

Intraspecific Variation in Ejaculate Traits of the Kuhl's Pipistrelle *Pipistrellus kuhlii* (Kuhl, 1817) (Mammalia: Chiroptera: Vespertilionidae) in Iran

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Abstract: Sperm competition is recognised as a widespread phenomenon in mammals. It occurs when more than one male competes over the fertilisation of the same female and influences on whole ejaculate traits (i.e., proportion viable, number and length of sperm). Bats have interesting sperm adaptations for competition, e.g. they can store fertile sperm for up to 200 days. We examined intraspecific sperm competition in the temperate vespertilionid bat *Pipistrellus kuhlii*. It can store spermatozoa for an extended period, a strategy allowing bats to interrupt their reproductive cycle by hibernating. Sperm was obtained from the epididymis of sexually mature bats. Viability, number and total length of sperm were assessed and compared between males. We found significant difference in sperm traits among individual *P. kuhlii* ($p < 0.05$) but these differences were not related to body condition and testis length variables ($p > 0.05$). In addition, no relation was observed between sperm length and sperm number or sperm viability. We could speculate that the difference in sperm traits might correlate with other body conditions such as hematocrit or other physiological traits.

Key words: bat, *Pipistrellus kuhlii*, sperm competition, testis

Introduction

Sperm competition is a prevalent phenomenon in mammals (GOMENDIO et al. 1998); it occurs when a female mates with more than one male. The theory predicts that sperm competition influences the penis morphology, ejaculate parameters and behaviour of many species (GINSBERG & HUCK 1989, BIRKHEAD & MOLLER 1998). Empirical studies have proven that sperm quality traits such as sperm number, size, velocity, motility and longevity, play a substantial role in sperm competition (SNOOK 2005). In evolutionary models, males are known to allocate most resources in mating and adjust ejaculate traits according to females' fecundity condition (MALLARD & BARNARD 2003). Males that experience high levels of sperm competition enhance their competitive ability and, therefore, will have a selective advantage (PITNICK et al. 1999).

Sperm competition can lead to variation in sperm quality traits among species and perhaps within species (SNOOK 2005, FITZPATRICK & LUPOLD 2014). Males can enhance their competitive ability, e.g. sperm length (PITNICK et al. 1999) and testicular size and function (RAMM et al. 2014), which may lead to sexually selected adaptations and elimination of rival sperm or the whole organism (BIRKHEAD & MOLLER 1998, GAGE et al. 2004). Typically, males that experience sperm competition are expected to produce greater numbers of sperm. Consequently, they have larger testes than monogamous species (PARKER & PIZZARI 2010) and testis size can be used as a parameter to study the level of sperm competition (SIMMONS & FITZPATRICK 2012). Some species of bats from the temperate latitudes store viable

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spermatozoa in the caudal epididymis, beyond the cessation of the annual spermatogenic period for 6-7 months (RACEY 1972).

Kuhl's pipistrelle *Pipistrellus kuhlii* (Kuhl, 1817) is a small bat, distributed from the entire Southern Europe (BENDA et al. 2006) through the entire Middle East to Pakistan as well as in the southern regions of Central Asia (HORACEK et al. 2000). In Iran, this bat is ranked as a common species (SHARIFI et al. 2000); it occurs both in Mesopotamian semi-deserts and in mountainous regions of the Zagros Range (DEBLASE 1980). Several important traits of its reproductive biology suggest sperm competition in this species. These are massive number of spermatozoa in the uterus of females during winter (SHARIFI et al. 2004) along with multiple mating during winter, short gestation period, late parturition in early summer and preserving viable spermatozoa for a long time.

In the present study, we have examined several ejaculate traits (sperm length, sperm viability, sperm number) in males of the Kuhl's bat. First, we assessed the intraspecific variation in ejaculate traits, focusing especially on sperm length because previous studies suggested that this was an important trait affecting sperm competition success in animals (GOMENDIO & ROLDAN 1991). Second, we examined ejaculate traits dependence with testis length and ratio of body mass (g) to length of forearm (mm) that was used as an index of body condition (health status).

Materials and Methods

Sixteen males of *P. kuhlii* were collected in western Iran in late summer and kept in separate cages for few days. For each bat, length of forearm, body mass and testis length was measured. One of the two caudal epididymides for each bat was used to conduct sperm quality. Bats, after recovery, were released in their natural habitat. None of them died as a consequence of the treatment.

