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Male age, mating probability and mating costs in the fly *Sepsis cynipsea*

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ABSTRACT

Can females obtain good-genes benefits for their offspring by mating with older males? Age-based mate choice is controversial and relatively infrequently studied experimentally. In addition, it has recently become apparent that conflict over mating can generate substantial costs for both sexes. There is overt conflict over mating in the fly *Sepsis cynipsea*, and copulation dramatically reduces female longevity. We investigated whether male age influences the probability of mating, as well as its effect on several direct and indirect fitness measures, while further scrutinizing mating costs in *S. cynipsea*. Male age did not affect mating probability, female fertility, fecundity, offspring development time or survival. The fact that male age did not influence the likelihood of copulation is entirely consistent with the lack of detectable fitness benefits to females based on male age. However, the delay between copulation and egg laying was weakly positively associated with offspring survival as adults, as well as with clutch size. Interestingly, copula duration was negatively associated with the number of offspring that emerged as adults. The latter result resembles findings in *Drosophila melanogaster*, and may be indicative of additional costs of mating in this species.

Keywords: conflict, fecundity, fertility, fitness, sexual selection, survival.

INTRODUCTION

Sexual selection results from variation in fertilization success due to mate competition or choice, which are often difficult to disentangle in practice. Females are typically the choosy sex and there is often close concordance between females on which mates are most attractive (Andersson, 1994). Choice can either be direct or indirect (Wiley and Poston, 1996) and the benefits of choice have traditionally been divided into either genetic or direct benefits (but see Hosken and Stockley, in press). Direct benefits of mate choice include increased fertility or survival, and there is general acceptance that benefits of this nature could favour mate choice (Andersson, 1994; Kirkpatrick and Barton, 1997). Genetic benefits of mate choice are more controversial, but may be the result of good-genes or sexy sons (Fisher, 1930; Hamilton and Zuk, 1982; Andersson, 1994; and see Kokko *et al.*, 2002). Recent meta-analyses provide evidence for both direct and genetic benefits to mate choice (Møller and Alatalo, 1999; Møller and Jennions, 2001), although the effect sizes may be small.

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One potential way females could obtain good-genes benefits is by mating with older males, since, by surviving, these males have demonstrated their superior genes (Trivers, 1972). Using different modelling approaches, recent studies have indicated that an association between old-age survival and genetic quality is possible under certain conditions and hence age-based mate choice is conceivable (Kokko, 1998; Beck and Powell, 2000; Beck *et al.*, 2002; see also Kokko and Lindström, 1996). Additionally, there are many examples of female choice for older males (e.g. Grant and Grant, 1987; Zuk, 1988; reviewed in Kokko, 1998; Brooks and Kemp, 2001). Nevertheless, despite theoretical and empirical support, age-based mate choice remains controversial (Brooks and Kemp, 2001). This is due in part to a lack of evidence that the offspring of older males are fitter. Nevertheless, recent work on two fly species has shown that male age influences female fitness, although not entirely in accordance with the age-based indicator mechanism (also older-males-as-good-genes theory). In *Drosophila melanogaster*, the offspring of older males were found to have reduced survivorship, the opposite relationship expected, but the mating success of offspring did not vary with sire age (Price and Hansen, 1998). However, there is no evidence in this species that older males are preferred mates. In the sandfly, *Lutzomyia longipalpis*, females prefer to mate with males of intermediate age, and reduced egg hatchability has been found when females were paired with older males. However, no measures of post-hatching offspring performance were reported (Jones *et al.*, 2000), even though if females are to benefit via mate choice, then some aspect of offspring performance (e.g. survival) needs to be enhanced.

In addition to elements of cooperation, mating and mating decisions can involve considerable conflict between the sexes (Parker, 1979; Alexander *et al.*, 1997). Intersexual conflict of this kind may lead to sexually antagonistic co-evolution. This can involve chase-away sexual selection, where adaptations in one sex are followed by counter-adaptations in the other and so on, thus generating cycles of antagonistic co-evolution (Rice and Holland, 1997; Holland and Rice, 1998). Under this scenario, sexual conflict over mating favours males evolving means to entice or coerce females into mating beyond the female optima, and females are then selected to evolve resistance to males to reduce mating costs. Quantitative genetic modelling suggests that mate choice can evolve as a side-effect of selection on females to reduce direct mating costs (Gavrilets *et al.*, 2000), although benefits via the females' sons were not included in this model. As above, it is often difficult to differentiate between Holland and Rice's chase-away conflict-driven model of sexual selection and more traditional sexual selection models. Nevertheless, conflict over mating can generate substantial fitness costs to both sexes (e.g. Chapman *et al.*, 1995; Rice, 1996; Hosken, 2001). For example, in *Drosophila melanogaster* seminal fluid proteins transferred to females during copulation stimulate egg production and laying and facilitate sperm storage. However, they also reduce female longevity and can reduce egg hatching (reviewed in Kubli, 1996; Wolfner, 1997; Chapman *et al.*, 2001).

