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Original Article

Sex-specific offspring growth according to maternal testosterone, corticosterone, and glucose levels

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Sex allocation theory in vertebrates has greatly benefited from the recent advances in studies on the physiological mechanisms of birth sex ratio variation (e.g., maternal glucose, stress, and testosterone levels). The same physiological mechanisms may, however, also mediate permanent sex-specific effects on individuals after birth. Together with biased maternal postpartum investment on sex allocation, they can have long-term fitness consequences for the offspring. We studied whether maternal preconception levels of serum glucose and testosterone, and fecal corticosterone metabolites differently influenced male and female pup weight until weaning in field voles (*Microtus agrestis*). In this species, high maternal preconception serum glucose and testosterone levels have previously been associated with the excess of male pups at birth. Our results suggest that male, but not female, pup weight increases with higher maternal preconception testosterone level whereas high maternal serum glucose level promoted female pup weight only. The level of maternal fecal corticosterone metabolites was not related to pup weight in either sex. These findings suggest that in field voles the same physiological mechanisms influencing sex ratio at birth may also influence offspring postnatal weight; however, such influences can act in conflict, as seen in the case of maternal glucose level.
Key words: fitness, maternal effects, *Microtus agrestis*, prenatal, sex-biased investment. *[Behav Ecol]*

INTRODUCTION

Despite the accumulating evidence for parental sex allocation in vertebrates with chromosomal sex determination, the lack of knowledge on proximate mechanisms of sex determination has hampered our understanding of sex allocation (West and Sheldon 2002). At present, the most promising candidate mechanisms include maternal testosterone, glucose, and glucocorticoid concentrations (reviewed, e.g., in Alonso-Alvarez 2006; James 2008; Grant and Chamley 2010; Navara 2010). In both monotocous and polytocous mammals, high maternal testosterone level, the effect of which is assumed to occur during ovum development or during fertilization, has been suggested to bias offspring sex ratio towards males (Grant and Irwin 2005; Grant et al. 2008, 2011; Helle et al. 2008; Shargal et al. 2008). Not all studies, however, have been able to replicate the finding that such an association arises during ovum development (Díez et al. 2009; Garcia-Herreros et al. 2010). Maternal glucocorticoids have also been suggested to skew offspring sex ratio in mammals via sex-biased embryonic mortality. In mammals, only few studies to date have examined whether glucocorticoids mediate offspring sex ratio variation. In polytocous species, Geiringer (1961) found that in albino rats (*Rattus norvegicus*) stressed mothers produced female-biased litters, whereas Ryan et al. (2012) reported that in ground squirrels (*Urocitellus richardsonii*) mothers with high levels of glucocorticoids during gestation produced male-biased litters. Maternal preconception fecal corticosterone metabolite level was not, however, associated with litter sex ratio in field voles (*Microtus agrestis*) (Helle et al. 2008). In addition to these hormonal mechanisms, a high level of maternal circulating glucose has been found to bias offspring sex ratio towards males in polytocous mammals (Machado et al. 2001; Cameron et al. 2008; Helle et al. 2008; Dunn et al. 2010). In humans, diabetic mothers seem to also overproduce sons (James 2006). This hypothesis invokes sex-biased early mortality as a mechanism responsible for male-bias.

Previous studies examining the role of testosterone, glucose, and glucocorticoids on birth sex ratio have rarely assessed their potential consequences on offspring later-life performance. Ryan et al. (2012) found indirect evidence suggesting that stressed mothers produced more males, which was correlated with smaller litter size that instead correlated with higher male mass at emergence. Ignoring potential postnatal influences is likely an important shortcoming because it is possible that the prenatal maternal effects influencing offspring sex have also permanent organizational sex-specific effects on offspring performance later in life, affecting their fitness (Cohen-Bendahan et al. 2005; Robinson 2006). For instance, steroids (including both testosterone and glucocorticoids) in vertebrates have been linked to postnatal growth, reproductive success, and survival and such effects may be stronger in one sex than in other (Lerner and Mason 2001; Bian et al. 2005; Groothuis et al. 2005; Müller et al. 2005; Saino et al. 2006; John-Alder and Cox 2007; Sockman et al. 2008; Cox et al. 2009; Pitala et al. 2009; Warner et al. 2009; Monclùs and Blumstein 2012). Testosterone, for one, is assumed to promote muscular and skeletal growth of males in vertebrate species in which males are the larger sex (Cox et al. 2009). However, this evidence has largely been acquired from

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research examining postnatal testosterone-mediated effects, although in terms of sex allocation theory the effects of prenatal steroids, potentially reflecting maternal effects, are more important as they are directly suggested to relate to sex ratio variation at birth.

