

Original Article

# Stress-induced sex ratios in ground squirrels: support for a mechanistic hypothesis

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Systematic deviations in sex ratio may be adaptive in the face of the prevailing environmental or physiological cues experienced by the mother; yet some theoretical and mechanistic hypotheses remain at odds and are rarely examined noninvasively under natural conditions. Conventional interpretations of the Trivers and Willard (Trivers RL, Willard DE. 1973. Natural selection of parental ability to vary the sex ratio of offspring. *Science* 179:90–92) hypothesis (TWH) predict that higher stress should be associated with female-biased litters; yet, several mechanistic hypotheses predict the opposite. We tested the predictions of the TWH and 2 stress-related mechanistic hypotheses in a free-living polygynandrous sciurid, Richardson's ground squirrel, *Urocitellus richardsonii*. We examined the relationship between litter sex ratio and indicators of maternal condition and investment. These included litter size, juvenile mass at emergence, as well as maternal age, changes in maternal body mass, and maternal fecal glucocorticoid (cortisol and corticosterone) levels during gestation, lactation, and post-weaning. Males born of small litters were significantly heavier at emergence than males from larger litters, which were female biased, whereas females showed no significant change in mass with litter size. Mothers with higher fecal cortisol levels during the gestation period (but not during lactation or post-weaning) were more likely to produce male-biased litters, whereas females producing larger litters showed significantly higher cortisol levels during lactation (but not gestation or post-weaning) than mothers producing smaller litters. Our results provide support for both the proximate glucose-mediated mechanistic model of Cameron (Cameron EZ. 2004. Facultative adjustment of mammalian sex ratios in support of the Trivers-Willard hypothesis: evidence for a mechanism. *Proc R Soc Ser B Biol Sci.* 271:1723–1728) and the broad-sense TWH, where social and life-history characteristics play an integral role. *Key words:* advantaged matriline, glucocorticoids, litter size, maternal condition, sex ratio, stress, *Urocitellus richardsonii*. [*Behav Ecol* 23:160–167 (2012)]

## INTRODUCTION

Skewed sex ratios at birth, weaning, or fledging in vertebrates have been attributed to the differential costs of successfully rearing offspring of a given sex and to condition-dependent differences in the relative reproductive value of each sex (Hardy 1997). In polygynous mammals, the fitness returns associated with high-quality male offspring may exceed those derived from producing high-quality female offspring because of the huge variability in male reproductive success. As a result, females with the greatest capacity for investment are predicted to bear more male offspring, whereas the opposite should be true for poorer quality females (Trivers and Willard 1973). Among mammals, the costs for producing male versus female offspring in any given species are often difficult to quantify (Frank 1990), but the immediate energetic investment into male offspring is ordinarily greater because of their higher developmental rates, larger size, and greater nutritional demands (Gomendio et al. 1990; Redondo et al. 1992; but see Koskela et al. 2009).

Despite evidence for sex ratio manipulation in mammals, the study of adaptive sex allocation (ASA) is plagued by incon-

sistent results (Charnov 1982; Clutton-Brock and Iason 1986; Frank 1990; Hardy 1997). Because the nature or timing of measurements used in sex allocation studies appear critical to the outcome, resolving these inconsistencies may hinge on elucidating the proximate physiological mechanisms for ASA, in addition to the ultimate factors responsible for them (Linklater 2007). Indeed, numerous potential mechanisms have been proposed but remain a source of contention and constitute the major limitation of arguments in support of ASA (Krackow 1995). Understanding the mechanisms by which sex ratio is manipulated in mammals is therefore an important goal for interpreting published work and for effectively designing future experiments to study ASA.

Endocrine systems play an essential role in the translation of external events into physiologically relevant responses (Sapolsky et al. 2000), providing an interface between environmental stimuli (e.g., resource abundance, predation pressure, social interactions) and behavior and life-history traits, such as growth, development, and reproduction (Boonstra et al. 2007). As such, hormones may be of critical importance to the study of litter size and sex ratio manipulation in mammals, by coupling maternal state and parameters of offspring production (Kaiser and Sachser 2009). The hypothalamic-pituitary-adrenal axis (HPA or stress axis) is of particular interest as a candidate system providing the mechanistic underpinnings of ASA because it is profoundly linked to reproductive physiology and behavior (Wingfield and Sapolsky

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2003) and can orchestrate entire physiological systems, influencing the secretion and activity of other hormones (Ketterson et al. 1991).

Baseline glucocorticoid (or GC, cortisol, and corticosterone) levels can provide an indication of individual state or condition by quantifying the physiological effort to sustain homeostatic balance in the face of social or environmental perturbations (Palme et al. 2005). Stressors associated with increases in baseline GC levels (such as overcrowding, resource limitation, or intraspecific competition) also match the conditions predicted to lead to the adaptive manipulation of offspring sex ratio (Cameron 2004). Furthermore, GCs demonstrate marked inhibitory effects on reproductive physiology and behavior, as well as parental investment (Wingfield and Sapolsky 2003), providing a critical link between the conditions favoring ASA and the physiological and behavioral mechanism for accomplishing sex ratio manipulation. Indeed, measures of social or environmental stress have been linked to ASA (Pratt and Lisk 1989; Götz et al. 2008; Love and Williams 2008; Ideta et al. 2009); however, the actual role of GCs themselves in sex ratio manipulation remains poorly understood, particularly in free-living mammalian populations.

