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The evolution of sperm length in moths

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Sperm form and function remain poorly understood despite being of fundamental biological importance. An instructive approach has been to examine evolutionary associations across comparable taxa between sperm characters and other, potentially selective reproductive traits. We adopt this approach here in a comparative study examining how sperm lengths are associated with male and female reproductive characters across moths. Primary data have revealed Lepidoptera to be an ideal order for examination: there is profound variation in the dimensions (but not organization) of the reproductive traits between closely related species which all share a monophyletic ancestry, for example, eupyrene sperm length varies from 110 to 12 675 μm. Eupyrene (normal fertilizing) and apyrene (anucleate and non-fertile) sperm lengths are positively correlated across taxa and both sperm types show positive associations with mating pattern (as measured by the residual testis size). At fertilization, eupyrene sperm must migrate down the often elongated female spermathecal duct from storage to unite with the ovum. Across taxa, the elongation of this duct is associated with increased eupyrene sperm length, suggesting a positive female influence on sperm size since longer, more powerful sperm may be selected to migrate and/or compete successfully down greater ductal lengths. Apyrene sperm length is not associated with female reproductive tract dimensions. However, we found a positive relationship between the residual testis volume and spermathecal volume, suggesting coevolution between male investment in spermatogenesis and the extent of the female sperm storage capacity. Within males, there is a positive association between the two organs which form the ejaculate-containing spermatophore: the testes and the accessory gland. The 'trade-up' in investment to these components is discussed in relation to paternal investment and mating patterns.

Keywords: sperm competition; eupyrene; apyrene; Lepidoptera; spermatheca; testes

1. INTRODUCTION

Spermatozoa are traditionally characterized as being numerous and tiny. However, there exists enormous variation in the size of sperm across the animal kingdom, from 28 μm in the porcupine *Hystrix africaeaustralis* (Gage 1998) to 58 290 μm in *Drosophila bifurca* (Diptera: Drosophilidae) (Pitnick *et al*. 1995). The adaptive significance of similar size variation in eggs has been well studied (Smith & Fretwell 1974; Stearns 1992). However, why male gametes show such variability remains poorly understood. Although there is good evidence that sperm number is an important fitness-related trait (e.g. Birkhead & Møller 1998), increasing sperm size may also be adaptive where the processes of fertilization and/or competition demand increased flagellar forces (Katz & Drobnis 1990) or more effective filling or positioning within the female reproductive tract (Briskie *et al*. 1997). Direct experimental testing of the significance of sperm form and function presents particular technical challenges and, therefore, a comparative approach has been instructive in understanding the evolutionary relationships between spermatozoa and their associated reproductive parameters (e.g. Pitnick *et al*. 1995; Briskie *et al*. 1997; Gage 1998). We adopt a similar approach in this study by examining the relationships within a large and consistently collected data set of sperm lengths and reproductive traits across moths.

Sperm fundamentally function to carry the male haplotype to unite with the ovum. However, this ultimate goal must be achieved in the sometimes complex environment of the female reproductive tract and there may be competition between rival males' sperm to fertilize (Parker 1970; Birkhead & Møller 1998). The forces arising from these interactions may select for sperm architecture (e.g. Gomendio & Roldan 1991; Briskie *et al*. 1997). Sperm competition, when sperm from two or more males compete for the fertilization of a female's ova (Parker 1970), is an important force in the evolution of male reproduction. There is evidence across some species that sperm competition is associated with variance in sperm size: birds (Briskie & Montgomerie 1992), butterflies (Gage & Cook 1994) and primates and rodents (Gomendio & Roldan 1991) show positive associations between sperm size and sperm competition, while across fishes the reverse relationship is seen (Stockley *et al*. 1997). Since relative testis size is significantly correlated with the degree of polyandry across a variety of taxa (primates (Harcourt *et al*. 1981), mammals (Kenagy & Trombulak 1986), birds (Møller 1988; Briskie & Montgomerie 1992), equids (Ginsberg & Rubenstein 1990), amphibians (Jennions & Passmore 1993), fishes (Warner & Robertson 1978; Stockley *et al*. 1997) and bats (Hosken 1997)) including Lepidoptera (Gage 1994; Gage & Cook 1994), it has been argued that the relative investment in testes is a good measure of the level of sperm competition a species has evolved (e.g. Briskie & Montgomerie 1992; Short 1997). We therefore also used this indirect measure to explore the associations between sperm length and mating patterns across a large data set of moth sperm lengths.

