Male reproductive senescence as a potential source of sexual conflict in a beetle

Pau Carazo, Pau Molina-Vila, and Enrique Font
Instituto Cavanilles de Biodiversidad y Biología Evolutiva, Universidad de Valencia, Apto. 22085, 46071 Valencia, Spain

The link between senescence and reproductive success is a contentious yet crucial issue to our understanding of male choice, sexual conflict, and the evolution of ageing. By imposing direct (i.e., male fertility) or indirect (i.e., zygote viability) reproductive costs to females, male senescence may lead to sexual conflict at different levels. For example, ageing may affect male ability to deliver sperm, thus setting the scene for sexual conflict over mating, and/or may affect the quality of individual sperm cells, generating the potential for sexual conflict over fertilizing strategies. We addressed these issues by studying the mating behavior, reproductive fitness, and fertilization patterns of females mated to young versus old males in a beetle (Tenebrio molitor). Our results show that male senescence imposes direct fertility costs on females and that females mated to old males produce offspring of lower quality (i.e., smaller) than those mated to young males. Compared with females mated to young males, females mated to old males were less receptive and decreased their allocation to spermatophore guarding (a crucial determinant of male reproductive success in this species), increasing the risk of sperm competition by other males. In contrast, old males increased their own investment in spermatophore guarding, which suggests the existence of antagonistic selection over sperm competition strategies. These findings lend support to the recent notion that ageing may act as an evolutionary source of sexual conflict. [Behav Ecol 22:192–198 (2011)]
reproductive fitness (i.e., number and quality of offspring), male and female mating behaviors (e.g., male vs. female’s relative allocation to mate guarding), and female fertilizing patterns in staged matings with either young or old males. *Tenebrio molitor* constitutes an excellent system in which to study the possible role of male ageing as an evolutionary source of sexual conflict. It is a relatively long-lived beetle with a highly polychaenorous mating system characterized by an evolutionary history of intense sperm competition, and it exhibits a unique type of short-term spermatophore guarding in which female and male allocation can be measured separately (Carazo et al. 2007; Fedina and Lewis 2008). Spermatophore guarding in *T. molitor* is a behavioral strategy aimed to reduce the risk of spermatophore inhibition by other males. Spermatophore inhibition results in the complete loss of a male’s sperm and takes place when a female remates with a second male before the sperm from the first male’s spermatophore has been released into its bursa copulatrix (7–10 min; Drnevich et al. 2000). Males achieve spermatophore guarding by increasing copula duration above that necessary for complete sperm transfer (Gage and Parker 1991) and by remaining in close contact with females after the end of copulas (postcopulatory associations; PCA) during which time they aggressively fend off any copulation attempts by other males (Carazo et al. 2007). Increased copula duration is not related to the amount of sperm transferred in this species (Gage and Parker 1991), and males seem to have a certain control over the duration of copulas because their aedeagus is covered with spines that are thought to have evolved to maintain genital contact during matings (Siva-Jothy et al. 1996). Although it is usually in the female’s best interest to allow long enough PCA so as to avoid spermatophore inhibition by other males, the duration of PCA depends less on male than on female behavior, which can easily escape postcopulatory mate guarding (Siva-Jothy et al. 1996; Carazo et al. 2007). Given this reproductive behavior, we hypothesized that, if sperm senescence affects female offspring quality in this species, females mating with old males would benefit by reducing their investment to spermatophore guarding, whereas males would benefit by doing the opposite. In *T. molitor*’s mating system, this should translate into significantly longer copulas (i.e., increase in male investment to spermatophore guarding) and significantly shorter PCA (i.e., decrease in female investment to spermatophore guarding) when females mate with old males.

**MATERIALS AND METHODS**

**Beetle maintenance**

We used beetles originating from 4 genetically heterogeneous stock cultures maintained in the Ethology Laboratory at the University of Valencia (Spain). These cultures have been running for more than 10 years with regular contributions from other cultures and from wild stock. All growth stages are kept together in single plastic containers with a rearing medium consisting of white flour and wheat bran to which chunks of fruit, bread, and various vegetables are added periodically. The cultures are covered with filter paper that is sprayed with water to provide moisture on a daily basis. All containers are stored in well-ventilated dark places at ambient humidity and under temperature-controlled conditions (22–25°C). We collected beetle pupae from the stock cultures and sexed them by examining developing genitalia on the ventral side of the eighth abdominal segment (Bhattacharya et al. 1970). Individuals were examined under a dissecting scope both as pupae and after eclosion and beetles with malformations were discarded. All the beetles extracted from each culture were isolated in 9-cm Ø plastic Petri dishes containing a surplus of wheat bran, pellets of rat chow, and chunks of fresh apple (replaced every 48 h) and henceforth haphazardly divided into 2 groups that were allocated to the “young” treatment (i.e., left to mature for 17–18 days posteclosion) or to the “old” treatment (i.e., left to mature for 83–85 days posteclosion).

