Size dependence in non-sperm ejaculate production is reflected in daily energy expenditure and resting metabolic rate

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ABSTRACT

The non-sperm components of an ejaculate, such as copulatory plugs, can be essential to male reproductive success. But the costs of these ejaculate components are often considered trivial. In polyandrous species, males are predicted to increase energy allocation to the production of non-sperm components, but this allocation is often condition dependent and the energetic costs of their production have never been quantified. Red-sided garter snakes (Thamnophis sirtalis parietalis) are an excellent model with which to quantify the energetic costs of non-sperm components of the ejaculate as they exhibit a dissociated reproductive pattern in which sperm production is temporally disjunct from copulatory plug production, mating and plug deposition. We estimated the daily energy expenditure and resting metabolic rate of males after courtship and mating, and used bomb calorimetry to estimate the energy content of copulatory plugs. We found that both daily energy expenditure and resting metabolic rate were significantly higher in small mating males than in courting males, and a single copulatory plug without sperm constitutes 5–18% of daily energy expenditure. To our knowledge, this is the first study to quantify the energetic expense of size-dependent ejaculate strategies in any species.

KEY WORDS: Energetic costs of reproduction, Size dependence, Copulatory plug, Ejaculates, Thamnophis

INTRODUCTION

In systems where females are polyandrous, males are selected to increase sperm and seminal fluid production (Parker, 1970, 1984, 1990, 1998; Parker and Pizzari, 2010; Tazzyman et al., 2009). Limits on energy allocation are a fundamental assumption in sperm competition models that predict context-dependent ejaculate allocation strategies (size dependence: e.g. Parker, 1990; Parker and Pizzari, 2010; condition dependence: e.g. Perry and Rowe, 2010; Rahman et al., 2013; Simmons et al., 1996; Simmons and Kotiaho, 2002; Simmons and Parker, 1992). In general, these models predict that male ejaculate allocation is affected by the cost of mate acquisition (Tazzyman et al., 2009). Male phenotypes that are more likely or have lower costs to obtain a mate are expected to expend less on ejaculates than male phenotypes that are less likely or have higher costs to obtain a mate (Parker and Pizzari, 2010; Tazzyman et al., 2009). These differences in allocation strategies should be reflected in energetic costs associated with courtship and mating (i.e. ejaculates). For example, a recent analysis of eutherian mammals demonstrated that higher basal metabolic rate (BMR: the energy required for basic bodily functions in endotherms) is positively associated with better quality ejaculates (higher sperm motility, viability and length; Lüpold, 2013; Tourmente et al., 2011). Sperm motility and viability can be greatly enhanced by non-sperm components of the ejaculate, such as seminal fluid proteins (Poiani, 2006).

In most animals, sperm is only a tiny fraction of the ejaculate (Cameron et al., 2007; Eberhard and Cordero, 1995; Pitnick et al., 2009; Poiani, 2006). The non-sperm components of the ejaculate (e.g. ions, proteins, sugars and steroid hormones; Poiani, 2006) are vital to male fertilization success in the context of sperm competition and cryptic female choice (Cameron et al., 2007; Eberhard and Cordero, 1995; Pitnick et al., 2009). These non-sperm components may constitute a substantial energetic expense that is predicted to trade off against other sperm traits (Cameron et al., 2007; Parker and Pizzari, 2010; Pitnick et al., 2009; Wedell et al., 2002) and mate acquisition (Kvarnemo and Simmons, 2013; Parker et al., 2013), and may contribute to the evolution of alternative and/or size-dependent mating tactics (Neff and Svensson, 2013; Oliveira et al., 2008). At a fundamental level, energy is likely to mediate such trade-offs (Lüpold, 2013), but empirical data of the energetic costs of producing the non-sperm components of the ejaculate are lacking (Parker and Pizzari, 2010; Tazzyman et al., 2009; Wedell et al., 2002). Measuring the energetic cost of these components is difficult, in part because spermatogenesis and seminal fluid synthesis usually occur simultaneously. However, this difficulty may be overcome when spermatogenesis is temporally dissociated from mating and seminal fluid production, as is the case in the species in this study.

Red-sided garter snakes (Thamnophis sirtalis parietalis Say 1823) are an exceptional model species for the study of energetic costs associated with courtship and mating because males fast during the spring mating season (O’Donnell et al., 2004). As a consequence of fasting, mating is segregated from other physiological activities that would confound interpretation of metabolic measurements (e.g. foraging, digestion and absorption). In addition to the temporal separation of mating and feeding, red-sided garter snakes display another convenient characteristic for the study of the costs of seminal fluid production: they exhibit a dissociated reproductive pattern (Crews, 1984), in which spermatogenesis occurs during late summer and ceases as the testes regress in the autumn; males then store sperm over winter for use in the spring mating season (Crews, 1984; Crews et al., 1984; Krohmer et al., 1987). While the ductus deferens and testes contribute a few substances to semen in the spring, most are produced in the late summer and autumn when spermatogenesis is ongoing (Marinho et al., 2009); however, the largest non-sperm contribution to the seminal fluid is produced during the spring (Friesen et al., 2013; Krohmer, 2004a).
The predominate component of the seminal fluid of the red-sided garter snake forms a large, gelatinous copulatory plug (hereafter ‘plug’) that is deposited within the female’s vaginal pouch during copulation (Devine, 1975; Friesen et al., 2013; Shine et al., 2000a). The plug is a spermatophore that encases the sperm (sensu Mann, 1984) and thus prevents sperm leakage or ejection while also delaying females from remating, and thus is crucial for male reproductive success (Devine, 1975, 1984; Friesen et al., 2013; Shine et al., 2000a). The plug material is produced in the kidneys (Friesen et al., 2013) within acocrine cells of the distal tubules collectively called the renal sexual segment (Aldridge et al., 2011). The acocrine cells of the renal sexual segment are sexually dimorphic and hypertrophied and actively produce plug material in males during the spring mating season (Krohmer, 2004a,b). Storage is limited to secretory vesicles in the acocrine cells (Aldridge et al., 2011) and, therefore, accessory seminal products (i.e. the plug) are replenished after mating if they are exhausted (Bishop, 1959; Krohmer, 2004b; Krohmer et al., 2004). Males are limited in the number of plugs they can deposit over a short period (1–2 plugs per day; Friesen et al., 2013; Shine et al., 2000a), but males will still copulate and not leave a plug (Friesen et al., 2013). In this system, the temporal separation of the key physiological processes that contribute to male mating success allows us to separate the cost of producing non-sperm ejaculate components and the cost of courtship from the cost of sperm production.