Among the proposed mechanisms for sex ratio manipulation in mammals (likely to differ from those operating in birds due to fundamental differences in sex determination), 2 in particular suggest an indirect role for the stress axis and GCs (Cameron 2004; Grant 2007). Both models make predictions that appear to conflict with those most commonly associated with the Trivers and Willard hypothesis (TWH; i.e., that stressed females in poor condition with high GC levels should produce a greater proportion of females). The question that remains, therefore, is to what extent these mechanistic models are empirically supported and if they can be reconciled in the face of what appear to be the contrary predictions of conventional sex ratio theory.

To address these questions, we quantified the relationships between measures of maternal quality (age, spring mass, and gain in mass over active season), maternal investment (litter size, sex ratio, and offspring size), and “stress” or “allostatic load” (in the form of fecal GC levels) in free-living Richardson’s ground squirrels (*Urocitellus richardsonii*). Traditional interpretations of sex ratio manipulation (so called “narrow-sense TWH; Cockburn et al. 2002) predict that poor quality females with the lowest capacity to invest (i.e., the lightest, most “stressed”, or the youngest) should produce female-biased litters. Under these conditions, high GC levels would also be predicted to be associated with female-biased litters and potentially with other aspects of female condition (e.g., age, mass; Clutton-Brock and Iason 1986).

On the other hand, support for both mechanistic hypotheses requires that GC levels be negatively associated with sex ratio (i.e., the ratio of males to the total number of offspring produced within a litter). The mechanism proposed by Cameron (2004) predicts that sex ratio will be positively associated with litter size, due to the differential mortality of developing female embryos, whereas the model of Grant and Irwin (2005) does not predict this relationship. The novelty of this study is that it tests the predictions of both mechanistic (proximate) and adaptive (ultimate) hypotheses using noninvasive techniques under ecologically relevant conditions.

Our study species, Richardson’s ground squirrels, are semi-fossorial, hibernating colonial rodents found in the northern plains and intermontane valleys of North America (Michener and Koepl 1985). Following emergence from hibernation, males compete intensely, often fatally, for mating opportunities (Michener 1983b). Although all females are typically inseminated in a given year, many males do not successfully reproduce (Michener and Locklear 1990). This single annual

reproductive event, coupled with the relatively low interyear recovery rates of *U. richardsonii*, results in enormous selective pressure for males to maximize their reproductive output during this time. The intense competition between males, and extreme variability in male reproductive success, fits criteria outlined by Trivers and Willard (1973) when discussing conditions that would lead to differential fitness consequences for high-quality males versus high-quality females in a population and thus selection for sex ratio manipulation. By carrying out this study using numerous, noninvasive fecal GC measurements throughout the active season, in an environment similar to that in which the behavior would have evolved, we investigate the proximate mechanisms of sex ratio manipulation without sacrificing the ultimate ecological relevance of the results.

## MATERIALS AND METHODS

### Study site and data collection

Our research focused on individuals belonging to a population of Richardson’s ground squirrels, *U. richardsonii*, residing on mowed fields and berms within the grounds of the Assiniboine Park Zoo in Winnipeg, Manitoba (lat 49°52’N, long 97°14’W). This free-living population has been extensively studied since 2003, and detailed genealogical information and data regarding the social behavior in this colony are available (e.g., Hare et al. 2004; Wilson and Hare 2004).

We commenced trapping on 18 March 2009, when male squirrels began to emerge from hibernation, and continued trapping until early July when most adults and yearlings had descended into their burrows for hibernation. Females emerged from hibernation after the males and were trapped, on average, twice weekly beginning in early April by placing National or Tomahawk live traps (Tomahawk Live Trap Co., Tomahawk, WI) baited with peanut butter (No Name Smooth Peanut Butter; Loblaw’s Inc., Toronto, Ontario, Canada) at or near burrow entrances. Using Pesola spring balances, we weighed females to the nearest 5 g at each capture and collected feces from beneath the traps for GC assays. We applied commercially available hair dye (Clairol Hydrience 52S; Pearl Black, Stamford, CT) to the dorsal pelage of the animals in unique patterns to facilitate individual identification and inserted numbered metal ear tags (National Band and Tag Company, Monel no. 1, Newport, KY) through the right pinna of each squirrel for permanent identification.

Because *U. richardsonii* females utilize and defend small core areas with little overlap with the core areas of other females during pregnancy and lactation (Michener 1979), the offspring belonging to a given mother were determined by which burrow the litter emerged from and when. We trapped young of the year as soon as possible, typically within 2 days of their initial emergence from their natal burrow. The juvenile squirrels were then sexed, marked, and released until all pups in the litter were accounted for. We recorded litter sex ratios for each mother, which are henceforth described in the conventional manner, as a ratio of males to total offspring produced (Charnov 1982). We carried out all trapping, marking, and handling of animals in accordance with the guidelines set forth by the Canadian Council on Animal Care as approved under protocol F08-012 of the University of Manitoba, Fort Garry Campus Protocol Management and Review Committee.

