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The costs of mating and egg production in *Callosobruchus* seed beetles

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The net cost of reproduction and the trade-off between reproduction and life span are affected by many male and female adaptations. Because several of these adaptations are sexually selected, we expect the cost of reproduction to be affected by sexual selection. For example, traits favoured in males by sexual selection may increase costs of mating for females. We conducted a series of experiments where we independently varied female exposure to males and access to oviposition substrates in six congeneric seed beetle species (*Callosobruchus* spp.). These experiments allowed us to partition the cost of reproduction for females into the cost of mating and the cost of egg production. The costs and benefits of a single mating in terms of effects on female life span varied markedly between species. In some species, females lived for longer after mating once but others had a shortened life span. Lifelong cohabitation with males resulted in a shortened life span for females of all species, but the extent to which cohabitation reduced female life span varied between species. We also found partial support for a depressed lifetime egg production as a result of cohabitation with males. Collectively, our results show that the costs and benefits of mating vary markedly within this clade of closely related and ecologically uniform species. We conclude that key traits, which influence the economics of sexual interactions and reproduction, have evolved rapidly in this model system.

Experimental data on the economics of reproduction are crucial for an understanding of the interactions between the sexes and, in particular, for understanding male–female coevolution. The costs of reproduction include the cost of mating itself and the costs of offspring production (i.e. producing eggs, giving birth, rearing offspring, Chapman et al. 1998). The costs of these two components are affected by traits in both males and females (Arnqvist & Nilsson 2000). Since many such traits are sexually selected, the costs of reproduction should evolve in response to evolution of the traits. By measuring interspecific variation in the trade-offs involved in mating and reproduction, we can gain new important insights into how these trade-offs and associated traits have evolved (Chapman et al. 1998).

The cost of mating has been studied in several empirical systems (Daly 1978; Bell & Koufopanou 1985; Fowler & Partridge 1989; Chapman et al. 1995; Arnqvist & Nilsson 2000). In *Drosophila*, for example, mating and egg production reduce longevity and virgins live longer than nonvirgins (Fowler & Partridge 1989; Chapman et al. 1995). This is also true for female butterflies, *Colias eurytheme* (Kemp & Rutowski 2004) and giant water bugs, *Belostoma flumineum* (Gilg & Kruse 2002). Costs such as these can, for example, be caused by toxic substances transferred to the female through the ejaculate (*Drosophila*: Chapman et al. 1995). Mating costs can also arise as a result of infliction of direct physical injuries, as in the dung fly, *Sepsis cynipsea*, where males are equipped with armoured genitalia that cause injuries to the female's internal genital tract (Blanckenhorn et al. 2002). Mated dung fly females show a high rate of mortality, presumably because of these injuries. Female dung flies housed with a male also have lower survivorship than females housed with another female, presumably as a result of the cost of copulation itself (Muhlhauser & Blanckenhorn 2002). Impairment of female immune function is another cost of mating (mealworm beetle, *Tenebrio molitor*: Rolff & Siva-Jothy 2002; damselfly, *Matrona basilaris japonica*: Siva-Jothy et al. 1998). Costs such as these derive from various traits in males (e.g. ejaculate composition, genital morphology) and from the way in which

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such traits interact with female morphology and physiology.

However, mating itself is also often associated with various benefits to females. These benefits are perhaps best understood in those species where males provide nuptial gifts (reviewed in Vahed 1998). In butterflies, for example, males transfer a nutrient-rich ejaculate that gives multiply mated females benefits in terms of fecundity, egg size and longevity (Wiklund et al. 2001). In one butterfly species, *Pieris napi*, females 'forage' for matings and multiply mated females attain almost twice the fecundity of those mated only once (Kaitala & Wiklund 1994). In a review, Arnqvist & Nilsson (2000) showed that female insects generally gain directly from mating more than once (in terms of an increased lifetime offspring production). However, species without nuptial gifts seemed to have an optimal female mating rate, where an intermediate number of matings maximized female fitness.

