

# Sperm competition and ejaculate investment in red squirrels (*Tamiasciurus hudsonicus*)

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**Abstract** Sperm competition is widespread in mammals and occurs when ejaculates from two or more males compete within the female's reproductive tract to fertilize the ova. Enlarged testes are associated with sperm competition because they produce sperm, but the accessory glands produce fluids and proteins that are also important for fertilization success. Sperm morphology can also have consequences for fertilization success because of its influence on sperm motility. Red squirrels engage in multiple mating, and thus sperm competition is likely. Here, we assess levels of multiple paternity in a natural population of red squirrels, test the prediction that testis size is correlated with size of the accessory glands and sperm morphometry, and test the prediction that ejaculate investment is condition-dependent. Five of six litters (83%) showed evidence of multiple paternity, indicating that sperm competition is likely to have occurred. Testis size was correlated with the size of all three accessory glands (prostate, seminal vesicle, epididymides), and there was a generally positive relationship between the size of the accessory glands and sperm length. Sperm morphology showed significant variation in size and shape among individual male squirrels. There was no evidence of condition dependence of testis size or the size of the accessory glands, but sperm midpiece length was negatively related to body condition. Further work should include determining the fitness consequence of variation in sperm morphometry, testis size, and accessory gland size, and determining the effects of variation in ejaculate investment on sperm motility.

**Keywords** Accessory glands · Midpiece · Multiple paternity · Sperm morphometry

## Introduction

Sperm competition is a widespread phenomenon in mammals and occurs when ejaculates from two or more males compete within the female's reproductive tract to fertilize the ova (Møller and Birkhead 1989; Birkhead and Møller 1998). Sperm competition will result in one (or more) male's sperm "winning" the contest and achieving fertilization of some or all of the female's ova. The presence of multiple paternity within litters (when a female carries a litter fertilized by more than one male) indicates that females mate with multiple males and thus implies that sperm competition occurs (Gomendio et al. 1998; Dean et al. 2006).

The testes produce sperm and reflect investment in ejaculates, and thus one of the predicted consequences of sperm competition is that the testes should be relatively large when the probability of sperm competition is high (Parker 1998). This prediction has been supported in numerous comparative studies across a variety of taxa (mammals—Stockley 2004; Harcourt et al. 1981; frogs: Byrne et al. 2002; birds—Briskie and Montgomerie 1992). Using testes size relative to body size as an index of the degree of sperm competition in a species is now ubiquitous in sperm competition studies (e.g., Short 1979; Stockley et al. 1997; Gage and Freckleton 2003; Anderson et al. 2005).

In addition to sperm, ejaculates contain components formed from the secretions of a variety of reproductive accessory organs (Simmons 2001; Poiani 2006). The fitness consequences of accessory gland size, as well as the influence of accessory glands on sperm competition, have been demonstrated in insects (Chapman 2001; Simmons

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2001; Fairn et al. 2007). In mammals, the accessory glands and associated structures include the seminal vesicles, the prostate gland, the epididymides, and the bulbo-urethral glands (Ramm et al. 2005). While the sperm are critical to the fertilization success of an ejaculate, the fluids and proteins provided by the accessory glands have a number of functions that can influence success in sperm competition including maintaining sperm viability and enhancing sperm motility (Ramm et al. 2005). Comparative analyses of both primates and rodents have found that a positive relationship exists between accessory gland size and the level of sperm competition among species (Dixson 1998; Ramm et al. 2005). Nonetheless, little is known about intraspecific variation in accessory gland size relative to other aspects of ejaculate investment.

Sperm morphology may be a particularly important aspect of ejaculate quality because a male's reproductive success is based, in part, on his sperm's ability to reach and fertilize the ova before his rivals' sperm do. Sperm swimming speed is a major determinant of fertilization success in domestic fowl (*Gallus gallus*) (Birkhead et al. 1999), Atlantic salmon (*Salmo salar*) (Gage et al. 2004), and red deer (*Cervus elaphus*) (Malo et al. 2005). Selection should therefore be strong and favor sperm that swim rapidly and are able to reach the egg before the sperm from rival males. Both flagellum length and the size of the midpiece have been the focus of much interest because of their effects on sperm swimming speed. For example, sperm swimming speed is expected to increase with flagellum length, and it has been shown that in some taxa, species that experience heightened levels of sperm competition have longer sperm (Gomendio and Roldan 1991, Briskie et al. 1997; LaMunyon and Ward 1998; Morrow and Gage 2000). The midpiece houses the helical array of mitochondria that power the flagellum and because a larger midpiece houses larger or more mitochondria (Cardullo and Baltz 1991), sperm with larger midpieces are expected to have greater swimming velocity (Bedford and Hoskins 1990; Turner 2003; Immler and Birkhead 2007). Larger midpieces are associated with species with high levels sperm competition in some birds (Immler and Birkhead 2007), primates (Anderson and Dixson 2002), and in mammals in general (Anderson et al. 2005, but see Gage and Freckleton 2003). Until recently, the shape of the sperm head has received little attention as a determinant of sperm swimming speed despite the relative importance of the head for sperm hydrodynamics. In red deer, sperm with elongated heads swam faster (Malo et al. 2006). Thus, sperm shape and size are important phenotypic traits that should be considered when examining aspects of ejaculate quality.

The North American red squirrel (*Tamiasciurus hudsonicus*) is a common diurnal tree dwelling sciurid. The red squirrel is a seasonal breeder with a mating system that

involves a mating chase in which several males (two to seven) gather around a female in estrus and subsequently chases her (Layne 1954). Female red squirrels are promiscuous and mate with multiple males on the day of estrus (Layne 1954; Lane et al. 2007). This mating system makes sperm competition a probable occurrence (Wauters et al. 1990; Koprowski 1993; Lane et al. 2007).