In this study, we focused on the cost of producing the copulatory plug. There are two principal lines of evidence suggesting that plug production is energetically costly. Firstly, the plug of the red-sided garter snake is the largest such plug among reptiles (Olsson and Madsen, 1998). On average, larger males deposit slightly larger plugs and longer copulations tend to produce larger plugs as well. Nevertheless, there is no relationship between male size and copulation duration, indicating that larger males deposit plug material more quickly, perhaps because they have greater storage capacity and associated delivery ducts than smaller males (Friesen et al., 2013, 2014c,d; Shine et al., 2000a). Secondly, copulating males have increased blood lactate compared with courting males, which suggests that mating incurs an additional energetic cost over courtship alone (Shine et al., 2004b).

Furthermore, energy allocation to plug production may differ among males based on male size and/or body condition as these factors affect male mating success in this species, with larger males being more likely to mate (Shine et al., 2001c, 2000b, 2006b). When male phenotypes differ in their likelihood and/or costs of acquiring mates, it is predicted that males with lower costs of obtaining a mate will also have reduced ejaculate expenditure (Parker and Pizzari, 2010; Tazzyman et al., 2009).

We addressed two specific questions by measuring daily energy expenditure and post-activity resting metabolic rates of courting males, and courting and mating (hereafter ‘mating’) males: (1) is copulatory plug production energetically more costly?; and (2) is the energetic cost of courtship and/or mating dependent on body condition and/or body size? These questions address fundamental assumptions of models of sperm competition and how energy budgets and male size may affect the evolution of ejaculate traits (Hayward and Gillooly, 2011; Lüpold, 2013; Parker and Pizzari, 2010; Tazzyman et al., 2009).

RESULTS

Daily energy expenditure

Mean (±s.e.) daily energy expenditure (DEE; kJ day⁻¹) for all animals was 7.33±0.616 kJ day⁻¹. Our estimates of DEE are consistent with those previously published using doubly labelled water (DLW) in free-ranging garter snakes (Peterson et al., 1998, 1999). DEE values were normally distributed (Shapiro–Wilk test $W=0.972$, $P=0.312$) and variance among groups was homoscedastic (Levene’s test $F=0.782$, $P=0.510$). To specifically test for size effects on DEE we modelled mass-specific DEE (kJ day⁻¹ g⁻¹) with ANCOVA (mass-specific DEE ≈ male size×treatment×male size×treatment: ANCOVA; $R^2=0.287$, $F_{3,44}=5.893$, $P=0.002$).

Mean mass-specific DEE was significantly higher in the mating males than in the courting males ($F_{1,47}=5.643$, $P=0.022$). There was a significant treatment×male size interaction ($F_{1,47}=12.032$, $P=0.0012$); the mass-specific DEE of the courting males increased with male size while that of the mating males decreased with male size, meaning that smaller males expended more energy when they mated than did the larger males. The difference in DEE between courtship and mating was highly significant for males under 46 cm SVL (see Fig. 1; Johnson–Neyman technique; White, 2003).

Within the mating group, there were 52 matings spread among 24 males, an average of 2.17 matings per male. All but one male mated. Seven males mated once, eight males mated twice, one male mated four times, and two males mated five times. The males with five matings were the third and fifth largest males. However, the number of matings
was not associated with initial male mass (linear regression, $R^2=0.084$, $F_{1,22}=0.200$, $P=0.171$). There was no relationship between DEE and the number of matings a male achieved ($R^2=0.012$, $F_{1,22}=0.266$, $P=0.611$) nor between DEE and the sum of the copulation duration for all one male’s matings ($R^2=0.022$, $F_{1,22}=0.494$, $P=0.489$). There was a weak relationship between total mass loss and the number of copulations per male ($R^2=0.161$, $F_{1,23}=4.231$, $P=0.052$; Fig. 2), and total time in copulo ($R^2=0.149$, $F_{1,23}=3.850$, $P=0.063$). Removal of a large male (54.6 g initial mass) that lost 9.5 g, but only mated once, yielded a significant relationship between total mass loss and the number of copulations a male achieved ($R^2=0.294$, $F_{1,22}=10.151$, $P=0.004$), and between total mass loss and time in copulo ($R^2=0.258$, $F_{1,22}=7.302$, $P=0.013$). However, there was no relationship between the number of copulations, and (a) body size (SVL: $R^2=0.283$, $P=0.179$), (b) body condition index [residuals of regression of ln(mass) as function of ln(SVL): $R^2=0.110$, $P=0.606$], (c) DEE ($R^2=0.056$, $P=0.796$), or (d) proportional mass loss ($R^2=0.2235$, $P=0.267$).

