Snail sperm production characteristics vary with sperm competition risk

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Sperm competition is widespread and influences both male investment in spermatogenic tissue and ejaculate characteristics. Sperm competition models assume trade-offs between sperm size and number, although such trade-offs may be difficult to detect. This study examines the effects of sperm competition risk on the sperm production characteristics of the freshwater snail *Viviparus ater*. In this prosobranch, females mate frequently and store sperm, generating sperm competition. Males produce two sperm morphs, fertile eupyrene sperm and non-fertilizing oligopyrene sperm. Non-fertilizing sperm may play a role in sperm competition and therefore, like fertilizing sperm, the number produced could vary relative to sperm competition risk. In addition, trade-offs between sperm number and sperm size may be expected.

We manipulated the sex ratio of sexually mature snails and found the presence of rivals affected the ratio of oligopyrene–eupyrene sperm males produced. In experimental and natural populations, the number of oligopyrene sperm produced, but not the number of eupyrene sperm, was significantly higher when the sex ratio was male biased. Testis mass did not vary between experimental treatments. We also found a negative relationship between the number and size of oligopyrene sperm produced, which is consistent with evolutionary models of sperm competition, and is, to our knowledge, the first intraspecific demonstration of a trade-off between these traits.

Keywords: eupyrene; oligopyrene; sperm competition; sperm number; sperm size; *Viviparus ater*

1. INTRODUCTION

Sperm competition (Parker 1970) occurs in a wide range of taxa (Smith 1984; Birkhead & Møller 1992, 1998), and because sperm production is costly (Dewsbury 1982; Nakatsuru & Kramer 1982) males should optimize their spermatogenic investment according to the sperm competition risk (the probability that a female has mated previously or will mate again subsequently) (Parker 1982, 1990a,b; Parker *et al.* 1996). Theoretical models therefore predict that when sperm competition risk is high, males should invest proportionally more in spermatogenesis, assuming sperm compete numerically (Parker 1982, 1990a,b), and a large number of comparative studies support these predictions (e.g. Short 1979; Harcourt *et al.* 1981; Kenagy & Trombulak 1986; Møller 1988, 1991; Gage 1994; Hosken 1997; Stockley *et al.* 1997). Intraspecific studies also indicate that males can facultatively alter their ejaculates in relation to the perceived sperm competition risk, including the presence of rival males (e.g. Gage 1991; Simmons *et al.* 1993; Cook & Wedell 1996; Gage & Barnard 1996; Simmons & Kvarnemo 1997). For example, male butterflies increase the number of both fertilizing eupyrene and non-fertilizing apyrene sperm in second copulations when the probability of encountering non-virgin females is high (Cook & Wedell 1996). In addition, the proportion of eupyrene sperm in the ejaculate increases. Similarly, male moths increase the

numbers of eupyrene sperm ejaculated into previously inseminated females, whereas apyrene number do not vary with female mating history (Cook & Gage 1995). The exact function of the non-fertilizing apyrene sperm is unknown, but a role in sperm competition has been proposed (Silberglied *et al.* 1984; Cook & Gage 1995).

Non-fertilizing sperm are not unique to lepidopterans, and like other prosobranch snails, *Viviparus ater* produce two sperm morphs, fertilizing eupyrene and non-fertilizing oligopyrene (Meves 1902). However, unlike the non-fertilizing sperm of lepidopterans, oligopyrene sperm contain a single chromosome and are thick, long and extremely motile compared with eupyrene sperm, which contain a full haploid chromosomal complement. Moreover, unlike the Lepidoptera, there appear to have been no experimental investigations of the effects of sperm competition on sperm production in snails, but snail parasperm are produced in large numbers in a highly organized developmental process (Buckland-Nicks 1998), which suggests an adaptive function. If oligopyrene sperm play a role in sperm competition, as has been proposed for other non-fertilizing sperm (e.g. Silberglied *et al.* 1984; Cook & Gage 1995), the quantity produced may be affected by varying sperm competition risk. However, because non-fertilizing sperm morphs, and possibly functions, differ appreciably across taxa, the influence of sperm competition on sperm production may also vary.

