



Sexual Activity and Reproductive Isolation Between Age-specific Selected Populations of Seed Beetle

Biljana Stojković*†, Darka Šešlija Jovanović*, Jelena Perovanović† & Nikola Tucić*†

* Institute for Biological Research "Siniša Stanković", Department of Evolutionary Biology, Belgrade Serbia

† Faculty of Biology, University of Belgrade, Belgrade, Serbia

Correspondence

Biljana Stojković, Faculty of Biology, Institute of Zoology, University of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia.
E-mail: bilja@bio.bg.ac.rs

Received: April 17, 2011

Initial acceptance: June 4, 2011

Final acceptance: June 25, 2011

(T. Tregenza)

doi: 10.1111/j.1439-0310.2011.01936.x

Abstract

We evaluated the degree of reproductive isolation between laboratory populations of the seed beetle (*Acanthoscelides obtectus*) selected to reproduce early (E) or late (L) in life, where different levels of sexual activity and sexual discrimination have been detected. We found a significant level of behavioral isolation among populations within the E selection regime in which beetles showed enhanced early-life fitness traits and low sexual activity. In contrast, substantially higher levels of sexual activity and an indiscriminate mating system inhibited rather than promoted pre-zygotic isolation between the L populations. Our results indicate that the study of sexual activity levels may be crucial for understanding the first steps in the pre-zygotic isolation among allopatric populations subjected to uniform selection.

Introduction

The choice of sexual partners based on certain trait's value in potential mates (mating preference) has long been considered to be an important step in initiating speciation because the divergence of mating preferences across allopatric populations can generate behavioral (pre-zygotic) reproductive isolation (RI; Coyne & Orr 2004). In theory, divergent evolution of mating preference can occur even if populations are initially identical and exposed to uniform selection because different mutations can be fixed through genetic drift (Cohan 1984) or because the order in which mutations arise in isolated populations is different (Mani & Clarke 1990). Although these random processes are unlikely to cause substantial differentiation of preferences among populations (Coyne & Orr 2004), an initial divergence may be subsequently amplified by sexual selection (Rundle et al. 2005). As pointed out by Fricke & Arnqvist (2004) and Fricke et al. (2010), under uniform artificial or natural selection, sexual selection could generate erratic diversification in traits associated with reproductive behavior across populations. Given that

empirical data on evolution of RI under uniform selection are scarce (e.g., Cohan & Hoffmann 1989; Rice & Hostert 1993; Boake et al. 2003; Fricke & Arnqvist 2004; Gay et al. 2009; Maklakov et al. 2010) and, also, that the interplay of the above-mentioned forces may produce a great number of possible evolutionary trajectories, it is not surprising that we still have very little idea of the frequency at which RI might arise under such circumstances.

Here, we report the results of one laboratory evolution experiment in which we asked whether a divergence in mating preferences could arise between allopatric populations of the seed beetle (*Acanthoscelides obtectus*) originating from one source and subjected to selection for age at reproduction. Using age-specific selection regimes in which selection was imposed on replicate populations of the seed beetle either for early (E) or late (L) reproductive success, previous studies have shown that the L population beetles were bigger, lived longer, and had higher pre-adult viability, longer development time, lower early fecundity, and higher total fecundity than beetles from the E populations (Tucić et al. 1996, 2004). These data strongly support Williams