We tested the age-based indicator mechanism, and further investigated mating costs in the dung fly *Sepsis cynipsea*. These flies are one of the most common dung breeders found in cattle pastures and are unusual in that males guard mates before copulation, and copulate only after females have laid their current clutch (Parker, 1972a,b). Pre-copulatory mate guarding is very uncommon in insects (Alcock, 1994) and is not known to occur in other fly genera (Parker, 1972a). It is important to note, however, that male *S. cynipsea* apparently cannot force copulations as females are typically larger (Parker, 1972b) and must lift their abdomens to allow genital contact. Sexual conflict over mating is very obvious, with females being extremely reluctant to mate. Reluctance is manifested as vigorous shaking behaviour when males mount females (Parker, 1972a; Ward, 1983) and is in part due to injuries, and the resulting longevity costs, associated with copulation (Blanckenhorn *et al.*, 2002). Copulation greatly increases a female's likelihood of death, but there seems to be no fertility benefit to females associated with increased number of copulations. In addition, only about 40% of field pairs eventually copulate after pairing and oviposition, with the other 60% of females managing to reject males (Ward *et al.*, 1992). Nevertheless, females tend to copulate more quickly with larger males (Ward, 1983). In addition, while females determine to a large extent if copulation occurs, males appear largely to determine copula duration (Blanckenhorn *et al.*, 2000; Martin and Hosken, 2002). Female age and mating status also positively influence copula duration (Martin and Hosken, 2002), but the effects of male age on mating behaviour have not been investigated to date. As outlined above, there is overt conflict over mating in this species and copulation dramatically reduces female longevity. However, even if conflict is rife, females nevertheless have to decide which male(s) to copulate with. This is especially true if females may only get one or a few chances to mate, as may be the case in this species.

We carried out a series of experiments to determine the effect of male age on mating probability in *S. cynipsea*. Additionally, we examined the effect of male age on several direct and indirect measures of female fitness, while further scrutinizing potential mating costs.

METHODS

About 70 female flies were collected from the field and brought to the laboratory during the summer of 1999. They were allowed to lay their clutches and the offspring were then mixed and reared in several population cages ($n = 8$). Each cage contained about 250 flies; each generation individuals were transferred between cages to minimize inbreeding effects. Flies were provided with sugar, pollen and water *ad libitum*, and fresh dung was supplied approximately every third day. Flies had been in the laboratory for approximately 35 generations before the experiments were conducted.

For the experiments described below, vials of dung, the larval food, were removed from the population cages and emerging flies were separated by sex and housed until the experiments began. This was done daily as males take about 24 h to reach sexual maturity. Males were collected so that by the time the experiments took place, three male age classes were available: 2-week-old, 1-week-old and 2-day-old virgin males. This covers a wide range of ages the flies typically attain in our cultures: the maximum longevity of flies is about 5–6 weeks in the laboratory. All females were virgins and 5–7 days old.

Females were then randomly assigned to a single male from one age class ($n = 200$ males per class), and pairs of flies were placed into glass vials. We recorded (by direct observation) if the flies interacted (we used male mounting as a conservative measure of interaction) and/or copulated, as well as copulation duration for each copulation. This experimental design does not confound mating order with treatment as females are only confronted with one male from one age class. Accordingly, if male age influences the probability of mating, it should be apparent with this no-choice design.

