

**Richardson's ground squirrel, *Urocitellus richardsonii*, Adaptive Sex Allocation**

by

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## Abstract

Trivers and Willard proposed that female mammals should adjust their investment in male versus female offspring relative to the effect of their underlying body condition on their ability to produce high quality offspring. I conducted studies testing whether litter size-sex ratio tradeoffs predicted by adaptive sex allocation (ASA) theory occur among Richardson's ground squirrel (*Urocitellus richardsonii*) dams, and whether circulating plasma glucose levels of those dams influence offspring sex ratio as predicted by Cameron's glucose-metabolism hypothesis. The results of these studies validate the use of the Roche Diagnostics Accu-chek® Aviva glucometer in measuring Richardson's ground squirrel plasma glucose concentration, inform future studies attempting to test the glucose metabolism hypothesis, and provide evidence from long-term data of litter size-sex ratio tradeoffs supporting earlier reports of ASA among Richardson's ground squirrels from the same study population that have been called into question owing to small sample sizes derived over single breeding seasons.

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## Chapter 1: Introduction

Trivers and Willard (1973) suggested that selection will favour departures from a 1:1 sex ratio of offspring in species where females in good body condition, capable of producing high quality male offspring, produce male-biased offspring sex ratios owing to the reproductive benefit those high-quality males provide. While advanced over 40 years ago, the Trivers-Willard hypothesis continues to be the subject of active investigation (e.g. Hamel et al. 2016) and there is considerable evidence supporting its basic tenets (Cameron, 2004). Despite this, relatively few empirical studies have provided conclusive results supporting the Trivers-Willard hypothesis (Cameron, 2004). Austad and Sunquist (1986) found that female Virginia opossums (*Didelphis virginiana*) that received supplementary food (and thus with higher blood glucose levels) produced male-biased offspring sex ratios, directly supporting the hypothesis. While the majority of the existing literature supports the Trivers-Willard hypothesis when assessed under certain conditions, debate continues regarding its applicability to mammals in general (Sheldon & West, 2004).

Studies on free-living Richardson's ground squirrels (*Urocitellus richardsonii*) reveal that the sex biases predicted by the Trivers-Willard hypothesis are not uniformly observed, as high stress levels during gestation can result in the production of male-biased litters (Ryan et al. 2012). Ryan et al. (2014) subsequently quantified the concentration of glucocorticoids and testosterone in blood and fecal samples of pregnant Richardson's ground squirrels from the start of the breeding season to the end of lactation to test for any relationship between levels of those hormones and the number of pups of each sex that emerged from their dam's natal burrow. Elevated levels of glucocorticoids and testosterone were found in dams producing male-biased

litters, with the highest cortisol levels detected during early gestation and peak testosterone levels during lactation. Total plasma cortisol levels dropped over the course of the breeding season and remained low afterwards. Glucocorticoid levels measured throughout the summer revealed that the only time period where there was a significant positive correlation between glucocorticoid levels and the number of males in a litter was during gestation. This points to a potential gestational mechanism promoting adaptive offspring sex allocation in Richardson's ground squirrels in that Ryan et al. (2012; 2014) reported an inverse relationship between litter size and sex ratio, with stressed females producing smaller, male-biased litters. Gedir and Michener's (2014) work with Richardson's ground squirrels, however, failed to support the Trivers-Willard hypothesis, with litter sex ratios fluctuating randomly about parity, and with no apparent relationship between litter size and sex ratio based on her 25-year dataset for a Lethbridge, Alberta area Richardson's ground squirrel population.

Results for less social members of the tribe Marmotini, such as the Franklin's ground squirrel (*Poliocitellus franklinii*), conform with the predictions of the Trivers-Willard hypothesis. Pero and Hare (2018) reported that dams with reduced ectoparasite loads, and thus presumably in better condition, produced significantly more male offspring than control dams.

The field of adaptive sex allocation is broad, spanning many taxonomic groups. Among large mammals, elk (*Cervus elaphus*) mothers with high kidney fat, that are thus in better body condition, were more likely to give birth to male offspring, supporting the Trivers-Willard hypothesis (Kohlmann, 1999). Conversely, tammar wallaby (*Macropus eugenii*) mothers with elevated blood glucose levels after the dry season were more likely to bear female offspring (Schwanz & Robert, 2014). Adaptive sex allocation resulting in biased primary sex ratios has also been studied extensively in avian species including Snow Geese (*Chen caerulescens*),

Eclectus Parrots (*Eclectus toratus*), numerous songbirds, European Kestrels (*Falco tinnunculus*) and Tawny Owls (*Strix aluco*) (Komdeur & Pen, 2002; Oddie, 1998).

Previous experiments with captive laboratory rats (*Rattus norvegicus*) have provided evidence for sex biasing in rodents due to artificially elevated levels of stress hormones, though lab conditions may introduce additional stresses that would not be encountered in nature, leading to artefactual findings (Bourke et al. 2013). Elevated blood glucose has been suggested as a potential cause of sex-biased offspring production in mice, in that increased circulating blood glucose purportedly reduces the survival of female blastocysts (Larson et al. 2001; Cameron, 2004). Under this mechanism, the presence of genes on the X-chromosome coding for a pentose-phosphate metabolic pathway, combined with elevated blood glucose levels, can cause a buildup of toxic byproducts in female blastocysts owing to the second X-chromosome in the trophoctoderm not being inactivated until blastocoel formation (Larson et al., 2001). Larson et al. (2001) found no evidence for this, though the relationship between circulating blood glucose levels, litter size and sex ratio documented by Ryan et al. (2012) for Richardson's ground squirrels render this a viable candidate mechanism worthy of further empirical study.

Beyond the mechanistic level considering the potential function of the glucose-based biases documented by Ryan et al. (2012, 2014), the Local Resource Competition hypothesis suggests that in order to reduce competition between mothers and female offspring, mothers are prone to having male-biased litters when they are young, or in poor body condition, and shift towards female-biased ones as they get older (Silk, 1984). This notion makes intuitive sense in Richardson's ground squirrels where males of stressed mothers would tend to disperse from the natal area, at least in some cases establishing themselves in a habitat patch promoting greater survivorship and reproductive success. The Local Resource Competition and the Trivers-Willard

hypothesis are not mutually exclusive, and can have highly complex interactions as pointed out by Wild and West (2007).

According to the International Union for Conservation of Nature's (IUCN) Red List of Threatened Species, Richardson's ground squirrels are listed as of Least Concern with a stable, non-fragmented population and a habitat range spanning most of the North American prairie (Cassola, 2016). Richardson's ground squirrels are also prolific breeders, weaning litters ranging from 1-14 pups every spring, with a median litter size of 6-8 pups (Michener, 1989). With such a wide range in litter size, they make an excellent model for studies exploring the influence of external factors on litter sex ratio and litter size (Gedir & Michener, 2014). The colonial nature of female Richardson's ground squirrels and other closely related squirrels makes them easily observable; hence, collecting life-history data is relatively simple and has led to a wealth of information pertaining to their sociality (Hare & Murie, 2007; Michener, 1983; Michener & Sheppard, 1972), communication (Davis, 1984; Hare, 1998; Sloan, Wilson, & Hare, 2005), life history and ecology (Hare, Todd, & Untereiner, 2004; Michener, 1977; Michener, 2000; Ryan et al., 2012; 2014).

Because previous research on Richardson's ground squirrel adaptive sex allocation has implicated elevated blood glucose as a factor promoting smaller, male-biased litters, the first component of this thesis (Chapter 2) focuses on validation of an Accu-chek<sup>®</sup> Aviva handheld blood glucose meter (Roche Diagnostics, Laval, Quebec) for use in the field with Richardson's ground squirrels. The veterinary literature is divided on the use of off-the-shelf portable glucose meters, with some seeing it as a convenient monitoring tool (Dingbo, Harris, Stutzman, Guido, Davidson, & Takemoto, 2007; Shiota, 2012; Woderer, Henninger, Garthe, Kloetzer, Hajnsek, Kamecke, Gretz, Kraenzlin, & Pill, 2007) while others suggest that more specialized equipment

is necessary to obtain reliable results (Acierno, Schnellbacher, & Tully, 2012; Summa, Eshar, Lee-Chow, Larrat, & Brown, 2014). In the latter case however, many of these devices are specialized for use in veterinary or medical labs and are often stationary benchtop analytical devices, such as the VITROS<sup>®</sup> 250 chemistry analyzer (Ortho Clinical Diagnostics, Raritan, NJ). By contrast, handheld glucometers are portable, commercially-available, affordable, reusable, and have been used productively in studies measuring blood glucose levels in lab rats (Clarke & Foster, 2012; Dingbo et al., 2007; Lieske, Ziccardi, Mazet, Newman, & Gardner, 2002; Woderer et al., 2007). These monitors require only a droplet of blood (0.6  $\mu$ l per test) to obtain a reading, eliminating the need to draw blood via a syringe, and minimizing discomfort and stress to the squirrels. To date, no validation studies have been done for glucometer use with Richardson's ground squirrels or any other squirrel species.

The second component of my thesis (Chapter 3) summarizes my attempt to perform a controlled field experiment directly manipulating maternal blood glucose in Richardson's ground squirrels to test whether that manipulation would affect litter size and sex ratios as per the predictions of the Trivers-Willard hypothesis. Tests of this hypothesis have traditionally been conducted on captive specimens in a lab setting (Cameron, Lemons, Bateman, & Bennet, 2008; Gray, Long, Green, Gardiner, Craigon, & Gardiner, 2013; Green, Spate, Parks, Kimura, Murphy, Williams, Kerley, Green, Keisler, & Roberts, 2008; Larson et al., 2001), or in free-living populations indirectly via food supplementation (Austad & Sunkist, 1986; Enright, Spicer, Kelly, Culleton, & Prendiville, 2001; Schwanz & Robert, 2014). My test was novel in the sense of attempting to directly manipulate circulating blood glucose concentrations of subject squirrels by administering controlled glucose doses to otherwise free-living ground squirrels via subcutaneously implanted osmotic minipumps. This manipulative field experiment was designed

to test whether blood glucose concentration during early gestation provides a mechanistic basis for adaptive sex allocation among Richardson's ground squirrels, or whether the changes in the concentration of glucose are a correlate of the mechanism promoting trade-offs between litter size and sex ratio documented by Ryan et al. (2012; 2014). Data presented in this chapter also explore any potential relationship between maternal cortisol and litter size and sex ratios, via analysis of fecal glucocorticoid metabolites, thus independently replicating the earlier studies of Ryan et al. (2012; 2014). This was the first attempt, to my knowledge, to test the Trivers-Willard hypothesis directly by manipulating blood glucose in a free-living mammal. While the results obtained did not prove informative in this regard, they do highlight various technical problems with this approach, thus paving the way for productive future research documenting the mechanism underlying adaptive sex allocation.

The final data chapter of my thesis (Chapter 4), and perhaps the most powerful given the problems that were encountered with the manipulative experiment, is a test for trade-offs between litter size and sex ratio using data amassed by Dr. Hare and his students at the Assiniboine Park Zoo site over the past 14 years. Gedir and Michener (2014) correctly point out that if adaptive sex allocation occurs during gestation, as suggested by Ryan et al. (2012; 2014), then we would expect there to be an inverse correlation between litter size and the extent of male bias within litters (based on the selective resorption/abortion of female blastocysts and/or embryos). While Gedir and Michener (2014) failed to detect such a trend in 24 years of data from Michener's Lethbridge, Alberta area Richardson's ground squirrel site, that site is surrounded by cultivated agricultural fields, and thus females residing there are unlikely to exhibit the extensive range of body condition of Richardson's ground squirrels at the zoo, where individuals have varying degrees of access to supplementary food. In effect then, Gedir and

Michener's (2014) failure to detect the predicted relationship between litter size and sex ratio may reflect the limited degree of body condition variability in Michener's study population. I thus performed a similar test for a correlation between litter size and sex ratio using 10 years of data from the Assiniboine Park Zoo site, where squirrels vary extensively in body condition, and relationships between litter size and sex ratio have been detected in two independent years of study (Ryan et al. 2012; 2014).

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## Chapter 2: Validation of the Accu-chek<sup>®</sup> Aviva glucometer for Richardson's Ground Squirrel, *Urocitellus richardsonii*

### Introduction

Glucose measurement is of great importance in physiological, ecological and behavioural research, in that glucose constitutes the body's primary fuel source. Traditional glucose analysis methods require time-consuming laboratory assays (Moodley, Ngxamngxa, Turzyniecka, & Pillay, 2015), and rely on field-collected blood samples that are prone to clotting or spoilage owing to adverse conditions (Olbrich, Muhrer, Cooper, & Martz, 1972). Transporting the organism to a lab or location where blood can be sampled and stored reliably, or assayed immediately, is complicated by stress-induced elevation of blood glucose (Haynes & Lu, 1969). The use of a hand-held glucometer in the field alleviates such concerns and may provide immediate and reliable blood glucose concentrations.

Hand-held glucometers are readily available and inexpensive, often being provided at no cost with purchase of test strips. Portable glucometers require only a small droplet of blood to measure blood glucose concentration, with some brands allowing blood to be added to the test strip where the initial sample proves insufficient (Bayer Inc., 2014). The current Accu-chek<sup>®</sup> Aviva handheld glucometer (Roche Diagnostics, Laval, Quebec) requires only 0.6 µl of blood per test and provides a result in approximately 5 seconds. This meter is described by Roche as having more than 150 system integrity checks to avoid unreliable results, and being "ready to use right out of the box" with no calibration required before meter use (<https://www.accu-chek.ca/en/meter-systems/aviva>, accessed 27 August 2017; <https://www.accu->

[chek.com/meters/aviva-meter](http://chek.com/meters/aviva-meter), accessed 25 July 2017). Though a control solution is available, it is not included with the device or with the disposable test strips, and thus results may be unreliable. In a study of five commercially available glucometers employing human blood samples, only three (the GlucoPlus™, OneTouch® Horizon™, and Accu-chek® Active) fell within the acceptable performance range set by the International Standardization Organization (ISO) of < 20% variation from lab-derived values (Essack, Hoffman, Rensburg, Van Wyk, Meyer, & Erasmus, 2009).

Studies involving humans (Mahmoodpoor, Hamishehkar, Shadvar, Sanaie, Iranpour, & Fattahi, 2016), cats and dogs (Dobromylskyj & Sparkes, 2010; Min-Hee, Do-Hyung, In-Seong, Gab-Chol, & Hee-Myung, 2016), ferrets (Summa, Eshar, Lee-Chow, Larrat, & Brown, 2014), grey seals (Bennet, Turner, Millward, Moss, & Hall, 2017), and parrots (Acierno, Schnellbacher, & Tully, 2012) have reported issues with both the accuracy and precision of portable glucometer readings. While falling within the ISO's accepted error range, the glucometers tested rarely produced results representative of the glucose values obtained from lab analysis. Given that these glucometers are marketed primarily as a medical point-of-care device, there should be little room for error in readings. They are also routinely used and are considered reliable in diabetes research employing rodents, including both laboratory mouse and rat lines (Dingbo, Harris, Stutzman, Guido, Davidson, & Takemoto, 2007; Shiota, 2012; Woderer, Henninger, Garthe, Kloetzer, Hajnsek, Kamecke, Gretz, Kraenzlin, & Pill, 2007).

While the Accu-chek® Aviva meter has been validated for use with humans, it has not been validated for squirrels (Mahmoodpoor, Hamishehkar, Shadvar, Sanaie, Iranpour, & Fattahi, 2016; Milton, Herman, Aiello, Danielson, Mendoza-Avelarez, & Piette, 2010). No validation of a portable glucometer has been conducted to date for Richardson's ground squirrels, *Urocitellus*

*richardsonii*. These squirrels are semi-fossorial, obligate hibernators that occupy shortgrass prairie and human-modified areas across western Canada and the northern United States of America (Michener & Koepl, 1985). These and other ground squirrel species are subject to intensive research exploring sociality (Hare & Murie, 2007; Michener, 1983; Michener & Sheppard, 1972), communication (Davis, 1984; Hare, 1998a; Sloan, Wilson, & Hare, 2005), life history, and ecology (Michener, 1977; Michener, 2000; Ryan, Anderson, Berkvens, & Hare, 2014). They are listed as being of least concern on the International Union for Conservation of Nature's (IUCN) Red List, however they are an important prey species (Cassola, 2016; Michener, 2000; Schmutz & Hungle, 1989), and play a pivotal role in structuring pastureland plant communities (Newediuk, Waters, & Hare, 2015). Here, we assess the accuracy and precision of the Accu-chek<sup>®</sup> Aviva glucometer for use in field research with Richardson's ground squirrels.

## **Methods**

Fifteen sexually mature ( $\geq 1$  year-old) female Richardson's ground squirrels were sampled from the population located at the Assiniboine Park Zoo in Winnipeg, Manitoba (49°52'11.0"N 97°14'30.0"W). This is a well-established population that has been studied extensively and is located in close proximity to an on-site veterinary hospital, minimizing stress on the squirrels due to transport (Clary, Skyner, Ryan, Gardiner, Anderson, & Hare, 2014; Ryan, Anderson, Berkvens, & Hare, 2014; Ryan, Anderson, Gardiner, & Hare, 2012). Squirrels were captured using Tomahawk live traps (Tomahawk Live Trap Co., Tomahawk, WI) baited with peanut butter (No Name<sup>™</sup> Brand, Loblaw Companies Ltd., Toronto, ON) and transported on foot within 5 min of capture to the veterinary hospital in a pillowcase-covered live-trap, thereby reducing

stress on the squirrel (Hare, 1998b; Ryan, Anderson, Gardiner, & Hare, 2012). Inside a procedure room at the veterinary hospital, the captured squirrel was coerced into a cloth handling bag, weighed to the nearest 5 g with a Pesola<sup>®</sup> spring balance (Pesola AG, Schindellegi, Switzerland), and then extracted from the handling bag with a gloved hand and manually restrained on its back on a towel-covered surgical table. Up to 1 ml of blood was drawn from each unanaesthetized squirrel from the medial saphenous vein of the left or right hindleg by veterinary staff using a 29-gauge insulin syringe. Upon removal of the needle, pressure was applied to the vein for a period of roughly 1 min to ensure coagulation and to prevent hematoma formation.

Droplets of blood from that sample were applied in immediate succession via the needle tip to 6 Accu-chek<sup>®</sup> Aviva test strips, to obtain 6 glucometer-based blood glucose concentration measurements from each sample. The remaining blood sample was then ejected via the syringe tip (with the needle removed) into additive-free Monoject<sup>™</sup> Covidien red stopper blood collection tubes (non-silicone coated tube) (Covidien Inc, 15 Hampshire Street, Mansfield, MA USA). After sitting at room temperature for approximately 20 minutes (until the clot was well formed), samples were centrifuged at 1642 g for 15 min to separate serum from cells. Serum samples were kept refrigerated overnight at 4 °C before being sent to the Provincial Veterinary Laboratory for colorimetric glucose concentration quantification in duplicate using a VITROS<sup>®</sup> 250 Chemistry Analyzer (Ortho Clinical Diagnostics, Raritan, NJ). Serum glucose concentrations from the VITROS 250 chemistry analyzer have been reported to be well-correlated with those from other biomedical-grade instruments in previous studies (Geffré, Braun, Germain, Palanché, Kueper, & Trumel, 2008; Trumel, Diquélou, Germain, Palanché, & Braun, 2005), and thus were adopted as the standard for comparison in our study. Subsequent to

sampling, squirrels were transported back to the field site in a pillowcase-covered live-trap, and released at their point of capture.

Major axis regression explored the relationship between the mean glucose concentrations of each of the 15 blood samples obtained using the VITROS<sup>®</sup> and Accu-chek<sup>®</sup> systems, while the correlation between those paired concentration measurements was calculated using a Spearman's rho test. Further, mean glucose concentrations from the two systems for each of the 15 samples obtained were compared using the Wilcoxon paired-sample test, in that the distribution of differences obtained from those samples was unlikely to have been drawn from a normally-distributed distribution of differences. Coefficients of variation for glucose concentration measurements were calculated for the first two samples measured with the VITROS<sup>®</sup> and the Accu-chek<sup>®</sup> systems, and for each of the subsequent concentration measurements made with the Accu-chek<sup>®</sup> glucometer. This assessed not only how variability was affected by the method employed to measure glucose concentration, but also how it changed with repeated sampling using the Accu-chek<sup>®</sup> glucometer. Further, we calculated Spearman's rank-order correlation coefficients (rho) between concentrations measured from the 15 samples with the mean VITROS<sup>®</sup>- versus Accu-chek<sup>®</sup>-derived glucose concentration values independently for the first through the sixth test strip readings, to assess how repeated glucometer readings affect accuracy.

The Wilcoxon paired-sample test was performed using Statview<sup>®</sup> 5.0.1, while all other inferential statistical tests were performed in R (R version 3.4.0, R Core Team 2013) using functions from the lmodel2 (Legendre, 2014) package and then plotted using functions from the ggplot2 (Wickham, 2009) package. Differences were considered statistically significant where  $P \leq 0.05$ .

## Results

Major axis regression revealed a positive relationship between the mean Accu-chek<sup>®</sup> and VITROS<sup>®</sup> blood glucose readings ( $R^2 = 0.341$ ; Fig. 1). The Spearman's rho test confirmed that blood glucose concentrations measured with the Accu-chek<sup>®</sup> Aviva portable glucometer were well correlated with those obtained from the VITROS<sup>®</sup> chemistry analyzer (Table 1). Further, a Wilcoxon paired-sample test contrasting the mean Accu-chek<sup>®</sup>-obtained blood glucose concentration with those from the VITROS<sup>®</sup> system for the 15 samples detected no significant difference between those readings ( $W_{14} = 88$ ,  $Z = -1.590$ ,  $P = 0.112$ ). The mean  $\pm$  SE difference between the Accu-chek<sup>®</sup> and VITROS<sup>®</sup> concentrations within samples was  $0.852 \pm 0.100$  mmol/L, though concentrations measured using the Accu-chek<sup>®</sup> glucometer were more variable than those obtained from the VITROS<sup>®</sup> system, with standard errors of 0.159 and 0.07 mmol/L respectively. The coefficient of variation for the Accu-chek<sup>®</sup> was greater than that for the VITROS<sup>®</sup> system based upon two measurements from each underlying sample, and increased more or less linearly with successive measurements through the sixth measurement made using the Accu-chek<sup>®</sup> on each blood sample (Table 1). Based on the mean  $\pm$  SE difference between the Accu-chek<sup>®</sup> and VITROS<sup>®</sup> concentrations within samples, accuracy did not stabilize until after 5 samples and peaked at 6 samples, with a mean difference of  $0.852 \pm 0.100$  mmol/L (Table 2). Concentrations measured at this point using the Accu-chek<sup>®</sup> glucometer were more variable than those obtained from the VITROS<sup>®</sup> system, with standard errors of 0.159 and 0.07 mmol/L respectively.

