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ORIGINAL ARTICLE

Attila Hettyey · J. Dale Roberts

Sperm traits of the quacking frog, *Crinia georgiana*: intra- and interpopulation variation in a species with a high risk of sperm competition

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Abstract Sperm traits often show extreme variation both between and within species. Between-species variation may often be interpreted in the context of a sperm competition theory, but within-species variation has remained unexplained. Previous studies on intraspecific variation in sperm traits have focused on a limited set of variables and may have failed to explain observed variation because of potential trade-offs between different sperm traits. We report on variation in number, size, motility and longevity of sperm in the frog *Crinia georgiana*, a species where sperm competition is common. We found intrapopulation variation in sperm size and motility and interpopulation variation in relative sperm number and size. When we combined relative sperm number and size into one variable, and motility and longevity into another, we found significant interpopulation variation in these variables as well. We also detected considerable intra- and significant interpopulation variation in cumulative sperm quality, a combination of all four sperm traits. Furthermore, a significant effect of the interaction between population origin and male size on sperm characteristics indicated interpopulation variation in the strength of selection acting on sperm traits of males adopting different mating strategies. We discuss heterogeneity in the reproductive environment, a complex genetic background in the determination of sperm characteristics and varying levels of developmental noise as potential contributors to the observed variation in sperm traits.

Keywords Alternative reproductive strategy · Intraspecific variation · Gamete evolution · Sperm quality · Trade-off

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A. Hettyey (✉)
Behavioural Ecology Group,
Department of Systematic Zoology and Ecology,
Eötvös Loránd University,
Pázmány Péter sétány 1/c,
Budapest, 1117, Hungary
e-mail: hettyeya@freemail.hu
Tel.: +36-1-2090555
Fax: +36-1-3812194

A. Hettyey · J. D. Roberts
School of Animal Biology (MO92),
The University of Western Australia,
35 Stirling Highway,
Western Australia, 6009, Australia

Introduction

Sperm number and morphology show extreme variation across species, ranging from numerous tiny amoeboid sperm of nematode worms (LaMunyon and Ward 1998) to the giant (58 mm) flagellate sperm of *Drosophila bifurca* (Pitnick et al. 1995). The adaptive significance of interspecific variation in these sperm traits has been examined by theoretical models (e.g. Ball and Parker 1997) and the comparative approach (sensu Harvey and Pagel 1991). There is increasing evidence from interspecific comparisons that sperm competition, occurring whenever the sperm from different males compete for access to the same eggs (Parker 1970), can significantly influence testis size and thus, sperm number (Harcourt et al. 1981; Møller and Briskie 1995; Hosken 1998) and even morphometry (Johnson and Briskie 1999; Anderson and Dixson 2002; Byrne et al. 2003). However, factors other than sperm competition such as variation in the fecundity of females or in agonistic behaviour between males can have significant consequences for testis size and thus, sperm number as well (Emerson 1997). Further, studies on the relationship between sperm morphometry and competition yielded equivocal results in mammals (Gomendio and Roldan 1991; Harcourt 1991; Hosken 1997; Gage and Freckleton 2003) and fish (Stockley et al. 1997; Balshine et al. 2001). Consequently, our knowledge of the causes of variation in sperm traits remains ambiguous, and it seems that selection driven by sperm competition may often be overruled by other factors affecting gamete evolution.

Within-species studies represent a more direct way of examining the variation in sperm traits (cf. Ward 1998) as

phylogenetic relationships, large interspecific differences in physiology, behaviour, reproductive anatomy, egg morphology and the fertilization environment may confound results of between-species comparisons (Garrido et al. 1989; Harvey and Pagel 1991; Gomendio and Roldan 1993; Stockley et al. 1996; Pitnick et al. 2003). Significant intraspecific variation in sperm traits has been reported in a wide range of taxa (e.g. sperm size: Snook and Karr 1998; Ward 1998; Morrow and Gage 2001a). Nevertheless, as all males within a species are selected for competition and fertilization within a common reproductive environment, we would expect sperm traits to be under intense stabilizing selection (Morrow and Gage 2001b), and it remains difficult to identify causes for intraspecific variation in sperm traits. One possible resolution that has been proposed to this paradox is the presence of alternative mating strategies; individuals adopting different strategies may exhibit different optima in sperm traits (Simmons et al. 1999; Leach and Montgomerie 2000; Vladiæ et al. 2002; Neff et al. 2003). Differences in the developmental environment or in the genetic background controlling spermatozoal traits may also lead to intraspecific variation in sperm characteristics (Ward 2000; Morrow and Gage 2001b; Blanckenhorn and Hellriegel 2002; Hellriegel and Blanckenhorn 2002).