In species with parental care, sex allocation does not end at birth, as parents can modify their sex-specific investment in offspring after their birth, in order to gain fitness benefits (West 2009). For example, Koskela et al. (2009) and Rutkowska et al. (2011) showed experimentally that bank vole (*Myodes glareolus*) females showed higher postpartum investment in daughters in terms of offspring weight at weaning, milk production, daily energy expenditure, and defensive behavior that may be selectively advantageous in this species. Such maternal effects may strengthen the direction of sex allocation produced by the mechanisms responsible for birth sex ratio bias. It is, however, possible that organizational prenatal and maternal postnatal effects on sex allocation show conflict: for example, a physiological mechanism may produce maladaptive or neutral variation in birth sex ratio that is compensated by sex allocation in the opposite direction during early offspring development.

To address this idea, we examined whether maternal levels of circulating serum testosterone and glucose and fecal corticosterone metabolites prior to breeding were differently associated with male and female pup weight up until weaning in a small mammal, the field vole. We have previously shown that in this species high maternal serum glucose and testosterone levels were associated with the excess of male pups at birth (Helle et al. 2008). Here we continued to examine whether these associations have downstream effects on the sex-specific growth of the pups that could be seen as compensatory or additive to the effects on birth sex ratio. We assume that these preconception (or baseline) hormonal levels reflect, at least to some degree, those experienced by developing fetuses, because in mammals experimental manipulation of maternal circulating testosterone levels have downstream demasculinized and masculinized influences of female and male offspring, respectively (e.g., Mann and Svare 1983; Manikkam et al. 2004; Bánszegi et al. 2010). The field vole shows sexual size dimorphism, adult males being, on average, 15% heavier than adult females (Innes and Millar 1994). Offspring weight prior to weaning is an important estimate of postnatal maternal investment and relevant for sex allocation theory, because it is likely a good correlate of adulthood body size that, in turn, affects fitness in many small mammal populations (Koskela et al. 2009). Disentangling the effects of prenatal organizational versus postnatal maternal investment on pup growth is difficult using correlative data we have at hand. Because we have measured here maternal preconception physiological parameters and not directly the amount of postnatal maternal sex-specific investment, we controlled for factors that potentially were related to (or constrained) maternal investment on pups' weight. These were litter size and litter sex ratio (Uller et al. 2005; Curtis 2010) and preconception maternal body weight. Note that litter sex ratio may also relate to postnatal pup growth due to steroid leakage between fetuses owing to their intrauterine position during prenatal development (Ryan and Vandenbergh 2002).

MATERIALS AND METHODS

Study species and design

The methods have already been described in detail in Helle et al. (2008). In brief, we used first-generation laboratory-born offspring of field voles trapped from the wild in southwestern Finland during spring 2005. The study was conducted during autumn 2005. We paired females (n = 29) with a random nonsibling male for 14 days. Pairing was preceded by the measurement of female body mass and the collection of, in the following order, fecal and vaginal samples for measuring corticosterone metabolites and the phase of estrus cycle, respectively, and blood samples for measuring testosterone and glucose (always conducted between 08:00 and 10:00 h). We recorded estrus cycle phase using vaginal cytology, because the levels of circulating testosterone and corticosterone may potentially vary according to female reproductive state (Cavigelli et al. 2005). All measurements were made by O.H., with all measures taken to minimize discomfort to the animals.

