Litter sex composition affects life-history traits in yellow-bellied marmots

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Summary

1. The presence of siblings might have long-lasting fitness consequences because they influence the early environment in which an animal develops. Several studies under laboratory conditions have shown long-lasting consequences from the presence of male siblings in utero on morphology and life-history traits. However, in wild animals, such effects of litter sex composition are unexplored.

2. We capitalized on a long-term study of individually marked yellow-bellied marmots (Marmota flaviventris) and documented the effects of weaned litter sex composition and anogenital distance on several life-history and fitness traits.

3. First, we demonstrated that the number of males in a litter influenced anogenital distance. Then, we found that masculinized females, those with larger anogenital distances, were less likely to survive their first hibernation, were more likely to disperse and were less likely to become pregnant and wean young. Males from male-biased litters had lower growth rates, but we failed to detect longer-term consequences.

4. Taken together, our results show profound sex-dependent effects of litter sex composition, probably due to differential prenatal exposure to androgens, in free-living animals. We conclude that masculinization might constitute an alternative mechanism explaining variation in different demographic traits. This finding highlights the importance of studying these maternal effects, and they enhance our concern over the widespread use of endocrine disrupting compounds.

Key-words: anogenital distance, fitness, litter sex ratio, testosterone, yellow-bellied marmots

Introduction

Litter sex ratio has important consequences for parental fitness because male or female offspring might contribute differently to their lifetime reproductive success (Clutton-Brock & Iason 1986). Therefore, parents might invest differently in sons and daughters, by biasing the sex ratio of the litter, sex-dependent resource allocation processes or through mechanisms of behavioural facilitation (Trivers & Willard 1973; Armitage 1987; Silk & Brown 2008). Whereas many studies have attempted to test the different hypotheses that might explain biased sex ratios under different conditions (Armitage 1987; Hofer & East 1997; Nunn & Pereira 2000; Cameron 2004), most have overlooked an important issue. Not all sons (or daughters) are equal, and therefore, the pay-off would differ depending on the characteristics of the individuals forming the litter. It is well known that the conditions experienced early in life might explain differences within and between litters (Lindström 1999). The presence of siblings might be an important source of variation. Siblings are known to have long-term fitness consequences, for example through social facilitation (Palanza et al. 2005; Rödel et al. 2008b), but the effects may begin in the uterus: male foetuses produce steroid hormones that diffuse through the amniotic fluid or travel through the mother’s bloodstream affecting their siblings’ morphological, behavioural and life-history traits (vom Saal & Bronson 1980; Ryan & Vandenberg 2002; Vandenberg 2009). These effects may persist until adulthood (vom Saal 1989; Clark, vom Saal & Galef 1992).

The effects of early exposure to testosterone because of the presence of male siblings have been mostly studied under laboratory conditions. A few studies have been carried out in wild species under semi-natural conditions, but importantly, some results differed from those obtained in the laboratory (Zielinski, vom Saal & Vandenberg 1992; Drickamer 1996; but see Uller et al. 2004). Whereas many studies under laboratory conditions have worked with the relative position of male foetuses in the uterine horns, under field conditions, this approach is not feasible as it requires caesarean section. However, there is good evidence that not only the relative position of the males, but also the mere presence of males, can have strong effects in the masculinization of the litter.

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The vast literature in this topic suggests that litter sex ratio could be used as a proxy of prenatal exposure to testosterone (Cantoni, Glaizot & Brown 1999; Ryan & Vandenbergh 2002; Lummaa, Pettay & Russell 2007), and an individual’s anogenital distance (the distance between the anus and the genital papilla, hereafter referred to as AG distance) as a proxy of masculinization (Vandenbergh & Huggett 1995; Hotchkiss & Vandenbergh 2005). The AG distance is the result of the elongation of the perineal tissue during the first weeks of embryonic development and is influenced by testosterone levels (vom Saal & Bronson 1980; Ryan & Vandenbergh 2002). In rodents, males have larger AG distance than females, and within a sex, AG distance is correlated with the early exposure to testosterone.

