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Interspecific variation in ejaculate allocation and associated effects on female fitness in seed beetles

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Abstract

When ejaculates are costly to produce, males are expected to allocate their ejaculate resources over successive matings in a manner that optimizes their reproductive success and this may have important consequences for their mates. In seed beetles (Coleoptera; Bruchidae), ejaculates vary in size across species from weighing less than 1%, up to as much as 8%, of male body weight. Ejaculates contain not only sperm but also a range of additional substances and females in some species gain benefits from receiving large ejaculates. Male ejaculate allocation may thus affect female fitness. Here, we first characterized the pattern of male ejaculate allocation over successive matings in seven-seed beetle species. We then assessed how this allocation affected female fitness in each species. Although females generally benefited from receiving large ejaculates, the interspecific variation observed both in ejaculate allocation patterns and in their effects on female fitness was remarkably large considering that the species studied are closely related. Our analyses suggest that variation in ejaculate composition is the key, both within and across species. We discuss possible causes for this variation and conclude that coevolution between male ejaculates and female utilization of ejaculate substances has apparently been rapid in this clade.

Introduction

Sperm competition occurs when sperm from two or more males compete over the fertilization of a given set of ova (Parker, 1998), and sperm competition theory predicts that several factors should affect the evolution of the mean amount of resources that males invest in sperm and ejaculate production (Engqvist & Reinhold, 2005). A key factor is clearly the sperm competition regime, in particular the sperm competition risk (i.e. the probability of female remating) and the intensity of sperm competition (i.e. the average number of ejaculates competing for a given set of ova). For example, Parker *et al.* (1996) modelled systems where male fertilization success is directly proportional to its contribution to the total number of competing sperm (i.e. a fair raffle), and showed that total ejaculate expenditure should increase with increasing sperm competition intensity. A subsequent model by Parker *et al.* (1997), addressing situations where male fertilization success depends on mating order (i.e. a loaded raffle), also predicted that ejaculate expenditure should increase with increasing sperm competition risk in most cases. Engqvist & Reinhold (2006) corroborated these predictions, but showed that this will depend on the degree of last male sperm precedence in a given taxa. Empirical support for these predictions comes from, for example, comparative studies across species showing correlated evolution between testis size and female remating rate (see Parker *et al.*, 1997; Simmons, 2001) and artificial selection experiments (Hosken & Ward, 2001; Pitnick *et al.*, 2001).

By tradition, males have been considered to have a more or less unlimited supply of sperm and the only limiting factor for male fitness was considered to be the number of mates (Bateman, 1948). This view has, however, gradually shifted. Many experimental studies have shown that sperm and ejaculate production costs can be considerable (Dewsbury, 1982; Nakatsumu & Kramer, 1982; Van Voorhies, 1992; Paukku & Kotiaho,

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2005) and that males may face ejaculate/sperm depletion (Arnqvist & Danielsson, 1999; Preston *et al.*, 2001; Ambriz *et al.*, 2002; Rigaud & Moreau, 2004; Damiens & Boivin, 2006). Given that the total amount of ejaculate resources is limited, males should evolve to allocate their ejaculate resources strategically across different females simply because a male's ejaculate allocation should be the key to its reproductive success (Dewsbury, 1982; Birkhead & Møller, 1998; Bonduriansky, 2001; Engqvist & Sauer, 2001; Wedell *et al.*, 2002). In line with this, many empirical studies have indeed shown that ejaculate allocation patterns are markedly plastic within species (Eady, 1995; Ofuya, 1995; Engqvist & Sauer, 2001; Wedell *et al.*, 2002). Parker *et al.* (1996) showed that sperm competition intensity is important: males should allocate most resources in matings where the perceived intensity of sperm competition is intermediate, such that maximal allocation is observed when the number of competing males equals two (see also Engqvist & Reinhold, 2005; Parker & Ball, 2005). Experimental support for this comes from studies showing that male ejaculate allocation depends on perceived sperm competition risk and intensity (Wedell, 1992; Wedell & Cook, 1999; Siva-Jothy & Stutt, 2003; Simmons *et al.*, 2007; Thomas & Simmons, 2007).

