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# Maternal androgens and behaviour in free-ranging North American red squirrels

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Variation in maternal behaviour can have profound consequences on offspring phenotype, survival, reproductive success, as well as maternal fitness. Understanding the mechanisms that underlie variation in maternal care can provide us with a more thorough understanding of the evolution of parental care. In some taxa, the effects of steroid hormones such as testosterone on paternal care have been well studied. However, patterns of female androgens in natural populations are rarely documented and relationships between maternal androgens and behaviour remain poorly studied. In this study, we first validated an enzyme immunoassay to measure faecal androgen metabolites (FAM) in territorial North American red squirrels, *Tamiasciurus hudsonicus*. We validated this assay by demonstrating that (1) our antibody reacts with testosterone metabolites, (2) reproductive females have significantly higher FAM than nonreproductive females, and (3) patterns of FAM were largely mirrored in plasma androgen levels. Second, we tested the hypothesis that androgen concentrations drive behavioural trade-offs in free-ranging breeding female red squirrels during gestation and lactation using 3 years of FAM data and 10 years of behavioural observations. FAM increased after conception and parturition, peaked during mid-lactation around the time of juvenile emergence, and then declined during the remainder of lactation and after weaning. Around the peak of FAM during mid-lactation, nest use was lowest, while territory defence and time spent foraging were at their highest levels. These associations between maternal androgens and behaviour support the hypothesis that androgens may play an important role in mediating maternal behaviour in free-ranging animals.


The extended period of maternal care is one of the more interesting and unique features of mammalian reproduction. The proper development and survival of mammalian offspring depend upon the presence of mothers and adequate provisioning of maternal care. Because of the relatively prolonged period of postnatal interaction between mothers and their offspring, variation in maternal care can have profound and long-lasting consequences on offspring phenotype, survival and reproductive success, as well as influence maternal lifetime fitness (Clutton-Brock 1991). For example, the time that females spend nursing their offspring can have long-term effects on body mass and sexual ornamentation (horn size) in male offspring of a sexually dimorphic ungulate species, in which body and horn size are likely to be major determinants of reproductive success (Festa-Bianchet et al. 1994). Variation in the amount of maternal care provided by laboratory rats in the form of arched-backed nursing and licking/grooming of offspring can also have life-long consequences on neural development, physiological responsiveness to stressors, and behaviour (reviewed by Meaney 2001; Champagne 2008; see also Maestripieri et al. 2007). This variation in maternal behaviour can then be preserved within genetic lineages across multiple generations via epigenetic mechanisms (reviewed in: Champagne 2008; Champagne & Curley 2009). Despite its potential ecological and evolutionary importance (Mousseau & Fox 1998), maternal behaviour has not been well documented in many species of free-ranging mammals, and the physiological mechanisms that may underlie variation in maternal care are relatively unexplored.

Variation in steroid hormone concentrations such as androgens may be one major source of differences in parental behaviour. The activational effects of testosterone clearly influence paternal behaviour (reviewed in Lonstein & De Vries 2000). For example, in mammals, testosterone can reduce paternal care (reviewed in:

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Lonstein & De Vries 2000; Nunes et al. 2000a, 2001; Schradin et al. 2009; for an opposite result, see Trainor & Marler 2001, 2002) but increase mating effort (Clark et al. 1997). Similarly, in male birds, elevated plasma testosterone simultaneously decreases levels of paternal care and increases aggression and mating effort (Hegner & Wingfield 1987; Wingfield et al. 1990; Ketterson & Nolan 1999; Hau 2007; McGlothlin et al. 2007). However, few studies have examined whether androgens influence maternal behaviour, which is surprising for two reasons. First, females also produce testosterone and its androgen precursors in the gonads, the adrenals (Staub & De Beer 1997), and potentially in the brain (Baulieu 1991; Soma et al. 2008; Pradhan et al. 2010). Second, androgens may play an important role in basic female reproductive physiology (reviewed in Walters et al. 2008) and behaviour (Sandell 2007).

Recent studies in natural populations have suggested an important but equivocal role for androgens in mediating maternal behaviour. For example, experimental elevation of testosterone decreased the time that female dark-eyed juncos, *Junco hyemalis*, spent brooding eggs (O'Neal et al. 2008; but see Clotfelter et al. 2004 for an opposite result), but did not affect nest defence or nestling provisioning rates (Clotfelter et al. 2004; O'Neal et al. 2008). In captive mammals, correlative studies suggest that high concentrations of testosterone inhibit or perhaps lessen the quality of maternal care (Bridges et al. 1982; Lonstein & De Vries 2000; Fite et al. 2005), which could increase the probability of infant death (Altmann et al. 2004). As such, variation in maternal androgens could have important effects on maternal behaviour and perhaps reproductive success, but these relationships have been rarely examined in free-ranging mammals. In one exception, Dloniak et al. (2006) demonstrated that maternal androgen concentrations during gestation in female spotted hyaenas, *Crocuta crocuta*, influenced the aggressive behaviour of their offspring, which is known to have important consequences for offspring fitness.

In this study, we investigated patterns of maternal behaviour and androgen concentrations in free-ranging North American red squirrels, *Tamiasciurus hudsonicus*, using behavioural observations collected in 10 years between 1994 and 2008 and using faecal samples collected over 3 years (2006–2008). We tested the hypothesis that androgen concentrations drive behavioural trade-offs in breeding female red squirrels. Specifically, we assessed the relationship between faecal androgen metabolites (FAM) and behavioural measures of territory defence, foraging and maternal behaviour (nest use) during reproduction (time from conception to weaning). Based on previous studies (Fite et al. 2005), we predicted that periods of heightened maternal androgens would be associated with increased time spent foraging and engaged in territory defence and decreased time allocated towards maternal behaviour.

## METHODS

### *Study Area and Species*

We studied a free-ranging population of red squirrels near Kluane Lake in the southwest Yukon, Canada (61°N, 138°W) that has been monitored continuously since 1987 (see McAdam et al. 2007 for details). The study area is dominated by white spruce (*Picea glauca*) trees whose seeds are the major food source for red squirrels in this region. In the autumn of each year, red squirrels collect and cache newly matured spruce cones in a larder hoard in the centre of their territory (Fletcher et al. 2010). Both male and female red squirrels individually defend a cache of cones from all male or female conspecifics year-round except during periods of oestrus, when females tolerate males on their territories (Smith 1968).

Most red squirrels in our study populations were permanently marked with uniquely numbered metal eartags (National Band and Tag, Newport, KY, U.S.A.) after being temporarily removed from their natal nest when they were around 25 days old. As part of a larger study, squirrels in our study population were frequently live-trapped using Tomahawk live traps ($48 \times 13 \times 13$ or $48 \times 15 \times 15$ cm, Tomahawk Live Trap Co., Tomahawk, WI, U.S.A.). During behavioural observations, we determined the identities of squirrels from a distance based on unique combinations of coloured electrical wire that was threaded through the metal eartags.

