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ORIGINAL ARTICLE

Tina Nilsson · Claudia Fricke · Göran Arnqvist

The effects of male and female genotype on variance in male fertilization success in the red flour beetle (*Tribolium castaneum*)

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Abstract The number of eggs fertilized by a male at any given copulation (fertilization success) is affected by a large number of factors. Male insemination and sperm competition success and various female structures and/or processes that bias paternity in favor of some males over others (cryptic female choice) are all likely to affect fertilization success. We suggest that more comprehensive measures of male fertilization success can increase our understanding of postcopulatory sexual selection. To improve our understanding of the importance of various sources of variance in male fertilization success, we conducted a series of experiments using flour beetles. Different wild-type strains were used in reciprocal double mating experiments, against a phenotypic marker strain. We assessed the relative effects of female genotype, male genotype and mating order on independent and inclusive measures of male defense ability (P_1), male offense ability (P_2), and female remating behavior. Female genotype influenced both P_1 and P_2, and male genotype interacted strongly with female genotype in its effect on P_2. We also documented an interaction between female and male genotypes in the effects of mating on female remating behavior, such that females tended to remate most rapidly when mated to males of their own genotype. It is clear from our experiments that cryptic female choice influences the pattern of fertilization success in flour beetles, and we suggest that cryptic female choice may often be an important component of postcopulatory sexual selection. Future investigations would benefit from studying the multiple components of variance in male fertilization success.

Keywords Sperm competition · Cryptic female choice · Speciation · Reproductive isolation · Sexual selection

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T. Nilsson (✉)
Animal Ecology, Department of Ecology
and Environmental Science, Umeå University,
901 87 Umeå, Sweden
e-mail: Tina.Nilsson@eg.umu.se
Tel.: +46-90-7866637
Fax: +46-90-7866705

C. Fricke · G. Arnqvist
Department of Animal Ecology, Evolutionary Biology Centre,
Uppsala University, Norbyvägen 18D,
752 36 Uppsala, Sweden

Introduction

In polyandrous species, a male's net reproductive success is determined both by his success in acquiring mates and copulations (i.e., mating success) and by the number of eggs fertilized at each mating (i.e., fertilization success). Variance in fertilization success generates postcopulatory sexual selection and can be due to differential insemination success (Tadler 1999), relative sperm competitive ability (see Parker 1970; Smith 1984; Birkhead and Møller 1998; Simmons 2001) or any of a series of female processes that bias paternity towards some of her mates over others (i.e., cryptic female choice) (Thornhill 1983; Eberhard 1996). Most studies of postcopulatory sexual selection have focused only on sperm competition, and the proportion of offspring sired by the second male to mate (hence P_2; Boorman and Parker 1976) has often been used as the sole measure of variance in male fertilization success. This might be insufficient for at least two reasons (see Simmons 2001).

First, postcopulatory sexual selection generates opposing evolutionary pressures on males; males are selected to achieve fertilizations when mating with already mated females (male offense ability) and to ensure other males do not fertilize the eggs of their mates (male defense ability) (Parker 1970). Phenotypic traits in males that increase their offense ability need not affect their defense ability. For example, Clark et al. (1995) showed that these two components of fertilization success were uncorrelated in *Drosophila melanogaster* and stressed that both should thus be assessed. Similarly, a recent study of *Tribolium castaneum* (Bernasconi and Keller 2001) reported a potential trade-off between these two components, suggesting that male investment in defense

may even be negatively correlated to that in offense (see also Arnqvist and Danielsson 1999). If this is commonly the case, restricting studies of postcopulatory sexual selection to measures of P_2 alone can clearly be misleading. The fertilization success of males when mating as first males (P_1) may greatly affect male reproductive success, and attempts should be made to disentangle these two components.