The long-term consequences of litter sex composition in free-living animals are largely unknown. We focused on yellow-bellied marmots (Marmota flaviventris), a large ground-dwelling sciurid rodent, that are the subject of a long-term study (Armitage 2010; Blumstein et al. 2010). Marmots have been studied since 1962, and many aspects of their biology, ecology and life-history are known (Armitage 1991). However, most studies have overlooked sibling effects as a driver of variation. In this study, we aimed to assess whether part of the unexplained variance in life-history traits might be due to litter sex composition. We assumed (but cannot formally test in the field) that weaned sex composition was equivalent to birth sex composition.

We then tested the relationship between AG distance and litter sex composition. If AG distance is a good proxy of prenatal exposure to testosterone, we would expect to find a relationship between AG distance and litter sex composition: males and females from male-biased litters would have larger AG distance than juveniles from 50:50 litters, and these two would have larger AG distance than juveniles from female-biased litters (Ryan & Vandenbergh 2002). We then explored how the masculinization of the litter was related to different life-history traits, such as growth, dispersal, survival and reproduction. We hypothesized that females from male-biased litters, and therefore, with larger AG distance than expected, would show other signs of masculinization, such as higher growth rates, which might decrease the probability of survival, enhanced dispersal and reduced reproduction, compared with females with shorter AG distance and coming from female-biased litters (vom Saal & Bronson 1980; Ryan & Vandenbergh 2002). Finally, we explored how litter composition affects male marmots, and whether a similar pattern though in the opposite direction to that in females might occur (i.e. males from female-biased litters show female-like characteristics such as smaller AG distance and lower dispersal rates).

**Materials and methods**

**STUDY SITE AND STUDY POPULATION**

The study was carried out in and around the Rocky Mountain Biological Laboratory, in the Upper East River Valley, CO, USA. Marmots from 10 different colonies have been studied for nearly 50 years (Blumstein et al. 2010). Animals are individually marked and social and family relationships are known. Marmots are studied along an elevational gradient. Down-valley sites (n = 5) have earlier snow melt, marmots emerge earlier from hibernation, they begin reproduction sooner, and there is a longer vegetative growing season compared to up-valley sites (n = 5) (Van Vuren & Armitage 1991).

**DATA COLLECTION**

Data for this study were collected from 2002 to 2009. We followed the fate of juvenile yellow-bellied marmots, from emergence from the burrow to dispersal or death. We determined pup emergence date by direct observations with spotting scopes. Pups were trapped shortly after emergence using Tomahawk live traps. We collected hair samples from each animal for later DNA parentage assignment. The pups were sexed, weighed using a spring scale or digital scale (accurate to 25 g), and AG distance (centre of genital papilla to centre of anus) was measured with dial or digital callipers (accuracy 1 mm).

**DNA parentage assignment**

Details of DNA parentage assignment are published elsewhere (Blumstein et al. 2010). Briefly, hair samples were plucked from marmots and stored in clean envelopes at −20 °C. DNA was extracted, amplified and genotyped across 12 microsatellite loci. Parentage was assigned in the program CERVUS 3.0 at the 95% and 80% confidence levels, with full parentage available for 91% of sampled animals.

**AG distance index**

We only used those individuals that were trapped within their first 10 days following natal emergence because AG distance at that age is a good indicator of the intrauterine position (Vandenbergh 2009). We calculated an AG distance index by calculating the standardized residuals of the relation between AG distance and body mass.

**Life-history traits**

**Growth rate.** We calculated the growth rate for each juvenile for which we had more than three measures of body mass collected over a span of time of more than 20 days. Growth rate was defined as the slope of the regression between age and mass because juvenile marmots gain mass linearly during their first weeks (Lenihan & Van Vuren 1996).