In terms of male–female coevolution, males can clearly evolve to affect the costs and benefits of mating and offspring production, such that the net cost of reproduction evolves (Wiklund et al. 2001). This would, for example, be the case under sexual conflict and sexually antagonistic coevolution. Males may then evolve characters that increase the cost of mating to females, thereby lowering the females' fitness (Holland & Rice 1998; Arnqvist & Rowe 2005). Such costs to females could arise as pleiotropic side-effects of the sexually selected function in males (Parker 1979). According to this hypothesis, male traits that are essentially harmful for females evolve despite causing harm if they confer net benefits to males. This scenario has recently gained support (*Tribolium castaneum*, *Callosobruchus maculatus*, *Drosophila melanogaster* and *S. cynipsea*: Hosken et al. 2003; Morrow et al. 2003).

Direct evidence of how specific traits that are associated with these costs and benefits have evolved is scarce and there have been few systematic studies of closely related species (Wiklund et al. 2001). We partitioned the cost of reproduction into different components corresponding to mating and offspring production in six closely related species belonging to the same seed beetle genus, *Callosobruchus*. This genus is currently becoming established as a new model system in evolutionary ecology, but there are no previous comparative studies of the costs of reproduction among different species. Most of the earlier studies have been on *C. maculatus* (Eady 1995; Messina & Slade 1999; Arnqvist et al. 2005) and a few on *C. chinensis* (Yanagi & Miyatake 2003; Miyatake & Matsumura 2004) and *C. subinnotatus* (Mbata et al. 1997). In *C. maculatus*, it is known that females suffer injuries from mating, but it is not clear whether this translates into a net cost of mating (Crudgington & Siva-Jothy 2000). By conducting the same experiment on all six species, we aimed to gain important insights into the evolution of traits and behaviours associated with the costs of reproduction. Since the costs and benefits of mating and offspring production are key to our understanding of interactions between the sexes in general, especially with regards to male–female coevolution, such information is much needed.

METHODS

Species and Rearing

We used six species of *Callosobruchus*: *C. maculatus* (provided by P. Credland, University of London), *C. subinnotatus*, *C. analis*, *C. rhodesianus*, *C. chinensis* (all provided by R. Smith, University of Leicester) and *C. phaseoli* (provided by Y. Toquenaga, University of Tsukuba). To assess the relative degree of intraspecific variation in the costs of reproduction, we included two populations of *C. maculatus*, one standard stock originating from Brazil, which has often been used in recent studies of mating interactions (Crudgington & Siva-Jothy 2000; Arnqvist et al. 2005) and another deriving from three adjacent populations in Nigeria. The beetles were all maintained on cowpea beans, *Vigna unguiculata*, except *C. chinensis* and *C. phaseoli* which were maintained on adzuki beans, *Vigna angularis*, and *C. analis* which were reared on mung beans, *Phaseolus aureus*. The generation time in the laboratory is approximately 3–4 weeks for all species. Females cement their eggs on to the beans and the larvae then bore themselves into the beans where they go through several larval instars and develop into a pupa (Southgate 1979). As adults, the beetles require neither food nor water to reproduce successfully (Wightman 1978; Savalli & Fox 1999). Our experimental design was built on the fact that female *Callosobruchus* spp. lay no, or only very few, eggs if they are deprived of beans (Møller et al. 1989; Messina & Slade 1999; Umeya 1987, cited in Yanagi & Miyatake 2003).