Plasma testosterone level was measured by a direct radioimmunoassay (DiaSorin, Stillwater, MN, USA). The intra-assay variations for testosterone were less than 8.1% and the inter-assay variations below 7.6%. The lower limit of testosterone assay sensitivity was 20 pg/ml defined as 2 standard deviation (SD) above 0. Variation due to estrus cycle was removed from the testosterone level before analyses (Helle et al. 2008). To measure baseline blood glucose, we placed females into hay only cages without food and corrected the blood glucose with the fasting time before sampling (Helle et al. 2008). During this procedure, approximately 20 fecal pellets per animal were collected to obtain samples for fecal corticosterone analysis. The rationale for measuring fecal corticosterone metabolites instead of from blood was to avoid the short-term confounding (e.g., due to handling) of baseline corticosterone level and to maintain a proper hematogical status of females (Harper and Austad 2000). Fecal samples were collected into calibrated tubes for the measurement of wet mass and the content was analyzed using a commercial double antibody ^{125}I radioimmunoassay kit (MP BioMedicals, Orangeburg, NY, USA). The results are expressed as the concentration of corticosterone metabolites (ng) per dry mass of feces (mg) per individual. The lower limit of corticosterone assay sensitivity was 7.7 ng/ml defined as 2 SD above 0. The intra-assay coefficient of variation (CV) for corticosterone was below 10.2%, and the inter-assay CV below 7.2%. The concentration of corticosterone metabolites was unrelated to fasting time (β [95% CI] = −0.0075 [−0.0229, 0.008], t = −0.99, P = 0.33). Mean (±SD) were 88.3 (±44.9) pg/ml, 7.2 (±3.6) mmol/L, and 0.7 (±1.2) ng/mg for serum testosterone, serum glucose, and fecal corticosterone metabolites, respectively.

Pups born (n = 129) from 29 litters were monitored daily until the pups reached the age of 16–23 days (median = 22 days) and the study was terminated. The sex of all pups was verified by necropsy at this stage. The birth sex ratio was significantly female-biased (0.40 ± 0.29 SD, see Helle et al. 2008). Nineteen pups (15%), including a litter of 8 pups that was found dead immediately after the birth, died prior to completion of the study. Their sex was also verified by necropsy. We measured the body mass of pups from parturition to the end of monitoring period (see above) a total of 6 times (due to some mortality, this ranged slightly between individuals), measurement interval usually being 3–5 days. No other procedures were applied to the pups during the study. A total of 120 pups (53 males and 67 females) were included in the analyses. Mean weight (±1 SD) at the last measurement time was 16.2 (±2.3) and 16.9 (±2.3) g for males and females, respectively. The study was approved by the Lab-Animal Care and Use Committee of the University of Turku (license number 1534/05).

Statistical analyses

Pup weight in relation to maternal testosterone, glucose, and fecal corticosterone metabolite concentrations was examined

using general linear mixed model with restricted maximum likelihood (REML) estimation (Littell et al. 2006). Due to nonconstant measurement intervals and period, the weight of individual pups was modeled with random regression coefficients, where random intercepts and slopes for age at measurement nested within the identity of their mother were allowed for each pup (Littell et al. 2006). All pups that were measured at least once were included in the analysis, because excluding the individuals that died before the last measurement event would likely have resulted in reduced statistical power to detect individual weight curves (Martin et al. 2011). Because the preliminary inspection of individual weight with age hinted towards an accelerating weight gain with advancing age, we started by fitting quadratic weight curves to the data using unstructured covariance matrix. The model allowing for quadratic weight gain indeed showed a better fit to the data than linear weight gain only (ΔAICc = 736.6). Furthermore, a model not allowing for correlated random intercepts with linear and quadratic slopes performed markedly worse (ΔAICc = 135.5). Our analysis controlled for litter size and sex ratio (i.e., proportion of males) (Curtis 2010) and maternal weight prior to breeding. Because our main goal was to examine sex differences on pup weight, we included interactions between sex and all these traits. All continuous predictors were grand mean centered (i.e., effects sizes are estimated at the average value of predictors) to obtain interpretable regression coefficients in the presence of interactions (Gelman and Hill 2007). This also alleviates the need for model simplification due to interactions. We thus base our inference on a global model, which provides the most accurate point estimates and their confidence intervals (CIs) (Harrell 2001). Individual sex was not centered, because value 0 has a meaningful interpretation (i.e., an individual is male) and because its levels are restricted to 2 (Gelman and Hill 2007). *F*-tests were used to determine the significance of fixed effects (Bolker et al. 2009). The covariance matrix of fixed parameter estimates was adjusted by the Kenward–Roger method (Littell et al. 2006). Multicollinearity among explanatory variables was assessed with variance inflation factors and condition indices. The largest variance inflation factor was 1.71 and the largest condition index was 2.40, suggesting no bias in the CIs of regression coefficients. The marginal residuals of the model fitted were symmetric and showed no patterns indicative of heteroscedasticity. R^2 statistics reported here are based on maximum likelihood model comparisons (Magee 1990). All analyses were conducted with SAS statistical software version 9.3 (SAS Institute Inc., Cary, NC, USA).