## Discussion

Our findings suggest that the Accu-chek<sup>®</sup> Aviva glucometer can be used to obtain reliable blood glucose concentration measurements from Richardson's ground squirrels. However, due to the sizable but non-significant difference between the Accu-chek<sup>®</sup> glucometer and VITROS<sup>®</sup> glucose concentration readings, we recommend that Accu-chek<sup>®</sup>-derived concentrations be adjusted relative to those obtained from known concentration standards, or by adjusting measured concentrations according to their relationship to results obtained from a calibrated instrument such as the VITROS<sup>®</sup> system employed in our study. Here, more accurate blood glucose concentrations for each subject squirrel can be obtained by inserting the Accu-chek<sup>®</sup> Aviva-derived measurement into the regression equation defining the relationship between the Accu-chek<sup>®</sup>-derived and VITROS<sup>®</sup>-derived concentration measurements.

Further compounding problems encountered with the Accu-chek<sup>®</sup>, the variability of the measurements derived from it (as measured by the Standard Error of the Mean) were more than double that for repeated concentration measurements derived from the VITROS<sup>®</sup> system. In that coefficients of variation for measurements derived from the Accu-chek<sup>®</sup> Aviva increased in an essentially linear fashion up to and including 6 samples, without any notable asymptote being reached, it is apparent that repeated sampling up to and including six samples does not resolve the problem of extensive variability in the glucose concentration measured with the portable glucometer. Repeated sampling with the Accu-chek<sup>®</sup> Aviva does, however, improve accuracy, as is evident from the increasing correlation between Accu-chek<sup>®</sup> Aviva and VITROS<sup>®</sup>-based measurements with an increasing number of test-strip measurements per sample. For blood samples obtained from the medial saphenous vein of Richardson's ground squirrels, our study reveals that while variability does not diminish with repeated measurement up to and including 6

test strips, accuracy does not appear to stabilize and peak until results from 5 test strips have been obtained. We thus recommend that future research involving Richardson's ground squirrels employ at least 5 repeated Accu-chek<sup>®</sup> Aviva test strips for each sample to ensure that the concentrations obtained are accurate. A validation study such as that reported here would prove prudent, however, to determine the optimal number of test strips required to obtain a representative mean blood glucose concentration for individuals of a given species at a particular point in time.

In general terms, our findings parallel those of Mahmoodpoor et al. (2016) in their validation of the Accu-chek<sup>®</sup> Aviva with diabetic humans, though their glucometer measurements were consistently higher than the laboratory-measured values (Mahmoodpoor, Hamishehkar, Shadvar, Sanaie, Iranpour, & Fattahi, 2016), while ours fell both above and below laboratory-determined concentrations. This difference may be attributable to the differences in hematocrit (the volume of red blood cells in a blood sample) values of humans and ground squirrels (Hedin, 1891), although Roche reports that the Accu-check<sup>®</sup> Aviva is designed for a hematocrit range from 10% to 65% (<https://www.accu-chek.ca/en/meter-systems/aviva>, accessed August 27, 2017). Thus, the measurements obtained with this glucometer should be robust to variation in hematocrit concentration since ground squirrel hematocrit concentrations fall within this range (Halikas & Bowers, 1973; Maginniss & Milsom, 1994; Yousef & Bradley, 1971). While such variability raises concerns about the comparability of concentration measurements among units, batches of test strips, and the potential applicability of this handheld glucometer model across species, it does appear that with the benefit of validation results, these portable glucometers can prove a valuable research tool.

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## Figures

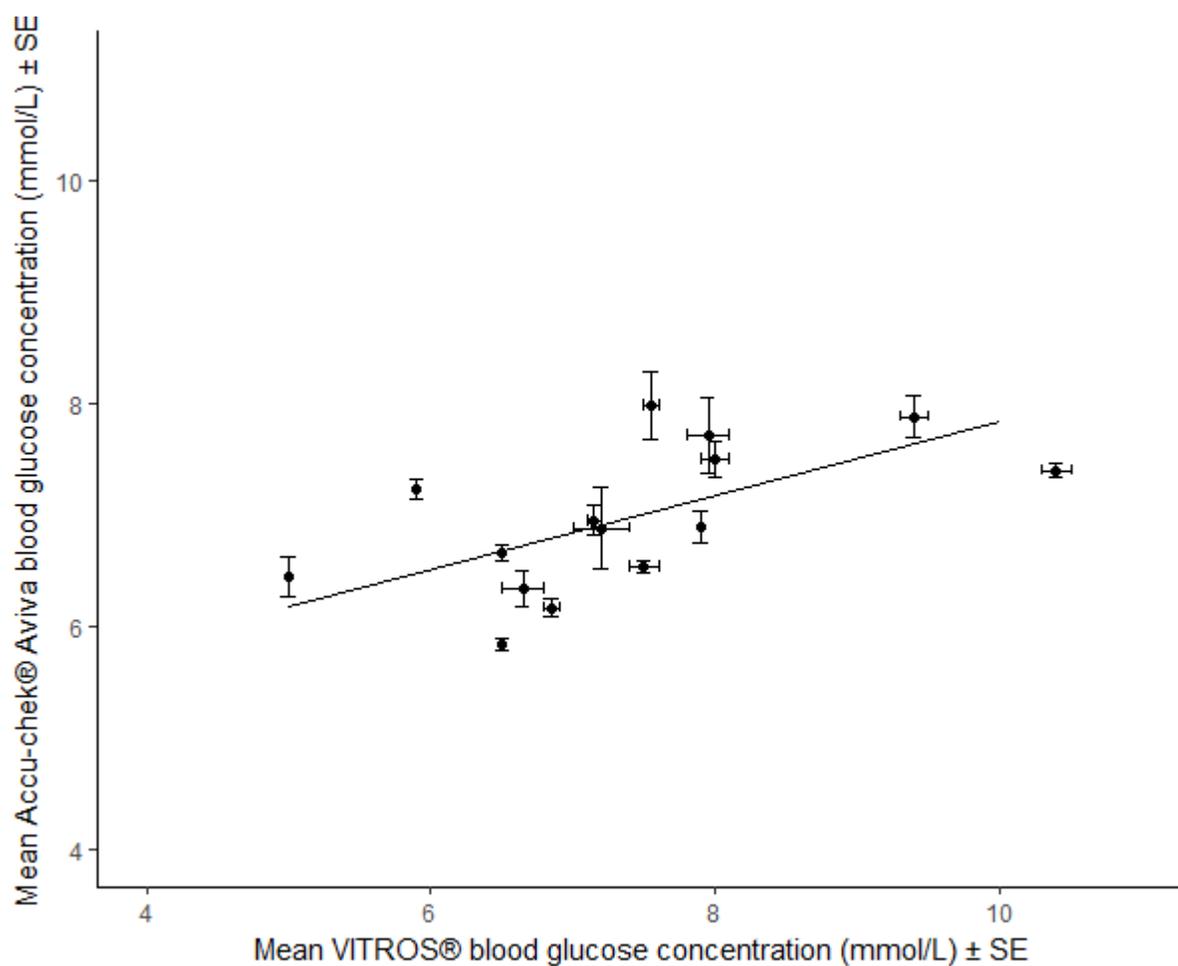


Figure 1: Mean blood glucose concentrations measured with the Accu-chek® Aviva versus the VITROS® systems. Major axis regression resulted in a best-fit linear relationship of  $y = 0.334x + 4.506$ ,  $R^2 = 0.341$ .

## Tables

		Number of Tests					
		1	2	3	4	5	6
<b>Spearman's Rho</b>	$\sigma$	0.631	0.680	0.618	0.606	0.686	0.681
	P	0.012	0.005	0.014	0.017	0.005	0.005
<b>Accu-chek® Aviva</b>	CV		0.030611	0.036034	0.037345	0.044397	0.054763
<b>VITROS®</b>	CV		0.012841				

Table 1: Spearman's Rho values revealing the correlation between Accu-chek® Aviva versus VITROS®-derived blood glucose concentrations for increasing numbers of tests run on each sample alongside the corresponding mean coefficients of variation for concentrations derived from the two instruments.

Mean Difference Between Accu-chek® and VITROS® Readings		Number of Tests					
		1	2	3	4	5	6
<b>Difference in concentration</b>	mmol/L	0.903333	0.854444	0.885556	0.863333	0.855333	0.852222
<b>Difference in SE</b>	mmol/L		0.116667	0.10158	0.095625	0.095544	0.099812

Table 2: Mean difference between Accu-chek® and VITROS® concentration readings and standard errors within samples for increasing numbers of tests run on each blood sample.

### **Chapter 3: Toward an experimental test of the glucose metabolism hypothesis as a mechanism underlying Richardson's ground squirrel, *Urocitellus richardsonii*, adaptive sex allocation**

#### **Introduction**

Adaptive sex allocation, as proposed by Trivers and Willard over 40 years ago (1973), has been investigated repeatedly (e.g. Austad & Sunquist 1986; Ryan, Anderson, Gardiner, & Hare, 2012; Ryan, Anderson, Berkvens, & Hare, 2014; Fishman, Vortman, Shanas, & Koren, 2018). While there is substantial evidence supporting its basic tenets, very few controlled experiments have provided conclusive results demonstrating adaptive sex allocation (Cameron, 2004). Further, it remains unclear how generally adaptive sex allocation applies across mammals (Sheldon & West, 2004; Gedir & Michener 2014). Ryan et al. (2012; 2014) detected relationships between serum glucocorticoid and glucose levels during early gestation and both litter size and sex ratio in free-living Richardson's ground squirrels at Winnipeg Manitoba's Assiniboine Park Zoo. These findings are consistent with Cameron's glucose metabolism hypothesis (Cameron, 2004), which purports to provide a mechanistic basis for mammalian adaptive sex allocation.

The glucose metabolism hypothesis suggests that mothers with high blood glucose will be more likely to produce smaller, male-biased litters owing to the presence of genes on the X-chromosome that influence the pentose-phosphate metabolic pathway (Cameron, 2004; Larson, Kimura, Kubisch, & Roberts, 2001). Since the second X-chromosome is not reduced to a Barr body in the trophectoderm until later stages of blastocyst development, it is postulated that

by having both copies of the pathway active, overexpression of the mRNA encoding glucose 6-phosphate dehydrogenase and hypoxanthine phosphoribosyl transferase may lead to a buildup of oxygen free-radicals and lead to the decreased viability of female blastocysts (Gutiérrez-Adán, Oter, Martínez-Madrid, Pintado, & De La Fuente, 2000; Larson, Kimura, Kubisch, & Roberts, 2001). I attempted a field-based experimental test of whether elevated circulating blood glucose in females lead to male-biased litter sex ratios via litter size reduction by administering controlled doses of glucose, in the range observed by Ryan et al. (Ryan, Anderson, Berkvens, & Hare, 2014), via implanted osmotic minipumps during early gestation.

## **Methods**

During the summer of 2016, I executed manipulations designed to increase circulating blood glucose concentrations, and collected both blood and fecal samples from otherwise free-living Richardson's ground squirrel females over the course of gestation and lactation at Winnipeg Manitoba's Assiniboine Park Zoo (49°52'11.0"N 97°14'30.0"W). I received approval from the Fort Garry Campus Protocol Management and Review Committee (F16-002) to capture 40 breeding female squirrels for use in my study. Owing to limited veterinarian availability to perform minipump implantation surgeries, only 20 breeding female squirrels were captured and subsequently followed through their annual reproductive cycle. The 20 breeding female squirrels captured served as subjects in my experiment, and were delivered controlled doses of glucose via subcutaneously implanted Alzet 1002 osmotic minipumps (Durect Corporation, Cupertino, CA) or were assigned to control groups. Of the 20 breeding females, 5 received a high glucose dose (7.14 mg/kg/day), 5 received a moderate dose (3.57 mg/kg/day), 5 received normal saline solution (0.9%), and 5 served as unmanipulated controls. Due to the small sample size, data from

the 4 treatment groups were analyzed both across their four cardinal treatment groups and also collapsed into glucose-supplemented (high and moderate glucose dose) versus control groups (saline and unimplanted) for analysis in order to maximize the likelihood of detecting any effect of supplementary glucose on litter size and/or sex ratio. The glucose dosages delivered were tailored to the dam's body mass within 2 d of conception, with the moderate dose being based on the difference between the mean and the upper 75<sup>th</sup> percentile of the range in blood glucose concentration measured from free-living Richardson's ground squirrels by Ryan, Anderson, Berkvens, & Hare (2014), and the high dose being double that. Fresh fecal samples were collected from each squirrel from underneath traps, body mass was recorded, and blood glucose was quantified at 4-day intervals over the gestation and lactation periods. Fecal samples were frozen at -20°C until I started processing them for measurement of fecal glucocorticoid metabolite concentration via radioimmunoassay (RIA) in Dr. W. Gary Anderson's lab at the University of Manitoba. For blood sampling, squirrels were live-trapped and conveyed to the zoo's on-site veterinary hospital in their pillowcase-covered live trap. Once in the procedure room, squirrels were removed from their live trap, weighed to the nearest 5 g with a Pesola<sup>®</sup> spring balance (Pesola AG, Schindellegi, Switzerland) and manually restrained while 0.5-1 ml of blood was drawn from the medial saphenous vein using an insulin syringe with a 29 gauge needle. Blood glucose concentration was quantified using an Accu-chek<sup>®</sup> Aviva handheld glucometer and test strips (Roche Diagnostics, Laval, Quebec). As outlined in Chapter 2, a correction factor was applied to blood concentration readings obtained from the Accu-chek<sup>®</sup> Aviva glucometer to arrive at reliable blood glucose concentration measurements. A total of 90 juvenile squirrels were captured within 3 d of their initial emergence from the 14 study litters. These juveniles were weighed, sexed based upon anogenital distance (Vom Saal & Bronson,

1980), and the spinal length measured in cm from the base of the nuchal crest to the base of the tail by positioning a flexible measuring tape along each restrained squirrel's spinal cord and reading the length obtained. Within 9-24 days after juveniles were weaned (mean 18 days) and hence capable of independent survival (Freeman et al., in review), dams were live-trapped and transported to the Assiniboine Park Zoo's Veterinary Hospital in live traps covered with a pillowcase to reduce stress (Hare, 2004). They were then humanely euthanized in the veterinary hospital under isoflurane anaesthesia using Pentobarbital Sodium solution (Euthansol<sup>®</sup>, Virbac AH, Inc., Fort Worth TX). Each dam was then necropsied for placental scar counts and recovery of minipumps. Placental scars were counted for each uterine horn and the distances between those were measured.

Fecal samples obtained from individual subjects over the course of gestation were hand ground using a mortar and pestle after drying in an oven overnight. Before grinding, wet and dry pellet weight and number were recorded. Between 2000 mg to 2500 mg of ground fecal matter was then measured into a 2 ml microcentrifuge tube and 2000  $\mu$ l of 70% ethanol was added to extract cortisol. The tubes were then centrifuged for 30 minutes at 2500 x g at 4°C, after which the supernatant was pipetted off into new tubes. This supernatant was then dried down in 300  $\mu$ l aliquots as needed for use in the RIA. Remaining dried fecal matter from samples collected was combined and the cortisol extracted from it to generate a pooled sample.

Samples were run in triplicate with three pooled samples in each assay to allow quantification of inter-assay variation. Intra-assay variation was tested by running 15 pooled samples in a single assay and inter-assay variation was tested by running a pooled sample in triplicate within each assay. Extraction efficiency was tested by spiking 6 tubes of dried pooled fecal matter with 200  $\mu$ l of 0.78 ng/ml cortisol and running those samples against 6 tubes of

pooled fecal matter with an additional 200  $\mu$ l of ethanol added as well as three tubes containing 200  $\mu$ l of the 0.78 ng/ml cortisol used for spiking. A test for parallelism was performed using 5 assays each consisting of a series of 7 dilutions run in triplicate. Due to the limited sample of 3 unimplanted control squirrels, comparison of FGM concentration in surgically-manipulated squirrels versus unmanipulated squirrels was unwarranted.

GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA) was used along with Microsoft Excel (2010) to convert raw radioisotope count data to FGM concentrations via  $\log(x)$  transformation, as well as for assessment of interassay variation, intraassay variation, and parallelism. All other statistical tests were performed using R statistical software (R version 3.4.0, R Core Team 2013). Kruskal-Wallis tests were performed on maternal blood glucose and FGM between focal groups, to determine if there was a significant difference between treatments, using the `kruskal.test` function. Model II regressions comparing maternal blood glucose and FGM to litter size and sex ratio, to examine potential tradeoffs, were performed using the `lmodel2` package in R (Legendre, 2014).

Scar counts from the 14 dams were compared to litter counts at emergence from the natal burrow via a Wilcoxon signed-rank test using the `wilcox.exact` function from the `exactRankTests` package (Hothorn & Hornik, 2017) and the `qnorm` function for the associated Z value to see if the count at emergence is representative of the litter size during gestation. A Wilcoxon rank-sum test was also performed, using the same function, on the difference between scar counts and litter counts between glucose supplemented and unsupplemented control groups to see if supplementation resulted in a greater deviation from the expected 1:1 pup to scar ratio. Bar graphs of maternal blood glucose and FGM as well as plots of the Model II regressions were produced using the `ggplot2` package (Wickham, 2009).

## Results

Given natural mortality of dams and their young, only 14 of the 20 subject squirrels ultimately weaned young; 4 in the high glucose treatment group, 3 in the moderate glucose group, 4 in the normal saline treatment, and 3 non-implanted controls. Kruskal-Wallis tests run on the data detected no significant difference in blood glucose concentrations among the 4 treatment groups in the first 12 days of gestation following implantation of the minipumps (Figure 1;  $H_{3,6} = 1.033$ ,  $P = 0.793$ ), or over the entire gestation period (Figure 1;  $H_{3,6} = 2.132$ ,  $P = 0.546$ ). Among the glucose-supplemented squirrels, no significant relationships were detected between blood glucose measured during the first 12 days of gestation and litter sex ratio at juvenile emergence (Figure 2;  $R^2 = 0.027$ ,  $P = 0.308$ ) or between blood glucose concentration measured over the full gestation period and litter sex ratio at juvenile emergence (Figure 2;  $R^2 = 2.148e^{-4}$ ,  $P = 0.396$ ). Further, among those glucose-supplemented squirrels, no significant relationships were detected between litter size at juvenile emergence and maternal blood glucose over the first 12 days of gestation (Figure 3;  $R^2 = 8.508e^{-4}$ ,  $P = 0.469$ ) or over the entire gestation period (Figure 3;  $R^2 = 0.099$ ,  $P = 0.444$ ). Among control squirrels, no significant relationships between litter sex ratio at juvenile emergence and maternal blood glucose were evident during the first 12 days of gestation (Figure 4;  $R^2 = 0.267$ ,  $P = 0.126$ ) or over the full gestation period (Figure 4;  $R^2 = 0.070$ ,  $P = 0.293$ ). Finally, control squirrels showed no relationship between litter size at juvenile emergence and maternal blood glucose during the first 12 days of gestation (Figure 5;  $R^2 = 0.367$ ,  $P = 0.078$ ) or over the entire gestation period (Figure 5;  $R^2 = 0.128$ ,  $P = 0.209$ ).

No statistically significant difference was detected ( $W_{14} = 60.5$ ,  $Z = -1.254$ ,  $P = 0.105$ ) between the number of juveniles at emergence ( $6.429 \pm 0.581$ ) and scar counts ( $7.643 \pm 0.589$ ),

though there were fewer emerged juveniles than there were scar counts among the dams constituting this sample. Additionally, no statistically significant difference was observed ( $W_{14} = 20.5$ ,  $Z = 0.391$ ,  $P = 0.652$ ) between the discrepancies in litter size and scar count of glucose-supplemented squirrels ( $1 \pm 0.956$ ) and discrepancies in litter size and scar count of control squirrels ( $1.429 \pm 0.922$ ).

Analysis of intra-assay and inter-assay variation provided coefficients of variation of 9% and 11% within and between assays respectively. Extraction efficiency was found to be 128%. The assays testing for parallelism showed that dilution of the sample does not lead to biased glucocorticoid metabolite measurements (Figure 6). Kruskal-Wallis tests comparing fecal glucocorticoid metabolite (FGM) measurements among the 4 treatment groups found no significant difference between mean maternal FGM in the first 12 days following implantation of the minipumps (Figure 7;  $H_{3,6} = 1.929$ ,  $P = 0.587$ ) or over the full gestation period (Figure 7;  $H_{3,6} = 1.852$ ,  $P = 0.604$ ). Comparison of the fecal glucocorticoid metabolite (FGM) measurements with the litter sex ratio data across all squirrels revealed no apparent trend between maternal FGM concentrations during the first half of gestation (Figure 8;  $R^2 = 0.035$ ,  $P = 0.167$ ) or over the full gestation period (Figure 8;  $R^2 = 0.061$ ,  $P = 0.157$ ). Further, no relationship was detected between the mean maternal FGM concentration over the first half of gestation and litter size for all squirrels (Figure 9;  $R^2 = 0.005$ ,  $P = 0.358$ ) or between maternal FGM concentration over the full gestation period and litter size (Figure 9;  $R^2 = 0.006$ ,  $P = 0.361$ ). FGM data were also examined within the glucose-supplemented and non-supplemented sample groups. No significant relationship was found between FGM in the glucose-supplemented squirrels during the first 12 days of gestation and litter sex ratio (Figure 10;  $R^2 = 0.140$ ,  $P = 0.168$ ) or between FGM over the full gestation period and litter sex ratio (Figure 10;  $R^2 = 0.206$ ,  $P = 0.127$ ). No significant

relationship was found between FGM in the glucose-supplemented squirrels and litter size in either the first 12 days of gestation (Figure 11;  $R^2 = 0.194$ ,  $P = 0.194$ ) or over the full gestation period (Figure 11;  $R^2 = 0.103$ ,  $P = 0.232$ ). Additionally, no significant relationship was found between FGM in the non-supplemented control squirrels and litter sex ratio in the first 12 days of gestation (Figure 12;  $R^2 = 0.028$ ,  $P = 0.246$ ) or over the full gestation period (Figure 12;  $R^2 = 0.018$ ,  $P = 0.285$ ). Finally, no significant relationship was found between FGM in the non-supplemented control squirrels and litter size in the first 12 days of gestation (Figure 13;  $R^2 = 0.002$ ,  $P = 0.427$ ) or over the full gestation period (Figure 13;  $R^2 = 0.003$ ,  $P = 0.454$ ). There is a consistent yet non-significant trend toward increasingly male-biased litters with increasing mean FGM concentration in both the first half of gestation and over the full gestation period for all focal squirrels (Figure 7). Additionally, there is a non-significant trend toward diminishing litter sizes with increasing FGM over both the first half of gestation and the full gestation period (Figure 8).

