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Intra-populational variation of ejaculate traits and sperm depletion in red-sided garter snakes

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Abstract

Female sexual promiscuity is a prevalent element of mating systems. One consequence of female sexual promiscuity is that male-male competition often continues post-copulation within the female's reproductive tract. According to theory, the number of sperm a male inseminates relative to his rivals strongly predicts his fertilization success. However, sperm quality is also important, especially when males are sperm limited and female sperm storage is prevalent. In this study, we examined intra-populational variation in sperm numbers and ejaculate quality (sperm mobility) in male red-sided garter snakes (*Thamnophis sirtalis parietalis*) and determined whether these traits varied with male body size and condition over successive matings. We obtained sperm by dissolving copulatory plugs collected from natural matings, which enabled us to also test whether males allocated more sperm to larger, more fecund females. We found significant variation in ejaculate quality among males and that small males transferred as many sperm as large males. Total sperm numbers declined significantly from a male's first to second ejaculate suggesting that males may become significantly sperm depleted across successive matings. The mass of the relatively sperm-free posterior portion of the copulatory plug that remained after liberation of sperm was correlated with copulation duration. Males copulated longer

with larger females; however, longer copulation durations did not correlate with total sperm. Thus, males may allocate more copulatory plug material to larger females to guard against her remating, instead of allocating more sperm.

Introduction

Sexual selection continues after copulation when females mate with multiple males whose sperm compete to fertilize ova (Parker, 1970; Simmons, 2005). Under competitive conditions, variation among male ejaculate traits such as the number and percentage of motile sperm, sperm morphology, velocity and longevity have all been demonstrated to affect fertilization success (Birkhead, Martinez, Burke & Froman, 1999; Boschetto, Gasparini & Pilastro, 2011; Casselman, Schulte-Hostedde & Montgomerie, 2006; Dziminski, Roberts, Beveridge & Simmons, 2009; Gage, Macfarlane, Yeates, Ward, Searle & Parker, 2004; Miller & Pitnick, 2002; Smith & Ryan, 2010). According to sperm competition theory, the number of sperm a male inseminates relative to his rivals is the primary determinant of male fertilization success (Parker, 1990; Parker & Pizzari, 2010). Males may be limited in the number of sperm they can produce because their ejaculates represent a substantial energetic expenditure (Dewsbury, 1982; Nakatsuru & Kramer, 1982; Olsson, Madsen & Shine, 1997; Parker, 1982). Depletion of sperm over successive matings has also been demonstrated to affect male fertilization success in many taxa (e.g, Birkhead, Veiga & Moller, 1994; Hines, Jivoff, Bushmann, van Montfrans, Reed, Wolcott & Wolcott, 2003; Preston, Stevenson, Pemberton & Wilson, 2001). Furthermore, sperm depletion may be especially important in species that have a compressed seasonal breeding period because males have restricted storage capacity and less time to replenish sperm stores (Wedell, Gage & Parker, 2002). In males, sperm storage is necessary when spermatogenesis and mating are temporally separated, which may affect pre- and postcopulatory selection (Uller, Stuart-Fox & Olsson, 2010) and ejaculate allocation strategies (Wedell et al., 2002). For example, in many species, larger males may be able to afford greater investment in successive matings because they have more sperm storage capacity while smaller

males may increase investment in individual ejaculates to compensate for lower chances of mating (Bissoondath & Wiklund, 1996; Simmons & Parker, 1992).

