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The evolution of sperm morphometry in pheasants

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ABSTRACT

Postcopulatory sexual selection is thought to be a potent evolutionary force driving the diversification of sperm shape and function across species. In birds, insemination and fertilisation are separated in time and sperm storage increases the duration of sperm-female interaction and hence the opportunity for sperm competition and cryptic female choice. We performed a comparative study of 24 pheasant species (Phasianidae, Galliformes) to establish the relative importance of sperm competition and the duration of sperm storage for the evolution of sperm morphometry (i.e. size of different sperm traits). We found that sperm size traits were negatively associated with the duration of sperm storage but were independent of the risk of sperm competition estimated from relative testis mass. Our study emphasises the importance of female reproductive biology for the evolution of sperm morphometry particularly in sperm storing taxa.

Keywords: sperm competition, female reproductive biology, sperm storage duration, sperm morphometry, pheasants, comparative study

1. INTRODUCTION

Postcopulatory sexual selection consisting of sperm competition (Parker, 1970; Birkhead & Parker, 1997) and cryptic female choice (Eberhard, 1996) is thought to be an important evolutionary force for many reproductive traits including sperm morphometry (Simmons, 2001; Miller & Pitnick, 2002; Snook, 2005). However, it is often difficult to disentangle male and female influences on sperm form and function. One reason for this is the difficulty of investigating the interaction between sperm and the female reproductive tract after insemination.

Morphometric sperm traits, including sperm length and midpiece volume are known to be influenced by sperm competition in a variety of taxa. Total sperm length is associated with the risk of sperm competition in insects (Gage, 1994; Morrow & Gage, 2000) amphibians (Byrne *et al*., 2003), fishes (Stockley *et al*., 1997; Balshine *et al*., 2001), birds (Johnson & Briskie, 1999) and mammals (Gomendio & Roldan, 1991; Breed & Taylor, 2000; but see Hosken, 1997; Gage & Freckleton, 2003). Similarly, midpiece size is positively related to the risk of sperm competition in mammals (Anderson & Dixson, 2002; Anderson *et al*., 2005; but see Gage & Freckleton, 2003). On the other hand, in taxa where females store sperm after insemination (Birkhead & Møller, 1993a, b) there is growing evidence for the influence of female reproductive biology and cryptic female choice on the evolution of sperm morphometry: the coevolution of the size (and number) of sperm storage organs and sperm size has been demonstrated in beetles (Dybas & Dybas, 1981), drosophilids (Miller & Pitnick, 2002), stalk-eyed flies (Presgraves *et al*., 1999), Scatophagidae (Minder *et al*., 2005), snails (Beese *et al*., 2006) and birds (Briskie & Montgomerie,1992; Briskie *et al*., 1997).

The aims of this study were twofold: first, to test the hypothesis that sperm morphometry has evolved in response to the risk of sperm competition (inferred from relative testis mass; Møller, 1991; Møller & Briskie, 1995) in pheasants (Phasianidae, Galliformes). Pheasants typically exhibit intense precopulatory sexual selection characterised by polygynous, promiscuous or lek mating systems (Johnsgard, 1986; McGowan, 1994; Höglund & Alatalo 1995) and the risk of sperm competition may therefore vary markedly across species. Second, to test the hypothesis that sperm morphometry has evolved in response to female reproductive biology, specifically in response to the duration of sperm storage inferred from clutch size and the interval between successive eggs. Across a wide range of bird species, a significant relationship exists between sperm storage duration and the duration of egg laying (Birkhead & Møller, 1992). In many bird species, copulation ceases before egg laying starts and females store sperm for days or weeks prior to fertilise their eggs (Birkhead & Møller, 1993a, b). Therefore, in species with larger clutch size sperm have to survive longer to ensure the fertilisation of all eggs. In the peafowl for example, females cease copulating 12 days before the first egg is laid (Birkhead & Møller, 1993b); and females lay five to six eggs on average at two day intervals (Birkhead & Petrie, 1995). Sperm therefore have to survive for an average of 26 days inside the female tract. In contrast, female Palawan peacock pheasants *Polyplectron emphanum* lay only two eggs at two day intervals (McGowan, 1994) and sperm have to survive for a much shorter period. Sperm storage duration has two implications for postcopulatory sexual selection both of which depend strongly on copulation frequency: sperm storage duration may (i) influence the likelihood that sperm of rival males meet inside the female reproductive tract (Parker,

1970) and (ii) may be a way for females to select high quality (e.g. long lived) sperm (Birkhead *et al*., 1993).

