

The Effects of Copulatory Courtship on Differential Allocation in the Red Flour Beetle *Tribolium castaneum*

Martin Edvardsson^{1,2} and Göran Arnqvist¹

Accepted July 26, 2004; revised November 5, 2004

Mate attractiveness is known to sometimes influence female reproductive investment (i.e. differential allocation) and the sex ratio of her offspring (i.e. sex allocation). Males of the red flour beetle *Tribolium castaneum* rub the lateral edges of the females' elytra with their tarsi during copulation. This behavior is important for paternity success when females have mated with two males. We manipulated female perception of the leg rubbing behavior by tarsal ablation and tested whether this behavior is also favored through differential allocation and whether it affects sex allocation. We found some support for an increase in female oviposition rate in response to intensive leg rubbing but failed to find any support for an effect on sex allocation. The overall sex ratio of offspring was slightly male biased but females did not appear to regulate the sex ratio of their offspring.

KEY WORDS: cryptic female choice; copulatory courtship; differential allocation; sex allocation; sexual selection; *Tribolium castaneum*.

INTRODUCTION

Postmating female influence over male paternity success, i.e., cryptic female choice, is receiving more and more interest (Eberhard, 1996). Altering reproductive investment, such as number of eggs or the investment in each

¹Department of Animal Ecology, Evolutionary Biology Centre, Uppsala University, Uppsala, Sweden.

²To whom correspondence should be addressed at Evolutionary Biology Centre, Uppsala University, Norbyvägen 18d, SE-762 36 Uppsala, Sweden. e-mail: martin.edvardsson@ebc.uu.se.

offspring, in response to male characters is one way through which females can exercise such cryptic choice (Thornhill, 1983; Eberhard, 1996). According to the differential allocation hypothesis, females facing trade-offs between investment in current and future reproduction should vary their reproductive investment depending on the attractiveness of their partner and hence the expected value of each offspring (Burley, 1986; Sheldon, 2000). In polyandrous species without paternal care of offspring, males will benefit from any trait that increases female egg production since this will elevate the number of offspring they sire before their mates remate with other males. To test whether a male character affects female reproductive investment it is necessary to manipulate female perception of the character in question. Simply demonstrating a correlation between a male character and female reproductive investment is not sufficient because this can be caused by correlations between the character value and other traits which influence female investment, such as quality of male territory and nuptial gifts, etc. (Sheldon, 2000). Several studies have attempted to test the differential allocation hypothesis experimentally (see Sheldon, 2000 for a review). Among these, studies on two bird species have involved physical manipulation of male characters that proved to be important for female reproductive investment: leg band color and leg band asymmetry in the zebra finch *Poephila guttata* (Burley, 1986, 1988; Swaddle, 1996) and tail length in the barn swallow (de Lope and Møller, 1993). Very few studies have looked at differential allocation in response to male characters in invertebrates, but it has been demonstrated in scorpionflies (Thornhill, 1983), field crickets (Simmons, 1987), and butterflies (Wedell, 1996).

Sex allocation theory predicts that when the expected fitness of male and female offspring differs, parents should, if possible, invest differently in sons and daughters depending on which sex has the highest expected fitness (Trivers and Willard, 1973; Charnov, 1982; Frank, 1990). Males generally have a greater potential reproductive rate than females and hence also a larger variance in fitness with the fittest males being fitter than the fittest females. Therefore, if attractiveness were important for male fitness and also heritable, then sons of attractive males would be expected to have a higher fitness than daughters of attractive males. Females would then benefit from skewing the sex ratio of their offspring toward sons if they had mated with a male that was relatively attractive (Ellegren *et al.*, 1996). Again, to test whether females bias the sex ratio of their offspring in response to a male character, it is necessary to manipulate female perception of that character. A significant association between female perception of the male character and offspring sex ratio would show both that females are able to manipulate the sex ratio of their offspring and that they do so in response to the male character. This has been done in blue tits where it has been found

that variation in male ultraviolet color influences the sex ratio of offspring (Sheldon *et al.*, 2000).

Males of the red flour beetle, *Tribolium castaneum*, rub their legs along the edges of the female elytra in bouts of one to a few strokes during copulation (Wojcik, 1969; Edvardsson and Arnqvist, 2000). Female perception of this copulatory courtship behavior can be manipulated by cutting off the outer parts of male legs. A previous study has shown that female perception of male leg rubbing is important for sperm precedence and paternity success (Edvardsson and Arnqvist, 2000). Females apparently favor males that perform more active leg rubbing, i.e., more bouts per minute, and thus this behavior is under selection by cryptic female choice. The possibility to manipulate female perception of a male trait important for female choice, makes *T. castaneum* an ideal system for testing hypothesis about both differential allocation and sex allocation. By manipulating female perception of the male trait it is possible to isolate the effects of the trait in question while avoiding effects of any correlated traits.

