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Correlated evolution between male ejaculate allocation and female remating behaviour in seed beetles (Bruchidae)

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Abstract

Sperm competition theory suggests that female remating rate determines the selective regime that dictates the evolution of male ejaculate allocation. To test for correlated evolution between female remating behaviour and male ejaculate traits, we subjected detailed experimental data on female and male reproductive traits in seven-seed beetle species to phylogenetic comparative analyses. The evolution of a larger first ejaculate was positively correlated with the evolution of a more rapid decline in ejaculate size over successive matings. Further, as predicted by theory, an increase in female remating rate correlated with the evolution of larger male testes but smaller ejaculates. However, an increase in female remating was associated with the evolution of a less even allocation of ejaculate resources over successive matings, contrary to classic sperm competition theory. We failed to find any evidence for coevolution between the pattern of male ejaculate allocation and variation in female quality and we conclude that some patterns of correlated evolution are congruent with current theory, whereas some are not. We suggest that this may reflect the fact that much sperm competition theory does not fully incorporate other factors that may affect the evolution of male and female traits, such as trade-offs between ejaculate expenditure and other competing demands and the evolution of resource acquisition.

Introduction

Morphological traits involved in post-mating sexual selection, such as sperm morphology and the dimensions of the female reproductive tract, have been shown to coevolve in several vertebrate and invertebrate taxa (e.g. Dybas & Dybas, 1981; Briskie & Montgomerie, 1993; Briskie *et al.*, 1997; Pitnick *et al.*, 1999, 2003; Presgraves *et al.*, 1999; Ilango & Lane, 2000; Morrow & Gage, 2000; Koene & Schulenburg, 2003; Minder *et al.*, 2005; Brennan *et al.*, 2007; Rönn *et al.*, 2007). Such correlated evolution is normally thought to be generated by sperm competition, which occurs when sperm from two or more males compete for the fertilization of a given set of ova (Parker, 1998). Comparative studies across species, as well as experimental studies within species, have shown that sperm competition affects the evolution of several male reproductive traits (see Simmons, 2001). For example, artificial selection experiments have shown that male testes size evolves in response to alterations in the level of sperm competition (Hosken & Ward, 2001; Pitnick *et al.*, 2001, 2003). Further, comparative studies have found associations between female remating rate (a proxy for the intensity of sperm competition; see definition below) and both male ejaculate expenditure (Svärd & Wiklund, 1989; Gage, 1994) and genital morphology (Arnqvist, 1998). However, comparative studies are complicated by the fact that interactions and trade-offs between different ejaculate traits in males can confound the relationship between any single male trait and female remating rate (Simmons, 2001).

Early sperm competition models predicted that increased female remating rate should be associated with increased total ejaculate expenditure across species (Parker, 1998). This prediction holds both for an increasing risk (the probability of female remating) and intensity (the absolute number of ejaculates competing

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for a given set of ova) of sperm competition (Simmons, 2001). In a more recent model, Parker & Ball (2005) predicted that male ejaculate weight should increase with female remating at low risks of sperm competition (i.e. when remating is relatively infrequent) but should decrease with female remating at high sperm competition intensities (i.e. when remating is very frequent). Relative testes size, however, was predicted to increase monotonically with both increased risk and intensity of sperm competition.

It has become increasingly clear that male ejaculate production may carry substantial costs to males and that male reproductive success can at least in part be limited by ejaculate production capacity (e.g. Dewsbury, 1982; Bonduriansky, 2001). Because of trade-offs between the size of each ejaculate and the number of ejaculates, males should show prudence in ejaculate allocation over successive matings (Wedell *et al.*, 2002). Given everything else equal, allocation of a large amount of ejaculate resources (i.e. sperm and other ejaculate substances) to the first mating is expected to result in a more rapid decrease in the size of the ejaculate over successive matings (i.e. accelerated depletion). By contrast, a more prudent allocation to the first mating should result in a slower ejaculate depletion rate. Thus, we expect more prudent allocation under more intense sperm competition (Wedell *et al.*, 2002). Theory also predicts that males should allocate their ejaculate resources more equally over matings if females vary in quality (for example, fecundity), largely because of an increased effect of the chance of encountering females of high quality compared with the current mate (Galvani & Johnstone, 1998; Reinhold *et al.*, 2002), but no comparative data have directly tested this prediction. Variation in female quality within species may be caused by, for example, variation in female body size, age and mating status (Wedell *et al.*, 2002) or, across species, variation in the relationship between female size and fecundity.