(1957) hypothesis that aging may result from trade-offs between early- and late-life history traits. We also found that the reproductive behavior of females and males was changed in the E and L populations. Early-life mating frequencies and mating speed of both sexes, as well as females' re-mating rates, were substantially higher in the late-reproducing populations rather than in the early-reproducing populations (Maklakov et al. 2006; Šešlija et al. 2009). Thus, selection for early-life fitness did not result in elevated mating efforts in either sex. This result indicated that the evolution of mating behavior proceeded in a direction opposite to that of life-history evolution. These findings are also in contrast to those obtained on another seed beetle, *Callosobruchus maculatus*, in which populations selected for early reproduction mated more frequently early in life than beetles subjected to selection for late reproduction (Maklakov et al. 2010). Recently, Stojković et al. (2010) further characterized the evolution of mating behavior in *A. obtectus* and found that the levels of heterosexual mating behavior in the E and L populations were positively correlated with the homosexual activity of both sexes. The observed link between hetero- and homosexual activity supports the 'perception error hypothesis' (Thornhill & Alcock 1983) for the maintenance of homosexual behavior in insects. This hypothesis proposes that increased mating attempts, despite the risk of making some mistakes, may outweigh the cost of choosing a partner of the right sex. In other words, the L females and males not only show higher heterosexual activity than E beetles but also have a higher level of same-sex mating behavior because of a lower degree of sexual discrimination. These findings raise the question of whether pre-zygotic RI evolves more rapidly among populations with elevated sexual activity of both sexes or high mating activity hampers the evolution of pre-zygotic RI. We predict that replicate populations in the L selection regime, where beetles exhibit higher levels of indiscriminate sexual behavior, will show a lower degree of pre-zygotic RI than populations in the E selection regime.

To assess the role of sexual activity in promoting RI between allopatric populations, we chose to perform experimental crosses between populations within the age-specific selection regimes. Copulation frequency, mating speed, and copulation duration were tested on pairs of beetles consisting of a female and male from the same replicate population (homotypic crosses) and pairs consisting of a female and male from different populations within the

same selection regime (heterotypic crosses). Because both selection regimes were replicated four times, this procedure allowed us to test the hypothesis that the populations within the E regime had a higher level of pre-zygotic RI than those within the L regime.

Methods

Life History and Experimental Conditions

The seed beetle, *A. obtectus* (Coleoptera: Chrysomelidae: Bruchinae), is a cosmopolitan pest of stored legumes. Populations of *A. obtectus* are most commonly detected in stores of dried legumes; their life cycle appears well adapted for reproduction in a storage environment. This beetle is aphagous as an adult, and the resources gained during larval development determine the supply available for allocation later in life. Larval development takes place in plant seeds, and adults emerge at the final molt. Egg-laying behavior in *A. obtectus* is triggered by mating and the presence of beans. Female resistance to re-mating is very high in this species; in fact, some authors regard *A. obtectus* as being monoandrous (cited in Maklakov et al. 2007). *Acanthoscelides obtectus*, like a number of other insect species, exhibits a transfer of male secretions from the spermatophore to the female hemolymph (Das et al. 1980).

All experiments reported here were performed in a dark incubator at approx. 30°C and 70% humidity. All seeds were obtained from one source and were frozen before use in the experiments. No food or water was offered to the experimental adults.

Experimental Populations

The base population, from which all selection lines were originally derived, was obtained by mass-mating beetles from three different locations in the vicinity of Belgrade (Tucić et al. 1996). Experimental females and males originated from the lines of *A. obtectus* on which selection had been imposed for early (E) and late (L) reproduction. Four replicates per selection treatment were maintained under adult aphagy.

Throughout the course of selection for early reproduction (E), in each replicate population, 300–500 individuals (sex ratio approx. 1:1) were divided into 3–5 separate Petri dishes (approx. 50 females and 50 males on 100 bean seeds), where they reproduced freely for 48 h after emergence. The adults were then removed. After approx. 3 wks, beans with

inoculated larvae from all Petri dishes in each replicate population were collected in a single large bottle. The new generations were founded from the beetles that emerged in these bottles. This regime gave rise to beetles with enhanced fitness during early life and a short lifespan. Because the females of this beetle species usually do not remate until a few days after their initial mating, the rate of female re-mating within the E treatment was very low, approx. 13% (Šešljija et al. 2009). In the present study, we used E populations selected for 222 generations.