For studying sperm quality, the principal storage medium was TALP (MORRIS et al. 2001) containing calcium chloride (anhydrous) 0.2 g/L, magnesium chloride 0.2 g/L, potassium chloride 0.2 g/L,

sodium chloride 8 g/L, sodium phosphate monobasic 0.05 g/L and D-Glucose 1 g/L. The epididymis was carefully separated from the testis and the caudal region was separated from its remaining part. The caudal epididymis was quickly transferred to a pre-warmed TALP solution (37°C and pH 7.8) and then lacerated with a razor. We attempted to remove from the sperm solution any epididymal tissue and non-sperm cells that may have resulted from the squashing of the epididymis.

Sperm viability was measured as the proportion of live sperm at stripping, using the Eosin-Y staining test (LIN et al. 1998). Each sample was immediately diluted in 1 ml of TALP solution. Sperm counts were performed by mixing, on a microscope slide, 10 µl of the sample solution with 10 µl of Eosin-Y stain. The stain penetrates the plasma membrane of dead cells, which appear pink, whereas live cells are colourless. For each male, two slides were prepared, using the above-described method, three times. The proportion of live sperm was calculated per 100 sperm per slide, using the two slides for each male and the mean value was used for the analyses.

For the estimation of sperm number, an aliquot of the sperm suspension was fixed in 0.9% NaCl, 0.1% formaldehyde and then counted using a modified Neubauer chamber using light microscope. Sperm number was determined using a calibrated sperm counting chamber and using this WHO (*World Health Organization*) sperm numbering formula: Number of sperm (million/ml) = Number of Cells × 10.000/ Number of square × dilution.

Thirty randomly chosen sperm cells were measured for each male from images of the sperm (magnified 400 X) using Windows computer running Image J (available at <http://rsb.info.nih.gov/nih-image/>). Total sperm lengths were measured to the nearest 0.1 µm (calibrated using the grid scale of a haemocytometer under the same magnification) on the digitised images of the sperm. We calculated the median sperm length for each individual. Single-factor analysis of variance (ANOVA) was used to test for significant differences between sperm quality in different traits.

Table 1. Descriptive statistics for measured traits of male Kuhl's Pipistrelle *Pipistrellus kuhlii* used in the analysis of intraspecific variation.

Parameters	NO.	Mean± SD	Range (min-max)	p-value
Body condition	16	0.16±0.16	0.16-0.18	0.001
Testis length (mm)	9	3.64±0.51	3.25-4.03	0.001
Sperm length (µm)	16	39.74±0.50	39.74-40.01	0.001
Sperm viability	16	95.13±2.52	93.79-96.48	0.001
Sperm number (million/ml)	16	102.27±32.10	85.16-119.38	0.001

Table 2. Correlation between log-transformed body condition (ratio of mass to forearm), testis length and sperm length with log-transformed sperm length, sperm viability and sperm number in the Kuhl's Pipistrelle *Pipistrellus kuhlii*.

Parameters		p-value
	Log sperm length	0.41
Log body condition	Log sperm viability	0.007
	Log sperm number	0.41
	Log sperm length	0.62
Log testis size	Log sperm viability	0.50
	Log sperm number	0.78

Results

The descriptive statistics of sperm parameters in the Kuhl's bat *P. kuhlii* are shown in Table 1. There was a significant variation among individuals in body condition ($p=0.001$), testis length ($p=0.001$), sperm length ($p=0.001$), viability ($p=0.001$) and number ($p=0.001$; Table 1). We included log-transformed body condition (ratio of mass to forearm), sperm length and testis length as the response variable in a general linear model. Log sperm length was not significantly correlated with body condition ($p=0.41$, $R^2=0.05$) nor with testis length ($p=0.62$, $R^2=0.03$) (Table 2, Figs. 1A and 1D). Log sperm viability was significantly correlated with log body condition ($p=0.007$, $R^2=0.41$) but was not significantly correlated with log testis length ($p=0.50$, $R^2=0.07$; Table 2, Figs. 1B and 1E). Log sperm number was not significantly correlated with body condition ($p=0.41$, $R^2=0.05$) nor with testis length ($p=0.78$, $R^2=0.01$; Table 2, Figs. 1C and 1F). In addition, log sperm length was not significantly correlated with log sperm viability ($p=0.31$, $R^2=0.07$) or with log sperm number ($p=0.81$, $R^2=0.004$; Table 2, Figs. 2A and 2B).

Our results showed that the males in better condition, as measured by body mass or forearm length, produced more viable sperm, but did not produce higher number or longer sperm. However, we found significant difference in length, viability and number of sperm between individuals of *P. kuhlii*.