Surveys exploring interpopulation variation in sperm traits are rare and have not produced any general explanation of variation (Hosken et al. 2003). Snook (2001) examined sperm morphometry in 11 North American populations of *Drosophila subobscura* along a latitudinal transect of 1,750 km, but the significant interpopulation variation he detected was not explained by latitude. Hosken et al. (2003) hypothesized that testis size or sperm size or variation in these traits could be associated with levels of fluctuating asymmetry (FA) and thus, developmental noise in yellow dung flies, but in the three populations they examined (Switzerland, UK and Iceland), they could not detect any significant correlations between measures of FA and the sperm traits evaluated. Pitnick et al. (2003) studied potential co-evolution of sperm size with length of female sperm storage organs across eight populations in *Drosophila mojavensis* and found some evidence for correlated evolution between these traits. In frogs with external fertilization, male–female co-evolution may, however, be of less importance than in species with internal fertilization.

The studies above on interpopulation variation in sperm traits only report on variation in sperm number and/or morphometry. Other sperm traits such as motility and longevity, which are just as important in the determination of fertilization ability of males (Birkhead et al. 1995; Stockley et al. 1996; Levitan 2000; Gage et al. 2004), have not been included. At the same time, trade-offs between different sperm traits have often been documented (e.g. Pitnick 1996; Levitan 2000; Gage et al. 2002). It seems that the adaptive value of distinct sperm characteristics cannot always be maximized independently of each other. Consequently, no sperm trait can be investigated separately in the hope of being able to reliably estimate what ultimately matters—competitiveness and fertilization ability of sperm. Analysing number, size, motility and longevity simultaneously should generate a more complete picture than previous studies. Here, we analyse interpopulation variation in four sperm traits that are considered to primarily determine ejaculate quality: number, size, motility and longevity (e.g. LaMunyon and Ward 1998; Levitan 2000; Reyer et al. 2003) in the frog *Crinia georgiana*.

Material and methods

The study species

The quacking frog (*Crinia georgiana*, Tschudi 1838) is a small [19–47 mm mature snout–vent length (SVL)] myobatrachid frog which is widely distributed in south-western Australia (Smith and Roberts 2003a). The reproductive season lasts from June to September (Main 1965), when males aggregate in choruses and vocalize to attract females (Byrne and Roberts 2004). Spawning sites vary from slowly flowing streams through temporarily flooded grass to small water-filled depressions (5 cm in diameter, personal observation). Females are amplexused inguinally and lay, on average, 70 eggs (Barker et al. 1995). Sperm competition plays an important role in the mating system of *Crinia georgiana*, as around 50% of all matings are polyandrous, resulting in multiple paternity of egg clutches (Roberts et al. 1999; Byrne and Roberts 2004). Polyandry occurs consistently over the whole range of the species and between years (Byrne 2002), but there is large seasonal variation in the operational sex ratio and male density that affects the intensity of male–male competition and the frequency of group spawning (Byrne and Roberts 2004). High levels of sperm competition have led to a relative testis mass four times greater in *Crinia georgiana* than in any other species of the *Crinia* genus (Byrne et al. 2002). Male size is bimodally distributed (Smith and Roberts 2003a), with larger males usually winning competition for females and smaller males more likely encountered as sneak males in polyandrous matings under low-density conditions (Byrne and Roberts 2004). However, large and small males both call and are able to attract females (Smith and Roberts 2003b).