Postcopulatory sexual selection is probably a pervasive phenomenon among non-avian sauropsids as evidenced by widespread multiple paternity in the group (Olsson & Madsen, 1998; Uller & Olsson, 2008; Uller et al., 2010). Non-avian sauropsids at high latitudes often occur in dense mating aggregations that may be especially prone to sperm competition as the intensity of sperm competition is predicted to increase with both population density and male-skewed operational sex ratio (OSR) (Duvall, Schuett & Arnold, 1993; Emlen & Oring, 1977; Kvarnemo & Simmons, 2013; Parker & Birkhead, 2013). Furthermore, sperm production, mating, and fertilization are temporally dissociated in many temperate non-avian sauropsids, which favors the evolution of both male and female sperm storage (Olsson & Madsen, 1998; Uller & Olsson, 2008; Uller et al., 2010). Female sperm storage also increases the risk of sperm competition because the ejaculates of two or more males are more likely to overlap within the female reproductive tract prior to ovulation (Birkhead 1998; Birkhead& Møller 1993). Thus, certain species of non-avian sauropsids are especially well suited to investigations of the effects of male sperm depletion and female sperm storage on postcopulatory selection and male reproductive strategies.

Red-sided garter snake (*Thamnophis sirtalis parietalis*) populations in Manitoba, Canada exhibit high density and strongly male-biased OSR mating aggregations during their mating season, which occurs immediately after spring emergence from hibernacula (Gregory, 1974; Shine, Langkilde, Wall & Mason, 2006). In addition, *T.s.parietalis* display a dissociated reproductive

pattern in which sperm production occurs during late summer and does not coincide with peak mating behavior in the spring (Crews, Camazine, Diamond, Mason, Tokarz & Garstka, 1984). Because the testes are quiescent during the breeding season (April-May) (Crews et al., 1984; Krohmer, Grassman & Crews, 1987), males mating in the spring rely solely on stored sperm. Nevertheless, males will mate many times if given the opportunity (Blanchard & Blanchard, 1941; Friesen pers. obs.). Therefore, the reproductive success of male red-sided garter snakes may depend on the amount of sperm they can store and how they allocate that sperm as they become sperm-depleted during their intense, compressed breeding period. Because larger female garter snakes are more fecund than smaller females (Fitch, 1965; Larsen, Gregory & Antoniak, 1993), males may prudently adjust their ejaculates (sperm and/or copulatory plug) according to female size to increase their fitness (Wedell et al., 2002). Male red-sided garter snakes invest heavily in a large, gelatinous copulatory plug which occludes the female cloaca after mating (Friesen, Shine, Krohmer & Mason, 2013; Shine, Olsson & Mason, 2000). The copulatory plug may mitigate the limits on sperm allocation by reducing the chances of a female remating, and thus sperm competition (Shine et al., 2000). These copulatory plugs also prevent sperm leakage from the female's reproductive tract and thus are a functional spermatophores (Friesen et al., 2013).

To date, few studies have quantified ejaculate traits in snakes (Fahrig, Mitchell, Eilts & Paccamonti, 2007; Friesen et al., 2013; Mattson, Vries, McGuire, Krebs, Louis & Loskutoff, 2007; Schulte-Hostedde & Montgomerie, 2006; Tourmente, Cardozo, Bertona, Guidobaldi, Giojalas & Chiaraviglio, 2006; Tourmente, Cardozo, Guidobaldi, Giojalas, Bertona & Chiaraviglio, 2007; Tourmente, Giojalas & Chiaraviglio, 2011; Tourmente, Gomendio, Roldan, Giojalas &

Chiaraviglio, 2009). Most of these studies have only addressed sperm morphology, and all of these studies collected semen samples either by surgery from the caudal ductus deferens, hand-manipulation or from museum specimens; and only one from ejaculates produced from natural matings (Friesen et al., 2013). Tourmente et al. (2007, 2011), are two of the few studies that assess sperm quality in a snake. They measured % motility [total % of sperm moving (Cooper, Noonan, von Eckardstein, Auger, Baker, Behre, Haugen, Kruger, Wang & Mbizvo, 2010)] and straight line velocity with video and computer aided sperm analysis (CASA), and found that sperm from two different species of snake exhibited the highest motility at, or near, preferred body temperature (Tourmente et al., 2007; Tourmente et al., 2011). Tourmente et al. (2011) collected sperm from the caudal ductus deferens and not from natural ejaculates. The copulatory plug of the red-sided garter snake contains almost all of the sperm (Friesen et al., 2013), so we can collect whole ejaculates from natural inseminations to examine ejaculate traits [e.g., sperm mobility, (Froman & McLean, 1996)], total sperm numbers, and remnant plug mass, i.e., the posterior portion of the plug that does not contain many sperm (Friesen et al., 2013).