Reinhold *et al.* (2002) extended previous models of strategic ejaculate allocation by incorporating variation in female fecundity within species (see also Galvani & Johnstone, 1998). They showed, for example, that males should invest more in early copulations compared with later ones simply because the probability of attaining additional matings may decrease over time. They also predicted that males should conserve ejaculate resources for later copulations, and hence allocate more prudently over successive copulations, when variation in female quality/fecundity is larger. Males are, indeed, known to adjust sperm number or ejaculate size according to factors that correlate with female fecundity in some taxa (e.g. Engqvist & Sauer, 2001; Mallard & Barnard, 2003). Comparative studies that directly address the evolution of ejaculate allocation in butterflies have shown that the pattern of ejaculate allocation varies across species and that increased sperm competition is associated with a more even allocation of ejaculates over successive matings (Svärd & Wiklund, 1989; Bissoondath & Wiklund, 1996).

However, we note that an understanding of strategic ejaculate allocation patterns based solely on sperm competition effects may well be incomplete. In taxa where the size of the ejaculate, or the number of sperm, directly affects female fitness, male ejaculate allocation will have important effects on female lifetime reproductive success (Arnqvist & Nilsson, 2000). Such feedback effects should have important consequences for selection on male ejaculate allocation, simply because male reproductive success will be affected if its mate suffers a shortage of viable sperm (see Ridley, 1988) or an insufficient amount of ejaculate resources (e.g. Vahed, 1998) as a result of prudent ejaculate allocation. It is currently difficult to assess how general such effects may be, as there are very few systematic studies of the extent to which female reproductive success depends on male ejaculate allocation (Wiklund *et al.*, 1993; Wedell, 1996; Torres-Vila & Jennions, 2005).

This study forms a part of an integrative comparative study of mating system evolution in seed beetles (Rönn *et al.*, 2006, 2007; Katvala *et al.*, 2008). The goal of the current contribution is twofold. First, we assess ejaculate allocation over successive matings in seven related seed beetles (Tuda *et al.*, 2006): six congeneric *Callosobruchus* species and one member of the genus *Zabrotes*. Female remating rate varies across these species (Katvala *et al.*, 2008), but they all share a common basic mating system (i.e. polyandry; Miyatake & Matsumura, 2004; Arnqvist *et al.*, 2005; Katvala *et al.*, 2008), are ecologically similar (Southgate, 1979; Labeyrie, 1981; Fujii *et al.*, 1989) and show last male sperm precedence (Eady, 1991; Boshra, 1994; Takakura, 2001; Rugman-Jones & Eady, 2007). However, average ejaculate weight ranges from weighing less than 1% of male body weight up to as much as 8% of male body weight (Savalli & Fox, 1998 and below) and there is a striking interspecific variation in the economics of mating and reproduction (Rönn *et al.*, 2006, 2007). We, thus, predict that there is interspecific variation in the pattern of ejaculate allocation over successive matings. The first aim of these experiments was to characterize such variation. Second, we determine the effects of ejaculate allocation on a number of female fitness components in these species. A previous study showed that the effects of a single mating on female lifespan differed markedly across species (Rönn *et al.*, 2006). Adults of these species do not need to feed to reproduce successfully (Wightman, 1978; Savalli & Fox, 1999) and ejaculate size may therefore affect female fitness to a great extent. We therefore predict that patterns of male ejaculate allocation should affect female fitness. Here, our aim was to examine this hypothesis and assess whether such effects vary across these species. In a companion paper, we include these data as part of a larger data set in a series of phylogenetic comparative analyses of the extent to which male ejaculate allocation patterns have coevolved with testis size and female mating rate in this clade (Katvala *et al.*, 2008).

Methods

Species and rearing

We used six species of *Callosobruchus* seed beetles in our experiments: *C. maculatus* [PC], *C. subinnotatus* [RS], *C. analis* [RS], *C. rhodesianus* [RS], *C. chinensis* [RS] and *C. phaseoli* [YT] and one outgroup species: *Zabrotes subfasciatus* [PC] (stocks provided by PC: Peter Credland, University of London; RS: Robert Smith, University of

Leicester; YT: Yukihiko Toquenaga, University of Tsukuba). *Callosobruchus maculatus, C. subinnotatus* and *C. rhodesianus* were reared on cowpea beans (*Vigna unguiculata*), *C. chinensis* on adzuki beans (*Vigna angularis*) and *C. analis* and *C. phaseoli* on mung beans (*Phaseolus aureus*). *Zabrotes subfasciatus* was reared on white beans (*Phaseolus vulgaris*). The generation time in the laboratory is approximately 3–4 weeks for all species. Females cement their eggs to the surface of beans and the larvae bore themselves into the bean where they go through several larval instars before pupating (Southgate, 1979). As adults, the beetles require neither food nor water to reproduce successfully (Wightman, 1978; Savalli & Fox, 1999). All beetles were maintained under controlled laboratory conditions at 28°C and 55% (±10%) RH with a 12 h : 12 h light : dark cycle.