Here, we test several predictions related to sperm competition and ejaculate investment drawn from sperm competition theory in a population of North American red squirrels. To determine the extent of sperm competition, we assessed parentage of young-of-the-year red squirrels using genetic analyses of microsatellite DNA loci. The presence of multiple paternity within litters would support the existence of sperm competition in our study population, and thus provide an appropriate context for an investigation of ejaculate investment.

Although the testes have been the focal trait when examining ejaculate investment, Ramm et al. (2005) showed that the accessory glands covary with testis size in a comparative analysis among rodent species. We assess the relative size of accessory glands in red squirrels in relation to testis size, predicting that males with large testes (and thus enhanced ejaculate investment) will have large accessory glands. Ejaculate investment is also expected to be reflected in sperm morphology, and so we test the prediction that males with large testes and large accessory glands will have longer sperm, larger midpieces, and more hydrodynamically efficient sperm (i.e., more elongated heads) (Malo et al. 2006).

Spermatogenesis (the production of sperm) is costly (Wedell et al. 2002), and thus we predict that ejaculate traits will be dependent on body condition. Males in good condition (carrying more energy reserves than males in poor condition) are predicted to invest more heavily in ejaculates. The condition dependence of testis size has been established across multiple taxa (Simmons and Kotiaho 2002; Burness et al. 2008) including rodents (Schulte-Hostedde and Millar 2004; Schulte-Hostedde et al. 2005a). To determine if other aspects of ejaculate investment are condition-dependent, we test the condition dependence of ejaculate traits including accessory gland size and sperm morphology (Simmons and Kotiaho 2002).

## Methods

### Multiple paternity

A population of red squirrels in Algonquin Provincial Park, ON (45°30'N, 78°40'W) was sampled on a 23-ha grid of mixed deciduous–coniferous forest during May–August 2005 (Gorrell and Schulte-Hostedde 2008). Tomahawk live traps (Tomahawk, WI, USA) were placed 40 m apart at a

height of approximately 1.5–2 m from the ground on a platform constructed from a shelf bracket and a plank of wood (60×15×1.5 cm) attached perpendicular to a randomly assigned mature tree (D.B.H.>30 cm). Traps were baited with a mixture of oats and peanut butter and a slice of apple. Traps were set every 3 to 4 days and checked within 10 h. Each squirrel was removed from the trap with a cloth bag, marked with a numbered ear tag, aged (juvenile or adult based on mass), and sex was determined. Tissue samples (ear clippings) were collected from 45 adults ( $n=21$  males;  $n=24$  females) and 30 juveniles.

DNA was extracted using QIAGEN DNeasy extraction tissue kits (QIAGEN Inc. Mississauga, Ontario, Canada). Eight microsatellite loci (Thu03, Thu23, Thu 33, Thu55, Thu25, Thu32, Thu42, and Thu50; Gunn et al. 2005) (Table 1) were amplified using polymerase chain reaction (PCR) on an Eppendorf Mastercycler gradient thermal cycler. Each 10- $\mu$ l reaction contained 2- $\mu$ l DNA dilutions with 8 $\mu$ l PCR cocktail (1 $\mu$ l 1 $\times$  PCR buffer (BioShop), 10.3 mM MgCl<sub>2</sub> (BioShop), 0.2 mM dNTP's (BioShop), 0.2 mM labeled forward primer (IDT), 0.2 mM labeled reverse primer (IDT), 5.9 $\mu$ l ddH<sub>2</sub>O (Fisher Scientific), and 1 U of Taq DNA polymerase (BioShop)). PCR cycling reactions were performed according to Gunn et al. (2005) with some minor modifications (annealing temperature was 59°C for primer Thu03 and 62°C for Thu23).

The amplified PCR product was sent to the Natural Resources DNA Profiling and Forensic Center (NRDPFC; Peterborough, Ontario, Canada) for desalting and genotyping on a MegaBASE 1000 DNA Analysis System (Biosciences). Profiling results were then resolved and scored using MegaBASE Genetic Profiler, version 2.2 (Amersham Biosciences).

Paternity was assigned using the likelihood-based approach and simulation procedures from CERVUS 3.0 (Kalinowski et al. 2007). Deviations from Hardy–Weinberg

equilibrium for each microsatellite were tested using CERVUS 3.0. Frequency of rare alleles was set to 1 for testing Hardy–Weinberg equilibrium for CERVUS 3.0. Observed and expected heterozygosities and  $F_{IS}$  were calculated using Genepop 4.0 (Rousset 2008). The identities of both mothers and fathers were unknown for the offspring; thus, to determine paternity, maternity was first found using CERVUS 3.0. An algorithm in CERVUS 3.0 allows for maternity assignment to be performed under the assumption that paternity is unknown. The maternity assignments were confirmed using the location of capture of the putative mothers in relation to the offspring's location. Once maternity was determined, paternity was then assigned using the identity of the mothers. The delta criteria were determined for all potential mothers and fathers using the CERVUS 3.0 assignment simulations. Simulations were run using the parameters outlined in Schulte-Hostedde et al. (2002). The simulations were run for 10,000 cycles and the parameters used for each simulation included the number of candidate parents on the field sampling grid, the proportion of candidate parents sampled (0.95) for mothers and (0.90) for fathers, the proportion of loci typed (0.80), and the rate of typing error (0.01). The relaxed criteria were set at 80% confidence, and the strict criteria were set at 95% confidence for maternity and paternity assignment (Kalinowski et al. 2007).

