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CORRECTION

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

Christopher R. Friesen, Donald R. Powers, Paige E. Copenhaver and Robert T. Mason

A number of minor errors were published in *J. Exp. Biol.* **218**, 1410-1418.

The corrected sections are reproduced below, with changes highlighted in bold. These changes do not affect the conclusions of the paper.

RESULTS

Metabolic substrates: respiratory quotient

Mean respiratory quotient (RQ=$\dot{V}_{CO_2}/\dot{V}_{O_2}$, where $\dot{V}_{CO_2}$ is the rate of CO_2 production and $\dot{V}_{O_2}$ is the rate of O_2 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_1=24.091 (where the subscript 1 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 **courting** males having a significantly higher RQ **than small mating males** (Kruskal–Wallis test, K_3=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 **small, courting males**.

DISCUSSION

Size-dependent strategies of ejaculate expenditure

The shift in RQ, seen only in **small 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).

The authors apologise for any inconvenience this may have caused.

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RESEARCH ARTICLE

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

Christopher R. Friesen[1,2,*], Donald R. Powers[3], Paige E. Copenhaver[3,4] and Robert T. Mason[2]

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).

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List of symbols and abbreviations

BMR	basal metabolic rate
DEE	daily energy expenditure
DLW	doubly labelled water
RMR	resting metabolic rate
RQ	respiratory quotient
RSS	renal sexual segment
SMR	standard metabolic rate
SVL	snout–vent length
$\dot{V}_{CO_2}$	rate of carbon dioxide production
$\dot{V}_{O_2}$	rate of oxygen consumption

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 apocrine cells of the distal tubules collectively called the renal sexual segment (Aldridge et al., 2011). The apocrine 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 apocrine 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 (snout–vent length, SVL) 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

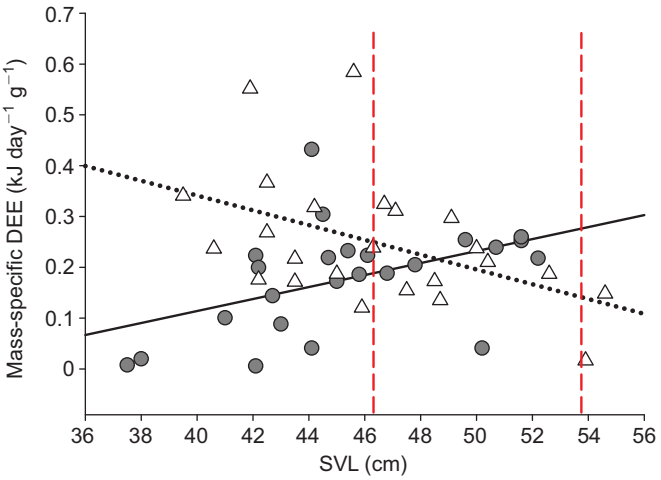

Fig. 1. Daily energy expenditure as a function of treatment and male size. DEE, daily energy expenditure; SVL, snout–vent length (a measure of male size). Solid circles and line represent courting males; triangles and dotted line represent mating males. The area between the dashed, vertical lines indicates the male size where the DEE between the treatments (courtship versus mating) was not significantly different (46.27 cm≥SVL≤53.80 cm) as determined using the Johnson–Neyman technique (White, 2003).

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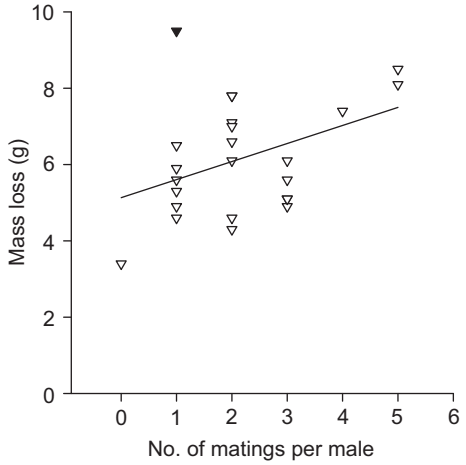

Fig. 2. Mass loss as a function of the number of matings a male achieved. Removal of a large male that lost 9.5 g (solid triangle) and only mated once yielded a significant relationship between mass loss and the number of copulations a male attained in the mating group of the doubly labelled water (DLW) experiment (R^2=0.294, $F_{1,22}$=10.151, P=0.004); otherwise, the slope of the regression is not significantly different from zero (P=0.052).