Experiments

Virgin beetles were obtained by isolating single beans infested with eggs, collecting individual beetles as they hatched, and keeping them isolated until the onset of the experiment. For each species, we collected 20 virgin males and 100 virgin females. Each male was then mated to five females in succession, with 2 h between successive matings. All individuals were between 24 and 48 h post-hatching when mated. Matings were performed under standard maintenance conditions (see above). The duration of every mating was recorded to the nearest second. Each male was weighed twice before and twice after each mating to the nearest 10^{-5} g, using a Sartorius® ME/SE analytical balance (Sartorius AG, Goettingen, Germany), and mean body weight before and after mating was calculated. We used the difference in body weight before and after mating as our measure of ejaculate weight. This method provides reliable estimates of ejaculate size in these beetles, as evidenced by correlations between male weight loss and female weight gain during copulations (Edvardsson & Tregenza, 2005). We note that in one of the species included (*C. subinnotatus*), a very low male remating propensity precluded five successive matings and our data for this species are thus restricted to three successive matings.

We used the length of the right elytra as a measure of body size (Wilson & Hill, 1989) for use in subsequent statistical analyses. This was measured by means of a digitizing tablet (Summsketch III™; Summagraphics Corp., Austin, TX, USA) placed under a dissecting microscope provided with a camera lucida (Leica® MZ8; Leica Microsystems GmbH, Wetzlar, Germany). After mating, females were isolated individually in Petri dishes (∅ 8 cm) containing 100 beans for egg laying (see above for host species). Petri dishes were checked daily around 12:00 hours and female lifespan (number of days) was determined. Seven days after the death of a given female, the number of eggs laid was counted and hatching rate of eggs was determined by dividing the number of eggs that hatched with the total number of eggs laid. Petri dishes were then kept in incubators (standard maintenance conditions; see above) to allow juvenile development. Approximately 5 weeks after a given female was introduced to a Petri dish, its entire hatched adult offspring were counted and juvenile survival was determined by dividing the number of eclosed adult offspring with the number of hatched eggs. Thus, five different female fitness components were measured for each female in our experiment: female lifespan, lifetime egg production, egg hatching rate, juvenile survival and lifetime offspring production.

Statistical analysis

We used univariate and multivariate analyses of covariance (ANCOVA and MANCOVA) to analyse our data. Body size, standardized within species to a mean of zero and a standard deviation of one to provide comparable effect sizes across species, was included as a continuous covariate in a subset of our models. Data for a single deviant male were excluded from the final analyses. Normality of residuals was assessed with Shapiro–Wilks tests. All analyses were performed using SYSTAT® 11 (Systat Software Inc., San Jose, CA, USA).

Results

We first analysed data from all species in a joint ANCOVA to assess differences in ejaculate allocation between species. Using ejaculate weight as a response variable, this model contained species as a fixed effect factor, mating number (i.e. whether a mating was a male's first, second, third, fourth or fifth mating), the squared term for mating number, the interaction between species and mating number, the interaction between species and the squared term for mating number and male identity as a random effects factor nested within species. This analysis revealed highly significant main effects of both species and mating number and its quadratic term on ejaculate weight ($F_{6,408} = 37.643$, $P < 0.001$, $F_{1,408} = 27.849$, $P < 0.001$ and $F_{1,408} = 6.449$, $P < 0.011$ respectively). Thus, ejaculate weight differed across species and generally decreased with mating number, although this decrease tended to be nonlinear over successive matings. The interaction terms between species and mating number ($F_{6,408} = 9.980$, $P < 0.001$) and between species and the squared term for mating number ($F_{6,408} = 4.812$, $P < 0.001$) were also highly significant, showing that species differ both in terms of the extent to which ejaculate weight decreases over successive matings and in the precise shape of this relationship (Fig. 1). We note that individual males within species also differed in ejaculate weight ($F_{137,408} = 1.267$, $P = 0.040$) and that the effect of species on ejaculate depletion rate was not caused by *Zabrotes* being different from *Callosobruchus*,