#### Testis size, accessory glands, and sperm morphometry

Red squirrels from an adjacent population in Algonquin Park were collected from early April to early May 2006. Tomahawk live traps (Tomahawk, WI, USA) were placed on the ground beside a large mature tree and secured to the ground using metal tent pegs. Traps were baited with a mixture of oats and peanut butter and a slice of apple at dawn

**Table 1** Descriptive statistics for the accessory glands (seminal vesicle, prostate gland, and epididymis), testes, and sperm morphology for male North American red squirrels (*Tamiasciurus hudsonicus*) sampled from Algonquin Provincial Park, Ontario ( $n=21$ ; 20 sperm measured per individual)

	Mean $\pm$ SD	Range	Between male CV
Seminal vesicle mass (g)	3.59 $\pm$ 1.06	1.94–5.48	–
Prostate gland mass (g)	0.65 $\pm$ 0.10	0.42–0.80	–
Epididymides mass (g)	0.99 $\pm$ 0.15	0.72–1.30	–
Total testes mass (g)	2.92 $\pm$ 0.39	2.36–3.69	–
Sperm head + acrosome length ( $\mu$ m)	20.48 $\pm$ 1.00	16.76–23.67	4.91%
Sperm head + acrosome width ( $\mu$ m)	16.76 $\pm$ 1.00	13.57–20.52	6.01%
Sperm head + acrosome length/width	1.22 $\pm$ 0.07	0.82–1.48	–
Sperm head length ( $\mu$ m)	11.16 $\pm$ 0.37	9.05–12.40	3.35%
Sperm head width ( $\mu$ m)	10.75 $\pm$ 0.30	9.88–11.35	4.71%
Sperm midpiece length ( $\mu$ m)	7.14 $\pm$ 0.60	5.82–10.79	8.35%
Sperm midpiece width ( $\mu$ m)	1.73 $\pm$ 0.18	1.25–2.40	10.08%
Flagellum length ( $\mu$ m)	134.08 $\pm$ 6.78	114.91–151.41	5.05%
Total sperm length ( $\mu$ m)	154.56 $\pm$ 6.91	134.86–172.85	4.47%

for 5 days each week for total of 4 weeks until the end of the breeding season (late May). Each squirrel was examined within the trap to determine the sex and the reproductive status of the squirrel. If the squirrel was male and had enlarged testes (reproductively active) then the trap was removed, covered in a black cloth and brought back to the Wildlife Research Station adjacent to the trapping grid. All females and non-target animals were released. Males were housed in these traps with food and water until they could be processed (<2 h); during this time, the squirrels were placed in a quiet room and covered with a dark cloth to minimize stress.

After euthanasia (overdose of isoflurane) individuals were measured to quantify body size. Each squirrel was weighed using a Pesola® scale ( $\pm 1$  g). Calipers were used to measure skull length (distance from occipital crest to tip of nose;  $\pm 0.1$  mm) and skull width (maximum width of the zygomatic arch;  $\pm 0.1$  mm). A ruler was used to measure right hind foot length from the heel to the tip of the longest nail ( $\pm 1$  mm). Carcasses were subsequently frozen at  $-40^{\circ}\text{C}$  for 122 days. Total body length was measured from the thawed carcasses. To standardize this measurement, all carcasses were thawed for 17 h. Total body length ( $\pm 1$  mm) was measured from the tip of the nose to the last vertebra of the tail, and tail length was measured ( $\pm 1$  mm) from the base of the tail to the last vertebra using a ruler. Body length was then calculated by subtracting tail length from total body length. All body size measurements were  $\log_{10}$ -transformed to improve normality. To minimize impacts of measurement error, all body size components were measured three times for each individual and the average used in all analyses (Schulte-Hostedde and Millar 2000).

Upon euthanasia, the epididymides, testes, seminal vesicles, and the prostate gland were dissected from each squirrel and identified based on Mossman et al. (1932). Briefly, the testes were found within the scrotum. The epididymis of each testis had a well-marked head (caput epididymis) and tail (cauda epididymis) connected by a narrow body (corpus epididymis). The paired lobulated seminal vesicles were fully engorged and were the most conspicuous gland in the reproductive tract. The seminal vesicles were located behind the urinary bladder where it was located distal to the entrance of the vas deferens extending past the anterior of the urinary bladder. The prostate gland was easily separated from the urethra, except the middle region, just caudal to the bladder. The prostate gland was shaped somewhat like an elongated egg that surrounded the urethra and was located near the entrance of the vas deferens. All connective tissues and fat were removed, and the organs were blotted dry with a paper towel and weighed ( $\pm 0.001$  g) using a digital scale (Acculab electronic precision scale, Edgewood, NY, USA). Sperm samples were collected from all males by making an incision in each epididymis at the junction with the testis and squeezing the contents into an Eppendorf tube. The fluid

was diluted with a 30% formaldehyde solution until a volume of 1 mL was reached. Collection of sperm post-mortem from the epididymis yields mature, normal sperm suitable for morphometric analysis (Olson et al. 2003; Perez-Garnelo et al. 2003).

A subsample of the diluted sperm was smeared on glass slides, dried, and fixed using a 30% formaldehyde solution (Fisher Scientific, Nepean, Ontario, Canada). Slides were stained by immersing them in methanol for 10 min then in eosin for 7 min. Slides were then mounted using Permount (Fisher Scientific, Nepean, Ontario, Canada). Using a Sony XC-ST50 camera mounted on an Olympus SZ61 compound microscope and Image-Pro Express 5.1 software (Media Cybernetics, Silver Spring, MD, USA), 20 sperm per individual were measured at 1,000 $\times$  magnification. Because the acrosome is so large in sciurids (Dvorakova et al. 2005), we measured maximum sperm head length (including the acrosome) and maximum sperm head width (including the acrosome) as well as the length and width of the sperm head alone. The ratio between maximum sperm length and maximum sperm width was calculated as an index of sperm hydrodynamics (Malo et al. 2006). In addition, midpiece length, midpiece width, total sperm length, and tail length (all in micrometer) for each sperm were measured. Each component was measured three times for each individual sperm. The ratio between maximum sperm head length and width (HL/HW) was calculated to determine the degree of head elongation for males (Malo et al. 2006).