RESULTS

We found that the associations between maternal preconception levels of serum testosterone and glucose and pup weight differed between the sexes (Table 1). In male pups, maternal testosterone level was positively associated with weight (β [95% CI] = 0.004 [0.001, 0.007], $F_{1,38.2}$ = 5.83, P = 0.021), whereas in female pups maternal testosterone level was unrelated to their weight (β [95% CI] = −0.001 [−0.005, 0.003], $F_{1,36.6}$ = 0.17, P = 0.68). For example, at day 21, the predicted male weight at the 3rd quartile of maternal testosterone level was 0.81 g higher compared with the 1st quartile (Figure 1). By contrast, maternal glucose level showed a positive association with pup weight in females (β [95% CI] = 0.105 [0.047, 0.163], $F_{1,30.1}$ = 13.76, P = 0.0008) but not in males (β [95% CI] = 0.025 [−0.033, 0.084], $F_{1,36.2}$ = 0.78, P = 0.38). For example, at day 21, the predicted female weight at the 3rd quartile of maternal glucose level was 0.49 g higher compared with the 1st quartile (Figure 2). Maternal preconception fecal corticosterone metabolite level was not related to pup weight in either sex (Table 1). All these associations were age independent (results not shown). Furthermore, there were sex differences in pup weight according to maternal preconception

Table 1

Pup weight (689 observations from 120 individuals) until age of weaning in relation to maternal preconception serum glucose and testosterone levels, fecal corticosterone metabolite levels, and relevant covariates

	Estimate	95% CIs	$df_{num, den}$	F	P
Random coefficients					
$\sigma^2_{intercept}$	1.205	0.838, 1.572			
$\sigma_{intercept \times linear\ slope}$	0.096	0.065, 0.127			
$\sigma^2_{linear\ slope}$	0.011	0.008, 0.015			
$\sigma_{intercept \times quadratic\ slope}$	−0.0018	−0.0037, 0.0001			
$\sigma_{linear \times quadratic\ slopes}$	0.00027	0.0001, 0.0005			
$\sigma^2_{quadratic\ slope}$	0.00004	0.00002, 0.00006			
Residual	0.351	0.299, 0.417			
Fixed coefficients					
Age	0.563	0.542, 0.584	1, 112.8	2787.3	<0.0001
Age^2	0.012	0.010, 0.013	1, 101	226.6	<0.0001
Sex (ref = females)	−0.099	−0.325, 0.128	1, 58.2	0.76	0.39
Litter size (LS)	0.018	−0.077, 0.115	1, 61.7	0.15	0.70
Litter sex ratio (LSR)	−1.826	−2.409, −1.242	1, 69.3	14.76	0.0003
Maternal weight (MW)	0.006	−0.020, 0.032	1, 58.9	7.44	0.0084
Maternal glucose (G)	0.107	0.057, 0.158	1, 61.8	9.69	0.0028
Maternal testosterone (T)	−0.001	−0.004, 0.003	1, 70.4	2.36	0.13
Maternal corticosterone (C)	0.057	−0.094, 0.209	1, 60.2	2.30	0.14
Sex × LS	−0.069	−0.234, 0.096	1, 61.7	0.70	0.41
Sex × LSR	1.780	0.809, 2.752	1, 69.3	13.36	0.0005
Sex × MW	0.044	0.002, 0.085	1, 58.9	4.47	0.0388
Sex × G	−0.090	−0.170, −0.009	1, 61.2	4.99	0.0291
Sex × T	0.005	0.000, 0.010	1, 70.4	3.99	0.0497
Sex × C	0.031	−0.161, 0.223	1, 60.2	0.10	0.75