To establish a new generation of each replicate population in the L selection regime, over 1000 beetles, whose sex ratio was approx. 1:1, were kept together in 10 separate vials (i.e., approx. 100 individuals per 3 cm diameter, 4 cm height vial; very high adult density) without beans and were allowed to mate from emergence until death. The beetles from all vials were mixed and introduced into bottles with approx. 100 bean seeds on day 10. Thus, eggs laid prior to this day did not contribute to the next generation (for details see Tucić et al. 1996). The adults of both sexes from the L treatment were bigger and lived longer than those from the E treatment. Because females and males were kept together during their whole life, there was the opportunity for re-mating of females and for the evolution of a polyandrous mating system. The estimated rates of female re-mating in replicate populations within the L treatment varied from 59% to 66% (Šešljija et al. 2009). Although both the E and L populations were established at the same time (in 1986), because of the 10-d lag period and longer pre-adult developmental time, the L beetles used in the present experiments were selected for 155 generations, i.e., 67 generations fewer than the E populations.

Although the numbers of founding individuals of each generation were larger in the L than in the E selection regime, we presumed that the effective sizes of the E and L populations did not differ significantly. In the E populations, where individuals commonly have only one opportunity to mate, female/male mating ratios could be close to one, whereas in the L populations, where male competition and last-male sperm precedence are expected, lower effective sizes could be observed (Snook et al. 2009).

Prior to the start of the present experiments, we kept all replicate populations at a population size of approx. 1000 individuals under common housing regimes and completely relaxed selection for two generations.

Mating Experiments

Mating trials were no-choice in design and were performed at approx. 26°C. A no-choice design was used because it avoids confounding mating propensity with mating preference (Rolan-Alvarez & Caballero 2000). All beetles were virgins and 24 h old at the start of the experiments. Experimental females were placed individually into observation chambers (30 mm Petri dishes without beans) with single males from either their own (homotypic crosses) or from the alternative replicate populations (heterotypic crosses) for 30 min (we refer to homotypic and heterotypic crosses as 'mating type'). The pairs were then observed, and copulations were recorded. Crosses were always nested within selection treatment and never crossed between treatments. For each pair of virgin beetles, we measured three frequently used indices of reproductive behavior in insects: copulation frequency, mating speed (the elapsed time to initiation of copulation), and copulation duration (the time spent in copula).

For each mating type within the E and L selection treatments (8 within- and 12 between-replicate populations), the behavior of approx. 80 mating pairs was observed. Between replicate populations, matings were designed as reciprocal crosses, e.g., approximately, 40 E₁ females were paired with 40 E₂ males, and 40 E₂ females were paired with 40 E₁ males, and so on. Because 'RI is best examined by performing a matrix of allopatric and sympatric crosses, whereas measurement of divergence requires crosses with a tester line' (Gay et al. 2009, p. 693), we chose to cross the replicate populations within selection treatments rather than use a common tester (i.e., the base population), even though such an experimental design necessarily involves some level of pseudoreplication (see also Rolan-Alvarez & Caballero 2000 for the advantages of using this design in testing RI).

Statistical Analysis

We used two statistics to evaluate the degree of prezygotic RI. The first analysis was based on a simple measure of copulation frequencies in different mating-pair combinations (homotypic and heterotypic crosses) within treatments. Proportions (percentages of copulation) were compared using a *G*-test of independence (Sokal & Rohlf 1995). The *G*-value is compared with the critical value of χ^2 for (*n* - 1) degrees of freedom. To examine whether mating speed and copulation duration differed between mating types,

we used separate mixed-effect nested ANOVAs (SAS Insitute, Inc. 2003).