Second, female postcopulatory behavior can be important for male fertilization success but is not captured by typical measures of P_1 or P_2. For example, the ability of males to induce a period after mating during which females are unreceptive to further matings (hence, refractiveness) will influence male fertilization success (Simmons and Gwynne 1991; Eberhard 1996). Very few studies have so far assessed the relationship between male characters and variance in female refractiveness, and this might be the least understood component of postcopulatory sexual selection. Recent studies of both house flies (Andrés and Arnqvist 2001) and bean weevils (Brown and Eady 2001) have, however, shown that male genotypes differ in their ability to elicit refractiveness in females. Similarly, female reproductive rate following mating will also affect male fertilization success (cf. Burley 1988; Sheldon 2000). A male that is able to increase the reproductive rate of his mates immediately after copulation will overall have a greater fertilization success than males unable to do so (Chapman et al. 1995; Eberhard 1996). Again, relatively few studies have dealt with differential effects of males on female reproductive rate as a component of postcopulatory sexual selection, but a few studies have reported effects of male genotypes on female reproductive rate (Andrés and Arnqvist 2001; Brown and Eady 2001; Nilsson et al. 2002).

The last decade has seen a growing awareness of the complexity and richness of the various processes that can contribute to variance in male fertilization success (Eberhard 1996; Birkhead and Møller 1998; Simmons 2001). Studies of postcopulatory sexual selection now face two challenges. We need measures of variance in male fertilization success that avoid some of the limitations with traditional uses of P_2. This can be achieved by using measures of variance in fertilization success that are more inclusive, provided that we regard any processes that occur during or after copulation as being part of postcopulatory sexual selection (Eberhard 1996). One such integrative measure would simply be the total number of eggs fathered by a male per mating under natural conditions, which would then reflect insemination success, female sperm utilization, and female postcopulatory behavior. However, this is often problematic for practical reasons. This goal can instead be achieved by simultaneously acquiring independent measures of P_1, P_2, female refractiveness, and female reproductive rate. Provided that the experimental protocol is such that it ensures that females copulate once to each focal male, it is then important to include also cases where successful insemination of either of two males cannot be verified post-experimentally (i.e., P_1=1/0 or P_2=1/0) in subsequent analyses. Such cases have been routinely excluded from analysis in studies of sperm competition, but should be included in more inclusive estimates of fertilization success primarily because they may represent variation in insemination success [due to male genital malfunctioning, female sperm dumping, etc. (Eberhard 1996; Tadler 1999; Pizzari and Birkhead 2000)]. Note that when used this way, P_1 and P_2 will not be measures of sperm competition success in the conventional sense (since sperm may not even be in competition) but rather quantify components of male postcopulatory fertilization success.

Variance in fertilization success can be due to male and/or female characteristics and distinguishing between sperm competition and cryptic female choice is another major challenge for students of postcopulatory sexual selection (see Birkhead 1998, 2000; Eberhard 2000; Kempenaers et al. 2000; Pitnick and Brown 2000; for an illuminating discussion of sperm choice). Some insights can be gained by using experimental designs that allow separation of the effects of male phenotype/genotype, female phenotype/genotype and their interaction on variance in fertilization success (Pitnick and Brown 2000). In particular, studies demonstrating male×female interactions (Clark and Begun 1998; Arnqvist and Danielsson 1999; Clark et al. 1999; Andrés and Arnqvist 2001; Brown and Eady 2001) show that male fertilization success depends on female traits, and so suggest a role for cryptic female choice.

This study represents an attempt at assessing the relative roles of the sexes in affecting variance in fertilization success in the red flour beetle, *T. castaneum*. To do this, we use discrete wild-type genotypes which are known to be partly differentiated with regards to the effects mating has on female reproductive rate (Nilsson et al. 2002). We conducted a double-mating experiment where all females were mated twice, once to a wild-type male and once to a phenotypic marker male, in all possible reciprocal combinations. We made an effort to use more inclusive measures of variance in male fertilization success (female refractory period and independent measures of P_1 and P_2), and use our results to discuss population divergence and possible causes of such evolution.