#### Statistical analysis

All data were tested for normality (Kolmogorov–Smirnov test) and all measurements of body size, body mass, accessory gland size, and sperm component size were  $\log_{10}$ -transformed to meet the assumptions of normality. An index of overall structural body size was determined using a principal components analysis (PCA) that reduced skull length, skull width, hind foot length, and body length to one principal component (PC1) (Table 2). Because testes mass significantly correlated with date of capture ( $r = -0.38$ ,  $P = 0.026$ ), all analyses with testes mass and accessory gland mass used capture date (Julian date) as a covariate. For all analyses of the testes, epididymides, and seminal vesicles, the sum of the mass of both the right and left sides were used.

To assess the relative size of the testes of our sampled red squirrels in relation to the Rodentia, we calculated the predicted testis mass of each individual male red squirrel using the allometric equation

$$Y = 0.031X^{0.77}$$

(Kenagy and Trombulak 1986) where  $Y$  is testis mass and  $X$  is body mass. We used a dependent  $t$  test to determine if the

**Table 2** Descriptive statistics for structural body measurements and component loadings (PC1) from a Principal Components Analysis for skull length (SKL), skull width (SKW), hind foot length (HFL), and body length (BL) of male North American red squirrels (*Tamiasciurus hudsonicus*) ( $n=21$ ) sampled from Algonquin Provincial Park, Ontario

	Mean±SD	Range	PC1
Skull length (mm)	45.8±2.10	41.6–50.4	0.50
Skull width (mm)	26.6±0.98	24.9–28.1	0.88
Hind foot length (mm)	41.9±2.04	37.0–45.0	0.35
Body length (mm)	180.8±11.4	150.0–201.0	0.79

The first principal component (PC1) explained 44% of the total variance

predicted testis mass was significantly different from the observed testis mass. In addition, we calculated the allometric equation

$$Y = 0.1043X^{0.584}$$

between testis mass ( $Y$ ) and body mass ( $X$ ) for the Sciuridae from data presented in Kenagy and Trombulak (1986) ( $n=11$  species). We again used a dependent  $t$  test to determine if the predicted testis mass was significantly different from the observed testis mass.

The relationship between the relative mass of each accessory gland and relative testes mass was examined using partial correlation coefficients determined with multiple regression (dependent variable—individual accessory gland mass; independent variables—testes mass, Julian date, and somatic body mass). Somatic body mass was calculated by subtracting the mass of the testes and all accessory glands from total body mass.

Multiple regressions were used to examine the effect of body condition on accessory gland mass and testes mass (dependent variable—testes, seminal vesicles, prostate, or epididymides mass; independent variable—somatic mass, capture date, and body size (PC1)). We interpreted the partial correlation coefficients of somatic body mass as “body condition” because it is the independent effect of somatic body mass corrected for size and date on the dependent variable. Using partial correlation coefficients for size-corrected mass (“body condition”) from a multiple regression is parallel to using residuals from a regression of somatic body mass on structural body size (Schulte-Hostedde et al. 2001) but has the advantage of taking into account all of the degrees of freedom (Schulte-Hostedde et al. 2005b). Size-corrected mass is correlated with protein and fat content in rodents (Schulte-Hostedde et al. 2001).

A one-way analysis of variance (ANOVA) was used to examine the inter-individual variation of sperm morphometric components. To assess inter-individual variation in

sperm shape, we conducted a discriminant function analysis (DFA) using seven sperm traits, maximum head length and width (including the acrosome), head length and width, midpiece length and width, and tail length. The DFA used a multivariate analysis of variance (MANOVA) to assess whether there was significant difference among individuals, then each sperm was classified to the individual it most likely belonged to. Mahalanobis’ distances between centroids associated with each individual squirrel were calculated, as were canonical vectors that maximized the variation in sperm components in discriminant space (Pimentel 1979).

Associations between reproductive accessory gland mass and sperm morphometry were investigated using multiple regression (dependent variable—sperm morphometry traits; independent variables—capture date, somatic body mass, and accessory gland mass) to produce partial correlation coefficients. Similar analysis was performed to examine the associations between testes mass and sperm morphometry (dependent variable—sperm morphometry traits; independent variable—capture date, somatic body mass, and testes mass).

Multiple regressions were used to examine the effect of body condition on sperm morphometry. Body condition of reproductively active males was determined as the partial correlation coefficients of somatic body mass after controlling body size, thus, was the independent effect of body mass on sperm morphometry (dependent variable—sperm morphometry traits; independent variable—somatic body mass and PC1).

STATISTICA 6.0 © (StatSoft, Inc. Tulsa, OK, USA) was used for all statistical analyses.

## Results

### Multiple paternity

All females produced only one litter during the sampled breeding season. One microsatellite locus (Thu40) was removed from the genotype analysis because of inconsistent allele peaks between individuals, making scoring of the locus unreliable. CERVUS 3.0 found that all seven remaining microsatellites were in Hardy Weinberg equilibrium (Appendix 1). Heterozygosity of the seven loci ranged from 0.84 to 0.88 and the number of alleles ranged from 10 to 13.

Using 95% confidence, CERVUS assigned maternity to 25 of the 30 (83%) offspring sampled and, of those 25, 16 came from litters with two or more offspring. In total, 15 females were identified as mothers of the 25 offspring, and six of these females were assigned two or more offspring. We confirmed the assignment of maternity by using the location of first capture of the offspring acquired from the

mark-recapture study and the location of the corresponding lactating mother. We found a general concordance in maternity assignment between the mark-recapture data and the genetic data. Only four of the 25 offspring had assigned maternities that did not match the closest lactating mother, as determined by the capture data. However, the genetically identified mothers were found 20–30 m away from the juveniles' sites of first capture.

Using the assigned maternities of litters with two or more offspring, we determined paternity for 13 of the 16 (81%) offspring. Eight of 12 scrotal males were identified as fathers of offspring with the assigned mothers. Five litters with more than one offspring (out of six litters) showed evidence of multiple paternity (83%)—that is, more than one father sired the offspring of the litter. No offspring-paternity match could be made for the sixth litter.