### Baseline (standard) metabolic rate

There was a significant increase in standard metabolic rate (SMR) over the temperature range 5–30°C [mixed model with temperature as a fixed effect, mass as a covariate and male ID as a random effect, reduced maximum likelihood (REML) estimation method: Type III test of fixed effects temperature: $F_{1,42}=128.05$, $P<0.0001$; mass: $F_{1,42}=1.29$, $P=0.262$]. Our SMR values for this population closely match previously published data with the exception of the absence of a sharp downward shift between 15 and 20°C as was seen in Aleksiuk (1971). Differences in SMR among temperatures were tested after Bonferroni correction for multiple comparisons. We were not able to resolve differences in SMR between 5 and 15°C, although an upward trend is apparent. Mean SMR values at all other temperatures were significantly different from one another (Fig. 3).

### Metabolic rate (resting metabolic rate) associated with copulatory plug production

We were specifically interested in size effects based on the results of the DLW experiment. Therefore, we had deliberately selected large and small males for this experiment, which resulted in a bi-modal size distribution; hence, we used ANOVA instead of ANCOVA to test for differences among size classes and treatments. Mean mass of males in the small size class was 17.6±0.9 g (11.3–29.9 g) and for males in the large size class it was 45.8±0.7 g (32.3–60.3 g). There were significant differences in mean mass-specific resting metabolic rate (RMR ($\dot{V}_{O_2} \cdot g^{-1} \cdot min^{-1}$)) among treatments and size classes (ANOVA $F_{3,74}=16.625$, $P<0.001$); specifically, mean RMRs of all groups differed from one another except between large mating males and small courting males (Fig. 4). Mean SMRs of males at
30°C (N=10) were compared with mean RMRs of the treatments separated by size class. Note that mass was not a significant predictor of SMR (see results above), 30°C is the preferred body temperature for this species, and this was the temperature at which RMR was measured. Mean RMRs of mating, but not courting, males were significantly higher than mean SMRs (ANOVA F_{4,83}=14.515, P<0.001; pairwise comparisons of RMR in each size class with SMR; Table 1). There was no relationship between mean RMR and copulation duration of mating males regardless of size class (ANCOVA: R^2=0.064, F_{2,46}=1.573, P=0.218; Type I sum of squares analysis: copulation duration: F_{1,47}=0.221, P=0.641; size class: F_{1,47}=2.926, P=0.094). This suggests that recovery from copulation did not affect RMR.

Metabolic substrates: respiratory quotient
Mean respiratory quotient (RQ=\frac{V_{CO2}}{V_{O2}} where \(V_{CO2}\) is the rate of CO2 production and \(V_{O2}\) is the rate of O2 consumption) across treatments and size classes was 0.743. Mean RQ of the mating males (median=0.71) was significantly lower than that of the courting males (median=0.76) [Kruskal–Wallis test, K_i=24.091 (where the subscript i indicates d.f.), P<0.001]. A non-parametric test was used because these data failed a normality test (Shapiro–Wilk, P<0.05). This difference in RQ between courting and mating males was driven by small mating males having a significantly higher RQ (Kruskal–Wallis test, K_i=31.394, P<0.001; multiple comparisons using Dunn’s method; Fig. 5). This suggests that small, mating males were using different metabolic substrates after mating from those used by the larger males or small, courting males.

Energy content of the copulatory plug
The dry plug mass collected for microbomb calorimetry ranged from 0.015 to 0.046 g with a mean mass of 0.028±0.002 g. Water content ranged from 0.042 to 0.178 g with a mean of 0.075±0.007 g. The constant volume heating value ranged from 5.01 to 36.51 kJ g\(^{-1}\) with a mean value of 22.90±3.15 kJ g\(^{-1}\). There was no difference in dry mass (in g: t_{17}=1.160, P=0.262), water content (in g: t_{17}=0.507, P=0.618), or total energy (in kJ: t_{17}=2.039, P=0.057) between plugs produced by vasectomized or control males. However, it is interesting that the energy density of plugs produced by vasectomized males (25.75±1.40 kJ g\(^{-1}\)) was ~26% greater than that of control males (20.35±1.97 kJ g\(^{-1}\)) (t_{17}=2.196, P=0.042), perhaps because sperm are mostly water. However, the proportion of water in the plugs did not differ between treatments (water mass/total wet mass of the plug: t_{17}=0.706, P=0.490). We multiplied the energy density and the mass of each plug produced by a vasectomized male to obtain the total energy in a plug without sperm, which ranged from 0.36 to 1.35 kJ. Accounting for treatment, male size did not affect energy density (in kJ g\(^{-1}\): male mass, F_{1,18}=0.0785, P=0.393) or total energy per plug (in kJ: male mass, F_{1,18}=2.057, P=0.177). Female mass did not strongly affect energy density (in kJ g\(^{-1}\): R^2=0.154, F_{1,18}=3.091, P=0.097), dry plug mass (in g: R^2=0.152, F_{1,18}=3.039, P=0.099), or total energy per plug (in kJ: female mass, F_{1,18}=3.704, P=0.072), but plugs from larger females contained significantly more water (in g: female mass, F_{1,18}=9.897, P=0.006). We note that this study has smaller sample sizes than those that have found significant effects of male and female size on plug mass in this species (Friesen et al., 2013, 2014c,d; Shine et al., 2000a).