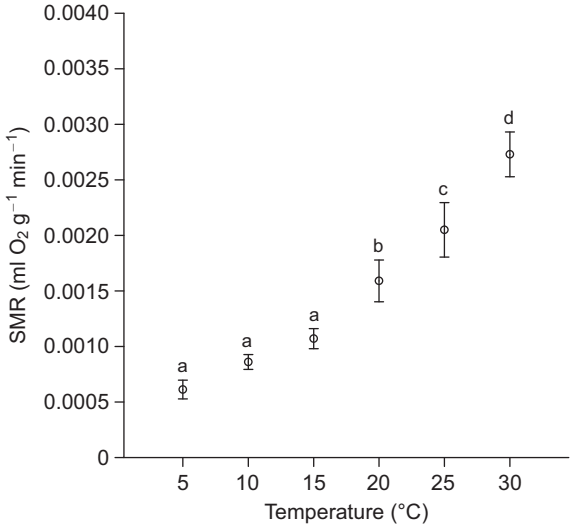

Fig. 3. Standard metabolic rate (SMR) at a range of biologically relevant temperatures that these snakes encounter in the wild. SMR is given as $\dot{V}_{O_2}$. The bars represent standard errors. Significant differences (P<0.05) among temperatures are indicated by different letters.

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 ($\dot{V}_{O_2}$, g^{-1} 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

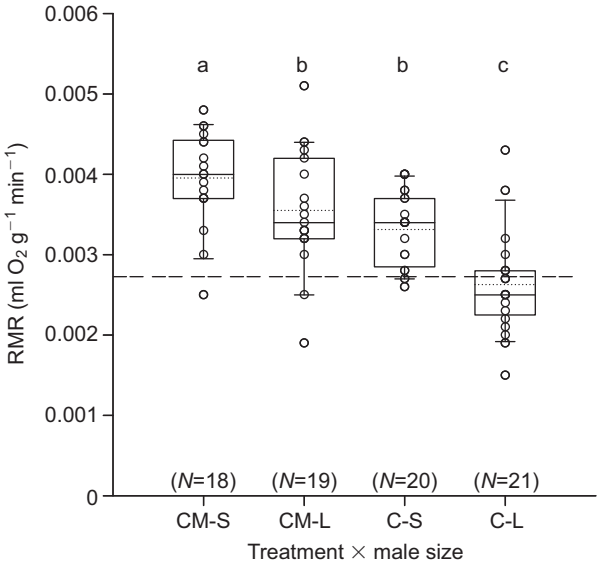

Fig. 4. Resting metabolic rate (RMR) among treatments and size classes. RMR is given as $\dot{V}_{O_2}$ at 30°C. The boxes enclose 50% of the data, the whiskers contain 80% of the data, the solid line within the boxes represents the median and the dashed line is the mean. Significant differences (P<0.05) among groups are indicated by different letters (see supplementary material Table S1). 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 dashed line that runs the width of the graph represents mean SMR at 30°C ($\dot{V}_{O_2}$=0.0027 ml g^{-1} min^{-1}). Mean SMR is significantly lower than RMR for all groups except large courting males.

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

Group	*N*	LS mean±s.e.m. ($\dot{V}_{O_2}$, ml g^{-1} min^{-1})	*t*-value	Comparison with SMR
CM-S	18	0.00396±0.00014	5.098	<0.0001
CM-L	19	0.00355±0.000169	3.454	0.005
C-S	20	0.00331±9.98E-05	2.478	0.060
C-L	21	0.00263±0.000135	0.433	0.666

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. Comparisons were made with the Holm–Sidak method.

