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Intraspecific variation in ejaculate traits of the northern watersnake (*Nerodia sipedon*)

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Abstract

Sperm competition occurs when the sperm of more than one male compete to fertilize the eggs of a female. In reptiles, sperm competition is particularly prevalent and is an important agent of sexual selection in males. Spermatogenesis in reptiles can be energetically expensive, suggesting that there may be costs to producing high-quality ejaculates. The northern watersnake *Nerodia sipedon* has a mating system characterized by aggregations in which a single female mates with multiple males, resulting in high levels of multiple paternities. Under these circumstances sperm competition is likely important, and selection should favour sperm and ejaculate traits that enhance a male's reproductive success. In this study, we examined intraspecific variation in ejaculate quality (sperm length, motility, sperm density, spermactocrit) in male watersnakes and determined whether ejaculate traits varied with body size and condition, using both size-corrected mass and haematocrit as indices of condition. We found large variation among males in all these traits, except for sperm length. Although there was significant variation in sperm length among males, the majority of variation in sperm length occurred within rather than among individuals. Males with high haematocrit had sperm that were less variable with respect to length, and large males produced ejaculates that were less concentrated with respect to sperm than small males. The lack of condition dependence of most ejaculate traits is consistent with previous studies that indicate that male reproductive effort in this species is generally not energy limited, perhaps because of opportunistic foraging during the mating season.

Introduction

Sperm competition is a common phenomenon across the animal kingdom (for reviews, see Birkhead & Parker, 1997; Birkhead & Møller, 1998), and occurs when a female copulates with more than one male – the sperm from those males will compete within the female's reproductive tract to fertilize the ova. Theoretical models predict that males should adjust ejaculate size and quality in response to the likelihood of sperm competition (Parker, 1990*a*,*b*, 1993, 1998) and the quality of the female. The mechanisms and traits underlying success at sperm competition are not well understood in reptiles, made especially difficult because they are internal fertilizers.

Intraspecific variation in ejaculate traits can influence fertilization success under sperm competition (Birkhead *et al*., 1999; Gage *et al*., 2004). Moreover, good-sperm models of polyandry suggest that females may gain indirect benefits from multiple matings if male condition is genetically correlated with ejaculate quality (Yasui, 1997). By mating with multiple males and thus encouraging sperm competition, a female can potentially ensure that her eggs are fertilized by the sperm of a high-quality male. Males that produce sperm with traits that enhance their competitive ability (e.g. sperm length; Pitnick, Markow & Spicer, 1999) will therefore have a selective advantage.

Sperm competition is a pervasive phenomenon in reptiles (Birkhead & Møller, 1993; Olsson & Madsen, 1998) and appears to be a major factor affecting sexual selection in male reptiles, particularly snakes (Shine, 2003). In addition, the energetic demands of spermatogenesis appear to be particularly onerous in reptiles. At the onset of the mating season, fat levels decrease markedly, in synchrony with a dramatic increase in testis size (Olsson & Madsen, 1998).

Food resources appear to mitigate the energetic costs of spermatogenesis in reptiles. For example, lizards that are supplementally fed maintain body fat during testes growth (reviewed in Olsson & Madsen, 1998). Also, in the adder *Vipera berus*, spermatogenesis and mating activities such as mate searching and male–male combat are temporally separated, and the rate of mass loss (presumably due to energetic expenditure) is as high during the spermatogenic phase as in the active phase of mating (Olsson, Madsen & Shine, 1997), indicating a high cost of sperm production in at least some snakes.

The northern watersnake *Nerodia sipedon* is a highly aquatic natricine snake with a mating system characterized by mating aggregations in which several males attempt to copulate with a single female by insinuating themselves between the female and other males (Weatherhead *et al*., 1995). The result of multiple mating is a high degree of mixed paternity within litters – Prosser *et al*. (2002) found that 58% of all litters were sired by more than one male, with one litter having five sires. Thus, variation in sperm competition ability is predicted to be important in explaining variation in male reproductive success. Indeed, other traits have been found to be unrelated to male reproductive success in this species, including body size, home range size, body condition and heterozygosity (Weatherhead *et al*., 2002).