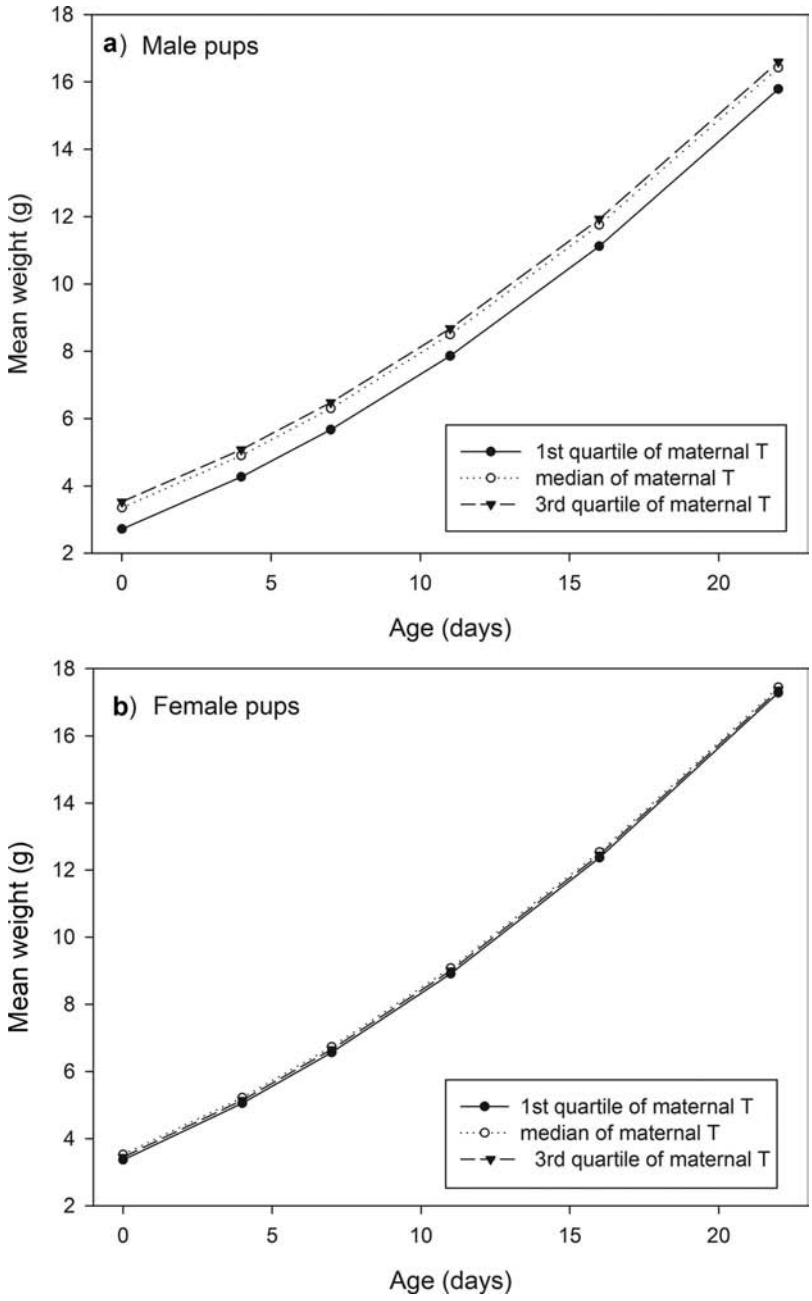

Figure 1
Estimated marginal means for male (a) and female (b) pup weight by age in relation to maternal preconception serum testosterone level (T), divided into quartiles.

weight and litter sex ratio (Table 1). Female pup weight was lower in male-dominated litters (β [95% CI] = −0.176 [−2.418, −1.110], $F_{1,40.9}$ = 29.71, P <0.0001) although the weight of male pups was unrelated to litter sex ratio (β [95% CI] = −0.157 [−0.084, 0.571], $F_{1,36.4}$ = 0.19, P = 0.67). Heavier females produced heavier male (β [95% CI] = 0.047 [0.016, 0.077], $F_{1,33.2}$ = 9.87, P = 0.035) but not female (β [95% CI] = 0.005 [−0.024, 0.035], $F_{1,29.7}$ = 0.13, P = 0.72) pups compared with lighter females. Pup weight was not related to litter size in either sex (Table 1). By looking at the standardized coefficients by sex (Figure 3), litter sex ratio seemed clearly the strongest predictor of female weight, whereas male weight was most influenced by both maternal weight prior to breeding and circulating testosterone level. The maternal attributes examined here explained 32.6% and 48.7% of variation in male and female weight until weaning, respectively.