Relaxation of the confidence level of parentage assignment to 80% did not change the number of assigned maternities or paternities.

#### Accessory glands, testis size, and sperm morphometry

##### *Structural body size*

Twenty-two reproductively active males were captured and euthanized. One male was removed from all analyses because his testes were malformed. PCA was performed on body size components from the remaining 21 males to establish overall structural body size with skull length, skull width, hind foot length, and body length (Table 1). The first principal component (PC1) explained 44% (eigenvalue 1.76) of the variation in male body size, and the component loadings for the first principal component were in a uniform direction (Table 3). The component loadings and proportion of variance explained by the morphological variables is similar to those used in other studies of red squirrels and

other rodents (e.g., Schulte-Hostedde et al. 2001; Gorrell and Schulte-Hostedde 2008).

##### *Accessory gland and testes size*

Males with relatively large testes had large reproductive accessory organs. After controlling for somatic body mass and date of capture, a significant positive correlation was found between seminal vesicle mass and testes mass (partial  $r=0.48$ ,  $t=2.27$ ,  $P=0.036$ ; Fig. 1a), prostate gland mass and testes mass (partial  $r=0.49$ ,  $t=2.33$ ,  $P=0.03$ ; Fig. 1b), and epididymides mass and testes mass (partial  $r=0.64$ ,  $t=3.59$ ,  $P=0.002$ ; Fig. 1c). Accessory gland mass and testes mass were unrelated to body condition ( $r<0.3$ ,  $P>0.3$  for all organs). To determine the statistical power ( $1-\beta$ ) of our analysis of body condition and testes and accessory gland size, we used G-Power (Faul et al. 2007) to assess the actual power of our analyses of condition dependence for testes and accessory gland size. Statistical power was relatively low (0.078–0.108) and given our sample size, a partial  $r$  of 0.552 would have been required to find a significant effect at  $\alpha=0.05$ .

Mean predicted testis mass calculated from the allometric equation for the Rodentia based on that presented by Kenagy and Trombulak (1986) was 1.76 g ( $\pm 0.10$  SD), whereas the mean observed testis mass was 2.93 g ( $\pm 0.39$  SD). This difference was significant ( $t_{20}=-13.20$ ,  $P<0.001$ ). Thus, male red squirrels had testes that were, on average, 66.5% heavier than predicted. In relation to the Sciuridae specifically, the mean predicted testis mass was 2.22 g ( $\pm 0.095$  SD). The difference between observed and predicted testes mass was significant ( $t_{20}=-7.98$ ,  $P<0.001$ ).

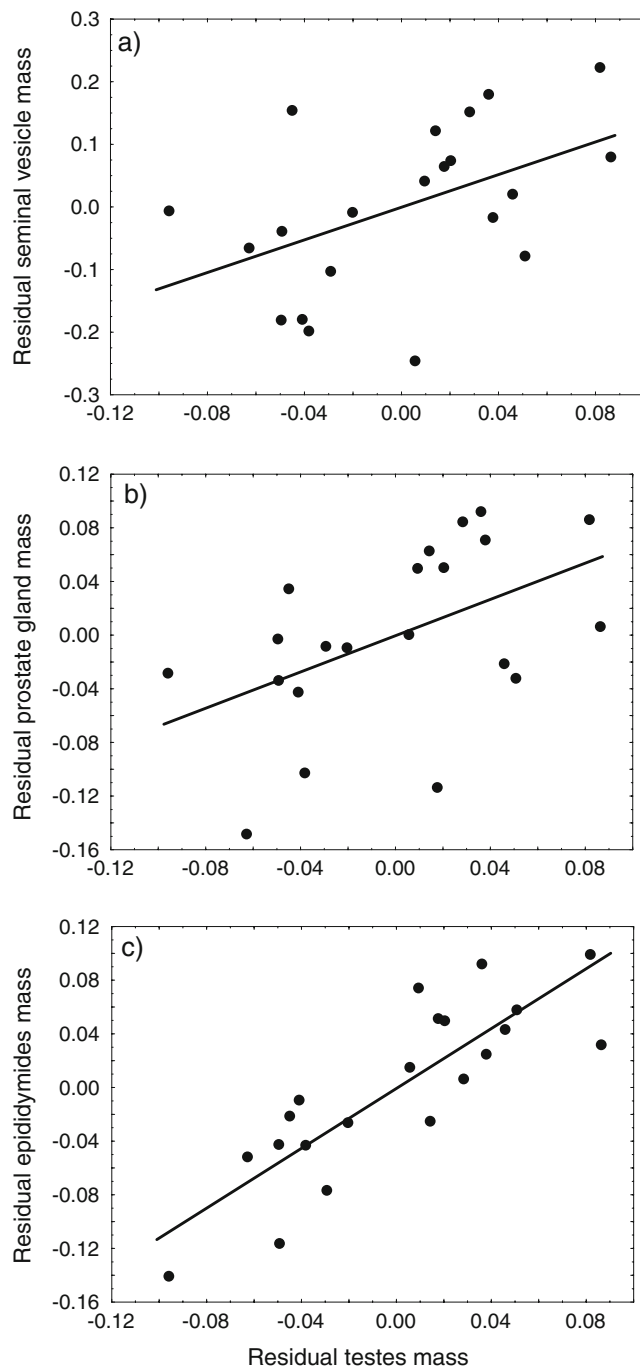
Observed testes mass was 1.57% of somatic mass (body mass minus testes mass only) (range 1.18–2.00%). This value should be interpreted cautiously because testes mass declined with date of capture, and our estimate of relative testes mass of 1.57% is not adjusted for date of capture.