Females that copulated were allowed to lay their eggs in a dung portion large enough to ensure maximal larval survival (O.Y. Martin, unpublished). They were provided with fresh dung every 2 days for 10 days and were then frozen, but some females that copulated did not lay eggs. We counted the number of eggs laid and the number of offspring emerging, and recorded the delay between copulation and egg laying, development time and post-emergence survival. Emergence and survival were assessed approximately every 12 h (morning and evening). We did not allow offspring to feed post-emergence, but they were supplied with water *ad libitum*. Thus survival was purely dependent on the quantity of food stored during the larval phase. This maximally stressful treatment increases the likelihood of detecting effects that may be obscured by more benign environments (e.g. Wilkinson, 1984; Hoffman and Parson, 1991; Moret and Schmid-Hempel, 2000). The body sizes of males and females that copulated were also recorded, as were those of a random sample of non-copulating pairs. The experiment was carried out in two blocks 1 week apart with 300 pairs being tested in each block.

The data were screened to check the assumptions of parametric tests, transformed when necessary, and analysed using logistic regression (copulation = yes/no) and GLM. However, for two variables, development time and delay, we could not normalize the data distribution with any transformation. In addition, for development and survival durations, we used family means because there is no really satisfactory way to deal with unequal sample sizes in repeated-measures analysis. Similarly, we did not use multivariate analysis to test for overall effects of male age, since some of the dependent variables were likely to influence each other. For example, egg number may be associated with mean offspring survival if egg number and size trade-off, or if in larger clutches there is increased food competition. Therefore, in the analysis of survival we included egg number, and so on (see Results).

RESULTS

Mounting, copulation and copula duration

Logistic regression indicated that female size influenced whether flies interacted (i.e. males mounted the females) ($n = 264$, $\beta = 2.8$, $r = 0.12$, Wald = 6.43, $P = 0.011$), with larger females being more likely to be mounted, but neither male age nor male size had a significant effect (Wald < 1.60, $P > 0.21$). We restricted our analysis of copulation to only those pairs that interacted, since we could be sure that they had been aware of each other during the mating trial. This logistic regression indicated male age had no influence on whether copulation occurred ($n = 264$, $r = 0.03$, Wald = 4.33, $P = 0.11$). However, both male and female size did, with larger members of both sex more likely to copulate (female: $\beta = 5.33$, $r = 0.23$, Wald = 16.27, $P = 0.0001$; male: $\beta = 3.5$, $r = 0.11$, Wald = 5.23, $P = 0.022$). If we included size difference in the analysis, this was not significant (Wald = 2.96, $P = 0.08$); if the whole data set was employed (i.e. not only those that interacted), again only male and female body size had a significant effect. Overall, about 14% of the variance in whether or not copulation occurred was explained by our model.

GLM (analysis of covariance) indicated copulation duration was weakly influenced by male age ($F_{2,86} = 3.33$, $P = 0.046$). This effect was significant regardless of whether male and female size or male and female size differences in a pair were included as covariates. Fisher's PLSD indicated the difference was between young and middle-age males only ($P = 0.002$), with other comparisons not statistically significant (young *vs* old, $P = 0.137$; middle *vs* old, $P = 0.141$) (Fig. 1). Neither female nor male size influenced copula duration ($F < 0.07$, $P > 0.78$), nor did their size difference ($F_{1,86} = 0.22$, $P = 0.637$).

Fig. 1. Copulation duration for the three male age classes used in the experiments (mean ± standard error). Young males were 2 days old, middle-age males 1 week old and old males 2 weeks old.

Potential female benefits: egg laying, clutch size, development time, emergence rates and survival

The delay between copulation and egg laying was negatively associated with copula duration ($\beta = -4.07$, $F_{1,55} = 8.84$, $P = 0.004$) (Fig. 2). However, this effect appears to be driven by one pair with a very short copulation (solid circle in Fig. 2); when this is removed, the association is no longer significant ($P = 0.062$). (*Note*: This pair produced no offspring, so it appears the copulation was not successful.) Delay was positively associated with the number of eggs laid [with or without the outlying datum: $F_{1,55(54)} = 26.51$ (24.3), $P = 0.0001$ (0.0001)]. There was also a block effect, with females from our first block laying faster ($F_{1,55} = 71.3$, $P = 0.0001$), but none of male age, male or female size, or size difference had a significant effect (all $F < 2.7$, all $P > 0.1$). However, after removal of the one pair with a very short copulation (solid circle in Fig. 2), there was a trend for a negative association between female size and delay ($F_{1,54} = 3.75$, $P = 0.058$).