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= $\dot{V}_{CO_2}/\dot{V}_{O_2}$, where $\dot{V}_{CO_2}$ is the rate of CO_2 production and $\dot{V}_{O_2}$ is the rate of O_2 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_1=24.091 (where the subscript 1 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_3=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 22.903±1.35 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, *Hyphessobrycon pulchripinnis*; Nakatsuru 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*: $\dot{V}_{O_2}$=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 ($\dot{V}_{O_2}$=0.0025 ml g^{-1} min^{-1}). Furthermore, we demonstrate that mating males have even higher energetic expenditures than males engaged only in courtship,

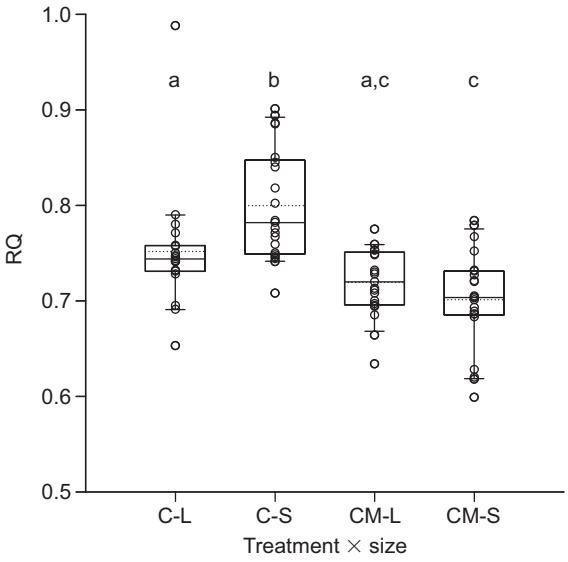

Fig. 5. Respiratory quotient (RQ) among treatments and size classes. CM, males that courted and mated (mating); C, males that courted only (courting); L and S, size class where L>35 g and S<30 g. The boxes enclose 50% of the data, the whiskers contain 80% of the data, the solid line within the boxes 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).

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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 Beaupre, 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 (Gregory, 2006; Gregory and Stewart, 1975), 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 mass$^{1.07\pm0.07}$; 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.

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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 Birkhead and Møller, 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 ($\dot{V}_{O_2}/\dot{V}_{CO_2}$). 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 alone 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 ^{18}O kg^{-1} body mass (^{18}O cat. no. 329878, Sigma, St Louis, MO, USA) and 0.12 g ^{2}H kg^{-1} body mass (^{2}H 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 un-taped 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

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(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 unreceptive 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 ($\dot{V}_{O_2}$, ml 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 from the total of 16 males, providing 10 measurements of SMR at each temperature. To measure SMR, 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 $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$. The percentage O_2 and CO_2 of our injected samples was measured using a FoxBox O_2/CO_2 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, and CO_2 was scrubbed using soda lime (cat. no. 266434, Sigma) prior to measurement of percentage O_2. As sample injections do not yield equilibrium $\dot{V}_{O_2}$ or $\dot{V}_{CO_2}$ 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):

$$V_{snake} = V_{measured} \times (V_{chamber}/V_{sample}),$$

where V_{snake} is total ml O_2/CO_2 consumed/produced by the snake during the measurement interval, $V_{measurement}$ is the ml O_2/CO_2 change in the sample as calculated by integration, $V_{chamber}$ is the chamber volume (ml) and V_{sample} is the sample volume (10 ml). $V_{chamber}$ 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 at the head and vent. $\dot{V}_{O_2}/\dot{V}_{CO_2}$ was then calculated as:

$$\dot{V}_{O_2} \text{ or } \dot{V}_{CO_2} = V_{snake}/t,$$

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, 2000b) 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 $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$. 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 SigmaPlot12.0 (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 and as mass-specific DEE (DEE/male mass) with male size (SVL) as a covariate using ANCOVA because we had a normal distribution of male sizes included in each treatment group. Furthermore, we defined the range of the covariate (SVL) at which there are significant differences between treatments (courting and mating) using the Johnson–Neyman technique (White, 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.

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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.

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Supplementary material

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