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Intraspecific variation of testis size and sperm length in the yellow-pine chipmunk (*Tamias amoenus*): implications for sperm competition and reproductive success

Received: 27 May 2003 / Revised: 8 October 2003 / Accepted: 9 October 2003 / Published online: 31 October 2003
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Abstract Comparative analyses have found that relative testis size is a strong predictor of the prevalence of sperm competition for many taxa, including mammals, yet underlying this pattern is the assumption that intraspecific variation in testis size is related to individual fitness. Because intraspecific variation in ejaculate investment underlies interspecific patterns, it is critical to understand the causes and consequences of intraspecific variation in ejaculate investment. We examined relationships between ejaculate investment (testis size and sperm length) and reproductive success, body size and condition in the yellow-pine chipmunk (*Tamias amoenus*), a small ground squirrel in which sperm competition occurs. We examined genetic estimates of male reproductive success from a wild population of yellow-pine chipmunks and determined that males with large testes had higher annual reproductive success than males with small testes. This result provides empirical support for the numerous comparative studies that indicate testis size is associated with the intensity of sperm competition. In addition, males in good condition had relatively larger testes than males in poor condition, but there was no evidence of sperm length being dependent on condition. Finally, contrary to many predictions, males that invested more in sperm production (relatively heavy testes) produced shorter sperm, not longer sperm, than males that invested less.

Keywords Body size · Condition dependence · Mammals · Sperm competition · Testis size

Introduction

Sperm competition is a common phenomenon across the animal kingdom [see Birkhead and Parker (1997) and Birkhead and Møller (1998) for reviews]. If a female copulates with more than one male, the sperm from these males will compete within the female's reproductive tract to fertilize the egg(s). Theoretical models predict that males should adjust general levels of ejaculate investment, as well as specific investment with respect to a particular female, depending on the likelihood of sperm competition (Parker 1990a, 1990b, 1993, 1998). Because sperm is produced in the testes, one of the predicted consequences of sperm competition is that the testes should be relatively large when the likelihood of sperm competition is high. This prediction has borne true across multiple taxa in many comparative analyses (e.g. mammals Harcourt et al. 1981; Ginsberg and Rubenstein 1990; Heske and Ostfeld 1990; birds Møller 1991; Møller and Briskie 1995; fish Stockley et al. 1997). Similar predictions have been made with respect to intraspecific variation in testis size, although these predictions are not consistently supported by the data (e.g. Rising 1987; Ribble and Millar 1992).

Sperm competition is prevalent in mammals (Møller and Birkhead 1989; Gomendio et al. 1998) and appears to influence penis morphology, ejaculate parameters and behaviour of many species (Ginsberg and Huck 1989). Intraspecific variation in testis size has been implicated as an important factor in male reproductive success because larger testes produce higher quality ejaculates (number of sperm, ejaculate volume and motility) and have higher rates of sperm production (Ginsburg and Huck 1989; Stockley and Purvis 1993; Gomendio et al. 1998). Thus, males with larger testes are assumed to have higher reproductive success than males with small testes in species with sperm competition. Although interspecific

Communicated by P.M. Kappeler

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patterns of testis size and sperm morphometry have often been examined in the context of sperm competition, an understanding of intraspecific variation in these parameters is critical to the evaluation of hypotheses regarding ejaculate investment and sperm competition.

A hypothesis developed by evolutionarily stable strategy (ESS) modelling suggests that subordinate males, which are less likely to copulate with a female at the optimal time for achieving fertilization, should invest more heavily in ejaculates than dominant males (i.e. male roles are fixed rather than random) (Parker 1990a). In this way, small subordinate males can compensate for their reduced likelihood of copulating with a female by maximizing their overall investment in sperm. If testis size is correlated with ejaculate investment, then large dominant males should have relatively small testes compared with small subordinate males. Indeed, a comparative analysis using 17 mammalian species found that small males invest disproportionately more in sperm production than large males (Stockley and Purvis 1993).