Discussion

Sperm competition plays a major role in sperm morphology even among closely related species and may be influenced by reproductive advantages. In our experiment, all measured sperm features were significantly variable between males of the Kuhl's Pipistrelle bats ($p<0.05$). An intraspecific difference in sperm competition is expected to influence the

evolution of specific sperm quality (SNOOK 2005), including sperm morphology, swimming speed, number and viability (PIZZARI & PARKER 2009). In general, our findings are consistent with other studies (SCHULTE-HOSTEDDE & MONTGOMERIE 2006, LASKEMOEN 2010) that have revealed substantial individual variation in ejaculate traits between males. The reasons of this intraspecific variation in sperm morphology for most species remain unclear. It has been suggested that complex reasons, involving phylogenetic constraints, co-evolution with the female reproductive tract (BRISKIE & MONTGOMERIE 1992) and the degree of multiple mating by the female (SCHULTE-HOSTEDDE & MONTGOMERIE 2006). However, the significant intraspecific variation in sperm length has been well described in many taxa. Across various species – field crickets *Gryllus bimaculatus* (De Geer, 1773), wood mice *Apodemus sylvaticus* (Linnaeus, 1758), rainbow trout *Onchorhynchus kisutch* (Walbaum, 1792) (MORROW & GAGE 2001), various passerine species (LASKEMOEN 2010) and long-tailed finch *Poephila acuticauda* (Gould, 1840) (ROWE et al. 2015) – significant sperm length variability within males were reported. The reason for this variation and for the unstable selection is somewhat unclear. TILLBOTTRAUD et al. (2005) have reported many examples of species that produce heteromorphic sperm, when animals produce both fertile and non-fertile sperm morphs. COOK & WEDELL (1999) argue that in insects, there is some evidence that non-fertilising sperm can control female remating rates. In addition, HOLMAN & SNOOK (2008) have found that non-fertilising sperm cells can protect sperm from immunological attacks from the female's reproductive tract.

We explored the correlation between variation in primary sexual characters (sperm length, sperm number and sperm viability) with difference in male condition (body condition) and testis length. Our findings revealed that the variation of sperm competition (with the exception of sperm viability that was correlated) was independent of testis length and body condition. Previous results of comparative studies across many taxa, including mammals, have revealed a positive (BALSHINE et al. 2001, MONTOTO et al. 2011), negative (STOCKLEY et al. 1997, SIMPSON et al. 2014) or no relationship (HOSKEN 2010) between sperm length and sperm competition. A comparative study of 17 species of mammals has shown that small males allocate disproportionately more resources in sperm production than large males (STOCKLEY & PURVIS 1993). Nevertheless, our results are in agreement with experimental data indicating that sperm length is unrelated to sperm competi-