## Discussion

Visual inspection of the reproductive failure rate data suggests that neither surgical pump implantation, nor the nature of the dose delivered, contributed to litter failure in general. There are multiple possible reasons for the failure of my glucose supplementation to induce an increased circulating blood concentration in the glucose-supplemented squirrels. First, the pumps may have failed to release their full dosage. While the technical information for the Alzet 1002 osmotic minipump indicates that the pump can deliver a glucose solution (Durect Corporation; [http://www.alzet.com/research\\_applications/AGEN.html](http://www.alzet.com/research_applications/AGEN.html), accessed 17 June 2018), it is possible, albeit unlikely, that glucose at the high concentrations employed came out of solution after

filling and the full dosage was not delivered. Further tests would need to be done to determine if this is the case. A second possibility is that the squirrels have a greater capacity for mounting an insulin response to elevated blood glucose, and thus regulating their circulating blood glucose concentration, than anticipated based on the observed range of blood glucose concentrations among free-living squirrels (Ryan, Anderson, Berkvens, & Hare, 2014). This interpretation could explain the observed similarity between glucose measured in the unimplanted and saline-implant control groups relative to the glucose-supplemented squirrels, though verification that this is the case would require further experimentation and histological methods that extend beyond the scope of the present research.

The non-significant trends toward decreasing litter size and increasingly male-biased litters with increasing FGM concentrations during gestation are consistent with the predictions of the local resource competition hypothesis, which suggests mothers in poor condition should bias litters towards the dispersing sex (Clark, 1978). It also agrees with the findings of Ryan et al. (2012; 2014), who noted trends towards increasingly male-biased litters with increasing maternal cortisol concentrations and a negative relationship between maternal gestational cortisol concentration and litter size in two non-sequential years of data from Richardson's ground squirrels in the Assiniboine Park Zoo population (Ryan, Anderson, Gardiner, & Hare, 2012; Ryan, Anderson, Berkvens, & Hare, 2014). In both those data sets and my own, sampling bias (Gedir & Michener 2014) could influence the results obtained as these studies employed subsets of females from within the full population.

The Wilcoxon signed-rank test run on the litters allowed to progress through emergence from the natal burrow at weaning shows no significant difference between litter counts and placental scar counts, similar to other findings for Richardson's ground squirrels (Gedir & Michener,

2014; Sheppard, 1972). There was, however, a difference of 1.214 between the average number of pups and scars in these litters, with more scars than pups, supporting the notion that litter reduction may be occurring at some point. The Wilcoxon rank-sum test, run on deviations of litter counts from scar counts between the glucose supplemented and control groups, shows no difference between treatments suggesting that if the pumps did deliver their dosage, it had no effect on litter reductions.

Considerable variation was noted in both glucose and cortisol responses among individuals in the population. The lack of any statistically significant trends may in part be attributable to this variation coupled with the small sample size obtained. Indeed, in that measured concentrations of fecal glucocorticoid metabolite concentrations were higher among unmanipulated control squirrels than in any of my minipump-implanted treatment groups during gestation (Figure 7), it would seem that the assignment of subjects to groups may have confounded the detection of any glucose treatment effect. Between the lack of significant results and the potential problems encountered with glucose dosing, I shifted my focus to examining potential tradeoffs in litter size and sex ratio in the full 10-year population dataset (Chapter 4) rather than replicating my manipulative study, thereby making more effective use of time and resources. Should this manipulative experiment be replicated in the future, tests should be done to determine the maximum dosage of glucose deliverable by the minipumps. Further, pancreatic tissue should be collected from all euthanized subjects and subjected to histological analysis to resolve beta cell numbers and potential pathology associated with mounting a regulatory response to elevated blood glucose levels. Finally, it may prove more practical to test the glucose metabolism hypothesis through activation and suppression of the hypothalamic-pituitary-adrenal axis (via osmotic minipump delivery of physiologically-relevant doses of adrenocorticotrophic hormone

and dexamethasone respectively), thereby circumventing the problems that arose in attempting to manipulate circulating blood glucose concentrations directly.

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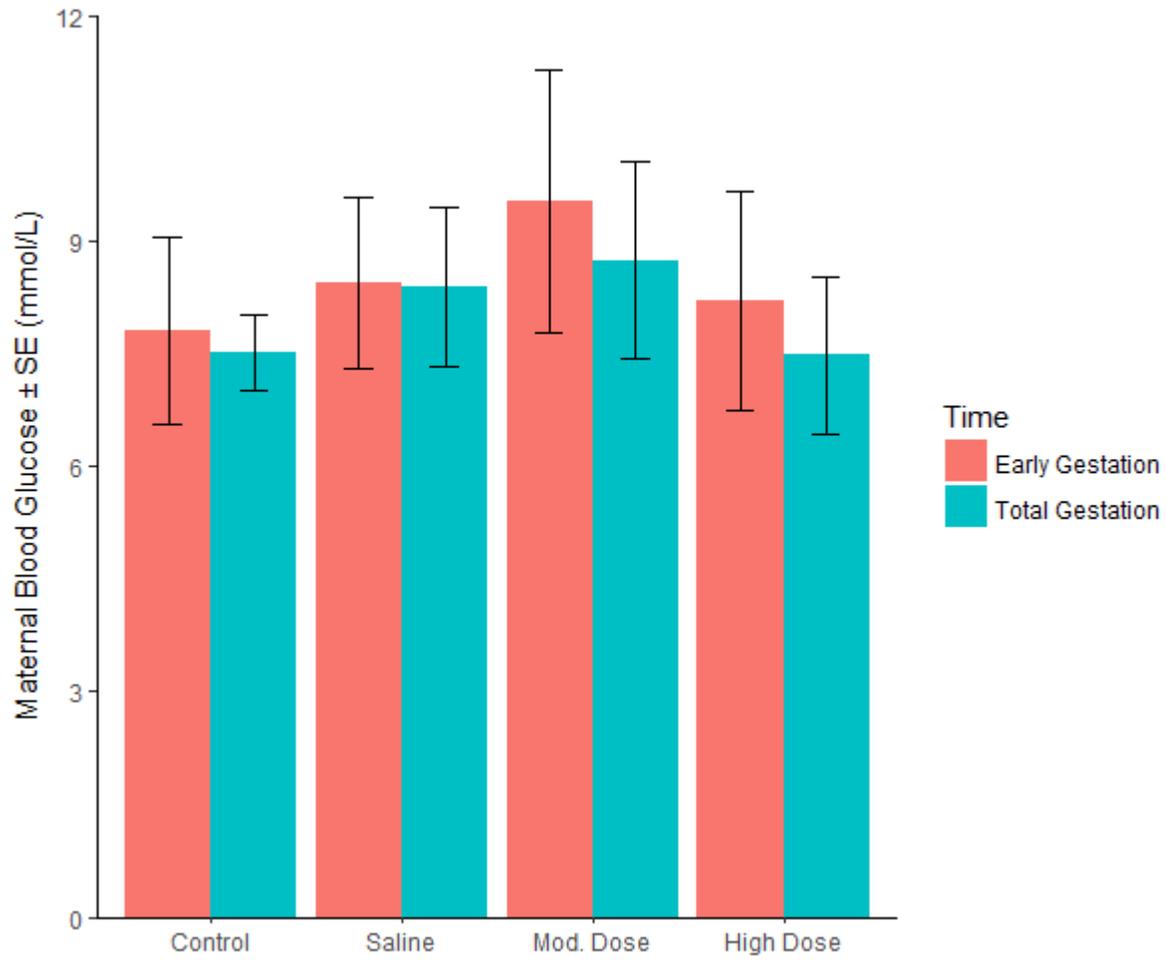
**Figures**

Figure 14: Comparison of maternal blood glucose concentrations measured during first 12 days of gestation and over the full 24 days of gestation for all squirrel treatment groups.

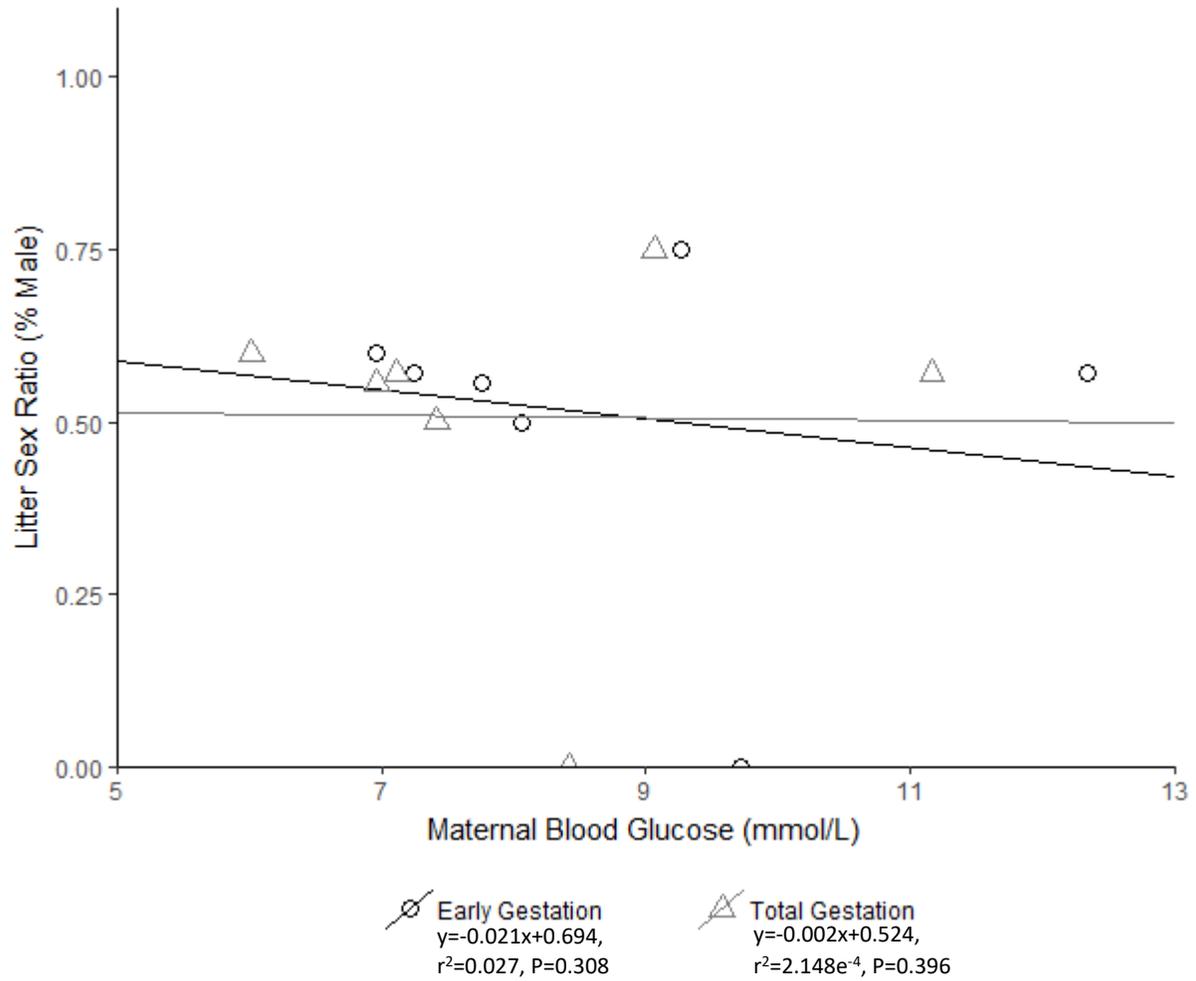


Figure 15: Model II major axis regression comparing maternal mean blood glucose concentration and litter sex ratio, expressed as percentage of the litter that was male, of glucose-supplemented squirrels ( $n = 7$ ) during the first half of gestation and over the full gestation period.

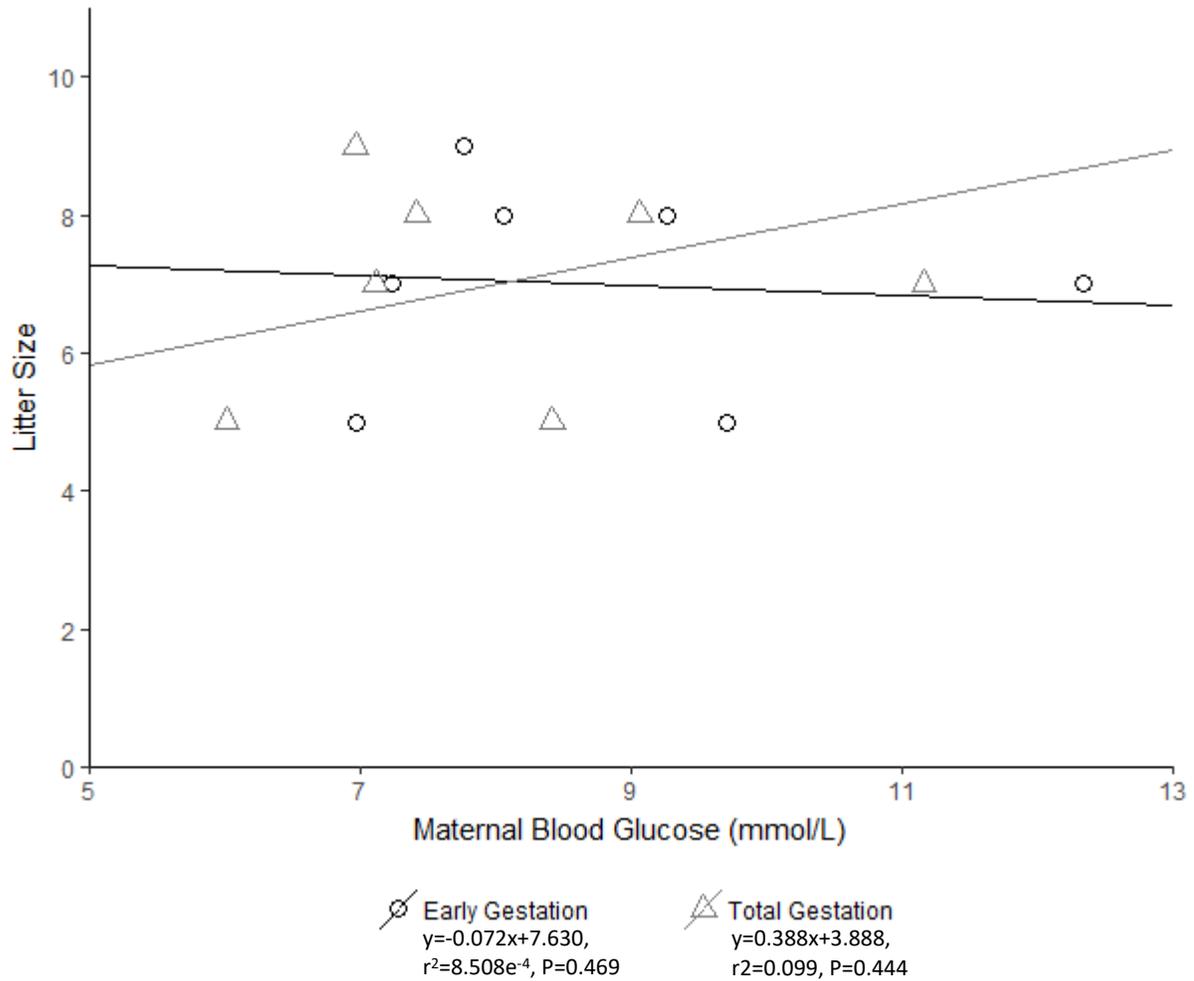


Figure 16: Model II major axis regression comparing maternal blood glucose concentration and litter size of glucose-supplemented squirrels ( $n = 7$ ) during the first half of gestation and over the full gestation period.

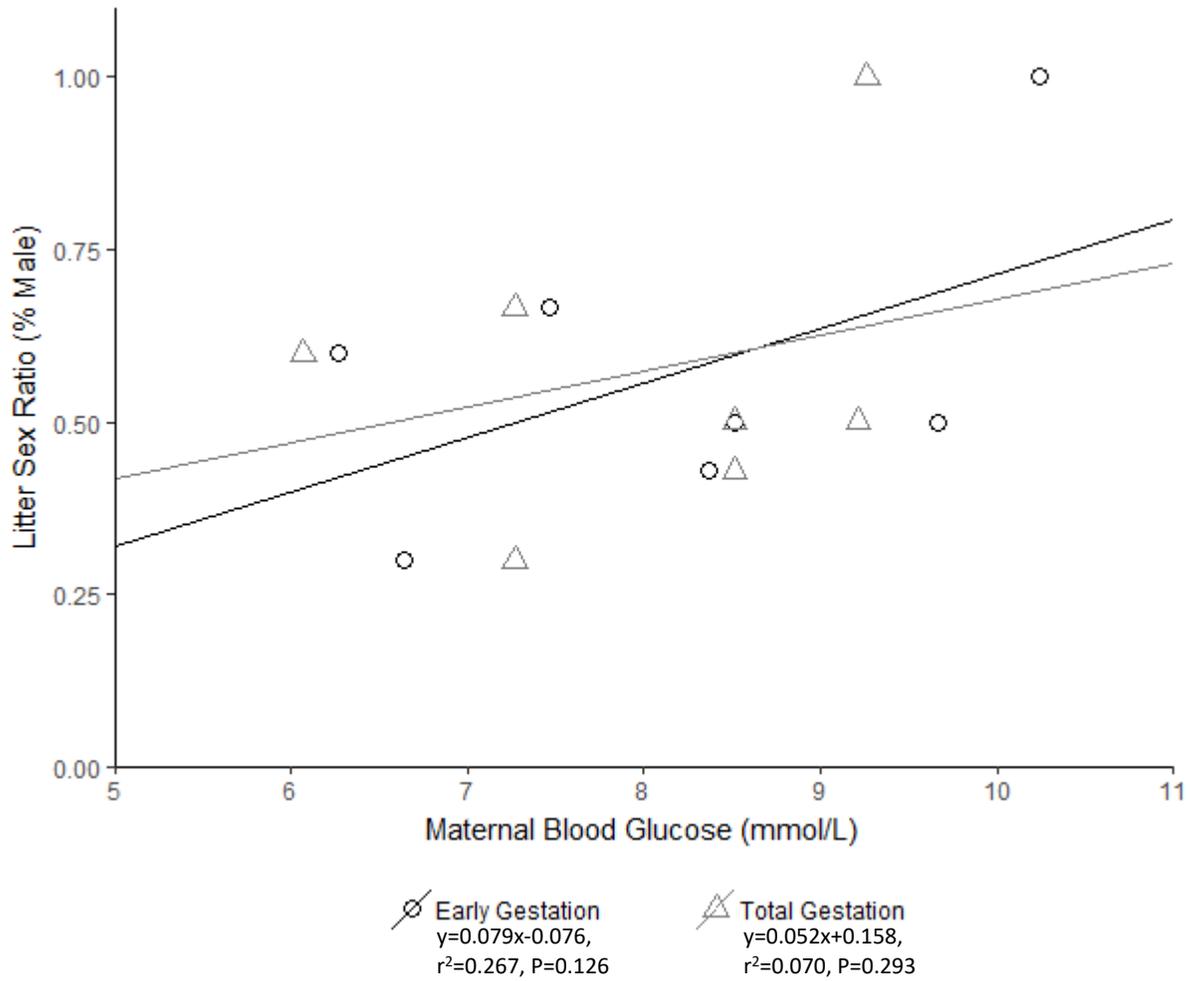


Figure 17: Model II major axis regression comparing maternal blood glucose concentration and litter sex ratio, expressed as percentage of the litter that was male, of control squirrels ( $n = 7$ ) during the first half of gestation and over the full gestation period.

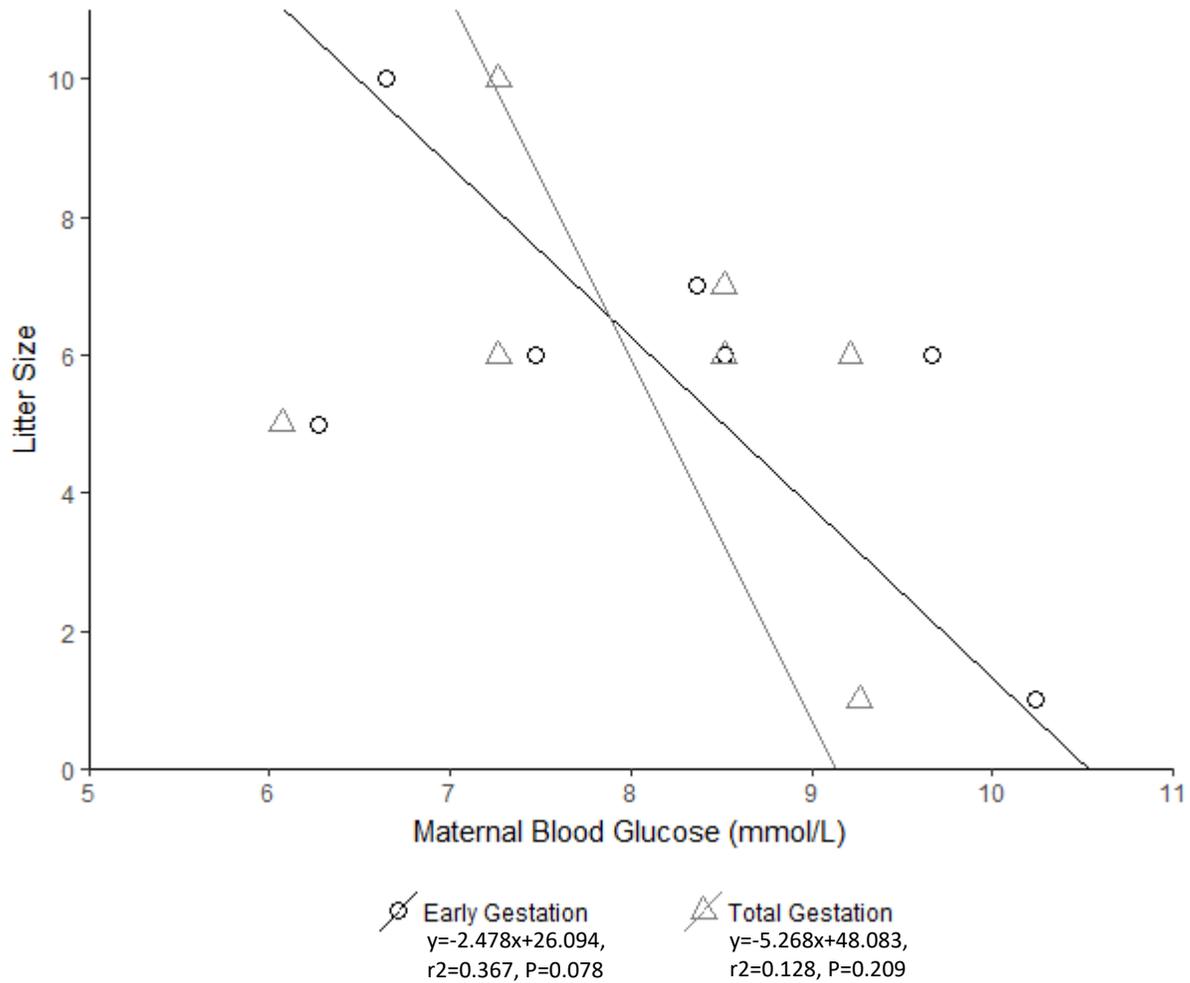


Figure 18: Model II major axis regression comparing maternal blood glucose concentration and litter size of control squirrels ( $n = 7$ ) during the first half of gestation and over the full gestation period.