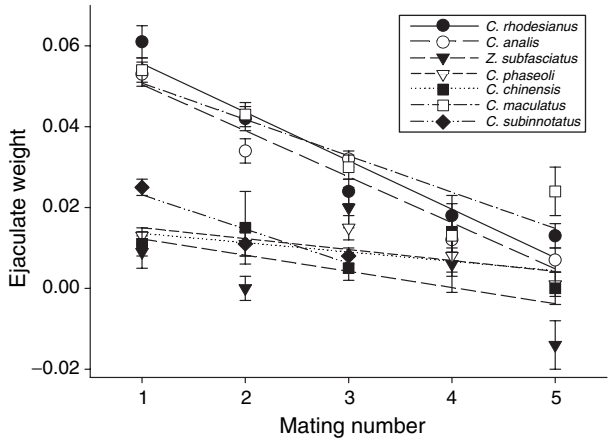

Fig. 1 Ejaculate allocation over successive matings. Figure shows mean (±SE) relative ejaculate weight for all species (i.e. ejaculate weight at each mating divided by male weight before that mating) and lines represent linear regressions.

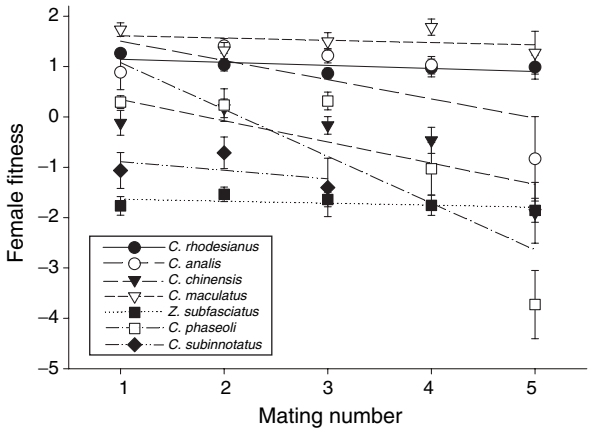

Fig. 2 The relationship between male mating number and mean female fitness (±SE) across all species. Female fitness represents score along the first principal component from a common principal component analysis of all five female fitness components measured. This composite measure of female fitness correlates well with lifetime offspring production ($r = 0.91$). Lines represent separate linear regressions for each species.

as depletion rate in *Z. subfasciatus* fell within the range of rates observed across species in *Callosobruchus* (Fig. 1).

Following this global model, we ran separate ANCOVAS for each species to further characterize interspecific variation, including also female size (see Table 1). These analyses showed that mating number had a significant effect on ejaculate weight in three (*C. maculatus, C. rhodesianus* and *C. analis*) of seven species. In *C. chinensis*, *C. phaseoli*, *C. subinnotatus* and *Z. subfasciatus,* none of the factors included in our model had significant effects on ejaculate weight. *Callosobruchus maculatus* and *C. analis* showed a similar pattern where ejaculate weight decreased over successive matings, but in a linear fashion. In *C. rhodesianus*, all factors showed significant effects on ejaculate weight. We also tested whether mating duration had additional affects on ejaculate weight, by inclusion in the inferential models. However, mating duration did not significantly affect ejaculate weight in any of our species (range of $P = 0.056$–0.861).

To test for global effects of our variables on female fitness, we first pooled data for all species in a common MANCOVA where all five female fitness components (see above) were treated as the response variables. Analogous to the global model of ejaculate allocation above, this MANCOVA model contained effects of species, mating number, the squared term for mating number, the interaction between species and mating number, the interaction between species and the squared term for mating number and male identity nested within species. This MANCOVA revealed highly significant effects of all included factors on female fitness ($P < 0.001$), showing that mating number generally affects female fitness and that such effects differ across species (Fig. 2). Again, the species effect seen was not caused by *Z. subfasciatus*, as this species was not markedly different from the *Callosobruchus* species. We then performed separate MANCOVAS for each species, where we also included female size as a covariate (Table 2). These analyses showed remarkably strong effects of mating number, widely defined to include also its quadratic term and its interaction with male identity, on female fitness in all *Callosobruchus* species except *C. maculatus*. Notably, the size of the effect of mating number on male ejaculate weight was not correlated with the size of the effect of mating number of female fitness across species (Spearman rank correlation of *F*-values for the linear term in both cases; $r_s = -0.50$, $P > 0.25$).

Table 1 Results of analyses of covariance of the effect of mating number on ejaculate weight for each species.