There was also a block effect on clutch size, with females from block 1 tending to lay more eggs ($F_{1,55} = 16.24$, $P = 0.0002$). As expected from the previous analysis, delay was positively associated with clutch size (we included it in this analysis since it is not clear if the delay influences, or is influenced by, clutch size) ($F_{1,55} = 26.51$, $P = 0.0001$) (see Fig. 3). There was also a trend for a positive copulation duration effect on egg number ($F_{1,55} = 3.43$, $P = 0.0691$). Male age again had no influence ($F_{2,55} = 0.05$, $P = 0.94$), and neither did male body size nor size differences ($F < 0.38$, $P > 0.54$), but there was a weak tendency for larger females to lay more eggs ($F_{1,55} = 2.7$, $P = 0.106$) (and see Ward, 1983). If the pair with the short copulation is removed from this analysis, there are no qualitative changes, except the copula duration effect becomes much weaker ($P = 0.096$).

A GLM model with mean, minimum and range of development times as the dependent variables and male age, block, male and female body sizes, copula duration, clutch size and delay between copulation and egg laying was not significant ($P = 0.68$). Univariate analyses also revealed these variables were not influenced by male age ($F_{2,40} < 1.72$, $P > 0.19$) or by any of the other variables (e.g. size, copula duration, clutch size, all F-values < 1.7, all $P > 0.2$; $t < 1.7$, $P > 0.10$), except for a block effect, which was significant ($F_{1,40} = 41.71$, $P = 0.0001$). Univariate analysis indicated the block effect was significant only for mean

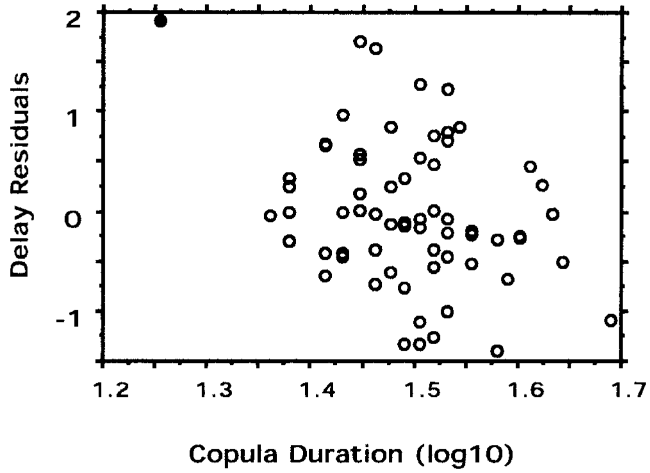

Fig. 2. The negative association between copula duration and the delay between copulation and egg laying. Shown are the delay residuals after controlling for the other effects in the analysis. The solid circle in the top left-hand corner of the figure is a pair with an exceptionally short copulation; when this is removed from the analysis, the association is no longer significant.

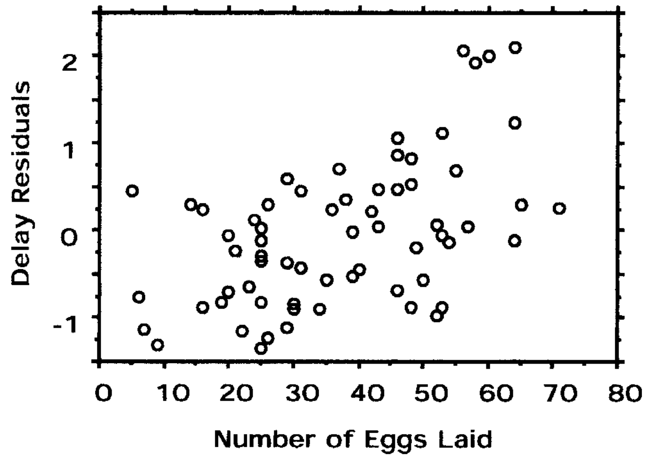

Fig. 3. The positive association between clutch size (number of eggs laid) and the delay between copulation and egg laying. Shown are the delay residuals after controlling for the other effects in the analysis.

and minimum development times (both $P < 0.001$) [again note the pair with the short copulation (solid dot in Fig. 2) had no offspring that emerged].