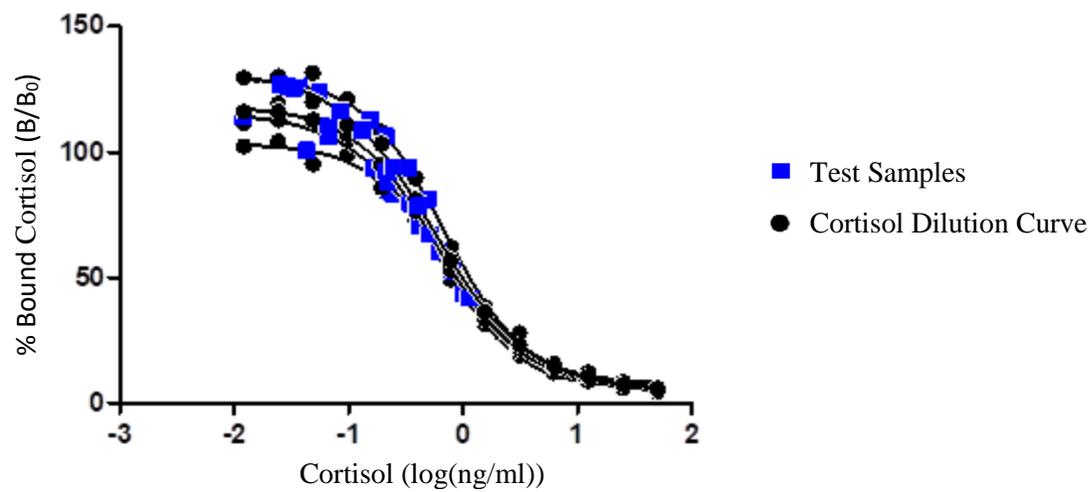


Figure 19: Combined representation of the 5 fecal cortisol metabolite assays run to test for parallelism.

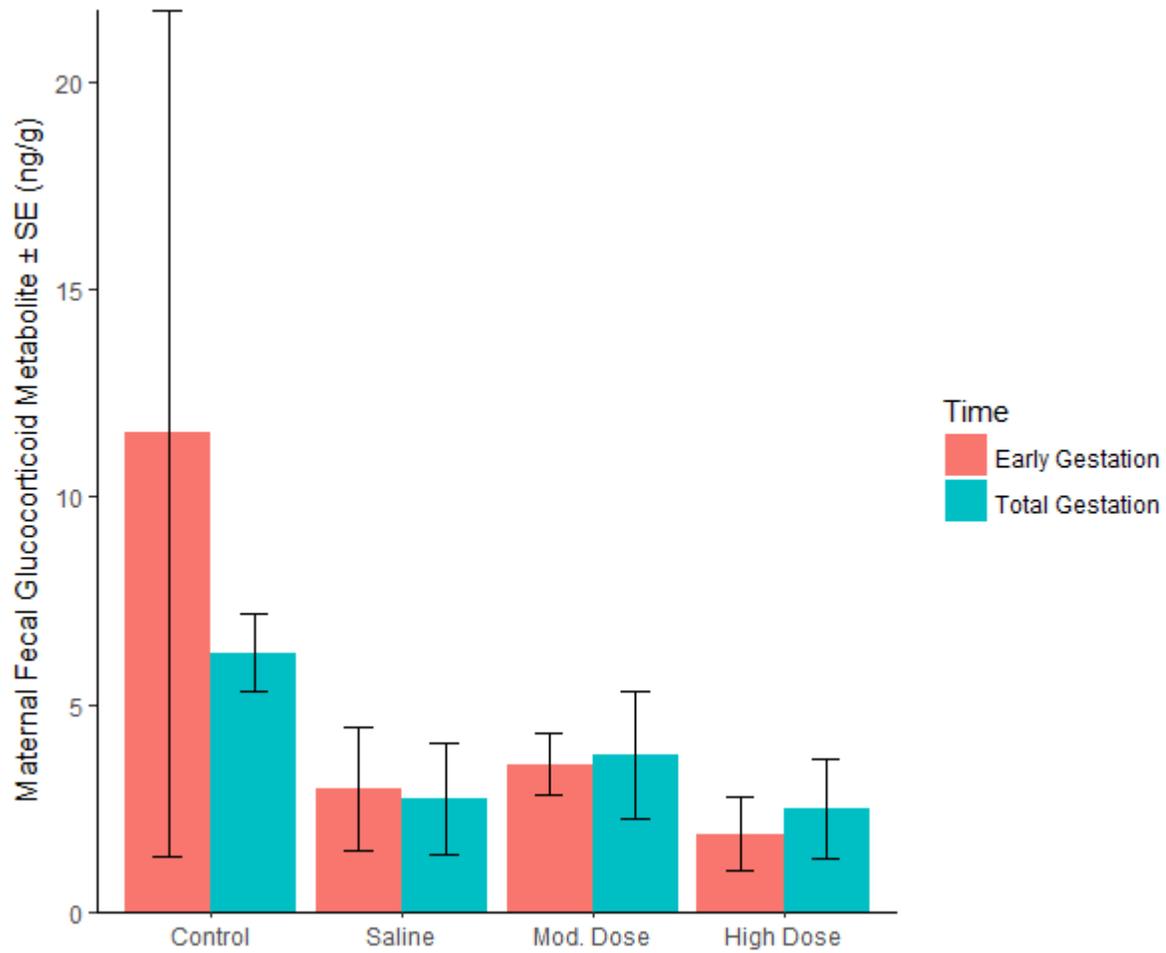


Figure 20: Comparison of mean maternal fecal glucocorticoid metabolite concentrations during the first 12 days of gestation and the full 24 days of gestation for all treatment groups.

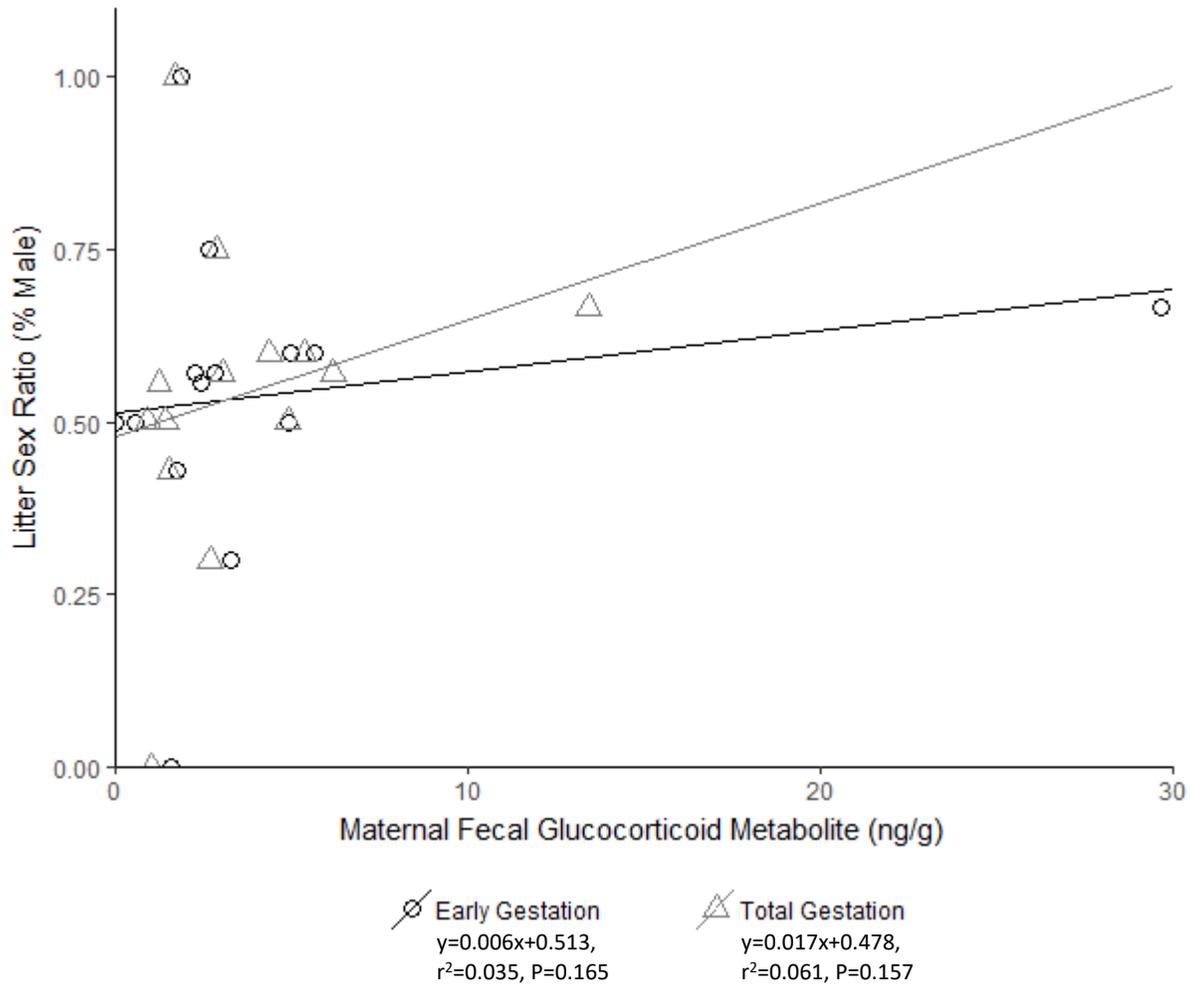


Figure 21: Model II major axis regression comparing maternal fecal glucocorticoid metabolite concentration and litter sex ratio, expressed as percentage of the litter that was male, of all focal squirrels ( $n = 14$ ) during the first half of gestation and over the full gestation period.

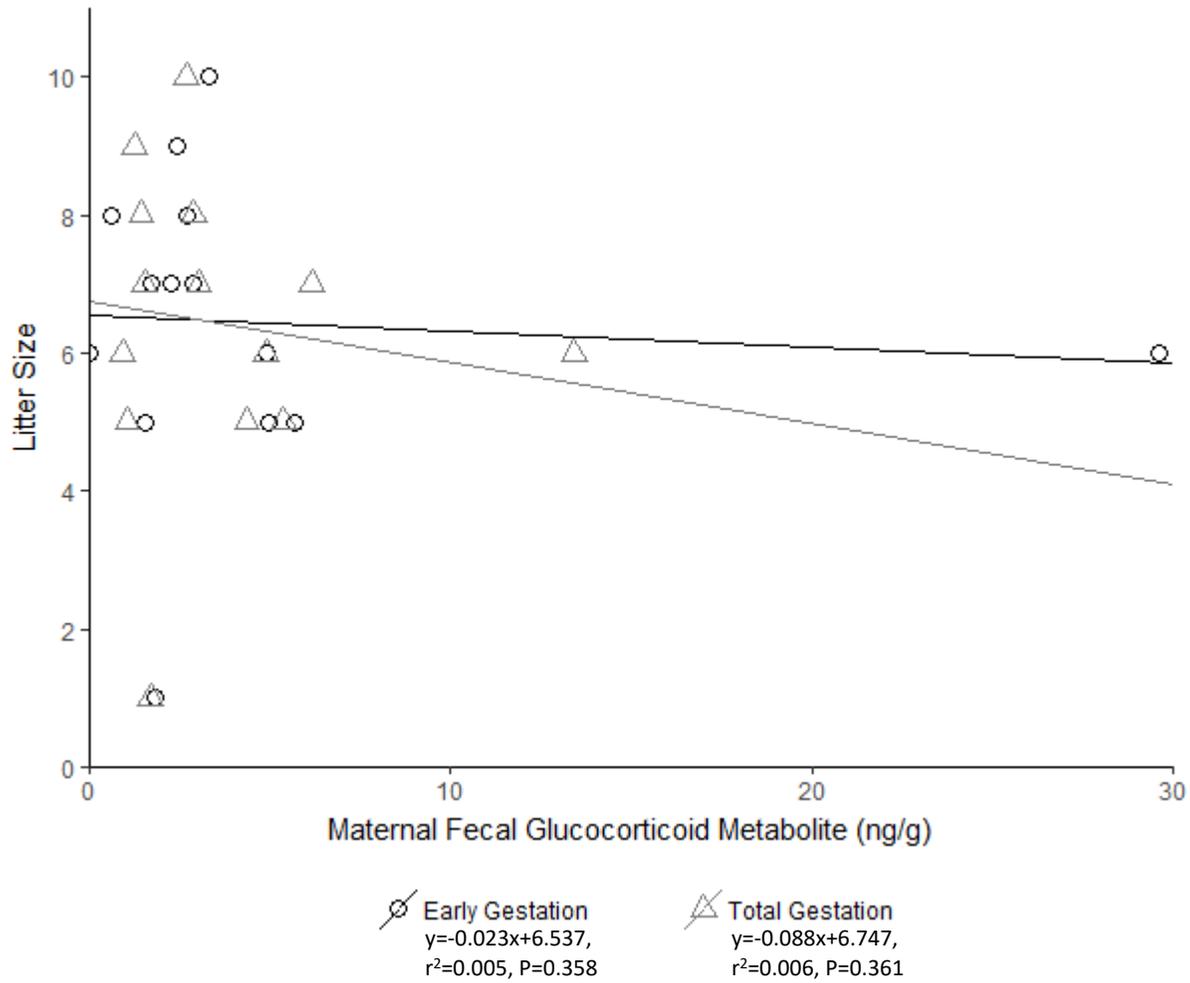


Figure 22: Model II major axis regression comparing maternal fecal glucocorticoid metabolite concentration and litter size of all focal squirrels ( $n = 14$ ) during the first half of gestation and over the full gestation period.

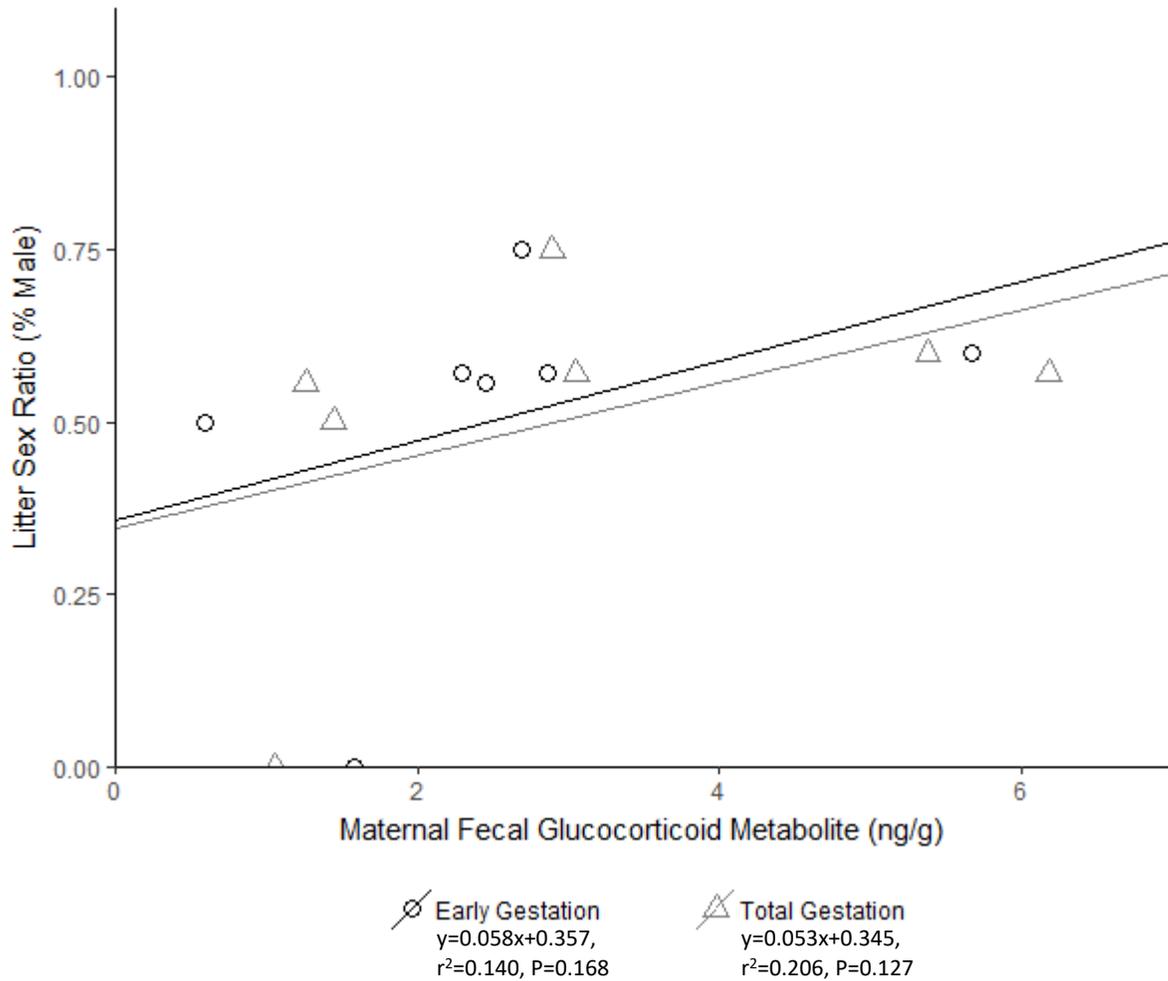


Figure 23: Model II major axis regression comparing maternal fecal glucocorticoid metabolite concentration and litter sex ratio, expressed as percentage of the litter that was male, of glucose-supplemented squirrels ( $n = 7$ ) during the first half of gestation and over the full gestation period.

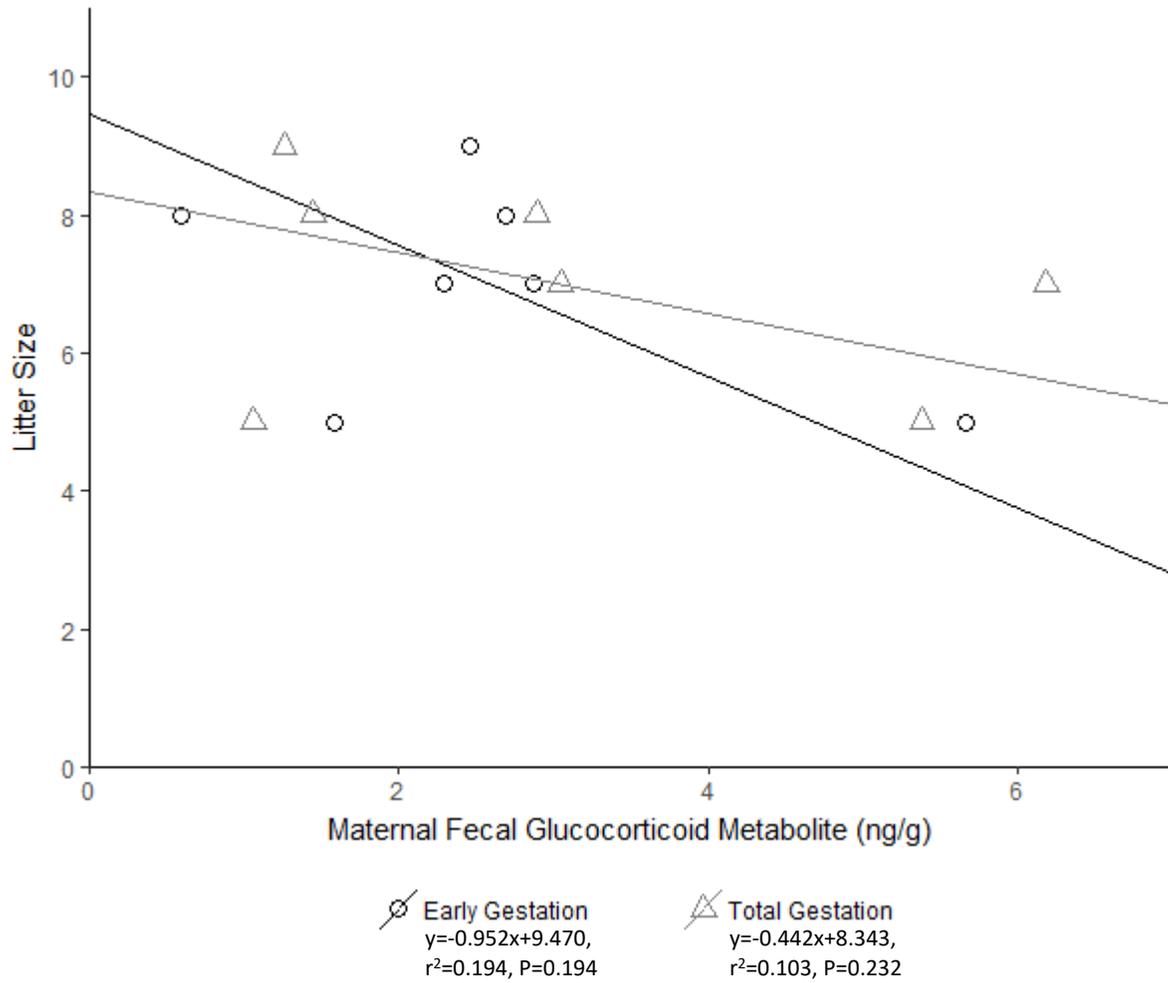


Figure 24: Model II major axis regression comparing maternal fecal glucocorticoid metabolite concentration and litter size of glucose-supplemented squirrels ( $n = 7$ ) during the first half of gestation and over the full gestation period.