**Survival.** Juvenile marmots and yearlings immediately following emergence from hibernation do not disperse and maintain small home ranges around their natal burrow. Our intensive and extensive observations and trapping allowed us to either observe or trap all animals from the population. Therefore, we were confident that an animal did not survive the winter when it was trapped or observed shortly before hibernation and was not trapped nor observed soon after emergence the next spring.

**Dispersal.** Yellow-bellied marmot yearlings disperse one or two weeks before natal emergence of the new cohort of juveniles (Van Vuren & Armitage 1994). We considered that a juvenile dispersed if: (i) it survived the first winter, and (ii) it was observed or trapped until shortly before the pups from its colony emerged, and (iii) it was then neither trapped nor observed after the emergence of the next cohort.
of juveniles. Philopatric individuals were observed or trapped following juvenile emergence (typically through the end of the season).

Reproduction. Female marmots are able to reproduce as two year olds. Some females initiate reproduction (as seen by swollen nipples – (Armitage 2003)), but they fail to wean young. We trapped all adult females at the beginning of the season and assessed whether they were pregnant or not by nipple inspection. Successful reproduction (for both sexes) was determined by DNA parentage assignment of the young weaned that year. For females that reached maturity (age = 2) and during their life span, we quantified (i) whether that individual attempted reproduction (i.e. became pregnant), independently of the final outcome (wean young or not) and (ii) whether the reproductive event was successful.

STATISTICAL ANALYSES

We determined that the variances were homogeneous and that the residuals of all the models followed normal distributions by visually checking normal probability plots and by a Shapiro–Wilk test.

Litter sex composition might be the consequence of maternal condition and prior experience. Therefore, we determined whether the proportion of males in a litter was explained by maternal characteristics. To do so, we used a subsample of our data that contained all adult females where the numbers of previous litters, body mass and exact age were known. We fitted a general linear mixed model where the proportion of male siblings was the dependent variable, and maternal age, number of previous litters and body mass were included as fixed factors, and maternal identity was included as a random factor. We also included the interaction between maternal age and number of previous litters. This analysis, and the ones detailed below, was performed with the function lmer from the package lme4 from the software package R, version 2.10.1 (R Development Core Team, 2009). The significance of model parameters was estimated by comparisons to a probability distribution obtained by 10,000 Markov Chain Monte Carlo simulations with the function pvals.fnc in the package language R (for further details, see Baayen, Davidson & Bates 2008). We found no effect of maternal characteristics on litter sex ratio (maternal age: β = -0.035; n = 80; pMCMC = 0.197; maternal body mass: β = 0.001; n = 80; pMCMC = 0.622; previous litters: β = 0.038; n = 80; pMCMC = 0.335; maternal age x previous litters: β = -0.008; n = 80; pMCMC = 0.312).

To study the effect of the proportion of male siblings on AG distance index, as well as to determine whether AG distance index was associated with juvenile growth rates, we fitted general linear mixed models. For the first analysis, we included AG distance index as the dependent variable, and the proportion of male siblings, litter size, the interaction of both terms and maternal age as fixed factors. Growth rates might be related to the AG distance index, the location where the animal grows, the litter size and maternal characteristics (Cantoni, Glaizot & Brown 1999; Rödel et al. 2008a). However, because litter size is correlated with the location of the litter, up-valley or down-valley (β = 11.47; n = 92; pMCMC = 0.003), we included location, together with AG distance index, maternal age and the interaction between the AG distance index and location as fixed factors. Whenever the interactions were not significant, we removed them from the final model (Engqvist 2005). Maternal identity and year were included in both models as crossed random factors to control for litter effects.

The effect of AG distance index on survival and dispersal was analyzed fitting generalized linear mixed models, because the response variables followed a binomial distribution. In the first analysis, we included AG distance index, maternal age and the location (up-valley or down-valley) as fixed factors, and the interaction between AG distance index and location (Côté & Festa-Bianchet 2001). In the second analysis (dispersal), we considered AG distance index and litter size and the interaction between both terms as fixed factors, because individuals from larger litters might experience higher pressures to disperse than those from smaller litters. In both models, mother identity and year were included as crossed random factors.