The effects of sperm competition risk on sperm size have received less attention than relationships between sperm competition and ejaculate investment (Parker

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1993). It is predicted that sperm size should evolve independently of sperm competition risk, because maintaining sperm size at some arbitrary but optimal minimum enables sperm number to be maximized within a fixed resource budget, and producing more gametes is considered adaptive when sperm compete numerically (Parker 1993). However, Parker's models do suggest that under special conditions, such as when sperm size increases survivorship and the delay between mating and fertilization increases sperm competition risk, selection can favour increased sperm size with increasing risk. Furthermore, it has been argued that increased sperm size is adaptive under conditions of sperm competition because longer sperm generate greater flagella force and possibly swim faster (Katz & Drobnis 1990; Gomendio & Roldan 1991). There is evidence for and against positive associations between sperm size and sperm competition risk. For example, Gage (1994) found a positive relationship across butterflies, although across microchiropteran bats no association was found (Hosken 1997; and see Harcourt 1991; Briskie & Montgomerie 1992; Dixon 1993; Briskie *et al.* 1997; Stockley *et al.* 1997). Nevertheless, a trade-off between sperm size and number is still a key assumption to many evolutionary models of reproduction (e.g. Parker 1982, 1990a,b, 1993; Parker & Begon 1993), although some authors suggest that trade-offs may not be realized (Gomendio & Roldan 1991, 1993). Pitnick (1995) provides evidence for a trade-off between size and number across *Drosophila*, but other intraspecific studies have found no relationship between sperm size and number (e.g. Gage & Cook 1994; Radwan 1996).

The purpose of the current study was to investigate sperm production characteristics in *V. ater* under varying sperm competition risks. This dioecious freshwater snail mates promiscuously and frequently; females remate about every 3.5 days, from April until October (Staub & Ribí 1995), and females store sperm in a spermatheca for up to two years (Trüb 1990). This combination of attributes is likely to generate sperm competition. We experimentally examined the effect of varying the sex ratio of sexually active adults (the operational sex ratio or OSR) on testis mass and the number and size of oligopyrene and eupyrene sperm produced by yearling and adult *V. ater*. We then investigated the relationship between the adult sex ratio (*ca.* OSR) of natural populations and the oligopyrene–eupyrene sperm ratio and sperm size in *V. ater*.

2. MATERIALS AND METHODS

(a) *Experimental design*

Snails were collected during May 1997 from a natural population in Lake Zürich (Switzerland). Approximately 800 adults and yearlings were sexed (the right tentacle of the male is thickened and functions as a penis) and randomly assigned to one of the experimental treatments (a random sample of adult females was retained for experiments). The two treatments were: groups with a female-biased sex ratio (4 males and 20 females; 16 replicates) or with a male-biased sex ratio (20 males and 4 females; 16 replicates). To test for age effects, all the males in eight groups of each treatment were younger or older than 2 years. Each experimental population was kept in rectangular cages (40 cm × 60 cm) in Lake Zürich from the end of May until

the end of October 1997. In June, August, September and October, one male was removed from each experimental replicate and its sperm characteristics were assessed. However, towards the end of the experiment (September and October), five males were excluded from analysis because they were infected with castrating trematode parasites, and three other males had died.

(b) *Assessment of sperm characteristics*

Males whose sperm was to be evaluated were killed by freezing, then shell size and body weight were measured. The shell was broken open and sperm were collected directly from the testis, and always from the same place (just above the point at which the testis joins the ejaculatory duct), with a pasteur pipette. Sperm extract (10 µl) was diluted in 1000 µl of physiological serum (0.9% NaCl), homogenized and a drop of this dilution was placed on two slides. One slide was fixed in methanol, stained with colorap stain (Bioreac, Lausanne) and was then used to assess the numbers of the two sperm types and hence the ratio of oligopyrene to eupyrene sperm (OER). OER was estimated by counting the number of both types of sperm under a microscope (×400) using a grid-marked ocular eyepiece. For each male, we counted approximately 250 spermatozoa. OER was counted twice for a number of males to estimate the accuracy of our method. Regression analysis indicated high repeatability ($n=13$, $F=82.8$, $p<0.001$, $r^2=0.88$). From the dilution we counted the number of oligopyrene sperm using an improved Neubauer counting chamber. However, because of their small size, eupyrene sperm were often difficult to detect and could easily be missed during sperm counts (but see above). Thus, eupyrene number were estimated using the calculated number of oligopyrene sperm and the observed OER. The total number of oligopyrene sperm was not assessed for the first sample of the experimental study, nor in the natural population (see below), but the OER was calculated as above. The other slide was used to measure sperm length. Length was measured from microscope images conveyed to a PC running BIOSCAN[®] OPTIMAS[™] software. For each male, we measured the length of eight oligopyrene and eight eupyrene sperm. After sperm collection, the testis was dissected and weighed. The mass reduction due to sperm sampling was so small relative to testes mass that it had no detectable effect on the results.