2. MATERIAL & METHODS

Data collection

We collected sperm samples from 24 pheasant species. "Males used for this study were all bred in captivity and held in the "Parc zoologique de Clères". Several species belong to populations managed by the European Endangered Programmes (EEP) to avoid inbreeding effects (Saint Jalme, 2002; Saint Jalme, *et al*. 2003; see also: Gomendio *et al*., 2000; Gage *et al*., 2006). Ejaculates were collected using the massage technique described in Saint Jalme *et al*. (2003). Data on sperm traits, testis mass and clutch size are summarised in the electronic Appendix 2. Data on testis mass and some morphometric sperm traits were not available for some species which explains variation in sample size in the analyses.

a) Sperm morphometry

Sperm from one ejaculate from one male per species were fixed in a 5% formalin solution. Intraspecific and intra-male repeatabilities (Lessells & Boag, 1987) for morphometric sperm traits were high, justifying the assumption that a single male is representative for a species (see Electronic Appendix Table 2). Furthermore, we compared the species mean for total sperm length obtained from an earlier dataset derived from several males per species by M. Saint Jalme (unpublished data) with the data from a single male per species used in this study. MSJ's species means and the data

used in the present study were highly correlated ($r = 0.94$, $p < 0.0001$, N = 25) and not significantly different (paired sample t test: $t_{10} = 1.17$, $p = 0.27$).

For morphometric analyses, a sub-sample of sperm was placed on a microscope slide and stained using the fluorescent dye Mitotracker green FM (Molecular Probes) to make the midpiece evident for measurement. Two digital pictures of fifteen sperm per male were taken at 400x magnification: one picture was taken using bright-field and one picture was taken using fluorescence. The following sperm traits were measured using the image analysis software Leica IM50 Image manager: (i) the length of the head, (ii) the length of the midpiece, and (iii) the length of the flagellum including the part wrapped by the midpiece. Total sperm length was calculated by adding head length and flagellum length.

b) Testis mass, body mass and sperm storage duration

Testis mass and body mass were measured during the peak breeding season of each species from the same males used for sperm measurements. Testis mass was obtained by measuring testis dimensions and converting volume into mass using Møller's formula (1991). This method is widely used and provides an accurate measurement of testis mass if applied properly (Calhim & Birkhead, in press). Testis dimensions were obtained using laparotomy: maximum testis length and width were measured by inserting an endoscope and a calliper through the abdominal air sac wall. Only the left testis was measured to minimise stress. The measurements of the dimensions of both testes in one Indian peacock *Pavo cristatus* revealed minimal differences between left and right testis (right testis: 2.34g and left testis: 2.46; see also Friedmann, 1927; Kimball *et al*., 1997).

For each species the left testis was measured and multiplied by two to obtain an index of total testis mass.

Information on clutch size and the mean interval between successive egg was obtained from the literature (Johnsgard, 1986; McGowan, 1994; MSJ, unpubl. data) and was used to estimate the duration of sperm storage: average clutch size was multiplied with the mean interval between subsequent eggs and one subtracted at the end.