**Mating trials**

We staged matings between mature young (17–18 days posteclosion) virgin females and 1) mature young (17–18 days posteclosion) males (n = 40 pairs) and 2) old (83–85 days posteclosion) males (n = 40 pairs). Males extracted from stock cultures were haphazardly divided in 2 groups assigned to different treatments. To ensure they were reproductively active, males were mated to a virgin female 24 h before each trial. Only males that exhibited normal courtship behaviors and achieved matings within 10 min since their introduction were used in subsequent trials. The following behaviors were recorded during mating trials: latency to courtship, courtship duration, probing duration, copula duration, and duration of PCA. Male precopulatory behavior was divided into 2 phases: 1) “courtship,” characterized by male intense antennal tapping of the body of the female and rapid stroking movements of its prothoracic legs against the female’s sides and 2) “probing,” the male everts its aedeagus and moves it from side to side across the female’s rear end until achieving intromission (Font and Desfils 2003; Carazo et al. 2004). Courtship and probing probably represent functionally distinct behavioral phases within the mating behavior of this species. Antennal tapping and leg scratching are typical courtship behaviors exhibited by numerous beetles, and they have been shown to influence female cryptic choice (Tallamy et al. 2003). On the other hand, probing lasts until the female lowers its last abdominal sternite and relaxes the muscles of its vaginal duct, thus allowing intromission (Font and Desfils 2003; Carazo et al. 2004). Hence, although courtship duration quite evidently reflects male investment, probing duration is much more likely to reflect female acceptance or reluctance to mate. *Tenebrio molitor* females respond to courtship and copula by remaining quiescent or not, so we recorded the amount of time females spent in locomotion during courtship and/or copula as an additional measure of female rejection or reluctance to mate. We found that probing duration was significantly correlated with female escape behavior (n = 70, r = 0.608, P < 0.001, Pearson’s correlation), which supports our idea that both these behaviors can be used to gauge female receptivity.

**Senescence effects on female reproductive fitness and fertilization strategies**

After mating trials, females were left to lay eggs in 9-cm Ø plastic Petri dishes (lined with filter paper and containing excess food) that were kept in an incubation chamber at 26 °C, 60% humidity, and a 14:10 h light:dark light cycle (although trays holding the Petri dishes were covered with a dark mesh to provide a dim lighting). Previous studies suggested that *Tenebrio molitor* females lay all their eggs within the first 3 weeks (Vainikka et al. 2006). Thus, females were transferred to new Petri dishes every 48 h during the first 20 days after matings. As a precautionary measure, after day 20, females were transferred to a new Petri dish and left to lay eggs for an additional 16 days. Thus, for each female, we collected a total of 11 Petri dishes during a period of 36 days following single matings (i.e., 10 collected over the first 20 days and an additional one with all the eggs laid during the subsequent 16 days).
Petri dishes containing eggs were left in the incubation chamber and examined every 2–3 days until hatching. At this time, we proceeded to count the number of larvae in each Petri dish (Vainikka et al. 2006). Females that participated in aspermic matings (old males, n = 5 and young males, n = 1) were discarded from reproductive fitness analyses along with a few females from each experimental group (old males, n = 4 and young males, n = 2) that did not survive throughout the whole oviposition period. Unexpected contingencies resulted in the loss of substantial data for 3 of the females in the old males’ experimental group, which were consequently also excluded (final sample sizes: young males, n = 34 and old males, n = 21). Emerged beetles were isolated with excess food in 5-cm Ø plastic Petri dishes for 10 days until fully mature (Font and Desfilis 2003) at which time they were weighted to the nearest 0.1 mg. We also noted whether mature beetles had any observable external malformations (e.g., absence of segments in the antennae, damaged elytra) and calculated the average proportion of mature adult beetles with malformations. Thus, we recorded the following variables for each female: number of offspring (i.e., live larvae produced), oviposition pattern (of offspring), larvae survival (i.e., proportion of larvae that reached adult mature stage), brood sex ratio, mean weight of offspring (of emerged beetles at sexual maturity), and proportion of offspring with malformations (at sexual maturity).