We determined maternal stress levels using a noninvasive measure of GCs (cortisol and corticosterone) in the feces. Fresh feces were collected from females during trapping, which we carried out between 1100 and 1600 h Central Daylight Time to minimize circadian fluctuations in GC hormones

(Boonstra et al. 2007). We then transferred fecal pellets to 20-ml polycarbonate sample vials using disposable wooden sticks (Puritan, Guilford, ME), carefully discarding fecal pellets contaminated with urine, another excretory route for circulating GCs (see Mateo and Cavigelli 2005). We numbered vials sequentially to allow cross-referencing to field notes providing the time of sample collection, date, and female identity and then stored them temporarily at  $-4^{\circ}\text{C}$  in a freezer located at the Assiniboine Park Zoo. Samples were later transferred to a  $-20^{\circ}\text{C}$  freezer for storage until required for GC assays.

### GC assays

We used radioimmunoassay (RIA) to ascertain fecal cortisol and corticosterone levels in our focal individuals, based on methods described by Mateo and Cavigelli (2005) but modified and validated for *U. richardsonii* (Hare JF, Skyner L, Enright C, and Anderson WG, unpublished). Briefly, we dried fecal samples overnight at  $60^{\circ}\text{C}$  before combining approximately 0.2 g of the dried fecal matter with 1.5 ml 95% ethanol. We then vortexed each sample vigorously before centrifugation at  $4^{\circ}\text{C}$  for 10 min at  $13\,000 \times g$ . After centrifugation, we drew off the resulting supernatant, which was then stored at  $-80^{\circ}\text{C}$  until measurement in the RIA, at which time we removed 100  $\mu\text{l}$  of supernatant and evaporated the ethanol in a sample concentrator (Savant speed vac; Thermo Scientific, Waltham, MA). The resultant pellet was then resuspended in RIA buffer (0.1 M phosphate buffer, 0.9% NaCl [w/v], and 0.5% bovine serum albumin [w/v]), and each sample was vortexed to ensure complete dissolution.

For the cortisol RIA, we combined 100  $\mu\text{l}$  of a cortisol-specific antibody (1:16 000 dilution; Fitzgerald Industries, North Acton, MA) in an assay tube with 5000 disintegrations per minute/100 ml (dpm) of tritiated cortisol (GE Healthcare, Piscataway, NJ) and 100  $\mu\text{l}$  of either sample or a known concentration of cortisol (Steraloids, Newport, RI). We then incubated the assay at room temperature for 1 h and then at  $4^{\circ}\text{C}$  overnight, after which time we added dextran (0.5% w/v)-coated charcoal (5% w/v) (100  $\mu\text{l}$ ) to the solution to terminate the reaction. After 15 min on ice, we centrifuged assay tubes for 30 min at  $4^{\circ}\text{C}$  ( $2500 \times g$ ) and decanted the resulting supernatant into a 7-ml scintillation vial. To each vial, we added 4 ml of Ultima Gold scintillation fluid (Perkin Elmer) and measured radioactivity using a liquid scintillation counter (LS6500; Beckman Coulter, Brea, CA). By interpolating from a standard curve made from known concentrations of cold cortisol, we determined cortisol concentration (nanograms per gram of dry mass of fecal matter) for each sample. For accuracy, we processed all samples in duplicate and standards in triplicate. We carried out corticosterone assays using identical techniques but with corticosterone antibody (Antibodies-online, Aachen, Germany), standards (Steraloids, Newport, RI, USA), and labelled hormone (GE Healthcare) in place of cortisol-specific analogs. Cortisol inter-assay and intra-assay variations were  $10.9 \pm 6\%$  and  $15.3\%$ , respectively, and the minimum detectable level was 0.051 ng/ml. Inter-assay and intra-assay variations for corticosterone were  $14.7 \pm 8.9\%$  and  $8.8\%$ , respectively, and the minimum detectable level for this hormone was 0.005 ng/ml. Samples demonstrated good parallelism for both hormones (data not shown), and extraction efficiency was 90% and 91% for cortisol and corticosterone, respectively.

### Data analysis

Prior to statistical analyses, we assessed the distribution of each variable independently and ranked models according to

Akaike's Information Criterion, corrected for small sample sizes (AICc). We ultimately chose models with the lowest AICc value and least number of parameters, as in Servanty et al. (2007). Based on the AICc values for distribution goodness-of-fit, most of our data did not depart significantly from those expected to come from a population following a Gaussian distribution, and so we did not transform data prior to analysis using standard parametric tests. Our GC assay values were not normally distributed but did approximate both log- and generalized log-normal distributions. Based on the nature of the data (derived from a biological assay and possessing a nonconstant variance; SAS Institute Inc. 2007), as well as the high  $P$  value for goodness-of-fit ( $P > 0.80$  for both cortisol and corticosterone), we used generalized logarithm transformed (Glog) values for all GC analyses. We then analyzed the transformed hormone data using standard parametric tests, including Pearson's correlation for the relationships between stress and sex ratio and litter size and one-way analysis of variance for categorical variables.