Here, we examine several ejaculate traits (sperm length, motility, sperm density, spermatocrit) in male northern watersnakes. First, we assess intraspecific variation in ejaculate traits, focusing especially on sperm length because other studies have suggested that this is an important trait affecting sperm competition success in animals (Gomendio & Roldan, 1991). Second, because some evolutionarily stable strategy models predict that small males should invest more in ejaculates (Parker, 1990*a*), and because the energetic costs of spermatogenesis appear to be high in reptiles, we examined ejaculate traits for size and condition dependence, using both size-corrected mass and haematocrit as indices of condition. Haematocrit is often used as an index of condition in many species, including birds (Merilä & Svensson, 1995; Brown, 1996; Svensson & Merilä, 1996; Hõrak, Ots & Murumägi, 1998; Ots, Murumägi & Hõrak, 1998) and tortoises (Peterson, 2002).

Methods

We hand-captured northern watersnakes just before the breeding season (mid to late April 2002) from three marshes (Barb's, Beaver and Lindsay Lake Marsh) located *c*. 10 km from the Queen's University Biological Station (45°37′N, 76°13′W) in Ontario, Canada (for details of habitat, see Brown & Weatherhead, 1999). Snakes were weighed and measured [snout–vent length (SVL) and tail length], and 300 μL of blood was taken from the caudal vein with a sterile 26-gauge needle. Blood from each of 26 male snakes was placed on a glass slide and collected in three heparinized micro-haematocrit capillary tubes. The tubes were immediately centrifuged for 2 min at 8000 rpm (Readacrit^{TM} centrifuge, Clay Adams, Parsippany, NJ, USA) and the layer of compacted cells and total blood volume measured to the nearest 1 mm. Haematocrit was calculated as the mean fraction of total volume made up of compacted cells, expressed as a percentage.

We obtained samples of ejaculate from male watersnakes by manually massaging ejaculate from the cloaca until a relatively large volume of ejaculate (10–30 μL) was expressed (Mengden *et al*., 1980). In some cases, insufficient ejaculate was expressed or a large proportion of ejaculate was contaminated by faecal material, and so not all ejaculate traits could be measured for all males. Immediately upon collection, 1 μL of ejaculate was placed in 800 μL of blood-bank saline (Fisher Scientific, Cat. No. SS442, 20D) and agitated until the ejaculate was uniformly distributed in the saline (*c*. 30 s). A drop of the dilute ejaculate was then placed on a sperm counting chamber slide (80 μL depth) and a coverslip placed on top. The sperm were then videotaped from a CCD video camera (Sony model XC-ST50; Toronto, Canada) mounted on an Olympus CH30 microscope at $\times$ 100 magnification (Olympus, Melville, NY, USA).

We analysed the videotape of swimming sperm for each male using the public domain program Image J (available at http://rsb.info.noh.gov/ij/). Sperm counts were conducted on images paused on the computer screen, and motility was estimated from the videotape immediately after the videotape had begun. Motility was scored by eye on a scale of 1–5, where 1 = 0–20%, 2 = 21–40%, 3 = 41–60%, 4 = 61–80% and 5 = 81–100% of sperm that were motile.

Sperm from each of 25 males were prepared for morphological measurement for each male by diluting 1 μL of ejaculate in 750 μL of fixative (3% glutaraldehyde in 0.1 M cacodylate buffer at pH 7), spreading the sample thinly across a microscope slide and allowing it to air dry. Ten haphazardly chosen sperm were measured for each male. We recorded and digitized images of the sperm (magnified $\times$ 400) using the microscope-mounted video camera and a Macintosh computer running Image J (available at http://rsb.info.nih.gov/nih-image/). Total sperm lengths were measured to the nearest 0.1 μm (calibrated using the grid scale of a haemocytometer under the same magnification) on the digitized images of the sperm. We calculated median sperm length for each individual for all analyses.

We collected any remaining ejaculate in a micro-haematocrit tube and immediately centrifuged the tube for 2 min at 5900 *g*. The sizes of the layer of compacted cells and the total ejaculate were measured to the nearest 1 mm. Spermatocrit was calculated as the fraction of total volume made up by the compacted cells.