Contrary to what previous work suggested, some females continued laying eggs beyond the first 20-day oviposition period. Thus, to test for differences in oviposition patterns between both experimental groups, we compared the average day in which females begun oviposition and the total amount of offspring resulting from the first 10 days, the second 10 days, and the last 16 days of oviposition. At all stages during the experiment, larvae and adult beetles were periodically supplied with fresh apple, wheat bran, and rat chow to guarantee excess of food and sufficient moisture.

Statistical analyses

To test for treatment effects in behavioral data from mating trials, we fitted a 1-way analysis of variance (ANOVA) model using SPSS 17.0. Graphical inspection of the data prior to statistical analyses (i.e., boxplots of observations) indicated the presence of outliers in behavioral data. To comply with distributional assumptions and avoid undue influence of outliers on estimates of group effects or variances, we Winorized these data at 90% by setting extreme values to the corresponding adjacent 5th or 95th percentile value (Wilcox 1997). Inspection of residuals against group means revealed a slight heterogeneity of variance in several behavioral variables (i.e., escape attempt, courtship, and probing; between group ratio of standard deviations <2.5), but ANOVA models are robust to moderate unequal variances if sample sizes are similar (Quinn and Keough 2002).

We used a $G$ test to analyze whether the proportion of aspermic matings was independent of male age. To test for treatment effects on reproductive output variables, we fitted a multivariate analysis of covariance (MANCOVA) model with male age as a fixed effect; female weight as a covariable (i.e., to control for putative maternal effects); and number of larvae, larvae survival, sex ratio, percentage of offspring with malformations, and offspring mean weight as response variables. Prior to pooling the brood weight data in the MANCOVA, we used a $t$-test for paired replicates to test for significant differences between male and female brood weight. Inspection of residuals revealed that all data conformed to homoscedasticity assumptions. All fitness variables showed a slightly positive skew in their distribution, but transformation of data was judged unnecessary, given that ANOVA is robust to slight deviations from normality when there is homogeneity of variance (Quinn and Keough 2002). We used Box’s tests to check for sphericity assumptions before conducting multivariate statistics.

Finally, oviposition period data were analyzed by fitting a partly nested repeated measures ANOVA model with oviposition period nested within male age. We also performed a 1-way ANOVA to test for the existence of significant treatment effects in the first oviposition day. As previously, residuals were graphically explored against group means to check for homogeneity of variance between different treatment groups. Partial squared Eta values ($\eta^2$) are provided along with the results of ANOVA models as an estimation of size effects. The significance level for rejection of the null hypothesis was set at $\alpha = 0.05$, and all reported probabilities are 2 tailed.

RESULTS

ANOVA analysis (Figure 1) revealed that matings involving old males were characterized by lower female receptivity as evidenced by longer probing duration ($F_{1,66} = 8.107, P = 0.006, \eta^2 = 0.106$) and female escape behavior ($F_{1,66} = 12.334, P = 0.001, \eta^2 = 0.157$) and by increased copula duration ($F_{1,66} = 15.142, P < 0.001, \eta^2 = 0.187$), whereas matings involving young males resulted in significantly longer PCA ($F_{1,66} = 9.970, P = 0.002, \eta^2 = 0.151$). We did not find significant treatment effects for either male latency to courtship initiation ($F_{1,66} = 0.290, P = 0.592$) or courtship duration ($F_{1,66} = 1.220, P = 0.273$).

We found that females mated to old males suffered a significantly higher proportion of aspermic matings than females mated to young males (aspermic/spermic matings: young males 1/34, old males 5/21; $G_{20} = 4.307, 0.05 > P > 0.02$). MANCOVA statistics revealed the existence of significant effects of male age on female reproductive success (Pillai’s trace, $F_{1,56} = 3.654, P = 0.009, \eta^2 = 0.337$), but the effect of male age on offspring survival was not significant ($P = 0.164$), or the presence of aspermic matings ($F_{1,56} = 2.408, P = 0.055$). However, univariate planned comparisons showed significant effects of female weight on offspring weight ($F_{1,40} = 7.845, P = 0.008$) but not on brood sex ratio ($F_{1,40} = 3.527, P = 0.068$), number of larvae ($F_{1,40} = 0.712, P = 0.404$), larvae survival ($F_{1,54} = 1.002, P = 0.323$), or percentage of offspring with malformations ($F_{1,54} = 1.209, P = 0.278$). Male age had a significant effect on offspring weight ($F_{2,34} = 12.233, P = 0.001, \eta^2 = 0.234$), but not on percentage of aspermic matings ($F_{1,34} = 0.394, P = 0.534$; Figure 2), larvae survival ($F_{1,40} = 3.268, P = 0.078$; Figure 2), and sex ratio of offspring ($F_{1,40} = 0.091, P = 0.748$; Figure 2), or percentage of offspring with malformations ($F_{1,40} = 1.545, P = 0.221$; Figure 2).