(c) *Natural population*

The sperm characteristics of males from natural populations were estimated by collecting 260 snails from six sites in Lake Zürich between 3 and 10 June 1996. At each site, the sex ratio was estimated by counting the number of males and females randomly collected (we assume equal 'trapability' of the sexes). Then the sperm characteristics of 21–24 males per population were assessed, except in one small population where only nine males could be collected. Male and sperm characteristics were measured as above. The density of each population was also estimated by counting the number of snails found within 10 m² using 100 cm × 100 cm frames.

(d) *Statistical analyses*

The effect of experimental treatment and age on the number of oligopyrene and eupyrene sperm was examined using an analysis of covariance (ANCOVA, Systat statistical package (Wilkinson 1989)). Shell size and date of analysis (months) were used as covariates. To test the effect of treatment on gonad investment, regressions of log-transformed testis weight versus

Figure 1. (a) Mean ($\pm$ s.e.) oligopyrene-eupyrene ratio produced by *V. ater* males subjected to high (open circles) or low (black squares) sperm competition risk treatments plotted monthwise. Numbers indicate the number of males sampled. (b) Mean ($\pm$ s.e.) number of oligopyrene (Oligo.) and eupyrene (Eup.) sperm produced by *V. ater* males subjected to high (black bars) or low (open bars) sperm competition risk treatments plotted over three months.

body weight were performed and the residuals from this common regression were used as an index of gonad investment. For natural populations, we analysed the effect of the sex ratio on the OER with a logistic regression model (SAS Institute 1990). We used the GENMOD procedure for the binary response of sperm ratio (i.e. oligopyrene or eupyrene sperm). Because the population density varied and as the size of males and relative testis weight (index of gonad investment) also varied, we included 'density', 'shell size' and 'index of gonad investment' as factors in the model. Significance levels were determined from the changes in deviance of the null model after addition of the independent variables. The change in deviance was approximated by a χ^2 distribution with corresponding degrees of freedom. The relationship between the proportion of sperm and the size of the both types of sperm was analysed with a Pearson correlation coefficient. The proportions of oligopyrene to eupyrene sperm were arcsine-transformed before analysis.

3. RESULTS

(a) Experimental manipulations

Males in the male-biased treatments produced more and a larger proportion of non-fertilizing oligopyrene sperm than males subject to the female-biased treatment, whereas eupyrene sperm number did not change significantly with treatment (figure 1a,b). The sampling date also had a strong effect upon the number of oligopyrene but not eupyrene sperm produced by males (figure 1b). Shell size, male age and the interaction between age and treatment had no significant effect (oligopyrene number: treatment, $F_{1,81}=11.379$, $p=0.001$; date, $F_{2,81}=7.692$, $p=0.001$; age, $F_{1,81}=1.057$, $p=0.307$; shell size, $F_{1,81}=3.076$, $p=0.083$; age $\times$ treatment, $F_{1,81}=0.109$, $p=0.742$; eupyrene number: treatment, $F_{1,81}=0.804$, $p=0.373$; date, $F_{2,81}=0.112$, $p=0.894$; age, $F_{1,81}=0.046$, $p=0.831$; shell size, $F_{1,81}=0.079$, $p=0.779$; age $\times$ treatment, $F_{1,81}=0.362$, $p=0.549$).

When we looked separately at the effect of sampling date on each treatment group, analysis showed that the date only affected the number of oligopyrene sperm produced by males from the high male density (=high sperm competition risk) group (high intensity: date, $F_{2,43}=13.731$, $p<0.001$; low intensity: date, $F_{2,39}=0.157$, $p=0.855$). When we analysed the effect of treatment monthwise, results indicated that males subject to high sperm competition risk produced a higher number of oligopyrene sperm in all months except for October when almost all mating had ceased (table 1). We have no data on total sperm number in June but the sex ratio had a significant effect on the oligopyrene-eupyrene sperm ratio ($F_{1,27}=8.433$, $p=0.006$).