Using the mobility assay and sperm counts, we tested whether sperm-depletion occurs by comparing sperm counts and ejaculate quality of first and second matings. In addition, we assessed the effect of male size on sperm numbers and ejaculate quality. To test the hypothesis that males adjust their ejaculate in response to the quality of their mates (Wedell et al., 2002), we evaluated the effect of female size on sperm numbers and ejaculate quality. Finally, we evaluated the effect of copulation duration on sperm numbers and the remnant mass of the copulatory plug that remains after the sperm are liberated from the plug.

Methods

Model system

Red-sided garter snakes are small (adult males average 45 cm in snout-vent length [Msvl], and females 68 cm Fsvl), natricine colubrids. Our study population is located near Inwood, Manitoba, Canada (50° 31.58'N 97°29.71'W). This population contains approximately 35,000 individuals (Shine et al., 2006). Males emerge from underground winter brumation sites in late April and form large, dense aggregations around the emergence sites. As females emerge they are met with vigorous courtship from up to 62 males (Shine et al., 2006).

Animal collection

One hundred actively courting males and 50 newly emerged, females that had not mated during that particular spring, but may have mated in previous seasons (e.g., autumn), were collected by hand the day before mating trials, which began on 19 May, 2009. The animals were transported to Chatfield Research Station, 16 km north of the collection site, and housed outdoors in nylon (1Mx1Mx1M) arenas and provided water *ad libitum*. Males and females were housed separately until mating trials began.

Mating trials

Small circular arenas (45 cm dia. x 75 cm tall) were set up indoors at the Chatfield Research Station with each placed under a 250W heat lamp 1m above the animals. We measured external body temperatures with a laser thermometer and adjusted the output of each lamp individually using dimmer switches to maintain optimal body temperature at 29-30°C (Hawley & Aleksiuk, 1975; Kitchell, 1969). Twenty males were randomly assigned to each of the arenas and were allowed to court and mate with newly emerged, unmated females that were

collected the same day as the male. A sex ratio of twenty males to one female is common in and around the dens (Shine, Elphick, Harlow, Moore, LeMaster & Mason, 2001; Shine et al., 2006) and a male-skewed sex ratio facilitates vigorous male courtship behavior (Joy & Crews, 1985). Courtship was observed continuously to allow the timing of copulation duration (±10s) when mating commenced. After copulation was initiated and had lasted one minute, the pair was gently removed to a separate, empty, circular arena so that they could copulate without interference from the other males; this separation also allowed easy observation of the termination of copulations. Thirty of these males mated on the first day (first matings) and these 30 males were allowed to court and mate with another set of newly emerged, unmated females on the next day. Of the 30 males that mated on the first day, 15 mated a second time the next day (second matings) and three of those males mated a third time. Males and females were weighed and measured within one hour after the matings trials terminated each day.

Ejaculate collection

Less than 30s after copulation terminated, each female was inspected for a copulatory plug. Each plug was removed by gently running a blunt probe around the plug to separate it from the walls of the vaginal pouch (Friesen et al., 2013; Shine et al., 2000). Once removed, the plug was placed in a 1.5 ml microcentrifuge tube in 1ml of Modified Ham's F-10 medium and 10 μg/ml of the antibiotic Gentamicin sulfate (Cat # 99175, Irvine Scientific; 21 mM HEPES buffer, 4 mM sodium bicarbonate, 1 mM calcium lactate, 0.5 mM magnesium sulfate, 5 mg/ml (0.5%) human albumin; e.g., (Friesen et al., 2013; Mattson et al., 2007). The females' vaginal pouch was lavaged with the same Ham's F-10 medium using a 20ga. intubation needle affixed to a 1ml syringe and subsequently added to the 1.5ml tube with the plug. The fluid from the vaginal