Despite the widespread importance of sperm competition in the evolution of male reproductive function (Birkhead & Møller 1998), in internal fertilizers sperm

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have primarily evolved to function in the female. It has been proposed that this fundamentally selective force has been neglected in past investigations of the evolution of sperm form (Briskie *et al*. 1997). Five studies have examined how sperm size has evolved in relation to female reproductive tract dimensions. Across eight species of ptilid beetles, sperm length was positively correlated with the female sperm storage site size and its duct length (Dybas & Dybas 1981). Similarly, sperm length was positively correlated with female sperm storage organ size across 32 species of *Drosophila* (Pitnick *et al*. 1995) and, subsequently, across 46 species of *Drosophila* (Pitnick *et al*. 1999). Briskie & Montgomerie (1992) found that sperm length was positively correlated with the sperm storage tubule (SST) length across 20 species of passerine birds. Finally, both long and short morphs of sperm were positively correlated with the sperm storage organ size across 13 species of diopsid flies (Presgraves *et al*. 1999).

This study explores the associations between sperm lengths and female reproductive tract dimensions across a maximum of 178 moth species. Lepidoptera are ideal for such a study since their reproductive characters vary widely in dimension but not organization and there is profound morphometric variation between closely related species. In addition to sperm lengths and the dimensions of the female tract in which sperm must migrate, the associations between male testis and accessory gland sizes and the sizes of the site of insemination and sperm storage were examined.

Lepidoptera produce two distinct types of sperm which differ in morphology and behaviour (Meves 1902): the nucleate eupyrene sperm are capable of fertilization whereas the apyrene sperm lack functional nuclear material (Meves 1902; Silberglied *et al*. 1984). Both sperm types are transferred to the female and both migrate to and persist within the female sperm storage organ (Drummond 1984). Recent evidence in a butterfly showed that apyrene sperm delay female remating and, hence, sperm competition (Cook & Wedell 1999) since the female remating propensity is controlled by the mass of motile sperm in storage and apyrene sperm are numerous and highly motile (Drummond 1984; Silberglied *et al*. 1984) (but note that some moth species show brief refractory periods; LaMunyon & Eisner 1994). Accordingly, the specific evolutionary relationships between apyrene sperm and female reproductive tract and spermathecal dimensions were examined.

Lepidoptera (and many insects) transfer sperm packaged in a specialized spermatophore. How the spermatophore functions in the context of sperm competition also warrants investigation. Spermatophores may function as either a source of nutrients or energy for the female (Gwynne 1984) or a mating plug which delays or prevents the success of subsequent rematings (and, hence, sperm competition) (Ehrlich & Ehrlich 1978). The spermatophore is created from different products of the testes and accessory glands (Drummond 1984) and the relationship between the relative male investment in these two organs may aid in the understanding of spermatophore function (Wedell 1993). If spermatophore size influences the female remating interval (and, thus, the level of sperm competition) then we may predict a positive relationship between testis size and accessory gland size, i.e. the 'ejaculate protection' hypothesis (Simmons & Siva-Jothy 1998). However, if spermatophore size has evolved in response to providing nutritional resources for the female then we may expect a negative relationship between these organs since increased confidence in paternity (in monogamous mating patterns with relatively smaller testes) is likely to allow the evolution of increased paternal investment (Gwynne 1984).

2. MATERIAL AND METHODS

(a) *Sampling and microdissection*

Moths were sampled using an ultraviolet lamp at several sites within the UK, Panama and Costa Rica. Species were either identified from the literature or by comparison with named specimens in collections at the British Natural History Museum, the Liverpool Museum, the Smithsonian Tropical Research Institute (Panama) and the Instituto Nacional de Biodiversidad (INBIO, Costa Rica). All moths were weighed and then the dimensions of their reproductive characters were measured after microdissection using a binocular microscope. Testes are spherical and, therefore, their volume was calculated from the mean of three equidistant diameter measures (x-, y- and z-axes). The paired male accessory glands are cylindrical, blind-ending tubules; their length and mean diameter (measured at five equidistant points along each tubule) enabled the total tubule volume to be calculated. Since we sampled wild-caught moths we were not able to control for mating history which might have influenced testis and accessory gland size and spermathecal diameter. However, random sampling will only introduce a random increase in the variance of these traits with no directional errors.