In contrast, relationships between sperm competition and sperm size have not shown a consistent pattern. Long sperm are presumed to swim faster than short sperm, and thus males that produce long sperm should have an advantage in sperm competition (Gomendio and Roldan 1991; Gomendio et al. 1998). ESS models predict that sperm size should increase, decrease or not change under sperm competition, depending on the parameters of the particular model (Parker 1993, 1998; Parker and Begon 1993). Comparative analyses have found a positive (Gage 1994; Briskie et al. 1997; Balshine et al. 2001), negative (Stockley et al. 1997), and no relationship (Hosken 1997) between sperm length and sperm competition across many taxa, including mammals. In a limited analysis comprising rodents and primates Gomendio and Roldan (1991) found a positive relationship between the level of sperm competition and sperm length. In contrast, Gage and Freckleton (2003) found no relationship between sperm competition and sperm length when analyzing 12 orders of mammals and correcting for phylogeny. Thus, despite the potential for intraspecific variation in sperm length to affect the outcome of sperm competition (Ward 1998), interspecific patterns of sperm length relative to sperm competition remain unclear, and explanations for intraspecific variation in sperm length of mammals remain largely unexplored.

The yellow-pine chipmunk (*Tamias amoenus*) is a small, ground-dwelling sciurid in which females are in oestrus for 1 day each year in late April or early May. Females advertise their oestrous state through vocalizations 3–5 days prior to its onset (Callahan 1981). Several males (2–6) aggregate near the female's den and pursue her on the day of oestrus. The female copulates with one or more males during this "mating chase" (Callahan 1981), and there is genetic evidence that the resulting litter is sired by more than one male (Schulte-Hostedde et al. 2003). Thus, sperm competition is probably a pervasive consequence of the breeding system of this species, as well as in other chipmunks and tree squirrels that

engage in a "mating chase" (Elliott 1978; Yahner 1978; Wauters et al. 1990; Koprowski 1993).

It is important to test predictions related to intraspecific variation in testis size in order to understand the underlying processes governing any observed interspecific patterns. Here, we test several predictions related to the fitness consequences of testis size, and relationships among body size, body condition and ejaculate investment (testis size and sperm length) in male yellow-pine chipmunks.

A fundamental assumption of sperm competition theory is that individual variation in ejaculate investment translates into fitness benefits under sperm competition (Parker 1998; Preston et al. 2003). We evaluate the fitness consequences of variation in testis size by examining the relationship between testis size and genetic estimates of annual reproductive success in a wild population of yellow-pine chipmunks (Schulte-Hostedde et al. 2002). We additionally obtained ejaculates from a sample of male chipmunks to examine relationships among body size, condition, testis size and sperm length. In particular, we tested the prediction that males with large testes will produce high quality (longer and thus faster) sperm (Gomendio and Roldan 1991). We also tested the hypothesis that the cost of ejaculate investment is high. There is evidence of condition-dependence of ejaculate parameters (Simmons and Kotiaho 2002, but see Gage and Cook 1994), and when females mate with multiple males, information from ejaculates, such as sperm length, may be used to assess male quality and thus aid in post-copulatory female choice (Pitnick et al. 1999). We therefore examined the relationship between male body condition, and testis size and sperm length.

Methods

Fitness consequences of testis size

To determine if there was any effect of testis size on male reproductive success, we re-examined genetic estimates of male reproductive success from yellow-pine chipmunks monitored in the Kananaskis Valley, Alberta, Canada (51°N, 115°W) (see Schulte-Hostedde et al. 2002 for details). Briefly, we conducted a mark-recapture study of yellow-pine chipmunks from 1998 to 2000. Body length and body mass were measured for individually marked chipmunks, and pregnant/lactating females were radio-tracked to their maternal den, where the offspring were captured upon emergence. Ear tissue samples were taken from putative fathers, mothers and offspring, and microsatellite DNA loci used to assign parentage (Schulte-Hostedde et al. 2000). Reproductive success was defined as the number of emerged offspring assigned to a particular parent. In 1999 and 2000 we measured the length of the right testis (± 1 mm) through the scrotum for all males captured during the breeding season (May). For many individuals, more than one measurement was taken over the course of the breeding season. We used the maximum testis length in our analysis when multiple measurements had been taken. To assess the relationship between external testis length (testis length measured through the scrotum) and actual testis mass, we measured external testis length of the chipmunks used in the electroejaculations (see below) and examined length relative to testis mass. External testis length was correlated with testis mass ($r=0.45$; $P=0.01$; $n=24$).

Size and condition dependence of testis size and sperm length

We collected ejaculates and measured morphometric traits of yellow-pine chipmunks trapped during the breeding season (late April to late May) of 1999 and 2000. Male yellow-pine chipmunks are generally sexually mature after their first winter (Sheppard 1969) and all males in this study were in breeding condition (i.e. the testes were enlarged and hair surrounding the testes was dark brown). All males were captured over 1–2 weeks during each year ($n=15$ in both 1999 and 2000) from seven sites (separate from the mark-recapture study described above) in the Kananaskis Valley, and kept at the University of Calgary Kananaskis Field Station. Chipmunks were housed individually in plastic or metal cages (40×30×15 cm; L×W×H). Food (sunflower seeds and rat chow) and water were provided ad libitum, and apple was occasionally provided.