Red flour beetles can live for many months and during their lifetime females mate with many males (Sokoloff, 1974). They should therefore trade current with future reproductive investment and this tradeoff may be influenced by male copulatory courtship. Males with more active copulatory courtship achieve a high paternity success when mating with already mated females. If the intensity of the copulatory courtship behavior is inherited, females could benefit from biasing the sex ratio of their offspring toward sons when they have mated with males with active copulatory courtship behavior. It is not known whether females of *T. castaneum* are able to control the sex ratio of their offspring. Previous studies have found the average sex ratio to be close to unity although sometimes either significantly female or male biased (Sokoloff, 1974).

In the current study, we test whether differential allocation and sex allocation occur in response to male leg rubbing in female red flour beetles. To do this, we manipulate female perception of male leg rubbing, quantify this behavior, and relate it to offspring production and the sex ratio of the offspring. To our knowledge, the current study is the first attempt to link a copulatory courtship behavior to differential allocation and also the first to investigate whether females exercise sex allocation based on their perception of copulatory courtship.

METHODS

The *Tribolium castaneum* wild-type strain Georgia (GA-1) was used in this experiment. This strain was provided by the US Grain Marketing

Research Laboratory in Manhattan, Kansas, USA and had been kept in our lab for 2 years at the time of this study. Georgia is a wild-type strain that was originally collected in a farmer's corn bin in Georgia (USA) in 1980 and is genetically heterogeneous (R. W. Beeman, personal communication). All beetles were maintained at 30°C and 70% relative humidity in dark incubators. A mixture of 19 parts whole-wheat flour and 1 part dry brewers yeast was used as a culture medium (Sokoloff, 1972). All individuals used in the experiment were sexed as pupae. They were mated as virgins at 10–18 days posteclosion. Mating experiments and behavioral observations were carried out at room temperature (20–25°C) and under diffuse room lighting.

Each female was mated to either a normal male or a male that had his two midlegs manipulated. All males were anesthetized, and males assigned to the manipulation treatment had their midlegs truncated at the midpoint of the tibia. Anesthetization was achieved by exposing males to CO₂ for 60 s. Manipulated males perform the leg rubbing behavior but do not reach the edges of the female elytra with the truncated legs. The manipulation and anesthetization does not shorten copulation duration, affect number of sperm inseminated, or impair the males' vigor (see Edvardsson and Arnqvist, 2000).

All matings were carried out in circular arenas (10 mm high, 35 mm diameter) where the bottom was covered with a thin layer of flour-yeast medium. All individuals were preconditioned prior to matings, by keeping beetles of both sexes isolated individually in separate arenas at room temperature for 24 h. Males assigned to the manipulation treatment were manipulated prior to this isolation period. Males were introduced into the females' arenas and the behavior of the beetles was observed under 6× magnification in a dissecting microscope. When beetles mated, copulation duration and every bout of strokes of each leg performed by the males were recorded using the EthoLog 2.2.5 software (Otoni, 2000). Pairs that had not mated within 1 h were discarded from the experiment.

When a pair had mated, the female was transferred to a vial containing 30 g fresh medium and the male was frozen. Females were allowed to oviposit for 4 days and were then transferred to a new vial. Four days later they were transferred to a third vial and after 4 more days the females were frozen. Adult offspring were counted and sexed through examination of their extruded genitalia.

The body size of mated males and females was measured as the distance between the anterior edge of the pronotum and the posterior end of the last sternite (number VII) on the ventral side, using a digitizing tablet placed under a side-mounted camera lucida attached to a dissecting microscope.

Statistical analyses were carried out using SYSTAT 10 and GLIM. An analysis of the egg production data from the sperm precedence experiment of Edvardsson and Arnqvist (2000) suggested that female perception of male leg rubbing was indeed important for her offspring production. In that experiment, females were mated first to a normal male and then, 24 h later, to either a normal male or a male that had had one or two of his legs ablated. Females that had mated to two normal males produced significantly more offspring over a 7-day period than did females that had mated to one normal male and one male with ablated legs (mean 102.4 vs. 89.1; $t_{69} = 2.31$, $P = 0.024$). Therefore, we expected leg rubbing to have a positive effect on offspring production *a priori*, and consequently used one-tailed *p*-values to evaluate the effect of leg rubbing on offspring production.