wash contains any sperm not embedded within the plug during copulation. The tubes were placed in a refrigerator at 4°C for two days and were gently agitated three times daily (every eight hours) to aid the liberation of sperm embedded within the plug (Friesen et al., 2013). *Thamnophis sirtalis parietalis* experience and survive temperatures during winter brumation below 1°C , and summer temperatures over 30°C (Hawley & Aleksiuk, 1975; Hoskins & Aleksiuk, 1973; Kitchell, 1969; Lutterschmidt, LeMaster & Mason, 2006). In addition, it takes two days for the spermatophore to dissolve under natural conditions (i.e., within the female's cloaca) (Shine et al., 2000). During this period, females are often exposed to temperatures below 4°C for days, and/or wide temperature swings (Hawley & Aleksiuk, 1975, Friesen unpublished data). The dissolution of the plug was evidenced by a dense "cloud" of sperm above the plug (Friesen et al., 2013). In this way, we collected 45 ejaculates for sperm counts and the mobility assay. Once the sperm were liberated, a small piece of the posterior portion of the plug remained. This portion contains almost no sperm (Friesen et al., 2013) and thus represents the investment in passive mate guarding.

Mobility assay

Sperm mobility has been demonstrated to be a heritable determinant of fertilization success and sperm competitive ability under fluctuating social environments in fowl (Birkhead et al., 1999; Froman, 2007; Froman, Feltmann, Rhoads & Kirby, 1999; Pizzari, Cornwallis & Froman, 2007; Pizzari, Froman & Birkhead, 2002; Pizzari, Worley, Burke & Froman, 2008b). Sperm mobility measures the net movement of a population of sperm through a dense medium that depends on % sperm motility, straight line velocity (Froman & Feltmann, 2000) and mitochondrial function (Froman & Kirby, 2005). The mobility assay captures important features

of whole ejaculate quality that sperm motility alone does not assess. When more expensive and less portable computer-aided sperm analysis systems are not readily available, the mobility assay is easy and objectively quantified under field conditions using an inexpensive, battery operated, portable spectrophotometer (Froman & McLean, 1996).

The mobility assay measures the ability of a population of sperm cells to swim against resistance through dense medium, in this case 3% (wt/vol) Accudenz® in Modified Ham's F-10 medium (Mattson et al., 2007). The number of sperm that have adequate velocity to penetrate the medium is proportional to the absorbance. Absorbance was measured using a spectrophotometer (ARS 596A Sperm Mobility Analyzer) at 550 nm, which correlates with the number of sperm that penetrate the medium. On the third day after the mating trials (when the sperm were liberated from the plug), we conducted a mobility assay modified from Froman and McLean (1996). The sperm samples were kept in an insulated plastic container with ice packs until one hour before the mobility assay, and then placed in a 28-30°C water bath for an hour before recording absorbance readings. Volumes (1.5ml) of 3% (wt/vol) Accudenz® were pipetted into each of two standard polystyrene cuvettes, each cuvette was covered with 1 cm^2 piece of Parafilm®, and placed in the 28-30°C water bath one hour before the assay. During spring emergence, both males and females experience temperature swings from 2-20°C within a 24 hour period and cold snaps (-6°C to 4°C) that last for days, before temperatures quickly increase the next day (Friesen unpublished data), and males engage in courtship at body temperatures as low as 5°C. Furthermore, *T.s.parietalis* can warm their bodies, and thus the sperm to their preferred body temperature of 29-30°C, in less than 20 minutes. Therefore, these incubation conditions are biologically relevant.