In the current contribution, we explore correlated evolution between male ejaculate allocation patterns and female remating rate using phylogenetic comparative analyses. A common limitation of comparative studies is the inability to infer evolutionary process from detected patterns (see Martins, 2000), but comparative analyses that combine observational data with experimental data offer at least a partial remedy for this problem (Autumn *et al.*, 2002; Rowe & Arnqvist, 2002). Here, we adopt such an approach. We combine morphological male and female traits with those that require experimentation for their quantification, such as ejaculate allocation pattern over successive matings and female remating rate. As a model system, we use six congeneric *Callosobruchus* spp. seed beetle species and the Mexican seed beetle *Zabrotes subfasciatus* as a closely related outgroup (Tuda *et al.*, 2006). In a companion paper, we show that these species differ in their pattern of ejaculate allocation and in how ejaculate allocation affects female fitness (Rönn *et al.*, 2008). Here, we test the following four coevolutionary predictions. The first three predictions derive directly from theory suggesting that increased intensity of sperm competition (increased female remating rate) should be associated with males allocating more resources to ejaculate production and that these resources should be allocated more prudently in species where females mate more often (see references above): (P1) when males evolve to allocate more resources to their first ejaculate, males should simultaneously evolve to become ejaculate depleted more rapidly as a result of the trade-off between the size of the first and of successive ejaculates; (P2) the evolution of increased female remating rate should lead to a lower weight of the first ejaculate and a decelerated ejaculate depletion rate; (P3) the evolution of increased female remating rate should be associated with the evolution of larger male testes; and finally (P4) elevated variation in female fecundity within species should be associated with smaller ejaculates and, most importantly, with males allocating their ejaculates more equally across matings.

Methods

Model system

We used six species of *Callosobruchus spp*. seed beetles (Coleoptera, Bruchidae) (*C. maculatus* [PC], *C. subinnotatus* [RS], *C. analis* [RS], *C. rhodesianus* [RS], *C. chinensis* [RS] and *C. phaseoli* [YT]) and one species from the closely related genus *Zabrotes* (*Z. subfasciatus* [PC]) in our study (see Fig. 1 for their phylogenetic relationship). Beetles were provided by P. Credland [PC, University of London], R. Smith [RS, University of Leicester] and Y. oquenaga [YT, University of Tsukuba]. Each species was maintained on their natural host: *C. maculatus*, *C. rhodesianus* and *C. subinnotatus* on cowpea beans (*Vigna*

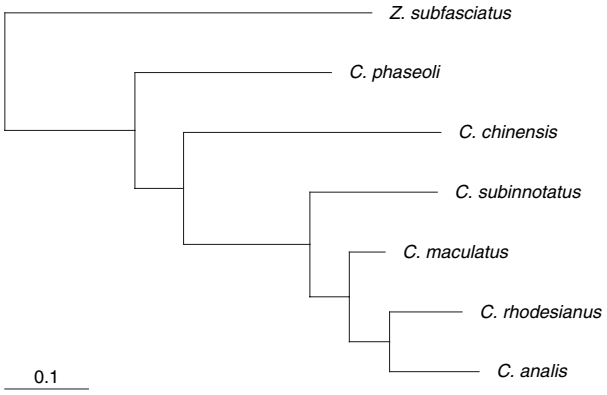

Fig. 1 Phylogenetic relationship between the seed beetle species included in this study. Modified after Tuda et al. (2006). The horizontal bar represents substitutions per site.

unguiculata), *C. chinensis* on adzuki beans (*V. angularis*), *C. analis* and *C. phaseoli* on mung beans (*Phaseolus aureus*) and *Z. subfasciatus* on white beans (*P. vulgaris*). All beetles were kept in climate chambers at 28°C and 55% (±10%) RH under a 12 h : 12 h light : dark cycle. Under these conditions, the generation time of all species is approximately 3–4 weeks.