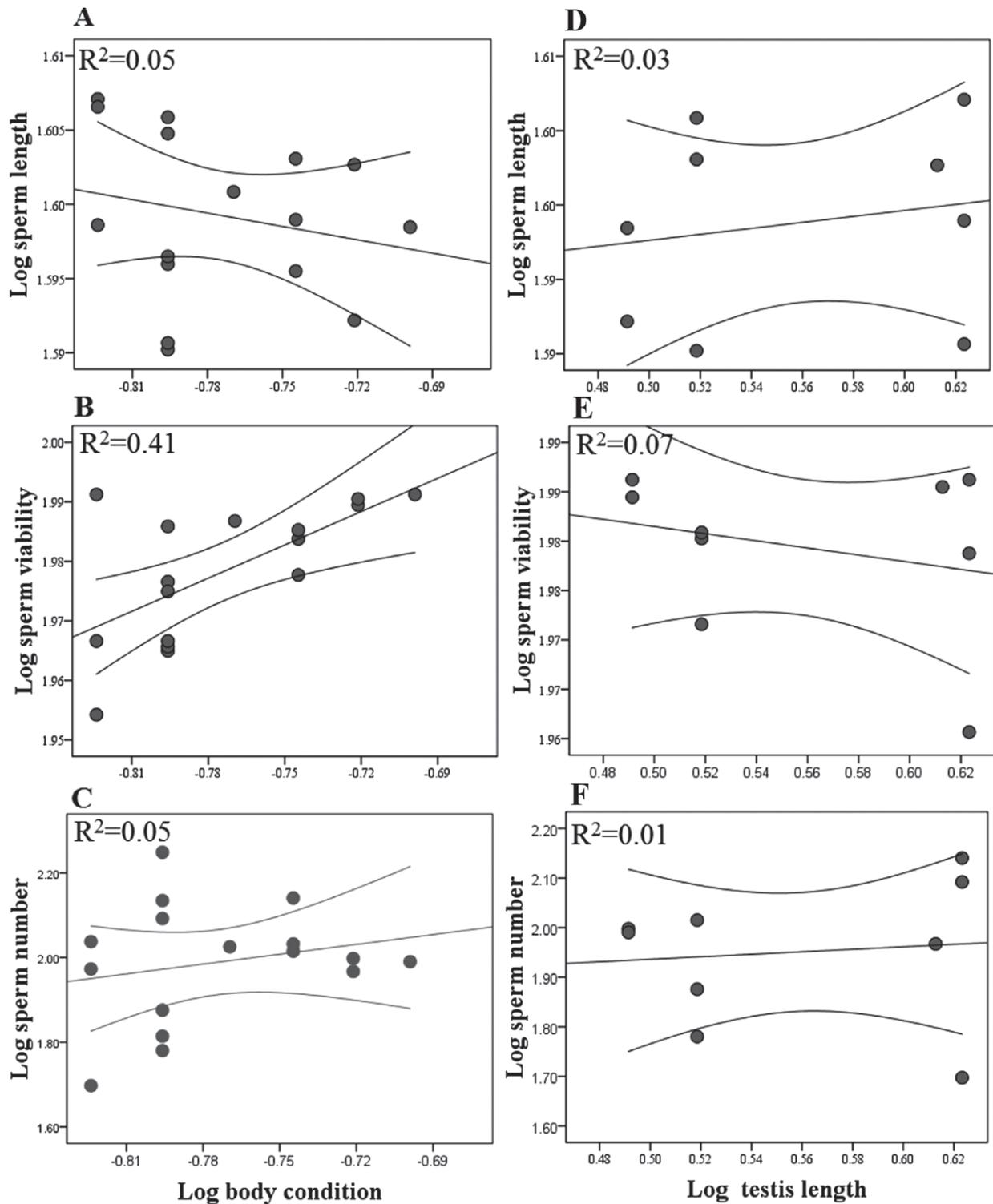


Fig. 1. Relationship between body condition (ratio of mass to forearm) and (A) sperm length, (B) sperm viability and (C) sperm number and testis length and (D) sperm length, (E) sperm viability and (F) sperm number in the Kuhl's Pipistrelle *Pipistrellus kuhlii*. All variables are log-transformed.

tion (GOMENDIO & ROLDAN 2008, MONTGOMERIE & FITZPATRICK 2009, SIMMONS & FITZPATRICK 2012, FITZPATRICK & LUPOLD 2014). These results have suggested that in this species sperm length and number are evolutionary independent of body condition and testis length (HOSKEN 2010).

One of the most characteristic evolutionary responses to sperm competition is the increase in relative testis size (BIRKHEAD & MOLLER 1998). Males that transfer more sperm per insemination are expected to have an evolutionary advantage (PARKER 1982). Similarly, a positive relation between testis