Source	*Callosobruchus maculatus*	*Callosobruchus chinensis*	*Callosobruchus rhodesianus*	*Callosobruchus analis*	*Callosobruchus phaseoli*	*Callosobruchus subinnotatus*	*Zabrotes subfasciatus*
Male	$1.38_{17,28}$	$0.82_{15,42}$	$\mathbf{5.35_{16,32}}^{***}$	$0.87_{19,52}$	$0.88_{19,51}$	$2.08_{19,4}$	$0.49_{19,51}$
Mating number	$\mathbf{11.52_{1,28}}^{**}$	$0.03_{1,42}$	$\mathbf{54.23_{1,32}}^{***}$	$\mathbf{14.22_{1,52}}^{***}$	$0.15_{1,51}$	$0.79_{1,4}$	$1.22_{1,51}$
Mating no. × mating no.	$2.87_{1,28}$	$0.01_{1,42}$	$\mathbf{16.62_{1,32}}^{***}$	$2.79_{1,52}$	$0.47_{1,51}$	$0.73_{1,4}$	$2.88_{1,51}$
Male × mating no.	$1.35_{17,28}$	$0.70_{15,42}$	$\mathbf{4.22_{16,32}}^{***}$	$0.68_{19,52}$	$1.16_{19,51}$	$2.91_{19,4}$	$0.65_{19,51}$
Female size	$0.44_{1,28}$	$0.39_{1,42}$	$\mathbf{13.60_{1,32}}^{***}$	$3.09_{1,52}$	$0.07_{1,51}$	$0.05_{1,4}$	$0.40_{1,51}$

Given are *F*-values and degrees of freedom (bold indicates statistical significance: $^{**}P < 0.01$; $^{***}P < 0.001$).

Table 2 Results of multivariate analyses of covariance of the effect of mating number on five female fitness components for each species.

Source	*Callosobruchus maculatus*	*Callosobruchus chinensis*	*Callosobruchus rhodesianus*	*Callosobruchus analis*	*Callosobruchus phaseoli*	*Callosobruchus subinnotatus*	*Zabrotes subfasciatus*
A: Male	**$3.915_{85,120}$*****	**$1.560_{85,125}$***	$1.262_{95,165}$	$1.145_{95,213}$	$0.765_{100,233}$	**$2.483_{95,53}$*****	$0.933_{95,233}$
B: Mating number	$0.752_{5,24}$	**$3.756_{5,25}$***	$1.314_{5,33}$	**$2.691_{5,43}$***	**$4.576_{5,47}$****	**$4.750_{5,10}$****	$1.347_{5,45}$
C: Mating no. × mating no.	$0.875_{5,24}$	**$6.852_{5,25}$*****	$2.022_{5,33}$	**$3.892_{5,43}$****	**$7.849_{5,47}$*****	**$4.744_{5,10}$****	$1.839_{5,45}$
D: Male × mating no.	$0.840_{85,120}$	**$1.881_{85,125}$*****	**$1.489_{95,165}$***	**$1.439_{95,213}$***	$1.305_{100,233}$	–	$1.193_{95,223}$
E: Female size	$2.524_{5,24}$	$0.372_{5,25}$	**$5.194_{5,33}$*****	**$3.335_{5,43}$***	**$4.608_{5,47}$****	$1.189_{5,10}$	$0.655_{5,45}$
Results of univariate ANCOVAS	A affects: Hatching rate, Juv. survival	A affects: no. of eggs Hatching rate, Juv. survival, no. offspring B affects: no. of eggs No. offspring C affects: no. of eggs Lifespan D affects: no. of eggs Hatching rate Juv. survival No. offspring	E affects: no. of eggs D affects: Juv. survival†	B affects: no. of eggs No. offspring C affects: no. of eggs No. offspring Female lifespan D affects: Hatching rate No. offspring Juv. Survival Female lifespan E affects: no. of eggs No. offspring	B affects: no. of eggs Hatching rate No. offspring Female lifespan C affects: no. of eggs Hatching rate No. offspring Juv. survival Female lifespan E affects: no. of eggs Hatching rate Female lifespan	A affects: no. of eggs Female lifespan Juv. survival B affects: Female lifespan C affects: Female lifespan	

Given are *F*-values (from Wilk's λ) and degrees of freedom (bold indicates statistical significance: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). We also give the results of univariate ANCOVAS (lower part of table), listing the female fitness components that were significantly (at $\alpha = 0.05$) affected by any of the factors included in the model.
†$P = 0.071$.