Methods

Experimental organisms

Three wild-type strains and one phenotypic marker strain of the red flour beetle, *T. castaneum*, were used as experimental organisms. The strains were provided by the *Tribolium* stock center at the U.S. Grain Marketing Research Laboratory in Manhattan, Kansas, United States of America (http://bru.usgmrl.ksu.edu/proj/tribolium/index.asp). The three wild-type strains were Georgia-1 (G), Tiw-6 (T), and CTC-485 (C). The G strain was collected in Georgia (USA) in 1980, the T strain in India in 1989, and the C strain in Australia in 1988, and all have been cultured in the laboratory since. The phylogeny of the different strains (hence genotypes) is not well established but genetic sequence data have shown that the C and G genotypes are more closely related to each other than

either are to the T genotype (Beeman et al. 1996). Previous experiments have documented a slight difference in female fertility following matings with males of these genotypes (see Nilsson et al. 2002), but the fertility rates are generally very high (G males=96%, T males=92%, C males=93%) and male and female genotypes do not interact in their effect on fertility. Previous experiments using a larger series of crosses between wild-type strains (including G and a variant of T) have also shown that both average egg hatchability (84.7%) and larval-adult survival (96.8%) are very high, and that males of different strains do not differ in these parameters (G. Arnqvist and C. Fricke, unpublished data). Thus, our measures of non-random fertilization success (see below) are not likely contaminated by differential egg-adult survival (cf. Gilchrist and Partridge 1997).

The phenotypic marker strain used, Black, is homozygous for a semi-dominant autosomal mutation causing black body coloration (Sokoloff et al. 1960). All beetles were maintained in a dark climate chamber at 29–30°C and at 60 (±10)% relative humidity. We used a standard mixture of 95% whole-wheat flour and 5% brewers' yeast as medium (Sokoloff 1972).

The experiments

We performed a series of double mating experiments to test the effects of genotype and mating order on male fertilization success. Each experimental female (wild-type) was mated to 1 wild-type and 1 Black male on 2 consecutive days with 24±0.5 h intermating interval, in all possible reciprocal combinations (yielding a total of 18 treatment combinations; median N=17 per combination). Males of the Black phenotypic marker strain were used throughout to enable paternity determination of offspring and to standardize the postcopulatory competitive background. The experiment was divided into two parts, aimed at independently assessing: (1) male defense ability (measured as P_1 with wild-type males mating as the first mate) and female refractiveness, and (2) male offense ability (measured as P_2 with wild-type males mated as the second mate).

To ensure virginity, all wild-type beetles used in the experiment were sexed as pupae and males and females were kept separately during emergence. In order to increase male persistence, all males and females were placed individually in separate vials 20–24 h prior to matings. Females were marked with a small drop of enamel paint on their pronotum in order to enable recognition. At mating, each wild-type female (10–15 days post-emergence) was placed in a mating vial (3.5 cm petri dish with filter paper in the bottom) together with a male, and the pair was continuously observed for 1 h. We defined copulations as those interactions in which genital contact was maintained for at least 35 s (the minimum time required for insemination; see Edvardsson and Arnqvist 2000). When copulation took place within 1 h, the male was removed directly after the termination of copulation. If the pair failed to copulate within the hour, the female was replaced by another virgin female and the male was given a second opportunity to mate. Only females that were successfully mated during the 1st day were paired the next day. The same procedure was used on the 2nd day, with the exception that if mating did not take place during the 1st hour, the male was replaced and the female was given a 2nd hour to mate. In the male defense experiment, the time until copulation after introduction of the second male (Black) was measured to provide a measure of female willingness to remate (i.e., refractiveness induced by the first male). Copulation duration was recorded in all matings.