The analysis of reproduction was a repeated measures analysis that followed a binomial distribution. We fitted two generalized linear mixed models, one for pregnancy, and one for successful reproduction. AG distance index and the age of the animal were included as covariates, and the location (up-valley or down-valley) and the year of birth as fixed factors. We included the interaction between the two covariates (AG distance index x age). Marmot identity was included as a random factor, to control for the repeated measures (given that a single marmot may reproduce in several years), and it was crossed with the random factor year (to control for annual effects).

For significant mixed-effects models, we calculated Nagelkerke’s Pseudo-\(R^2\) (based on maximum likelihoods), which can be used as a measure of explained variance for this kind of analysis (Nagelkerke 1991).

Results

We followed the fate of 183 juvenile female marmots from 65 different litters and of 261 male juveniles, from 68 different litters. Litter size ranged from 1 to 9, average = 5 ± 1.79 SD, median = 5. In the analyses, we only considered those litters with at least two siblings.

ANOGENITAL DISTANCE AND THE PROPORTION OF MALE SIBLINGS IN THE LITTER

The proportion of male siblings in the litter had a significant and positive effect on female AG distance index (pseudo-\(R^2 = 0.18\); \(β = 2.037\); \(n = 183\); pMCMC < 0.001; Fig. 1a). Litter size had a marginal and negative effect (pseudo-\(R^2 = 0.002\); \(β = -0.001\); \(n = 183\); pMCMC = 0.047). Neither maternal age (\(β = 0.054\); \(n = 183\); pMCMC = 0.563) nor the interaction between the proportion of male siblings and litter size was significant, and the latter was removed from the final model (\(β = 0.201\); \(n = 183\); pMCMC = 0.225).

Similarly, juvenile males born in male-biased litters had a longer AG distance index (pseudo-\(R^2 = 0.02\); \(β = 0.519\); \(n = 261\); pMCMC = 0.041). Neither litter size (\(β = -0.052\); \(n = 261\); pMCMC = 0.207) nor maternal age (\(β = -0.052\); \(n = 261\); pMCMC = 0.105) had any effect on the AG distance index. The interaction between AG distance index and litter size was not significant (\(β = 0.0237\); \(n = 261\); pMCMC = 0.259) and was removed from the final model (Fig. 1b).

GROWTH RATE

We found no effect of AG distance index on female growth rates (\(β = -0.226\); \(n = 108\); pMCMC = 0.663). The location where the marmots lived was a good predictor of
growth rates (pseudo-$R^2 = 0.33; \beta = 2.306; n = 108;$ $pMCMC = 0.019$): as expected, females born down-valley, where pups emerged earlier and have a longer vegetation period, had higher growth rates than those born up-valley. The age of the mother had no effect on the pups’ growth rate ($\beta = 0.028; n = 108; pMCMC = 0.604$). The interaction between AG distance index and location was not significant ($\beta = -1.919; n = 108; pMCMC = 0.311$) and was removed from the final model.

However, male juveniles with a larger AG distance index had slower growth rates than males with shorter AG distance index ($\beta = -1.155; n = 128; pMCMC < 0.001$). Moreover, the location where the animals were born was positively related to growth rates ($R^2 = 0.09, \beta = 4.892; n = 128; pMCMC < 0.001$): those born down-valley had higher growth rates than those born up-valley. However, maternal age had no effect on growth rates ($\beta = 0.238; n = 128; pMCMC = 0.236$). The interaction between AG distance index and location was not significant ($\beta = -0.664; n = 128; pMCMC = 0.533$) and was removed from the final model. Whereas the location where the animal lived explained 9% of the variance (pseudo-$R^2 = 0.089$), the inclusion of AG distance in the model increased the explained variance to 12% (pseudo-$R^2 = 0.119$).