We partitioned stress hormone data into 3, biologically relevant time periods, termed "gestation", "lactation", and "post-weaning." We defined the gestation period, roughly 23 days (Michener 1980b), as the time immediately prior to conception and ending with the inferred date of parturition. Because females give birth to their litter in their burrows, we inferred parturition date based on the date of juvenile emergence, which is on average 29.2 days following parturition (Michener 1985). The lactation period was the time period between parturition and litter emergence, whereas the post-weaning period consisted of the remainder of the summer following litter emergence, ending with female immergence into hibernation.

Early season mass was female mass on 18 or 19 April, dates on which the most females (15/21) were trapped and which corresponded to the time prior to conception for the majority (>85%) of females, based on the inferred parturition described above. We determined summer weight gain by subtracting the early season mass from the prehibernation mass. Prehibernation mass was the mass on 2 July, a date on which masses for most (18/21) females were recorded and which was within 1 week of immergence into hibernation for all but 1 female. We were able to trap and weigh most (>90%) juveniles within 2 days of their first emergence from the natal burrow. If more than 3 days passed between the first and the last juvenile trapping from a given litter, data from those litters (2/21 litters) were not used for these analyses as juveniles gain mass rapidly within the first 2 weeks of emergence (Hare JF, unpublished data).

We tested sex ratios for deviations from parity for individual litters and for all juveniles combined using cumulative distribution binomial statistics, whereas sex differences in offspring mass at emergence were analyzed using an unpaired  $t$ -test. Age-specific differences in sex ratio, litter size, GC levels, and offspring mass were analyzed using one-way analysis of variance, where females were categorized into yearlings, 2-year-olds, and 3 years and older. We excluded 1 immigrant female of unknown age from all age comparisons. Pearson's product-moment correlation was used to examine the relationships between all continuous variables, including the relationship between litter size and sex ratio, dam mass gain over summer, and juvenile masses at emergence. All pairwise correlations were based on the residual maximum likelihood method, with significance set to 5% ( $\alpha = 0.05$ ). Analyses were carried out using JMP, Version 8.0.2.2 (SAS Institute Inc., Cary, NC) with significance set to  $\alpha = 0.05$ , and figures were created using Graphpad Prism Software, version 5.0 (San Diego, CA, www.graphpad.com) or R (version 2.12.2 1.36 GUI (5691); R Development Core Team, 2011).



**RESULTS**

**Age-effects on maternal condition and offspring production**

To examine the potential for age-related effects on maternal condition, we compared age classes separately for spring emergence mass and weight gain over the summer. We were also interested if another measure of maternal condition, GC levels are affected by the age of the female. Yearling females emerged significantly lighter ( $259.5 \pm 12.5$  g) than two 2-year-olds or females 3 years and older ( $325.0 \pm 15.3$  g and  $329.9 \pm 11.5$  g, respectively;  $F_{2,14} = 9.85, P = 0.002$ ), but there were no age-related differences in weight gain from spring to prehibernation ( $F_{2,12} = 0.481, P = 0.629$ ).

We assessed potential age-related effects on investment in the form of litter size, sex ratio, and the mean of juvenile masses. Neither litter size ( $F_{2,18} = 0.974, P = 0.397$ ), sex ratio ( $F_{2,18} = 0.0145, P = 0.986$ ) nor mean juvenile mass ( $F_{2,17} = 0.0747, P = 0.928$ ) differed significantly among the 3 age classes. Furthermore, age class had no significant effect on cortisol or corticosterone levels for any of the 3 periods (gestation, lactation, or post-weaning;  $P > 0.190$  in all cases).

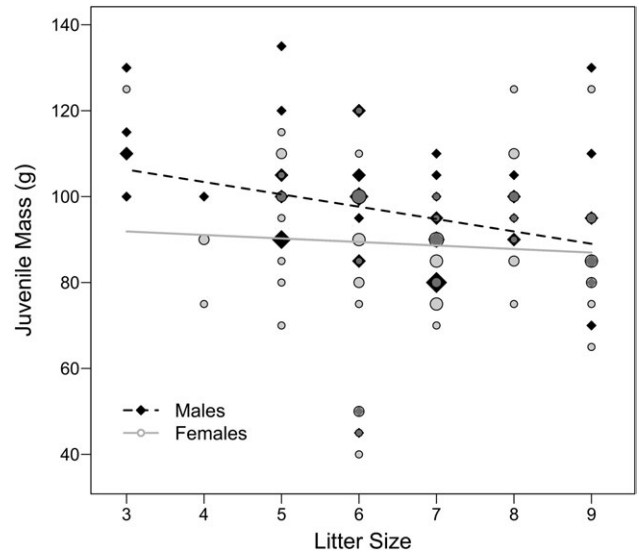
**Age-independent maternal condition and offspring production**

We considered the associations between parameters of maternal investment, including litter size and sex ratio, juvenile mass, and maternal seasonal changes in mass, to examine the effects of maternal condition on litter production and vice versa. Independent of age class, spring emergence mass was significantly correlated with subsequent litter size, with heavier mothers tending to produce larger litters ( $P = 0.019$ ; Table 1). Although mothers producing larger litters exhibited neither greater nor lesser gains in mass over the active season ( $P = 0.276$ ; Table 1), weight gain was significantly related to litter sex ratio; mothers that produced more males gained more mass over the summer ( $P = 0.038$ ; Table 1).