Statistical analysis

Spermatocrit was arcsin transformed and morphological measurements were log transformed to improve the fit to normality. All data were otherwise normal. Individual variation in sperm length met assumptions of homogeneity of variance (Levene's test $F_{24,285} = 0.98$, $P = 0.50$).

Results

Sperm size

Sperm length in northern watersnakes is not particularly variable [coefficient of variation (CV)] $<1\%$ across all sperm measured; $n = 250$) compared with other body and ejaculate traits (Table 1). Moreover, a higher proportion of the total variance in sperm length occurred within (77.5%) rather than among (22.5%) individuals. Nonetheless, there was significant variation among individuals in sperm length

Table 1 Sample size (*n*), mean and estimates of intraspecific variation (standard deviation and range) for morphological and ejaculate traits of male northern water snakes *Nerodia sipedon*

Trait	*n*	Mean	SD	Range
Snout–vent length (cm)	26	56.3	4.6	47.0–62.3
Body mass (g)	26	106	27	55–170
Haematocrit (%)	26	30.8	5.4	15.3–38.3
Sperm length (μm)	25	112	1.1	110.2–113.9
Motility score (1–5)	23	3	1.4	1–5
Sperm density ($\times 10^6\,\mu L^{-1}$)	23	3.34	1.4	1.10–6.31
Spermatocrit (%)	16	62.7	15	31.2–89.5

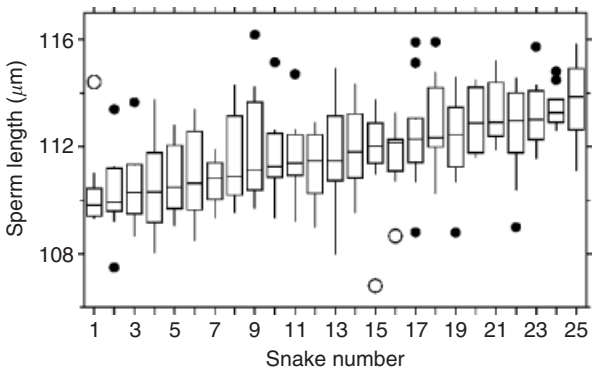

Figure 1 Sperm length variation within and among male northern water snakes *Nerodia sipedon*. Box plots show medians, 1 interquartile range (IQR) as boxes, whiskers extending to the extreme values within the median, 1.5 IQR, and outliers as circles (>1.5 IQR), with ○ indicating extreme outliers (>3 IQR).

(one-way ANOVA: $F_{24,225} = 4.35$, $P < 0.001$; Fig. 1), with a continuous distribution of median sperm sizes (Fig. 1). The distribution of median sperm lengths was not significantly different from normal (Shapiro–Wilk test, $W = 0.96$, $P = 0.51$).

In 14 of the 25 males that we sampled, there were outliers in the sample of sperm measured (Fig. 1), suggesting some possible anomalies during spermatogenesis. Although we were careful to avoid measuring sperm with broken tails, it is possible that some of the low outliers may be due to undetected breakage. This cannot explain the many positive outliers ($n = 10$ males).

Median sperm length was not related to any of the body size or condition variables that we measured, either singly or when controlling for variation in the other variables (Table 1). However, variation in sperm length, as measured by the within-individual standard deviation in sperm length, was significantly and negatively correlated with haematocrit ($r_S = -0.44$, $P = 0.03$, $n = 25$; Fig. 2). Thus males that were in better condition, as measured by haemaocrit, produced less variable spermatozoa. The magnitude of this relation is unaffected by removing the three males with extreme outliers ($r_S = -0.45$, $P = 0.04$, $n = 22$) or all males with any outliers ($r_S = -0.46$, $P = 0.15$, $n = 11$), although the latter is no longer statistically significant. Thus this result appears to be biologically meaningful. The standard deviation of sperm