DISCUSSION

By simultaneously contrasting the associations of maternal circulating testosterone and glucose levels and concentration of fecal corticosterone metabolites with pup weight until weaning, we found that in field voles male and female weight seemed to be differently influenced by the preconception testosterone level of their mother. Mothers with higher preconception testosterone level produced heavier male but not female offspring. High maternal preconception level of glucose seemed to promote offspring weight gain in females only. In addition, male-dominated litters produced female pups with lower weight and heavier mothers tended to raise heavier male but not female offspring. All these associations persisted beyond birth, as no evidence for age dependency was observed. Maternal preconception concentration of

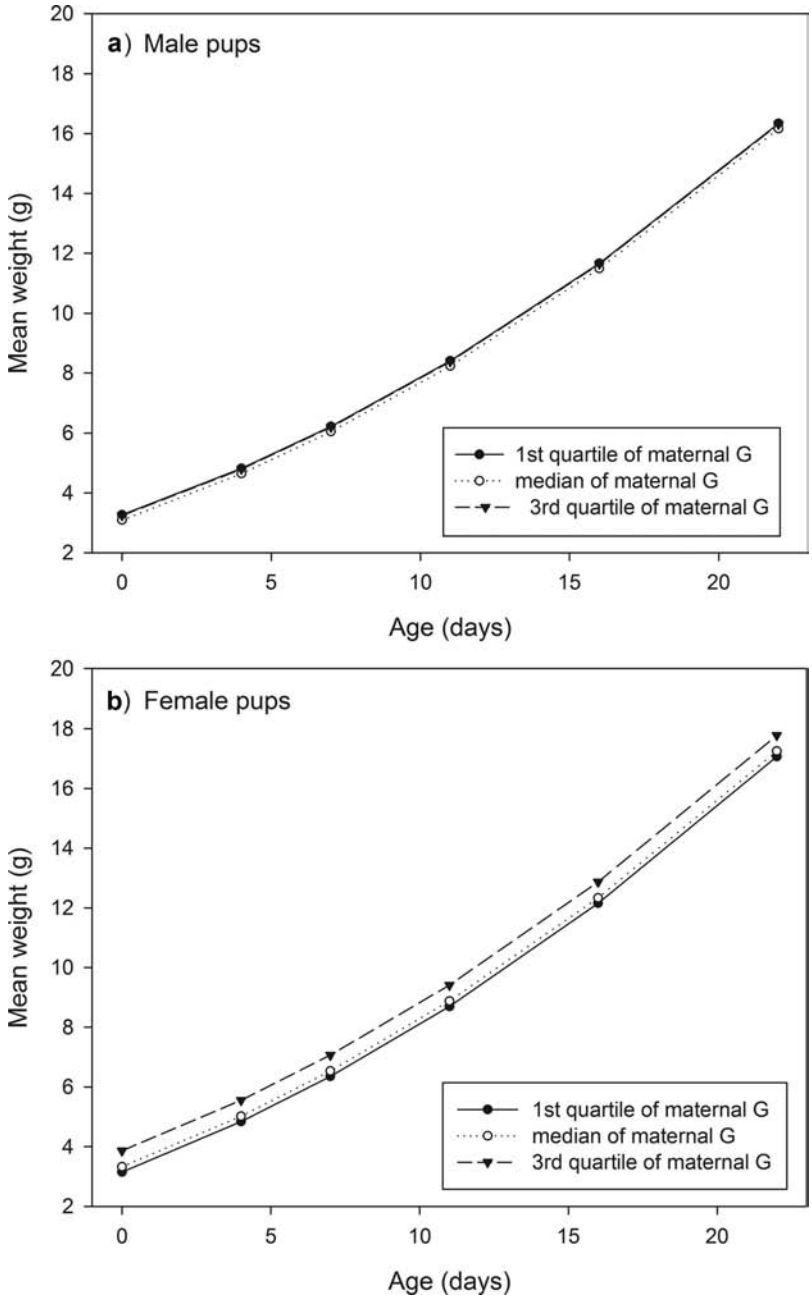

Figure 2
Estimated marginal means for male (a) and female (b) pup weight by age in relation to maternal preconception serum glucose level (G), divided into quartiles.