Individual litter sex ratio varied considerably and was inversely correlated with the number of offspring in the litter ( $P = 0.0496$ ; Table 1). A pooled analysis of juvenile masses at emergence (29–31 days old) revealed significant differences between males and females (Mann–Whitney  $U = 1543, n_m = 73, n_f = 60, P = 0.003$ ), with males significantly heavier than juvenile females at emergence ( $96.5 \pm 2.2$  g versus  $88.9 \pm 2.2$  g). Male mass decreased significantly (Spearman’s  $\rho = -0.359, df = 54, P = 0.007$ ) with increases in litter size, whereas female mass did not (Figure 1).

**GCs, maternal condition, and offspring production**

To test the predictions of both the mechanistic and the theoretical hypotheses, we quantified the association between ma-



**Figure 1**  
Relationship between litter size and emergence mass for *Urocitellus richardsonii* juveniles. Emergence mass was not related to litter size for females (degrees of freedom [df] = 64,  $P = 0.366$ ; grey circles, solid grey line), but males from larger litters were significantly lighter than those from smaller litters (Spearman’s  $\rho = -0.359, df = 54, P = 0.007$ ; black diamonds, broken black line). Number of observations for each sex represented by symbol size, between 1 and 5 observations. Least-squares regression lines are based on parametric correlations (not used due to the data distribution) but are provided for illustrative purposes.

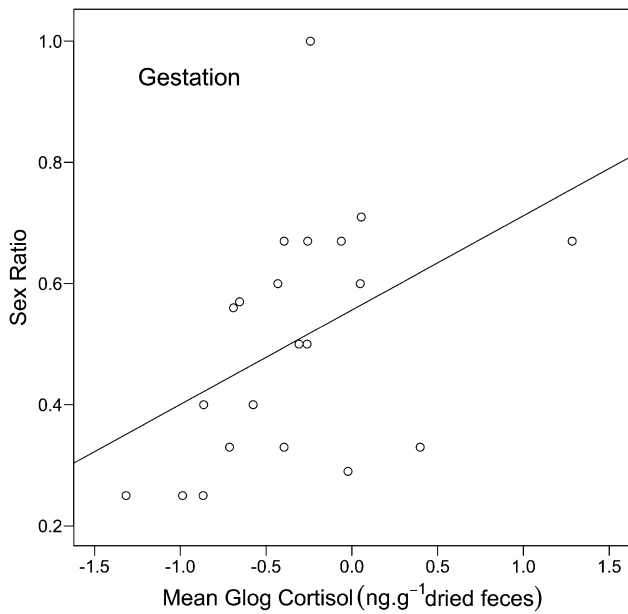
ternal GC levels and measures of condition, as well as parameters of investment during 3 distinct reproductive periods. We observed a significant relationship between cortisol levels during gestation and the subsequent litter sex ratio of the gestating mother (Figure 2). Mothers with a high mean cortisol level during gestation were significantly more likely to produce male-biased litters ( $P = 0.049$ ; Figure 2). We did not observe a relationship between corticosterone and sex ratio in gestating mothers ( $P = 0.450$ ; Table 2) nor did we find a significant correlation between either corticosterone or cortisol and litter sex ratio during the lactation or post-weaning periods ( $P > 0.326$  for all; Table 2).

Neither cortisol nor corticosterone levels during gestation were significantly related to litter size ( $P > 0.424$  for both; Table 2). This was also true of post-weaning GC levels ( $P > 0.188$  for both; Table 2); however, higher cortisol levels during lactation were associated with the production of larger litters ( $P = 0.014$ ; Figure 3). Corticosterone levels during lactation were not significantly correlated with litter size ( $P > 0.118$ ;