Female remating rate

To assess female remating rate, we performed a series of remating experiments. Thirty virgin females of each species (24–48 h post-eclosion) were first mated once with one virgin male each. Each female was then given the opportunity to remate once every second day for the duration of her life, during exposure that lasted for 1 h (or less if remating occurred) where we recorded if females remated or not. For each species, half of the females ($N = 15$) were exposed to one virgin male during each exposure and the other half ($N = 15$) to three virgin males. Between mating trials, females were kept individually in 8-cm ∅ petri dishes provided with 100 beans of their preferred host for oviposition in climate chambers. All mating trials were performed at 22 ± 1°C. As a measure of female remating rate, we simply used the mean number of matings observed per female for each species. We thus used absolute remating rate rather than a measure that is relative to female lifespan, because the former should better quantify the intensity of sperm competition. In the comparative analyses reported below, we use only remating rate for females exposed to one male. The average number of matings in the two treatments was strongly correlated across species ($r = 0.93$), and the two measures yielded qualitatively identical and quantitatively very similar results. Female remating rate was significantly different across species ($F_{6,95} = 18.44$, $P < 0.001$).

Ejaculate characteristics and variance in female quality

The methods employed to measure the average weight of the first ejaculate, the rate at which ejaculate weight drops over successive matings (i.e. ejaculate depletion rate) and mean body size are detailed in our companion paper (all three traits are significantly different across species) (see Rönn *et al.*, 2008). In brief, the ejaculate depletion rate represents the species-specific slope from regressions describing the decrease in ejaculate weight over five successive matings. This metric will thus be more negative for more rapid rates of depletion. Quantifying variance in female quality within a taxon is a nontrivial task. Here, we define female quality as female lifetime offspring production and (using data on the reproductive performance of females mated once to virgin males from Rönn *et al.*, 2008; $N = 20$ females of each species) extract three related measures of variation in female quality within each species: (1) the coefficient of variation in female lifetime offspring production; (2) the Pearson correlation between female elytra length and lifetime offspring production; and (3) the Pearson correlation between female body weight and lifetime offspring production. The relationship between female size and female fitness differs markedly across species (Rönn *et al.*, 2008), and we report only analyses involving the last of these three measures below but note here that all generated qualitatively identical and quantitatively very similar results.

Testes size

A number of virgin males were collected from each species ($N = 7–9$). For each male, we recorded elytra length (see Rönn *et al.*, 2008; for methodology) and the two pairs of testes were dissected out. The testes were then transferred to a drop of glycerin on a microscope slide (76 × 26 mm) and covered with a standard coverslip (18 × 18 mm). The outline of each testes was then traced twice for each male, using a side-mounted camera lucida attached to a dissecting microscope (Leica® MZ8; Leica Microsystems GmbH, Wetzlar, Germany), and the area of the testes (sum of all four testes for each male) was recorded (using ImageJ 1.36b; Rasband, 2006). For each male, we used the average of the two repeated measures. The average testes size, elytra length and male body weight was recorded for each species (see Rönn *et al.*, 2008 for measuring protocol). Male testes size differed markedly across species, as revealed by an ANCOVA with testis size as response variable and species as a factor ($F_{6,47} = 38.689$, $P < 0.001$) and male size within species as a continuous covariate ($F_{1,47} = 0.214$, $P = 0.645$).

Comparative analyses

We assessed covariation between species (see Appendix 1 for species-level data) using regressions based on both species-level data and on phylogenetically independent contrasts (hence, PICs; Felsenstein, 1985). We also replicated all of our comparative analyses using a phylogenetic generalized least squares (GLS) approach (Rohlf, 2001), as implemented in the MatLab program REGRESSION.M (see Blomberg *et al.*, 2003), but the GLS models generated results that were qualitatively identical and quantitatively very similar to conventional regressions using PICs with one single exception (see Results section). Data on phylogenetic relationships between our species, including branch length information, were retrieved from the recent phylogenetic hypothesis by Tuda *et al.* (2006; Fig. 1). Briefly, this is a well-resolved phylogeny based on a total of 1651 bp of sequence data from three different mitochondrial genes. Species-level data were transformed into PICs using the Contrast module within PHYLIP (version 3.64; Felsenstein, 2005) and COMPARE (version 4.6b; Martins, 2004). To assess the amount of phylogenetic signal present in our data,

we calculated the descriptive statistic K using the MatLab program PHYSIG.M (see Blomberg *et al.*, 2003). A K less than one indicates that relatives resemble each other less than expected under Brownian motion model of evolution, which one would expect if there is adaptive evolution that is uncorrelated with the phylogeny. A K greater than one implies that relatives resemble each other more than expected under a Brownian motion model. This could be the result of either phylogenetic constraints or adaptive evolution that is correlated with the phylogeny.