Shell size had no effect upon sperm proportion except in September when shell size was significantly negatively correlated with the number of oligopyrene sperm ($r=-0.50$, $p=0.007$, $N=28$). Treatment and age had no effect on the index of gonad investment (treatment, $F_{1,116}=0.859$, $p=0.356$; age, $F_{1,116}=0.150$, $p=0.699$; treatment $\times$ age, $F_{1,116}=0.560$, $p=0.456$).

We observed a significant negative correlation between the size and the number of oligopyrene sperm produced ($r=-0.332$, $p=0.002$, $N=88$; figure 2a), but there was no significant correlation between size and number of eupyrene sperm ($r=-0.026$, $p=0.812$, $N=88$; figure 2b). The negative relationship between size and number of oligopyrene sperm remained significant when controlling for treatment or month of sampling (oligopyrene sperm number regressed against treatment or month and residuals regressed against length: $r=-0.336$ and -0.317 , $p=0.001$ and 0.003 , respectively; $n=88$). In addition, there was significant variation in the length of both sperm types across males (ANOVA: eupyrene, $F_{87,605}=20$, $p<0.001$; oligopyrene, $F_{87,616}=22.6$, $p<0.001$).

(b) Natural populations

The OER increased as the number of males to females increased (figure 3). Analyses of the relationship between OER and sex ratio indicated that the proportion of oligopyrene sperm produced was positively correlated with the risk of sperm competition (i.e. with the ratio of males to females) ($\chi^2=22.79$, d.f.=1, $p<0.001$). Shell size

Table 1. ANCOVA table for the effect of treatment (high or low intensity of sperm competition) on the number of oligopyrene sperm produced by male *Viviparus ater*

(Shell size was used as a covariate. Primary data in figure 1*b*. MS is mean squared.)

source	d.f.	August		September		October	
		MS	F	MS	F	MS	F
treatment	1	14544	10.957 ^a	3393	5.122 ^a	19.22	0.026
age	1	2.108	0.002	128.28	0.194	265.6	0.353
treatment × age	1	21.714	0.016	481.9	0.727	58.52	0.079
shell size	1	255.628	0.193	4093	6.178 ^a	424	0.563
error		1327.421	—	0.068	—	0.071	—
d.f.		27	—	23	—	23	—

^a $p < 0.05$.

Figure 2. Relationship between the number and length of (a) oligopyrene, and (b) eupyrene sperm produced in experimental populations. Each point represents the mean length of eight sperm of an individual. Least-squares regression lines have been included for illustrative purposes only.

Figure 3. Mean ($\pm$ s.e.) oligopyrene/eupyrene ratio produced by *V. ater* at different natural sites plotted against the sex ratio at the site. Numbers indicate the number of males sampled.

was also negatively related to OER ($\chi^2=17.83$, d.f.=1, $p<0.001$), with small snails producing a higher proportion of oligopyrene sperm than big snails (figure 4). Neither population density, relative testis mass nor any of the variable interactions had a detectable effect on OER (density, $\chi^2=2.32$, d.f.=1, $p=1.12$; testis weight, $\chi^2=0.20$, d.f.=1, $p=0.65$). In free-living snails we also observed a significant, negative correlation between the length and the proportion of oligopyrene sperm produced ($r=-0.193$, $p=0.034$, $N=121$; figure 5*a*), but we observed no significant relationship between the length and the proportion of eupyrene sperm ($r=0.194$, $p=0.091$; figure 5*b*).