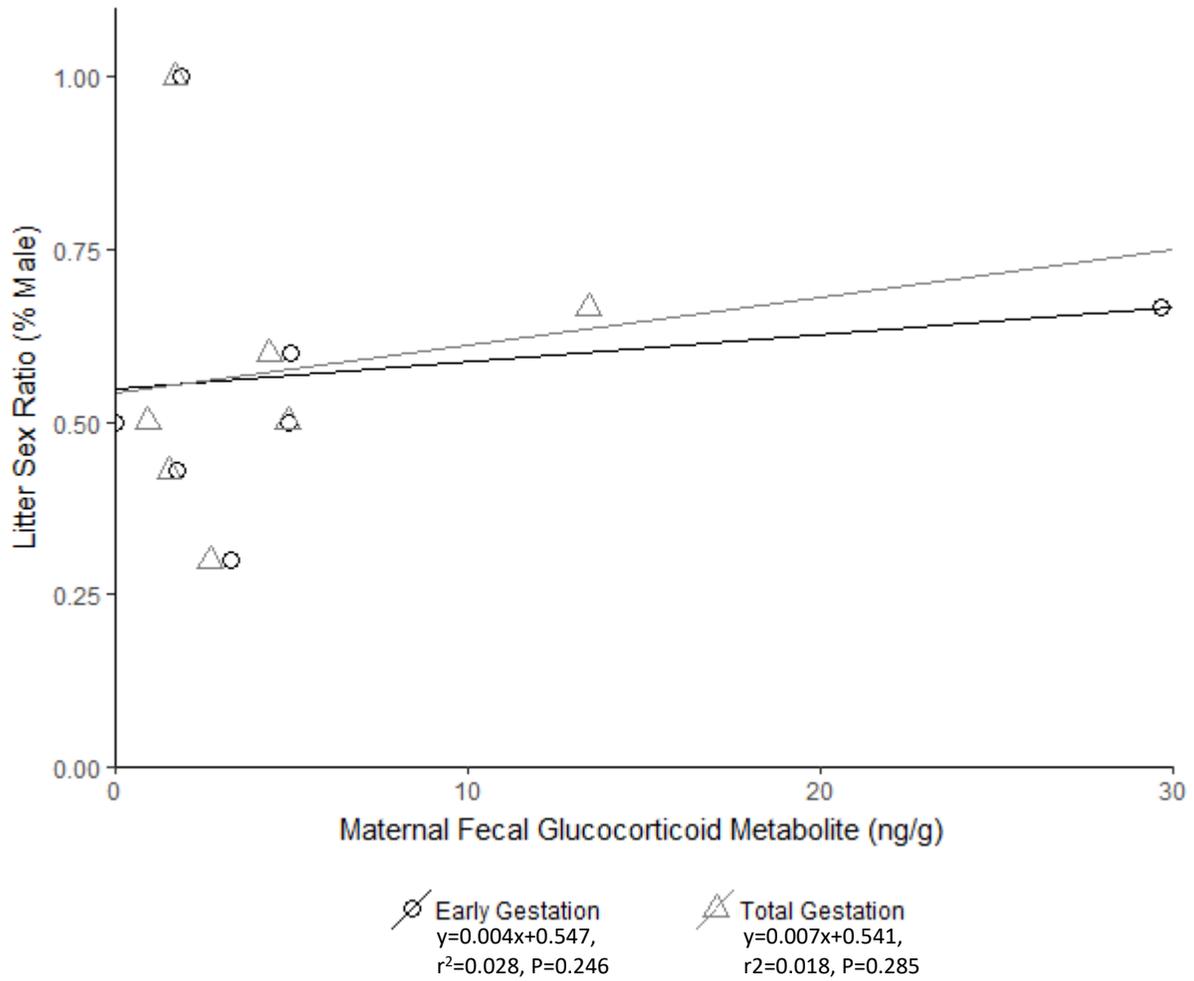


Figure 25: Model II major axis regression comparing maternal fecal glucocorticoid metabolite concentration and litter sex ratio, expressed as percentage of the litter that was male, of control squirrels ( $n = 7$ ) during the first half of gestation and over the full gestation period.

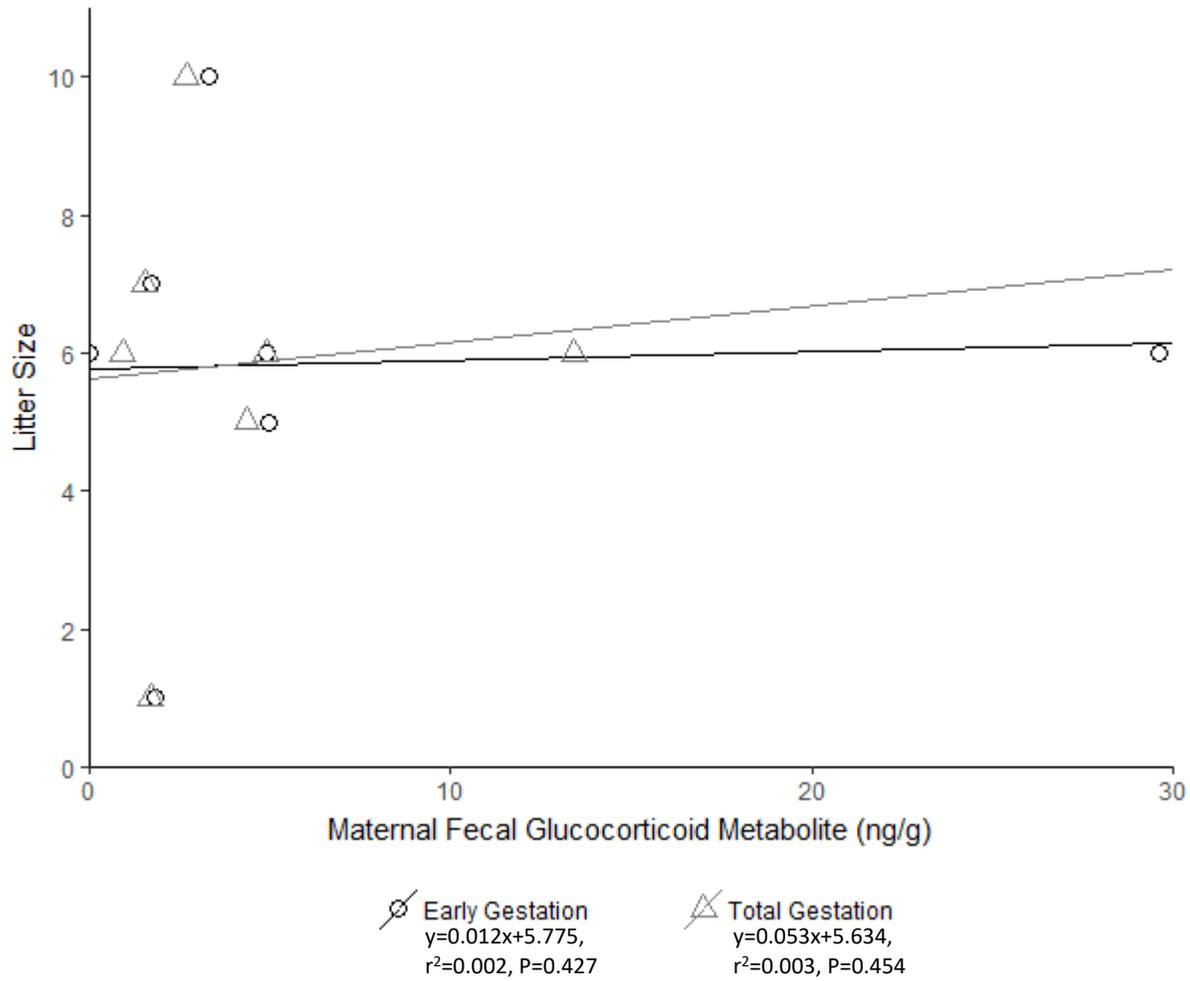


Figure 26: Model II major axis regression comparing maternal fecal glucocorticoid metabolite concentration and litter size of control squirrels ( $n = 7$ ) during the first half of gestation and over the full gestation period.

## **Chapter 4: Analysis of long-term Richardson's ground squirrel litter data: Evidence of a trade-off between litter size and sex ratio**

### **Introduction**

Fisher's fundamental sex ratio theorem (Fisher, 1930) offers a general explanation for the prevalence of 1:1 sex ratios among animals. This hypothesis, however, does not explicitly take into account the selective premium placed on fitness maximization, particularly where one sex may impose a greater resource cost on the parent than the other, or where one sex may better serve parental fitness interests in propagating like copies of parental genes. This is where the adaptive sex allocation hypothesis proposed by Trivers and Willard (1973) offers a more comprehensive explanation. Based on their observations, they suggested that mothers in better condition, relative to others in the population, should bias the production of offspring towards the sex that will have a better reproductive payoff with additional investment of resources (Trivers & Willard, 1973).

Williams (1979) subsequently refined Trivers and Willard's original idea, making it applicable to polygynous species by taking into account tradeoffs in litter size and sex ratio (Williams, 1979). In polygynous species, it can prove advantageous to have male-biased litters when male and female offspring experience differential variance in reproductive success. Male reproductive success can be more variable than female reproductive success during their lifetime, as males can potentially have multiple reproductive partners in any given breeding season or could have none (Darwin, 1871). In Red Deer, males were found to have three times the reproductive success of females when considered over their lifetime (Pemberton, Albon,

Guinness, Clutton-Brock, & Dover, 2004; Rose, Clutton-Brock, & Guinness, 1998). Mothers able to invest more resources in their offspring tend to invest more in male offspring than females, and males have been shown to be more affected by additional investment than females (Austad & Sunquist, 1986; Clutton-Brock, Albon, & Guinness, 1984). Birth weight has been found to be correlated with lifetime reproductive success in sexually dimorphic species such as Red Deer, with heavier males siring more offspring (Kruuk, Clutton-Brock, Rose, & Guinness, 1999). In social species, males in better body condition may be better able to compete for dominance and secure mates (Alberts, Buchan, & Altmann, 2006).

In some species, parents can preferentially produce and rear one sex over the other. Werren and Charnov (1978) demonstrated how in animal populations where there is overlap in generations, differences in life history between sexes, and a means by which parents can vary offspring sex ratio in response to changes, parental fitness may benefit from producing litters biased toward one sex rather than equally investing in both sexes. Manipulation of the offspring sex ratio can occur prior to conception, as in cattle (Roche, Lee, & Berry, 2006) and pigeons (Pike, 2005). During gestation, offspring sex ratio can be varied via changes to the environment the embryo is developing in and result in sex-differential offspring mortality (Austad & Sunquist, 1986; Cameron, 2004; Myers, Master, & Garrett, 1985). Following gestation and while the offspring are still dependent on the parents, sex ratio can be adjusted directly by the parents (Voland, 1984; McClure, 1981). Aside from pre-conception manipulation, offspring sex ratio manipulation can only occur through reduction of the litter or clutch size in some manner (Gedir & Michener, 2014; Helmreich, 1960; Krackow, 1992).

In the case of Richardson's ground squirrels, where males disperse by the time of sexual maturity (Michener & Michener, 1977), the local resource competition hypothesis suggests that

it would be beneficial for mothers in poor condition to invest in male offspring (Clark, 1978). This hypothesis posits that in order to avoid competition with progeny for limited resources, mothers should produce litters biased towards the dispersing sex. Female Richardson's ground squirrels are capable of mating with multiple males during the breeding season (Michener & McLean, 1996), and as a result of multiple insemination there is multiple paternity within litters (Hare, Todd, & Untereiner, 2004). Prior to reaching maturity and during the lactation period, nursing up to 14 pups (Risch, Michener, & Dobson, 2007) imposes significant costs on the mother due to the offspring putting on mass at a rate six times higher than during gestation, supported only by what their mother can provide (Michener, 1989a).

Producing offspring is energetically expensive, with lactation being the most costly part of female mammalian reproduction (Clutton-Brock, Albon, & Guinness, 1989). Depending on the species being studied, male and female offspring place different amounts of stress on the mother (Clark, Bone, & Galef, 1990; Festa-Bianchet, 1989; Gomendio, Clutton-Brock, Albon, Guinness, & Simpson, 1990). Investing resources into offspring detracts from the mother's own reserves, as well as the time she could be spending adding to said reserves (Bell, 1977; Fowler & Partridge, 1989; Reznick, 1983; Williams, 1966a; Williams, 1966b). In the case of hibernating mammals, this can impact over-wintering mortality rates (Neuhaus, 2000). Beyond the impact incurred during the immediate breeding season, in some cases, having male offspring can influence future offspring. This has been documented in bighorn sheep ewes, where mothers were more likely to have female lambs in seasons following raising a male lamb (Berube, Festa-Bianchet, & Jorgenson, 1996). Milk quality has also been seen to change in dairy cattle depending on the sex of previous calves, potentially impacting the ability to provide nutrients to subsequent offspring (Hinde, Carpenter, Clay, & Bradford, 2014).

While many studies support either the Trivers-Willard (Austad & Sunquist, 1986; Berube, Festa-Bianchet, & Jorgenson, 1996; Cameron, 2004; Clark, Bone, & Galef, 1990; Clark, Waddington, & Galef, 1991) or local resource competition hypotheses (Caley & Nudds, 1987; Clark, 1978; Cockburn, Scott, & Dickman, 1985; Johnson, 1986), some also find the forces influencing litter sex ratio are unclear, with more than one adaptive sex allocation method being present simultaneously (Dušek, Bartoš, & Sedláček, 2011; Fisher, 1999; Isaac, Krockenberger, & Johnson, 2005). There have also been studies reporting contradictory findings from within the same species (cf. Ryan, Anderson, Gardiner, & Hare, 2012; Ryan, Anderson, Berkvens, & Hare, 2014; Gedir & Michener, 2014; Schwanz & Robert, 2014). Other studies fail to find evidence for adaptive sex allocation of any kind in their study species, calling into question whether adaptive sex allocation applies universally across species (Krackow, 1997; MacLeod & Clutton-Brock, 2013; Silk & Strum, 2010; Silk, Willoughby, & Brown, 2005).

The potential causes of variation in litter size and sex ratio have been investigated extensively in mammals, and many factors have been implicated in influencing those. Variation in local temperature and rainfall during early pregnancy have been found to have a notable effect on prairie deer mouse (*Peromyscus maniculatus*) litters. There, increased mortality of female embryos occurs where mothers are exposed to high temperatures before implantation, while after implantation, exposure to high temperatures increased male embryo mortality, and offspring mass decreased among mothers exposed to rainfall (Myers, Master, & Garrett 1985). Access to supplemental food, fructose, salt, and calcium were also found to bias opossum, rat, and mouse litters towards male offspring (Austad & Sunquist, 1986; Gray, Long, Green, Gardiner, Craigon, & Gardner 2013; Schmidt & Hood, 2012). Diets high in saturated fats, as compared to low fat but high carbohydrate diets, have been found to yield male-biased litters in lab mice (Rosenfeld,

Grimm, Livingston, Brokman, Lamberson, & Roberts, 2003). Maternal hormone levels have been correlated with litter sex ratios, with both circulating testosterone and plasma cortisol during early gestation in Richardson's ground squirrels being found to be significantly correlated with smaller, male-biased litters (Ryan, Anderson, Berkvens, & Hare, 2014). Potential effects can even be carried over between generations as prenatal testosterone during the mother's development has been correlated with smaller, male-biased litters in rabbits (Banszegi, Szenczi, Dombay, Bilko, & Altbacker, 2012). Genetic factors may also play a role, as it has been reported that carriers of the genes for chryptorchidism (undescended testes) in dogs tend to produce male-biased litters, and litters containing chryptorchid offspring tend to be male-biased in both dogs and pigs (Dolf, Gaillard, Schelling, Hofer, & Leighton, 2008; Gubbels, Scholten, Janss, & Rothuizen, 2009). Selective pressure to bias litters to optimize fitness has been observed in multiple species, including; rabbits (Verts, Carraway, & Green, 1997), mice (Gaukler, Ruff, & Potts, 2016), possums (Isaac, Krockenberger, & Johnson, 2005), and roe deer (Macdonald & Johnson, 2008). Studies of domesticated rodent species have revealed sex-biased litters in the context of multiple different treatments (Rosenfeld & Roberts, 2004).

In addition to *in utero* influences on litter size and sex ratio, there are also numerous factors that can impact litter size and sex ratio immediately following birth. Mothers in poor physical condition have been known to reduce interactions with offspring, such as limiting milk access for one or more offspring, or even abandonment of offspring in order to improve their own fitness (Fairbanks & McGuire, 1995; McClure, 1981; Tait, 1980). A study of bushy-tailed wood rats (Moses, Boutin & Teferi, 1998) found that even in the absence of differential treatment of male versus female offspring, male offspring suffered higher pre-weaning mortality rates than female offspring. This was attributed to greater energetic demands in male offspring development

compared to female offspring due to sexual dimorphism. Infanticide is common in rodents, and often appears to be the result of high stress on the mother while pregnant, or elevated exposure to testosterone when young (Miley, Blustein, & Kennedy 1982). Competition for shared resources with siblings, namely milk or body heat, may also lead to malnourishment and potentially death in weaker offspring (Bautista, Drummond, Martinez-Gomez, & Hudson, 2003).

Richardson's ground squirrels are a semi-fossorial, burrowing, obligate hibernating sciurid, classified in the IUCN Red List as being of least concern (Cassola, 2016) and found throughout the North American prairies (Michener & Koepl 1985). Within these ecosystems they serve as a keystone species, providing a food source for both avian and terrestrial predators (Cassola, 2016; Michener, 2000; Schmutz & Hungle, 1989) and modifying the landscape to create habitat for myriad plant and animal species (Michener & Koepl, 1985; Newediuk, Waters, & Hare, 2015). This species is well suited to studies exploring adaptive manipulation of litter sex ratio in that their reproductive cycle is highly predictable. They breed once per year, in a roughly two week window shortly after their emergence from hibernation, during which time females are sexually receptive only for a couple of hours on a single afternoon. Gestation takes 22-23 days and the litter is sequestered below ground during lactation for a further 28-30 days. Litter size ranges from 3-14 offspring, with a median litter size of 6-8 (Michener, 1989a).

In two studies conducted in non-sequential years, Ryan, Anderson, Gardiner, & Hare (2012) and Ryan, Anderson, Berkvens & Hare (2014) found correlations between maternal fecal glucocorticoid metabolite concentrations and both litter size and litter sex ratios in Richardson's ground squirrels. In addition to this, they observed statistically significant trends in the relationship between litter size and sex ratio directly within their sample group (Ryan, Anderson, Gardiner & Hare, 2012; Ryan, Anderson, Berkvens & Hare, 2014). In both of these published

studies, however, only a single year's data were examined, and data were subsampled to improve model fit. Ryan, Anderson, Berkvens, & Hare (2014) also concede that the model used does not fit the data well, owing to violations of the model's underlying assumptions, and that the relationship between litter size and sex ratio disappeared when data for all breeding females in the 2013 population were analyzed. It has also been suggested that these results supporting the adaptive sex allocation hypothesis for Richardson's ground squirrels may be a product of sampling bias, rather than an actual trend within the population (Gedir & Michener, 2014).

The limited time period over which studies tend to be conducted, often encompassing only one or two generations, is recognized as a common problem in ecological studies (O'Neill, DeAnglis, Waide, & Allen, 1986; Maxwell & Jennings, 2005; Sergeant, Moynahan, & Johnson, 2012; Sullivan, Sullivan, Lindgren, & Ransome, 2013). In the case of studies of litter size and sex ratio trade-offs, this could lead to over-reporting of trade-offs observed by chance alone (Clutton-Brock & Sheldon, 2010; Gedir & Michener, 2014), and may account for some of the variation in results reported within some species (Cameron, 2004). The trade-offs between litter size and sex ratio within litters predicted by adaptive sex allocation theory and reported for Richardson's ground squirrels by Ryan et al. (2012; 2014) were subjected to an empirical test using long-term multigenerational data for Richardson's ground squirrels by Michener and Gedir (2014). By comparing their 24-year dataset from the second of Michener's two field sites to various models, they found that random sex allocation provided the best fit to their data, and did not observe the tradeoff between litter size and sex ratio reported in the two independent one-year studies by Ryan (Ryan, Anderson, Gardiner, & Hare, 2012; Ryan, Anderson, Berkvens, & Hare, 2014). Gedir and Michener's study, however, was conducted in a highly uniform environment of grazing land surrounded by planted crop fields (see Michener, 1989b and

Michener, 1996 for a more complete description of site characteristics). This imposes a problem in that access to food and predation pressure were likely more or less uniform across all squirrels on her study site. These conditions may not provide sufficiently strong differences in body condition and stress among individuals to elicit noticeable variation in litter size and sex ratios (Boonstra, Hik, Singleton, & Tinnikov, 1998; Hik, 1995; Sheriff, Krebs, & Boonstra, 2009). By comparison, the Richardson's ground squirrels studied by Ryan et al. (Ryan, Anderson, Gardiner, & Hare, 2012; Ryan, Anderson, Berkvens, & Hare, 2014) that occupy mowed fields and berms within the Assiniboine Park Zoo in Winnipeg, Manitoba are exposed to much greater variation in food availability, owing both to access to zoo animal feed in some areas, "hand-outs" of food items from zoo visitors in others, or no supplementary food whatsoever.

The goal of my study was to determine if trade-offs between litter size and sex ratio are evident from long-term, multigenerational data within the same population of Richardson's ground squirrels where such trade-offs have been reported based on relatively small samples of squirrels in two independent short-term studies (Ryan, Anderson, Gardiner, & Hare, 2012; Ryan, Anderson, Berkvens, & Hare, 2014). Failing that relationship, where random allocation best fits the long-term data (Gedir & Michener, 2014), I would conclude that the findings of Ryan (Ryan, Anderson, Gardiner, & Hare, 2012; Ryan, Anderson, Berkvens, & Hare, 2014) represent an artefact of sampling bias given the restriction of their data sets to select individuals for which physiological data were obtained within single summers.