If needed, data were transformed to meet the assumptions of the inferential models used. In a few of our regression models, residual plots nevertheless revealed potential outliers. Therefore, we also assessed all models (involving both PICs and species-level data) by calculating bootstrap estimates of the 95% confidence limits for the regression coefficients based on 1000 bootstrap replicates. However, the resulting confidence intervals are not reported here as this analysis, with one single exception (see below), revealed a complete congruence between the two inferential methods: the bootstrapped 95% CI did not enclose zero in all cases where conventional t-tests deemed an effect as being significant, whereas the opposite was true in cases where t-tests deemed an effect as being nonsignificant at $\alpha = 0.05$. In this sense, the results reported below are robust. We analysed our data by fitting analogous models of both species-level data and PICs. In the Results section, the results of the species-level analysis are immediately followed by the corresponding results for the PICs within square brackets. We note that in no case did the absolute value of PICs correlate significantly with the standard deviation of the PICs, suggesting that the standardization method used was appropriate (Garland *et al.*, 1992). All inferential regressions involving PICs were forced through the origin (Garland *et al.*, 1992), following full regressions (i.e. including a constant) revealing that in no case was the intercept significantly different from zero (Harvey & Pagel, 1991). All statistical analyses were carried out with SYSTAT® 11.0 (Systat Software Inc., San Jose, CA, USA).

Results

Phylogenetic signal

For testes size, female remating rate, male body weight and variation in female quality, the values of K were all less than one (0.77, 0.46, 0.66 and 0.58 respectively). For weight of the first ejaculate and the rate of ejaculate depletion, however, K values were larger than one (2.49 and 2.12 respectively). Hence, although the phylogenetic signal was weak for most traits studied here, it was relatively high for the pattern of ejaculate allocation.

Weight of the first ejaculate vs. ejaculate depletion rate

A large first ejaculate was strongly associated with a high rate of ejaculate depletion (Fig. 2; $r^2 = 0.98$ [0.96], $\beta = -0.001$ [−0.001], SE = 0.001 [0.001], $t = 12.02$ [7.41], $P < 0.001$ [< 0.001]). Thus, as males evolve to provide females with a large first ejaculate, the rate at which ejaculate size decreases over successive matings will also increase. The relationship between the weight of the first ejaculate and ejaculate depletion rate was not confounded by effects of male body weight, as adding male body weight did not improve the fit of the model (partial F-test: $F_{1,4} = 0.078$ [0.108], $P = 0.794$ [0.929]).

Female remating rate vs. the weight of the first ejaculate and ejaculate depletion rate

Although neither the weight of the first ejaculate nor the rate of ejaculate depletion was significantly related to female remating rate in univariate analyses ($\beta = 0.001$ [0.001], SE = 0.001 [0.001], $t = 1.15$ [0.87], $P = 0.300$ [0.425]; and $\beta = -17.2$ [−29.9], SE = 10.2 [18.5], $t = 1.69$ [1.61], $P = 0.152$ [0.168] respectively), a multiple regression analysis revealed a relationship between female remating rate and the independent effects of the weight of the first ejaculate and ejaculate depletion rate, both when using species-level data and PIC data (see Table 1). Again, the addition of male body weight did not improve the fit of this model (partial F-test: $F_{1,3} = 0.96$ [1.23], $P = 0.399$ [0.348]). Interestingly enough, increased female remating rate was associated with a decreased size of the first ejaculate but also with a more rapid rate of ejaculate depletion. However, the condition index (Belsley *et al.*, 1980) for this multiple regression model suggested that collinearity between the two independent variables