##### *Sperm morphometry*

The sperm measurements for some sperm components were incorrectly recorded, and so for five males, the number of sperm measured for some sperm components was 19 instead of 20. ANOVA of sperm morphological traits (Table 1) revealed significant differences among individual males for all sperm components ( $F_{20,399}>2.23$ ,  $P<0.001$  for all traits; Fig. 2). Discriminant function analysis (DFA) was used to assess inter-individual variation in sperm length. The initial MANOVA found a significant difference among individuals (Wilk's  $\lambda=0.071$ ,  $F_{140,2581}=9.037$ ,  $P<0.001$ ). The average Mahalanobis distance among males was 7.786 (range 0.538–37.505). The DFA correctly classified 38.2% of sperm with the appropriate individual red squirrel.

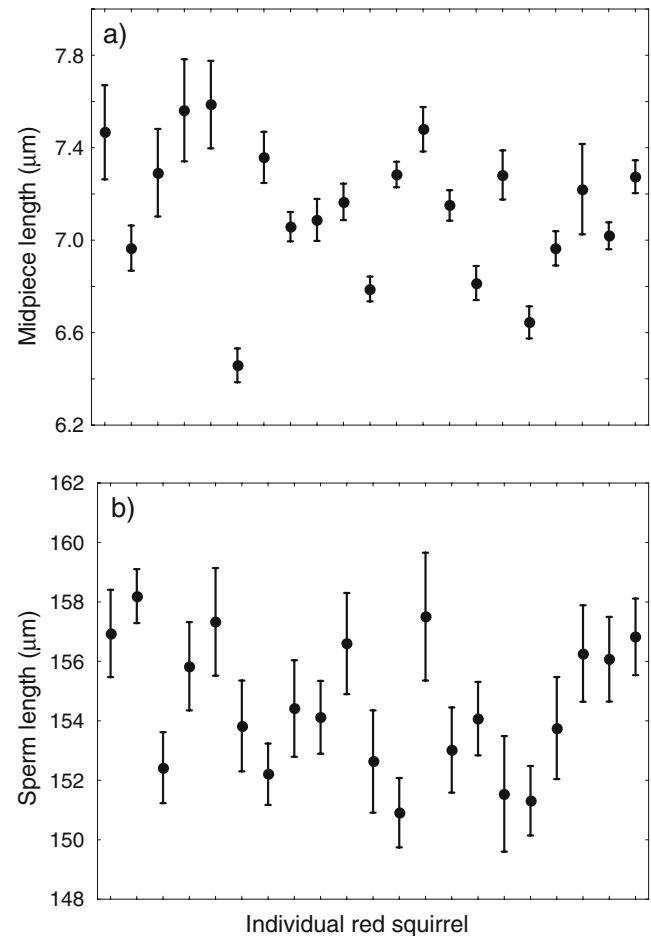
**Table 3** Factor structure of canonical vectors CV1 and CV2 for seven sperm components from male red squirrels ( $n=21$ )

Sperm morphological trait	CV1	CV2
Max. head length	-0.031	0.614
Max. head width	0.295	0.619
Head length	0.309	-0.049
Head width	-0.939	0.189
Midpiece length	0.067	0.146
Midpiece width	-0.197	0.275
Tail length	0.021	-0.194
Eigen value	0.721	1.038
Cumulative proportion of variance explained	0.461	0.730



**Fig. 1** Plot of residual testes mass (corrected for date of capture and somatic mass) and mass of the individual accessory glands (**a** seminal vesicles ( $P=0.036$ ), **b** prostate gland ( $P=0.03$ ), **c** epididymides ( $P=0.002$ )). In all cases, males with relatively large testes had large accessory glands

The first two canonical vectors (CV1 and CV2) explained a cumulative proportion of variation in the sperm morphological traits of 73.0%. Both CV1 and CV2 (Fig. 3) described variation in sperm shape, with CV1 most strongly associated with variation in head width and to a lesser

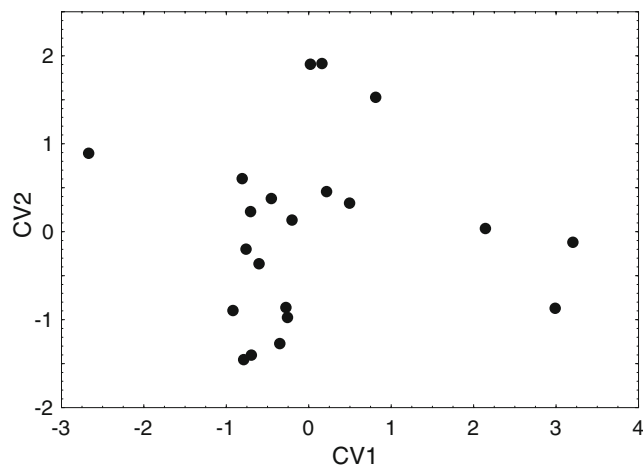


**Fig. 2** Inter-individual variation of representative sperm traits (**a** midpiece length, **b** sperm length;  $P<0.001$  for both traits). Mean values per individual are represented. Error bars represent 95% confidence intervals

extent head length and maximum head width. CV2 was most strongly associated with variation in maximum head length and maximum head width, and to a lesser extent midpiece width.

In general, larger accessory glands were associated with longer sperm. Males with relatively heavy prostate glands had longer sperm in terms of total sperm length (partial  $r=0.46$ ,  $P=0.047$ ). As well, males with relatively heavier epididymides had longer flagella (partial  $r=0.43$ ,  $P=0.05$ ). A similar but not significant relationship was found for seminal vesicle mass and total sperm length, (partial  $r=0.42$ ,  $P=0.07$ ). Males with relatively heavy prostate glands tended to possess longer midpieces (partial  $r=0.43$ ,  $P=0.067$ ).

Maximum sperm width (including acrosome) was negatively but not significantly related to testes mass after correcting for somatic mass and date of capture (partial  $r=-0.445$ ,  $P=0.056$ ). Thus, males with large testes tended to have narrow sperm heads.