We then employed a hierarchical model developed by Rolan-Alvarez & Caballero (2000), which has the advantage of providing information on the biological causes of sexual selection and sexual isolation. Defining sexual selection as a change in genotypic or phenotypic frequencies of mated vs. total population frequencies (Hartl & Clark 1989) allows the estimation of the pair sexual selection index (PSS) for each pair type [there are four pair types in two populations' crosses – two homotypic (e.g., $E_1\text{♀} \times E_1\text{♂}$ and $E_2\text{♀} \times E_2\text{♂}$) and two heterotypic (e.g., $E_1\text{♀} \times E_2\text{♂}$ and $E_2\text{♀} \times E_1\text{♂}$)]. Under the assumption of random mating between females and males from the same or different populations, sexual selective differences between copulating and non-copulating samples (PSS) are evaluated as the ratio of expected number of matings in each pair type obtained from the pool of mated individuals and expected pairs from total numbers, i.e., population frequencies. PSS evaluates an intrinsic tendency of different phenotypes/genotypes to mate more frequently than others (Pérez-Figueroa et al. 2005) and can be considered the mating propensity parameter. On the other hand, assortative and disassortative matings have been defined as deviations from random mating in mated individuals (Merrell 1949) and when considered in a context of incipient speciation, it is called sexual isolation (Spieth & Ringo 1983; Rolan-Alvarez & Caballero 2000). Thus, in this model, sexual isolation effects are mainly caused by intersexual choice and can be calculated for every pair type as the number of observed copulating pairs divided by the expected pairs from mated individuals. This parameter, which provides more precise estimation of mate choice, is named 'pair sexual isolation' (PSI). Simple measures of copulation frequency in homotypic and heterotypic crosses, commonly used in RI studies, are theoretically similar to the pair total index ($PTI = PSI \times PSS$), although basic mating proportions are not statistically compared with expected values under the assumption of random mating as they are in the hierarchical analysis. According to Rolan-Alvarez & Caballero (2000), the decomposition of mating behavior into sexual selection and sexual isolation is possible in laboratory experiments where controlled matings between males and females originating from the same or different populations are monitored and the total numbers of each mating combination, as well as their copulation frequencies, are known.

The total sexual isolation between two populations, the I_{PSI} parameter, is calculated from the PSIs of four

pair types (Rolan-Alvarez & Caballero 2000; Carvajal-Rodriguez & Rolan-Alvarez 2006). It quantifies the sexual isolation component based mainly on mate choice by removing the effects of different mating propensities of distinct populations. I_{PSI} ranges from -1 to $+1$, with -1 representing disassortative mating, 0 representing random mating, and $+1$ representing complete assortative mating (complete sexual isolation). The analyses were performed using software JMating software, version 1.0.8 (Carvajal-Rodriguez & Rolan-Alvarez 2006), with 10 000 re/samplings of the data to calculate the standard deviation and the significance of these indices in different mating combinations. The significance of mating propensity coefficients (PSS) was examined by the G -test (GS) using JMating software. The G -test can also be used to compare I_{PSI} values, both between selection treatments and within each selection treatment, to detect heterogeneity. Following the suggestion of Coyne et al. (2005), we assessed the differences in I_{PSI} within and between the E and L selection treatments using heterogeneity G values (G_h ; see also Sokal & Rohlf 1995, pp. 697–715).

Results

Mating Frequencies and RI Indices

Late-reproducing females and males (L populations) copulated at significantly higher frequencies compared with early-reproducing females and males (for pooled data $G_{(1)} > 35$ for both homotypic and heterotypic crosses; Table 1). However, the analyses of copulation frequencies between homotypic and heterotypic crosses within both treatments, using a replicated G -test, revealed no significant differences for each of the four replicate populations or pooled within-treatment data. A higher copulation frequency in homotypic than heterotypic crosses was found only for replicate 2 in the E treatment. Therefore, with relative frequencies of mating within and between replicate populations, we found no evidence for sexual isolation (i.e., a higher proportion of successful mating in homotypic compared with heterotypic pairs) in either treatment.

However, when we employed the model proposed by Rolan-Alvarez & Caballero (2000), in which mate choice could be disentangled from population differences in mating propensity, we found significant sexual isolation (parameter I_{PSI}) between replicate populations within the E treatment but not among the L populations (Table 2). Although the degree of isolation depended on which pairs of replicate

Table 1: G-test examining the pattern of copulation frequencies (in %) in homotypic (within replicate population) and heterotypic (among replicate populations) mating types within early reproduction (E) and late reproduction (L) selection treatments

Replicate population	Mating type		G
	Homotypic	Heterotypic	
E1	37.50	32.39	0.63 (1)
E2	51.02	35.09	4.00 (1)*
E3	45.45	38.18	1.25 (1)
E4	41.34	47.46	1.07 (1)
Pooled	42.86	39.13	1.28 (1)
Heterogeneity			5.67 (3)
L1	64.08	61.69	0.18 (1)
L2	56.52	67.69	3.01 (1)
L3	72.84	66.81	1.03 (1)
L4	67.01	57.14	2.86 (1)
Pooled	65.42	63.49	0.43 (1)
Heterogeneity			4.37 (3)

The number after the letter indicates the replicate population (1–4).