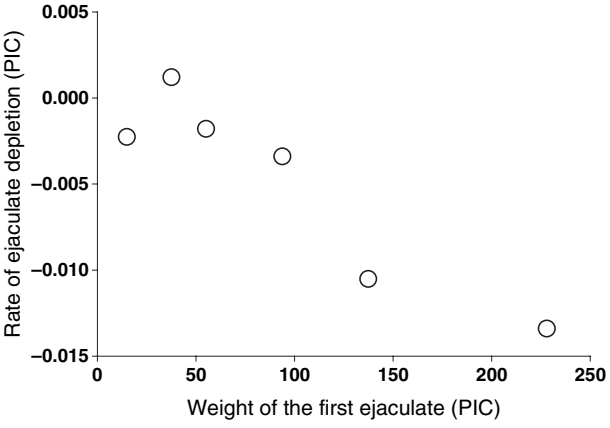

Fig. 2 The relationship between the weight of the first ejaculate and the rate of ejaculate depletion, shown as phylogenetically independent contrast values (positivized for the weight of the first ejaculate). Note that more negative values along the Y-axis correspond to a more rapid rate of ejaculate depletion.

Table 1 Multiple regression models of female remating rate, using the weight of the first ejaculate and ejaculate depletion rate as explanatory variables.

	Coefficient	SE	*t*	*P*
Weight of first ejaculate	−0.006 [−0.007]	0.001 [0.002]	4.58 [3.15]	0.010 [0.034]
Ejaculate depletion rate	−129.1 [−145.8]	24.9 [38.5]	5.19 [3.79]	0.007 [0.019]

Results are given for both species-level data and phylogenetically independent contrasts (the latter in square brackets). See text for additional analyses. $r^2 = 0.95$ [0.90], $N = 7$ [6].

could represent a possible inferential problem in our species-level analysis (ci = 24.5) but not in the analysis of PIC data (ci = 6.8). We use two different routes to overcome possible collinearity problems. First, we used principal component analysis to converte the relationship between the weight of the first ejaculate and the rate of ejaculate depletion to a first (PC1) and second (PC2) principal component. Here, PC1 represents an axis describing the negative covariance between the two variables and PC2 is orthogonal to this axis and thus represents the deviation from the negative covariance. A multiple regression model, using the uncorrelated PC1 and PC2 as independent variables, showed both an effect of the negative covariance between the two variables (PC1: $\beta = 0.06$ [0.19], SE = 0.02 [0.06], $t = 3.34$ [3.32], $P = 0.029$ [0.030]; standardized $\beta' = 0.53$ [0.65]) and an effect of the deviation from this covariance (PC2: $\beta = 0.91$ [1.40], SE = 0.18 [0.42], $t = 4.90$ [3.30], $P = 0.008$ [0.030]; standardized $\beta' = 0.78$ [0.65]) on female remating rate. Univariate regressions showed no significant effect of PC1 ($\beta = 0.06$ [0.19], SE = 0.04 [0.10], $t = 1.41$ [1.92], $P = 0.218$ [0.112]) but a significant effect of PC2 for species-level data ($\beta = 0.91$ [1.40], SE = 0.32 [0.74], $t = 2.82$ [1.90], $P = 0.037$ [0.115]) on female remating rate. Second, we replicated our conventional multiple regression model for species-level data using ridge regression, a common way to overcome collinearity problems. The ridge regression coefficients ($\beta = -0.006$ and -119.8), using the Hoerl–Kennard–Baldwin (HKB) estimator of the ridge complexity parameter (λ), were very similar indeed to those estimated in our conventional model (see Table 1). These computational exercises both confirm that female remating rate covaries with the weight of the first ejaculate and the rate of ejaculate depletion in a manner which to some extent is orthogonal to the covariance between the latter two variables. They thus provide support for the conclusion that increased female remating rate was indeed associated with a decreased size of the first ejaculate and a simultaneous increase in the rate of ejaculate depletion.

Testis size vs. weight of the first ejaculate and ejaculate depletion rate

Testis size was positively related to the weight of the first ejaculate, when controlling for male body weight in a multiple regression model (testis size: $\beta = 0.002$ [0.002], SE = 0.001 [0.001], $t = 4.52$ [3.83], $P = 0.011$ [0.019]; body weight: $\beta = 0.15$ [0.15], SE = 0.02 [0.01], $t = 8.72$ [12.42], $P < 0.001$ [< 0.001]; $r^2 = 0.98$ [0.99]). Testis size was also negatively related to ejaculate depletion rate ($\beta = -29.3$ [−28.2], SE = 4.3 [5.9], $t = 6.8$ [4.8], $P = 0.002$ [0.009]), when controlling for effects of male body weight ($\beta = 0.14$ [0.15], SE = 0.01 [0.01], $t = 12.2$ [14.7], $P < 0.001$ [< 0.001]; $r^2 = 0.99$ [0.99]). These models, thus, show that the evolution of relatively larger testes is associated with a larger first ejaculate and a higher ejaculate depletion rate. In both regression models, we found a strong allometric relationship with testis size and male body weight.