Red squirrels in the Yukon are seasonal breeders and, in most years, females are in behavioural oestrus for 1 day and produce one litter after a ~35-day gestation period followed by a ~70-day lactation period (Steele 1998; McAdam et al. 2007; S. Boutin, personal communication). Litter size ranges from one to seven pups per litter but is generally three or four pups per litter (mean ± SE: 3.10 ± 0.043 pups per litter: McAdam et al. 2007). Juveniles typically emerge from their natal nest around 77 days postconception (range 71–86 days postconception), but they continue to nurse until ~106 days postconception (Humphries & Boutin 1996). During each capture event, we determined the reproductive status of males (abdominal versus scrotal testes) and females (pregnant, lactating, or neither). We determined the dates of parturition by frequent trapping (every 3 days), weighing and palpation of pregnant females, or by assigning an age to neonates based upon their mass when they were accessed in their natal nest soon after birth (for calculation of parturition dates, see: Becker 1993; Boutin & Larsen 1993). Dates of conception were estimated by subtracting 35 days from known dates of parturition.

### *Measuring Maternal Behaviour in Free-ranging Red Squirrels*

We recorded the behaviour of radiocollared (model PD-2C, 4 g, Holohil Systems Limited, Carp, Ontario, Canada) breeding females with instantaneous sampling at 30 s intervals (Altmann 1974). We conducted a total of 903 7-minute observation sessions on 125 female red squirrels that were radiocollared over a 10-year period between 1994 and 2008 (1994–1997, 1999, 2001–2004, 2008). This corresponded to an average of 7.2 sessions per female squirrel (range 1–48 sessions), and the interval between behavioural observation sessions on the same individual was at least 28 min. Behavioural observations on each female were conducted opportunistically during the entire reproductive period with the goal of maximizing the spread of observations for each female across different reproductive conditions and dates. We performed a total of 41 sessions during gestation, 588 during lactation, and 274 after weaning. Behavioural focal sessions occurred from 0600 to 2300 hours, but the majority of these observations occurred from 0700 to 1200 hours (743 of 903), which generally corresponded to the period of highest activity of red squirrels in this region (B. Dantzer, personal observations).

We categorized and analysed female behaviour in a similar way as previous studies of red squirrels (Stuart-Smith & Boutin 1995; Humphries & Boutin 2000; Anderson & Boutin 2002) including whether the squirrel was in or out of its nest, feeding, foraging, travelling, resting, vigilant, vocalizing ('barking' and 'rattling': Smith 1968), or out of sight. In lactating squirrels, we interpret time spent in the nest with offspring as an indirect measure of maternal behaviour as mothers are ensuring proper thermoregulation of offspring, grooming, nursing and interacting with their dependent offspring. Foraging and feeding observations were identified as separate behaviours in the field but were grouped together to calculate the proportion of time spent self-provisioning and we refer to these two behaviours simply as foraging. Rattling is the territorial vocalization of red squirrels, whereas barking is an alarm call (Smith 1968).

The same observer (B.D.) collected all behavioural observations in 2008, but 39 observers collected the data between 1994 and 2004. Although collection of observations was similar from 1994 to

2004 and in 2008, there were some important differences that affected our analysis of the data. First, rattling was recorded continuously (all-occurrences) only in 2008 and not from 1994 to 2004. Second, we only recorded whether the focal squirrel was on or off its midden (i.e. its hoard of cached white spruce cones) during observations recorded in 2008 but not in those from 1994 to 2004. As a result of these differences, we treated these two data collection periods as distinct data sets and analysed them separately (see Statistical Analyses section below).

### *Validation of an Enzyme Immunoassay to Measure Faecal Androgen Metabolites*

We validated an enzyme immunoassay (EIA) to measure faecal androgen metabolites (FAM) in this species as follows. (1) We conducted a radiometabolism study to determine the route of excretion (faeces or urine) and time delay of excretion of testosterone metabolites (sensu Palme et al. 2005) by injecting captive female red squirrels with radiolabelled testosterone and collecting urine and faeces over the next 72 and 120 h, respectively (Appendix 1). (2) We performed reverse-phase high performance liquid chromatography (RP-HPLC) to characterize the structure of the faecal testosterone metabolites and demonstrate that our EIA antibody detects the testosterone metabolites (Appendix 1, Fig. A1). (3) We demonstrated that in female red squirrels, FAM mirrored plasma androgen concentrations, with lactating females having significantly higher FAM and plasma androgen levels than nonbreeding females (Appendix 2, Fig. A2). More details of these procedures and their results can be found in Appendix 1 and 2.

### *Collection and Extraction of Faecal Samples*

Between 2006 and 2008, we collected a total of 384 faecal samples from 88 female squirrels prior to conception ($N = 16$), during gestation (range 1–37 days postconception: $N = 123$), lactation (range 35–106 days postconception: $N = 196$) and post-weaning (after 106 days postconception: $N = 49$). Faecal samples were collected from underneath live-traps and placed in individual 1.5 ml vials in a −20 °C freezer within 5 h of collection. Squirrels were in traps for less than 2 h prior to handling, which is not long enough for FAM concentrations to be affected by trap-induced stress from the current capture event (Appendix 1; Dantzer et al. 2010; see also Harper & Austad 2001). Furthermore, we only used faecal samples from squirrels that had not been trapped or handled within the previous 72 h to avoid confounding effects of previous trapping and handling events. Faecal samples collected from January to April were generally already frozen at the time of collection and remained so while in the field (B.D., personal observation). In the warmer months (May–September), faecal samples were placed into insulated containers filled with wet ice until they were transferred to a −20 °C freezer. FAM in faecal subsamples left at room temperature for 5 h (4.28 ± 0.27 ln ng/g dry faeces) did not differ from the FAM in subsamples of the same faecal sample that were frozen immediately (4.29 ± 0.13; paired $t$ test: $t_{10} = 0.02$, $P = 0.98$). As such, there should be no systematic bias in FAM in faeces collected in the field and kept under cold conditions until frozen.

All faecal samples were lyophilized (LabConco, MO, U.S.A.) for 14–16 h, frozen in liquid nitrogen, and then pulverized using a mortar and pestle. Between samples, we rinsed the mortar and pestle with 5 ml of 80% methanol. We then extracted 0.05 g of the dry ground faeces by adding 1 ml of 80% methanol. This solution was then shaken with a multivortexer at 1450 revolutions per minute for 30 min, and finally centrifuged for 15 min at 2500$g$ (24 516.625 m/s$^2$; Touma et al. 2003; Palme 2005). The resulting supernatant was stored at −80 °C until analysis via EIA.