The repeated measures ANOVA model did not reveal a significant effect of male age on the total number of offspring laid in each treatment ($F_{2,54} = 0.010, P = 0.920$) but revealed significant differences in the number of offspring produced during the different 3 oviposition periods (i.e., first 10 days, second 10 days, and last 16 days; $F_{2,54} = 56.737, P < 0.001$; Figure 3) and a significant interaction between oviposition period and male age ($F_{2,54} = 4.004, P = 0.027$; Figure 3). The existence of a significant interaction between oviposition period and male age strongly suggests that oviposition patterns between old and young male females differed. A planned trend analysis (Quinn and Keough 2002) included in the model confirmed that the oviposition pattern of females mated to old males departed significantly from the linear trend exhibited by females mated to young males, in that the former shifted their fertilizations toward an earlier phase ($F_{1,52} = 8.837, P = 0.009$; see Figure 3). The first oviposition day was not different between...
both groups of females (±SEM; old males, 2.4 ± 0.51 days, n = 20 and young males, 2.9 ± 0.48 days, n = 28; F1,41 = 1.082, P = 0.304, single factor ANOVA).

DISCUSSION

Direct and indirect fitness costs of male and sperm senescence.

Our results suggest that male age imposes direct fertility costs to females as females mating with old males are more likely to incur aspermic matings (i.e., matings in which males fail to transfer sperm; Jones et al. 2006) than those mating with young males. Available evidence in this species shows that mating reduces female attractiveness (probably due to a male-produced antiaphrodisiac and/or to pheromonostasis; Carazo et al. 2004), so aspermic matings may be particularly costly for females seeking multiple copulations. Also, we offer direct empirical evidence that females mated to old males produce offspring of significantly smaller size than those mated with young males (Figure 2). There is abundant

Figure 1
Mean (±SEM) for the behavioral variables measured during mating trials. Probing and female escape behaviors were recorded as a measure of female receptivity (see METHODS). As expected, matings with old males resulted in lower female receptivity, a drastic increase in copula duration (i.e., extended copulas are a male-controlled spermatophore-guarding mechanism in this species; 21) and a drastic decrease in PCA duration (i.e., spermatophore-guarding mechanisms that is not under male control; 21). Asterisks indicate level of significance (*P < 0.05, **P < 0.01, and ***P < 0.001). All probabilities are 2 tailed.

Figure 2
Mean (±SEM) of measures of reproductive fitness in females mated to old versus young males: (a) number of offspring (i.e., live larvae produced), (b) larvae survival (percentage of larvae that survive to sexual maturity), (c) percentage of offspring with malformations at sexual maturity, and (d) mean offspring weight at sexual maturity. Asterisks indicate level of significance (*P < 0.05, **P < 0.01, and ***P < 0.001). All probabilities are 2 tailed.
evidence that body size is an important determinant of male and female reproductive success in *Tenebrio molitor* (Rantala et al. 2003; Vainnikka et al. 2006; Carazo et al. 2007), strongly suggesting that male ageing in this species may also impose indirect reproductive costs on females. Alternatively, the larger offspring of females mated to young males may also reflect differential female allocation (e.g., Sheldon 2000). However, we did not find significant differences in the number of offspring laid by females mated to old versus young males, which seems to argue against this possibility as differential allocation usually results in larger clutch sizes (Simmons 1987; Sheldon 2000). In contrast to our results, traditional good genes models of mate choice predict the existence of female preferences for old males (reviewed in Brooks and Kemp 2001). This is based on the assumption that viability selection will produce old males with higher somatic genetic quality within the cohort they were born in (Kokko and Lindstrom 1996). If age is a good indicator of male genetic quality, females mating with old males will garner indirect benefits by siring high-quality offspring, thereby leading to the evolution of female preferences for old males. However, the available empirical evidence is not conclusive as some females seem to prefer young over old males (Brooks and Kemp 2001).