Experiment

To obtain virgin beetles we first isolated beans infested with eggs and then collected individuals as they hatched, keeping them isolated until the experiment. For each species, we collected 60 virgin females and assigned them randomly to one of four treatment groups (A, B, C and D; $N = 15$ females per treatment). In treatment A, the females were placed singly into empty petri dishes. These females experienced no costs of mating or egg production. In treatment B, the females were mated once and then placed into an empty petri dish. Females in this treatment experienced the effects of mating once, but not of egg production. In treatment C, females were also mated once but their petri dishes contained 100 beans. These females thus experienced both the cost of a single mating and the cost of egg production. Finally, in treatment D, the females were mated once and then placed into a petri dish containing 100 beans and two virgin males. We then replaced these males every third day with new virgin males, to standardize male harassment across females. In this treatment, the females thus experienced both the cost of mating multiply and the cost of reproduction but also the costs that may derive from cohabiting with males (i.e. male harassment and additional matings). We assumed that the balance between the various costs and benefits of reproduction will be manifested as differences in female life span between treatments (Chapman et al. 1998; Wagner et al. 2001; Yanagi & Miyatake 2003; Reguera et al. 2004). Once a day we checked the petri dishes, recorded dead females and determined their life span (number of days). After the females died we measured their body size, using a digitizing tablet placed under a dissecting microscope provided with a camera lucida, and counted all the eggs laid. All beetles were maintained under controlled laboratory conditions at 27 °C and 45 ± 10% relative humidity with a 12:12 h light:dark cycle. All individuals were 24–48 h old when mated. The experiment was done at a temperature of 22–25 °C.

Statistical Analysis

The data were analysed by analyses of covariance (ANCOVA), where body size (standardized within species to a mean of zero and a standard deviation of one) was included as a continuous covariate, following a log transformation of the response variable to fulfil the assumptions made under ANCOVA. A few deviant observations ($|\text{studentized residual}| > 3$) were excluded from the final analyses. Normality of residuals was assessed with Shapiro–Wilks tests. In a first step, all the data was entered into one common ANCOVA. For further analyses of (1) cost of mating, (2) cohabitation with males and (3) egg production we conducted three separate focused ANCOVAs that included only the relevant treatments. In the first and second models, the number of eggs laid was included as a covariate to control for any differences in life span that might be caused by egg production.

Our data set included six species, of which one was represented by two populations (see above). To avoid inflating the degrees of freedom for 'species' effects, we performed two full sets of analyses, one set including the Brazil population of *C. maculatus* and another set instead including the Nigeria population. Below, we report the results only from the analyses including the standard Brazil population as a representative of *C. maculatus*. However, in only one case did the results of the two sets of analyses differ in terms of our ability or inability to reject null hypotheses at $\alpha = 0.05$, and this case is commented on below. Data for both populations are, however, included in all figures. The residuals from all models were not significantly different from normality ($P > 0.05$ in all cases), except for the model comparing lifetime egg production between treatments C and D ($P < 0.05$). In this case, transformations failed to stabilize the residual distribution, but graphic inspection of the residuals showed that they were homogeneous and that the residual distribution was symmetrical. Nevertheless, we also assessed these models by using the bootstrap procedure suggested by ter Braak (1992), based on 1000 bootstrap replicates. For all analyses we used SYSTAT 11 (Systat Software Inc., Port Richmond, CA, U.S.A.).

RESULTS

An ANCOVA including all species and treatments revealed highly significant main effects of both species and treatment on female life span (species: $F_{5,329} = 32.45$, $P < 0.001$; treatment: $F_{3,329} = 433.96$, $P < 0.001$). The interaction term between species and treatment was also highly significant ($F_{15,329} = 15.91$, $P < 0.001$), showing that treatment effects on female life span indeed differed between species.

In the analysis of the cost of mating (treatments A versus B; Table 1), life span differed significantly between species but there was no main effect of treatment. However, the interaction term between species and treatment was highly significant, showing that species differed in their response to our treatment. A single mating prolonged life span in some species but reduced it in others (Fig 1a).