Statistical analyses

a) *Comparative methods*

To control for statistical non-independence of traits due to phylogeny in our analyses (Felsenstein, 1985; Harvey & Pagel, 1991) we used the approach of generalised least-squares in a phylogenetic framework (GLS: Pagel, 1999; Freckleton *et al*., 2002). The GLS approach allows the performance of regression and correlation analyses and the use of maximum-likelihood models takes phylogeny into account by referring to an internal matrix of expected covariances among species based on their degree of shared ancestry. The maximum-likelihood approach also allows the estimation of the phylogenetic dependence parameter λ which ranges between zero and one indicating the relative importance of phylogeny in explaining the similarities between traits. Values of λ close to zero indicate that traits are likely to have evolved independently of phylogeny whereas values of λ close to one indicate strong phylogenetic relationships of traits. Analyses were performed using a code developed by R. Freckleton for the statistical package R V.2.1.0 (R Foundation for Statistical Computing 2005). The phylogeny used

in the analyses was obtained from Kimball *et al*. (1999, 2001) and Randi *et al*. (2000: Electronic appendix).

b) *Multiple regression analyses*

We performed multiple regression analyses in a phylogenetic framework (GLS) as described above. Morphometric and functional sperm traits were included separately as dependent variables and testis mass, body mass and clutch size as independent variables. Stepwise removal of non-significant terms resulted in the minimal adequate model. The performance of multiple regression analyses including testis mass, body mass and clutch size allowed to control for possible allometry between both testis mass and body mass (Dunn *et al*., 2001) and clutch size and body mass (Bennett & Owens, 2002). We tested for collinearity between specific independent variables (body mass and testis mass, body mass and clutch size) as described in Belsley *et al*. (1980). The highest condition index was 6.65 and only condition indices >30 indicate collinearity to be a problem. Where necessary, data were normalised using the appropriate transformation to obtain optimal fit of the GLS model.

In addition, we examined the inter-relationships among functional sperm traits, among morphometric sperm traits and between sperm functional and morphometric sperm traits using GLS. To assess the allometric relationships between morphometric sperm traits we calculated the slope v of a Reduced Major Axis regression (RMA: Ricker, 1973; McArdle, 1988). As an approximation we used the standard error of the GLS regression (Sokal & Rohlf, 1995) to perform a t test of v against one.

The use of Bonferroni correction seemed inappropriate as it enhances the chance of Type II errors since *p* values strongly depend on sample size (Nakagawa, 2004). We calculated the effect size *r* from *t* values from the multiple regression analyses (Cohen, 1977; Nakagawa, 2004) and used Cohen's (1988) benchmarks to estimate the strength of the observed pattern. 95% non-central confidence limits (CL) for *r* indicate statistical significance if zero is not included in the CLs (Smithson, 2003).

3. RESULTS

Sperm morphometry

None of the morphometric sperm traits showed any relationship with relative testis mass either when including testis mass, body mass and sperm storage duration or when excluding sperm storage duration (Table 1; Figure 1). Flagellum length showed a negative association with sperm storage duration which was significant when testis mass and body mass were removed from the model (Table 1; Figure 1). Similarly, total sperm length was also significantly negatively associated with sperm storage duration in both the model including testis mass, body mass and sperm storage duration and in the model including sperm storage duration only. The model AICs suggested that the minimum models including sperm storage duration only were more adequate than the maximum model including all three independent variables (flagellum: maximum model AIC = -40.3; minimum model AIC = -46.1; sperm total length: maximum model AIC = -41.9; minimum model AIC = -46.5). Head length and midpiece length showed no relationship with sperm storage duration. Effect sizes for flagellum length and total sperm length

were medium to large indicating that the association between sperm morphometry and sperm storage duration was substantial (Table 1).

Maximum likelihood (ML) values of λ were low for head length and high for midpiece length, flagellum length and total sperm length when testing the relationship between morphometric triats and testis mass and body mass. However, they were intermediate when testing the relationship between both flagellum length and total sperm length against sperm storage duration indicating that factors other than phylogeny plays an important role in explaining the observed pattern (Table 1).