After incubation, we estimated % motility (ranged from 70-99%) and inspected the samples for bacterial infection (0% infected) using a microscope at 50x and 400x magnification respectively. Bubbles were gently tapped from the Accudenz® just prior to blanking the cuvette in the spectrophotometer. Each ejaculate sample in its 1.5 ml tube was gently inverted three times to mix the sperm, and then set aside for 30s to allow undissolved plug debris to settle. A 150 µl sample of the "ejaculate" was overlaid on the surface of the Accudenz® solution, and the cuvette was returned to the 28-30°C water bath, the preferred body temperature of *T.s.parietalis* (Aleksiuk, 1976). Tourmente *et al.* (2011) demonstrated that sperm motility was highest near the preferred body temperature in two different species of snake. After a 5 min interval (T1), the cuvette was transferred to the spectrophotometer and absorbance at 550 nm recorded, and again at a 10 min interval (T2). Finally, we made one final absorbance reading after vigorously mixing the contents of the cuvette ("Mix") to control for variation in sperm numbers within and among samples. This procedure was repeated with the same sample for a duplicate reading.

Sperm counts

After absorbance was measured, the contents of each cuvette and the original tube containing the plug were collected in separate 1.5 ml tubes, centrifuged for 10 minutes at 600 rpm. Sperm from each cuvette and the tube containing the plug were preserved for counts in 3% paraformaldehyde in phosphate buffered saline (pH 7.2). Sperm counts were made in triplicate for each cuvette as well as the tube containing the sperm and the plug using a Petroff-Hausser Counter (cat. # 3900, Hausser Scientific). The total number of sperm in each ejaculate was estimated as the sum of the average counts for each replicate cuvette and from counts made

on the tube containing the plug and the remaining sperm. The plug mass was not recorded immediately after copulation because we felt it was important to minimize handling of the ejaculate prior to the mobility analysis. However, once the sperm were liberated (dissolution), the mass of the relatively sperm-free posterior portion of the plug was recorded to the nearest 0.01g (Mettler BB2400).

Statistical analysis

Statistical analyses were conducted in Sigmaplot 11.0 and/or XLSTAT 2012.6.02 (GLM mixed models). Sperm counts were ln transformed to equalize variance. Body Condition Index (BCI) was calculated as the residual derived from the linear regression of male mass on male SVL (Bradshaw, 1986; Moore, Lerner, Lerner & Mason, 2000) fitted in Sigmaplot 11.0 (Adj. R^2 = 0.875, $P < 0.0001$). The average within-pair coefficient of variation ($SD/\bar{X}$) for all pairs of absorbance readings (i.e., mobility scores, N = 35 pairs) was 0.061. Absorbance readings of mixed samples (Mix) and sperm counts for each replicate cuvette were highly correlated (R = .921, Adj. $R^2 = 0.846$, $P < 0.001$).The plugs were allowed to dissolve for the same period (± 1h), i.e., the interval between mating and when the assay was run, and not correlated with the time of day when the assay was run (hereafter "start time") ($R^2 = 0.018$, $P = 0.540$). However, start time was positively correlated with absorbance readings at 5 min. (T1: $R^2 = 0.236$, $P = 0.019$) but not 10 min. (T2: $R^2 = 0.146$, $P = 0.072$) or those of the samples after mixing (Mix: $R^2 = 0.006$, $P = 0.732$). The effect of start time became pronounced after controlling for sperm numbers, *i.e.*, T2 absorbance/Mix absorbance (T2/Mix: $R^2 = 0.414$, $P = 0.001$). Therefore, the effect of start time of the assay was statistically removed by using the standardized residuals from a regression analysis of T2/Mix as a function of start time for most of the analyses. We used a

generalized linear regression, mixed models to conduct repeated measures analysis on mobility scores, with mate number as a fixed effect, male identity as a random effect and start time of assay as a covariate (REML estimation method, and compound symmetry covariance structure in XLSTAT).