A potential confusing variable could be the proportion of male siblings, because slower growth rates might result from competition with more male siblings. We compared a set of candidate models following an information-theoretic approach. In particular, we used the second-order Akaike’s information criterion ($AIC_c$) because Burnham and Anderson (2002) suggested the use of this modification when the relation between the sample size and the number of parameters of the global model is < 40. The global model included AG distance index, the proportion of male siblings, the location where the animal lived and the maternal age. We compared the global model with two models where we removed one of the variables of interest (AG distance index or proportion of male siblings). We found that the model that best fitted the data was the one with only the AG distance index. Whereas all the models were supported with the data ($\Delta AIC_c < 4$; Table 1), the one including only AG distance index was three times more likely to be the candidate model than the one with only the proportion of male siblings.

### Survival

Females with a smaller AG distance index had higher over-winter survival rates than those with larger AG distance index (pseudo-$R^2 = 0.03; Z = -2.599; n = 177; P = 0.009$; Fig. 2a). Neither the location where the female lived ($Z = -0.931; n = 177; P = 0.352$) nor the age of the mother ($Z = 0.362; n = 177; P = 0.717$) had any effect on survival rates. The interaction between AG distance index and location was not significant ($Z = -1.072; n = 177; P = 0.284$) and was removed from the final model.

For males, only location predicted survival to hibernation (pseudo-$R^2 = 0.04; Z = -3.941; n = 260; P < 0.001$): males living down valley were more likely to survive. AG distance index ($Z = -1.951; n = 260; P = 0.055$) and maternal age ($Z = 1.332; n = 260; P = 0.183$) had no effect on over-winter survival. The interaction between AG distance index and location was not significant and was removed from the final model ($Z = 0.224; n = 260; P = 0.823$).

### Dispersal

From those that survived the winter, females with larger AG distances were more likely to disperse than those with shorter AG distances (pseudo-$R^2 = 0.07; Z = 2.431; n = 98,$ $P = 0.012$).
all values > 0. The presence of siblings has profound implications in an animal’s life (Drummond, Gonzalez & Osorno 1986; Hofer & East 1997; Palanza et al. 2005; Rödel et al. 2008b). Apart from direct effects, trait-mediated indirect effects, such as litter sex ratio, may play an important role. We have identified important long-lasting morphological and life-history consequences of litter sex ratio in yellow-bellied marmots. Individuals from male-biased litters showed signs of masculinization. Masculinized females had larger AG distances, were less likely to survive, dispersed more and had reduced fecundity, compared to females from female-biased litters. For males, the presence of other male siblings had shorter-term consequences: males from male-biased litters had larger AG distances and lower growth rates. Although the consequences of masculinization were apparent when female marmots reached maturity (2 years), the effects were stronger during the early stages of development. While litter composition has been widely studied in small rodents under laboratory conditions (vom Saal & Bronson 1980; Galea, Ossenkopp & Kavaliers 1994; Clark & Galef 1998), this is the first study in a large rodent under natural conditions, where we could follow the fate of the animals from weaning to death and account for the long-lasting consequences of their litter sex ratio.

We assumed that weaning litter sex composition might be a good estimator of birth litter sex composition. The few studies that have been able to test this assumption in semi-natural conditions have shown that, in most cases, nest mortality affected all the litter, because the main causes of nest mortality were predation, infanticide, floods or maternal abandonment (Millar, Havelka & Sharma 2004; Rödel et al. 2009), similar to what might happen to yellow-bellied marmots in our study area.

The mechanism explaining masculinization in male-biased litters relies on the flow of testosterone among foetuses (Ryan & Vandenbergh 2002). In small litters, as in our study, where the average litter size was five, foetuses might be evenly spaced along the uterine horns, and it is possible that the foetal membranes of adjacent foetuses are separated from each other (Even, Dhar & vom Saal 1992). Under these circumstances, testosterone diffusion between foetuses might
be too low to influence morphological parameters. However, we found a clear effect of the proportion of males in the litter on the AG distance, explaining 22% of the variance in females, whereas only 3% in males. Similar results have been found in California mice (*Peromyscus californicus*): females from male-biased litters had larger AG distances, though there was no detected effect on males’ morphology (Cantoni, Glaios & Brown 1999).