4. DISCUSSION

Male snails apparently altered sperm production characteristics when large numbers of potential competitors were present: the relative proportion of oligopyrene sperm was positively associated with the ratio

Figure 4. Mean oligopyrene–eupyrene ratio produced by *V. ater* at different natural sites plotted against shell size. Least-squares regression lines have been included for illustrative purposes only.

of male to female snails in both experimental and natural populations, and there was a concurrent and significant negative relationship between the size and number of non-fertilizing sperm produced. An alternative explanation for this relationship is that at high male densities mating opportunities are reduced leading to a build up of oligopyrene sperm reserves. However, this seems unlikely as there was no association between fertilizing eupyrene sperm number, or proportion or size, and sperm competition risk (as indicated by sex ratio). In addition, poor-quality males may have had fewer copulations and produced smaller oligopyrene sperm. However, there was no association between sperm and shell size (a likely indicator of male quality), so we do not believe this to be the case (in addition, see Sait *et al.* (1998), who found no effect of infection or body size on parasperm length in a moth). Thus it appears males altered sperm production in response to rivals. This conclusion seems especially likely as spermatogenesis appears to be continuous throughout active adult life (i.e. when adults are not hibernating) as indicated by the presence of immature sperm during dissections (A. Oppliger, unpublished data). In addition, relative testis mass did not vary with sperm competition risk, which is perhaps unsurprising as all animals were sexually mature when experiments began.

Viviparus ater males and females mate promiscuously, and females store sperm for prolonged periods; thus when large numbers of males are present the risk of sperm competition increases (a finding characteristic of other studies (e.g. Gage 1991, 1995)). In addition, sperm competition intensity (the number of competitors with which one must compete for fertilizations (Parker *et al.* 1996)) may also increase. The predicted optimal male ejaculate-investment strategies differ between risk and intensity models of sperm competition. Risk models predict

Figure 5. Association between the oligopyrene–eupyrene sperm ratio and (a) oligopyrene sperm length and (b) eupyrene sperm length produced by *V. ater* at different natural sites. Each point represents the mean length of eight sperm of an individual. Least-squares regression lines have been included for illustrative purposes only.

positive associations between the probability of sperm competition and male gametic expenditure, whereas intensity models predict males should decrease expenditure when the number of rivals in a competition increases above two (with models assuming fertilization success is proportional to the number of sperm a male has in competition) (see also Hosken & Stockley 1998). We have no information on the mechanism of sperm competition, the males' abilities to evaluate competitor number or how sperm production translates to ejaculates in *V. ater*, but males did not increase production of fertilizing eupyrene sperm when numbers of potential rivals increased. How sperm production relates to ejaculates and fertilization success will be the subject of future investigation.

However, the increased production of oligopyrene sperm, both in number and proportion of sperm produced, when in the presence of large numbers of potential rivals strongly suggests these sperm play a role in sperm competition, as proposed for other non-fertilizing sperm (e.g. Silberglied *et al.* 1984). Similar results have been reported for the armyworm *Pseudaletia seperata*. Males reared at high larval densities increase production of apyrene sperm but did not alter eupyrene sperm number in copulations with virgin females compared with larva raised alone (He & Miyata 1997). High male density also increased sperm output in the moth *Plodia interpunctella*, but did not alter the apyrene–eupyrene ratio (Gage 1995). In addition, other lepidopteran studies have shown increased numbers of eupyrene sperm and either increased or unaltered apyrene numbers when females had, or were likely to have, previously copulated (Cook & Gage 1995; Cook & Wedell 1996).

Various hypotheses of the role of non-fertilizing sperm have been proposed (reviewed in Silberglied *et al.* (1984)); these include aiding eupyrene sperm transport, acting as nutrients, disabling or displacing rival sperm or delaying female receptivity. The ‘cheap filler’ hypothesis suggests that the highly motile apyrene sperm, which persist in the spermathecae along with the eupyrene sperm, deceive the sensory system of the female’s spermathecae, delaying receptivity (Silberglied *et al.* 1984; Cook & Gage 1995). Moreover, because the apyrene sperm of lepidopterans are smaller and simpler than eupyrene sperm, they are likely to be cheaper to produce (Gage & Cook 1994; Cook & Gage 1995). In contrast, *V. ater* oligopyrene sperm are much longer than the eupyrene morph and are highly complex (Hanson *et al.* 1951). They exhibit their most vigorous motility in the testis, and although they migrate to the females seminal receptacle with the eupyrene sperm, they do not persist there for long; in females dissected soon after copulation, large numbers of oligopyrene sperm are present and motile in the seminal receptacle, but 7–15 days later none are present (A. Oppliger, personal observations), although fertile eupyrene sperm are stored for up to two years (Trüb 1990). However, it is unknown how soon after insemination that ova are fertilized and females remate approximately every 1–7 days (Staub & Ribi 1995), so oligopyrene sperm could still fill some defensive role with remating possibly occurring sooner in their absence. Hanson *et al.* (1951) suggested oligopyrene sperm disintegrate in the female storage organ releasing substances that nourish, or stimulate the eupyrene sperm, but it is difficult to envisage how oligopyrene sperm could distinguish between self and non-related eupyrene sperm to circumvent cuckoldry of nutrients. However, the parasperm of some snails do secrete products that may create a hostile insemination site for subsequent males, and in some instances plug the female tract (Buckland-Nicks 1998), so offensive functions are possible. The reduced OER at the approach of winter may relate to the relatively short lifespan of oligopyrene sperm, as it would be wasteful to produce large amounts of parasperm that would not survive overwinter storage.