### *Determination of Immunoreactivity in Faecal Samples*

To quantify FAM, we used a previously developed testosterone EIA that measures 17β-OH androgens (Palme & Möstl 1994), which has been successfully validated in some primate species (Möhle et al. 2002) and guinea pigs (Bauer et al. 2008). Details of this procedure (Möhle et al. 2002) and cross-reactivities of the antibody can be found elsewhere (Palme & Möstl 1994). We are confident that our EIA antibody detected FAM from testosterone because it showed a high affinity for 17α-hydroxyandrogens compared to other androgens (cross-reactivity with 17-oxo- or 17α-hydroxyandrostanes was below 0.1%: Palme & Möstl 1994). Samples were run in duplicate, and the intra- and interassay coefficients of variation were 6.3 and 16.5% ($N = 30$ plates). The assay had a sensitivity of 0.3 pg per well.

### *Statistical Analyses*

To determine how behaviour varied among breeding female red squirrels, we used a principal components analysis (PCA) to reduce our multiple behavioural response variables (see Table 1 for list of behaviours) into a fewer number of synthetic variables. We conducted separate PCAs for behavioural observations conducted from 1994 to 2004 ($N = 627$ trials) and those conducted in 2008 ($N = 276$ trials) for the two reasons described above (see also Table 1 for differences in PCA loadings). We used the unrotated first principal component (PC1) of a PCA of the behavioural correlation matrix (Table 1), which explained 22 and 19.9% of the total variation for the 1994–2004 and 2008 behavioural observations, respectively. While there is no general agreement about how strongly an individual variable should load onto a principal component to warrant interpretation (Budaev 2010), we selected behaviours with loadings greater than 0.30. All PC1 loadings were greater than 0.37 except for rattling from the PCA for the 1994–2004 behavioural observations (Table 1).

We examined changes in maternal behaviour and FAM during reproduction by modelling changes in PC1 scores and FAM concentrations as a function of days since conception. We first used three separate linear mixed models (LMM) with the same fixed effects to examine the variation across reproduction (time from conception to weaning) in (1) PC1 scores from the 1994–2004 behavioural observations, (2) PC1 scores from the 2008 behavioural observations and (3) FAM concentrations. We then examined changes in specific maternal behaviours during reproduction by modelling the proportion of time spent (1) in the nest, (2) foraging and (3) rattling as a function of days since conception using three

**Table 1**
Loadings of the first axis from principal components analysis using correlation matrices from 7 min behavioural observations conducted on breeding female red squirrels in 1994–2004 and 2008

| | Year(s) of behavioural observations | |
|---|---|---|
| Behaviour | 1994–2004* | 2008† |
| Barking | −0.08 | −0.1 |
| **Feeding** | **−0.37** | **−0.38** |
| **Foraging** | **−0.42** | **−0.42** |
| **In nest** | **0.63** | **0.41** |
| On midden | | 0.14 |
| Out of sight | −0.16 | 0.22 |
| **Rattling** | **−0.23** | **−0.45** |
| Resting | 0.04 | 0.11 |
| **Travelling** | **−0.43** | **−0.45** |
| Vigilant | −0.07 | −0.02 |

Behaviours in boldface font indicate loadings that were used in our interpretation for principal component 1. No observations were made 'On midden' in the 1994–2004.
* These models are based on 627 observation sessions on 44 squirrels.
† These models are based on 276 observation sessions on 81 squirrels.

separate generalized linear mixed models (GLMM) with binomial errors (logit link, models fit with Laplace approximation). Because we recorded rattling continuously in 2008, we only analysed the behavioural focal data from 1994 to 2004 to determine how the proportion of time rattling varied among breeding females. Because maternal behaviour and androgens may vary nonlinearly across the reproductive period, we included both a linear and a quadratic term for days postconception (hereafter, days postconception$^2$) in our statistical models when preliminary analyses using nonlinear regression showed that there were significant nonlinearities between our response and predictor variables (general additive models: Hastie & Tibshirani 1990).

In all of these mixed effects models, we accounted for the repeated sampling of behaviour or FAM from the same squirrels or by the same observers by including a random intercept term for squirrel ID and a random intercept term for observer using the lme4 package (Bates et al. 2008; Pinheiro & Bates 2009) in R (version 2.9.2, R Development Core Team 2009). We calculated the proportion of residual variance in the LMMs that was due to the individual squirrel (i.e. repeatability: Lessels & Boag 1987) by dividing the proportion of variance explained by the random effect (among-individual variance) by the total residual variance (among-individual variance + within-individual variance) and used likelihood ratio tests to determine whether the random effects improved the fit of each of the models (Pinheiro & Bates 2009). We used Wald $t$ tests to test the significance of the fixed effects of the GLMMs (Bolker et al. 2008).

To meet assumptions of normality, we ln-transformed FAM and PC1 values prior to analysis. Diagnostic plots after the transformations revealed that the residuals from the models described above were normally distributed and homoscedastic, and there were no outlying observations with high leverage. FAM concentrations are expressed as ln-transformed ng/g dry faeces. For all of the LMM and GLMMs described above, days postconception was standardized, but we present the raw days postconception in the figures.

We also used segmented regression (segmented package in R: Muggeo 2008) to determine whether changes in maternal behaviour and FAM occurred around the same time period, which would suggest covariation between maternal androgens and behaviour. Segmented or broken-line regression is typically used in data sets where the relationship between independent and dependent variables exhibits an abrupt change past some threshold (breakpoint) of the independent variable. In this approach, many separate regressions are performed on different intervals of the independent variable to identify whether there are differences in the magnitude or sign of the linear relationship between the independent and dependent variables. Here, segmented regression identified where the change in relationship between FAM or the measures of maternal behaviour and days postconception occurred (breakpoint) with some estimate of uncertainty (SE) about the location of this breakpoint in the regression lines. This approach allowed us to identify the points at which androgens and allocation towards specific behaviours peaked during lactation.

We present all results as means ± SE and considered differences statistically significant at $\alpha = 0.05$. All statistical analyses were conducted using R (version 2.9.2, R Development Core Team 2009).

### *Ethical Note*

All animal care protocols complied with both the Canadian Council on Animal Care and the ASAB/ABS *Guidelines for the Use of Animals in Research and Teaching*, and were approved by the Institutional Animal Care and Use Committees of the University of Toronto (no. 20006991) and Michigan State University (no. 04/08-046-00).

## RESULTS

### *Maternal Behaviour*

We interpreted PC1 as an index of time allocation of breeding females as the loadings reflected a trade-off between maternal behaviour (time in the nest interacting with offspring) and time engaged in other behaviours (foraging, rattling, travelling; Table 1). In both data sets, larger values of PC1 represented sessions in which females spent more time in their nest with their pups and less time foraging, rattling and travelling (Table 1).

Maternal behaviour varied significantly before and after parturition in the analyses of PC1 for both the 1994–2004 and 2008 data sets. For the 1994–2004 behavioural observations, PC1 scores decreased linearly with increasing days since conception (slope on ln scale for days postconception = $-0.068 \pm 0.015$; $t_{626} = -4.49$, $P < 0.0001$; Table 2) indicating that nest use decreased, whereas foraging, rattling and travelling increased. Maternal behaviour in 2008 showed a similar pattern (slope on ln scale for days postconception = $-0.015 \pm 0.005$; $t_{275} = -2.81$, $P < 0.0001$; Table 2) except that there was also a significant nonlinear relationship between PC1 scores and days postconception (slope on ln scale for quadratic term for days postconception = $0.00007 \pm 0.00003$; $t_{275} = 2.20$, $P = 0.014$; Table 2).