RESULTS

Males copulated successfully within an hour in 94 out of 209 cases. This is similar to what we have observed in previous experiments. Manipulated males had a somewhat lower rate of success than normal males with 43 out of 108 manipulated males mating successfully compared to 51 out of 101 for normal males but the difference was not significant ($\chi^2_1 = 2.4$, $P = 0.12$).

We analyzed variance in offspring production in a multivariate general linear model (Table I). Average copulation duration was 61.5 s with a standard deviation of 38.9 s. There was no significant difference in copulation duration between males of the two treatments. Copulation duration had no significant effect on offspring production. Although offspring production did increase with female size there were no significant effects of either female or male size. There was a significant interaction between the rate of

Table I. The Results of a General Linear Model with Offspring Production as the Response Variable

Source	SS	df	F-ratio	P
Copulation duration	410.1	1	0.26	0.616
Leg manipulation	1097.9	1	0.68	0.206*
Female size	3249.5	1	2.02	0.160
Male size	667.5	1	0.42	0.522
Rate of leg rubbing	19.1	1	0.01	0.457*
Copulation duration ⁽²⁾	178.2	1	0.11	0.740
Leg manipulation × rate of leg rubbing	4755.8	1	2.96	0.045*
Error	133261.7	83		

**P*-values are one-tailed.

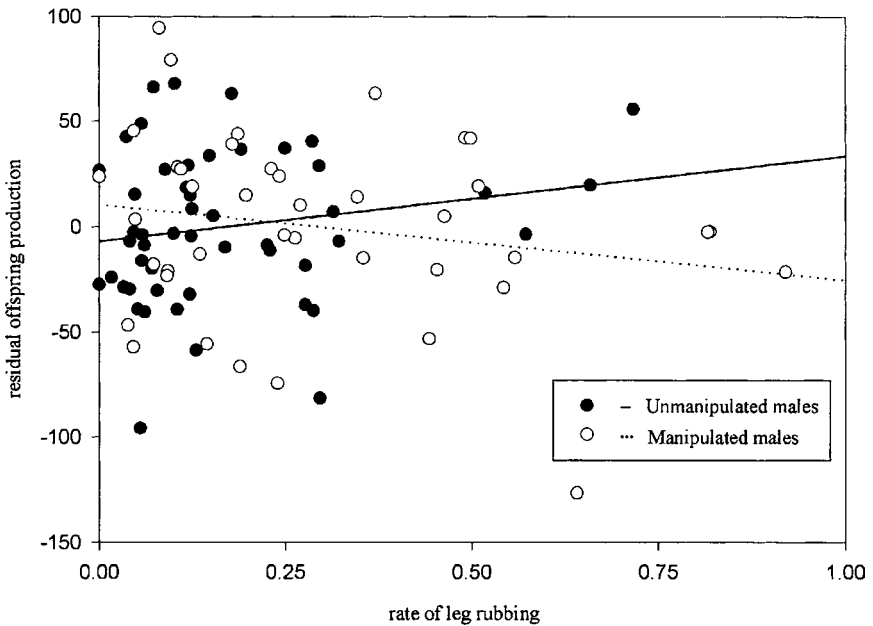


Fig. 1. The relationship between offspring production and rate of male leg rubbing (bouts of rubbing per second) in unmanipulated and manipulated males. Residual offspring production was generated by taking the residuals from a general model, identical to the one presented in Table I apart from the exclusion of the rate of leg rubbing and its interaction with leg manipulation. See Table I for statistical evaluation.

male leg rubbing and leg manipulation (see Fig. 1). Offspring production increased with rate of male leg rubbing in unmanipulated males but not in manipulated males. However, when including only unmanipulated males, the relationship between leg rubbing and offspring production was not quite significant (linear regression, $P_{\alpha/2} = 0.099$). There was a tendency for a decrease in offspring production with increased leg rubbing in manipulated males (Fig. 1).

We analyzed the variance in sex ratio with a generalized linear model of the number of sons, using a logit link function and binomial errors with the total number of offspring per female as the binomial denominator. The model including leg rubbing, leg manipulation, and the interaction between leg rubbing and leg manipulation was not significant ($\chi^2_3 = 2.71$, $P = 0.44$). The overall sex ratio, calculated from all offspring ($N = 13962$) was 0.51. This is a significant male bias ($\chi^2_1 = 5.94$, $P = 0.015$). There was, however, no repeatability within females over the three time periods and the sex ratio did not change over time (Table II).