To interpret these multivariate effects, we lean on the results of univariate ANCOVAS (see Table 2). In general, there were striking differences between species with regard to the size of the effect of mating number on female fitness. Whereas four species showed strong effects, one showed only effects that differed across males and two showed no significant effects. Further, mating number (and its squared term) primarily had effects on female productivity, affecting female lifespan, lifetime egg production and lifetime offspring production. Lifetime offspring production is our most integrative measure of female fitness, and we note that this was significantly affected by mating number in three species. The individual males used in this experiment differed in terms of their effects on female fitness in five of seven species, either through a main effect or through an interaction between male identity and mating number. In contrast to mating number, male identity also affected measures of offspring performance, such as juvenile survival and hatching rate of eggs (see Table 2).

In an attempt to disentangle the general and independent effects on female fitness of ejaculate weight on one hand and ejaculate composition on the other, we modelled female lifetime offspring production in an ANCOVA using species and male identity nested within species as factors and ejaculate weight, mating number and female size as covariates. The focal variables in this analysis were ejaculate weight and mating number. Mating number had a very strong effect on female fitness ($F_{1,425} = 64.52$, $P < 0.001$), whereas ejaculate weight did not ($F_{1,425} = 0.09$, $P = 0.76$). Inclusion of the full set of interactions in this model did not affect this overall result.

Discussion

The seven-seed beetle species included in this study are ecologically relatively similar and share a common mating system: all species live in arid environments, exhibit multivoltine lifecycles and lay their eggs on legumes where the larvae develop inside the seed (Southgate, 1979; Labeyrie, 1981; Fujii *et al.*, 1989) and all exhibit varying degrees of polyandry (Miyatake & Matsumura, 2004; Arnqvist *et al.*, 2005; Rönn *et al.*, 2006; Katvala *et al.*, 2008). Despite these similarities, we found remarkably high levels of interspecific variation in ejaculate allocation patterns and the effects that these patterns had on female fitness. This shows that these species have evolved distinct ways of allocating their ejaculates over successive matings. With regard to the

effects of ejaculate allocation on female fitness, females generally benefited from mating early in the succession although ejaculate allocation affected different female fitness components in different species. Here, we first discuss the underlying factors for variation in ejaculate allocation and the rate of ejaculate depletion across species and, second, how this variation affected female fitness. We also discuss how these results can shed light on the evolution of ejaculate size. Finally, we discuss some of the implications our results have for our understanding male–female coevolution in this model system.

In general, we found that ejaculate weight decreases over successive matings. This is a common finding in insects (e.g. Eady, 1995; Lauer, 1996; Lewis, 2004; Damiens & Boivin, 2006) which has also been documented previously in one of the species included here, *C. maculatus* (Savalli & Fox, 1999). However, the seed beetle species included in this study clearly allocates their ejaculate resources differently over successive matings. In some species, males transfer a large ejaculate in the first mating followed by a rapid decrease in ejaculate weight. In others, males transfer a more similar sized ejaculate over successive matings (see Fig. 1). We note that this grouping does not entirely reflect shared ancestry or host plant species. For example, although *C. chinensis* and *C. phaseoli* share a similar pattern of ejaculate allocation, they are the most distant relatives within this genus (Tuda *et al.*, 2006). Similarly, seed beetle species using *V. unguiculata* as a host are found in both groups and the same is true for those using *P. aureus* as a host. Hence, our data indicate that the ejaculate allocation patterns have evolved quite rapidly within this clade, a conclusion supported by the phylogenetic comparative analyses in Katvala *et al.* (2008).

Ejaculate allocation

The potential costs and benefits of ejaculate allocation have been investigated both empirically and theoretically. Three factors have been previously implicated to explain why ejaculate weight decreases over successive matings. First, ejaculate allocation may simply reflect constraints in ejaculate production rate (Arnqvist & Danielsson, 1999; Moore *et al.*, 2004). Although a range of costs must constrain ejaculate production in some sense, and thus potentially contribute to the basic pattern of decreased ejaculate weight over successive matings, it cannot fully account for the patterns seen in this study. If species were constrained in how males allocate their ejaculate resources, we would expect to see a more uniform pattern of allocation across species. The fact that ejaculate allocation differs across species implies that selection has played a role during this evolution (see Fig. 1). Although the weight of the first ejaculate and the rate by which ejaculate weight decreases over successive matings show correlated evolution in this clade (Katvala *et al.*, 2008), this does not preclude the possibility that ejaculate allocation *per se* is adaptive. The total amount of resources allocated to ejaculate production clearly differs across species (see Fig. 1) and, more importantly, the phylogenetic comparative analyses of Katvala *et al.* (2008) reveal coevolution between ejaculate allocation and female remating rate that is independent of ejaculate weight.