The relationship between shell size and oligopyrene sperm production in field populations may also relate to sperm competition risk if smaller males occupy unfavoured mating roles and typically always copulate

with previously inseminated females. Such effects on spermatogenic/ejaculate expenditure have been noted in other taxa (e.g. Gage *et al.* 1995; Stockley *et al.* 1994). Overall, in our experimental populations there was no effect of shell size on OER as competition between small and large males did not occur. However, a positive relationship was detected towards the end of experiments when growth rate differences among young individuals led to high variance in shell size and thus sperm competition could occur between small and large males.

The trade-off between oligopyrene size and number is consistent with the predictions of many evolutionary models of sperm production (e.g. Parker 1982, 1990a,b, 1993) and is, to our knowledge, the first demonstration of an intraspecific trade-off between these traits. In contrast, there was no trade-off between size and number for eupyrene sperm, and while theoretical models have only considered fertilizing sperm, predictions should also apply to parasperm because production will also be energetically limited. Other intraspecific studies have found no relationship between sperm size and number (e.g. Radwan 1996), but Pitnick (1995) found evidence for a trade-off between sperm size and numbers across *Drosophila* species. Moreover, because males may have different energetic investments in spermatogenesis (e.g. Gage *et al.* 1995; Stockley *et al.* 1994), it was suggested that trade-offs may not be apparent in comparative studies (Pitnick & Markow 1994; Pitnick 1995). The trade-off detected in our study suggests oligopyrene numbers are important during sperm competition, whereas size is important when sperm competition risk is low. This is similar to findings across fish (Stockley *et al.* 1997), but is in contrast to some other studies that have shown sperm size, albeit fertilizing sperm, to be positively associated with sperm competition risk or fertilization success during sperm competition (e.g. Gomendio & Roldan 1991; Gage 1994; Radwan 1996; Briskie *et al.* 1997). Unless sperm length affects differ with varying probability of sperm competition, optimal sperm length should be unaffected by sperm competition risk. In *V. ater*, perhaps larger sperm facilitate transfer of self-sperm, whereas oligopyrene sperm number mediates displacement of rival ejaculates. Further investigations in *V. ater* are warranted, but it seems oligopyrene number must increase fertilization gains more steeply than size when sperm competition risk is high. In addition, inter-male variation in sperm length has been noted in other taxa (e.g. Ward & Hauschteck-Jungen 1993; Sait *et al.* 1998), with length possibly influencing female sperm storage (e.g. Otronen *et al.* 1997).

In conclusion, we provide, to our knowledge, the first evidence of a size-number trade-off in the production of oligopyrene sperm. Moreover, we have shown that the number and proportion of non-fertilizing sperm produced is positively related to sperm competition risk. It therefore appears that the number of non-fertilizing sperm is more important than their size during sperm competition, as suggested for fertile sperm (Parker 1982).

Thanks to Paula Stockley, Matt Gage, Paul Ward and four anonymous reviewers for insightful comments which greatly improved this manuscript. We also thank Paul Ward for generously supplying equipment, J. Brunnschweiler, U. Hauser

and B. Müller for help with the snails, and H. Maag, P. Steinmann, L. Modolo and C. Vorburger for the scuba diving. This work was supported by Swiss National Science Foundation grant numbers 31-46861.96 (D.J.H.) and 31-46951.96 (G.R.).

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