After the second mating, each female was placed in an oviposition vial (9 cm petri dish) with 12 g of standard medium (sifted to enable offspring counting) and kept in the rearing chambers. The oviposition vials were replaced after 1 week and the females were allowed to lay eggs for a 2nd week before being removed. The females were then frozen and the body size of all females was subsequently measured using a digitizing tablet under a side-mounted camera lucida attached to a dissecting microscope. After 7 weeks, all adults in the oviposition vials were counted and paternity was determined/scored according to body color. Females that failed to produce offspring were discarded from further analyses.

Statistical analysis

The effects of our experimental variables on wild-type male copulation duration and female refractiveness were analyzed with conventional analyses of variance, using SYSTAT. Response variables were transformed prior to analysis, if needed, to stabilize variances and meet the assumptions of the models used. Data on male defense ability (P_1) and male offense ability (P_2) represent proportions, and general linear models are thus inappropriate (cf. Arnqvist and Danielsson 1999). We therefore analyzed male offense and defense data with generalized linear models (GLIM), using binomial errors and a logit link function. In cases where data showed signs of overdispersion (McCullagh and Nedler 1989), we applied William's adjustment for overdispersion (Crawley 1993) prior to inference. Means are presented below with ±SE.

Results

Offspring production was measured over 2 consecutive weeks, giving two estimates of the proportion of offspring fathered by the last male. However, this proportion was highly repeatable over the 2 weeks (data for both offense and defense experiments; r=0.909, P<0.001) and total offspring production during the 2 weeks was therefore used for each replicate female.

Copulation duration

An analysis of the copulation duration of wild-type males is presented in Table 1. Variance in copulation duration was primarily determined by female (G=91.4±4.4, T=116.5±8.9 and C=139.0±13.7 s, respectively) and male genotypes (G=108.0±10.2,T=149.9±9.7 and C=73.9±2.7 s, respectively), but they also interacted in their effect. Mating order also had an effect on copulation duration,

Table 1 The results of an analysis of variance of the effects of our factorial variables on the copulation duration of wild-type males. Residuals from this model did not differ significantly from normality (Kolmogorov-Smirnov One Sample Test; P=0.339)

Source	SS	*df*	*F*	*P*
Female genotype	0.012	2	10.561	<0.001
Male genotype	0.051	2	45.712	<0.001
Mating order	0.000	1	0.201	0.654
Female genotype×male genotype	0.006	4	2.496	0.043
Female genotype×mating order	0.001	2	1.107	0.332
Male genotype×mating order	0.004	2	3.766	0.024
Female genotype×male genotype×mating order	0.003	4	1.466	0.212
Error	0.172	310		

Table 2 The results of a multivariate generalized linear model of variance in P_1. The full model was significant (*LLR*=33.32, *df*=2, *P*<0.001). The contribution of each source was tested in an analysis of deviance, by deletion of (1) each main factor from a model including all main factors only, and (2) the interaction from the full model

Source	*LLR*	*df*	*P*
Female genotype	8.78	2	0.012
Male genotype	4.79	2	0.091
Female genotype×male genotype	5.29	4	0.259
Copulation duration	10.87	4	0.028

Table 3 The results of an analysis of variance of the effects of our factorial variables on female reluctance to remate. Residuals from this model did not differ significantly from normality (Kolmogorov-Smirnov One Sample Test; *P*=0.058)

Source	SS	*df*	*F*	*P*
Female genotype	6.084	2	2.325	0.100
Male genotype	0.139	2	0.053	0.948
Female genotype×male genotype	16.564	4	3.165	0.015
Error	283.942	217		

but this effect varied across male genotypes. G and T males copulated for longer when mating as second males with non-virgin females, whereas C males copulated for a shorter time in such matings.