Second, theory suggests that variation in female fecundity may affect the evolution of male ejaculate allocation (see Reinhold *et al.*, 2002). Here, predictability of female fecundity over time is the key. If females with high and low residual fecundity are always present in a population, as would be the case if generations are overlapping and females of different ages thus co-occurring, this should favour prudence and males should allocate a more equal amount of ejaculate resources over successive matings. By contrast, if females with high residual fecundity dominate early in males' life and females with low residual fecundity later, as would be the case if generations are discrete and young females dominate early in each generation, the amount of ejaculate resources should decrease more rapidly over time. The species included in this study are reared under discrete generations in the laboratory, a scenario that probably also reflects their natural conditions (Southgate, 1979). Thus, variation in female fecundity may have contributed to the pattern of decreased ejaculate weight over successive matings. We note that empirical evidence suggesting that males indeed allocate ejaculates according to variation in female fecundity comes from studies showing effects of female body size on ejaculate size (Wedell, 1992; Simmons *et al.*, 1993; Engqvist & Sauer, 2001; Mallard & Barnard, 2003). Female size is often a good predictor of fecundity in insects (Gage, 1998; Wedell, 1998; Wedell & Cook, 1999). As pointed out by Galvani & Johnstone (1998), this could be seen as cryptic male choice for females of higher reproductive value. We did not find strong support for this in our experiment, as males did not generally seem to allocate their ejaculate according to female size. One exception is *C. rhodesianus*, where males allocated larger ejaculates to larger females. Interestingly enough, this is also the species that shows the strongest relationship between female size and fecundity.

Third, male ejaculate weight may decrease over successive matings as a result of changes in sperm competition intensities. Most importantly, matings early in life might be more profitable for males simply because the proportion of virgin females may be higher and the degree of sperm competition lower (Parker *et al.*, 1996, 1997; Galvani & Johnstone, 1998; Simmons, 2001). Further, males are predicted to invest more ejaculate in early copulations than in later ones if the probability of achieving additional matings decreases over time (Reinhold *et al.*, 2002). In *Callosobruchus* seed beetles with discrete generations, the frequency of virgin females decreases with time. Thus, sperm competition intensity

will clearly increase, and the probability of achieving additional matings will decrease, over time as already mated females are much more reluctant to mate compared with virgins (personal observation, but see also Eady, 1995). The general pattern of ejaculate allocation seen among our species is thus in agreement with predictions from sperm competition theory.

It is important to note that sperm competition may shape the pattern of ejaculate allocation in ways other than those deriving from variation in the number of sperm transferred. For example, males may benefit from transferring a large ejaculate to females because large ejaculates delay female remating (Eady, 1995; Savalli & Fox, 1999), thereby serving a defensive function in sperm competition. Similarly, large ejaculates may also represent offensive adaptations to sperm competition, because they may aid in removing previously stored sperm (Eady, 1995). Paternal investment may also select for larger ejaculates (Thornhill, 1976; but see Wickler, 1985; Takakura, 2004), and studies of one of the seed beetle species included in this experiment (*C. maculatus*) have indicated that females may be able to metabolize either nutrients (Fox, 1993) and/or water (Arnqvist *et al.*, 2005; Edvardsson, 2007) in ejaculates received and that they benefit from doing so.

In conclusion, the general pattern of ejaculate allocation documented here is probably an adaptation to decreased female fecundities over time and/or to an increase in sperm competition intensity with time. The results of an accompanying study (Katvala *et al.*, 2008), however, suggest that sperm competition intensity is a better predictor of evolution of male ejaculate allocation than is variation in female fecundity.