Inter-relationships between sperm traits

The GLS regression slope $b = 0.78$ (± 0.37 s.e.) and the RMA regression slope $v = 2.16$ indicated a positive relationship between midpiece length and flagellum length with a slope (v) was significantly different from one ($t = 3.13$, $P < 0.01$), indicating a positive allometric relationship between these two traits.

4. DISCUSSION

In pheasants, female reproductive biology seems to have a major influence on the evolution of sperm morphometry whereas we found no evidence for an influence of sperm competition on sperm morphometry. Our results suggest that the duration of sperm storage inferred from clutch size and spread of laying may have a major impact on pheasant sperm morphometry. To our knowledge, this is the first evidence for a relationship between sperm morphometry and sperm storage duration. A relationship between the evolution of sperm morphometry and female reproductive biology has also

been demonstrated in passerine birds where sperm size was found to have coevolved with the number and size of female sperm storage tubules rather than with sperm competition *per se* (Briskie & Montgomerie, 1992; Briskie *et al*., 1997).

The lack of a relationship between sperm morphometry and the risk of sperm competition inferred from relative testis mass might be due to a possible lack of variation in sperm competition risk across pheasants. Although mating systems vary markedly across species ranging from monogamy to polygyny (Johnsgard, 1986; McGowan, 1994), females appear to copulate only once or twice for a single clutch and hence the risk of sperm competition might be low and variation across species minimal. On the other hand, we found no evidence that variation in relative testis mass between species was any less in pheasants than in other avian taxa (S. Immler & T. R. Birkhead, unpubl. data).

A trade-off between sperm size and sperm longevity has been suggested in mammals and fish (Gomendio & Roldan, 1991; Stockley *et al*., 1997; Gage, 1998). Such a trade-off would be a plausible explanation for the negative relationship between the size of sperm traits and sperm storage duration in pheasants. In mammals, no relationship between sperm size and oestrus length (as an index of sperm survival inside the female reproductive tract) has been found (Hosken, 1997, 1998; Gage, 1998). This contrasts with an earlier finding in mammals where sperm lifespan inside the female reproductive tract and fertility span of females were positively correlated (Gomendio & Roldan, 1993). A possible explanation for this discrepancy is that the relative timing of copulation and ovulation varies across species (Asdell, 1964) and mechanisms controlling ovulation might influence the evolution of sperm longevity. The role of

sperm longevity after insemination is crucial for our understanding of the mechanisms in postcopulatory sexual selection and more detailed investigation is needed on the factors influencing sperm longevity.

The biological mechanisms underlying the trade-off between sperm size and longevity are highly debated (Cardullo & Baltz, 1991; Gage, 1998; Immler & Birkhead, in press). In mammals, it has been suggested that sperm longevity is influenced by the negatively allometric relationship between midpiece size and flagellum length which results in a relatively higher metabolic rate in longer sperm and thus a reduced lifespan (Cardullo & Baltz, 1991; Gage, 1998). However, in pheasants, the allometric relationship between midpiece length and flagellum length is positive, which means that unlike mammals, longer sperm have a relatively longer midpiece. The reason suggested for mammals, that increased metabolic rate accounts for reduced lifespan of longer sperm, therefore cannot apply to pheasants. One possibility is that midpiece size *per se* influences sperm longevity rather than the relationship between midpiece size and flagellum length (see also Immler & Birkhead, in press). In fowl, *Gallus gallus domesticus*, the duration of sperm storage appears to be determined in part at least by sperm metabolism, which in turn may be determined by mitochondrial (midpiece) function (Froman, 2003). The negative relationship between midpiece size and sperm storage duration in pheasants might indicate that a larger midpiece dissipates energy sooner than a smaller midpiece. However, the relationship between sperm size and sperm longevity needs further investigation in pheasants as well as in other taxa.