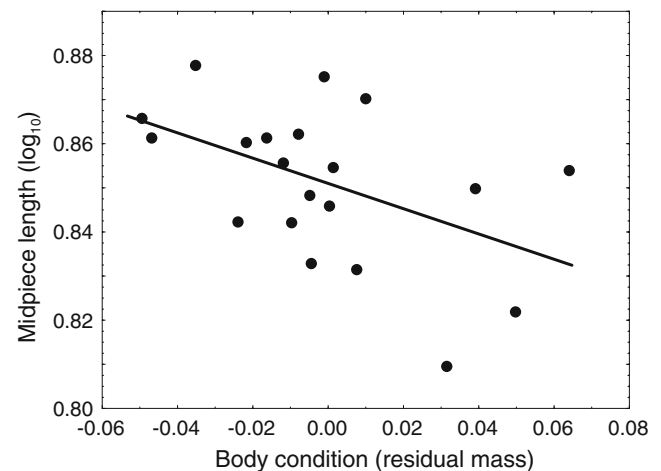
**Fig. 3** Centroids for conical vectors (CV) based on canonical analysis of sperm morphological traits (see Table 3 for vector loadings). Each data point represents a single male red squirrel. There is significant variation in sperm shape among males ( $P < 0.001$ )

A significant relationship was found between sperm midpiece length and body condition, where males in better body condition had smaller midpieces (somatic body mass partial  $r = -0.44$ ,  $P = 0.05$ , Fig. 4). No other sperm traits were associated with body condition.

## Discussion

The relatively high degree of multiple paternity (83% of litters with  $\geq 2$  offspring) observed in this population of red squirrels provides ample evidence that sperm competition occurs in this species. Thus, investment in ejaculates should be important in the context of individual fitness for male red squirrels. The estimate of multiple paternity from our population of red squirrels is somewhat inflated compared with Lane et al. (2007) who reported that 45 of 68 (66.2%) litters from red squirrels in the Yukon were multiply sired. This discrepancy may be the result of our modest sample size and the relatively low number of females that were assigned  $\geq 2$  offspring. Other squirrels, and rodents in general, have similar degrees of multiple paternity including 91.7% in yellow-pine chipmunks (*Tamias amoenus*) (Schulte-Hostedde et al. 2004), 78% in Belding's ground squirrels (*Spermophilus beldingi*) (Hanken and Sherman 1981), and 89% in California ground squirrels (*S. beecheyi*) (Boellstorff et al. 1994). Nonetheless, our estimate of the rate of multiple paternity should be interpreted cautiously because of our relatively small sample size ( $n = 6$  litters with  $\geq 2$  offspring) and because we did not bring pregnant females into the lab and collect DNA samples from the entire litter directly at parturition.

In addition, the predicted testes mass based on an allometric equation for rodents (Kenagy and Trombulak



**Fig. 4** Plot of body condition (residual body mass) and sperm midpiece length. Male red squirrels in poor condition had larger midpieces than males in good condition ( $P = 0.05$ )

1986) indicates that testes mass of red squirrels is about 66.5% greater than predicted. Using an allometric equation calculated from data provided in Kenagy and Trombulak (1986) for the Sciuridae alone indicates that testes mass is approximately 32% greater than predicted. The difference between these two relative values for testes size can be explained by the relatively high incidence of sperm competition within the Sciuridae that renders testes size larger among the Sciuridae than the rodents in general (e.g., Koprowski 2007). The relative testis mass expressed as a percentage of somatic mass (1.57%) is comparable to other sciurids that engage in sperm competition including chipmunks (e.g., yellow-pine chipmunks 1.2% (Schulte-Hostedde and Millar 2004), Townsend's chipmunk (*T. townsendii*)—1.2% (Kenagy and Trombulak 1986)) and other tree squirrels (Eurasian red squirrel (e.g., *Sciurus vulgaris*)—0.8% and gray squirrel (*Sciurus carolinensis*)—0.99% (Kenagy and Trombulak 1986)).

Accessory glands have been implicated as important contributors to ejaculate quality via the fluids they excrete and the proteins they produce (Ramm et al. 2005). It has been well established in insects that accessory glands and their products can affect fitness (Chapman 2001; Simmons 2001; Fair et al. 2007). The importance of the accessory glands to success in sperm competition in mammals has been highlighted by Ramm et al. (2005), who found a positive relationship between testes size and the size of several accessory glands including the prostate and seminal vesicles in a comparative study among rodents. Similar results focusing on primates and the seminal vesicles have been found (Dixson 1998). Underlying the comparative patterns that indicate that species with large testes have large accessory glands (Dixson 1998; Ramm et al. 2005) is the assumption that these patterns exist within species—that

is, that individuals that invest heavily in ejaculates (and thus succeed at sperm competition) will have concomitantly enlarged accessory glands. In our study, male red squirrels that had larger testes also had larger epididymides, seminal vesicles, and prostate glands. This pattern suggests that an increase in ejaculate investment requires not only larger testes to produce sperm but larger accessory glands to produce fluids and proteins. The increased size of the epididymides also suggests that individuals with larger testes have greater sperm storage capacity and thus have an increased number of sperm per ejaculation (Ramm et al. 2005). These results suggest that considerations of ejaculate investment should encompass structures beyond the testes and include the accessory glands and associated structures.

We originally hypothesized that investment in ejaculates would be reflected in sperm morphometry, and indeed we did find some patterns that indicated that males with large accessory glands had longer sperm. In addition, males with large testes tended to have sperm with narrower sperm heads. The hydrodynamic advantage afforded to a sperm with a narrower head presumably makes the sperm more competitive with rival ejaculates. Longer tails and narrow heads are associated with greater sperm swimming speeds (Gomendio and Roldan 1991; Burness et al. 2004; Malo et al. 2006) and thus our result is consistent with the idea that males that invest heavily in ejaculates produce elongated sperm with narrow heads—both properties presumably advantageous in a hydrodynamic context.