Mature sperm were recovered from the distal portion of the vasa deferentia where mature bundles of eupyrene and disassociated apyrene sperm are stored (Norris 1932; Drummond 1984). Sperm samples were dispersed in distilled water and then air-dried as smears on glass slides. Phase-contrast microscope images of up to five eupyrene bundles (median $= 5$ and mean $= 4.5 \pm 0.05$ sperm male^{-1}) and five individual apyrene sperm (median $= 5$ and mean $= 4.7 \pm 0.04$ sperm male^{-1}) from each male were relayed to a video monitor where they could be traced onto acetate sheets. Images of these tracings were measured using a digital map measurer. This method of measuring sperm length is highly repeatable both between measuring events (repeated-measures generalized linear model (GLM), $F = 155\,033$ and $p < 0.001$) and between observers (repeated-measures GLM, $F = 184\,710$ and $p < 0.001$).

The fragile spermatheca and its associated duct were carefully removed from the female and mounted on a slide in Aquamount. Lateral compression and distortion with the coverslip was avoided by using spacers. The dimensions of the spermatheca and its duct were again measured using identical techniques to sperm measurement (see above). Lepidopteran spermathecae are cylindrical and their volume was calculated accordingly, with a mean radius derived from ten equidistant diameter measures and length traced down the central axis of the organ. We did not count the accessory spermatheca as a site of sperm storage since sperm were never observed therein. The spermathecal duct length was measured along its entire central axis from the point of constriction at the base of the spermatheca to where it joins the oviduct. The bursa copulatrix is variable in shape and, therefore, the dry weight of the bursa was measured. The bursa was removed from the female at the point where the ductus bursae meets the sclerotized antrum.

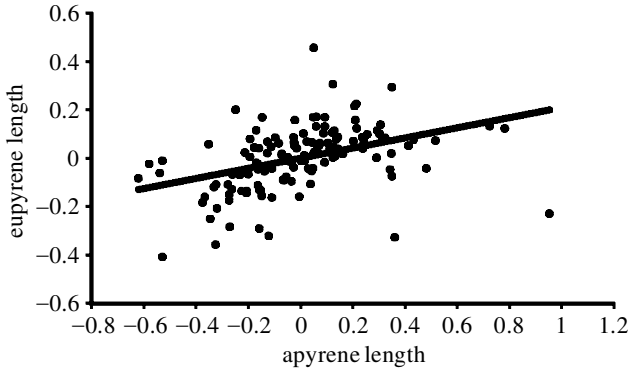

Figure 1. Apyrene sperm length is positively associated with eupyrene sperm length across 135 species after phylogenetic subtraction ($r^2 = 0.17$, $n = 135$ and $p < 0.001$). Slope exponent $= 0.21 \pm 0.04$ s.e.

Mean character values for each species were calculated from the means from each individual of that species (median = 2 and mean $= 3.0 \pm 0.2$ individuals species^{-1}). Sperm lengths were measured from 149 species (representing 17 families). Testis and accessory gland volumes were measured from 140 species. The spermathecal volume and duct length were measured from 77 species. The bursa copulatrix weight was measured from 79 species.

(b) *Comparative and statistical analysis*

Species data are not necessarily free of phylogenetic association since they may share character values as a result of a common ancestry rather than from independent evolution (Harvey & Pagel 1991). Such phylogenetic inertia must be controlled for by using modern methods of comparison as advocated by Felsenstein (1985) and Harvey & Pagel (1991). In the absence of a moth phylogeny we attempted to control for species relatedness using a recognized moth taxonomy (Scoble (1992) and references therein). Taxonomic organization does not provide information on historical changes in higher-order character values through evolutionary time. However, we controlled for taxonomic association at lower orders using Stearn's (1983) phylogenetic subtraction method. Phylogenetic subtraction removes the variation in species' character values which can be attributed to a higher-order taxonomic (or phylogenetic) association so that only residual values remain which are therefore free from the value attributed to the phylogenetic position. Accordingly, the family means for each character were calculated and these values subtracted from each species value. This method is appropriate for this comparative data set since character values vary profoundly among closely related species and some of these characters can show rapid selection responses (e.g. sperm morphometry; Beatty 1970) suggesting low levels of phylogenetic inertia.