## Methods

### *Ten year data set*

All data used in this study were obtained from a Richardson's ground squirrel population located at the Assiniboine Park Zoo in Winnipeg, MB (49°52'N, 97°14'W), which has been the subject of intensive study from 2004-2016 in the context of research exploring alarm communication and cognition (e.g. Freeman, Hare, Anderson, & Caldwell, 2018; Sloan & Hare, 2006; Sloan & Hare, 2008; Sloan, Wilson, & Hare, 2005; Swan & Hare, 2008a; Thompson & Hare, 2010; Wilson & Hare, 2006), personality (Clary, Skyner, Ryan, Gardiner, Anderson, & Hare, 2014) and reproduction (Hare, Todd, & Untereiner 2004; Ryan, Anderson, Gardiner, & Hare, 2012; Ryan, Anderson, Berkvens, & Hare, 2014 ). Trapping occurred during the three-week breeding season from mid-March through early April to the emergence of adults into hibernation by the end of July each year. Squirrels were captured using live traps (Tomahawk Live Trap Co., Tomahawk, WI) baited with peanut butter (No Name™ Brand, Loblaw Companies Ltd., Toronto, ON). Trapped squirrels were transferred into a cloth bag for weighing with a Pesola® spring balance (Pesola AG, Schindellegi, Switzerland), then handled by gripping the body around the scapulae using a leather glovecovered hand. Upon first capture, squirrels were marked for permanent identification by affixing a numbered metal ear tag (National Band and Tag Company, Monel no. 1, Newport, KY) through the right, left or both pinnae. Additionally, a unique identification mark was applied using a paint brush to the squirrel's dorsal pelage using black hair dye (Clairol Hydrience 52S; Pearl Black, Stamford, CT) for identification of individuals from a distance and to minimize the need to handle the squirrels every time they were captured in a trap. Newly emerged juveniles were assigned a sequential

number, yearling squirrels were assigned a dot above their number from the previous season, and adults of 2+ years or unknown age were assigned a unique mark.

Ten years of data were collected and tabulated, documenting litter size, sex ratio, mother, and the number of prior litters produced by each mother. All litters where the maternal parentage of one or more juveniles was questionable, were discarded from the data set. This left a sample of 420 litters from 301 distinct mothers, ranging in age from 1 to 6 years old and producing between 1 to 4 litters over the years they were trapped. A second unique name was assigned to each dam to avoid errors due to reuse of markings or ear tag numbers over the ten-year period, or the occasional renaming of individuals.

Average litter size and sex ratio were calculated for each year as well as overall to contrast those with litter sizes and sex ratios reported at the field site near Lethbridge, Alberta used by Gedir and Michener (2014). All statistical tests were conducted using R statistical software (R version 3.4.0, R Core Team 2013). Data were tested for normality using the Anderson-Darling normality test found in the R package kSamples (Scholz & Zhu, 2017). This test was selected over the more powerful Shapiro-Wilk test due to the large number of repeated values present in the data set. Model II major axis regressions (Ricker, 1973; Laws & Archie, 1981) were then employed to test for any potential relationships between litter size and sex ratio within the first, second, and third litters of each mother pooled across all years, as well as on an annual basis, to test for any apparent relationships within each calendar year. Additionally, regressions were run on litter size and sex ratio of litters from one, two, and three year-old mothers known to have had at least three observed litters over the 10-year sample period, to see if there are any apparent relationships within age groups. For any significant relationships found in the first, second, or third observed litters, average annual litter sizes and sex ratios were calculated and additional

major axis regressions were performed focusing solely on those litters in each year. Further regressions were performed comparing average litter sex ratio and litter size prior to and following the construction of the zoo's Australian Walkabout exhibit in 2013 for litters from the area of that enclosure, along with a two-sample Wilcoxon rank-sum test comparing average annual litter sex ratios before and after construction. This was deemed important to consider as the establishment of that exhibit introduced foods including carrots, beets, and various greens meant for the enclosure's Kangaroos but that were accessed liberally by the squirrels in the area, and hence likely promoted variable body condition depending on the placement of food relative to individual females territories.

Major axis regression analyses were performed using the R package lmodel2 (Legendre, 2014) and the two-sample Wilcoxon rank-sum test was run using the wilcox.exact function in the R package exactRankTests (Hothorn & Hornik, 2017) as well as the qnorm function to calculate the associated Z value. A Binomial test was performed to test for alternation of male- versus female-biased average litter sex ratios within the population between years of study to determine if there is a pattern to alternate biases toward the production of male or female offspring by dams, producing more of what had been the rarer sex produced in the previous year. This was done using the R (2013) binom.test function. For all tests, results were deemed significant at an alpha value of 0.05, and reported as mean  $\pm$  SE unless otherwise noted. All plots were done using the ggplot2 package in R (Wickham, 2009).

### *Placental scar counts*

In addition to the analysis of the ten years of litter data, 64 dams were humanely euthanized by Assiniboine Park Zoo staff as part of a ground squirrel control program and their uteruses were dissected out for placental scar or embryo counts in 2016. Fifty of these squirrels were

obtained from the pest control officer at the Assiniboine Park, and were collected during early gestation. The remaining 14 dams were from the study population within the zoo that had been manipulated as part of a failed experiment that attempted to manipulate circulating blood glucose levels during gestation seen in Chapter 3. Following euthanasia, the uterine horns were dissected out of each female under veterinary supervision, and placental scar counts were obtained.

## Results

### *Ten year data set*

Average litter size at the Assiniboine Park Zoo site over the duration of the study was  $5.976 \pm 0.104$  pups, and litter size ranged from 1-14. Over the 10 years from 2006 to 2016 for which sex ratio data were obtained, the average percentage of males produced was 49.1%. In any given year, as many as 55.8% (2012) and as few as 43.2% (2009) of the juveniles at emergence were males (Table 1). The proportion of males within years was unlikely to have been drawn from a normally distributed population of proportions ( $A = 6.333$ ,  $P = 1.000e^{-4}$ ). Average litter size in the first, second, and third breeding seasons for all mothers remained around 6 pups. Average litter sex ratio in the first and second breeding seasons were close to parity, while the average percentage of males produced per litter in the third breeding season dropped to 36.8% (Table 2). Major axis regressions run on the first, second, and third set of litters observed for all mothers detected an apparent relationship between litter size and sex ratio in the initial litters only ( $R^2 = 0.011$ ,  $P = 0.033$ ;  $R^2 = 0.003$ ,  $P = 0.301$ ;  $R^2 = 0.023$ ,  $P = 0.253$  respectively for first through third litters). Litters of primiparous mothers contained an average of  $5.940 \pm 0.116$  pups and ranged in size from 1-13. Average percentage of males produced by first-time mothers was 49.4% with

average annual litter sex ratios ranging from as many as 60.7% males (2012) to as few as 40.3% male (2009) juveniles at emergence (Table 3). Trend direction was not consistent between subsequent litters born to each dam, with first litters showing a significant trend in sex ratio with litters becoming more female-biased with increasing litter size (Figure 1), second litters showing a similar trend (Figure 2), and third litters becoming more male-biased with increasing litter size (Figure 3). No significant relationships were observed in the regressions looking at trade-offs in litter size and sex ratio with respect to the dam's age for the squirrels known to have had at least three litters ( $R^2 = 0.001$ ,  $P = 0.441$ ;  $R^2 = 0.028$ ,  $P = 0.253$ ;  $R^2 = 1.460e^{-4}$ ,  $P = 0.492$  for litters one through three respectively). The one-year-old dam's litters (Figure 4) and three-year-old dam's litters (Figure 5) show little to no sex-bias, while the two-year-old dam's litters show a strong trend towards female-biased litters as litter size increases (Figure 6).

Major axis regression of the litters by year for each of the 10 years of litter sex ratio data obtained detected statistically significant relationships between litter size and sex ratio only in 2013 ( $R^2 = 0.065$ ,  $P = 0.043$ ) and 2016 ( $R^2 = 0.220$ ,  $P = 3.900e^{-4}$ ), where increasing litter size was associated with a decreasing proportion of males within litters. In no other year did the proportion of variance in sex ratio explained by litter size exceed 0.71 (Table 4). Across the 10 years, the slope of the relationship between litter size and sex ratio departed from zero in a positive direction in 3 years, and in a negative direction in the remaining 7 years (Table 5). Looking only at the litters of all first-time mothers captured in each year, the only year with a significant trend is 2016 ( $R^2 = 0.276$ ,  $P = 0.002$ ) though the data from 2007 approached statistical significance ( $R^2 = 0.100$ ,  $P = 0.081$ ). In all years except 2015, the observed trend in the first-time litters is negative, with increasingly female-biased litters as litter size increases (Table 6). The regression specifically looking at trade-offs in litter size and sex ratio in the Australian

Walkabout enclosure before versus after construction show no significant relationships between litter size and sex ratio in either period ( $R^2 = 2.896e^{-4}$ ,  $P = 0.444$ ;  $R^2 = 0.009$ ,  $P = 0.169$  respectively). Data from before the walkabout was constructed and no supplementary food was available reveal no trend towards a skewed litter sex ratio in either direction with increasing litter size (Figure 7). Data from after construction of the walkabout, where pans provided supplementary forage items for squirrels show a slight trend towards increased numbers of female offspring with increasing litter size (Figure 8). The Wilcoxon rank-sum test contrasting average annual litter sex ratios from before construction of the walkabout to after construction showed no significant change in average annual litter sex ratios of squirrels in the area following construction and supplementation with food intended for the exhibit's inhabitants ( $W = 13$ ,  $Z = 1.368$ ,  $P = 0.914$ ). The binomial test run on the alternating male- versus female-biased average litter sex ratios between successive years detected no significant departure from the number of inter-annual alternations in sex ratio expected by chance alone ( $P = 1$ ), with litter sex ratio alternating between male- and female-biased 5 times over the 10-year period.

#### *Placental scar counts*

A total of 481 implantations were counted, 233 embryos and 248 scars. The average number of scars or embryos observed was 8.017, and ranged from 5 to 14 total implantations. Scars and embryos were not detected together within dams.

## **Discussion**

Our analysis of 10 years of Richardson's ground squirrel litter data from the Assiniboine Park Zoo site provides unambiguous evidence of a trade-off between litter size and the sex ratio

of offspring produced in the initial litters of Richardson's ground squirrel mothers studied, which constitute the vast majority of all litters observed (Table 2). The significant trade-off between litter size and sex ratio in the pooled first-time litters (Figure 1), combined with the fact that in all years except 2015 there were trends toward female-biased litters with increasing litter size in the first-time litters (Table 6), suggest that selection has favored the evolution of a mechanism in primiparous dams that promotes adaptive sex allocation via elimination of developing females, resulting in the production of smaller male-biased litters under certain circumstances. Our data do not, however, show the predicted male bias in average litter sex ratios for each successive litter of multiparous females following that first litter, or a change towards increasingly female-biased litters with increasing maternal age that would be predicted under the local resource competition hypothesis (Clark, 1978).

Our findings contrast with those of Gedir and Michener (2014), who found no evidence of trade-offs between litter size and sex ratio within litters. Their failure to detect any relationship could, in part, be attributable to the potential for error in their methods. Within their generated models, their window for categorizing previous litters as having an unbiased litter sex ratio ranged from 40% male to 60% male. In the three years, as opposed to the two years they report, where a statistically significant litter sex ratio bias was observed, said biases fell within this "unbiased" range. Additionally, while they do go on to look at litter sex ratios at the level of individual mothers, their model comparison treats the tested Trivers-Willard variants as well as the local-resource competition hypotheses as mutually independent, ignoring the fact that they can overlap and interact within a population (Wild & West, 2007). There is also a problem in their use of deviance information criterion (DIC) values, in that DIC favours models that make the least assumptions (Spiegelhalter, Best, Carlin, & Van Der Linde, 2002). Given that

Mendelian sex allocation will occur in the background of all tested models, and that the hypotheses tested can overlap, their analysis will automatically be biased in favor of random allocation as it is the least complicated model. Additionally, DIC is only applicable for a multivariate normal distribution, which they do not mention having tested for (Gedir & Michener, 2014).

I did find a significant trade-off in the population data for 2013, as reported by Ryan, Anderson, Berkvens, and Hare (2014), however I did not detect the trade-off they reported for this same population in 2011 (Ryan, Anderson, Gardiner, & Hare, 2012). This may be due to my use of non-parametric tests rather than the parametric tests they ran on the data set, or selection criteria for which dams to omit due to unreliability. Additionally, with the exception of glucocorticoid data, none of their data were transformed prior to analysis using parametric tests. The only other year in which a significant trend was observed was in 2016. This suggests that while the trends Ryan, Anderson, Gardiner and Hare (2012) and Ryan, Anderson, Berkvens, and Hare (2014) reported do exist, they do not apply uniformly to the population across years. Further, Ryan and his co-author's use of population subsamples in both years does lend credence to Gedir and Michener's assertion that their results were, at least in part, subject to sampling bias.

Primiparous and multiparous females exhibited weak relationships between litter size and sex ratio, with R-squared values decreasing from 0.011 for first litters to 0.003 for second litters before going up to 0.023 for third litters. This suggests that trade-offs between offspring sex and litter size may fluctuate over the dam's lifetime. Average litter sex ratio dropped significantly in latter litters, however, with first- and second-time mothers averaging close to parity while litters of third-time mothers averaged 36.8% male (Table 2). This is consistent with observations that in

philopatric iteroparous species, there is a benefit to having female-biased litters later in life as they will compete with the mother for resources for a shorter period of time as well as not requiring as heavy of an investment of resources as male offspring prior to weaning (Clutton-Brock, Albon, & Guinness, 1982; Clutton-Brock & Iason, 1986; Cockburn, Scott, & Dickman, 1985). Correlations between litter size and sex ratio were, however, non-significant for all one-, two-, and three-year-old dams from within the zoo's walkabout area (Figures 4, 5, 6). A long term study of bighorn sheep by Martin and Festa-Bianchet (2011), conducted over 33 years, found no correlation between environment and litter sex ratio in young to prime-aged sheep. Their study suggests that sex biasing, if present, may be reliant on maternal state, which constitutes a combination of body condition, current environment, past history, and age (Martin & Festa-Bianchet, 2011). It could be that the data collected may be skewed due to the majority of squirrels sampled being in their first three years of breeding, with only a few surviving to old age at 6 years.

A significant correlation between litter size and sex ratio was detected in two of 10 years (2013 and 2016) for data collected from Richardson's ground squirrels at the Assiniboine Park Zoo site (Table 5). Fecal cortisol levels have been studied in this population and did reveal a correlation with litter sex bias in a subsample of the population during early gestation in 2013 (Ryan, Anderson, Berkvens, & Hare, 2014). No relationship was detected, however, when fecal cortisol was compared to litter sex ratio again in 2016 (Yeo, Anderson, Berkvens, & Hare, Unpublished Data), though that analysis was based on a sample size of only 14 breeding females. It may also be the case that selective pressures in the area and forage conditions affecting maternal body condition were simply not sufficient to elicit a response from the mothers in certain years (Moore, Hayward, & Robert, 2015; Ryan, Anderson, Berkvens, & Hare 2014). This

is far from a definitive conclusion, however, as predatory attacks remain common on squirrels inhabiting the zoo grounds and the intensity of response to predators is comparable to populations in more natural areas (Sloan & Hare, 2006; Swan & Hare, 2008b). Further, squirrels were subject to occasional disruption due to construction, which can lead to variation in stress among individuals based on their proximity to physical disruption and to variation in stress experienced among years (O'regan, Kenyon, Seckl, & Holms, 2010; Schell, Young, Lonsdorf, & Santymire, 2013; Van Meter, French, Dloniak, Watts, Kolowski, & Holekamp, 2009). Four major construction events, two on-site and two off-site, occurred during the 10-year study period. The first on-site event was the opening of the Assiniboine Park Zoo's Australian Walkabout exhibit in May 2013, construction of which overlapped with the lactation period. The second was the renovation of the zoo's educational area at the Southern end of the study site in 2016, an event which again overlapped with that year's lactation period. In 2012, a new administration building was constructed to the Southwest of the site, and the zoo entrance building was built just to the South of the study site in 2014. There is also a road along the north side of the walkabout leading directly to the zoo's west gate, which is used by zoo and occasionally construction vehicles. My analysis of the overall litter data does not reveal any consistent association between these years and biased litters, with 2012 litters on average being 55.6% male and the remaining three years remaining around parity (Table 4). Our analysis of the primiparous litters, however, reveals that two of the four years having the greatest physical disruption also had the highest male-biased average sex ratios across all years at 60.7% (2012) and 56.2% (2016) (Table 3). Of all years studied, only 2013 and 2016 showed significant litter size-sex ratio tradeoffs ( $R^2 = 0.065$ ,  $P = 0.043$ ;  $R^2 = 0.220$ ,  $P = 3.900e^{-4}$  respectively), which also happen to be the years when major on-site disturbances occurred (Table 5). This supports the interpretation of

Ryan, Anderson, Berkvens and Hare (2014), who suggested that variation in maternal stress was correlated with detectable trade-offs in litter sex ratio and litter size.

Availability of supplemental food sources also varied on the site among years. Prior to construction of the Australian Walkabout in 2013, the study area was nominally a picnic area for visitors, though not a single zoo visitor was observed picnicking in that area in any year of our study. Following construction of the Australian Walkabout exhibit, squirrels in the Western half of the site had access to food put out for the exhibit's inhabitants, including Red Kangaroos, *Macropus rufus*, and Emus, *Dromaius novaehollandiae*. Additionally, the opening of the Shirley Richardson Butterfly Garden in 2009 provided access to butterfly larvae for squirrels at the Northern end of the study site. While access to supplemental food may also have reduced local resource competition, differential access to food should still have enhanced variation in body condition among dams, mirroring food supplementation studies exploring adaptive sex allocation in other species (Austad & Sunquist, 1986; Koskela, Huitu, Koivula, Korpimäki, & Mappes, 2004). While there is no significant difference between average litter sex ratios from before or after construction of the walkabout, litters from the area in the years after construction show a negative trend with increasingly female-biased litters as litter size increases ( $R^2 = 0.009$ ,  $P = 0.169$ ; Figure 8), compared to the lack of an apparent trend from prior to construction ( $R^2 = 2.896e^{-4}$ ,  $P = 0.444$ ; Figure 7).

While Gedir and Michener's (2014) analysis of a 24-year data set encompassing 1049 litters from a Richardson's ground squirrel population outside Lethbridge, AB detected no apparent trade-off between litter size and sex ratio, my present analyses employing 10 years of data from 409 litters weaned on the Assiniboine Park Zoo site does. Adaptive sex allocation has also been documented in long-terms studies of other ground-dwelling squirrel species. Results consistent

with the local resource competition hypothesis were obtained from an 18-year study of golden-mantled ground squirrels, *Callospermophilus lateralis* (Wells & Van Vuren, 2017). Wells and Van Vuren (2017) noted an increased incidence of male-biased litters from younger females when high densities of female kin were present in the area, which was reversed in older females. They did not, however, notice any trade-off in litter size with sex ratio (Wells & Van Vuren, 2017). Additionally, Armitage (1987) reported that young yellow-bellied marmot (*Marmota flaviventris*) dams in matrilineal colonies produced significantly more female offspring than male offspring compared to older dams in the colony, but noted that overall sex ratio post-weaning did not vary significantly from 1:1. Further, he did not detect any trade-off between litter size with sex ratio. Accounting for the density of female kin in the area during early gestation may be worth incorporating in future studies of Richardson's ground squirrels.

Of the 409 reliable litters trapped between 2006 and 2016 inclusive, 7 were single pup litters. Besides the remote possibility of litters with a single juvenile at weaning resulting from misattribution of emerging young to dams owing to juveniles mixing with nearby litters, these singleton litters could also be due the result of litter size reduction either via reabsorption (e.g. Conway, 1955; Owusu, Adu, Awotwi, & Awumbila, 2010) or abortion of certain young over the course of gestation (e.g. Cameron 2004; Grant 2008), or even selective nursing of preferred young by their dam (e.g. Moses et al. 1998). It is generally acknowledged that placental scars or similar localized pigmentation remain at the site of implantation, including in cases of resorption provided it occurred in mid to late gestation (Conway 1955). My placental scar counts taken immediately following weaning in the experiment in Chapter 3, as well as litter counts of embryos taken during early gestation in Richardson's ground squirrels failed to reveal any litters of 1, suggesting that reductions could be happening. Additionally, the lack of a significant

difference between litter counts and placental scar counts in Chapter 3, similar to other findings for Richardson's ground squirrels (Gedir & Michener, 2014; Sheppard, 1972), yet with noticeably more scars than pups, provides further support of this. This lack of statistically significant, yet still noticeable, litter size reductions during gestation suggests that adaptive sex allocation, if present, may not be occurring at this point in offspring production but rather later on during lactation and weaning. This is perplexing, however, as infanticide is not common in ground squirrels (Michener, 1982), and intra-specific aggression towards juveniles is rare in Richardson's ground squirrels (Michener, 1973). Another possibility is that litter size reductions are occurring in the earliest stages of gestation, prior to formation of the placental scar. If this is the case, it would agree with the findings of Ryan, Anderson, Gardiner and Hare (2012) and Ryan, Anderson, Berkvens and Hare (2014) correlating increased occurrence of male-biased litters with elevated circulating cortisol in mothers during early gestation.

The pattern of increasing and decreasing trends in litter sex ratio by year appears superficially to suggest that the benefits of producing male-biased or female-biased litters may shift depending on fluctuating external factors, such as the density of males in the area from previous litters. Gedir and Michener's (2014) data set also reveals a similar pattern of fluctuations about unity between years, but this pattern is not mentioned in their analysis (Gedir & Michener, 2014). This would fit with the Trivers-Willard hypothesis in the broad sense, as it suggests that mothers would bias their litter in favor of whichever sex conveys the greatest reproductive benefit for her investment of additional resources, which in itself can be highly context-specific (Trivers & Willard, 1973). According to the local mate competition hypothesis, analysis of such a pattern of alternating sex biases would ideally be done on a scale reflecting the available mates during estrus (Hamilton, 1967). The Hare lab's zoo population does this, with

males ranging across the site during the breeding season despite elevated male intrasexual aggression and territoriality during that time period. The result of my binomial test, however, revealed that the number of these inter-annual alternations do not deviate from what one would expect by chance, and thus I conclude that there is no pattern to the direction of oscillations about the 1:1 sex ratio relative to the previous year's average sex ratio within the population. It is unlikely that the advantages of producing male- or female-biased litters varies relative to the density of residual male offspring in the area from the previous breeding season.