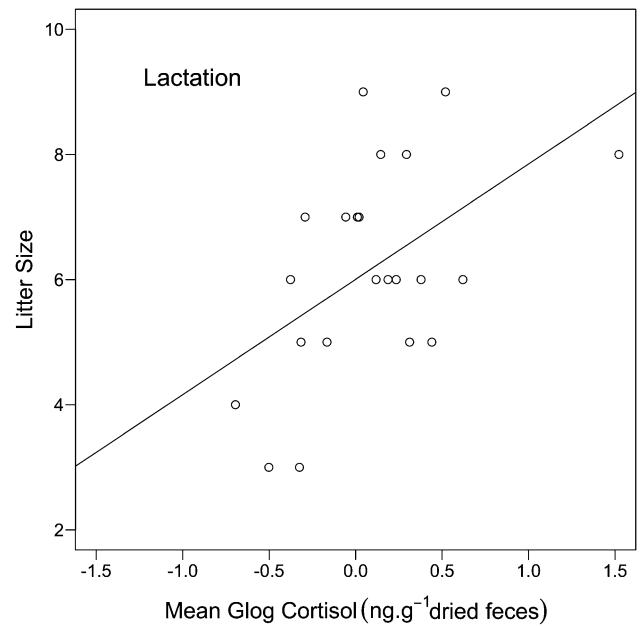
**Table 1**  
Relationship between indicators of maternal condition and litter composition in *Urocitellus richardsonii*

Variable	By variable	n	Correlation (=r)	Lower 95%	Upper 95%	Probability
Mass gain	Spring mass	16	-0.290	-0.687	0.240	0.2756
Litter size	Spring mass	18	0.548	0.109	0.808	*0.0186
Litter size	Mass gain	16	-0.286	-0.685	0.244	0.2822
Sex ratio	Spring mass	18	-0.339	-0.696	0.152	0.1690
Sex ratio	Mass gain	16	0.522	0.036	0.809	*0.0381
Sex ratio	Litter size	22	-0.423	-0.717	-0.002	*0.0496

Measures of maternal condition approximate the time of conception (spring mass) and the gain in mass between spring mass and descent into hibernation (mass gain). Correlations estimated by residual maximum likelihood method, with significance ( $P < 0.05$ ) indicated by an asterisk (\*).



**Figure 2** Relationship between fecal cortisol levels of gestating *Uroditellus richardsonii* females ( $n = 21$ ) and the sex ratio of the subsequent litter emerging from the maternal burrow. Generalized log-transformed (Glog) data fit a normal distribution and showed a significant relationship with litter sex ratio ( $r^2 = 0.19$ ,  $P = 0.049$ ;  $y = 0.16[\text{Glog-MeanCortisol}] + 0.56$ ).



**Figure 3** Relationship between fecal cortisol levels of female *Uroditellus richardsonii* ( $n = 22$ ) during lactation and the number of offspring in the litter at juvenile emergence. Generalized log-transformed (Glog) cortisol values showed a significant relationship with litter size ( $r^2 = 0.27$ ,  $P = 0.014$ ;  $y = 1.84[\text{Glog-MeanCortisol}] + 6.00$ ).

Table 2). Cortisol and corticosterone levels showed a strong positive correlation with each other for all 3 reproductive periods ( $P < 0.010$  for all; Table 2).

**DISCUSSION**

Support for the narrow-sense TWH led to the prediction that females in poorer body condition would have higher GC levels and fewer sons. Conversely, both mechanistic hypotheses sug-

gested that females with high GC levels should have more sons, and the mechanistic model of Cameron (2004) led us to predict that mothers with proportionally more sons would also tend to have smaller litters. We found substantial evidence supporting litter size and sex-ratio optimization with respect to maternal condition and fecal cortisol levels, supporting trade-offs in investment and a mechanism associated with sex-differential mortality. Increases in litter size were accompanied by a smaller proportion of males in the litter, but

**Table 2**

**Relationship between cortisol and corticosterone levels during distinct reproductive phases (gestation, lactation, and post-weaning) and reproductive allocation (sex ratio and litter size) in *Uroditellus richardsonii***

Variable	By variable	<i>n</i>	Correlation (=r)	Lower 95%	Upper 95%	Probability
<b>Gestation</b>						
Mean (Glog CORT)	Mean (Glog Cortisol)	21	0.607	0.238	0.823	*0.0035
Sex ratio	Mean (Glog Cortisol)	21	0.435	0.004	0.729	*0.0490
Sex ratio	Mean (Glog CORT)	21	0.174	-0.278	0.564	0.4502
Litter size	Mean (Glog Cortisol)	21	-0.184	-0.570	0.269	0.4249
Litter size	Mean (Glog CORT)	21	0.132	-0.318	0.534	0.5678
<b>Lactation</b>						
Mean (Glog CORT)	Mean (Glog Cortisol)	22	0.845	0.657	0.934	*<.0001
Sex ratio	Mean (Glog Cortisol)	22	-0.200	-0.574	0.242	0.4339
Sex ratio	Mean (Glog CORT)	22	-0.226	-0.592	0.216	0.3269
Litter size	Mean (Glog Cortisol)	22	0.549	0.166	0.788	*0.0143
Litter size	Mean (Glog CORT)	22	0.266	-0.175	0.619	0.1186
<b>Post-weaning</b>						
Mean (Glog CORT)	Mean (Glog Cortisol)	21	0.555	0.162	0.796	*0.0090
Sex ratio	Mean (Glog Cortisol)	21	-0.179	-0.567	0.274	0.4367
Sex ratio	Mean (Glog CORT)	21	0.064	-0.378	0.482	0.7832
Litter size	Mean (Glog Cortisol)	21	0.299	-0.153	0.647	0.1884
Litter size	Mean (Glog CORT)	21	-0.098	-0.508	0.348	0.6719