Data collection

In 2003 we collected male quacking frogs from four localities distributed over the species' range: Kangaroo Gully (32°11′S, 116°15′E), Harvey (33°06′S, 115°58′E), Bridgetown (34°00′S, 116°09′E) and Walpole (34°59′S, 116°42′E, Fig. 1). We collected sexually mature males that called, had well-developed, large forearms and a black throat (Smith and Roberts 2003b) from active choruses. Collections were made from 25 July to 3 August and from 15 August to 22 August.

We hand-collected eight males from each site at a time and brought them back to the laboratory. Until processing, we kept males individually in 500-ml plastic containers with 30 ml of reverse-filtered tap water at room

Fig. 1 A map showing the distribution of *Crinia georgiana* and the sites of collection

temperature. Three and 5 days after collection, we randomly selected two small and two large males and measured their SVL (to the nearest 0.5 mm) with a plastic ruler. Animals were killed by double pithing.

After weighing testes (to the nearest 0.1 mg), we immediately crushed them and released sperm into reverse-filtered tap water so that sperm density was always around 4×10^6/ml to avoid potential biases in sperm longevity or motility due to large differences in sperm concentrations. We estimated sperm number from testis mass using a pilot analysis regressing sperm number on testes mass. We weighed the sperm suspensions and pipetted 50 μl onto specially prepared microscope slides (described in Reyer et al. 2003) and recorded sperm movement for 5 min with a Hitachi HV-C20 3CCD video camera attached to a microscope at ×200 magnification for subsequent determination of sperm motility. After recording, we let slides air-dry for sperm size measurements.

After sperm release, we pipetted 50 μl of the sperm suspensions onto microscope slides 15, 30, 60 and 120 min after sperm activation and estimated the percentage of living sperm by counting 100 sperm using a microscope at ×400 magnification. We considered sperm to be alive if their tail membrane was undulating and dead if there was no movement of the tail membrane (for a detailed description of myobatrachid sperm, see Lee and Jamieson 1992). The percentage of live sperm after 60 min was used as our measure of mean sperm longevity. Data gathered at other time points were used to compare survival in the studied populations. We estimated true sperm densities in the sperm suspensions using a Neubauer chamber. We pipetted 10 μl into the chamber and counted sperm in ten quadrats. From the density estimates and the weights of the sperm suspensions, assuming 1 ml of sperm suspension weighed 1 g, we calculated total sperm numbers. We measured sperm length of 20 sperm from each male at ×800 magnification using OPTIMAS 6.5. Measurements on sperm size proved to be highly repeatable when we compared three replicate measurements on 200 sperm originating from 20 males (R=0.94; SE=0.62). To further enhance reliability of sperm size data, we repeated each measurement three times and used the average in the analysis. For estimates of sperm motility, videotapes were digitized. We measured swimming speed of all individual sperm visible for at least 5 s in the first 30 s of the recordings made on each male using AVI Digitiser, a Visual Basic based software programmed by and available from P.C. Withers (pwithers@cyllene.uwa.edu.au). We collected measurements of swimming speed for an average of 33 sperm (range 9–66) per male, each measured for an average of 19 s (range 5–27). We calculated per-male averages and used these as our measure of motility in analyses. Testing for repeatability of sperm motility measurements by tracing 12 sperm twice resulted in extremely high repeatability estimates with a low standard error (R=0.99; SE<0.001). All measurements were done in the laboratory at a constant temperature of 20°C.

Statistical analyses

Snout–vent length, testis mass, sperm size and motility were $\log_{10}$-transformed, sperm number was square-root transformed and survival percentage was arcsine-square-root transformed to achieve normality and enhance homogeneity of variances (Sokal and Rohlf 1995). For five males, sperm motility could not be measured; consequently, we excluded these from all analyses that included motility. Testis mass correlated with sperm number (Pearson correlation, r=0.934; n=64; p<0.001) and with longevity (Pearson correlation, r=0.319; n=64; p=0.01); hence, to prevent collinearity problems, we discarded testis mass from further analyses. Sperm number correlated with SVL (Pearson correlation, r=0.323; n=64; p<0.01). To avoid potential patterns in the variance in sperm number being overruled by differences due to variation in body size, we calculated relative sperm number as the residuals from the regression of sperm number on SVL. We created two size groups of males for each population separately (overall: small males, 20–32.5 mm SVL; large males, 31–42 mm SVL) to test for potential differences in sperm traits due to size-dependent alternative mating strategies of males (body size bimodality and size-related variance in mating tactics discussed above). This resulted in eight large and eight small males per population.