The lack of condition dependence of testis size and accessory gland size was a surprising result, especially given the large number of studies that have found this pattern. For example, a positive relationship between body condition and testes size has been found in deer mice (*Peromyscus maniculatus*), red-back voles (*Clethrionomys gapperi*), bushy-tailed woodrats (*Neotoma cinerea*) (Schulte-Hostedde et al. 2005a), yellow-pine chipmunks (Schulte-Hostedde and Millar 2004), and dung beetles (*Onthophagus taurus*) (Simmons and Kotiaho 2002). Males in good condition should invest heavily in their ejaculates, as better quality ejaculates improve reproductive success under sperm competition, ultimately resulting in fitness benefits for these males (Preston et al. 2003; Schulte-Hostedde and Millar 2004). The lack of evidence of condition dependence of testes size and other accessory glands may be because of low statistical power. The actual statistical power ( $1-\beta$ ) of our tests ranged from 0.078 to 0.183 and would have required a much larger effect size in order to gain a significant pattern at  $\alpha=0.05$ . Thus, we cannot accept the null hypothesis in the absence of an adequate sample size. Nonetheless, the sample size used here was similar to other studies that have found significant condition dependence (Schulte-Hostedde and Millar 2004, Schulte-Hostedde et al. 2005a), and the range of standard-

ized variation in residual mass was also similar. Thus, future work that enhances sample size, uses alternative estimates of condition (e.g., hematocrit—Schulte-Hostedde and Montgomerie 2006) and an experimental approach to enhance individual condition may be fruitful in definitively answering this question.

Consistent with other studies, we found substantial variation in sperm morphology among males. Significant inter-individual variation in all sperm components measured, including sperm length, has been found among males of several species including the northern water snake (*Nerodia sipedon*) (Schulte-Hostedde and Montgomerie, 2006), red deer (Malo et al. 2006), Atlantic salmon (Gage et al. 1998), and other vertebrates and invertebrates (Morrow and Gage 2001). Of particular interest was that the midpiece had the highest coefficient of variation of all sperm components. The midpiece houses the mitochondria that power the sperm's flagellum, and these mitochondria use ATP (adenosine triphosphate) as a source of energy. High phenotypic variance is often indicative of traits under sexual selection (Pomiankowski and Møller 1995), and so the midpiece may be a structure that is under stronger sexual selection than other sperm components.

The significance of the midpiece is highlighted by the negative relationship between body condition and midpiece length—males with longer midpieces were in poorer condition than males with shorter midpieces. Interestingly, a similar result was found in lake whitefish (*Coregonus clupeaformis*) (Burness et al. 2008) where ATP reserves (presumably housed in the midpiece with the mitochondria) were negatively related to body condition. The correlation between body condition and the midpiece/ATP observed in both lake whitefish and red squirrels may be mediated by a third, unmeasured variable. One possibility is testosterone, which elevates metabolism and thus reduce energy reserves, as well as enhancing reproductive investment (Bhasin et al. 1998, Buchanan et al. 2001).

The importance of the midpiece is further supported by the large size of the midpiece in species of primates in which females mate with multiple partners relative to primates where females mate with a single partner (Anderson and Dixson 2002). Complicating this interpretation is the recent finding that sperm with shorter midpieces had greater swimming velocity in red deer (Malo et al. 2006). Thus, sperm motility studies will be required to assess whether a short or long midpiece is advantageous in red squirrels, and how this is affected by variation in energy reserves, both at the level of individual sperm and of the individual animal.

There is no clear explanation of why variation in sperm components exists, especially if this variation reduces a male's overall ejaculate competitiveness via sub-optimal

sperm morphology and reduced sperm motility (Morrow and Gage 2001). It has been predicted that sperm form and function will experience intense selection and thus sperm size will be optimally distributed at the population level (Morrow and Gage 2001). All males in the population should produce this distribution and thus there should be little variation within and between males (Morrow and Gage 2001). It may, however, be advantageous for internal fertilizing males to have a mixed strategy for sperm morphology, perhaps in order to better overcome female reproductive barriers (Morrow and Gage 2001). Of particular interest was significant inter-individual variation in sperm shape, with sperm components varying in relative size across males. The adaptive significance of this variation is unclear, but as with variation in sperm size, a mixed strategy may be employed by males in an attempt to overcome any barriers present in the female's reproductive tract.

Several avenues are now open to pursue issues raised by the results of this study. First, as mentioned, an experimental approach to raising individual body condition would help address the question of condition dependence of testis size and the midpiece. Do supplementally fed males develop larger testes and produce sperm with larger midpieces? An assessment of sperm motility is also critical to determine the correlates of various sperm components to sperm motility and ultimately

fertilization success. Finally, understanding the fitness consequences of variation in sperm morphometry, testis size, and accessory gland size will elucidate the selection pressures on these phenotypic traits that are so important in sperm competition.

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## Appendix 1

Characterization of seven microsatellite loci in the North American red squirrel (*Tamiasciurus hudsonicus*) captured in Algonquin Provincial Park, ON. The number of alleles, allele size range (in bp), the observed ( $H_O$ ) and expected ( $H_E$ ) heterozygosities,  $F_{IS}$  and Hardy-Weinberg equilibrium P-values (HWE) are given for each locus. Locus name and accession number from Gunn et al. (2005).

Microsatellite	Accession #	Number of alleles	Allele size (bp)	$H_O$	$H_E$	$F_{IS}$	HWE (p)
Thu03	AJ585770	12	217–239	0.76	0.87	0.1	0.57
Thu33	AJ841910	10	143–161	0.82	0.86	0.05	0.80
Thu23	AJ841900	13	178–202	0.92	0.88	−0.06	0.78
Thu55	AJ843236	11	251–275	0.71	0.87	0.20	0.19
Thu25	AJ841902	11	175–203	0.88	0.86	−0.02	0.87
Thu32	AJ841909	11	264–286	0.88	0.87	−0.02	0.06
Thu42	AJ843223	12	236–260	0.78	0.84	0.07	0.62

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