Female remating rate vs. relative testis size

Our analyses of the relationship between female remating rate and relative testes size yielded partly incongruent results for species-level analyses and regressions involving PICs (testis size: $\beta = 0.77$ [0.99], SE = 0.21 [0.41], $t = 3.76$ [2.41], $P = 0.020$ [0.073]; body size: $\beta = -0.62$ [−0.84], SE = 0.18 [0.34], $t = 3.38$ [2.49], $P = 0.028$ [0.067]; $r^2 = 0.78$ [0.78]). The fact that the bootstrapped CIs enclosed zero for both independent variables motivated more detailed analyses. We first tested these regression models using nonparametric randomization tests (based on 2000 randomizations; Manly, 1997), which again yielded incongruent results ($P = 0.039$ [0.123] and $P = 0.055$ [0.136], for testes and body size respectively). Thus, regressions using species-level data consistently showed that relative testes size was positively related to female remating rate, whereas regressions involving PICs failed to reject the null hypothesis. We note that this model was also the only model for which regressions involving PICs and its phylogenetic GLS analogue gave different results: a phylogenetic GLS model showed a significant positive relationship between female remating rate and relative testes size (testis size: $\beta = 0.63$, SE = 0.15, $t = 4.13$, $P = 0.014$; body size: $\beta = -0.50$, SE = 0.12, $t = 4.09$, $P = 0.015$). In summary, species-level data showed a robust positive relationship, but this was only significant when shared ancestry was controlled for using the phylogenetic GLS approach. Because the phylogenetic signals for the traits involved were fairly low, we interpret our data as showing positively correlated evolution of relative testes size and female remating rate.

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Variation in female quality vs. weight of the first ejaculate and ejaculate depletion rate

Variation in female quality was not significantly related to the weight of the first ejaculate (β = 0.001 [0.002], SE = 0.001 [0.002], t = 0.71 [0.75], P = 0.510 [0.488]) nor to the rate of ejaculate depletion (β = −14.4 [−23.4], SE = 25.2 [38.9], t = 0.57 [0.60], P = 0.592 [0.574]). Adding male body weight as an independent variable to these models did not significantly improve either of them (partial F-tests: $F_{1,4}$ = 1.43 [2.53], P = 0.298 [0.187]; $F_{1,4}$ = 1.25 [2.46], P = 0.326 [0.192] respectively). These models, thus, provide no support for a coevolution between variation in female quality and either the weight of the first ejaculate or the rate of ejaculate depletion.

Discussion

Our study documents correlated evolution of ejaculate characteristics, relative male testis size and female remating rate. Most strikingly, we found correlated evolution between ejaculate weight and the rate of ejaculate depletion. This shows that as males evolve to provide a larger first ejaculate, they also evolve a more rapid exhaustion of ejaculate resources over successive matings. This finding confirms an important evolutionary trade-off, assumed in many theoretical models of sperm competition (Parker, 1998) (prediction P1). Although decreased size of successive ejaculates is a common finding in insects at the species level (see Simmons, 2001), we know of no previous comparative data on ejaculate depletion rates across species of any taxa. Because the species included here are closely related (Tuda *et al.*, 2006), our data suggest that ejaculate allocation strategies have evolved quite rapidly in this clade (Rönn *et al.*, 2008). The pattern of correlated evolution also shows that the evolution of relatively larger testes is associated with a heavier first ejaculate and, as a consequence, a more rapid rate of ejaculate depletion.

In general, sperm competition is considered one of the strongest forms of selection causing evolution of reproductive behavior and physiology among males (Birkhead & Møller, 1998; Simmons, 2001). Most models of sperm competition assume that the success of the last male to mate is sizeable and that increased investment in ejaculates leads to higher fertilization success. These assumptions are well supported in seed beetles. Eady (1991) found that the proportion of offspring fertilized by the last male to mate is generally high in *C. maculatus* (P2 = 0.83) and that the main source of variance in fertilization success among males is the number of sperm transferred by the last male (Eady, 1995). Similarly, Boshra (1994), Takakura (2001) and Rugman-Jones & Eady (2007) reported last male sperm precedence in *C. chinensis*, *Bruchidius dorsalis* and *C. subinnotatus* respectively.