fecal corticosterone metabolites appeared not to influence offspring weight in this species.

Studies examining the determinants of sexual size dimorphism in vertebrates have shown that in species where males are the larger sex, testosterone administration during development differentially stimulates male growth (Cox et al. 2009). However, the influence of prenatal testosterone on sex-specific growth after birth that is more directly relevant for sex allocation theory has been much less frequently examined, except in birds where the manipulation of yolk androgens is feasible (Groothuis et al. 2005). Therefore, by assuming that maternal preconception circulating testosterone levels mirror those experienced by the developing fetuses, our study provides a rare example of such an association in a semidomesticated/wild mammal because most of our previous knowledge on the issue comes from studies on livestock or laboratory rodents (Gill et al. 1998; Ryan and Vandenbergh 2002). However, the weight of female pups was found to be negatively associated with the proportion of male siblings. Our previous result in these voles showed that females with higher preconception testosterone level produced male-biased litters (Helle et al. 2008). Therefore, high maternal testosterone level may indirectly, through male-biased primary sex ratio and accordingly elevated between-fetus testosterone leakage (Ryan and Vandenbergh 2002), influence also the weight of female pups. Taken together, in terms of postnatal weight males seem to benefit and females seem to suffer from high maternal testosterone levels in this species.

Our previous work in this species (Helle et al. 2008) and current findings suggesting a growth-enhancing role of maternal testosterone level in offspring males only fit within the sex allocation framework of Trivers and Willard (1973). That is, mothers with high testosterone level may

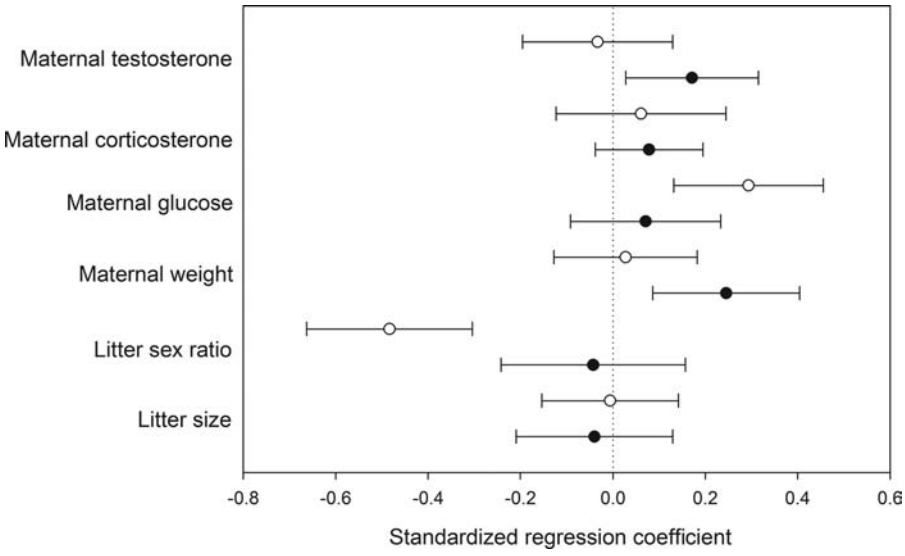

Figure 3
Standardized regression coefficients and their 95% CIs for female (open dots) and male (filled dots) pup weight according to the predictor variables examined. Regression coefficients were standardized to make the effect sizes comparable between the predictors.