Results

Sperm numbers

Five plugs were excluded from the analysis due to excessive clumping due to incomplete deliquescence of the plug, which precluded repeatable sperm counts. Sperm counts were estimated from the remaining 45 plugs which ranged from 1.19×10^7 to 7.01×10^8 [$\bar{X} = 7.98 \times 10^7$ (SEM = 1.89×10^7)]. Sperm counts were not affected by male size ($R^2 = 0.004$, $P = 0.673$) or copulation duration ($R^2 = 0.027$; $P = 0.451$) **Figures 1a and 2b respectively**. Males did not allocate more sperm to larger females (Fsvl, $R^2 = 0.047$, $P = 0.322$). However, copulation duration was positively correlated with female mass (Fmass, $R^2 = 0.187$, $P = 0.024$). There was weak evidence of a positive, but non-significant, correlation between the mass of the plug after liberation of the sperm (hereafter, remnant plug mass) and female size (Fsvl, $R^2 = 0.158$, $P = 0.060$), and remnant plug mass was significantly correlated with copulation duration ($R^2 = 0.221$, $P = 0.018$). Remnant plug mass was not related to the number of sperm inseminated ($R^2 = 0.011$, $P = 0.611$).

Sperm numbers decreased significantly from the first matings to the second, with first matings producing 5.8 times more sperm on average than second matings (N = 12, Repeated measures

ANOVA, $F_{1, 16} = 13.011$, P = 0.004), **Figure 2**. Sperm numbers from three plugs produced from third matings were $4.75x10^6$, $8.61x10^7$ and $3.66x\ 10^8$; this low number of plugs prohibits a meaningful statistical analysis.

Limiting the analysis to the male's first matings only, male size (snout to vent length: svl) and body condition (BCI: residuals of Mmass | Msvl generated by regression analysis) did not predict sperm numbers (Msvl, $R^2 = 0.105$, P = 0.131; BCI, $R^2 = 0.025$, P = 0.475)

Sperm mobility

The initial analysis was confined to the male's first matings. Seven individuals were removed from the analysis due to unusually high variance in absorbance readings that were attributable to debris from plug material. Time of day when the assay was conducted had a significant effect on mobility (T2/Mix) for first matings ($R^2 = 0.341$, P < 0.001), thus standardized residuals from this regression analysis were used for the remainder of the analyses. Among-male variation in mobility was significant (Kruskal-Wallis ANOVA on ranks; H = 37.265, df = 22, P = 0.022), **Figure 3**. Analysis of the first matings only revealed a significant negative relationship between sperm mobility and male size (Msvl: $R^2 = 0.122$; P = 0.042), **Figure 4**. Mobility was not correlated with total sperm inseminated ($R^2 = 0.035$, P = 0.392) or BCI ($R^2 = 0.039$, P = 0.324).

Mobility of first and second matings by 15 males was analyzed to assess sperm quality after successive matings (male mate #). Mobility improved significantly from first to second matings in these 15 males [GLM mixed model; $F_{1, 29} = 10.530$, P = 0.003), **Figure 5**. Female size was not significantly different between first and second matings (Fsvl, $t_{df=14} = 0.981$, P = 0.343) and the

difference in female size (Fsvl) from the first mating to the second was not correlated with the change in a male's sperm mobility (R^2 = 0.022, P = 0.595). Male size was not significantly correlated with mobility in the second mating (R^2 = 0.000, P = 0.856).

Discussion

We used the framework of sperm competition theory to investigate within-population variation in sperm traits. Uncovering correlates of variation in sperm number offers insight into how postcopulatory selection operates within a population (Birkhead & Pizzari, 2002; Parker, 1971). To our knowledge, this is the first direct quantitative investigation of the correlates of multiple ejaculate traits from natural inseminations in any snake and, more generally, only one of a few in squamates (e.g., Olsson, 2001; Tokarz, 1999; Tokarz & Slowinski, 1990).