Correlations were determined by residual maximum likelihood method using generalized log-transformed (Glog) hormonal data (Cortisol = cortisol and CORT = corticosterone) from fecal GC extractions. Significance ( $P < 0.050$ ) is indicated by an asterisk (\*).

males born of small litters were heavier at emergence than males from larger litters (Figure 1). Mothers with higher fecal cortisol levels during the gestation period (but not during the lactation or post-weaning periods) were more likely to produce male-biased litters (Figure 2), whereas females that produced larger litters showed significantly higher cortisol levels during lactation (but not gestation or post-weaning) than mothers producing smaller litters (Figure 3).

The positive correlation we observed between the mass of the mother shortly after emergence from hibernation and subsequent litter size (Table 1) corresponds to the time of ovulation, when reproductive allocation in response to maternal condition would prove most advantageous. In Richardson's ground squirrels and other ground-dwelling sciurids with low infant mortality, larger litters result in a greater number of surviving young and have not been associated with a decrease in future survivorship or reproductive output for dams (Michener and Locklear 1990; Hare and Murie 1992). Consistent with the predictions made under the "individual optimization" hypothesis (Perrins and Moss 1975; Pettifor et al. 1988), *U. richardsonii* mothers may produce optimally sized litters that match their capacity for rearing them, obscuring the apparent costs of producing larger litters (Risch et al. 2007). Accordingly, we observed no significant correlation between the size of litter a dam produced and her capacity to gain weight over the active season (Table 1). The fact that litter size was not significantly different for dams belonging to the different age classes—even though mass following spring emergence, around the time of mating—suggests that individual condition overrides age-related effects in this population (see also Broussard et al. 2005, 2006).

Although individual females may produce litters that complement their capacity to raise them, demonstrating an effect of quality or condition on litter production, evidence for the cost of litter size and offspring sex is still apparent. We found a significant relationship between the number of offspring and the sex ratio of the emerging litter, with larger litters becoming more female biased (Table 1). Michener (1980a) found similar relationships among captive *U. richardsonii* litters, and these results are supported by a growing body of evidence for other polytocous species, which gestate and give birth to multiple young (Krackow and Hoeck 1989; Krackow 1993; Servanty et al. 2007; Shibata and Kawamichi 2009). In our study, juvenile males were significantly heavier than females at the time of emergence, and their mass declined significantly with increases in litter size, whereas female mass did not (Figure 1). These data strongly imply a greater cost to mothers for producing male offspring, a greater cost to males for being born of larger litters, and a trade-off between offspring sex and the total number of young.

We also observed a significant positive relationship between mean cortisol levels during gestation and the sex ratio of the litter a mother subsequently produced (Figure 2). Mothers with high gestational cortisol levels produced proportionally more males, even though GC levels during the rest of the summer showed no significant relationship to sex ratio (Table 2). The relationships between sex ratio and both litter size and cortisol levels support 2 key predictions of the "glucose metabolism" hypothesis of Cameron (2004) as a mechanism for sex ratio manipulation, which can be summarized as follows.

Weight gain in gestating and lactating mammals is in part orchestrated by sustained moderate elevation of circulating GC levels, which stimulate foraging behavior and resource mobilization and are often accompanied by an elevation in circulating glucose (Sapolsky et al. 2000). In mice, experimentally reduced (Cameron et al. 2008) and elevated (Machado et al. 2001) glucose levels corresponded to female and male-biased litters, respectively, and naturally high serum glucose

levels were related to smaller male-biased litters in field voles *Microtus agrestis* (Helle et al. 2008). Moreover, elevated glucose has been shown to inhibit the development of female—but not male—blastocysts *in vitro* (Larson et al. 2001), by way of X-chromosome-linked increases in glucose metabolism accompanied by the build up of toxic by-products from the pyruvate pentose phosphate pathway (Kimura et al. 2005). Additional support for sex differential glucose-mediated mortality comes from supplementary feeding of high fat diets (Austad and Sunquist 1986; Rosenfeld 2004; Dama et al. 2011), as well as the effect of timing of conception on sex ratio (Sheldon and West 2004; James 2008), which relate to variation in circulating glucose levels and its availability to the developing conceptus (Cameron 2004). In *U. richardsonii*, the number of follicles ovulated determines the maximum litter size, and so restricts any mechanisms for sex-ratio and litter-size optimization to differential embryo mortality.