We used electroejaculation (Martin 1978; Concannon et al. 1996) to obtain ejaculate samples from male chipmunks (successful electroejaculations: 1999 $n=14$; 2000 $n=10$). Individuals were anaesthetized (Metofane, Jannssen Pharmaceutica, North York, Ontario, Canada) and a lubricated probe (No.1 probe, P-T Electronics, Boring, Ore.) was inserted into the anus to a depth of 2–3 cm. The depth of the probe was sometimes varied to achieve ejaculation. Using an electroejaculator (Model 303, P-T Electronics, Boring, Ore.), we applied an electric current through the probe in three series of 10–25 pulses with durations of 3 s, and 15–20 s between pulses: the first series at 1 V, the second at 2 V and the third at 3 V. Generally, 10 pulses of 1 V were conducted, followed by 10–15 pulses at 2 V and 10–25 pulses of 3 V until emission of ejaculate occurred. Ejaculate was collected using a capillary tube, which was placed directly on the urethra. Ejaculate samples were subsequently smeared on glass slides, dried, and sprayed with a fixative (CytoPrep, Fisher Scientific, Nepean, Ontario, Canada). Slides were then stained with eosin and mounted. We used the public domain program NIH Image (available at <http://rsb.info.nih.gov/nih-image/>) to measure tail lengths of ten sperm from each male.

Following the acquisition of ejaculates, chipmunks were euthanized [with an overdose of anaesthetic (isoflurane)] and measured to quantify body size (see Schulte-Hostedde and Millar 2000 for details). Total body length was measured to the nearest 1 mm from the tip of the nose to the last vertebra of the tail. Tail length was measured to the nearest mm from the base of the tail to the last vertebra using a ruler. Log-transformed body length (total body length minus tail length) was used as an index of body size. Body mass was measured using a pesola scale (± 1 g). Both testes were dissected and weighed (± 0.01 g). We used the total mass of the testes as an index of testis size.

Statistical analysis

We used a general linear model to examine the effects of testis length on genetic estimates of reproductive success after controlling for body length, body mass and year (1999 and 2000). We interpreted the partial correlation coefficient for testis length as the independent effect of testis length on reproductive success. Because some individuals were represented in both years, we avoided pseudoreplication by randomly selecting one year to represent an individual.

General linear models were also used to determine the factors (e.g. body length, body mass) influencing traits associated with sperm competition (testis mass, sperm length) (see Table 1 for descriptive statistics). Partial correlation coefficients were calculated to determine the independent effect of the predictor on the response variable.

Table 1 Descriptive statistics for measured traits of male yellow-pine chipmunks used in the analysis of intraspecific variation of testis mass and sperm length ($n=30$)

Trait	Mean	SD
Body length (mm)	125	5.65
Body mass (g)	51	3.15
Total testis mass (g)	0.61	0.1
Sperm tail length (μm) ^a	85.3	1.88

^a $n=24$

Results

Fitness consequences of testis size

Males with long testes had greater annual reproductive success than males with short testes (partial $r=0.65$; $P=0.005$; $n=19$; Fig. 1), after controlling for body length, body mass and year.

Size and condition dependence of testis size

We included body mass and body length as predictor variables and testis mass as the response variable in a general linear model. We interpreted the partial correlation coefficient of body mass as “condition” because the coefficient represents the effect of body mass on testis mass, after controlling for body length. This is analogous to using residuals from a regression of body mass on body length as an index of condition and reflects variation in body composition: males in good condition have relatively more muscle mass and fat than males in poor condition (Schulte-Hostedde et al. 2001). Males in good condition had heavier testes than males in poor condition (partial $r=0.45$; $P=0.014$; $n=30$; Fig. 2). Small males had heavier testes than large males had, although this relationship was not significant (partial $r=-0.31$; $P=0.095$; $n=30$).