Changes in the three important female behaviours (proportion of time in the nest, foraging and rattling) corroborated the observed changes in PC1 scores. Soon after parturition (35 days postconception), maternal behaviour was highest ($55.9 \pm 8.5\%$ of time in nest), declined into early lactation, was lowest during mid-lactation when juveniles were first emerging from their natal nest ($15.2 \pm 2.9\%$ of time in nest), and then slightly increased following juvenile emergence (slope on ln scale for quadratic term for days postconception = $0.11 \pm 0.02$; $t_{902} = 5.29$, $P < 0.0001$; Fig. 1a, Table 3). The proportion of time females spent rattling was low soon after parturition ($0.4 \pm 0.28\%$ of time spent rattling), increased into early lactation and peaked during mid-lactation during the period of juvenile emergence ($2.3 \pm 0.54\%$ of time), and then remained fairly constant throughout the rest of lactation and postweaning (slope on ln scale for quadratic term for days postconception = $-0.21 \pm 0.079$;

**Table 2**
Effects of days since conception on the behaviour of breeding female red squirrels

| Model | | | | |
|---|---|---|---|---|
| | **Random effect** | **Variance** | $\chi^2_1$ | ***P*** |
| PC1 (1994–2004)* | ID | 0.0076 | 22.36 | <0.0001 |
| | Observer | 0.0019 | 1.3 | 0.30 |
| PC1 (2008)† | ID | 0.05 | 13.32 | 0.0002 |
| | **Fixed effect** | **Parameter ±SE** | ***t*** | ***P*** |
| PC1 (1994–2004)* | Intercept | 1.04±0.02 | 48.34 | <0.0001 |
| | Days postconception | −0.068±0.015 | −4.49 | <0.0001 |
| PC1 (2008)† | Intercept | 0.15±0.2 | 0.682 | 0.25 |
| | Days postconception | −0.015±0.005 | −2.81 | 0.0027 |
| | Days postconception$^2$ | 0.00007±0.00003 | 2.2 | 0.014 |

Maternal behaviour is represented by the first axis of a principal components analysis (PC1) of all behaviours recorded during behavioural observations of breeding female red squirrels collected over 10 years from 1994 to 2008. A nonlinear term for days postconception (days postconception$^2$) was included to examine the nonlinear relationship between days since conception and maternal behaviour (PC1). Results are from separate generalized linear mixed effects models conducted for data collected from 1994 to 2004 and those collected in 2008. Random intercept terms were included to account for repeated observations of individual squirrels (ID) and by different observers (Observer).

* These models are based on 627 observation sessions on 44 squirrels by 39 observers.

† These models are based on 276 observation sessions on 81 squirrels by 1 observer.

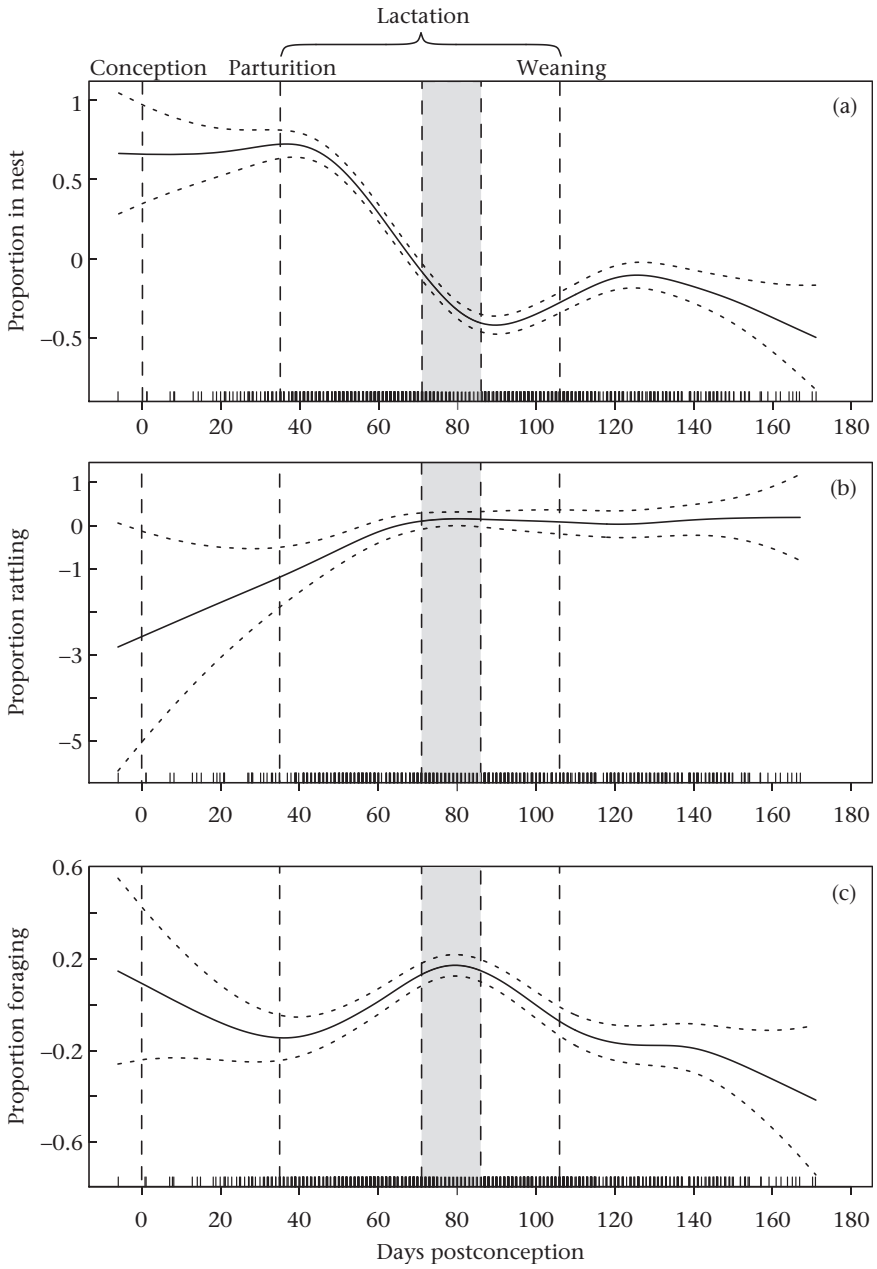


**Figure 1.** Maternal behaviour of female North American red squirrels across the reproductive cycle. Behavioural variables represent the proportion of time females spent (a) in the nest, (b) rattling (territorial vocalization) and (c) foraging during 7 min behavioural observations. Values on *Y* axes represent standardized residual values from generalized additive models to visualize all nonlinearities, but the significant nonlinear relationships were analysed using generalized linear mixed models. Dashed lines represent the standard errors and the grey box represents the range of juvenile emergence from the natal nest (71–86 days postconception). Dashes on the *X* axis represent each behavioural session performed. $N = 903$ (a, c), $N = 627$ (b).