Female fitness

Female seed beetles are likely to be affected by the ejaculate, as they are adapted to arid conditions (Tuda *et al.*, 2006) and do not need to feed or drink to reproduce successfully (see Labeyrie, 1981). In a previous study, we showed that the cost of mating differs across these species (Rönn *et al.*, 2006) and we suggested that this variation may in part be a result of differences in ejaculate size and/or content or in female ability to metabolize components in the ejaculate. Insect ejaculates may also contain toxic seminal fluids (Chapman *et al.*, 1995). In the seed beetle *Acanthoscelides obtectus*, the ejaculate transferred to females has been shown to contain toxic compounds that affect female fitness negatively (Das *et al.*, 1980). Thus, ejaculates can clearly have a variety of effects, both positive and negative, on female fitness.

Our results show that female fitness generally decreases with the number of times that its mate has mated previously. Moreover, there is no apparent association between host plant species and the presence of such effects, as seed beetles using *V. unguiculata*, *V. angularis* and *P. aureus* all show a decrease in female fitness with mating number. Because ejaculate weight decreases over successive matings, one might conclude that the decreasing size of the ejaculate is causing these effects. Yet, the effects on female fitness may be caused by differences either in the size and/or in the composition of the ejaculate. Our results suggest that it is actually general differences in the composition rather than the size of the ejaculate that play the largest role. First, the general effect of mating number seen on female fitness was not primarily caused by variance in ejaculate weight, suggesting that changes in ejaculate composition over successive matings is important. Second, we found no correlation across species between how rapidly ejaculate weight drops and how rapidly female fitness decreases over successive matings: the species in which ejaculate size decreased most rapidly (i.e. *C. rhodesianus*, *C. analis* and *C. maculatus*) were actually among the species where female fitness was least affected by mating number (see Fig. 2). Third, the fact that species differed to such a great extent with regard to which female fitness components that were affected by male mating history also suggests that changes in ejaculate composition over successive matings is the key. Most species showed effects primarily on female fecundity and/or female lifespan, but others also showed effects on hatching rate of eggs and/or juvenile survival (Table 2). As a caveat, we note that females of different species may also vary in how they metabolize and/or utilize the ejaculate.

The finding that females need to secure large enough amounts of ejaculate to maximize their fitness also has implications for the evolution of female mating rates. Our results show that males continue to mate with virgin females even beyond the point where male ejaculate depletion results in female fitness being compromised. In an experiment involving more severe ejaculate depletion, Ofuya (1995) showed that the fecundity of *C. maculatus* females was significantly dependent on the number of previous matings that the male had performed that day, presumably because of a lack of ejaculate substances. We show here that this can incur direct fitness costs for females (see also Hunter *et al.*, 1993; Pérez-Staples & Aluja, 2004; Damiens & Boivin, 2006) and may thus lead to sexual conflict over mating decisions. As a response, females may increase the rate of remating and/or discriminate against males who are ejaculate depleted (Savalli & Fox, 1999). The latter has been shown in, for example, cockroaches, where females discriminate against males who have mated multiply (Harris & Moore, 2005). These results, thus, reverberate the view that direct effects are important in shaping female mating patterns (Thornhill & Alcock, 1983; Arnqvist & Nilsson, 2000). It is worth noting that in one of the species studied here (*C. phaseoli*), male ejaculate depletion significantly affected both hatching rate of eggs and subsequent juvenile survival. These female fitness components can be considered either

direct or indirect effects, but are often thought to be affected by male genetic quality and/or genetic compatibility. Yet, the effects of mating number in *C. phaseoli* must, of course, be caused by phenotypic attributes of the ejaculate via maternal or paternal environmental effects.

In three of the species studied here (*C. maculatus*, *C. chinensis* and *C. subinnotatus*), we also found main effects of male identity on female fitness (see 'A' in Table 2). This could be because of males differing in genetic quality and/or in the direct effects they have on females. Although we cannot disentangle the relative importance of these two sources of variance given our data, the fact that males to a large extent varied in the effect they had on hatching rate and juvenile survival at least suggests that there is genetic variation in male quality (*sensu* Hunt *et al.*, 2004) in our laboratory populations.

In conclusion, whereas previous studies of ejaculate allocation have focussed on intraspecific variation, we are concerned with interspecific variation within a group of closely related species. We found striking interspecific variation in the pattern of ejaculate allocation over successive matings. Ejaculate allocation also had effects on female fitness, but the magnitude and type of these effects varied across species. Our experimental data thus show that ejaculate characteristics and the effects that these have on female fitness components can evolve rapidly. In a companion contribution, we analyse these results further in a phylogenetic comparative framework to gain insights into the coevolution of male ejaculate allocation and female remating rate (Katvala *et al.*, 2008).

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