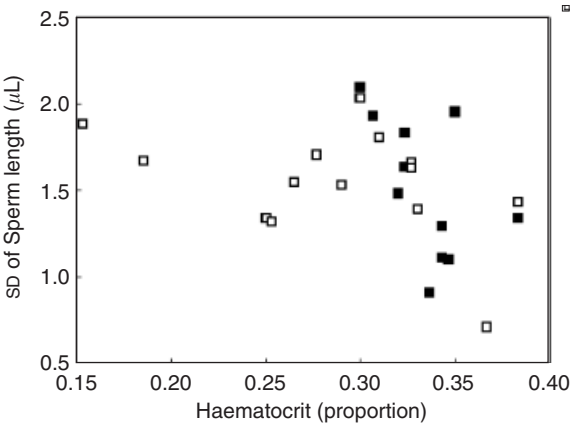

Figure 2 Relation between intraindividual variation in sperm length and body condition (as measured by haematocrit). Data from males with outliers are shown by □.

length was not related to either residual mass ($r_S = -0.25$, $P = 0.24$, $n = 25$) or mean sperm length ($r_S = -0.13$, $P = 0.54$, $n = 25$).

Sperm concentration in ejaculates

Spermatocrit was significantly correlated with sperm concentration, as measured with a haemocytometer ($r = 0.57$, $P = 0.02$, $n = 16$). Because these two variables estimate the same thing (number of sperm per unit volume of ejaculate), we might expect this correlation to be much higher, but spermatocrit is also influenced by sperm size (larger sperm occupy a larger volume than smaller sperm). Thus, including both sperm length and sperm density in a multiple regression model to explain spermatocrit explains a higher proportion of the variation in spermarocrit ($R^2 = 0.48$), although about half of the variation in spermatocrit still remains unexplained in this model. Thus, we used the residuals of the regression of spermatocrit (angular transformed) on sperm length as an index of sperm concentration.

Residual spermatocrit was significantly negatively correlated with male SVL (Spearman rank correlation, $r_S = -0.55$, $P = 0.03$, $n = 16$; Fig. 3), but not with any other male morphological trait including body condition (i.e. tail length, mass, haematocrit, residual mass; $P > 0.30$ in each case). Three small males (SVL < 50 cm) were obvious outliers in this sample (Fig. 3), but removing them from the analysis does not affect our conclusion ($r_S = -0.57$, $P = 0.04$, $n = 13$) that larger males had lower sperm concentration in their ejaculates.

Sperm motility

Sperm motility score was not significantly correlated with any measure of male body size or condition ($P > 0.30$ in each case).

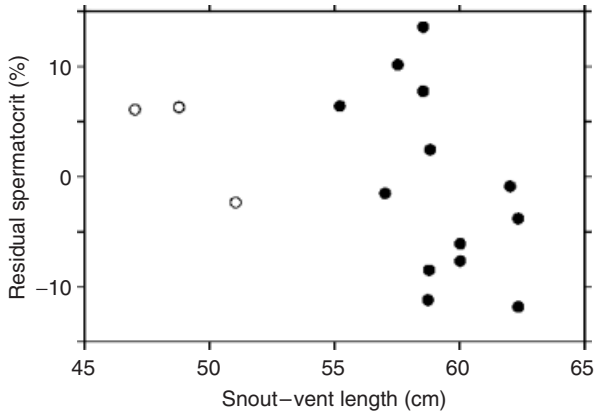

Figure 3 Relation between residual spermatocrit and male snout–vent length. Data from three small males that were outliers are shown by ○.

Discussion

Consistent with other studies examining intraspecific variation in ejaculate traits in both vertebrate and invertebrate taxa (e.g. Morrow & Gage, 2001), we found substantial interindividual variation in ejaculate traits among male northern watersnakes. In addition, we found that males in poor condition (as measured by haematocrit) had more variable sperm morphology, and that small males had ejaculates with a higher concentration of sperm than large males.

Watersnake sperm were relatively long compared with other vertebrate taxa. For example, mean sperm length in northern watersnakes is 111.9 μm, or about 2–5 $\times$ as long as the sperm of most fishes studied to date (Stockley *et al*., 1997) as well as 100 species of Australian myobatrachid frogs (Byrne, Simmons & Roberts, 2003). In mammals, sperm length rarely exceeds 100 μm except for some rodent species (Gage & Freckleton, 2003). The only vertebrate taxa that have sperm that are obviously larger than that of northern watersnakes are birds, with some species having sperm up to 290 μm in length (Briskie & Montgomerie, 1992; Briskie, Montgomerie & Birkhead, 1997). The reasons for this vast variation in sperm length are likely complex, involving phylogenetic constraints, coevolution with the female reproductive tract (Briskie & Montgomerie, 1992) and the degree of multiple mating by the female. In terms of intraspecific variation in sperm length, our estimates of variation in sperm length among males were comparable to other species reported by Morrow & Gage (2001). CV in flagellum length (calculated from standard error) for a variety of species (mostly mammals) ranged from 0.05 to 1.6%, whereas the CV for northern watersnakes was 0.98%, suggesting very similar patterns of intraspecific variation.