$t_{626} = -2.65$, $P = 0.008$; Fig. 1b, Table 3). The proportion of time females spent foraging was lowest soon after parturition ($12.9 \pm 0.3\%$ of time spent foraging), increased into early lactation, was highest during mid-lactation during the period of first juvenile emergence from their natal nest ($17.8 \pm 1.4\%$ of time), and then declined following juvenile emergence and throughout the rest of lactation and postweaning (slope on ln scale for quadratic term for days postconception $= -0.053 \pm 0.022$; $t_{902} = -2.44$, $P = 0.015$; Fig. 1c, Table 3).

Inclusion of individual squirrel identity as a random intercept term significantly improved the fit of all of our models for maternal behaviour (Tables 2 and 3). From the analyses of PC1 for the 1994–2004 and 2008 behavioural data, individual identity explained 7.1 and 16.4% of the residual variance in maternal behaviour not accounted by the fixed effects, respectively. This suggests that there were repeatable interindividual differences among females in the amount of time they allocated towards interacting in the nest with offspring, foraging, rattling and travelling.

### *Maternal Androgens*

Maternal androgen concentrations were significantly higher during lactation ($3.57 \pm 0.007$ ln ng/g dry faeces; $N = 196$) than during pregnancy ($3.01 \pm 0.008$; $N = 123$; $F_{1,317} = -8.52$, $P < 0.0001$). Our more detailed analyses, however, showed that

**Table 3**
Effects of days since conception on the proportion of time breeding female red squirrels spent in the nest, uttering rattle vocalizations and foraging during behavioural observations collected over 10 years from 1994 to 2008

| Model | | | | |
|---|---|---|---|---|
| | **Random effect** | **Variance** | $\chi^2_1$ | ***P*** |
| In nest* | ID | 4.85 | 2230 | <0.0001 |
| | Observer | 1.46 | 413 | <0.0001 |
| Rattle† | ID | 0.56 | 331 | <0.0001 |
| | Observer | 0.17 | 4.2 | 0.041 |
| Foraging* | ID | 1.98 | 2589 | <0.0001 |
| | Observer | 1.25 | 281 | <0.0001 |
| | **Fixed effect** | **Parameter ±SE** | **Wald *t*** | ***P*** |
| In nest* | Intercept | −1.8±0.38 | −4.76 | <0.0001 |
| | Days postconception | −0.43±0.035 | −12.09 | <0.0001 |
| | Days postconception$^2$ | 0.11±0.02 | 5.29 | <0.0001 |
| Rattle† | Intercept | −3.99±0.18 | −22.14 | <0.0001 |
| | Days postconception | 0.44±0.12 | 3.69 | 0.0002 |
| | Days postconception$^2$ | −0.21±0.079 | −2.65 | 0.008 |
| Foraging* | Intercept | −1.03±0.28 | −3.71 | 0.0002 |
| | Days postconception | −0.075±0.035 | −2.11 | 0.035 |
| | Days postconception$^2$ | −0.053±0.022 | −2.44 | 0.015 |

A nonlinear term for days postconception (days postconception$^2$) was included in these linear mixed effects models to examine the nonlinear relationship between days since conception and the specific maternal behaviours.

* These models are based on 903 observation sessions on 125 squirrels by 40 observers.

† These models are based on 627 observation sessions on 44 squirrels by 39 observers.

FAMs were lowest around conception, increased throughout gestation and after parturition, peaked during mid-lactation around juvenile emergence, and then declined during the latter part of lactation and after weaning (slope on ln scale for quadratic term for days postconception $= -0.21 \pm 0.024$; $t_{383} = -8.75$, $P < 0.0001$; Fig. 2, Table 4).

Similar to maternal behaviour, maternal FAM exhibited significant repeatable interindividual variation as inclusion of individual identity as a random intercept term significantly improved the fit of the detailed model using days postconception ($\chi^2_1 = 46.1$, $P < 0.0001$; Table 4) and explained 14.6% of the variance in FAM not accounted by the fixed effects.

### *Relationship between Maternal Androgens and Behaviour*

Using segmented regression, we found that maternal androgens peaked (breakpoint at 73 ± 4 days postconception; Fig. 2) during mid-lactation around the period when juveniles were first emerging from their natal nest (71–86 days postconception). During this same time period, the proportion of time that females spent in the nest declined to its lowest level (breakpoint at 66 ± 15 days postconception; Fig. 1a) and the proportion of time that females spent rattling (breakpoint at 74 ± 16 days postconception) and foraging (breakpoint at 79 ± 13 days postconception) increased to their highest levels (Fig. 1b, c).