We first investigated potential relationships between sperm quality measures with bivariate correlations using standardized scores. We further explored the relationships between sperm traits using principal component analysis (PCA), where all four variables measured, number, length, motility and longevity, were included. To assess within-population variation in sperm size, we entered sperm size as the test variable into Kruskal–Wallis tests using codes for individual males as the grouping variable. We repeated this for each population and adjusted α levels according to

the sequential Bonferroni technique (Rice 1989). We did the same for sperm motility. Analysis of variance (ANOVA) could not be used, as the assumption of homogeneity of variances was violated. We compared sperm longevity between populations with a repeated-measures general linear model (GLM) analysis of covariance (ANCOVA) by entering survival at 15, 30, 60 and 120 min as the repeated-measures factor, male size category and population code as fixed factors and time from collection as a covariate. To assess interpopulation variation in relative sperm number, size, motility and longevity, we used a GLM multivariate analysis of covariance (MANCOVA) with sperm metrics as dependent variables, population code and male size category as fixed factors and date of capture and time from collection as covariates. We entered relative sperm number instead of absolute sperm number into the model, as we were interested in differences relative to body size between males belonging to the two size categories applying sneak vs caller mating strategies (Byrne and Roberts 2004). As date of capture did not explain a significant proportion of variance in the model, we discarded it from the final model. Similarly, we performed two GLM ANCOVAs entering the PC 1 and PC 2 scores originating from the rotated solution of the PCA as the dependent variable. Finally, we tested the hypothesis that cumulative quality of sperm varied between populations. We added PC 1 and PC 2 scores for each individual male and used the sums as estimates of cumulative sperm quality. We entered cumulative sperm quality as the dependent variable into a GLM ANCOVA together with population code and male size category as fixed factors and time from collection as a covariate. All statistical tests were calculated using SPSS 12.0.1 for Windows.

Results

Sperm motility was positively correlated with sperm longevity [Pearson correlation; r=0.340; n=59; p=0.008; critical α after Bonferroni correction (Rice 1989) was 0.0083]. All other correlations between sperm characteristics were non-significant [relative number size r=0.317, n=64, p=0.011; critical α after Bonferroni correction (Rice 1989) was 0.01; relative number longevity r=0.184, n=64, p=0.15; relative number, motility r=−0.047, n=59, p=0.72; size, longevity r=−0.050, n=64, p=0.69; size, motility r=−0.023, n=59, p=0.87]. PCA resulted in two components with Eigenvalues exceeding 1, explaining 35 and 32.5% of the variance, respectively. After Varimax rotation, both components showed strong loadings: motility and longevity on component 1 (with loadings of 0.79 and 0.83, respectively; relative sperm number 0.16 and sperm size −0.14) and relative sperm number and sperm size on component 2 (with loadings of 0.83 and 0.78, respectively; motility −0.13 and longevity 0.15). The rotated solution explained a total of 67.5% of the variance, with component 1 contributing 34% and component 2 contributing 33.5%.

Kruskal–Wallis tests indicated significant within-population variation in sperm size (Kangaroo Gully χ^2_{15}=145.09, p<0.001; Harvey χ^2_{15}=141, p<0.001; Bridgetown χ^2_{15}=136.57, p<0.001; and Walpole χ^2_{15}=120.78, p<0.001) and in sperm motility (Kangaroo Gully χ^2_{15}=68.8, p<0.001; Harvey χ^2_{13}=82.66, p<0.001; Bridgetown χ^2_{15}=101.86, p<0.001; and Walpole χ^2_{12}=101.45, p<0.001) in all four populations (e.g. Fig. 2).