Taken together, my results provide evidence of a trade-off between litter size and sex ratio in the Assiniboine Park Zoo population of Richardson's Ground Squirrels within the first year of breeding. Through my examination of placental scars in mothers of known litters, I can also conclude that trade-offs are not likely to be occurring as a result of litter reduction during gestation, and thus must be occurring prior to or during the earliest stages of gestation, leaving no evidence of placental scarring, or during the post-partum period. As a result, I conclude that while random sex allocation appears to fit the population superficially, at finer scales, there is evidence of adaptive sex allocation. Given the complex interactions of many factors influencing the sex ratio of offspring produced (Austad & Sunquist, 1982; Bautista, Drummond, Martinez-Gomez, & Hudson, 2003; Cameron, 2004; Hardy, 1997; Myers, Master, & Garrett 1985), perhaps the selective pressures promoting adaptive sex allocation are collectively too weak, or exert effects at a much more local scale in biasing offspring sex ratio in the population studied. The latter part of this would be consistent with the discrepancies identified between long-term studies of populations approaching the western (Gedir & Michener 2014) and eastern limit (present study) of the Richardson's ground squirrel's range. Further studies are necessary to

document potential factors contributing to variation in the life history and behaviour of populations across this range.

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## Figures

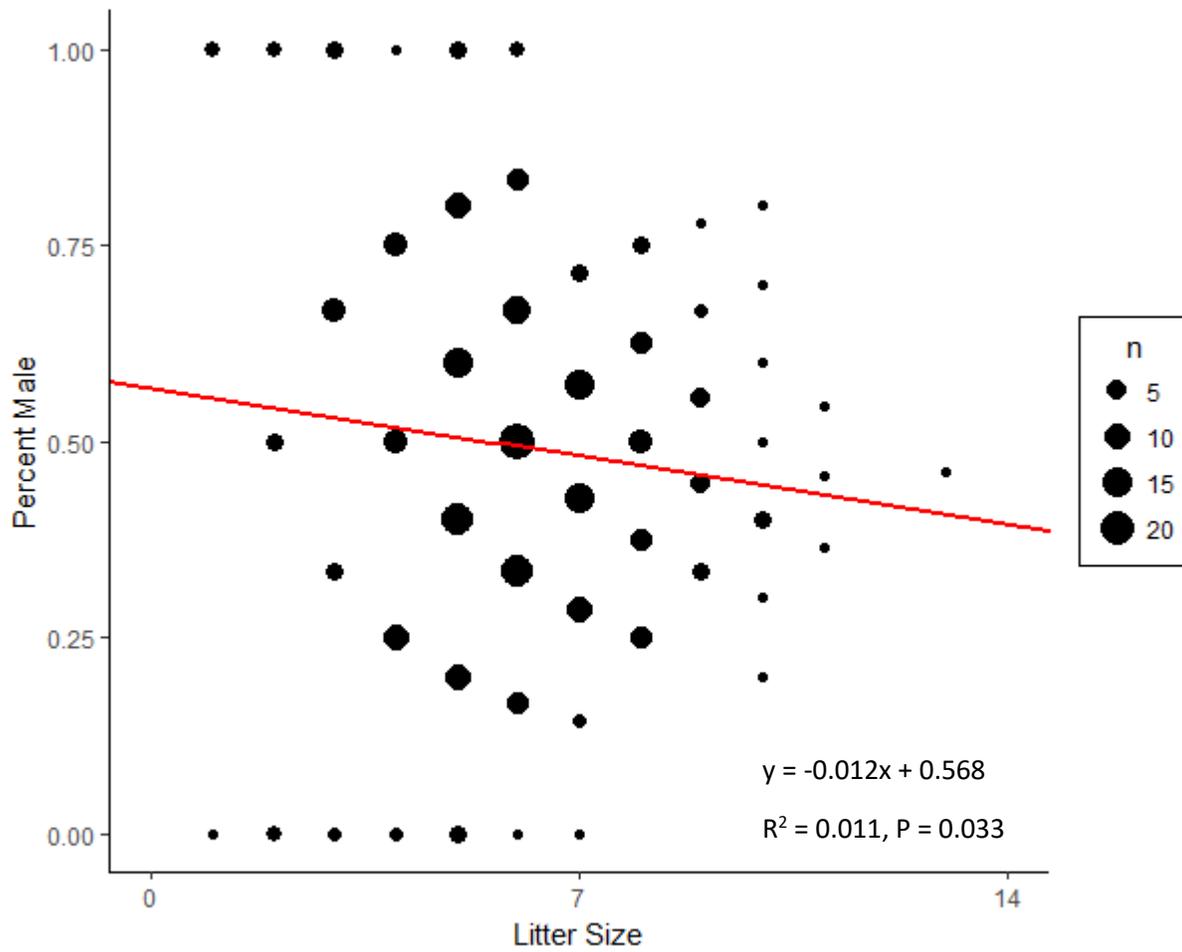


Figure 1: Major axis regression comparing litter sex ratio, expressed as the percentage of male offspring, and litter size in the first litters of all mothers observed (301 total litters). The red line represents the line of best fit, data points are scaled by size to reflect the number of tied observations.

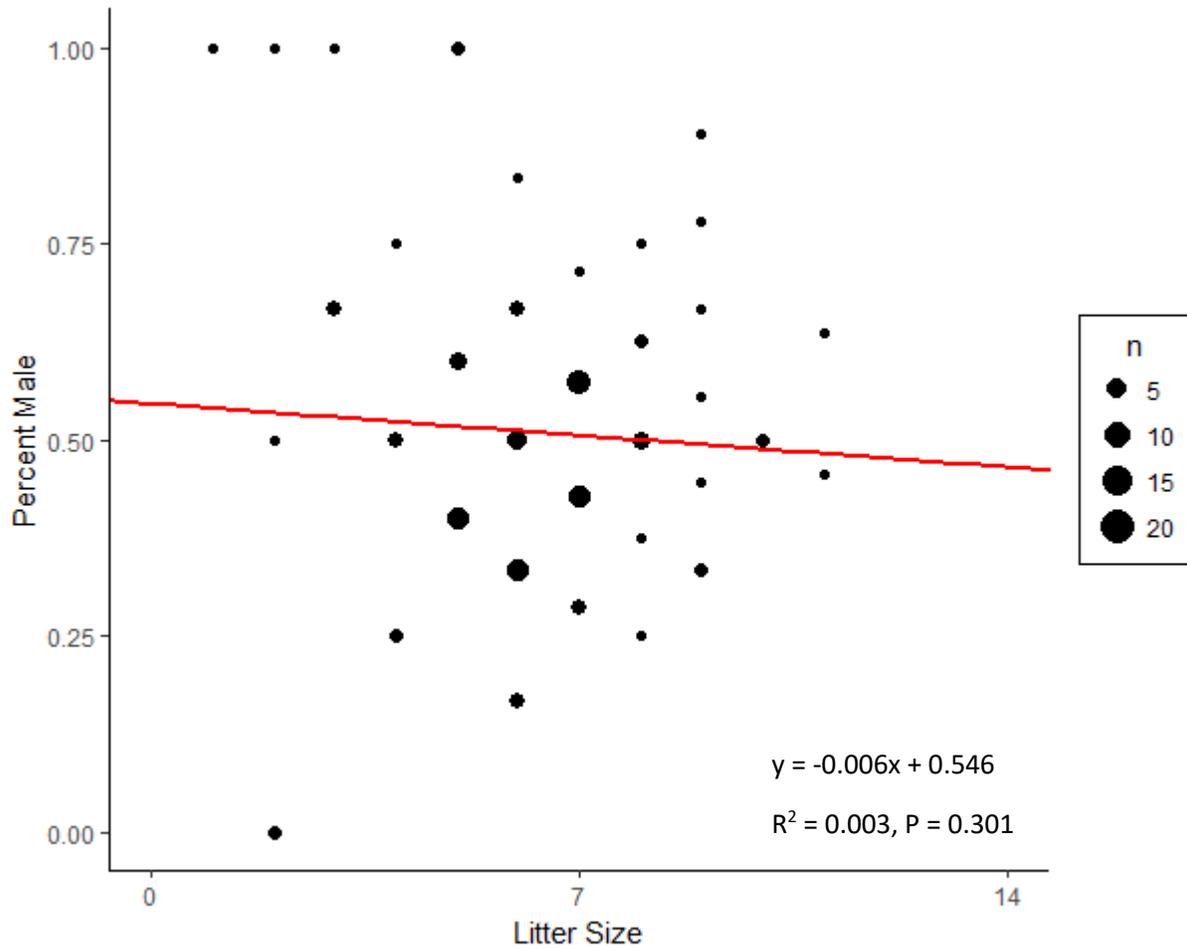


Figure 2: Comparison of litter sex ratio, expressed as the percentage of male offspring, and litter size for all mothers for which a second litter was observed (83 litters total). The red line represents the line of best fit, data points are scaled by size to reflect the number of tied observations.

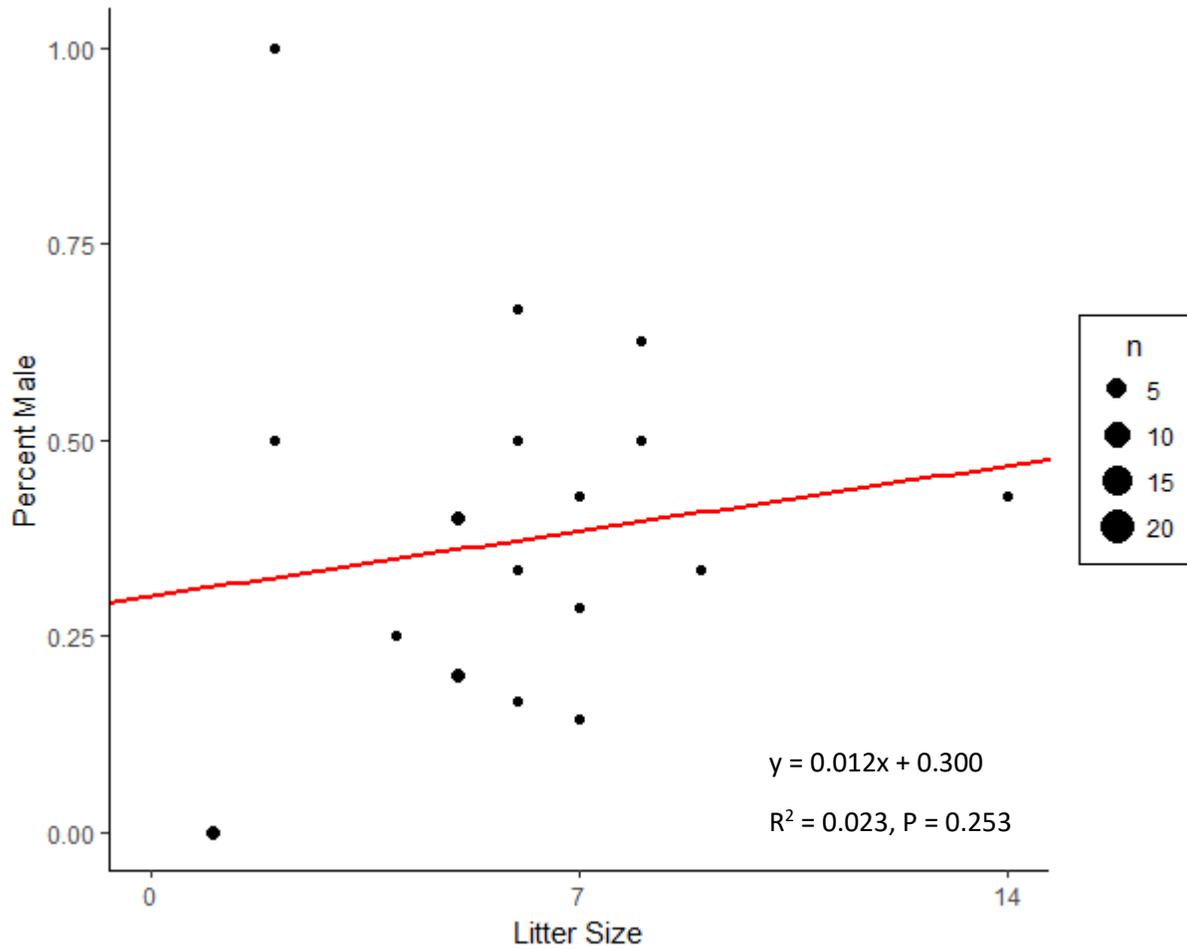


Figure 3: Major axis regression comparing sex ratio, expressed as the percentage of male offspring, and litter size for all mothers for which a third litter was observed (20 litters total). The red line represents the line of best fit, data points are scaled by size to reflect the number of tied observations.

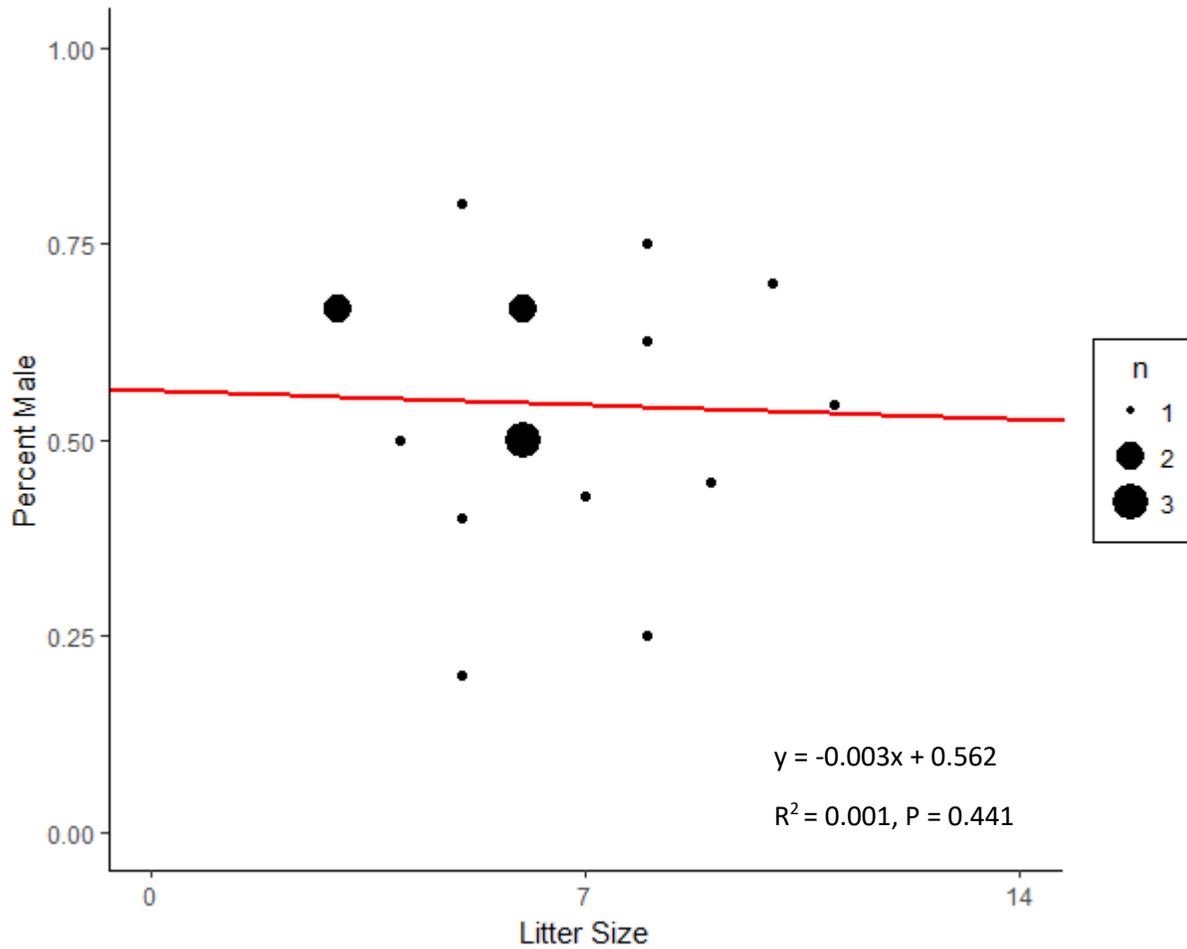


Figure 4: Major axis regression comparing litter size and sex ratio, expressed as percentage of the litter that was male, in litters of one-year-old dams for which at least three litters were observed on site across all years ( $n = 18$ ). Red line represents the line of best fit, data points are scaled by size to reflect the number of tied observations.

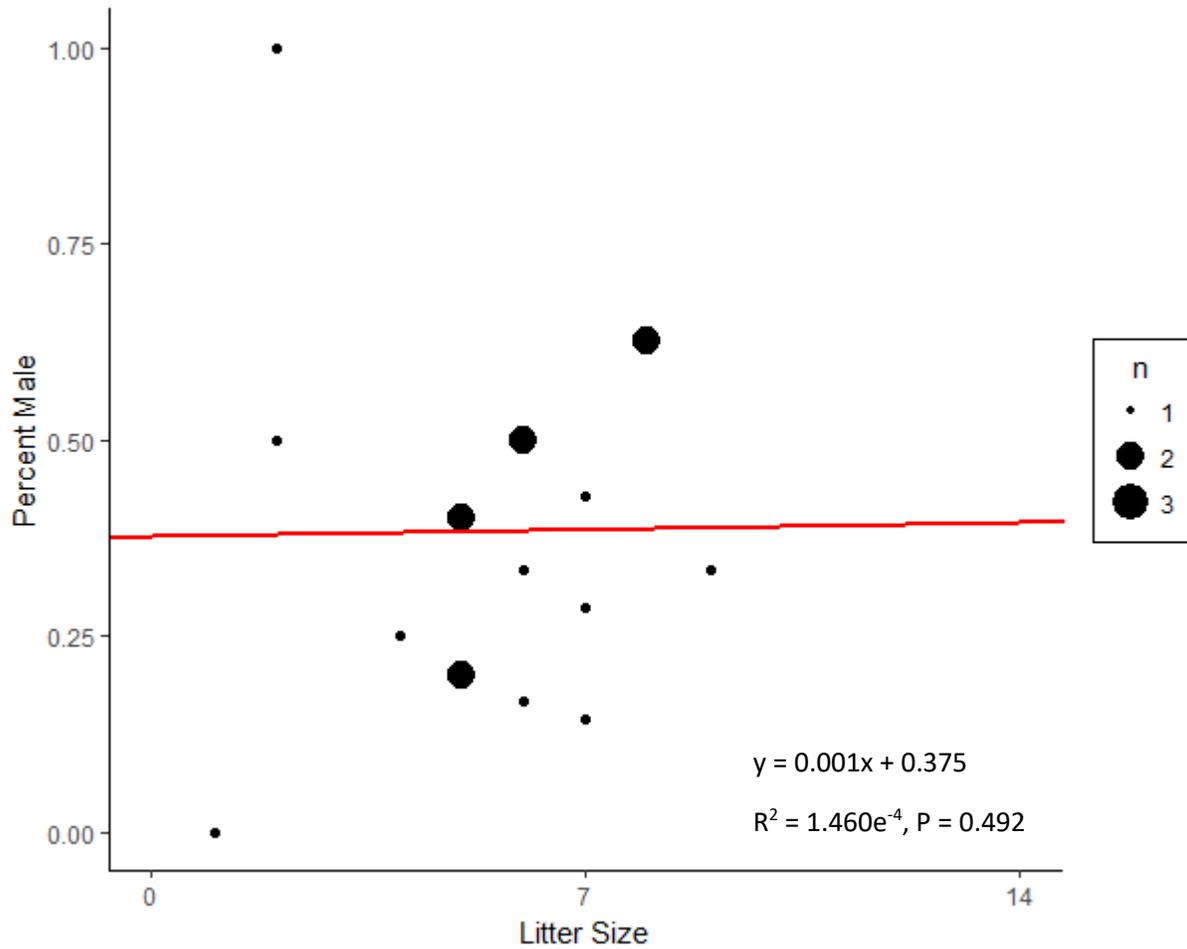


Figure 5: Major axis regression comparing litter size and sex ratio, expressed as percentage of the litter that was male, in litters of all three year old dams for which at least three litters were observed on site across all years ( $n = 18$ ). Red line represents the line of best fit, data points are scaled by size to reflect the number of tied observations.

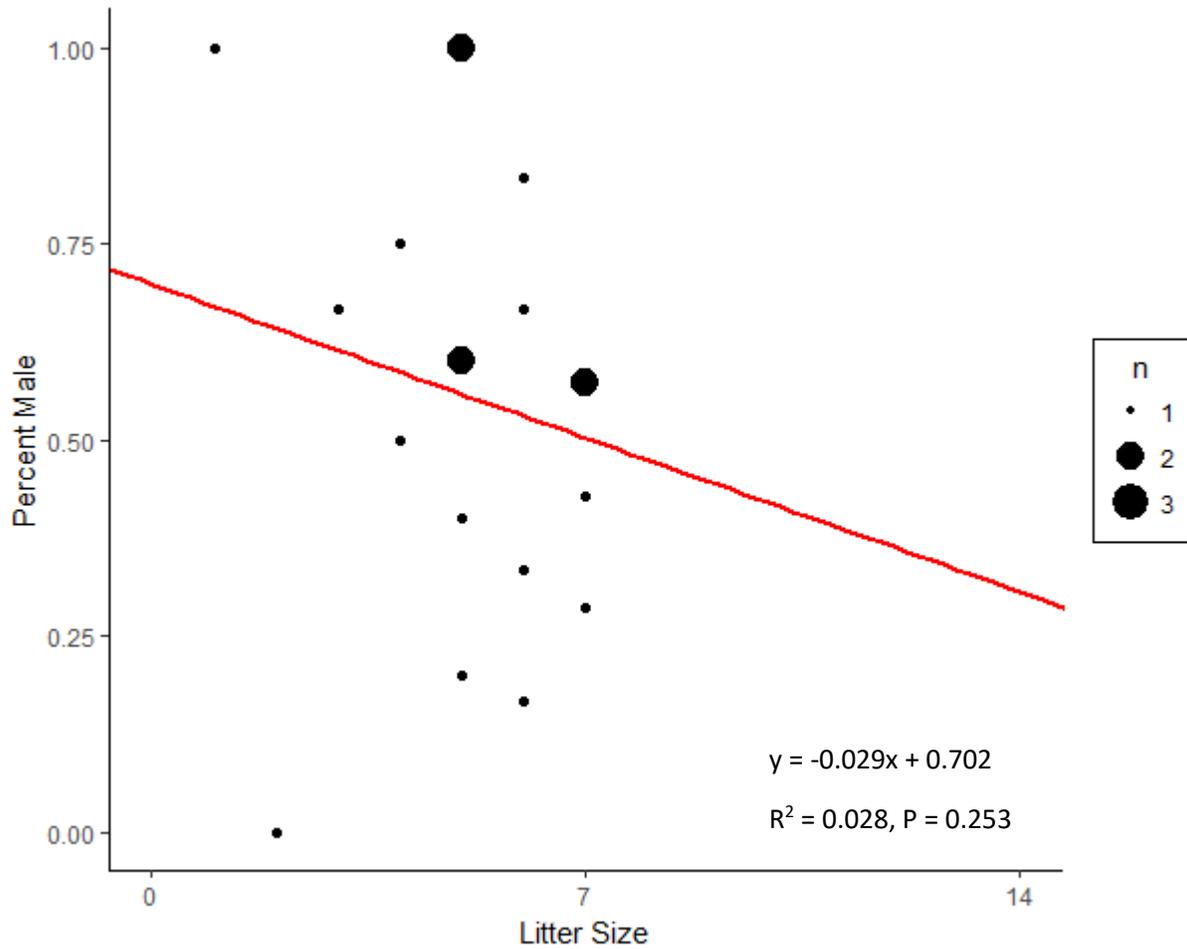


Figure 6: Major axis regression comparing litter size and sex ratio, expressed as percentage of the litter that was male, in litters of two-year-old dams for which at least three litters were observed on site across all years ( $n = 19$ ). Red line represents the line of best fit, data points are scaled by size to reflect the number of tied observations.