Male defense ability (P_1)

We analyzed variation in male defense ability (P_1) in a generalized linear model of the number of offspring produced by the wild-type male when mating as a first male (Table 2). The analysis showed that female genotypes differed with regards to the level of P_1 resulting from double matings (average P_1: G=0.45±0.05, T=0.71±0.07 and C=0.67±0.08), but we failed to find an effect of male genotype. Some female genotypes are thus more permissive overall to male defense than are others. We analyzed the influence of copulation duration by simultaneously adding linear and squared terms of the duration of both 1st and 2nd copulations to the model presented in Table 2. This showed that, as expected, long copulations of the first male (β=23.97±9.37, *P*=0.012) and short copulations of the second male (β=−18.66±10.09, *P*=0.066) were both associated with elevated levels of P_1.

Female refractiveness

The analysis of female reluctance to remate (Table 3) showed that female and male genotypes interacted strongly in their effect on female refractory period. The overall pattern of the interaction was such that females showed the shortest average refractory period when previously mated to males of their own genotype, in every case (Fig. 1), suggesting that females were generally less reluctant to remate when previously mated with males with which they were coevolved (i.e., males from the same strain). When female genotypes were analyzed separately, however, male genotype significantly affected female refractory period in only one female genotype (G). G females' response to their own males was significantly weaker than the response to C (*P*=0.025) but not to T (*P*=0.096) males (Tukey's HSD multiple comparisons).

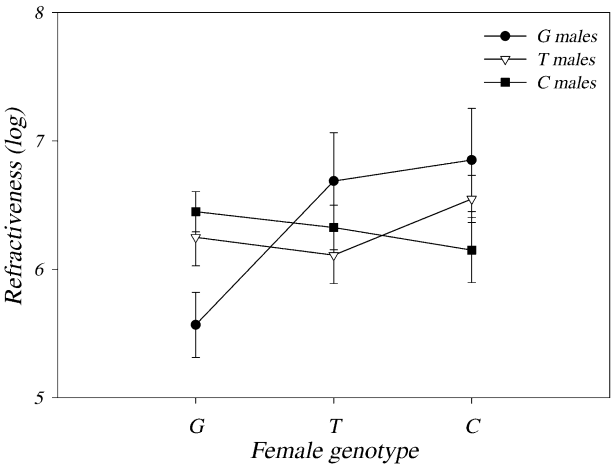

Fig. 1 The effects of female genotype and that of her first mate on average (±SE) time to remating during second matings involving Black males, in the male defense experiment. Note that females were least reluctant to remate with Black males when mated previously with males from their own genotype

Table 4 The results of a multivariate generalized linear model of variance in P_2. The full model was significant (*LLR*=32.29, *df*=12, *P*<0.001). The contribution of each source was tested in an analysis of deviance, by deletion of (1) each main factor from a model including all main factors only, and (2) the interaction from the full model

Source	*LLR*	*df*	*P*
Female genotype	7.21	2	0.027
Male genotype	6.23	2	0.044
Female genotype×male genotype	14.17	4	0.007
Copulation duration	10.99	4	0.027

Male offense ability (P_2)

Variance in male offense ability (P_2), measured as the relative number of offspring produced by the wild-type male when mating as second male, was affected by all factors analyzed (see Table 4 and Fig. 2). Overall, some females were more permissive of male offense success than others (average P_2: G=0.54±0.04, T=0.31±0.08 and C=0.53±0.07) and some male genotypes were generally more successful than others (average P_2: G=0.57±0.07, T=0.45±0.05 and C=0.49±0.05). However, male and female genotypes also interacted strongly, such that the success in male offense by a given male genotype to a

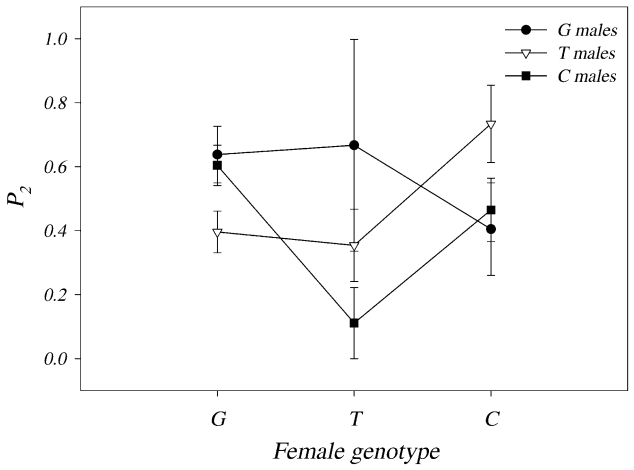

Fig. 2 The effects of male and female genotypes on male offense ability. The figure shows the average (±SE) proportion of offspring fertilized by the second male to mate (P_2)