Repeated-measures GLM ANCOVA confirmed the decrease of the number of active sperm over the time period of our investigation ($F_{3,53}$=27.08, p<0.001; Fig. 3). We found no interpopulation variation in sperm longevity ($F_{9,129.1}$=1.16, p=0.32) and got no significant difference according to male size category ($F_{3,53}$=2.74, p=0.052). Time from collection and the interaction between population code and male size category had no significant effect on sperm survival ($F_{3,53}$=0.32, p=0.81 and $F_{9,129.1}$=0.91, p=0.52, respectively).

General linear model MANCOVA indicated that sperm characteristics differed between the four populations (Table 1). Male size category had no effect on sperm traits, but the interaction between population origin and male size category was significant (Table 1). Time from collection had no significant effect on sperm traits (Table 1). Between-subject effects revealed that relative sperm number and size varied between populations, whereas sperm motility and

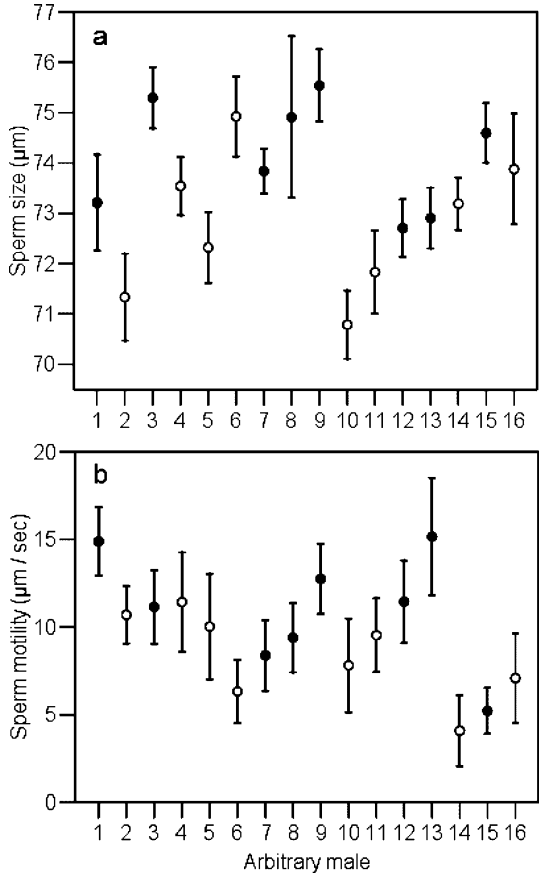

Fig. 2 An example of between-male variation in (**a**) sperm size and (**b**) motility within one of the four studied populations (Bridgetown: ○, small males; •, large males). Means±2 SE of untransformed data are shown

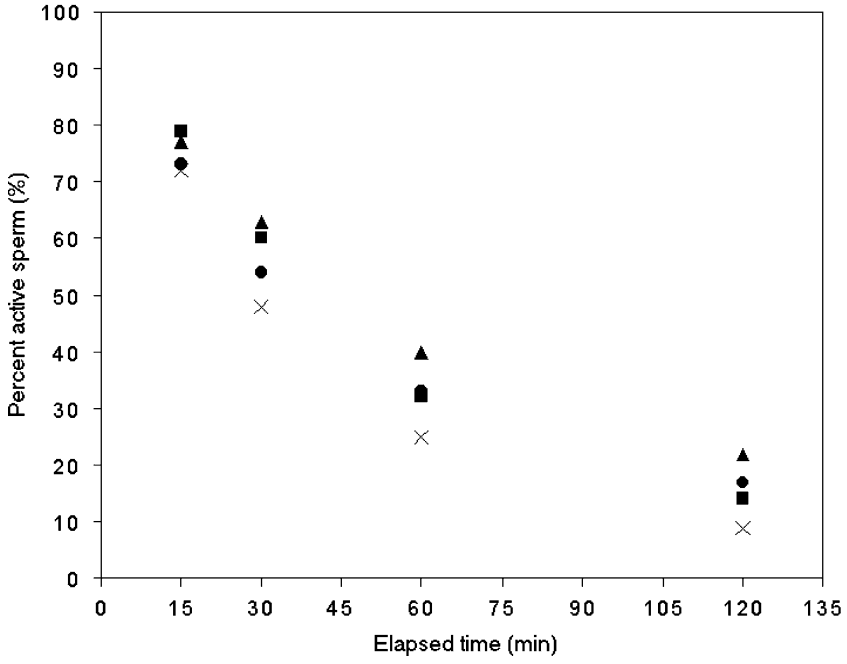

Fig. 3 Sperm survival as a function of time in the studied populations. For the ease of interpretation, population means of untransformed data are shown (▲, Kangaroo Gully; *x*, Harvey; ■, Bridgetown; and ●, Walpole)