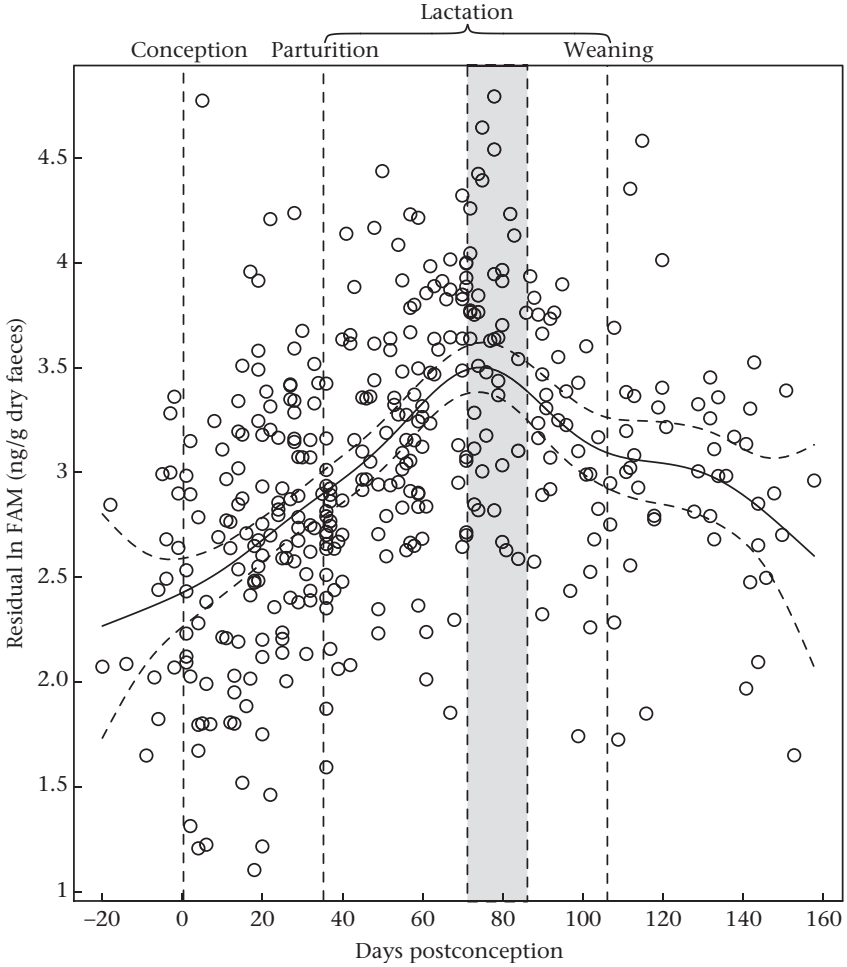


**Figure 2.** Faecal androgen metabolite (FAM) concentrations in female North American red squirrels ($N = 88$ squirrels) prior to conception ($N = 16$ samples), during gestation ($N = 123$ samples) and lactation ($N = 196$ samples), and after weaning ($N = 49$ samples). See text and Table 4 for results from these models. Dashed line represents the standard error and the grey box represents the range of juvenile emergence from the natal nest (71–86 days postconception).

**Table 4**
Effects of days since conception on faecal androgen metabolite (FAM) concentrations of breeding female red squirrels

| **Random effect** | **Variance** | $\chi^2_1$ | ***P*** |
|---|---|---|---|
| ID | 0.10 | 46.1 | <0.0001 |
| **Fixed effect** | **Parameter ±SE** | ***t*** | ***P*** |
| Intercept | 3.55±0.052 | 68.53 | <0.0001 |
| Days postconception | 0.31±0.03 | 9.9 | <0.0001 |
| Days postconception$^2$ | −0.21±0.024 | −8.75 | <0.0001 |

A nonlinear term for days postconception (days postconception$^2$) was included to examine the nonlinear relationship between days since conception and FAM. Results are from linear mixed effects models conducted for data collected from 2006 to 2008 that included a random intercept term for repeated samples of individual squirrels (ID).

## DISCUSSION

Our observations are consistent with the hypothesis that androgens influence behavioural trade-offs in breeding females, whereby periods of heightened androgens are associated with a reduction in time allocated towards maternal behaviour and an increase in time spent engaged in resource acquisition and territory defence. We found that FAM in breeding female red squirrels increased after conception and parturition, peaked during mid-lactation around juvenile emergence from the natal nest, and then declined during the remainder of lactation and after weaning. Around the same period when maternal androgens were highest, breeding female squirrels spent the least amount of time in the nest, but the highest amount of time rattling and foraging. Previous studies have also found evidence for this hypothesis (Fite et al. 2005). For example, the highest plasma testosterone concentrations during reproduction in female Belding's ground squirrels, *Spermophilus beldingi*, occur during peak aggressive and nest maintenance behaviour (Nunes et al. 2000b). Additionally, experimental elevation of testosterone in breeding female birds can inhibit some behavioural estimates of maternal investment in current offspring (time spent brooding: O'Neal et al. 2008).

In red squirrels, the observed changes in maternal androgens during reproduction may optimize parental investment in current offspring by decreasing costly maternal behaviour (nursing) and increasing time investment in territory defence and self-maintenance (foraging) to maximize maternal longevity. Costs of reproduction were previously found to be absent in prime-aged females in this population (survival costs of reproduction were only documented for young and very old females: Descamps et al. 2009). It is possible that changes in maternal androgens and adjustments to maternal behaviour during lactation are important for minimizing these costs of reproduction. During years of high food availability that occur every 4–6 years (LaMontagne & Boutin 2007), females have greater reproductive success due to increased juvenile survival (McAdam & Boutin 2003), and lifetime female fitness is heavily influenced by longevity (McAdam et al. 2007). The patterns of maternal androgens that we found in this study may be the result of natural selection for maternal behaviour that decreases the costs associated with reproduction and increases longevity to maximize female reproductive success by increasing the chance of encountering years of high food availability in which reproductive success is increased.

Changes in maternal behaviour are unlikely to be driven by seasonal changes in resource abundance or temperature. Red squirrels breed seasonally, so changes in reproductive status were associated with advancing Julian date. This caused collinearity between days postconception and Julian date that precluded the inclusion of both terms in our statistical models. However, mean parturition dates and hence dates of gestation and lactation vary by over 1 month from 1 year to the next (Boutin et al. 2006). In addition, breeding by red squirrels is asynchronous, such that parturition dates within a given year also span over 1 month or more (Lane et al. 2008, 2009). For example, 35 days postconception corresponded to the beginning of March for some females in some years, but late June for other females. In our study area, there are marked differences in snow pack, temperature and seasonal food resources between the months of March and June. This inter- and intra-annual asynchrony in breeding by red squirrels would probably eliminate the possibility that the consistent nonlinear relationships between maternal behaviour and days postconception from 1994 to 2008 that we observed were due to seasonal changes (e.g. food or temperature).

### *Maternal Androgens in Mammals*

We had expected that maternal androgens might be highest around weaning to reflect the absence of suckling. In many mammalian species, suckling by offspring inhibits gonadal activity, and consequently suppresses production of testosterone and oestradiol (Taya & Greenwald 1982; reviewed in McNeilly 2001). Prior to weaning, as offspring grow and become more independent, suckling stimulation declines and gonadal activity may increase and enable androgen production (Taya & Greenwald 1982). However, we found that FAM peaked during mid-lactation coinciding with juvenile emergence. Juvenile emergence occurs 25–35 days prior to average weaning date (Humphries & Boutin 1996) and does not represent a time when suckling stimulation by offspring is attenuated. In fact, suckling stimuli are probably highest at this time because of the high energetic demands of offspring due to their large size and activity, and because they are likely to be inefficient at foraging on their own. We also found that FAM did not decline after parturition when suckling was initiated, and it increased from parturition until mid-lactation. Lastly, from first emergence until weaning, the proportion of time that juvenile red squirrels spend feeding outside of the nest significantly increases (M. C. Andruskiw, personal communication) and consequently suckling presumably decreases, but we found that FAM actually decreased. Therefore, we do not think that patterns of FAM reflect suckling stimuli by offspring in this species.

Although ovulation can occur during the later stages of lactation in red squirrels (Boutin et al. 2006; McAdam et al. 2007), it is unlikely that the observed peak in FAM around juvenile emergence reflected a surge in oestradiol associated with ovulation (Shaikh 1971). The cross-reactivity of oestradiol in the testosterone EIA is very low (<0.1%: Palme & Möstl 1994). Additionally, if FAM reflects changes in oestradiol concentrations during ovulation cycles, we would expect a peak in FAM around conception (the first ovulation), which we did not see (Fig. 2).