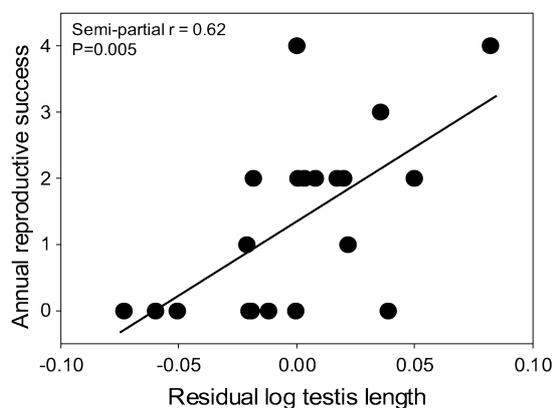


Fig. 1 Semi-partial correlation between annual reproductive success (number of offspring sired) and residual testis length (corrected for body mass and body length) for male yellow-pine chipmunks ($n=19$). Males with relatively large testes sired more offspring than males with relatively small testes

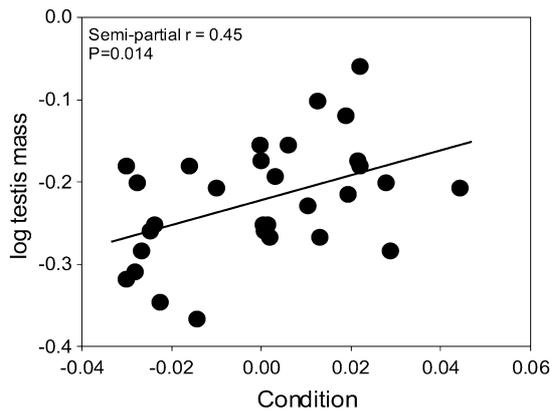


Fig. 2 Semi-partial correlations from multiple regression between testis mass and body mass for male yellow-pine chipmunks ($n=30$). Males in good condition (body mass corrected for body length) had relatively heavier testes than males in poor condition

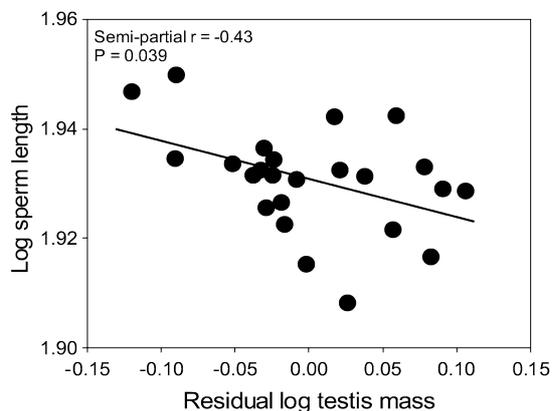


Fig. 3 Semi-partial correlation between mean sperm length and residual testis mass (corrected for body mass and body length) for male yellow-pine chipmunks. Males ($n=24$) with relatively large testes produce shorter sperm than males with relatively small testes

Effect of condition, size and testis mass on sperm length

There was significant variation among individuals in mean sperm length ($F_{23,225}=3.07$; $P<0.001$). We included body mass, body length and testis mass as predictor variables, and sperm length as the response variable in a general linear model. Only testis mass had an independent effect on sperm length: large testes produced shorter sperm, after controlling for body mass and body length (partial $r=-0.44$; $P=0.039$; $n=24$; Fig. 3).

Discussion

A fundamental assumption of sperm competition theory is that male fitness is enhanced by ejaculate investment (measured as testis size) under sperm competition (Parker 1998). Indeed, the assumption that males with large testes have a fitness advantage within populations underlies the large number of comparative studies that have found testis

size to be positively associated with the degree of sperm competition (e.g. Møller and Briskie 1995; Stockley et al. 1997; Byrne et al. 2002). Despite the importance of this assumption, there are few studies that have tested it (but see Bercovitch and Nürnberg 1996; Preston et al. 2003). The results of this study provide evidence that males with relatively large testes do have a fitness advantage under sperm competition. The breeding system of the yellow-pine chipmunk is a promiscuous one in which both males and females mate with multiple partners, and multiple paternity is evident at high levels (Schulte-Hostedde et al. 2003). Male yellow-pine chipmunks with relatively large testes had higher annual reproductive success than males with relatively small testes, consistent with the suggestion that sperm production influences male fitness. This result is similar to that found in Soay sheep (*Ovis aries*), a species in which females mate promiscuously, sperm competition is prevalent, and a large proportion of variation in male reproductive success is explained by testis size (Preston et al. 2003). These intraspecific patterns provide empirical support for the numerous studies of interspecific variation in testis size that indicate that testis size is associated with the intensity of sperm competition in mammals (Gomendio and Roldan 1998) and other taxa (e.g. Møller and Briskie 1995; Stockley et al. 1997).