DISCUSSION
Costs of reproduction for females far outweigh those of males (Hayward and Gillooly, 2011); however, evidence also suggests male investment in courtship, mate acquisition and territorial defence is not trivial (Galimberti et al., 2007; Kotiaho et al., 1998; Lane et al., 2010; Marler et al., 1995; Oberweger and Goller, 2001; Ryan, 1988; Vehrencamp et al., 1989). The few studies to quantify costs of ejaculate production have found that sperm production is often limited (e.g. tetra fish, Hypessobrycon pulchripinnis; Nakatsu and Kramer, 1982), that males undergoing spermatogenesis quickly lose mass, which suggests spermatogenesis is energetically taxing (adder, Vipera berus; Olsson et al., 1997; reviewed in Wedell et al., 2002), and that ejaculate production can be more energetically costly than courtship (salamander, Desmognathus ochrophaeus; Marks and Houck, 1989). With three separate experiments using three different methods, we show that copulatory plug production alone, sans spermatogenesis, generates increased metabolic rates similar to those induced during pregnancy in this species. The average RMR of gravid female garter snakes during late pregnancy (Thamnophis sirtalis: \(V_O2=0.0023\) ml g\(^{-1}\) min\(^{-1}\); recalculated into common units from Birchard et al., 1984) is similar to the post-activity RMRs of males that engaged in courtship (\(V_O2=0.0025\) ml g\(^{-1}\) min\(^{-1}\)). Furthermore, we demonstrate that mating males have even higher energetic expenditures than males engaged only in courtship,

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**Table 1. All pairwise multiple comparison procedures of RMR of the different size classes with SMR**

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>LS means±e.m. ((\dot{V}_{O2}) ml g(^{-1}) min(^{-1}))</th>
<th>t-value</th>
<th>Comparison with SMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-M</td>
<td>18</td>
<td>0.00396±0.00014</td>
<td>5.098</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>C-L</td>
<td>19</td>
<td>0.00355±0.000169</td>
<td>3.454</td>
<td>0.005</td>
</tr>
<tr>
<td>C-S</td>
<td>20</td>
<td>0.00331±0.00885</td>
<td>2.478</td>
<td>0.060</td>
</tr>
<tr>
<td>C-L</td>
<td>21</td>
<td>0.00283±0.000135</td>
<td>0.433</td>
<td>0.666</td>
</tr>
</tbody>
</table>

LS, least square; RMR, resting metabolic rate; SMR, standard metabolic rate; CM, males that courted and mated (mating); C, males that courted only (courting); L and S, size class where L>35 g and S<30 g.

**Fig. 5. Respiratory quotient (RQ) among treatments and size classes. CM, males that courted and mated (mating); C, males that courted only (courting); L and S, size class where L>35 g and S<30 g. The boxes enclose 50% of the data, the whiskers contain 80% of the data, the solid line within the boxes represents the median and the dashed line is the mean. Significant differences (two-tailed P<0.05) among groups are indicated by different letters and were derived from pairwise comparison (Dunn’s method).**
especially for small males. DEE measurements and focal observations in the field are essential to fully understand the energetic costs of courtship and scramble competition in red-sided garter snakes. Nevertheless, because plug production is dissociated from spermatogenesis in this species, males are aphagous and the renal sexual segment is hypertrophied and active during the spring breeding season, it is reasonable to attribute the increased energy expenditure and metabolic rates of mated males to the replenishment of non-sperm components of the ejaculate.

Our data show that production of a single plug can represent 2–18% of the DEE (assuming perfect anabolic efficiency) and the greater energy content of plugs without sperm suggests that sperm are less energy dense than other ejaculatory components. These DEE values are comparable to an average-sized female garter snake supporting 15 offspring during late pregnancy (~11.5% of DEE; calculated from Birchard et al., 1984, see their fig. 3). However, female energetic costs of reproduction are still likely to be higher than those for males in this species, as considerable energy (~40% increase in RMR) is required during vitellogenesis (Van Dyke and Beaufort, 2011). In addition, the vitellogenic phase and pregnancy last for 2–2.5 months in this population (C.R.F., unpublished data) compared with 1–1.5 months that males are engaged in courtship and mating during spring emergence (Gregory, 1974). Still, the energetic costs to females can be mitigated because they actively forage during vitellogenesis and through most of pregnancy (Van Dyke and Beaufort, 2011), whereas males are aphagous during breeding (O’Donnell et al., 2004). Therefore, energy usage during breeding explains the significant decrease in a male’s body condition found in other studies of this species, which in turn increases the risk of mortality and places a limit on male reproductive effort (Shine et al., 2001b; Shine and Mason, 2004, 2005). Although previous work in this system demonstrated that body condition is a factor that predicts mating success (Shine et al., 2004a), neither courtship nor ejaculate costs depended on body condition, as has been found in other species (e.g. Perry and Rowe, 2010).