Previous explorations of intraspecific variation in sperm length have consistently found significant variation among individual males. Thus, in species as diverse as field crickets *Gryllus bimaculatus*, wood mice *Apodemus sylvaticus* and rainbow trout *Onchorhynchus kisutch*, male sperm length varies significantly among males (Morrow & Gage, 2001). Why this variation exists, and why sperm length should not be subject to stabilizing selection (which would result in low intermale variation), is unclear. However, sperm length may be condition dependent in some species (Schulte-Hostedde & Millar, 2004; but see Gage & Cook, 1994). Our result that males in poor physiological condition (as measured by haematocrit) produce sperm that is more variable morphologically than that of males in good condition suggests that spermatogenesis is more stable in males in good physiological condition. Similarly, in yellow dung flies *Scathophaga stercoria*, intramale variation in sperm length was increased when food was limited (Hellriegel & Blanckenhorn, 2002), suggesting that body condition may play a role in spermatogenesis.

Despite the significant relation between sperm variability and body condition, the majority of ejaculate traits were not related to size-corrected mass or haematocrit. The lack of condition dependence of most ejaculate traits in northern watersnakes is consistent with documented patterns of reproductive effort that seem to place this species in an unusual position, at least with respect to other reptiles (Olsson & Madsen, 1998). During the mating season the home range of male northern watersnakes can average 0.5 ha when females are widely distributed (Brown & Weatherhead, 1999). Yet, despite this relatively large home range and the presumed energetic costs of spermatogenesis, males do not lose mass over the course of the mating season (Brown & Weatherhead, 2004). This observation has led to the conclusion that energy does not constrain male reproduction in this species because males continue to forage while seeking mating opportunities (Brown & Weatherhead, 2004). The fact that most ejaculate traits are independent of body condition underlines this conclusion and suggests that alternative factors may constrain male reproduction.

Parker (1990*a*) predicted that when males are in a disfavoured role (i.e. small, subordinate males), they should invest more heavily in ejaculates than males in a favoured role (i.e. large, dominant). Our results indicate that smaller males produce more concentrated ejaculates than large males, suggesting that small males may be in a subordinate role with respect to larger males. However, Weatherhead *et al*. (2002) found no evidence that male reproductive success was related to body size. This pattern may be explained if small males compensate for a reduced probability of copulation by investing more in ejaculates than large males, leading to more equitable reproductive success across different male body sizes.

These non-significant results with respect to condition may have occurred because they are real or of low statistical power, or because our measures of condition were crude. Our sample sizes (n = 16–26 males) are comparable to other studies that have found significant relations between ejaculate traits and indices of size and condition (e.g. Uglem *et al*., 2001; Casselman & Montgomerie, 2004; Schulte-Hostedde & Millar, 2004). Our estimates of condition in northern watersnakes were based on haematocrit and size-corrected mass (which is correlated with energy reserves; Weatherhead

& Brown, 1996), indices that may be coarse estimators of physiological condition.

This study represents a first step in examining sperm competition in a reptile species where multi-male mating by females is pervasive. Further research should investigate the effects of cryptic female choice and variation in sperm quality (e.g. swimming speed) on fertilization success in this species. Sperm storage may occur within a breeding season (a female may participate in more than one mating aggregation in a breeding season) in northern watersnakes (Weatherhead *et al*., 1995; Sever & Hamlett, 2002), so the potential for cryptic female choice exists. In addition, sperm swimming speed has been found to be related to fertilization success in domestic fowl (*Gallus gallus*, Birkhead *et al*., 1999; Froman *et al*., 2002), and so subsequent studies should examine this aspect of ejaculate quality in the context of fertilization success and condition dependence.

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