longevity did not (Table 2, Fig. 4). We found no significant effect of the interaction between population origin and male size category on any specific sperm trait [critical α adjusted according to Rice (1989); Table 2].

A GLM ANCOVA with PC 1 scores revealed significant differences between the studied populations ($F_{3,50}$=8.54, p<0.001; Fig. 5). Time from collection had a significant effect on PC 1 ($F_{1,50}$=5.04, p=0.029). The interaction between body size category and population code was significant ($F_{3,50}$=3.57; p=0.02). Another GLM ANCOVA, with PC 2 scores as the dependent variable, gave similar results; interpopulation differences ($F_{3,50}$=9.83, p<0.001; Fig. 5) and the effect of time from collection ($F_{1,50}$=5.37, p=0.025) were significant.

The cumulative quality of sperm varied between the four studied populations as shown by a GLM ANCOVA ($F_{3,50}$=19.25, p<0.001; Fig. 5). Male size category did not affect cumulative sperm quality ($F_{1,50}$=0.08, p=0.77) and neither did the interaction between population code and male size category ($F_{3,50}$=1.51, p=0.22) affect cumulative sperm quality. Time from collection had no effect either ($F_{1,50}$=0.0, p=0.99).

Discussion

Divergence between populations in gamete traits may be a common mechanism leading to reproductive isolation and speciation, as the development of ejaculate–female incompatibilities may lead to limitations in gene exchange (Pitnick et al. 2003). Despite its evolutionary significance, general and widely accepted explanations for interpopulation variation in sperm traits are still lacking (Hosken et al. 2003) especially because sperm traits are thought to be under strong stabilizing selection (Morrow and Gage 2001a). However, the ability of sperm to fertilize eggs and to compete with sperm of other males may not be defined by one sperm trait but by a combination of sperm quantity and quality (e.g. Levitan 2000). Consequently, instead of restricting a study to one characteristic of sperm, investigating sperm quantity and quality simultaneously may create a better basis for the study of the evolution of sperm.

We predicted that despite the likely occurrence of intra- and interpopulation variation within separate sperm traits, assuming trade-offs between these traits do exist, variation in different characteristics of sperm may extinguish each

Table 1 Results of a GLM MANCOVA testing interpopulation variation in relative sperm number, size, motility and longevity

Effects	Pillai's trace	*F*	*df*	*p*	$\partial\eta^2$	Observed power
Population origin	1.002	6.147	12, 147	**<0.001**	0.334	1.000
Male size category	0.086	1.109	4, 47	0.363	0.086	0.322
Time from collection	0.177	2.520	4, 47	0.054	0.177	0.670
Population origin × male size category	0.398	1.871	12, 147	**0.042**	0.133	0.884

Significant cases are in bold

Table 2 Between-subject effects for population origin and for the interaction between population origin and male size category

	Dependent variables	*F*	*df*	*p*	$\partial\eta^2$	Observed power
Population origin	Size	12.359	3	**<0.001**	0.426	1.000
	Relative number	1.699	3	**<0.001**	0.566	1.000
	Motility	0.899	3	0.448	0.051	0.233
	Longevity	1.450	3	0.239	0.080	0.361
Population origin × male size category	Size	0.793	3	0.503	0.045	0.209
	Relative number	3.490	3	0.022	0.173	0.746
	Motility	2.020	3	0.123	0.108	0.488
	Longevity	2.744	3	0.053	0.141	0.630