An analysis of the cost of cohabitation with males (treatments C versus D; Table 2) showed that females cohabiting with males generally suffered a reduced life span compared with those that did not, when differences in egg production were controlled. The effect of cohabitation on female life span differed significantly between species (Fig. 1b). Analyses of lifetime egg production differed slightly depending on which population of *C. maculatus* was included. The model including the Brazil population showed that females cohabiting with males produced fewer eggs than those mated only once (least-squares means: 54.80 versus 60.03; ANCOVA: $F_{1,163} = 4.68$, $P = 0.032$; bootstrap $P = 0.037$) and did not vary between species (test of interaction between species and treatment: $F_{5,163} = 0.69$, $P = 0.629$; bootstrap $P = 0.583$). In the model including the Nigeria population, females cohabiting with males again produced fewer eggs (least-squares means: 54.17 versus 56.73) but this difference was not statistically significant ($F_{1,160} = 0.91$, $P = 0.342$; bootstrap $P = 0.345$; test of interaction between species and treatment: $F_{5,160} = 0.64$, $P = 0.670$; bootstrap $P = 0.655$).

In terms of the cost of egg production (treatments B versus C) our analysis showed significant main effects of both species and treatment (Table 3). However, the strength of the treatment effect varied between species (Fig. 1c).

To assess whether the two populations of *C. maculatus* differed in their response to our treatments, we also performed a separate model including all treatments but only these two populations. This analysis revealed main effects of both population (ANCOVA: $F_{1,105} = 12.42$, $P = 0.001$) and treatment ($F_{3,105} = 280.87$, $P < 0.001$) on female life span. The interaction between population and treatment was also significant ($F_{3,105} = 2.71$, $P = 0.049$), showing that our treatments affected life span differently in the two conspecific populations. However, the magnitude of these differences was small compared to differences between species (Fig. 1, Table 4).

Table 1. Analysis of covariance of the effects of a single mating on female life span

Source	Sums of squares	*F*	*df*	*P*
Species	1.449	53.95	5	<0.001
Treatment	0.002	0.29	1	0.592
Species * Treatment	0.099	3.70	5	0.003
Number of eggs	0.032	5.89	1	0.016
Female size	0.020	3.71	1	0.056
Error	0.881		164	