Sperm limitation

Males are sperm limited as there was an average of over a five-fold decrease in sperm numbers from first to second matings. Sperm numbers decreased equally from first to second matings regardless of male size; thus, neither male size-class saved sperm for second matings, suggesting males allocate a maximum amount of their sperm to first matings. The decline in sperm numbers between matings may translate to a decline in paternity, but that outcome needs to be tested. Without knowledge of sperm attrition rates within the female reproductive tract, we do not know when a male should be considered sperm-depleted. Twenty percent of females remate in arenas (Shine et al., 2000), and if they are remating to receive adequate numbers of sperm to fertilize their ova, then this percentage may represent the fraction of

males that are sperm-depleted. An experiment could be designed to test for a correlation between female remating rate and male mate number.

Ejaculate quality

Ejaculate quality has also been shown to be important in sperm competition. In domestic fowl inseminated with experimentally manipulated heterospermic ejaculates, low quality sperm that had a numerical advantage fertilized eggs produced early in the laying order (Pizzari et al., 2008b). However, high quality sperm overcame the numerical disadvantage to fertilize most of the eggs over the whole clutch (Pizzari et al., 2008b). Findings such as this reveal the dynamic nature of sperm competition when sperm are stored within the female reproductive tract. In our study, there was significant among male variation in ejaculate quality, as measured by mobility. There are a few explanations that may explain the variation. All of the males we collected had seemed to be newly emerged, had good body condition and were vigorous courters. However, one could not know if a newly emerged male had mated the previous fall, which may account for variation in both sperm quantity and quality. Schulte-Hostedde and Montgomerie (2006) also failed to find significant effect of body condition on sperm concentration in water snakes, but did find positive correlation with hematocrit, which we did not measure. In our study, smaller males had higher mobility than larger males in first matings, but this effect disappeared in second matings. Overall, ejaculate quality improved on second matings and the size effect disappeared. It is conceivable that the improvement in quality makes up for the decline in sperm numbers from the first mating to the second in a competitive context. After correcting for the number of sperm (i.e. T2/Mix), an increase in sperm mobility

represents a larger proportion of sperm that moves against resistance (Froman & Feltmann, 2000), which has been shown to predict sperm competiveness in fowl (Birkhead et al., 1999; Pizzari et al., 2008b). Thus, an increase in the quality of the ejaculate, even with a decrease in sperm numbers, may explain why paternity was unaffected by male mate number (Friesen, Kerns & Mason, in review). However, a non-adaptive, mechanistic explanation for the increase in sperm mobility seems more straightforward. In *T.s. parietalis*, spermatogenesis occurs in August through September (Krohmer et al., 1987) and as sperm are produced, they first fill the most caudal portion of the ductus deferens (Friesen per. obs.); these sperm would also be the first to be inseminated. As sperm function declines with sperm age (Pizzari, Dean, Pacey, Moore & Bonsall, 2008a), earlier inseminations most likely contain older sperm. Larger males may have made more sperm and thus have more "old" sperm relative to new sperm in their first ejaculate.

Other factors that may affect sperm quality and quantity, such as parasite loads and intrinsic resistance and tolerance of parasites (e.g., Liljedal, Folstad & Skarstein, 1999; Måsvaer, Liljedal & Folstad, 2004) deserve immediate investigation. Males of this species harbor multiple genera of helminth parasites including trematodes that are found in close association with the gonads and fat stores (Gibson & Rabalais, 1973; Rau & Gordon, 1978, Uhrig pers. comm.). It is possible that these parasites directly damage the gonads, reduce energy availability for gamete production, and/or elicit an immune response that in turn alters gamete production, all consequences of parasitism observed in other host species (Figenschou, Folstad, Rudolfsen, Hanssen, Kortet, Skau, Killie, Oskam & Strand, 2013; Lafferty & Kuris, 2009). This question is being investigated currently.