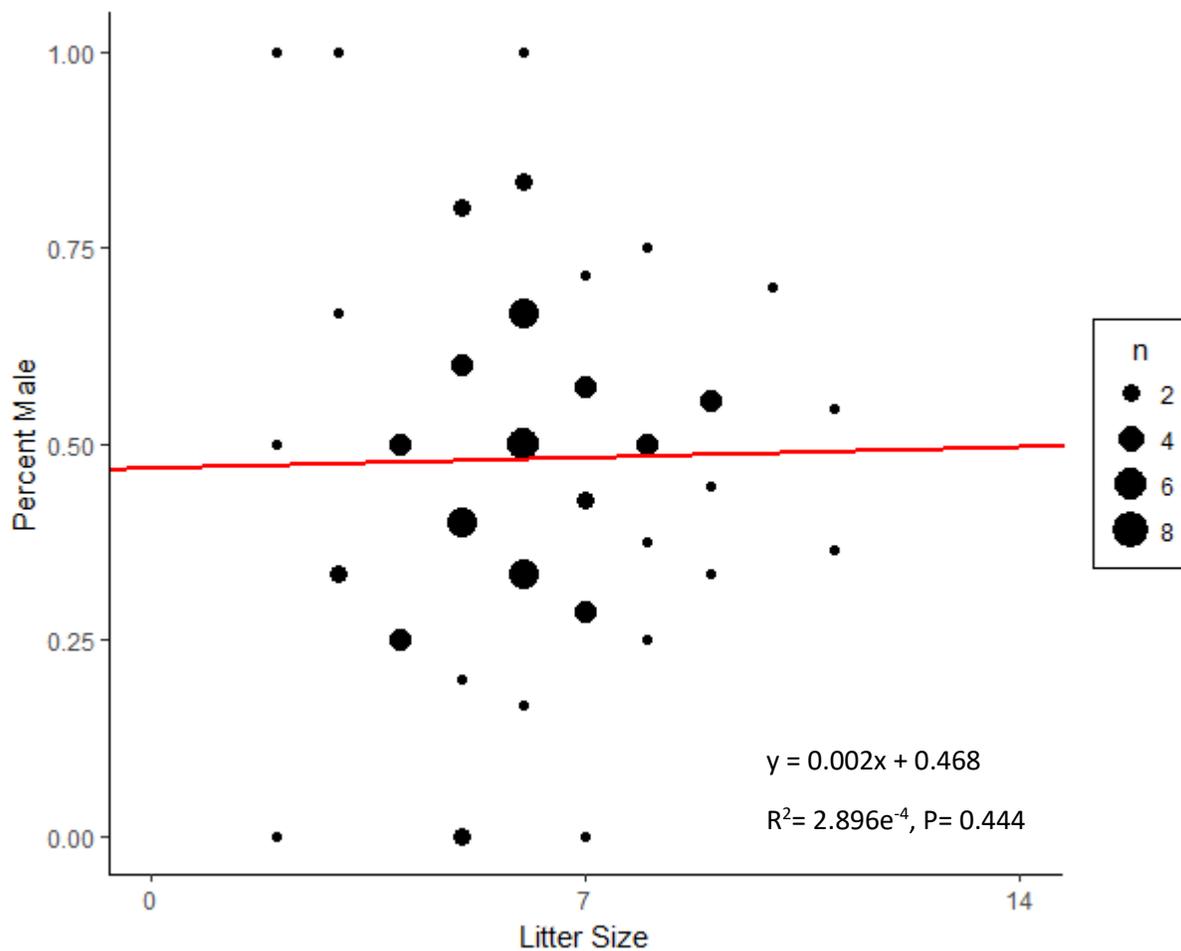


Figure 7: Major axis regression comparing sex ratio, expressed as the percentage of male offspring, and litter size for all litters occurring in the picnic area prior to construction of the Australian Walkabout (70 total litters). The red line represents the line of best fit, data points are scaled by size to reflect the number of tied observations.

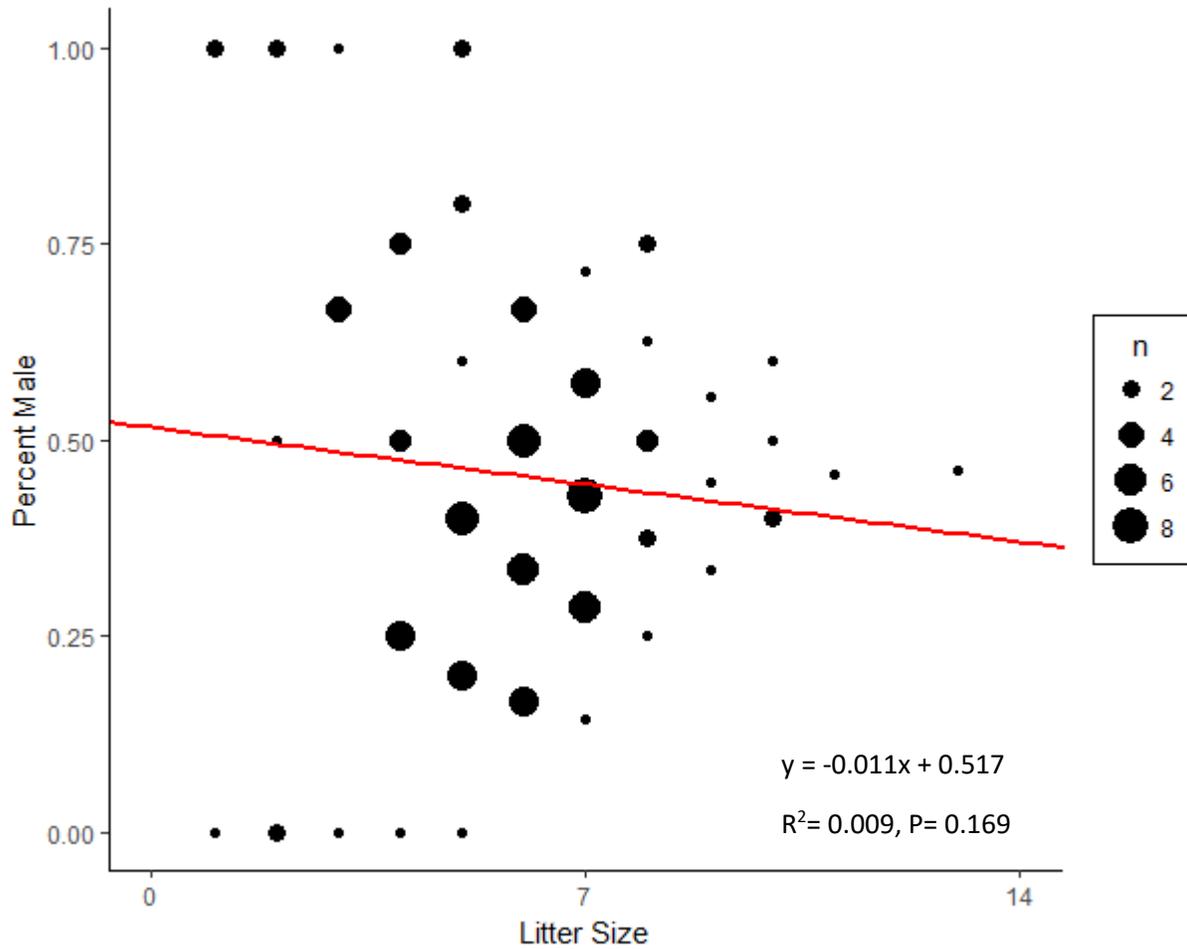


Figure 8: Major axis regression comparing sex ratio, expressed as the percentage of male offspring, and litter size for all litters occurring in the picnic area after construction of the Australian Walkabout (105 total litters). The red line represents the line of best fit, data points are scaled by size to reflect the number of tied observations.

**Tables**

<i>Year</i>	<i>Total Litters</i>	<i>Total Juveniles</i>	<i>Reliable Litters</i>	<i>Reliable Juveniles</i>
2006	46	260	33	212
2007	38	206	30	186
2008	81	466	67	407
2009	24	153	22	137
2011	31	170	27	153
2012	40	238	35	227
2013	57	289	43	240
2014	64	375	61	371
2015	59	329	43	229
2016	53	317	48	299
<i>Total</i>	493	2803	409	2475

Table 1: Total number of litters and juvenile Richardson's ground squirrels as well as the number of litters and juveniles that were deemed reliable from each year's trapping data in terms of ascribing all juveniles to their dam.

	<i>Number of Litters</i>	<i>Average Litter Size ± SE</i>	<i>Average Litter Sex Ratio ± SE (% Male)</i>
<i>First Litters</i>	301	5.940 ± 0.116	0.494 ± 0.014
<i>Second Litters</i>	83	6.217 ± 0.229	0.510 ± 0.023
<i>Third Litters</i>	20	5.700 ± 0.673	0.368 ± 0.053

Table 2: Average Richardson's ground squirrel litter size, litter sex ratio, and number of litters studied for data collected from 2006 to 2016 sorted by each dam's ordinal reproductive season.

<i>Year</i>	<i>Number of Litters</i>	<i>Average Litter Size ± SE</i>	<i>Average Litter Sex Ratio ± SE (% Male)</i>
2006	33	6.424 ± 0.308	0.485 ± 0.038
2007	21	6.238 ± 0.507	0.555 ± 0.054
2008	62	6.000 ± 0.256	0.464 ± 0.029
2009	10	6.100 ± 0.567	0.403 ± 0.052
2011	26	5.577 ± 0.369	0.456 ± 0.049
2012	24	6.458 ± 0.340	0.607 ± 0.039
2013	32	5.406 ± 0.291	0.493 ± 0.043
2014	37	6.432 ± 0.379	0.448 ± 0.037
2015	29	5.069 ± 0.435	0.461 ± 0.049
2016	27	5.704 ± 0.373	0.562 ± 0.047
<i>Total/Means ± SE</i>	301	5.940 ± 0.116	0.494 ± 0.014

Table 3: Average Richardson's ground squirrel litter size, litter sex ratio, and number of litters studied for all primiparous dams trapped from 2006 to 2016 arranged by year in which the data were collected.

<i>Year</i>	<i>Number of Litters</i>	<i>Average Litter Size <math>\pm</math> SE</i>	<i>Average Litter Sex Ratio <math>\pm</math> SE (% Male)</i>
2006	33	6.424 $\pm$ 0.308	0.485 $\pm$ 0.038
2007	30	6.200 $\pm$ 0.456	0.535 $\pm$ 0.044
2008	67	5.925 $\pm$ 0.246	0.464 $\pm$ 0.027
2009	22	6.091 $\pm$ 0.405	0.432 $\pm$ 0.039
2011	27	5.667 $\pm$ 0.366	0.496 $\pm$ 0.047
2012	35	6.486 $\pm$ 0.260	0.558 $\pm$ 0.031
2013	43	5.488 $\pm$ 0.325	0.485 $\pm$ 0.036
2014	61	6.082 $\pm$ 0.299	0.462 $\pm$ 0.034
2015	43	5.326 $\pm$ 0.363	0.490 $\pm$ 0.037
2016	48	6.229 $\pm$ 0.284	0.528 $\pm$ 0.032
<i>Total/Means <math>\pm</math> SE</i>	409	5.976 $\pm$ 0.104	0.491 $\pm$ 0.011

Table 4: Average Richardson's ground squirrel litter size, litter sex ratio, and number of litters studied from 2006 to 2016 arranged by year in which the data was collected.

<i>Year</i>	<i>Line equation</i>	<i>R</i>	<i>R<sup>2</sup></i>	<i>P</i>
2006	-0.018x + 0.597	-0.141	0.020	0.216
2007	-0.003x + 0.552	-0.028	0.001	0.441
2008	-0.012x + 0.536	-0.107	0.011	0.194
2009	0.015x + 0.338	0.158	0.025	0.240
2011	-0.01x + 0.551	-0.074	0.006	0.360
2012	-0.01x + 0.620	-0.078	0.006	0.327
2013	-0.029x + 0.642	-0.255	0.065	0.043
2014	0.011x + 0.396	0.096	0.009	0.230
2015	0.012x + 0.426	0.115	0.013	0.230
2016	-0.054x + 0.863	-0.471	0.220	3.900e <sup>-4</sup>

Table 5: Major axis regression line equations comparing litter size and sex ratio as percentage of the litter that is male as well as the corresponding R, R-squared, and significance values for all observed Richardson's ground squirrel litters by year in which they were observed.

<i>Year</i>	<i>Line equation</i>	<i>R</i>	<i>R<sup>2</sup></i>	<i>P</i>
2006	-0.018x+0.597	-0.141	0.020	0.215
2007	-0.034x+0.768	-0.316	0.100	0.081
2008	-0.007x+0.508	-0.064	0.004	0.312
2009	-0.020x+0.523	-0.212	0.045	0.275
2011	-0.011x+0.555	-0.077	0.006	0.357
2012	-0.018x+0.721	-0.154	0.024	0.233
2013	-0.029x+0.648	-0.188	0.035	0.153
2014	-0.003x+0.470	-0.035	0.001	0.42
2015	0.022x+0.351	0.191	0.036	0.161
2016	-0.067x+0.946	-0.525	0.276	0.002

Table 6: Major axis regression line equations comparing litter size and sex ratio as percentage of the litter that is male as well as the corresponding R, R-squared, and significance values for litters of all primiparous dams by year of trapping.

## Chapter 5: Conclusion

Based on prior research suggesting a relationship between circulating maternal plasma glucose during gestation and litter sex ratios in Richardson's ground squirrels (Ryan, Anderson, Berkvens, & Hare, 2014; Ryan, Anderson, Gardiner, & Hare, 2012), I undertook a series of studies quantifying maternal plasma glucose concentrations, fecal glucocorticoid metabolites (FGMs), and their relationship to potential tradeoffs between litter size and sex ratio. The first of my goals was to validate the portable, off-the-shelf, Accu-chek<sup>®</sup> Aviva handheld glucometer (Roche Diagnostics, Laval, Quebec) for use as a research tool for documenting circulating plasma glucose concentrations of Richardson's ground squirrels. The second, and original primary focus of my thesis, was to test the glucose metabolism hypothesis (Cameron, 2004) through direct manipulation of maternal plasma glucose concentration during gestation via surgically-implanted osmotic minipumps filled with a glucose solution. The third goal of my thesis was to determine if tradeoffs in litter size and sex ratio predicted by the glucose metabolism hypothesis are evident in long-term data for Richardson's ground squirrels at Winnipeg, Manitoba's Assiniboine Park Zoo. This tradeoff, along with a positive correlation between dam FGM concentration during early gestation and the proportionate representation of males within each dam's litter were previously reported by Ryan et al. in two independent single-breeding season studies of free-living Richardson's ground squirrels within the Assiniboine Park Zoo population (Ryan, Anderson, Berkvens, & Hare, 2014; Ryan, Anderson, Gardiner, & Hare, 2012). These findings were called into question by Gedir and Michener (2014), however, who found no relationship between litter size and sex ratio in their analysis of

24 years of Richardson's ground squirrel litter data from Michener's study population of Richardson's ground squirrels near Lethbridge, Alberta.

In Chapter 2, I performed a validation study of the Roche Diagnostics Accu-chek Aviva<sup>®</sup> glucometer for use in the field with Richardson's ground squirrels. While neither as accurate nor precise as measurements from the lab-based Ortho Diagnostics VITROS<sup>®</sup> 250 Chemistry Analyzer system to which measurements from the Accu-chek Aviva<sup>®</sup> were compared, my findings demonstrate that the portable glucometer provides blood glucose concentration readings that are well correlated with those from the less convenient and more costly lab-based system. As such, my results validate the use of the Accu-chek Aviva<sup>®</sup> as a tool for quantifying plasma glucose concentrations from Richardson's ground squirrels in a field setting. My results are similar to the findings of Mahmoodpoor, Hamishehkar, Shadvar, Sanaie, Iranpour and Fattahi (2016), who tested this portable glucometer for use by diabetic humans, but did not confirm the consistent overestimate of plasma glucose concentrations reported by Mahmoodpoor, Hamishehkar, Shadvar, Sanaie, Iranpour & Fattahi (2016). This difference raises concerns about the comparability of readings taken from different glucometers and test strip batches. Future research comparing the performance of the Accu-chek Aviva<sup>®</sup> with other portable glucometers on the market would also prove useful. While the utility of this device in obtaining reliable plasma glucose concentrations from other species remains unaddressed, validation studies such as mine across a range of species could ultimately reveal the general utility of the Accu-chek Aviva<sup>®</sup> as a tool for field research.

While the lack of significant results and the problems in establishing the desired circulating plasma glucose concentrations within the study reported in Chapter 3 ultimately prompted me to shift focus to looking at the 10 years of Richardson's ground squirrel litter data from the

Assiniboine Park Zoo, it did provide insights that will prove useful in future attempts to test the glucose metabolism hypothesis. First, there is considerable variation in both blood glucose and cortisol responses among individual Richardson's ground squirrels. It may be useful for future studies to obtain baseline responses for individual prior to manipulation to account for this. Given intrinsic variation among individuals, it is also apparent that much larger sample sizes than those obtained in the present study would need to be employed to obtain robust results where dams are divided among multiple treatment groups. There are also questions about the dosage of glucose the Alzet 1002 osmotic minipumps can effectively deliver, as there was no significant difference in blood glucose measurements between groups in the study. Tests should be conducted to determine the maximum concentration of glucose that can be passed through the pump's membrane. It is important to note, however, that the failure to establish differences in circulating plasma glucose concentrations among treatment groups may in fact be attributable to the squirrel's ability to regulate blood glucose concentrations. Either way, a more productive approach to testing the glucose metabolism hypothesis may be to manipulate the activation of the hypothalamic pituitary-adrenal (HPA) axis directly by administering controlled doses of adrenocorticotrophic hormone to activate the HPA axis, or dexamethasone to suppress that axis and then measure the effect of that manipulation on FGMs, circulating plasma glucose concentration and the resultant sex ratio within litters produced by the experimentally treated dams. The ability of Richardson's ground squirrels to regulate blood glucose levels should also be examined from a histological standpoint, with postmortem excision of pancreatic tissue and counting of the beta cells.

Results from my analysis of the full 10-year population data set from the Assiniboine Park Zoo in Chapter 4 support the findings of Ryan et al. (2012, 2014) in providing evidence of a

tradeoff between litter size and sex ratio in Richardson's ground squirrels during their first year of breeding, with a bias towards production of female offspring with increasing litter size. This differs from the findings of Gedir and Michener (2014), who suggested that random allocation provided the best fit for litter sex ratio data from Michener's Alberta Richardson's ground squirrel population. Further, and contrary to Gedir and Michener (2014) my analysis of placental scar counts from Richardson's ground squirrel dams of known litters revealed that litter reductions are likely occurring either in the earliest stages of gestation or post-partum. Given the striking differences between these Manitoba and Alberta Richardson's ground squirrel populations, future studies quantifying range-wide variation in life history and phenology would likely prove fruitful. More importantly, the difference between these populations highlights the context-dependent nature of the expression of adaptive sex allocation in Richardson's ground squirrels. It is likely that the extensive variation in female body condition resulting from unequal access to supplementary food allowed the detection of adaptive sex allocation in the Assiniboine Park Zoo population, but not in the Alberta population where homogeneity of forage would result in relatively limited variation in body condition among gestating dams. Given the complex interactions of factors that have been proposed to modulate adaptive sex allocation (Austad & Sunkvist, 1982; Bautista, Drummond, Martinez-Gomez, & Hudson, 2003; Cameron, 2004; Hardy, 1997; Myers, Master, & Garrett 1985), it is likely that the selection pressures experienced by local populations vary, resulting in variation in phenology, demography and life history. Future investigations of adaptive sex allocation may also benefit by accounting for social factors such as density, relatedness, dominance and personality variation among individuals.

A substantial amount of information exists regarding Richardson's ground squirrel life history, ecology and behavior (Davis, 1982; Davis, 1984; Hare, 1998a; Hare 1998b; Hare &

Atkins, 2001; Hare & Murie, 2007; Hare, Todd, & Untereiner, 2004; Hare & Warkentin, 2012; Michener, 1977; Michener, 1983; Michener, 2000; Michener & Sheppard, 1972; Ryan et al., 2012; 2014; Sloan, Wilson, & Hare, 2005; Warkentin, Keeley, & Hare, 2001). In addition to providing insight into the conditions under which we might expect to detect adaptive sex allocation in this species, my research highlights major knowledge gaps pertaining to the reproductive physiology of Richardson's ground squirrels. By validating the use of an affordable and readily available glucometer, and through insights gained in my failed attempt to directly test the glucose metabolism hypothesis, it is my hope that future researchers will employ the methods I have advocated to definitively test for the operation of the mechanism proposed under the glucose metabolism hypothesis. Only a few other long term studies have explored adaptive sex allocation in ground squirrels (Armitage, 1987; Gedir & Michener, 2014; Wells & Van Vurren, 2017). My findings from the analysis of 10 years of Richardson's ground squirrel litter data contrast meaningfully with the findings of Gedir and Michener (2014), the only other long-term study of adaptive sex allocation in Richardson's ground squirrels published to date. My findings support the earlier reports of Ryan et al. (2012, 2014) that adaptive sex allocation occurs in Richardson's ground squirrels, and that adjustment of offspring sex ratio is achieved via litter size reduction. Taken together, my thesis research contributes meaningfully to our knowledge regarding the reproductive ecology of Richardson's ground squirrels and provides additional tools and methods to advance that knowledge further.

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