Table II. Two-Way ANOVA with Female and Time Period as Factors and with Offspring Sex Ratio as Response Variable

Source	SS	df	F-ratio	P
Female	0.529	93	0.89	0.737
Period	0.008	2	0.65	0.524
Error	1.190	186		

Note. The residuals did not differ from normality (Kolmogorov-Smirnov test, $P = 0.504$).

DISCUSSION

Female response to male copulatory courtship differed significantly between females, which perceived and those which did not perceive male stimuli. While postmating oviposition rate did not increase with the intensity of male leg rubbing among females mated to males with ablated legs, these variables were positively related among females mated to unmanipulated males. The latter relationship was, however, only marginally significant when analyzed separately. In a previous study, females mated to two males produced significantly more offspring when the second male to mate had normal legs compared to when the second male had ablated legs (Edvardsson and Arnqvist, 2000). These effects cannot be attributed to males with ablated legs producing smaller ejaculates with less of substances stimulating female egg production than normal males. By freezing females in liquid nitrogen immediately after copulation, before they could discard any sperm, and then counting the number of sperms present in the female reproductive tract, we found that males with ablated legs do not transfer a lower number of sperms than do control males (Edvardsson and Arnqvist, 2000). Taken together, the results from the previous and the current study provide some support for differential allocation in red flour beetles.

We note, however, that the differential allocation effect does not appear to be strong, and we suggest that this mechanism will only generate weak selection under a natural range of variation in male copulatory courtship behavior. Nevertheless, it does provide an additional way in which the reproductive success of male red flour beetles can be influenced by cryptic female choice. Males with intensive copulatory courtship are thus rewarded both through sperm precedence and through a small increase in female egg production, raising the number of eggs the males' sperm can fertilize before they have to compete with the sperm from future matings. A few prior studies have reported a positive covariance between male sperm precedence and female offspring production rate in insects (Clark *et al.*, 1995; Clark and Begun, 1998; Arnqvist and Danielsson, 1999), but have not

been able to relate this covariance to male traits causally. A positive relationship between these sources of variance in male paternity success have important consequences in terms of postmating sexual selection, since successful males will have a twofold advantage: they will not only achieve a high relative fertilization success but will also enjoy a high female offspring production rate.

At the moment, it is only possible to speculate over the mechanism by which males with intensive leg rubbing are favored through differential allocation. However, one mechanism seems to be particularly plausible. In a previous study, we showed that female perception of male leg rubbing is important for male relative fertilization success (Edvardsson and Arnqvist, 2000). Furthermore, Bloch Qazi *et al.* (1998) provided evidence for an active female role in transferring sperm within the reproductive tract of female red flour beetles. This suggests that the intensity of male leg rubbing influences how much of the received sperm a female transfers to her sperm storage organs and how much she discards. The uptake and storage of any substances included in the ejaculate is likely to be affected in the same way as sperm uptake and storage. Males of many insect species have been found to include substances that stimulate female egg production in their ejaculates (see Eberhard, 1996 for a review). A substance that is thought to function in this way has been found in *Tenebrio molitor*, confamiliar to *T. castaneum* (Feng and Happ, 1996). Therefore, it seems likely that leg rubbing not only influences the uptake of sperm but also of substances that increase female egg production, causing the relationship between leg rubbing and number of offspring and also covariance between sperm precedence and female offspring production rate. More studies are needed to evaluate the effects of copulatory courtship in other species, but it seems plausible that these behaviors are favored in males because they increase uptake and/or retention of both sperm and secondary substances.

Female red flour beetles do not seem to manipulate the sex ratio of their offspring in response to the male copulatory courtship behavior. Nor do females appear to "control" the sex ratio of their offspring at all under the conditions in our study since the repeatability of offspring sex ratio within female was far from significant over the three time periods. This could be a consequence of physiological constraints but it could also be due to that the relative fitness of sons of attractive males simply has not been high enough for sex allocation to evolve in *T. castaneum*. The overall sex ratio in our study was slightly male biased (0.51). This could be caused by a number of factors such as sex-biased egg and/or larval mortality or an intraejaculate sperm competition advantage for sperm giving rise to males.

In all, female perception of male leg rubbing in *T. castaneum* has important effects on male paternity success mediated through sperm

precedence and, to some extent, female offspring production but females do not manipulate the sex ratio of their offspring in response to this male behavior.