Significant cases are in bold; critical α levels were adjusted according to Rice 1989

other, and the cumulative quality of sperm would be close to equal both within and between populations. Due to the lack of any detectable trade-offs between the studied sperm traits, our results do not support this hypothesis. We detected within-population variation in sperm size and motility. Relative sperm number and size, and also their combined measure estimating sperm quantity, varied between the four studied populations. Neither measure of sperm quality, motility and longevity varied between the studied populations one by one, but when we combined them together into a single measure, we got significant interpopulation variation. This was probably due to the positive correlation between sperm motility and longevity. The significant interaction between male size category and population origin in the determination of interpopulation variation in sperm characteristics indicated that males using different reproductive strategies may have different sperm characteristics in *C. georgiana*. The significant interpopulation variation in sperm quantity and quality in the studied populations was facilitated by the lack of trade-offs between the components defining them. Large within-species variation in sperm characteristics has been claimed to be hard to interpret (Morrow and Gage 2001a). We propose mechanisms that may have contributed to the evolution of the experienced intra- and interpopulation variation in sperm characteristics.

Differences in the risk and intensity of sperm competition may create variation in sperm quantity and quality on at least four different levels: (1) between species due to differences in the reproductive system (e.g. Harcourt et al. 1981; Hosken 1997; Byrne et al. 2003), (2) between populations of a given species, (3) between individual males adopting different mating strategies (e.g. Simmons et al. 1999; Leach and Montgomerie 2000; Neff et al. 2003; current study) and (4) between ejaculates within individual males engaging in consecutive matings (e.g. Birkhead et al. 1995; Oppliger et al. 2003; Byrne 2004). Despite the increasing evidence for 1, 3 and 4, we know nothing about

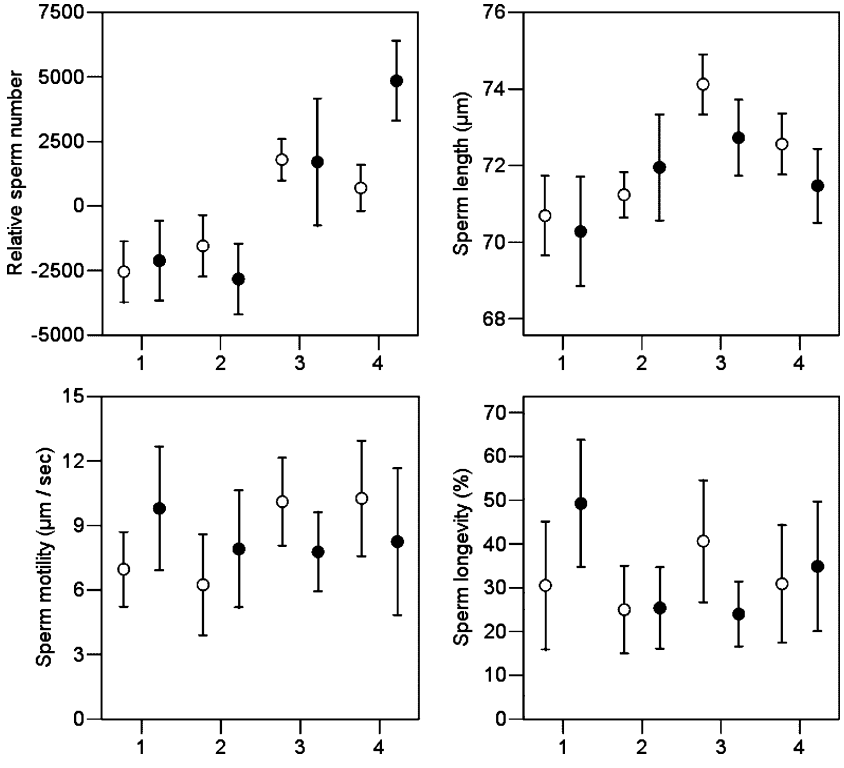

Fig. 4 Relative sperm number (**a**), size (**b**), motility (**c**) and longevity (**d**) of the two male size categories in the four studied populations (○, small males; •, large males; *1*, Kangaroo Gully; *2*, Harvey; *3*, Bridgetown; and *4*, Walpole). Means±2 SE of untransformed data are shown