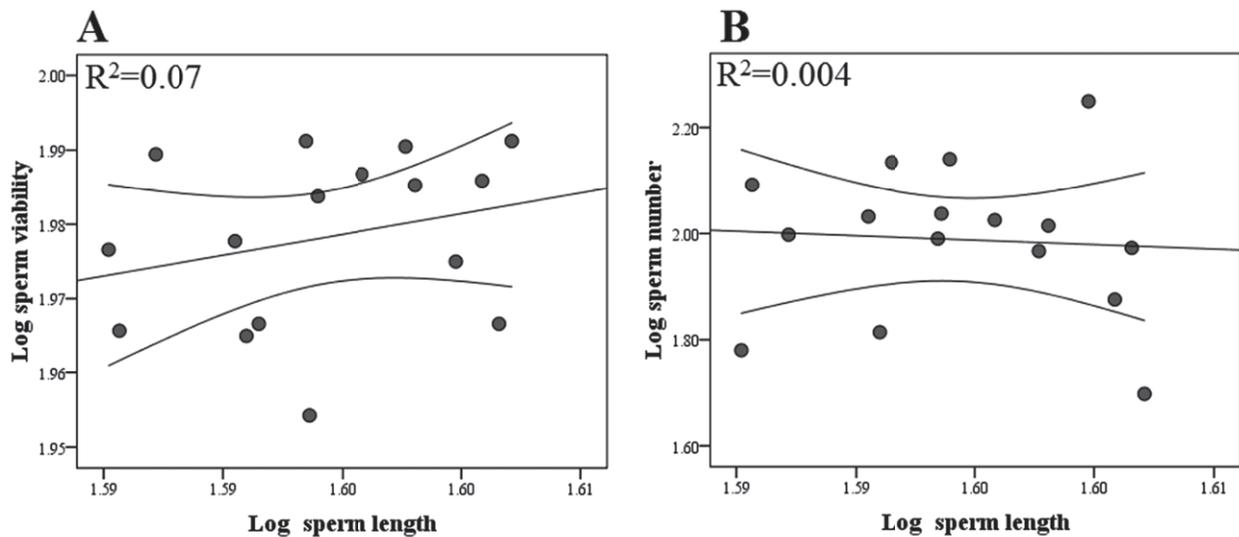


Fig. 2. Relationship between sperm length and (A) sperm viability and (B) sperm number in the Kuhl's Pipistrelle *Pipistrellus kuhlii*. All variables are log-transformed.

mass and sperm competition is known for a wide range of taxa (GAGE 1994). It has been suggested that males with disfavoured role (i.e. small, subordinate males) need to invest more heavily in ejaculates than males with a role (i.e. large, dominant) in sperm competition (PARKER 1990). In addition, body size can correlate with sperm number or ejaculate traits (WEDELL 1997, MALLARD & BARNARD 2003). Since male bats restrain uncooperative females (RACEY et al. 1987), the larger males may take longer mating and this may increase the chance of success of fertilization (see SCHWAGMEYER & FOLTZ 1990).

In a study on relations between sperm length and body mass of 232 mammalian species, it was demonstrated for all that the relationship was negative except for bats, for which it was positive (CUMMINS & WOODALL 1985). HOSKEN (1997, 2010) found sperm size to be independent of body mass and group size in bats. Similar results for 300 species of mammals, after correction for phylogenetic effects, was obtained (GAGE 1998). In a comparative study across 83 mammalian species (GAGE & FRECKLETON 2003), no general influence of sperm competition has been found on head, midpiece or flagellum lengths. It seems that stabilising selection acts as a salient evolutionary force and intraspecific variance in sperm length is reduced when the risk of sperm competition increases (FITZPATRICK & BAER 2011). Indeed, study on 22 species of passerine birds (LASKEMOEN 2010) have demonstrated that sperm swimming, speed and length were closely and negatively dependent of relative testis size and the frequency of extra-pair paternity (commonly used as an index of sperm competition, e.g. HOSKEN

1997, DUNN et al. 2001). However, in another study (LASKEMOEN 2010) applying phylogenetic methods on 42 passerine species, a positive relation was found between sperm length and swimming speed, with the frequency of extra-pair paternity.

In our study, we examined the relation of sperm length, viability and number with body condition and testis length as well as relation of sperm viability and number with sperm length. Our results showed that no significant ($p>0.05$) relation between these traits (except for relation of sperm viability with body condition, $p=0.007$). It seems these traits evolve independently in *P. kuhlii* as a response to sperm competition. Variation in sperm morphology has been previously found across species of bats (FORMAN & GENOWAYS 1979). HOSKEN (1998) showed that the sperm of *Nyctophilus geoffroyi* (Leach, 1821) could be still fertile after seven months and that one male was better in some respects during sperm competition; perhaps his sperm were competitively superior or he was able to pre-empt rival sperm independently of mating order, e.g. by producing larger ejaculates or copulating more frequently. It is reported that ejaculate size might be intraspecifically variable (e.g. primates: MARSON et al. 1989; fish: GAGE et al. 1995; insects: SIMMONS et al. 1993). However, bats are an exception among mammals because of the long period of sperm storage in male and female reproductive tracts and other aspect of sperm traits may be influence by sperm viability (GOMENDIO & ROLDAN 2008).

There is theoretical and empirical evidence that sperm numbers are an important sign of sperm competition (e.g. PARKER 1982, STOCKLEY et al. 1996).

In consistence with the above, we found a significant intraspecific difference in testis size and sperm number of *P. kuhlii* that could be interpreted as sperm competition. However, we recorded variation in sperm size, viability and sperm number that could not be explained by body size and other explanation should be sought. The fact that body condition was independent of some sperm traits, suggests that different factors may constrain male reproduction (SCHULTE-HOSTEDDE & MONTGOMERIE 2006).

While there has been substantial research on sperm competition in many groups of animals, few studies has been done on bats. Therefore, many features of sperm storage, sperm competition and sperm traits when regarding long-term sperm storage in the reproductive tracts of male and female bats are unclear. This study represents a first step in examining intra-sperm competition in a bat species by ejaculation traits, where multi-male mating with females is pervasive. Further research is needed to explore the other body conditions (e.g. hematocrit, spermatocrit) or genetic samples in fertilisation or reproduction success.

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