The data were $\log_{10}$ transformed before analysis to normalize the distributions. Allometric relationships were determined in each analysis and, where necessary, controlled for prior to analysis of the residuals. Regressions were carried out on the data obtained after phylogenetic subtraction and, thus, the sample sizes represent the number of species data points available after subtraction. A sequential Bonferroni test was used to control for type I error within groups of regressions where the same independent variable was tested against more than one dependent variable (Holm 1979; Rice 1989). For example, where the independent variable body size was regressed against seven dependent variables the conventional level of significance that must be satisfied ($p = 0.05$) was lowered accordingly (Rice 1989).

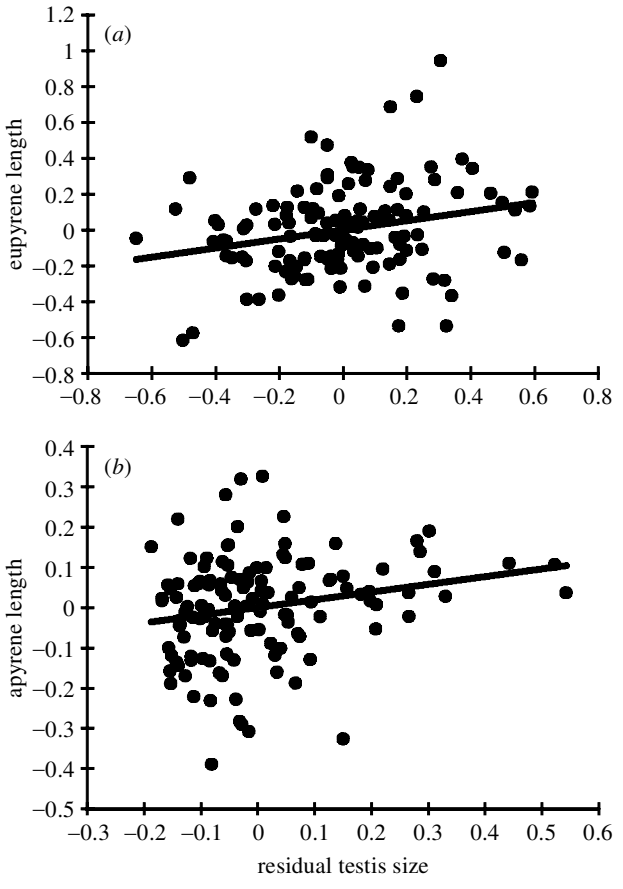

Figure 2. (*a*) Residual testis size is positively associated with eupyrene sperm length across 127 species after phylogenetic subtraction ($r^2 = 0.06$, $n = 127$ and $p = 0.005$). Slope exponent $= 0.25 \pm 0.09$ s.e. (*b*) Residual testis size is positively associated with apyrene sperm size across 131 species after phylogenetic subtraction ($r^2 = 0.05$, $n = 131$ and $p = 0.01$). Slope exponent $= 0.19 \pm 0.08$ s.e.

3. RESULTS

(a) *Interspecific variation*

Across moths, the variation found in the parameters measured was considerable: eupyrene length 110–12 675 μm, apyrene length 106–883 μm, spermatheca volume 0.06–2.01 mm^3, spermathecal duct length 797–17 530 μm, bursa copulatrix dry mass 5–87 mg, male accessory gland volume 0.11–218.93 mm^3, testis volume 0.07–36.75 mm^3, spermatophore count 1–12 spermatophores, body mass 0.01–4.54 g and forewing length 10–133 mm. This range of trait variance within a monophyletic taxon (Ackery 1984) is ideal for instructive comparative analyses.

(b) *Allometric associations*

There were significant positive associations between body size and testis size ($r^2 = 0.10$, $p < 0.0001$ and $n = 130$), accessory gland size ($r^2 = 0.14$, $p < 0.0001$ and $n = 130$) and bursa copulatrix size ($r^2 = 0.52$, $p < 0.0001$ and $n = 45$). We therefore controlled for these allometric relationships and calculated the residuals from the mean regression lines for subsequent analyses. However, we found no relationships between body size and eupyrene length ($r^2 = 0.001$, $p = 0.8$ and $n = 135$), spermathecal duct length ($r^2 = 0.02$, $p = 0.33$ and $n = 47$) or spermatheca size ($r^2 = 0.048$, $p = 0.07$ and $n = 69$) and so absolute values