The influence of female reproductive biology including female reproductive anatomy, physiology and behaviour on the evolution of sperm morphometry is still

poorly understood. Several studies have addressed the relationship between sperm size and various aspects of female reproductive biology: in some sperm storing taxa, sperm size was found to be positively related with the size and/or number of sperm storage organs (passerine birds: Briskie *et al*., 1997; insects: Dybas & Dybas, 1981; Miller & Pitnick, 2002; Presgraves *et al*., 1999; Morrow & Gage, 2000; Minder *et al*., 2005; snails: Beese *et al*., 2006). However, in sperm storing bats no relationship between female reproductive tract dimensions and sperm size was found (Hosken, 1997, 1998) possibly because unlike the other taxa, female bats do not appear to have specific sperm storage structures. These contrasting results emphasize the need for more detailed investigation of the interactions between sperm and the female reproductive tract.

Conclusions

As in other sperm storing taxa, sperm morphometry in pheasants appears to have evolved in response to female reproductive biology and to sperm storage duration in particular. Sperm storage is likely to be a strong selective pressure favouring sperm traits such as sperm survival and longevity rather than sperm swimming velocity.

Fundamental differences in female reproductive biology may lead to marked differences in the evolution of sperm morphometry between taxonomic groups. Future studies should attempt to disentangle the relative importance of sperm competition and cryptic female choice for the evolution of sperm morphometry and function.

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TABLES, FIGURES & LEGENDS:

Table 1: Multiple regression analyses controlling for phylogeny (GLS) to test the relationship between sperm morphometry and testis mass, body mass and sperm storage duration (storage dur.) across pheasant species. For head length and midpiece length results for maximum models are shown. For flagellum length and total sperm length results from separate models including (i) testis mass and body mass and (ii) sperm storage duration only are shown as indicated by broken line. The *t* values result from a test of the slopes against zero. The model including the maximum likelihood (ML) value for λ is compared against the models including λ = 1 and λ = 0 and supersrcipts after the ML value of λ indicate significance levels of the likelihood ratio tests (first position: against λ = 1; second position: λ = 0; significance levels: * = P <0.05; ** = P <0.01). We also calculated effect size *r* and 95% non central confidence limits (CL) from the *t* value given in the GLS analysis to test for the strength of the association between traits (lower CL: LCL, upper CL: UCL). CLs excluding zero indicate a significant relationship whereas CLs including zero indicate no statistical significance.

						effect size		
trait	slope	*t*	*P*	λ	n	*r*	LCL	UCL
head				<0.001**$^{n.s.}$	22			
testis mass	-0.13	1.41	0.18			0.32	-0.12	0.78
body mass	0.23	1.74	0.10			0.38	-0.05	0.85
storage dur.	-0.12	1.86	0.08			0.40	-0.03	0.87
midpiece				1.00 $^{n.s.,}$**	22			
testis mass	-0.07	1.04	0.31			0.24	-0.21	0.69
body mass	-0.12	1.04	0.31			0.24	-0.21	0.69
storage dur.	-0.01	0.08	0.94			0.02	-0.43	0.47
flagellum				0.90 $^{n.s.,}$**	24			
testis mass	-0.01	0.64	0.53			0.14	-0.29	0.57
body mass	-0.05	1.21	0.24			0.26	-0.16	0.69
storage dur.	-0.10	2.93	0.008	0.77 $^{n.s.,}$ *		0.53	0.16	1.02
total length				0.86 $^{n.s.,}$ **	24			
testis mass	-0.04	1.54	0.14			0.32	-0.10	0.76
body mass	-0.001	0.49	0.63			0.11	-0.32	0.54
storage dur.	-0.11	3.20	0.004	0.66 *,*		0.56	0.21	1.06