Our data strongly suggest that interspecific variation in sperm competition regimes has contributed to the divergent evolution of ejaculate allocation patterns in seed beetles. Increased female remating rate (i.e. increased sperm competition intensity) was associated with a decreased size of the first ejaculate in this clade. This matches an important prediction of the model by Parker & Ball (2005) (prediction P2), as female seed beetles mate multiply (> 3 times in their lifetime) even under conditions where mating opportunities are relatively restricted (Arnqvist *et al.*, 2005; this study). According to their model, ejaculate size should decrease when females mate on average with more than two males. Support for this general prediction has also been found in bushcrickets (Tettigonidae; Vahed, 2006). However, as noted by Vahed (2006), findings of negatively correlated evolution between female remating rates and ejaculate size do not demonstrate that smaller ejaculates result from higher remating rates. The causal relationship could, in fact, be reversed, as would be the case if males suppress female remating by evolving larger ejaculates (see Arnqvist & Rowe, 2005). Further, the negative association could be driven by variation in underlying ecological factors which affect both ejaculate size and remating rate (Williams *et al.*, 2005).

As mentioned above, increased female remating rates should be associated with smaller first ejaculates when associated with increased sperm competition intensity, but this means that males should also evolve a more prudent allocation as a consequence (Wedell *et al.*, 2002). Although we found that increased female remating rate indeed was associated with smaller first ejaculates and larger testes (prediction P2 and P3), it was simultaneously associated with a more rapid depletion of ejaculates over successive matings. The latter finding is seemingly contrary to predictions made by sperm competition theory (prediction P2) and suggests a somewhat more complex coevolutionary pattern. Although the coevolution of the weight of the first ejaculate and the rate of depletion of ejaculate resources is obviously affected by within-male trade-offs (see above), female mating rate seem to coevolve with these male traits in a manner which is largely orthogonal to their internal coevolution. We see two alternative, but not mutually exclusive, explanations for our findings. First, models of sperm competition typically do not allow for allocation to competing demands other than ejaculate traits (e.g. Parker *et al.*, 1996, 1997). It is possible that the relative profitability for males of, for example, allocating resources to mate searching vs. ejaculate production is altered as female remating is elevated. Thus, males may evolve to allocate relatively fewer resources to ejaculate production (resulting in a smaller first ejaculate and more rapid ejaculate depletion) and more to competing demands, such as mate searching, overt male–male competition and/or persistence in mating attempts, as

females evolve to remate more frequently. Trade-offs between ejaculate expenditure on one hand and mate attraction and/or search activity on the other have been documented in other insects (e.g. Simmons *et al.*, 1992; Gage, 1995). In this sense, our results suggest that the predictive power of sperm competition theory may be limited and that complex trade-offs between allocation to various competing demands, as well as evolution of resource acquisition (see below), need to be more explicitly incorporated into such models.

Second, Williams *et al.* (2005) recently showed that evolution of factors unrelated to sexual selection, such as resource availability, could drive concerted evolution of reproductive traits. According to this scenario, reduced resource availability could result in a decreased size of the first ejaculate, accelerated ejaculate depletion rate and increased remating by females. Here, correlated evolution would result from variation in total resource acquisition rather than from evolution of resource allocation between competing demands. We note that seed beetles are capital breeders (adults do not need water or food to reproduce successfully) and the amount of resources gained during larval development thus determine the resources available for allocation later in life. A possible example of how evolution of resource acquisition could generate correlated evolution is based on the fact that seed beetle females seem to benefit from nutrients and/or water received with ejaculates (Fox, 1993; Takakura, 1999), as long as mating rates are not too high (Arnqvist *et al.*, 2005; Rönn *et al.*, 2006), and that females in a sense sometimes 'forage' for matings (Takakura, 2004; Edvardsson, 2007). If males evolve to utilize their resources more efficiently, they could afford to produce both larger first ejaculates and to deliver ejaculates at a decelerated depletion rate (Williams *et al.*, 2005). This may then lead to a lower female remating rate, as females secure more ejaculate resources per mating. This scenario is, at least in part, supported in a companion paper (Rönn *et al.*, 2008) where we show that female fitness is indeed generally compromised when females receive smaller amounts of ejaculate resources (see also Savalli & Fox, 1999).