large extent depended on the genotype of his mate. It is worth noting that in no case were average male P_2 highest with females from their own strain (Fig. 2).

Discussion

We have demonstrated independent and interacting effects of male and female genotypes on several components of variance in male fertilization success. Here, we discuss the implications of our results for postcopulatory sexual selection and population divergence in reproductive characters.

Postcopulatory sexual selection

There has recently been a marked increase in attention to the role of females in studies of postcopulatory sexual selection (Eberhard 1996; Telford and Jennions 1998). Studies demonstrating effects of female genotype (e.g., Lewis and Austad 1990; Wilson et al. 1997) essentially show either that females "handle" males and/or their gametes differentially, and are thus in line with a more active role of females in postcopulatory sexual selection, or that males generally invest differentially in different types of females (i.e., "cryptic male choice"; Reinhold et al. 2002; Wedell et al. 2002). We found that female genotype indeed influenced male success both in male offense (P_2) and male defense (P_1). Together with other experiments, showing both that females actively influence sperm storage (Bloch Qazi et al. 1998) and that female perception of male copulatory courtship influences male fertilization success (Edvardsson and Arnqvist 2000), this suggests that cryptic female choice is important in shaping male fertilization success in *T. castaneum*. However, as pointed out by Pitnick and Brown (2000), male×female interactions provide more unambiguous evidence for cryptic female choice as such interactions demonstrate that female characteristics affect relative male fertilization success. The finding of significant male×female interactions for both success in male offense and in the ability to elicit female refractiveness (see below) thus add considerable strength to our interpretation of the results. Our study adds to a growing list of studies of other insect species showing an effect of male×female interactions on fertilization success of males (Clark and Begun 1998; Arnqvist and Danielsson 1999; Clark et al. 1999; Andrés and Arnqvist 2001; Brown and Eady 2001), and thus suggests that female traits may generally shape the pattern of postcopulatory sexual selection experienced by males.

While sperm competition is generally accepted as an important component of sexual selection (Birkhead and Møller 1998), this can hardly be said about cryptic female choice at this point (Telford and Jennions 1998; Birkhead 2000). Partly for these reasons, postcopulatory sexual selection has often been measured exclusively as P_2. We wish to stress that there are several components of postcopulatory sexual selection and that it is, in principle, necessary to quantify all in order to fully understand this form of sexual selection (Andrés and Arnqvist 2001; Brown and Eady 2001). One potentially important component is female refractiveness, i.e., the length of the male-induced period during which the female is unreceptive or reluctant to further matings. Depending on the rate of female egg-laying, a male can clearly increase his paternity success in any given female by simply inducing a longer refractory period (Simmons and Gwynne 1991; Eberhard 1996). Studies of male×female interactions for female refractiveness are unfortunately very rare, and most investigators have not assessed variance in female remating behavior in this context. However our results, together with two recent reports of similar male genotype×female genotype interactions for female remating rate in two other insect species (Andrés and Arnqvist 2001 in house flies and Brown and Eady 2001 in bean weevils), suggest that variance in female remating behavior may be a major but yet largely unexplored component of postcopulatory sexual selection by cryptic female choice.