ACKNOWLEDGMENTS

Thanks to Tim Hipkiss for suggesting that we should look at sex ratios. We would also like to thank Claudia Fricke for practical help with the experiment and José Andres, Urban Friberg, Claudia Fricke, Ted Morrow, Tina Nilsson, and two anonymous reviewers for comments on earlier drafts of this paper. Financial support was provided by the Swedish Research Council.

REFERENCES

- Arnqvist, G., and Danielsson, I. (1999). Postmating sexual selection: The effects of male body size and recovery period on paternity and egg production rate in a water strider. *Behav. Ecol.* **10**: 358–365.
- Bloch Qazi, M. C., Aprille, J. R., and Lewis, S. M. (1998). Female role in sperm storage in the red flour beetle, *Tribolium castanum*. *Comp. Biochem. Physiol.* **120**: 641–647.
- Burley, N. (1986). Sexual selection for aesthetic traits in species with biparental care. *Am. Nat.* **127**: 415–445.
- Burley, N. (1988). The differential allocation hypothesis: An experimental test. *Am. Nat.* **132**: 611–628.
- Charnov, E. L. (1982). *The Theory of Sex Allocation*, Princeton University Press, Princeton, NJ.
- Clark, A. G., Montserrat, A., Prout, T., Harshman, L. G., and Langley, C. H. (1995). Variation in sperm displacement and its association with accessory gland protein loci in *Drosophila melanogaster*. *Genetics* **139**: 189–201.
- Clark, A. G., and Begun, D. J. (1998). Female genotypes affect sperm displacement in *Drosophila*. *Genetics* **149**: 1487–1493.
- de Lope, F., and Møller, A. P. (1993). Female reproductive effort depends on the degree of ornamentation of their mates. *Evolution* **47**: 1152–1160.
- Eberhard, W. G. (1996). *Female Control: Sexual Selection by Cryptic Female Choice*, Princeton University Press, Princeton, NJ.
- Edvardsson, M., and Arnqvist, G. (2000). Copulatory courtship and cryptic female choice in red flour beetles. *Proc. R. Soc. Lond. B* **267**: 559–563.
- Ellegren, H., Gustafsson, L., and Sheldon, B. C. (1996). Sex ratio adjustment in relation to paternal attractiveness in a wild bird population. *Proc. Natl. Acad. Sci. U.S.A.* **93**: 11723–11728.
- Feng, X., and Happ, G. M. (1996). Isolation and sequencing of the gene encoding Sp23, a structural protein of spermatophore of the mealworm beetle, *Tenebrio molitor*. *Gene* **179**: 257–262.
- Frank, S. A. (1990). Sex allocation theory for birds and mammals. *Annu. Rev. Ecol. Syst.* **21**: 13–55.
- Ottoni, E. B. (2000). EthoLog 2.2: A tool for the transcription and timing of behavior observation sessions. *Behav. Res. Methods Instrum. Comput.* **32**: 446–449.
- Sheldon, B. C. (2000). Differential allocation: Tests, mechanisms and implications. *Trends Ecol. Evol.* **15**: 397–402.

- Sheldon, B. C., Andersson, S., Griffith, S. C., Örnborg, J., and Sendecka, J. (2000). Ultraviolet color variation influences blue tit sex ratios. *Nature* **402**: 874–877.
- Simmons, L. W. (1987). Female choice contributes to offspring fitness in the field cricket *Gryllus bimaculatus* (De Geer). *Behav. Ecol. Sociobiol.* **21**: 313–321.
- Sokoloff, A. (1972). *The Biology of Tribolium with Special Emphasis on Genetic Aspects*, Vol. 1, Oxford University Press, Oxford, UK.
- Sokoloff, A. (1974). *The Biology of Tribolium with Special Emphasis on Genetic Aspects*, Vol. 2, Oxford University Press, Oxford, UK.
- Swaddle, J. P. (1996). Reproductive success and symmetry in zebra finches. *Anim. Behav.* **51**: 203–210.
- Thornhill, R. (1983). Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps*. *Am. Nat.* **122**: 765–788.
- Trivers, R. L., and Willard, D. E. (1973). Natural selection of parental ability to vary the sex ratio of offspring. *Science* **179**: 90–92.
- Wedell, N. (1996). Mate quality affects reproductive effort in a paternally investing species. *Am. Nat.* **148**: 1075–1088.
- Wojcik, D. P. (1969). Mating behavior of eight stored-product beetles (Coleoptera: Dermestidae, Tenebrionidae, Cucujidae and Curculionidae). *Fla. Entomol.* **52**: 171–197.