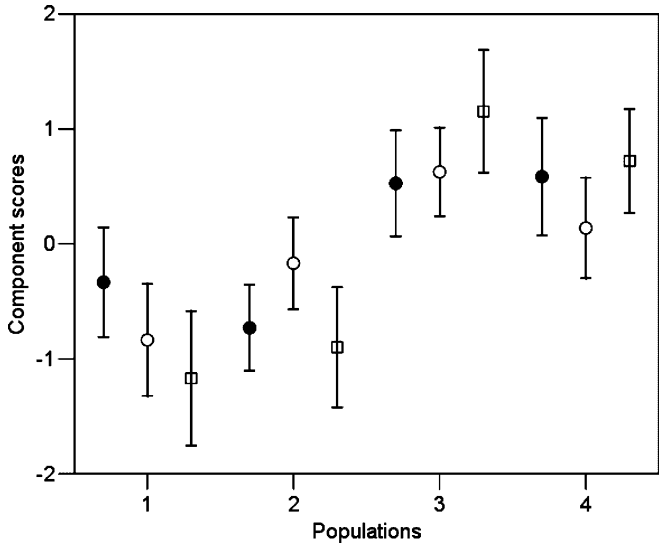

Fig. 5 Interpopulation variation in sperm quality (*full circles*, PC 1 correlating with sperm motility and survival) sperm quantity (*empty circles*, PC 2 correlating with relative sperm number and size) and in cumulative sperm quality (*transparent squares*, sums of PC 1 and PC 2). Means±2 SE are displayed (*1*, Kangaroo Gully; *2*, Harvey; *3*, Bridgetown; and *4*, Walpole)

the relationship between interpopulation variation in levels of sperm competition and that of sperm quantity and quality. However, we see no reason why this intermediate level should be different from other levels. There are many ways interpopulation variation in the levels of sperm competition may arise. More southern populations have extended periods of calling (J.D. Roberts, unpublished observations), which means that there are more opportunities for nights with highly skewed operational sex ratios (Byrne 2002; Byrne and Roberts 2004). Geographic variation in sex ratios per se may lead to differences in the frequency of polyandrous matings as well (however, see Byrne 2002).

Selection pressures driving evolution to create interpopulation variation in sperm characteristics may also originate directly from disparities in the fertilization environment. The fertilization environment is recognized to contribute to the evolution of interspecific differences in sperm characteristics (e.g. Stockley et al. 1996). Within species, the fertilization environment is generally referred to as homogenous (e.g. Morrow and Gage 2001a). This may be a good approximation for species with internal fertilization. However, in species with external fertilization, substantial between-population differences in water chemistry, volume or flux in the body used for egg deposition may require different adaptations in sperm characteristics to maximize fertilization efficiency and may thus lead to interpopulation variation in sperm traits.

Parker and Begon (1993) proposed that sperm size variation within species may reflect developmental noise. Recent empirical studies have failed to support this theory unequivocally (pro, Hellriegel and Blanckenhorn 2002; contra, Hosken et al. 2003; Pitnick et al. 2003). Based on our study, it is impossible to rule out the effect of developmental noise as a cause of between- and within-population variation in sperm traits. To eliminate such an effect would require "common garden" experiments.

Another important source of within-population variation in sperm characteristics may be the presence of more than one evolutionarily stable strategy for the investment into different sperm traits possibly linked with alternative reproductive strategies. Evidence for this comes from bluegill sunfish (Neff et al. 2003). In sneaker males, the ejaculate has a higher sperm density, but contains shorter-lived sperm, than ejaculates from parental males, where sperm density is lower but longevity is higher. Alternative allocation strategies into distinct sperm traits may be present in other species as well.

In summary, our results do not support the suggestion that within a species with external fertilization, sperm are adapted to a common reproductive environment and should be thus subjected to strong stabilizing selection, with the distinct sperm characteristics being selected towards a common overall mean and low variance. The reproductive environment is likely to be heterogeneous due to interpopulation variation on the strength of sperm competition and due to disparities in the fertilization environment, whereas varying levels of developmental noise and alternative allocation strategies into distinct sperm traits may explain some of the within-population variation and add to the interpopulation differences in sperm characteristics.

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