 0.09**	**-0.44 – -0.07**	**-2.84**	**48**	**0.0033**
	Rattle playbacks	**0.41 ± 0.18**	**0.02 – 0.75**	**2.24**	**48**	**0.015**

Results from a linear mixed-effects models (LMMs) that included either i) local population density (*10*) or a 2-level categorical variable for ii) food-addition treatment (on or off food-addition study area), or iii) playback treatment (rattle playbacks or control). A covariate for days post-conception was included in these models because of their effects on concentrations of FCM. Random intercept terms for individual and year were included in the model for the effects of natural variation in density on FCM (individual σ^2 = 0.15, year σ^2 = 0.2) and in the model for the effects of the experimental increase in actual density on FCM (individual σ^2 = 0.18, year σ^2 = 0.11). A random intercept term for individual was included in the model for the effects of the playback treatment on concentrations of FCM (σ^2 = 0.02). Concentrations of FCM in ln ng/g dry feces.

Table S5. Elevated concentrations of fecal cortisol metabolites (FCM) measured in pregnant female red squirrels were positively associated with the production of faster growing offspring.

Fixed effect	Parameter ± SE	95% CI	t	df	P
Intercept	1.77 ± 0.05	1.41 – 1.70	33.2	98	<0.0001
Previous cones	-0.01 ± 0.007	-0.02 – 0.09	-1.52	98	0.07
Current cones	**-0.13 ± 0.005**	**-0.04 – 0.05**	**-25.55**	**98**	**<0.0001**
Parturition date	**-0.23 ± 0.01**	**-0.16 – 0.01**	**-21.5**	**98**	**<0.0001**
Litter size	**-0.36 ± 0.01**	**-0.28 – -0.14**	**35.15**	**98**	**<0.0001**
FCM	0.01 ± 0.005	-0.08 – 0.08	1.94	98	0.028

Results from a linear mixed-effects model that contained covariates for white spruce cone abundance in the previous autumn (previous cones) and in the upcoming autumn (current cones: *14*) and litter characteristics (litter size, parturition date). Random intercept term for individual ($\sigma^2 = 0.17$) was included. Concentrations of FCM in ln ng/g dry feces.

Table S6. Offspring produced by female red squirrels fed cortisol (glucocorticoids: GCs) during pregnancy and early lactation grew significantly faster than those produced by control females.

Response Variable	Fixed effect	Parameter ± SE	95% CI	t	df	P
Neonate mass	Intercept	12.78 ± 1.76	11.02 – 14.49	7.23	127	<0.0001
	Estimated age	-0.38 ± 1.39	-1.59 – 1.11	-0.27	127	0.39
	Litter size	0.20 ± 1.65	-1.40 – 2.14	0.12	127	0.45
	Fed GCs	0.88 ± 2.80	-1.87 – 3.55	0.31	127	0.38
	Litter Size x Fed GCs	-0.03 ± 2.53	-2.56 – 2.61	-0.01	127	0.49
Litter Size	Intercept	3.87 ± 0.27	3.33 – 4.42	14.47	29	<0.0001
	Fed GCs	-0.09 ± 0.39	-0.89 – 0.71	-0.23	29	0.41
Offspring growth	Intercept	1.53 ± 0.12	1.39 – 1.66	12.64	26	<0.0001
	Litter size	-0.29 ± 0.17	-0.48 – -0.06	-1.68	26	0.052
	Fed GCs	**0.86 ± 0.17**	**0.61 – 1.03**	**4.98**	**26**	**<0.0001**

Results are from a linear mixed-effects models (neonate mass and offspring growth) or a general linear model (litter size) that included a 2-level categorical variable for glucocorticoid treatment (fed GCs or fed control). A random intercept term for individual was included in the model for neonate mass ($\sigma^2 = 28.4$) and a random intercept term for litter identity was included in the model for neonate mass ($\sigma^2 = 28.4$) and offspring growth ($\sigma^2 = 0.04$).

Table S7. The sex ratio of litters (proportion of males) produced by female red squirrels fed cortisol (glucocorticoids: GCs) during pregnancy and early lactation did not differ from control females.

Fixed effect	Parameter ± SE	z	P
Intercept	0.20 ± 0.28	0.72	0.23
Litter size	0.44 ± 0.31	1.41	0.08
Fed GCs	-0.30 ± 0.41	-0.74	0.23
Lit. Size x Fed GCs	-0.48 ± 0.46	-1.06	0.14

Results are from a generalized linear model that included a 2-level categorical variable for glucocorticoid treatment (fed glucocorticoids or control).

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