The peak in FAM around juvenile emergence may have also promoted the increase in female territorial vocalizations that we observed around juvenile emergence. Protecting altricial offspring from infanticide may be a major driver in the evolution of female territoriality in mammals (Wolff 1993, 1997). In many species, male testosterone concentrations are positively correlated with aggressive or territorial behaviour (reviewed in: Wingfield et al. 1990; Demas et al. 2007). As such, heightened concentrations of circulating androgens in females could increase antagonistic behaviour, which could reduce the risk of infanticide during the period of offspring dependence (sensu Ostner et al. 2002). Infanticide has been documented in this population (S. Boutin, unpublished data), but we know little about factors affecting rates of infanticide in red squirrels.

### *Repeatable Interindividual Variation in Maternal Behaviour and Androgens*

Recent studies in the burgeoning field of animal personality research have demonstrated the potential for consistent interindividual differences among individuals in behaviour to have important ecological and evolutionary consequences (Sih et al.

2004; Réale et al. 2007). We found that there were repeatable interindividual differences among breeding female red squirrels in how they allocated their time during the breeding season, including when they were interacting with their dependent offspring. Previous studies have demonstrated repeatable differences in maternal behaviour of mammals (e.g. 'mothering styles': Altmann 1980; Maestripieri 1993, 1994; Albers et al. 1999; Bardi et al. 2001). While our ability to observe how lactating red squirrels interact with their dependent offspring was limited because of their secretive behaviour, we did demonstrate that there were repeatable differences among females in the amount of time they spent in the nest with their offspring as opposed to the time spent foraging and defending their territory via vocalizations. Whether these individual differences in the amount of time spent in the nest with offspring are associated with variation in the style of care provided within the nest will require creative methods for observing behaviour within nests under natural conditions.

The organizational effects of prenatal exposure to androgens can have acute effects on offspring phenotype in oviparous (reviewed in Groothuis et al. 2005) and mammalian species (Seale et al. 2005; Dloniak et al. 2006). As such, the repeatable interindividual differences in maternal androgen concentrations that we found in this study could generate significant variation in offspring phenotype, which could have important ecological and evolutionary implications. Future studies in this species will estimate the heritability of FAM and could elucidate how maternal androgens influence offspring phenotype and survival, as well as relationships between maternal androgens, survival and reproductive success.

There is now growing evidence that androgens may play a major role in shaping maternal behaviour (Nunes et al. 2000b; Fite et al. 2005; Sandell 2007; O'Neal et al. 2008). Although our study is correlational, our data are consistent with the hypothesis that androgens may play a similar role in shaping behavioural trade-offs between resource acquisition and parental care in breeding females as has been found in males of some species (Hegner & Wingfield 1987; Wingfield et al. 1990; Ketterson & Nolan 1999; Hau 2007; McGlothlin et al. 2007). In red squirrels, androgens could be used to optimize maternal behaviour by reducing maternal care and enhancing the probability of survival and the future reproduction of breeding females. Definitive tests of these hypotheses, however, will depend on long-term experimental manipulations of maternal androgen concentrations.

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## APPENDIX 1. RADIOMETABOLISM STUDY AND RP-HPLC

### *Methods*

Radiometabolism studies are a vital part of the validation of an assay to measure hormone metabolites in faecal samples because they identify the route of excretion (urine or faeces) of the metabolites as well as the time delay of excretion (Touma & Palme 2005). From January to March 2008, we performed a radiometabolism study on red squirrels in captivity (for details about capture and husbandry of squirrels, see Dantzer et al. 2010). We injected four captive female red squirrels intraperitoneally with 1110 kBq of radiolabelled testosterone (1, 2, 6, 7- [$^3$H]; Amersham Biosciences, Quebec, Canada; specific activity = 1.55 TBq/mmol) dissolved in 0.1 ml physiological saline containing 5% ethanol and 5% toluene at 0800 hours on day 1 of this study. We collected urine (0–52 h postinjection) and faeces (0–120 h postinjection) approximately every 4 h (except from 2000 to 0800 hours) from pans underneath the cages that were covered with metal screening (0.5 × 0.5 cm mesh) to prevent faeces and urine from mixing. Between sampling periods, we rinsed the pans twice with a radioactive decontamination solution (Decon 75, Fisher Scientific, Pittsburgh, PA, U.S.A.). Faecal and urine samples were placed into a −20 °C freezer within 20 min of collection. We extracted faecal samples as described within the text above except that we also rinsed the mortar and pestle twice with a decontamination solution (Decon 75) between samples.

We dried down urine samples under air until only about 1 ml remained. To determine radioactivity in the urine and faecal extracts, we added 4 ml of ACS scintillation fluid (Amersham Biosciences, Quebec, Canada) to the concentrated urine or 100 μl of the faecal extract and quantified radioactivity using a liquid scintillation counter with quench correction (Packard Tri-Carb 2900TR, Boston, MA, U.S.A.).

Faecal extracts of samples containing peak radioactivity from female ($N = 2$) squirrels were dried under air and then subjected to reverse-phase high performance liquid chromatography (RP-HPLC). After separation, we measured both the radioactivity and immunoreactivity (see below) in the collected fractions. Details of this method can be found in Lepschy et al. (2007) and Touma et al. (2003). All of the procedures listed above for the captive red squirrels were approved by the Institutional Animal Care and Use Committees at the University of Toronto (no. 20006991).

*Results*

*Route of excretion and time to peak excretion of radiolabelled testosterone*

We collected 135 faecal samples and 84 urine samples from 10 squirrels (4 females, 6 males) over the 120 h radiometabolism study. Of the 1110 kBq of $^3$H-testosterone administered to the squirrels, we recovered 37.7 ± 0.04%, of which 43.7 ± 0.1% was in the urine and 56.3 ± 10.4% was in the faeces. The mean time to peak excretion of $^3$H-testosterone was 7.0 ± 0.9 h in the urine and 10.3 ± 1.3 h in the faeces. Therefore, trapping-induced stress could not have influenced FAM as traps were checked every 2 h and the peak in radioactive FAM occurred over 10 h after injection.

*Structure and polarity of testosterone metabolites from RP-HPLC analysis*

Injected $^3$H-testosterone was heavily metabolized, and polar metabolites resembling conjugated steroids dominated (Fig. A1). Several radioactive peaks beyond fraction 60 were found and two of these peaks (eluting around fraction 83 and 87) yielded the highest immunoreactivity in the testosterone EIA. No radioactivity with corresponding immunoreactivity (as determined by the testosterone EIA) at the elution position of testosterone (around fraction 80) was present. Thus, testosterone was entirely metabolized prior to excretion, as has been found in previous studies (Möhle et al. 2002). The results from the radiometabolism study and RP-HPLC demonstrate that testosterone metabolites were excreted in the faeces of North American red squirrels and that our EIA antibody reacted with testosterone metabolites around fractions 83 and 87 with a 17β-hydroxy-group.