GLM indicated the proportion of flies that emerged from each clutch was not significantly influenced by male age ($F_{2,49} = 0.64$, $P = 0.53$), but was strongly negatively associated with copula duration ($\beta = -1.62$, $F_{1,49} = 9.31$, $P = 0.0037$; Fig. 4). Other than a block effect ($F_{1,49} = 5.25$, $P = 0.0263$), no other variable (clutch size, male or female body size or size

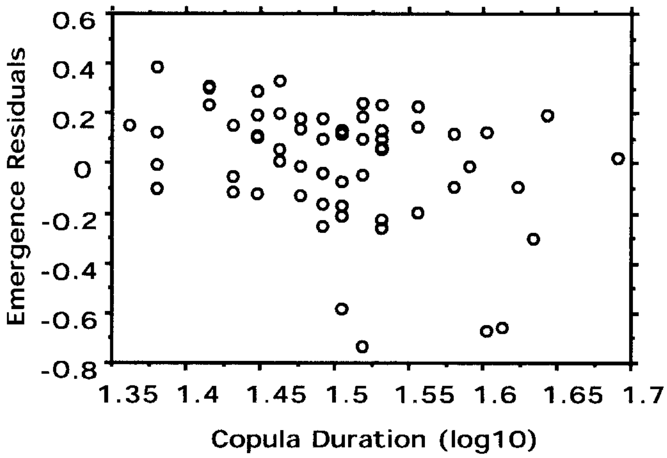

Fig. 4. The negative association between copula duration and the number of offspring to emerge. Shown are the emergence residuals after controlling for the other effects in the analysis.

differences, delay between copulation and egg laying) significantly influenced emergence (all $F < 3.2$, all $P > 0.08$).

GLM with mean, minimum and range of offspring survival as the dependent variables revealed that they were not significantly associated with any of our measured variables in multivariate or univariate comparisons (overall model with male age, block, male and female body sizes, copula duration, clutch size and delay between copulation and egg laying as independent variables: $F_{5,41} = 1.216$, $P = 0.238$; male age as predictor in a univariate analysis of survival: $F_{2,41} = 0.283$, $P = 0.754$; all other $P > 0.104$), except for a weak positive association between delay between copulation and egg laying and survival (mean delay = 6.6 days, range 5–8 days; $t = 2.077$, $P = 0.044$).

DISCUSSION

Male age has been proposed as one indicator mechanism that females could use to obtain indirect fitness benefits and, although it remains a controversial idea, recent ESS modelling suggests such a mechanism is feasible (Kokko, 1998; see Beck and Powell, 2000; Beck *et al.*, 2002). In addition, there is some evidence that females use male age in mate choice in some instances. For example, female red-winged blackbirds typically select older males for extra-pair copulations (Weatherhead and Boag, 1995; see Grant and Grant, 1987; Dickinson, 2001), and in a sandfly, females favour middle-aged males (Jones *et al.*, 2000). However, we could find no evidence that male age influenced the likelihood of copulation, which is largely determined by females in *Sepsis cynipsea*. As with all negative results, the failure to find an effect could represent Type II error. However, because we employed fairly large sample sizes (e.g. n for age and copulation probability = 264), we do not think this is the case. It is also possible that the use of non-virgin males would have revealed an effect (although more recent results indicate this is not the case: O.Y. Martin and D.J. Hosken, unpublished), or that after so long in the laboratory, flies no longer behave as they would in nature. We do not think this latter point provides a satisfactory explanation for the lack of effect though, since longevity would probably be more favoured in our laboratory environment, especially because the culture technique does not involve discarding living flies at any stage. That is, flies that live longer can potentially reproduce more often, although this may not be entirely true if reproductive output trades off against longevity (one of the criticisms of the male age indicator mechanism; Hansen and Price, 1995). Nevertheless, this lack of age effect is entirely consistent with the fact that male age apparently did not influence female fecundity or fertility, or offspring survival or development time. This is in spite of a weak age effect on copula duration which influenced some of these traits. However, *post-hoc* analyses indicated only middle-aged males and young males differed significantly in copula duration, so the lack of age effect on the offspring/female fitness traits is not surprising. Therefore, it would appear that, at least within the confines of our experiment, females have little to gain by paying attention to male age. This is in contrast to a previous study which showed males are sensitive to female age, increasing copula duration when females are older (Martin and Hosken, 2002). It should be noted, however, that females need not be sensitive to male age just because the opposite is true and that in a previous study of copula duration (Martin and Hosken, 2002) only 1-week-old males were used.