Ejaculate adjustment

Males did not adjust the quantity or the quality of their sperm in accordance to female size or mass in contrast to what has been found in numerous taxa (Delbarco-Trillo, 2011; Kelly & Jennions, 2011; Wedell et al., 2002). Males may inseminate as much sperm as they are able during their first mating regardless of female size because the risk of sperm competition is high in these populations. Eighty-five percent of litters produced by females that were allowed to mate only once during the spring exhibited multiple paternity (2-3 fathers, Friesen et al. in prep.). Furthermore, although most garter snakes form mating aggregations at spring emergence (Gregory, 1984), the aggregations of the Interlake region are by far the largest (Shine et al., 2006). Perceived risk of sperm competition (probability that a female is polyandrous) is perpetually high among rival males in these larger mating aggregations and in our arena trials as well (20 males per female). Males do prefer to court unmated females without copulatory plugs (Shine et al., 2000) suggesting that they reduce energetic investment in courtship based on perceived intensity of sperm competition (Kvarnemo & Simmons, 2013). Males are predicted to decrease ejaculate investment as the intensity of sperm competition (intensity = number of competing ejaculates Kvarnemo & Simmons, 2013) decreases, but this prediction has mixed support (Kelly & Jennions, 2011). Regardless, in red-sided garter snakes, sperm stored within the female reproductive tract from autumnal matings would leave no cues that would indicate the intensity of sperm competition for males to act on.

Although sperm quantity and quality were not correlated with female size, we did find that copulation duration was correlated with both the remnant plug mass and female body mass,

suggesting males allocate more copulatory plug material to larger females. The remnant plug mass represents the posterior-most portion of the copulatory plug, which contains relatively few sperm (Friesen et al., 2013). Shine *et al.* (Shine et al., 2000) found a similar trend between whole plug mass and female size (with presumably larger cloacae), but no relationship with copulation duration. The lack of a relationship between sperm numbers and copulation duration in the current study suggests it may be that the variation in copulation duration is explained by allocating more plug material to bigger females. To the extent that plugs act as passive mate-guarding devices (Shine et al., 2000), males may be allocating more mate-guarding resources to larger females instead of more sperm.

The findings of our current study, namely the decrease in the number of sperm inseminated across successive matings, suggest that there may be an upper limit on the number of matings in which male red-sided garter snakes can gain paternity. Depletion of sperm may be a general problem for males with a dissociated reproductive pattern (Crews et al., 1984). How variation in ejaculate traits translates to fertilization success has been extensively documented in other species (reviewed in Birkhead & Møller, 1998), and paternity analysis coupled with evaluation of each male's ejaculate would be useful to elucidate the effects of sperm limitation on males and females in this system. Males may trade-off investment in sperm for investment in the paternity assurance of the copulatory plug. The energetics mediating this tradeoff is fertile ground for exploration.

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Figure 1: Regressions plots of the relationship between sperm numbers from natural ejaculates and **a)** male size (Msvl; $R^2 = 0.004$; P = 0.673) **b)** The relationship between sperm inseminated and copulation duration ($R^2 = 0.027$; P = 0.451).

Figure 2: Before and after line plot of the decrease in sperm numbers from the first to second matings (Repeated measures ANOVA, $F_{1, 16} = 13.011$, P = 0.004).

Figure 3: Intrapopulational variation in ejaculate quality as measured by standardized mobility score of first matings.

Figure 4: Effect of male size on sperm mobility of first matings.

Figure 5: a) Relationship between male size (Msvl), first matings (open triangles and dotted line; $R^2 = 0.110$, P = 0.123), second matings (closed circles and solid line; $R^2 = 0.000$, P = 0.856) and sperm mobility. The slopes are not significantly different (ANCOVA, interaction of Msvl x Mate #, P = 0.198). **b)** Before and after line plot of the increase in mobility from the first to the second matings [GLM mixed model (REML)], with male mate # as a fixed effect, male ID as a random effect and start-time of assay as a covariate; $F_{1, 29} = 10.530$, P = 0.003).

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