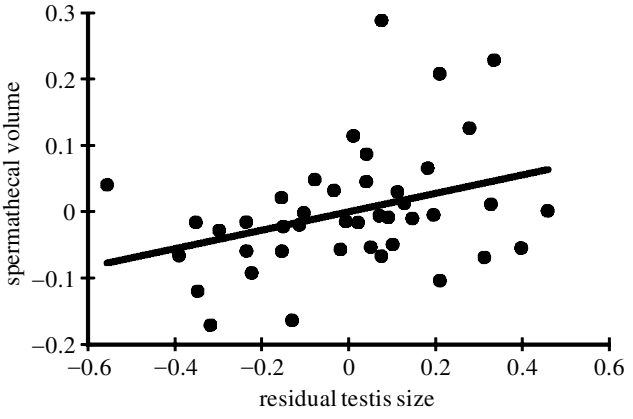

Figure 3. Residual testis volume is positively associated with spermathecal volume across 42 species after phylogenetic subtraction ($r^2 = 0.12$, $n = 42$ and $p = 0.025$). Slope exponent $= 0.14 \pm 0.06$ s.e.

were used for these in the subsequent phylogenetic subtractions. The relationship between body size and apyrene length ($r^2 = 0.03$, $p = 0.035$ and $n = 135$) becomes non-significant after sequential Bonferroni adjustment. Therefore, the absolute apyrene length was used in subsequent analyses (the outcome of these results was not affected by using the residual apyrene length in place of the absolute apyrene length).

(c) *Associations between sperm sizes*

There was a significant positive relationship between eupyrene length and apyrene length ($r^2 = 0.17$ and $p < 0.001$) across 135 species (figure 1).

(d) *Associations with testis size*

Both eupyrene and apyrene sperm lengths showed a significant positive relationship with the residual testis size (eupyrene $r^2 = 0.06$, $p = 0.005$ and $n = 127$ and apyrene $r^2 = 0.05$, $p = 0.013$ and $n = 131$) (figure 2*a*,*b*). There was also a significant relationship between the residual testis size and spermathecal volume ($r^2 = 0.12$, $p = 0.025$ and $n = 42$) (figure 3). There were significant positive associations between the residual bursa copulatrix size and residual testis size ($r^2 = 0.15$, $p = 0.009$ and $n = 44$) (figure 4*a*) and residual male accessory gland size ($r^2 = 0.15$, $p = 0.009$ and $n = 45$) (figure 4*b*). In addition, the residual testis size is positively correlated with the residual accessory gland size ($r^2 = 0.5$, $p < 0.001$ and $n = 130$) (figure 5).

(e) *Associations with female reproductive tract size*

The eupyrene sperm length was positively correlated with the spermathecal duct length ($r^2 = 0.11$, $p = 0.02$ and $n = 48$) (figure 6), but showed no association with the spermathecal storage volume ($r^2 = 0.014$, $p = 0.43$ and $n = 46$). The apyrene sperm length showed no association with the spermathecal duct ($r^2 = 0.01$, $p = 0.60$ and $n = 47$) or spermatheca volume ($r^2 = 0.03$, $p = 0.27$ and $n = 46$).

4. DISCUSSION

Our primary results (see § 3(a)) revealed that this study analyses comparative relationships across a profoundly variant series of reproductive parameters which have evolved within a monophyletic taxon (Ackery