Size-dependent strategies of ejaculate expenditure

There is evidence of a positive relationship between testes mass, male size and metabolic rate across taxa (Hayward and Gillooly, 2011, but only N=3 species of reptiles). However, small male red-sided garter snakes have higher mass-specific RMR and DEE than large males in both the mating and courting groups, which suggests smaller males are less efficient and/or allocate more energy to courting and to producing non-sperm components of the ejaculate than larger males. Rates of ejaculate production and allocation may also differ for males of different size classes within species/populations, if a male’s size lowers his probability of mating or generates greater costs of obtaining a mate (Parker and Pizzari, 2010; Tazzyman et al., 2009; Wedell et al., 2002). Larger male garter snakes have a mating advantage (Shine et al., 2004a, 2000b). Although the DLW study indicates that larger males accrue significant energy costs over time if they are not allowed to mate, small mating males expended more energy than larger males (Fig. 1). Although males are less likely to produce plugs and also become sperm deleted after successive matings (Friesen et al., 2013, 2014c), it is not known whether smaller males become deficient in plug material faster than larger males. The shift in RQ, seen only in small mating males (Fig. 5), provides support for the hypothesis that smaller males are investing in plug production, as a shift in the substrates used in metabolism could be due to shunting resources to plug production from muscular activity (i.e. mate searching and courtship). Kidney mass, of which the renal sexual segment is a part, scales isometrically with body mass (kidney mass=body mass1.07±0.07; C.R.F., unpublished data) and copulatory plug size is determined, in part, by female size (Shine et al., 2000a). Therefore, regardless of his size, a male must deposit a plug large enough to occlude the female’s cloaca. This may have a disproportionate effect on the metabolic rate of small males if they have less capacity than large males to store plug material, because when a small male mates, the plug material may need to be replenished immediately via synthesis. Larger males may have less need to ‘fill-up’ their stores of plug material if their larger storage capacity is enough for several matings.

Our results provide support for the prediction that as the cost of acquiring a mate increases, there is also an increase in (non-sperm) ejaculate expenditure (Parker and Ball, 2005; Tazzyman et al., 2009). We also show that courtship alone does not significantly increase RMR of larger males over the baseline metabolic rate (Fig. 4), but it does increase for small males. Therefore, the interaction between male size and DEE and between male size and post-activity RMR (Figs 1 and 4) may represent the outcome of selection acting on continuous variation in the costs of obtaining a mate for different sized males (Tazzyman et al., 2009).

These allocation strategies are likely to shift through ontogeny (Pianka and Parker, 1975). Greater energetic investment by smaller males is risky, as it may leave them in poor body condition that then increases their chances of mortality in the dens (Shine et al., 2001b) and further reduces their chances of remating (Shine and Mason, 2005). However, this strategy makes sense if the prospect for future matings is low and the costs of attaining a mating are high, as they seem to be for small males who have considerably high RMR after courtship. Given the frequent occurrence of random mortality events associated with extreme winter cold experienced in these high-latitude populations (Shine and Mason, 2004), selection may favour rapid sexual maturity at a small size and an ‘all-in’ strategy risking future reproduction for immediate fitness gains.

Prudent allocation of ejaculates

Selection for large and effective mating plugs is predicted to be strong when the male sex ratio is highly biased and males are sperm limited, as in this species (Fromhage, 2012). However, given the high cost of ejaculate production, and other risks associated with decreased body condition, we expect selection on males to mitigate these costs and allocate ejaculate in proportion to potential fitness gains (Wedell et al., 2002). For example, across taxa it is common for larger females to receive larger ejaculates (more sperm and seminal fluid) because female size often indicates higher fecundity with concomitantly higher fitness payoffs (Bonduriansky, 2001; Edward and Chapman, 2011; Wedell et al., 2002). Indeed, in red-sided garter snakes, total plug mass is positively correlated with male and female size (Friesen et al., 2014c,d; Shine et al., 2000a). Males allocate more plug material, but not more sperm, to larger females, and males tend to copulate longer with larger females (Friesen et al., 2014c). We found further support in the present study for larger females receiving larger copulatory plugs, but water content has the strongest relationship with female size. This suggests that males do allocate more ejaculate to larger females to fully occlude their larger cloaca, but much of the increase in plug mass comes from increased water content, rather than energetically expensive plug material.
Conclusion
Empirical evidence shows that the costs of male reproduction may be considerable and include the energy required for mate searching, courtship displays and territorial defence (e.g. Galimberti et al., 2007; Kotiaho et al., 1998; Lane et al., 2010; Marler et al., 1995; Oberweger and Goller, 2001; Ryan, 1988; Shine and Mason, 2005; Vehrencamp et al., 1989). By experimentally quantifying the energetic costs of non-sperm ejaculate production using robust physiological methods, we show for the first time that non-sperm ejaculate is a large energetic expense, which is comparable with increased metabolic rates of pregnant females. Further work on other species with dissociated reproductive patterns would provide useful comparative data on allocation to non-sperm components of the ejaculate. There are several taxa (fish, reptiles, mammals and amphibians) where males exhibit a dissociated reproductive pattern (see tables in Birkehead and Moller, 1993; Crews, 1984), and which could be used to assess the energetic costs of ejaculate production in different mating systems. Furthermore, in associated breeders, it is conceivable that experiments incorporating castration, hormone replacement treatments and vasectomies could be used to separate the cost of ejaculate production from spermatogenesis. Using laboratory-bred model organisms, such as mice, elegant experiments could be designed that genetically disrupt spermatogenesis and/or the development of sexual accessory glands (e.g. Dean, 2013), and these animals could be used to establish the energetic costs associated with the production of particular components of the ejaculate. This would be an interesting subject for future work in other species, as metabolic rates are a universal currency that allows ready comparison of reproductive costs between sexes and across taxa. Using this kind of broad comparative approach is essential for us to better understand the trade-offs among ejaculate components and other traits in response to sperm competition and sexual conflict across divergent taxa.