## APPENDIX 2. PHYSIOLOGICAL VALIDATION

*Methods*

A second step in validating an assay to measure FAM concentrations is to demonstrate that the effects of reproductive condition on FAM mirror those found in plasma androgen concentrations. We collected faecal and plasma samples from nonbreeding (not pregnant or lactating) and lactating females. Faecal samples were collected from 2006 to 2008, while plasma samples were collected in 6 years between 1996 and 2010. We obtained plasma samples either from cutting a small piece of the toenail or from the suborbital sinus. For the latter, we first anaesthetized squirrels using isoflurane ISP (3.5% in air) and bled them from the suborbital sinus using a heparinized glass pipette (Boonstra et al. 2008). Squirrels were completely anaesthetized within 15–30 s and blood samples were collected within 1 min. All plasma samples were collected within 2 h of live trapping and squirrels were released following collection of the blood samples. These procedures were approved by the University of Toronto Animal Care Committee (no. 20006991).

We measured plasma testosterone + dihydrotestosterone (DHT) via radioimmunoassay using a protocol based upon that of Abraham et al. (1971) that we have used previously (Boonstra & Boag 1992; Boonstra et al. 2008). Plasma samples (25 μl) were treated with 20 μl of $NH_4OH$ to saponify triglycerides. The antibody (P43/11) was produced by Croze & Etches (1980) and has relatively high cross-reactivity to 5α-dihydrotestosterone (62%) and low cross-reactivity to dehydroepiandrosterone (<0.8%: Boonstra et al. 2008). Assay sensitivity was 10 pg/25 μl plasma, and nondetectable samples

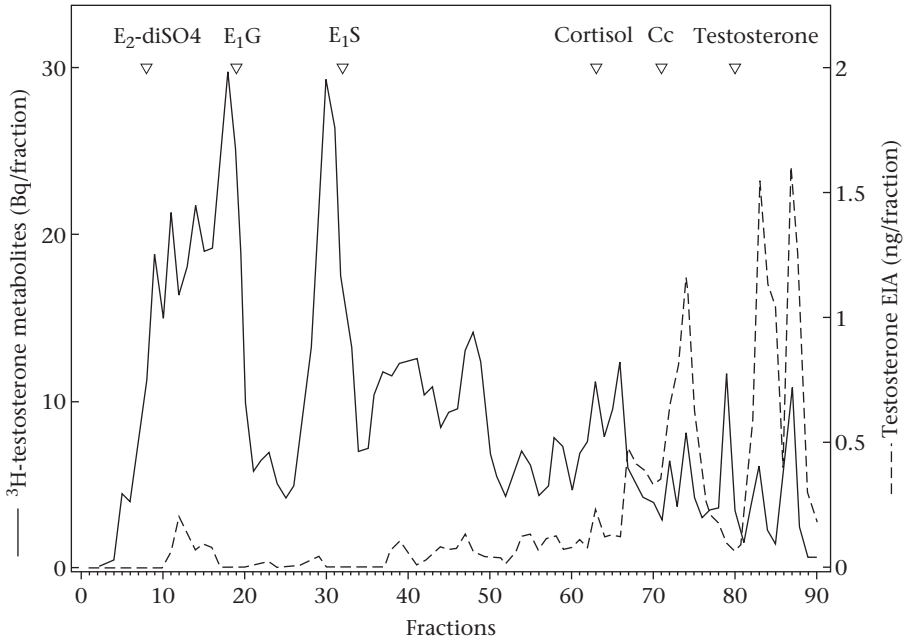


**Figure A1.** Reverse-phase high performance liquid chromatographic (RP-HPLC) separation of faecal $^3$H-testosterone metabolites (peak sample) in the faeces of female North American red squirrels. Open triangles mark the approximate elution positions of respective standards ($E_2$-di$SO_4$ = 17β-oestradiol-disulphate, $E_1G$ = oestrone-glucuronide, $E_1S$ = oestrone-sulphate, Cc = corticosterone).

were given a value of 10 pg. The intra- and interassay coefficients of variation were 5 and 6%, respectively ($N = 8$ independent assays).

To determine how the reproductive condition of female (nonbreeding or lactating) squirrels affected (1) plasma testosterone + DHT and (2) FAM concentrations, we conducted a general linear model (fixed effect: reproductive condition) and LMM (fixed effect: reproductive condition), respectively. To meet assumptions of normality, we ln-transformed plasma androgen ($x + 1$) and FAM concentrations. However, raw plasma androgen concentrations are presented below and in Fig. A2.

*Results*

The effects of reproductive condition on plasma testosterone + DHT concentrations were mirrored in FAM (Fig. A2). Female reproductive condition had a significant effect on plasma testosterone + DHT ($F_{1,35} = 9.064$, $P = 0.0048$), with lactating females having significantly higher plasma testosterone + DHT concentrations ($N = 18$; $0.98 \pm 0.15$ ng/ml) than nonbreeding females ($N = 19$; $0.53 \pm 0.05$ ng/ml; $t_{35} = -3.01$, $P = 0.0048$; Fig. A2). Reproductive condition also had the same significant effect on FAM ($F_{1,65} = 36.86$, $P < 0.0001$; Fig. A2), with lactating females having significantly higher FAM ($N = 36$; $3.86 \pm 0.09$ ln ng/g dry faeces) than nonbreeding females ($N = 94$; $2.81 \pm 0.04$ ln ng/g dry faeces; $t_{129} = -8.09$, $P < 0.001$). These results demonstrate that this assay is fully capable of detecting variation in FAM in female red squirrels that is directly related to plasma androgen concentrations.

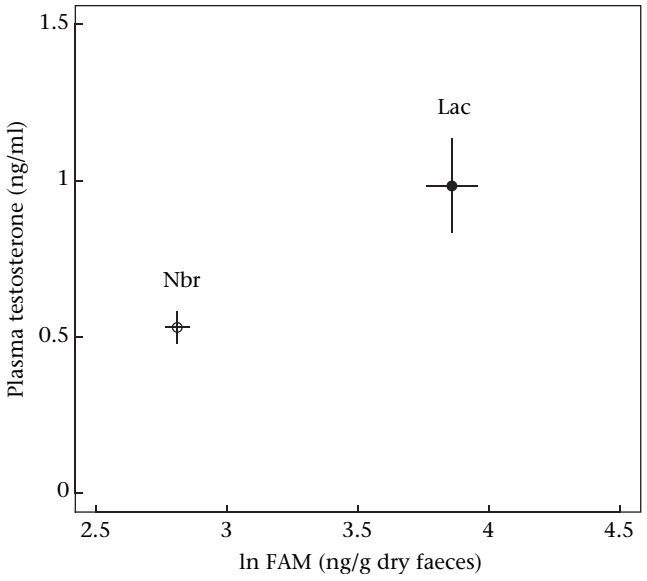


**Figure A2.** Effects of reproductive condition on plasma testosterone + dihydrotestosterone (DHT: 'plasma testosterone') and ln-transformed faecal androgen metabolite (FAM) concentrations in nonbreeding ('Nbr') and lactating ('Lac') female North American red squirrels (March–August). Raw plasma androgen concentrations are presented but ln-transformed ($x + 1$) values were used for the statistical analysis. Data presented are means ± SE.