While male age had no influence on the probability of copulation, as in previous studies (e.g. Ward, 1983), we found that male size influenced mating success and that larger females were also more likely to copulate. This may appear counterintuitive, since mating is very costly (Blanckenhorn *et al.*, 2002) and larger females should be able to thwart male mating attempts more easily than smaller females. However, larger females may also be less susceptible to injuries inflicted by the aedeagus if they have a larger reproductive tract, and there is evidence in other insects that features of the female reproductive tract scale with body size (e.g. Gage, 1998; Hosken and Ward, 2000). So larger females could obtain the sperm they require at a lower cost than smaller females. Although we did not examine the influence of female size on copulatory costs in our previous work, we did find that many females had no internal injuries after copulation (Blanckenhorn *et al.*, 2002), consistent with the above explanation. In the present study, we also found that copula duration was not influenced by size or size differences, as previously reported (Martin and Hosken, 2002). This lack of a size difference effect potentially contradicts the explanation presented above for the effect of female size. However, if male genitalia are negatively allometric (e.g. Eberhard *et al.*, 1998), a size difference effect would not be expected.

Although there was no male age effect on mating or our fitness surrogates, copulation duration was significantly associated with several variables we measured. Longer copulation led to shorter delays between copulation and egg laying, although this effect appears to be driven largely by one pair with a very short copulation that resulted in no offspring. Moreover, when this single datum was removed, the effect was no longer significant. Nevertheless, such an association could be due to longer copulations with females that are about to lay large clutches, as occurs in a moth where copulation is longer with fatter females (Gage, 1998), or longer copulations resulting in greater transfer of oviposition stimulant (e.g. Kubli, 1996). However, it should also be noted that, in free-living flies, many copulations occur after females have just laid their clutch. This renders the association between copula duration and delay somewhat enigmatic and for this reason we believe the association is an artefact. Nevertheless, delay was positively associated with clutch size, suggesting larger clutches take longer to develop.

Copulation duration was also negatively associated with the proportion of flies that emerged. We currently do not know if this was due to lower hatch or emergence rates.

Nevertheless, this is an intriguing result, especially since we previously showed that female fertility is maintained for at least two clutches with one copulation, and increasing copulation number does not appear to increase fertility (Blanckenhorn *et al.*, 2002). It is feasible that conflict over mating causes the negative association between copula duration and emergence. Perhaps this is due to longer copulations causing increased damage to females. Previous work has shown that increased copulation number decreases female longevity (Blanckenhorn *et al.*, 2002), so it is possible that longer copulations damage females more and cause the reduction in emergence. Previously, we found no effect of copulation number on emergence (Blanckenhorn *et al.*, 2002), although we did not look at copulation duration *per se* and hence the lack of a significant effect may be due to considerable overlap when using categorical assignment (i.e. some females with two copulations will have a total copula duration less than some single copulation females). It is also possible that lower-quality males have longer copulations and the observed outcome is a consequence of their poor quality (which would have to be inherited by the offspring), although this appears to be the least plausible explanation (why mate with low-quality males?). Therefore, costs of sexual conflict over mating in this species may be the explanation, although it is unclear whether this was caused by lower hatch rates, lower survival or both. However, similar effects have been documented in *D. melanogaster*, in which male seminal fluid proteins reduce egg hatching success, at least in the short term (Prout and Clark, 2000; Chapman *et al.*, 2001).

Development time was not influenced by any of our predictor variables (except for a block effect, possibly due to differences in dung quality) and even maternal size had no effect. In this species, development time appears to be relatively inflexible (Blanckenhorn, 1999) and, although we found a reasonable amount of variation, we could not explain any of it. It is possible that our measures were too coarse (i.e. every 12 h) and that more frequent observation would have revealed some effect. Similarly with offspring survival, it is possible that more frequent measurement may have found some statistically significant associations.

In conclusion, we found no influence of male age on mating success and we could not detect any direct or indirect benefits to females that mated with older males. Female *S. cynipsea*, therefore, do not appear to use male age as a criterion for mate choice and our results provide no support for the age-based indicator mechanism in this species. However, larger clutches appear to take longer to develop and, interestingly, when copulations were longer, fewer offspring emerged, which is consistent with collateral costs associated with sexual conflict over mating.

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