We also identified a significant positive correlation between the size of litter a dam produced and her fecal cortisol levels during lactation (Figure 3). This relationship was absent earlier in the summer during the period corresponding to gestation and later in the summer post-weaning period, suggesting that cortisol plays an important role in sustaining a key parameter of maternal offspring investment. Indeed, lactation itself has been described as the most energetically taxing component of reproduction, particularly in smaller animals, which are less able to rely on fat reserves (Gittleman and Thompson 1988). Compared with other sciurids, the costs of lactation in *U. richardsonii* are thought to be especially high and vary directly with litter size (Michener 1989) and are compounded by other maternal behaviors expressed during lactation (e.g., nesting, huddling, licking). Because the stress response is typically attenuated during lactation (Bauman and Currie 1980), it is unlikely that the higher cortisol levels that we observed during lactation for larger litters correspond to acute stressors but rather should be interpreted in the context of their effect on foraging and the mobilization of internal energy stores (Hill et al. 2003). Therefore, females with larger litters endure greater costs during lactation, and the "allostatic load" of nursing larger litters is evident in the dam's HPA and cortisol response.

Finally, we found a significant relationship between sex ratio and summer mass gain, with dams who produced more males gaining significantly more weight overall (Table 1). Interestingly, there was no significant relationship between gain in mass and litter size (Table 1). In the matrilineal societies of *U. richardsonii*, daughters tend to remain philopatric, whereas males often disperse, and dispersal is usually prior to hibernation in the first summer (Michener and Michener 1977). Post-weaning investment by dams in female offspring is likely to be greater than into dispersing male offspring (Michener 1980a). Mothers of males who disperse may therefore devote less time to vigilance and establishing juveniles within the colony and more time to foraging, making the production of small male-biased litters a strategy that favors recouping the costs of reproduction later in the summer.

Thus, GCs were positively related to sex ratio, which was negatively associated with litter size, but GCs showed no relationship to age or other predictors of maternal condition. These results support the glucose metabolism hypothesis, but conflict with conventional predictions of the narrow-sense TWH. Still, we believe that rejecting the TWH entirely based on support for the mechanistic hypotheses alone is premature for several reasons.

First, original predictions that poor condition mothers should invest more in females were made in species which typically give birth to 1 offspring in a given reproductive bout (Trivers and Willard 1973). Uncovering ASA is complicated

for iteroparous, polytocous species, where sex-differential costs and potential reproductive pay-offs interact with offspring number and a dam's future reproductive potential (Williams 1979). Thus, the production of male-biased litters, when in conjunction with a reduction in litter size, may still be a conservative strategy for small poor condition mothers (Myers 1978; McGinley 1984). Second, the shift in our general understanding of the role of GCs and "stress" from one of disease to one of "allostasis" and protective measures (McEwen 1998) has important implications in how these types of data are interpreted. For example, females nursing large litters required the energy mobilization and behaviors necessary to feed those litters (accompanied by high cortisol), but did not gain less mass, or have higher GC levels during the post-weaning period, demonstrating the transient effect of this hormonal response. Thus, GC levels are not always indicative of individual quality in an absolute sense but rather of the mediation of environmental and physiological challenges (Sterling and Eyer 1988). The fact that age and mass were not related to GC levels supports this conclusion. Finally, the ultimate adaptive function of sex ratio manipulation is not necessarily exclusive of the mechanistic hypotheses. Our predictions were aimed at testing the narrow-sense TWH, where condition plays the predominant role, but a number of predictions arise depending on a host of other variables (Cockburn et al. 2002). Based on the social structure and life history of Richardson's ground squirrels, we propose an ultimate explanation for how male-biased litters produced by stressed mothers with high cortisol levels could prove adaptive.

The establishment and maintenance of matrilineal kin clusters allow for the control of access to burrow systems essential for hibernation, predator avoidance, and rearing young (Michener 1983a; Hare and Murie 2007). Female members of such clusters benefit from an inclusive fitness payoff by producing daughters that become integrated into their existing kin cluster, potentially enhancing the overall competitive ability of their matriline relative to others (Michener 1980a). Females living outside the context of an existing matriline, on the other hand, would presumably have access to a poorer resource base, be subject to an increased risk of predation, and would be subject to more aggressive interactions with conspecifics (Michener 1983a; Hare and Murie 2007). The production of males, which disperse from the natal area to pursue breeding opportunities with females residing in existing matrilines elsewhere (Michener and Michener 1977), would be selectively favored for struggling females lacking an established matriline. This "advantaged matriline" hypothesis has seen theoretical (Altmann M and Altmann J 1991) and mixed empirical support derived from research involving nonhuman primates (Simpson MJA and Simpson AE 1982; Altmann et al. 1988), ungulates (Clutton-Brock et al. 1984), and canids (Sidorovich et al. 2007), where daughters often inherit maternal rank.

Our study provides evidence for a GC-mediated mechanism driving sex ratio manipulation. Due to interactions between litter size and sex ratio, our data best support the glucose metabolism model of Cameron (2004). Fine tuning of investment such that sex ratio is modified in conjunction with other factors, such as litter size, and in response to other variables, including those which are social or ecological in nature, means that the expression of ASA may be nearly as diverse as the species and environments responsible for shaping this behavior. Manipulative experiments testing presumptive mechanisms of sex ratio manipulation and their potentially adaptive role in free-living populations (*sensu* Ketterson et al. 1996) will prove particularly informative but should continue to be considered with respect to the social and life-history context in which they evolved.

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