MATERIALS AND METHODS
Experimental rationale
We used the DLW water method (Nagy, 1983), which indirectly measures CO_2 production (a measure of metabolic rate) over the course of a sampling period, and is expressed as energy expenditure over time (e.g. kJ day^{-1} or DEE). The DLW method provides us with an ecologically relevant measurement of the cost for an organism to engage in an activity for a period. When we found significant, size-dependent differences in the DEE of courting versus mating males, we then used respirometry to precisely measure CO_2 and O_2 simultaneously to determine whether mating (i.e. plug production) was the cause. There are several benefits of using respirometry: (1) the increased precision allowed for more confidence in the measurement of CO_2 production than the DLW method, (2) it allowed us to temporally isolate courtship and mating in a way that DEE estimates could not and (3) by measuring CO_2 and O_2 simultaneously, we could detect shifts in catabolism during plug production. Specifically, we measured metabolic rates in the post-activity recovery phase. We also measured baseline metabolic rates of males that had not engaged in any activity prior to measurement as a control to account for the energy required for basic bodily functions in a quiescent animal (i.e. the SMR, which is analogous to the BMR measured in a homoeothermic animal). We hypothesized that the metabolic rate of both courting and mating males would be elevated over baseline after the activity. Moreover, we hypothesized that mating would further increase metabolic rate over that of courting males if the mating males were replenishing seminal fluid (i.e. plug material). Respirometry also provides us with the RQ, which is the ratio of CO_2 production to O_2 consumption (V_{CO_2}/V_{O_2}). A respiratory quotient of 1.0 is associated with catabolism of carbohydrates, a respiratory quotient of 0.71 is associated with fat catabolism, and a respiratory quotient between 1.0 and 0.71 represents protein catabolism and/or a mixture of metabolic substrates. It is typically assumed that fasted animals will have a RQ of 0.71 due to their reliance on fat catabolism, but there is evidence from birds that extended fasts might result in somewhat higher RQ (~0.76) perhaps due to catabolism of tissue proteins (Walsberg and Wolf, 1995). The substrates being catabolized also determine the correct conversion factor used in DLW studies and thus it is important to validate these for estimates of DEE. The direction of a shift in RQ between mating and courting males is difficult to predict because we do not know the nature of metabolic pathways for plug production in reptiles, but it is possible that the use of particular substrates for synthesis removes them from circulation, which in turn shifts the balance of substrates available for energy production. Thus, for example, we might expect the use of proteins for plug production to result in a shift in the RQ downward or an upward shift if a fasting animal with dwindling fat reserves is catabolizing muscle.

DEE
We collected 48 recently emerged, actively courting male red-sided garter snakes from a hibernaculum 1.5 km north of Inwood, Manitoba, Canada (25 April 2007). Procedures performed on animals were approved by Oregon State University [IACUC; ACUP A3229-01 and ACUP-3738], and the research was conducted under permit from Manitoba Conservation [WSB 04004; WB1240]. Size-matched snakes were assigned to two different treatment groups: courting (N=24) and courting and mating (‘mating’; N=24). Individual size can affect reproductive success in small groups of males (2–4 males; Shine et al., 2000b); however, size matching should mitigate among treatment differences in DEE due to group composition along with 24 males.

Our DLW protocol was based on the methods of Nagy (1983). Briefly, blood samples (300–500 µl) were taken from the caudal vein before intraperitoneal injection of a mixture of 0.3 g ¹⁸O cat. no. 329878, Sigma, St Louis, MO, USA) and 0.12 g ^3H kg⁻¹ body mass (¹³C cat. no. 151882, Sigma) (0.379 µl mixture g⁻¹ body mass; Schoeller, 1983) with a precision 25 µl Hamilton syringe (Reno, NV, USA). A second blood sample (300–500 µl) was obtained as above after 4–6 h equilibration time (Peterson et al., 1998, 1999). Final blood samples (300–500 µl) from all males were taken 9 days later (Peterson et al., 1998). Plasma (150–300 µl) from each blood sample was immediately sealed in an auto-sampler vial and frozen (−20°C) until the samples were sent to the University of Arkansas Stable Isotope Lab for mass spectrometry analysis. We used Nagy’s (1983) calculations to check our results against those of the most recent DLW studies on garter snakes (i.e. Peterson et al., 1998, 1999). Calculations of CO_2 production were made using the formula from Nagy (1983), and conversion to an energy equivalent assumed to be 27.7 J ml⁻¹ CO_2. This value was selected because the snakes fast during spring courtship (O’Donnell et al., 2004) and are likely to be using fat stores. RQs calculated from measurements made using respirometry were roughly consistent with fat catabolism (see Results). Using the energy equivalent values, we obtained an estimate of the DEE (kJ day⁻¹) for each snake.

During the 9 day sample period, the weather allowed males to court and mate on 5 days (28 and 29 April, 2, 3 and 7 May). On each of these 5 days, the males were placed in semi-natural enclosures (one for each treatment group) and thus were able to court or court and mate with females all day (09:00 h to 17:00 h) as would occur in the den. These enclosures were side by side and shared a common wall; therefore, it is unlikely there were systematic differences between them (e.g. temperature or sun exposure). However, to further ensure the enclosure had no effect on DEE, the groups were rotated between the two cleaned enclosures on successive days. Animals were brought indoors and kept in cloth bags (11–12.5°C) at night when temperatures were forecast to be below 2°C to prevent them from freezing. Males exhibit intense courtship in semi-natural outdoor enclosures where they can be easily observed (e.g. Shine et al., 2004a, 2000a,b). Each group of males (N=24 in each) was kept in a separate enclosure (1×1×1 m). All of the courting males were placed in an enclosure together with females that had a 2×1 cm piece of Nexcare™ adhesive tape (3M, St Paul, MN, USA) affixed over their cloacae such that the males could not mate with them. All of the mating males were placed in an enclosure together with untaped females.