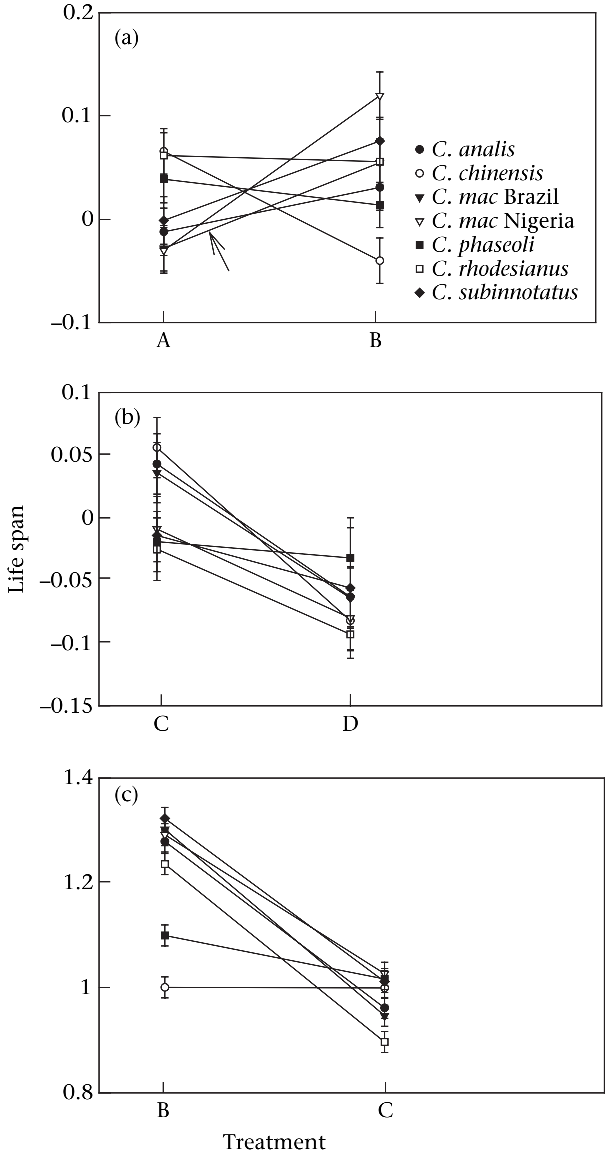

Figure 1. The effects of (a) a single mating, (b) cohabitation with males and (c) egg production on female life span ($\overline{X} \pm$ SE) in all species, after controlling for the effects of variation in female size within species and, in (a) and (b), the number of eggs laid. Treatment A: virgin females, kept without egg-laying substrate; treatment B: females mated once, kept without egg-laying substrate; treatment C: females mated once, kept with egg-laying substrate but without males; treatment D: females mated once, kept with egg-laying substrate and two males. Arrow in (a) indicates the line representing *C. maculatus* Brazil. *C. mac*: *C. maculatus*.

Table 2. Analysis of covariance of the effects of cohabitation with males on female life span

Source	Sums of squares	*F*	*df*	*P*
Species	0.339	13.57	5	<0.001
Treatment	0.193	38.49	1	<0.001
Species * Treatment	0.066	2.63	5	0.026
Number of eggs	0.003	0.64	1	0.427
Female size	0.013	2.51	1	0.115
Error	0.810		162	

Table 3. Analysis of covariance of the costs of egg production on female life span

Source	Sums of squares	*F*	*df*	*P*
Species	0.502	17.38	5	<0.001
Treatment	2.420	418.85	1	<0.001
Species * Treatment	0.833	28.83	5	<0.001
Female size	0.019	3.37	1	0.068
Error	0.954		165	

DISCUSSION

The six congeneric species studied here are ecologically relatively similar. For example, all live in arid environments, have multivoltine life cycles and lay their eggs on legumes where the larvae develop inside the seeds (Southgate 1979; Labeyrie 1981; Fujii et al. 1989). Despite this, our results document substantial variation in the costs and benefits of mating and reproduction among these species. The fact that they differ to this extent provides indirect evidence for rapid evolution of traits that directly affect the economics of sexual interactions. This conclusion is strengthened by the fact that we found significant differences in response to our treatments even between two conspecific populations. We first discuss possible underlying factors for this variation and then how these results can provide insights into the evolution of reproductive traits and behaviours.

In terms of the net effect of a single mating, there was substantial variation in the effect on female life span (Fig. 1a). In three species, copulation increased female life span, and this effect was most pronounced in *C. maculatus*. Benefits of mating to females, in terms of prolonged life span, have been documented in several insect species (reviewed in Arnqvist & Nilsson 2000) and the suggested cause for such an effect has in most cases been beneficial seminal fluid products (e.g. field crickets, *Gryllus lineaticeps*: Wagner et al. 2001; house flies, *Musca domestica*: Hicks et al. 2004). The ejaculate size of *C. maculatus* represents approximately 5% of male body weight (Fox 1993a) and females may benefit from nutrients (Fox 1993b) or water (Arnqvist et al. 2005) contained in the ejaculate. Since we focused on the net effects of a single mating, our results show that the beneficial effects must outweigh any costs to *C. maculatus* females that derive from mating (Crudgington & Siva-Jothy 2000), at least under our experimental conditions.