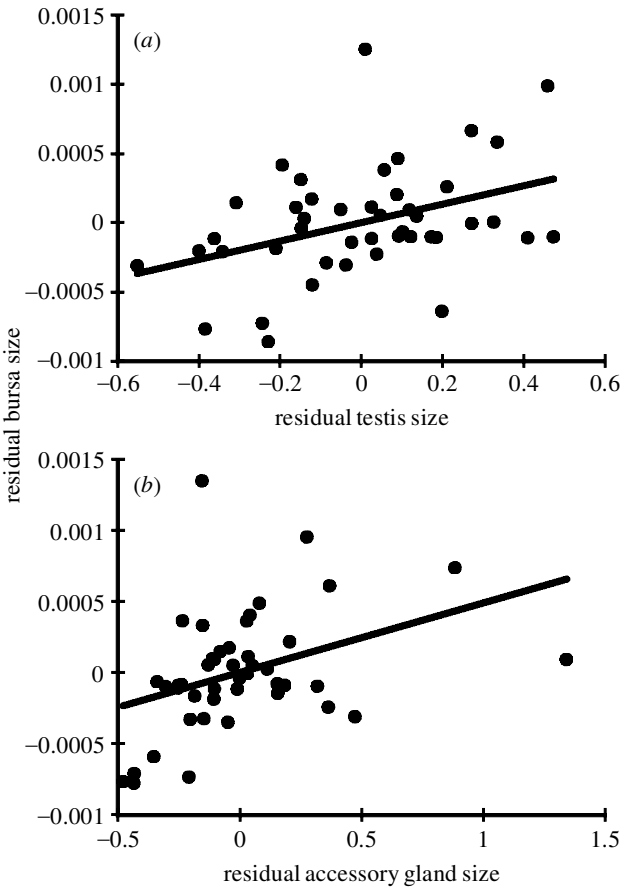

Figure 4. (*a*) Residual bursa copulatrix size is positively associated with residual testis size after phylogenetic subtraction ($r^2 = 0.15$, $p = 0.009$ and $n = 44$). Slope exponent $= 0.0007 \pm 0.0002$ s.e. (*b*) Residual bursa copulatrix size is positively associated with residual male accessory gland size after phylogenetic subtraction ($r^2 = 0.15$, $p = 0.009$ and $n = 45$). Slope exponent $= 0.0049 \pm 0.0002$ s.e.

1984). For example, eupyrene sperm length varies more than 100-fold from 110 µm to the relatively enormous 12.7 mm sperm of *Xenosoma geometrina* (Morrow 2000). This extreme variation across closely related species enables instructive evolutionary comparisons to be analysed.

Our results show that sperm length is associated with both female reproductive traits and, indirectly, male:male competition. Eupyrene sperm length is not associated with body size, but shows positive correlations with spermathecal duct length (figure 6). The spermathecal duct is likely to be an environment in which eupyrene sperm function is directly selected. Sperm movement into storage after spermatophore deposition is female mediated (Tschudi-Rein & Benz 1990). However, less is understood about the migration of sperm from storage to fertilization at oviposition. Sperm migration down the elongate spermathecal duct may place direct selection on axonemal length so that longer sperm may have evolved in species as a response to where females have evolved more elongate spermathecal ducts. Although experimental evidence remains lacking, flagellar elongation is predicted to enable increased forward propulsive power to be created (Katz & Drobnis 1990). Although the precise environment of the female tract in which sperm operate remains poorly understood, the narrow ducts we observed down which sperm migrate

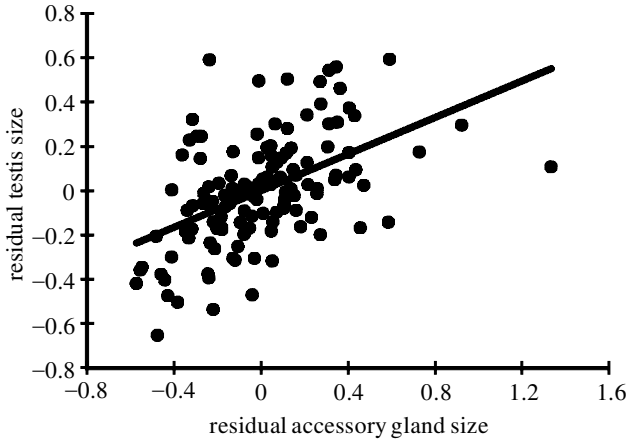

Figure 5. Residual accessory gland volume is positively associated with residual testis volume across 130 species after phylogenetic subtraction ($r^2 = 0.25$, $n = 130$ and $p < 0.0001$). Slope exponent $= 0.41 \pm 0.06$ s.e.

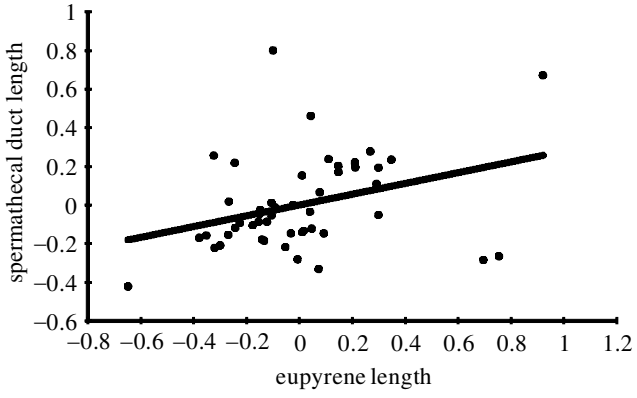

Figure 6. Eupyrene sperm length is positively associated with spermathecal duct length across 48 species after phylogenetic subtraction ($r^2 = 0.113$, $n = 48$ and $p = 0.02$). Slope exponent $= 0.28 \pm 0.12$ s.e.