Apart from the above components of postcopulatory sexual selection, variance in female postcopulatory reproductive rate also influences the relative reproductive success of males (Eberhard 1996; Sheldon 2000; Andrés and Arnqvist 2001; Brown and Eady 2001). We have showed elsewhere, in an experiment involving the same genotypes (Nilsson et al. 2002), that female reproductive rate is influenced by the interaction between male and female genotype. Again, this obviously adds strength to our more general conclusion of the role of females in determining male postcopulatory reproductive success in *T. castaneum*.

Finally, it is worth noting that we found no obvious correspondence between the role of male genotype in the male defense and male offense experiments. While the number of genotypes included in this study is too low to

allow a rigorous statistical evaluation of this pattern, it is in line with earlier findings showing that male offense and defense abilities are to a large extent determined by different loci (Clark et al. 1995; Bernasconi and Keller 2001).

Divergence in reproductive characters

The populations of *T. castaneum* used in this experiment have diverged in components that determine male fertilization success (see also Nilsson et al. 2002). Apart from random processes, such as founder effects and/or genetic drift, there are at least two adaptive evolutionary scenarios that can lead to such divergence. The divergence can either have evolved by sexually antagonistic coevolution (Rice 1996, 1998; Parker and Partridge 1998) or by male-female coevolution driven by selection on females to secure indirect genetic benefits. Clark et al. (1999) and Andrés and Arnqvist (2001) suggested that the pattern of male×female genotypic interactions should differ under these processes. In short, random processes should result in a variable pattern where females' relative reproductive response to males from their own population/strain should not differ overall from that to males from other populations/strains. In contrast, females should evolve resistance to males with which they are coevolved if divergence is driven by sexually antagonistic coevolution, due to fitness costs of antagonistic male adaptations, and hence responding more weakly than average to males with which they are coevolved (see also Parker and Partridge 1998). Under the alternative adaptive hypothesis, divergence through indirect benefits, females should evolve preference for male signals, thereby responding more strongly than average to males with which they are coevolved. However, as pointed out by Brown and Eady (2001) and Chapman et al. (2003), these seemingly contrasting predictions should be applied with caution, as their relevance will depend on the absolute magnitude of divergence between populations or genotypes (see also Price 1997; Howard 1999; Gavrilets 2000; Eady 2001).

The pattern of male×female interactions in our data was variable, and not entirely consistent. The interaction effect on copulation duration was relatively simple in that the relative order of male genotypes was the same in all female genotypes, and males only differed by the magnitude of variance in copulation duration across female genotypes. The pattern of the interaction for male offense ability is difficult to categorize, since in two out of three cases, males of the females' own genotype had an average success. In the remaining case, the males from the females' own genotype actually had the highest success. It is important to note, however, that in neither of these cases did the response of females to their own males differ significantly from the response to the other two male genotypes (focused post-hoc tests; $P>0.05$), despite overall significant interactions. The pattern of the male×female interaction for female reluctance to remate showed that males of the females' own genotype elicited the weakest average response in all genotypes. In the only case in which female response to their own males differed significantly from the response to the other two male genotypes, the response to their own males was weaker than the response to other males. This pattern is also expected if females are selected to remate with foreign males to avoid inbreeding (cf. Bateson 1983; Tregenza and Wedell 2002). However, fertility, hatchability and larval survival were all generally very high and did not differ between crosses in our experiments. Inbreeding avoidance is thus a highly unlikely cause of the pattern seen in female remating behavior.

In summary, our data are not entirely conclusive with regards to the predictions discussed above. Although we cannot exclude that random processes alone have caused the divergence observed here, this seems highly unlikely since the traits involved (components of male fertilization success) should have been exposed to selection. Instead our results, in combination with the results of Nilsson et al. (2002), suggest that females may at least in some regards have evolved resistance to postcopulatory manipulations of the males with which they are coevolved. This in turn implies sexually antagonistic coevolution as a generator of both postcopulatory sexual selection (Andrés and Arnqvist 2001; Gavrilets et al. 2001) and population divergence (Rice 1998; Howard 1999; Arnqvist et al. 2000).

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