In this species, the sex ratio at spring emergence is strongly male biased. Several dozen males will congregate around a newly emerged female, forming ‘mating balls’ in which males court her and attempt copulation.
RESEARCH ARTICLE


(Shine et al., 2001a, 2006a). Using an established ethogram of male garter
snake mating behaviour (Blanchard and Blanchard, 1941; Crews et al.,
1984; Moore et al., 2000; Noble, 1937), we recorded whether a male
was engaged in courtship on each of the 5 days that weather permitted courtship
and mating. The ethogram scores range from 1 to 5 as follows: (1) the male
investigates the female and tongue flicks her, (2) the male presses his chin
against the female and rapidly tongue flicks, (3) the male aligns his body
with the female and he continues chin rubbing and rapid tongue flicking, (4)
the male attempts cloacal apposition with the female, and active tail
searching (wraps his tail around her tail) and finally (5) copulation. When
presented with a female, all males in the mating and courting groups
indicated receptivity by engaging in courtship (≥3 on the ethogram) at some
point on every day that courtship and mating occurred (we could not
systematically record courtship throughout the day as timing copulation
duration took precedence over monitoring courtship scores).

Females were collected as they emerged and used within 2 days. We kept
the females in outdoor enclosures (1×1×1 m) and provided them with water
ad libitum. Males from both the mating and courting groups had access to
two females at a time from 09:00 h to 17:00 h each day. Although female
latency to mate can be longer than 2 h in enclosures (e.g. Whittier and
Crews, 1986, 1989), a receptive female typically mates in less than 20 min
and males become less interested in un receptive females (C.R.F. and
R.T.M., unpublished observation). We therefore set a 30 min threshold after
which a female was replaced with a new female in both the courting and
mating groups. In addition, if a female mated, she was replaced with a new
female. Thus, males of both groups were constantly exposed to new, attractive females that mimicked females emerging at the den. The 1×1×1 m
enclosures provided room for males to rest outside the mating ball. In the
mating group, when copulation occurred, the mating pair was then gently
moved to a smaller enclosure where copulation could be closely monitored
to record copulation duration (±10 s) (Friesen et al., 2013, 2014b,c,d). The male was reintroduced to the enclosure after copulation ended.

Baseline (standard) metabolic rate (SMR)

We used SMR ($V_{O2}$, mg g$^{-1}$ min$^{-1}$) as a baseline, which is the lowest rate of
metabolism, measured at a particular temperature, in an inactive and post-
absorptive ectotherm (McNab, 2002). For measurement of SMR, 16 male
red-sided garter snakes were collected from the den and transported to
George Fox University, Newberg, OR, USA. These animals were not the
same animals as those used for post-activity RMR measurements, but they
were still in the post-hibernation fasting phase of their annual cycle when the
renal sexual segment is hypertrophied (Krohmer et al., 1987).

We measured SMR at 5°C increments over a temperature range of 5–30°C.
We had 16 males for this experiment but only 10 spaces in the incubator;
therefore, at each temperature, 10 males were randomly selected for
measurement and calculated using the same protocol as for SMR (above).

To measure RMR, snakes were sealed in cylindrical plastic, air-tight
metabolism chambers (volume 550 ml) and placed in an environmental chamber (Model 36-VL, Percival Scientific,
Perry, IA, USA) to precisely control measurement temperature and maintain
a dark environment. After a 30 min equilibration period, the chambers were
flushed with fresh air and returned to the environmental chamber for
180 min. At the end of this period, 10 ml of chamber air was removed
from the chamber through a 3-way valve using a calibrated 10 ml syringe, and
injected into the inlet line of an open-flow respirometry system to measure
$V_{O2}$ and $V_{CO2}$. The percentage $O_2$ and $CO_2$ of our injected samples was
measured using a FoxBox $O_2$/CO2 analyser (Sable Systems, Inc., Las Vegas,
NV, USA) with its subsampling pump set at a flow rate of 125 ml min$^{-1}$.
Water vapour was scrubbed from inlet air with calcium sulphate
(W. A. Hammond Drierite Co. Ltd., Xenia, OH, USA), including the
sample, prior to measurement of percentage $CO_2$. CO$_2$ was scrubbed
using soda lime (cat. no. 266434, Sigma) prior to measurement of percentage
$O_2$. All sample injections do not yield equilibrium $V_{O2}$ or $V_{CO2}$ values, we
integrated the area under the output peaks to calculate total ml $O_2$/CO$_2$
change in the sample (Bartholomew and Lighton, 1986). Total $O_2$/CO$_2$
consumed/produced by the snake was calculated as (Lighton, 2008):

\[
V_{\text{snake}} = V_{\text{measured}} \times \left( \frac{V_{\text{chamber}}}{V_{\text{sample}}} \right).
\]

where $V_{\text{snake}}$ is total ml $O_2$/CO$_2$ consumed/produced by the snake during
the measurement interval, $V_{\text{measured}}$ is the ml $O_2$/CO$_2$ change in the sample as
calculated by integration, $V_{\text{chamber}}$ is the chamber volume (ml) and $V_{\text{sample}}$ is
the sample volume (10 ml). $V_{\text{chamber}}$ was corrected for the volume occupied
by the snake by assuming the volume of the snake is equal to a cylinder with
length equal to SVL and width equal to body diameter at the midpoint
between the snout and the vent. Omitting the tail from the calculation
compensated for the reduction in diameter in the head and vent. $V_{O2}/V_{CO2}$ was then calculated as:

\[
\frac{V_{O2}}{V_{CO2}} = \frac{V_{\text{snake}}}{t},
\]

where $t$ is the duration of the measurement interval in min.