Small male chipmunks tended to have relatively large testes compared with large males (although not statistically significant), and this result is consistent with a fundamental prediction made by sperm competition models (Parker 1990a). Males in a disfavoured role (i.e. small subordinate males) are predicted to invest more toward ejaculates than males in a favoured role (i.e. large dominant males) to compensate for their reduced likelihood of copulating with a female at the optimal time for fertilization (Parker 1990a). Our results with respect to body length and testis mass are consistent with a comparative analysis of mammals that indicated that, within species, small males tend to invest more in ejaculates than large males (Stockley and Purvis 1993). A caveat to this conclusion, however, is that negative allometry may produce a similar pattern (Kenagy and Trombulak 1986).

Male testis size was positively related to male condition (mass corrected for body length), indicating that males in good condition had relatively large testes. Simmons and Kotiaho (2002) presented similar results in dung beetles (*Onthophagus taurus*) in which males in good condition had larger testes and smaller sperm than those in poor condition. There is evidence of heritability of body condition (Merilä et al. 2001; Simmons and Kotiaho 2002), and therefore females may use ejaculate traits genetically correlated with condition to inform cryptic female choice and obtain indirect benefits for their offspring. Good-sperm models of polyandry (Yasui 1997) have suggested that females may gain indirect benefits from multiple matings when male condition is genetically correlated with ejaculate quality because the outcome of sperm competition ensures that their eggs are fertilized by

high quality sperm. Thus female chipmunks may engage in multiple matings (Callahan 1981; Schulte-Hostedde et al. 2003) in order to ensure that their offspring gain the genes associated with good body condition.

Our results indicated that males that invested heavily in ejaculates (measured as testis mass) produced shorter individual sperm. This is contrary to results obtained from interspecific comparisons in mammals which have suggested that sperm competition selects for larger sperm (Gomendio and Roldan 1991), or does not select for sperm size at all (Gage and Freckleton 2003). One possibility is that males that invest heavily in ejaculates trade-off large sperm against the total number of sperm produced (Parker 1998). Although intraspecific variation in sperm length is rarely examined in mammals, in several fishes no differences in sperm length have been found between alternative phenotypes that differ in the degree of ejaculate investment (e.g. Gage et al. 1995; Leach and Montgomerie 2000; Vladic and Jarvi 2001).

Finally, the results of this study may have consequences for the evolution of female-biased sexual size dimorphism in this species (Schulte-Hostedde et al. 2002). The general pattern in mammals is for males to be larger than females, and this is usually thought to result from the high mating success of larger males, leading to selection for large male size (Andersson 1994). Male reproductive success is independent of body size in yellow-pine chipmunks, and it has been hypothesized that the decoupling of the relationship between male reproductive success and body size has facilitated the evolution of female-biased sexual size dimorphism (Schulte-Hostedde et al. 2002). Indeed, despite evidence of size-dependent patterns of dominance (Schulte-Hostedde and Millar 2002), there is no evidence that male mating success (the number of genetic mates) or reproductive success (the number of offspring sired) is dependent on size (Schulte-Hostedde et al. 2002, 2003). The independence of body size and reproductive success, and the relatively low variation in male reproductive success (Schulte-Hostedde et al. 2003) may be explained by sperm competition, whereby multiple mating by females leads to successful matings by males regardless of size, and males in good condition that invest heavily in sperm are most successful in gaining paternity.

Acknowledgements We thank Dr. K. Goodrowe and D. Ryckman of the Toronto Zoo, and Prof. C. Platz of the International Canine Semen Bank for advice on the electroejaculation technique, and Dr. R. Cooper of the Calgary Zoo for the loan of the electroejaculation equipment. Dr. R.D. Montgomerie provided facilities for the measurement of sperm morphology. The University of Calgary's Kananaskis Field Station is gratefully acknowledged for logistical assistance. We are indebted to H.L. Gibbs and L. DeSousa for facilities and expertises in the genetic analysis of male reproductive success. Comments from G. Burness, S. Casselman and two anonymous reviewers greatly improved the manuscript. This study was supported by an Ontario Graduate Scholarship, Natural Sciences and Engineering Research Council of Canada (NSERC) post-graduate scholarship and two grants-in-aid of research from the American Society of Mammalogists to A.I.S.-H., and an NSERC operating grant to J.S.M. All procedures described herein were approved by the Canada Council on Animal Care.

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