in Lepidoptera present an opportunity for more elongate flagella to increase contact against the duct walls and generate greater progressive forces. If competition also proceeds along this duct (and most moth species generate sperm competition via polyandry and sperm storage) (see Drummond 1984), then intrasexual competition may generate directional sexual selection for further flagellar elongation (Briskie *et al*. 1997). Our results provide indirect evidence that both eupyrene and apyrene sperm lengths are associated with mating patterns across moths. Residual testes size is positively associated with the degree of polyandry (and, hence, level of sperm competition) in most comparative studies examining this relationship (Warner & Robertson 1978; Harcourt *et al*. 1981; Kenagy & Trombulak 1986; Møller 1988; Ginsberg & Rubenstein 1990; Briskie & Montgomerie 1992; Jennions & Passmore 1993; Gage & Cook 1994; Gage *et al*. 1995; Hosken 1997; Stockley *et al*. 1997). In moths we found that both eupyrene and apyrene sperm lengths are positively associated with residual testes size (figures 2*a*,*b*), providing indirect evidence that the level of sperm competition has a positive directional influence on the evolution of increasing sperm lengths across moths.

Although eupyrene and apyrene sperm lengths are positively associated across moths (figure 1), as is the case across butterflies (Gage 1994), recent work by Cook & Wedell (1999) showed that apyrene sperm function differently to eupyrene sperm by acting as a 'cheap filler' (Silberglied *et al*. 1984). Under the cheap filler hypothesis, the smaller but highly motile, anucleate apyrene sperm occupy the spermatheca and deceive the female into thinking that she has sufficient sperm in storage and this postpones remating. Therefore, the relationship between eupyrene and apyrene sperm length is probably the result of selection upon both sperm cells to function differently in the common environment of the female reproductive tract. As for eupyrene sperm, apyrene sperm length is also positively associated with residual testes size, implying general selection on both sperm types from the female mating pattern and sperm competition risk. However, in contrast to eupyrene sperm length, apyrene sperm length is not associated with the spermathecal duct length. These relationships suggest that, while eupyrene sperm length is directly (via duct length) and indirectly (via mating pattern) selected for competence in migration down the spermathecal duct, apyrene sperm length may be indirectly selected (via mating pattern) for competence in occupying and moving within the spermatheca (Cook & Wedell 1999). Neither eupyrene or apyrene sperm lengths are associated with spermathecal volume. However, there is a significant relationship between residual testis size and the volume of the sperm storage organ (figure 3). Thus, an increased volume of the sperm storage site may have exerted selection upon males to evolve larger testes to produce greater ejaculate masses (Svärd & Wiklund 1989) to fill and/or swamp the spermatheca.

Male resource allocation at the gonadal level is also associated with the female site of spermatophore deposition. The relative testes and accessory gland sizes, the two organs fundamentally creating the spermatophore, are positively associated with the size of the female bursa copulatrix. Spermatophores are deposited in the bursa and processes at this site may also be important in postponing future sperm competition. The enlarged spermatophore may act directly as a mating plug or obstacle which hinders the mating success of future males (Gwynne 1984). Further, females have evolved mechanisms to monitor the size of spermatophores using stretch receptors in the wall of the bursa: larger spermatophores (in addition to ejaculate size in the spermatheca) delay female remating receptivity (Sugawara 1979). These direct and indirect mechanisms of female assessment are associated with male investment in the two major organs which provide the materials which form the spermatophore: the testes and accessory glands. Interestingly, there is also a positive relationship between the sizes of these two male reproductive organs (figure 5). If the accessory glands function solely to provide nutriment to females (Drummond 1984) we would predict that, as polyandry increases and confidence in paternity decreases across species, there will be a trade-off between gonadal (sperm competition) and accessory gland (nuptial feeding) investment. Instead we see a positive correlation or a 'trade-up' in both organs, suggesting that accessory gland products in moths also function in ejaculate and, hence, paternity, protection which is consistent with previous comparative findings for bush crickets (Wedell 1993).

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