In light of these discrepancies, recent models have reexamined the validity of the age-based indicator or viability hypothesis (Hansen and Price 1995; Beck et al. 2002; Beck and Promislow 2007). Perhaps the greatest flaw of traditional indirect-benefit models is that, despite its enormous potential to hinder reproductive fitness, they rarely acknowledge the effects of ageing. As already discussed, the direct costs of mating with old males could reduce or even counter any possible indirect benefits associated with male age (Brooks and Kemp 2001; Jones and Elgar 2004; Dean et al. 2007; Pizzari et al. 2007). Furthermore, ageing places males under 2 potentially antagonistic selective forces with respect to genetic quality (i.e., indirect benefits): increasing somatic genetic quality through viability selection versus a decrease in germ line genetic quality via premeiotic and postmeiotic sperm senescence (Beck and Promislow 2007; Pizzari et al. 2007). The extent to which any of these forces prevails will probably vary among species, but recent theoretical work suggests that germ line quality may be the most important determinant of male genetic quality (Beck and Promislow 2007). Although previous evidence is scarce (Price and Hansen 1998), our results seem to support the notion that senescence affects over the genetic quality of offspring may override any potential viability benefits of mating with old males.

### Ageing and sexual conflict over mating and fertilization

Our results suggest that, in the mealworm beetle, male ageing effects (i.e., both direct and indirect) on female reproductive success may lead to sexual conflict at different levels (Dean et al. 2007, 2010). First, taken together, our findings seem to suggest that females try to avoid matings with old males. Old males took longer to achieve intromissions (i.e., longer probing duration), and females spent more time exhibiting escape behaviors in reproductive interactions with old males (see Figure 1). That females took longer to copulate with old males may reflect an age impairment on male ability to court females (e.g., decreased antennal and leg tapping rate; Fedina and Lewis 2008), but the fact that we did not find significant differences in the stimulatory phase of male courtship seems to argue against this possibility. Similarly, our finding that female escape behaviors were significantly longer in matings with old males could reflect that old males are less effective at manipulating female behavior (e.g., through accessory seminal products; Arntquist and Rowe 2005) than young males, but this would not explain why females exhibited longer escape behaviors during precopulatory phases with old males (see Figure 4).

Second, we found that male age differentially affected the allocation of males and females to spermatophore guarding, a crucial determinant of reproductive success in this species (Drnevich et al. 2000; Carazo et al. 2007). As we predicted, sexual conflict arising due to male and sperm ageing translated into longer copulas (i.e., increased male allocation to spermatophore guarding) and shorter PCAs (i.e., a decrease in female allocation to spermatophore guarding; see Figure 1). Female resistance to spermatophore guarding was further evidenced by the existence of female escape attempts during the final stages of copulas with old males (time attempting to escape, ±SEM; 13.57 ± 8.868 s, n = 33; Figure 4), a behavior we had rarely observed in previous experiments (e.g., Carazo et al. 2007), and that was virtually nonexistent in copulas with young males (±SEM; 0.06 ± 0.057 s, n = 37; Figure 4). Our findings agree with the idea that by affecting both male ability to deliver sperm and the quality of individual sperm cells, ageing may drive sexual conflict over mating and sperm competition strategies in *Tenebrio molitor*.

### Could the onset of female fertilization be an adaptive response to postmeiotic sperm senescence in old males?

An interesting result of our study is that females mated to old males advanced the fertilization of their ova with respect to females mated with young males (Figure 3). The most obvious effect of this shift in female oviposition is that, on average, sperm from old males will be stored in the female spermatheca for a shorter time and thus will be less likely to experience postmeiotic senescence than that from young males. The effects of postmeiotic sperm senescence have been rarely addressed (Pizzari et al. 2007), but recent studies show that it may affect the evolution of female fertilization strategies (Dean et al. 2007). Postmeiotic sperm senescence has been shown to reduce fertilizing efficiency in kittiwakes (*Rissa tridactyla*, Wagner et al. 2004), crickets (*Acheta domesticus*;...
Carazo et al. • Senescence and sexual conflict in a beetle

We wish to thank L. Gonzalez and M. Mellado-Silva for their help with the larvae counts, rearing, and maintenance of the beetles used in the present research, and M. Serra for useful feedback on the present study.

REFERENCES


Figure 4 Female escape behavior (EB) during male courtship, male probing, and during copulas. Bars represent the mean (±SEM) time females spent trying to escape males in copulas with old versus young males.