In another set of species, females that mated once suffered a decreased life span compared to virgins and this effect was most pronounced in *C. chinensis*. Our results regarding *C. chinensis* are in accord with those of Yanagi & Miyatake (2003) who also found that female *C. chinensis* that mated once had a decreased life span. What are the underlying causes for the interspecific variation in the net effect of a single mating observed here? One obvious possibility is that the composition and/or relative size of the ejaculate varies between species, and the ejaculate of some species may even contain substances that are toxic to females. Observations on another bruchid beetle (*Acathoscelides obtectus*) have shown that the

Table 4. Female life span, lifetime egg production and size for all species and treatments

Species	Treatment	Life span (days)	Number of eggs laid	Length of female elytra (cm)
C. maculatus, Nigeria				0.196±0.003
	A	18.53±1.15	0.53±0.47	
	B	18.93±1.04	22.79±5.60	
	C	9.85±0.60	70.31±5.96	
	D	7.93±0.34	73.71±4.32	
C. maculatus, Brazil				0.218±0.002
	A	18.40±0.79	0.20±0.15	
	B	19.20±0.61	4.60±1.70	
	C	7.93±0.30	90.33±3.57	
	D	6.60±0.19	77.00±2.96	
C. chinensis				0.211±0.001
	A	12.00±0.63	0.73±0.43	
	B	9.20±0.53	1.47±1.26	
	C	9.13±0.46	47.33±5.31	
	D	6.40±0.38	43.73±3.36	
C. rhodesianus				0.199±0.003
	A	15.47±0.74	11.47±2.68	
	B	16.40±0.64	8.33±3.07	
	C	7.00±0.35	67.33±5.77	
	D	5.93±0.29	67.57±7.35	
C. analis				0.216±0.002
	A	17.27±0.65	0.27±0.15	
	B	17.67±0.66	2.40±1.02	
	C	8.33±0.49	75.13±5.58	
	D	6.46±0.29	62.46±1.95	
C. subinnotatus				0.272±0.002
	A	18.86±0.72	0.00±0.00	
	B	19.73±1.59	3.60±1.82	
	C	10.27±0.95	33.27±4.02	
	D	8.53±0.39	34.67±5.16	
C. phaseoli				0.217±0.002
	A	13.67±0.75	5.07±2.66	
	B	11.73±0.49	16.13±3.93	
	C	9.53±0.42	45.93±6.22	
	D	9.13±0.36	42.67±2.17	

Means are given ± SE. Treatment A: virgin females, kept without egg-laying substrate; treatment B: females mated once, kept without egg-laying substrate; treatment C: females mated once, kept with egg-laying substrate but without males; treatment D: females mated once, kept with egg-laying substrate and two males.

ejaculate of this species contains substances that are toxic to females in the sense that they shorten female life span (Das et al. 1980). Differences in female physiology between species may also influence the net effect of a single mating. For example, females of some species may be better able to use ejaculate substances to increase their life span than others. Another type of trait that might affect the cost of mating in this group is the morphology of the male genitalia. In general, the morphological structures of male genitalia evolve very rapidly among insects (Eberhard 1985; Arnqvist 1998; Hosken & Stockley 2004), including bruchid beetles (Kingsolver 1970). Any costs that females suffer as a result of male genitalia might therefore also evolve rapidly. In *C. maculatus*, the male genitalia are equipped with sclerotized spines which harm females internally (Crudgington & Siva-Jothy 2000) and females that mate twice die at a younger age than females mated only once (Arnqvist et al. 2005). However, our results show that *C. maculatus* females enjoy a net benefit from mating once. Collectively, these results suggest that the relative costs and benefits of mating to females depend on their mating status (see also Fox 1993a, b; Savalli & Fox 1999). Arnqvist et al. (2005) suggested that complex interactions between several different costs and benefits of mating produce a nonlinear relation between the number of matings and female fitness in *C. maculatus*. By showing that a single mating is strongly beneficial to females of this species, our novel results add important weight to this suggestion.