Post-activity RMR associated with copulatory plug production

Animals were collected on 7–14 May 2011 from the same population as
those collected for the DLW experiment. Twenty males were allowed access
to females while placed in small cylindrical arenas (Friesen et al., 2014c).
In one arena we placed 20 large (>35 g) males and in the other arena we placed
20 small (<30 g) males. Average male mass is 32 g in this population (e.g.
Shine et al., 2006a, 2006b) and we wanted separation from this value to
focus on size effects. This also created a non-overlapping, bi-modal
distribution in male size (see Results), so we used ANOVA instead of
ANCOVA to test for size effects with each treatment (mating and courting)
separated into two size classes (large and small). We used 20 males in each
group as this approximates the size of the actively courting core of a large
mating ball in the field (Shine et al., 2004a; D.R.P., unpublished data).
A single female was placed in each arena with the 20 males who would then
court the female until one of the males mated with her. The mating pair, and
a single courting male that was also in position to mate with the female, were
gently removed from the arena to a second arena where courtship duration
could be recorded. To be selected, the courting male had to have exhibited
courtship at level 4 on the ethogram, which means the male was courting
vigorously, tail-searching for the female’s cloaca and in position to mate,
such that it is only the result of chance that he was not the mating male. To
eliminate bias related to female proximity or courtship intensity, the courting
male selected for measurement of RMR was removed from the arena at the
same time as the mating male and female. At the conclusion of copulation,
the mating male and the courting male were removed from the arena for
measurement of RMR. Measurement of RMR of the mating male was made
following separation from the female (and thus plug deposition).

Equilibration for RMR measurement of the courting male began within
5 min of its selection. No courting male was used more than once; in four
cases where the mating male had been allowed to court and mate more than
once, these males were not included in our analyses (sample sizes: mating,
N=37; courting, N=41).

To measure RMR, snakes were sealed in cylindrical plastic, air-tight
metabolism chambers, and submerged in a 30°C circulating water bath to
maintain constant temperature at 30°C. After a 30 min equilibration period,
the chambers were flushed with fresh air, and then resubmerged for
180 min. At the end of this period, 10 ml of chamber air was removed
from the chamber through a 3-way valve using a calibrated 10 ml syringe, and
injected into the inlet line of an open-flow respirometry system to measure
$V_{O2}$ and $V_{CO2}$. The percentage $O_2$ and $CO_2$ of our injected samples was
measured and calculated using the same protocol as for SMR (above).

Energy content of the copulatory plug

To measure the water and energy content of copulatory plugs, we collected
plugs from females (May 2014), weighed them immediately after collection
and again after drying them to constant mass (±1 mg), and then conducted
bomb calorimetry on each plug separately using a Phillipson Oxygen
Microbomb Calorimeter (Phillipson, 1964). We obtained sperm-free plugs
from females that had mated with males that had been vasectomized during
May 2014 (N=9; see Friesen et al., 2013 for surgery methods) and plugs
with sperm from females that had mated with intact males (N=10). Plugs
were collected within 30 s of the termination of copulation (e.g. Friesen
et al., 2014d).
Statistical analyses

Statistical analyses were conducted using SigmaPlot 12.0 (Systat Software Inc., San Jose, CA, USA) and XLSTAT 2012 (Statistical Innovations, Belmont, MA, USA). Heteroscedastic data were transformed where indicated or non-parametric tests were used. Body condition has been calculated as the residual deviation from the regression of body mass on SVL fitted in Sigma Plot 12.0 (e.g. Friesen et al., 2014a). We modelled DEE both as a function of treatment and male size using ANOVA as we had purposely created two size classes within each treatment to examine size effects, which generated a bimodal size distribution.

Acknowledgements

We thank the Manitoba Department of Natural Resources (especially D. Roberts) for logistical support, and the residents of Chatfield (especially the Johnson family) for hospitality and encouragement. We would like to thank S. J. Beaufre for advice on the DLW method in snakes and M. Thompson at the University of Sydney for the use of a microbomb calorimeter. M. R. Parker, R. Nesbitt and M. Tullero assisted with data collection for the DLW experiment, and E. J. Uhrig, M. K. Squire, S. L. Eddy, C. M. Whittington, J. U. Van Dyke, M. Olsson, T. Day, R. Bonduriansky, M. D. Dean, D. S. Siegel and three anonymous reviewers provided excellent comments on early drafts of this manuscript.

Competing interests

The authors declare no competing or financial interests.

Author contributions

C.R.F. conceived of the conceptual design of the whole project, conducted all of the DLW and bomb calorimetry studies, analysed all data and wrote the manuscript. D.R.P. helped to write the manuscript and designed the RMR and SMR experiments and conducted the work with the help of P.E.C. R.T.M. helped to write the manuscript and helped to conduct the DLW experiment.

Funding

This study was funded by National Science Foundation (NSF) grant IOS-06125 (R.T.M.), NSF DDIG IOS-1011727 (C.R.F.), George Fox University (GUF) Faculty Development Grant (D.R.P.) and a GUF Richter Scholar Grant (P.E.C.).

Supplementary material

Supplementary material available online at http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.120402/-/DC1

References

Aldridge, R.D., Jellen, B.C., Siegel, D.S. and Wisniewski, S.S. 2003. We modeled RMR using ANOVA as we had purposely created two size classes within each treatment to examine size effects, which generated a bimodal size distribution.


Table S1
Comparisons for factor: Treatment

<table>
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<th>Comparison</th>
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