facultatively skew birth sex ratio toward males, which are heavier and, presumably, have increased fitness as adults. In general, large body size is expected to be associated with higher fitness in the wild (e.g., Blanckenhorn 2000) and this assumption is likely to hold in voles as well (Koskela et al. 2009). However, whether the sexes differ in this respect remains ambiguous in field voles, because it is currently unclear whether males proportionally benefit more from large size than females. This might be the case given that adult males weigh, on average, 15% more than adult females (Innes and Millar 1994; see also Burthe et al. 2010). In line with this interpretation, we also observed a stronger correlation between maternal weight at breeding and pup weight in males compared with females, as heavier mothers raised heavier male but not female offspring. In addition, mothers with elevated testosterone level producing proportionally more and heavier male offspring may produce poorer quality female offspring as a side effect, because females exposed to high in utero testosterone levels may show reduced reproductive success as adults (Bánszegi et al. 2012). If this pattern holds more generally, the importance of a testosterone-related mechanism of a sex ratio variation may be related to the sexual size dimorphism of the species examined at least in mammals. In other words, we might expect birth sex ratio variation to be most strongly related to maternal testosterone levels in mammalian species with high sexual size dimorphism in favor of males.

We could not corroborate previous findings which suggest that corticosterone (either pre- or postnatal) have sex-specific influences on offspring growth in vertebrates (Bian et al. 2005; Uller et al. 2009; Warner et al. 2009). However, not all studies have found support for the sex-specific association between maternal stress and offspring postnatal growth. For example, in a related species, the bank vole, pup weight was unrelated to maternal stress during pregnancy (Marchlewska-Koj et al. 2003). One potential explanation for this may be that corticosterone has also downstream effects on other steroids such as testosterone (Klimek et al. 2005). It is thus possible that stress-related effects may actually be mediated by testosterone concentrations in mammals (Navara 2010). Because in this study we simultaneously contrasted the roles of maternal circulating testosterone and fecal corticosterone metabolites on pup weight, it is possible that when accounting for the potential direct influence of testosterone on offspring sex ratio, corticosterone has no longer sufficient explanatory power. We cannot exclude the possibility that the absence of stress-related effects on pup growth may be because the voles used in the study were experiencing relatively benign laboratory conditions compared with natural conditions, which may not have been stressful enough to illicit a detectable response in maternal investment. Finally, it is also possible that preconception fecal corticosterone metabolite levels correlated poorly with the corticosterone levels during pregnancy that may have had more relevance to the outcome.

High maternal glucose concentration has previously been shown to skew birth sex ratio towards males in this (Helle et al. 2008) and other rodent species (Machado et al. 2001; Cameron et al. 2008; Dunn et al. 2010) perhaps owing to female-biased mortality during early development. It is thus a bit surprising that we found evidence that high maternal glucose level seemed to increase postnatal weight of females only. Such an association has been rarely studied, but a study by Oh et al. (1988) using laboratory rats showed that experimentally induced hyperglycemia during the latter half of pregnancy resulted in accelerated pup growth after birth particularly in females. However, a recent study by Dunn et al. (2010) found that experimentally reduced plasma glucose levels during gestation in female rodents did not affect their offspring birth weight in males or females. These results suggest that maternal glucose level may influence differently early survival of male and female conceptuses by favoring male survival and skewing birth sex ratio towards males, but this effect seems to be reversed during subsequent growth where high maternal glucose level tends to favor female growth. It may be speculated that this initial male advantage of glucose-induced early growth and survival is diminished during the postnatal period owing to their higher energy expenditure (e.g., due to general activity and metabolic rate), leading to lower glucose storage and weight gain.

Together with our previous results showing that maternal preconception glucose and testosterone levels were associated with offspring birth sex ratio bias among these field voles (Helle et al. 2008), our current findings suggest that these factors may also bear postnatal consequences by influencing pup weight. In the case of maternal testosterone level, the current results strengthen its role on sex allocation in this species as mothers with high preconception testosterone levels produced an excess of heavy males. However, our results also suggest a potential conflict in these kinds of associations, because although high maternal preconception glucose level

skewed litter sex ratio towards males, it tended to increase only female weight after birth. More experimental work is needed to establish causality in these associations, as this research cannot determine whether the associations observed in pup weight arose from prenatal programming or postnatal maternal effects. Nevertheless, these results highlight the need to also consider the potential sex-specific postnatal consequences of maternal effects on offspring performance.

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