While some studies have highlighted that prenatal exposure to testosterone might retard intra-uterine growth and lead to low birth weight (Padmanabhan et al. 2006), the effect of testosterone on growth may vary. Testosterone increases growth in marmosets, *Callithrix jaccus* (Smith, Birnie & French 2010), females of the viviparous lizard *Lacerta vivipara* (Uller et al. 2004), as well as in several species of birds (Groothuis et al. 2005). In marmot females, there was no effect of AG distance on growth rate, whereas males with smaller AG distances had higher growth rates. Male and female growth was affected by the location where the animals were born. The individuals that experienced longer vegetation periods had higher growth rates, and for males, it also explained over-winter survival, as shown in other studies (Schwartz & Armitage 2005; Ozgul et al. 2010). Differential survival related to AG distance was apparent in females: females with shorter AG distances were more likely to survive the winter. While other studies have failed to find any relationship between AG distance and survival (Zielinski, vom Saal & Vandenbergh 1992; Drickamer 1996; Uller et al. 2004), the differential survival is potentially important because it will determine the characteristics of the population.

Testosterone levels might also influence dispersal. In grey-sided voles (*Clethriomys rufocanus*), females from male-biased litters were more likely to disperse (Ims 1989). By contrast, in female house mice (*Mus domesticus*), there was no effect of AG distance on dispersal, whereas male house mice with larger AG distance were more likely to disperse (Drickamer 1996). In marmots, about half the females disperse as yearlings (Armitage 1991), and we found that masculinized females were more likely to disperse. Our results suggest that philopatric females come from female-biased litters, whereas dispersing females come from male-biased litters.

The long-lasting effects of litter sex composition were even apparent when females reached maturity. Females with shorter AG distance were more likely to become pregnant and to successfully raise young. Therefore, females from female-biased litters might have higher lifetime reproductive success. However, the characteristics of the litters born from masculinized or feminized females might differ, which constitutes an interesting topic for further studies. Similarly, in other mammals, feminized females enter puberty sooner, are preferred by males and have a longer reproductive life span (vom Saal & Bronson 1980; vom Saal & Moyer 1985; Drickamer 1996; Zehr, Gans & McGlinchey 2001; Lummua, Pettay & Russell 2007).

One important implication of our findings has to do with sex-biased maternal investment. It is known that females might produce male-biased litters under different circum-

stances favouring the existence of masculinized females (Trivers & Willard 1973; Nunn & Pereira 2000; Silk & Brown 2008; Duckworth 2009). However, in some instances, masculinization of the litter can be the consequence of exposure to endocrine disrupting compounds during the early development, and thus, be beyond maternal control. Androgen and oestrogen-mimicking chemicals are widespread in the environment (Gray et al. 2002). It has been shown in many species how these chemicals negatively affect reproduction (Parks et al. 2001; Orlando et al. 2004; Andresen et al. 2006), and they might interfere with other life-history traits (Hotchkiss et al. 2003). Importantly, it is known that not all individuals respond to these chemicals in the same manner. For instance, feminized females are known to respond more to Bisphenol A, an oestrogen-mimicking chemical, than masculinized females (Vandenbergh 2003). The consequences would depend on to what extent females invest in any of the sexes.

Taken together, we have shown long-lasting maternal effects from pre-natal testosterone exposure have important demographic and life-history consequences in the wild. Whereas it seems that early exposure to exogenous androgens might have organizational effects in females, it might only have activational effects in males, because of the higher levels of endogenous synthesis in males. Our findings highlight the importance of studying these maternal effects, as well as the concern over the widespread use of endocrine disruptor compounds that may disrupt evolved fitness-maximizing strategies.

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