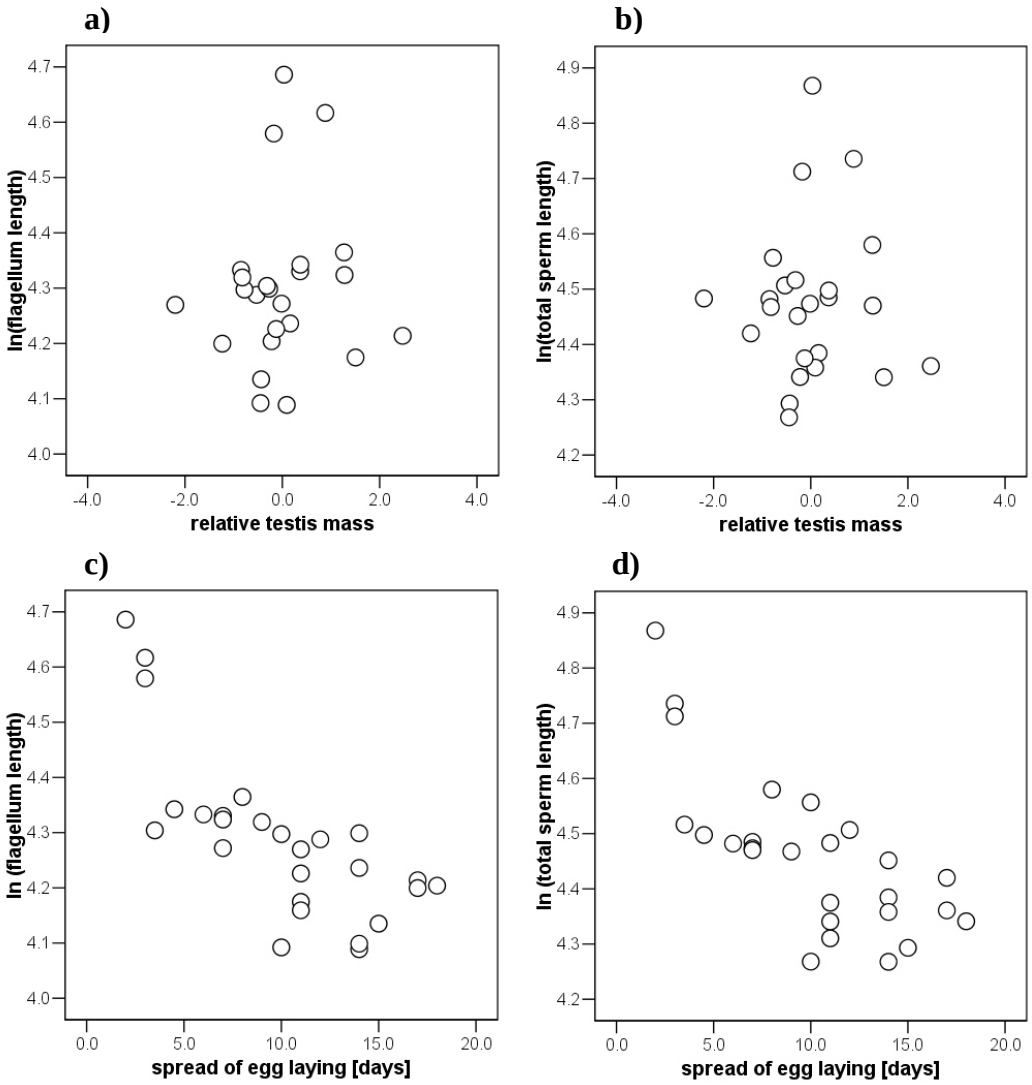

Figure 1: Association between morphometric sperm traits and (i) relative testis mass and (ii) sperm storage duration inferred from spread of egg laying. Relative testis mass indicates residual testis mass obtained form a regression between testis mass and body mass. Figures are not controlled for phylogeny: **a)** no significant association between flagellum length and relative testis mass (b = -0.01, t = 0.64, P = 0.53, λ = 0.90, n = 22); **b)** no significant association between total sperm length and relative testis mass (b = -0.04, t = 1.54, P = 0.14, λ = 0.86, n = 22); **c)** significant negative association between flagellum length and spread of egg laying (b = -0.10, t = 2.93, P = 0.008, λ = 0.77, n = 24); **d)** significant negative association between total sperm length and spread of egg laying (b = -0.11, t = 3.20, P = 0.004, λ = 0.86, n = 24).