Females had a lower lifetime egg production when kept with males compared to when mated once only and then isolated from males (Table 4), although this pattern was statistically significant only in the model where the Brazil population represented *C. maculatus*. It is clear, however, that females of all species suffered a much reduced life span when kept with males (Fig. 1b). In theory, these results could be caused by an elevated copulation rate and/or male harassment (Clutton-Brock & Parker 1995;

Arnqvist & Rowe 2005). Since females of all species included in this study do remate (Miyatake & Matsumura 2004; Arnqvist et al. 2005; M. Katvala & J. Rönn, unpublished data), we can safely assume that females kept with males mated more than once during their lifetime. Thus, the reduction in life span observed here could, at least in part, be caused by an elevated mating rate per se. However, detailed experiments on one of the species included here (*C. maculatus*) have shown that mating rate per se does not markedly affect female life span and that effects on lifetime egg production are much less dramatic than those observed here (Fox 1993b; Arnqvist et al. 2005). We thus suggest that our results are primarily caused by costs of male harassment. Costly male harassment of females has been found in many insects (Rowe et al. 1996; Clutton-Brock & Langley 1997; Watson et al. 1998; Muhlhauser & Blanckenhorn 2002; Martin & Hosken 2004), including *C. chinensis* (Lan & Horng 1999), but also in vertebrates (Le Boeuf & Mesnick 1991; Magurran & Seghers 1994; Reale et al. 1996; Shine et al. 2000; Pilastro et al. 2003; Plath et al. 2003). In most of these cases, costs to females derive from either lost feeding opportunities or direct risk of lethal injury. None of those, however, is likely to account for the costs seen in our experiments, since females are aphagous and do not risk suffering lethal injuries from male harassment. Male *Callosobruchus* are generally very persistent, and behavioural observations suggest that females are likely to incur considerable energy expenditure when fending off harassing males (Watson et al. 1998). In sum, our results show that females living in the presences of males suffer reduced fitness and we suggest that this is primarily due to energetic costs of resisting harassing males.

The cost of egg production can be seen as incorporating two distinct components: the cost of egg maturation (i.e. oogenesis) and the cost of egg laying (i.e. oviposition). In *C. maculatus* and *C. chinensis*, emerging females already have matured eggs in their ovaries (Wilson & Hill 1989; Yanagi & Miyatake 2003), and the cost of egg maturation should therefore be expended primarily during the larval stages. We thus predicted relatively small costs of egg production, largely representing the egg-laying component, in our experiments. However, most species showed a clear cost of egg production (Fig. 1c). This suggests that females do use body reserves to mature eggs in the adult stage, but the extent to which costly oogenesis occurs in adults apparently varies between species. Notably, *C. chinensis* and *C. phaseoli* showed a much smaller, or no, cost of egg production compared with the other species.

Our conclusions are based on experiments done in a laboratory setting and a potential concern is that our laboratory environment may poorly reflect natural conditions. We are less concerned about this possibility for two reasons. First, we reared our beetles on legumes that they also infest in the field, and they were kept at temperatures, humidities and densities that correspond to conditions encountered in natural populations (Southgate 1979; Labeyrie 1981; Fujii et al. 1989). Second, and more importantly, our conclusions are based on treatment effects and differences between species in how they respond to our experimental treatments. These effects should be much less sensitive to any differences between natural and laboratory environments than main effects of species.

One of the species included in this study, *C. maculatus*, has recently become established as a widely used model system in evolutionary biology and behavioural ecology. By setting *C. maculatus* into a wider comparative perspective, we wish to highlight two important points. First, our results show that species in this genus are surprisingly different in terms of the economics of male–female interactions. Thus, *C. maculatus* may not always be the most suitable species with which to address particular questions in this domain. For example, the fact that *C. maculatus*, perhaps somewhat surprisingly (cf. Crudgington & Siva-Jothy 2000), showed the most positive net effect of a single mating suggests that we should be cautious when drawing general conclusions from experiments on this species alone. Second, the extensive interspecific variation documented here suggests that more detailed and phylogenetically comparative future studies of the concerted evolution of male and female reproductive traits in this group of insects, and how this in turn affects the economics of reproduction, might provide important and more general insights into male–female coevolution.

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