The models of Galvani & Johnstone (1998) and Reinhold *et al.* (2002) both predict that males should evolve to allocate ejaculate resources more prudently in the face of increased variance in female quality (i.e. fecundity) (prediction P4). We failed to find any evidence for males allocating their ejaculates more equally over matings in species with a larger variation in female quality or in species with a stronger relationship between female size and fecundity. Several within-species studies have shown that males are able to tailor their ejaculates in response to variation in female quality (see Wedell *et al.*, 2002) but our study is, to our knowledge, the first assessment of this hypothesis using a comparative framework.

One may ask whether our experiments accurately quantify variation in female remating rate across species under more natural conditions. There are at least three reasons to believe that this is the case. First, in seed beetles, the laboratory environment is generally considered a good approximation of more natural environments, such as storage sites for cultivated legumes (Messina, 1991). Second, female remating rates as measured in our experiments differ significantly across species. Third, and more importantly, the fact that female remating showed correlated evolution with relative testis size suggests that our measure is biologically relevant. Associations between relative testes size and sperm competition intensity has been documented in several vertebrate and insect taxa (e.g. Karlsson, 1995; Birkhead & Møller, 1998; Hosken & Ward, 2001). However, in insects, this association may sometimes be blurred as a result of variation in sperm competition mechanisms (Simmons & Siva-Jothy, 1998; Simmons, 2001).

The two complementary levels of analysis used here (species-level data and analyses taking shared ancestry into account) gave almost completely congruent results, despite the presence of a fairly strong phylogenetic signal in parts of our data (Losos, 1999; Blomberg *et al.*, 2003). This fact adds significant weight to our main findings, as they apparently did not rely either on a particular phylogenetic hypothesis or on a particular distribution of species-level data (Carvalho *et al.*, 2006). We conclude that our analyses at both levels revealed a tightly correlated evolution between ejaculate size and ejaculate depletion rate, and gave evidence for coevolution between the pattern of ejaculate allocation and female remating rate. However, our results also illustrate the fact that comparative analyses, even when based on experimental data, suffer from limitations in terms of determining the causation of coevolutionary patterns (e.g. Martins, 2000). Further disentangling causal relationships between the concerted evolution of ejaculate weight, ejaculate depletion rate, female remating rate and testis size will prove a challenge (Williams *et al.*, 2005) that may require the employment of controlled selection experiments.

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Appendix

Mean values (SE; *N*) for all traits and species.

	Callosobruchus analis	*Callosobruchus rhodesianus*	*Callosobruchus maculatus*	*Callosobruchus subinnotatus*	*Callosobruchus phaseoli*	*Callosobruchus chinensis*	*Zabrotes subfasciatus*
Weight of the first ejaculate (μg)	213.4 (11.0; 20)	220.0 (16.0; 20)	203.1 (13.7; 18)	180.9 (16.0; 21)	45.5 (9.0; 22)	35.8 (7.0; 20)	17.2 (7.0; 20)
Ejaculate depletion rate	−0.012	−0.013	−0.013	−0.012	−0.002	−0.003	−0.003
Female remating rate	1.21 (0.11; 14)	1.31 (0.24; 13)	3.07 (0.32; 15)	2.0 (0.28; 15)	0.27 (0.12; 15)	1.07 (0.15; 14)	1.73 (0.12; 15)
Testes size	1.16 (0.08; 9)	1.16 (0.04; 7)	1.17 (0.09; 9)	2.95 (0.11; 7)	0.66 (0.04; 8)	0.46 (0.03; 8)	0.36 (0.02; 7)
Male elytra length (mm)	1.91 (0.03; 9)	1.88 (0.03; 7)	1.81 (0.04; 9)	2.69 (0.02; 7)	2.10 (0.02; 8)	1.63 (0.02; 8)	1.36 (0.02; 7)
Variation in female fecundity (CV)	0.25	0.16	0.14	0.63	0.27	0.29	0.27
Correlation between female fecundity and female body weight	0.67	0.83	0.88	0.33	0.85	0.07	0.69
Correlation between female fecundity and female elytra length	0.35	0.34	0.01	0.15	0.71	0.15	0.40