Degrees of freedom are given in parentheses.

* $p < 0.05$.

populations were compared, the total isolation index detected between replicate populations within the E treatment ($I_{PSI} = 0.29$, $p < 0.001$ by bootstrapping) was ten times greater than the total I_{PSI} between replicate populations within the L treatment ($I_{PSI} = 0.029$, $p = 0.458$ by bootstrapping). Additionally, there was significant heterogeneity in the I_{PSI}

indices between the two selection treatments ($G_h = 28.98$, $df = 6$, $p < 0.001$).

In four population crosses within the E treatment, the sexual isolation indices were significantly positive (ranging from 0.33 to 0.59). The positive values suggested that these combinations were assortative in mating if the effects of female and male propensity in each population were removed. Despite the fact that, for two pairings (2×4 and 3×4 ; Table 2), we did not find evidence for either assortative or disassortative mating, the insignificant heterogeneity among population crosses within the E treatment ($G_h = 2.37$, $df = 3$, $p > 0.05$) confirmed the overall sexual isolation between populations of beetles exhibiting low sexual activity (i.e., significant I_{PSI}).

In the L treatment, where the beetles demonstrated higher sexual activity, the numbers of successful copulations in three pairs of population crosses did not differ significantly from those expected under random mating; two population pairs showed significant positive I_{PSI} (assortative mating), and one cross type showed a significant negative (disassortative mating) value (Table 2). Considering these results and the heterogeneity detected in I_{PSI} between the L replicate populations ($G_h = 26.61$, $df = 3$, $p < 0.001$), it was not surprising that the total sexual isolation index was not significant within the L treatment.

Within both experimental treatments, we found no differences in the general willingness of individuals to mate with members of the same or different

Cross combination	E populations		L populations	
	Sexual isolation (I_{PSI})	Mating propensity (GS)	Sexual isolation (I_{PSI})	Mating propensity (GS)
1×2	0.488 ± 0.100 $p = 0.000$	2.77 (2) $p = 0.250$	-0.018 ± 0.071 $p = 0.799$	0.88 (2) $p = 0.644$
1×3	0.325 ± 0.094 $p = 0.000$	4.37 (2) $p = 0.112$	0.136 ± 0.067 $p = 0.040$	1.37 (2) $p = 0.504$
1×4	0.587 ± 0.084 $p = 0.000$	1.51 (2) $p = 0.470$	0.032 ± 0.063 $p = 0.621$	0.12 (2) $p = 0.942$
2×3	0.369 ± 0.099 $p = 0.000$	1.23 (2) $p = 0.541$	-0.193 ± 0.065 $p = 0.003$	3.21 (2) $p = 0.201$
2×4	-0.149 ± 0.082 $p = 0.065$	0.95 (2) $p = 0.622$	0.056 ± 0.075 $p = 0.461$	7.93 (2) $p = 0.019$
3×4	0.068 ± 0.082 $p = 0.409$	2.99 (2) $p = 0.224$	0.224 ± 0.069 $p = 0.001$	0.46 (2) $p = 0.795$
Total	0.285 ± 0.050 $p = 0.000$	6.30 (6) $p = 0.390$	0.029 ± 0.039 $p = 0.458$	3.90 (6) $p = 0.690$

For I_{PSI} standard deviation and two-tailed probability across 10 000 re/samplings are presented.

For mating propensity, probability of G statistics following the Chi-square distribution with degrees of freedom given in parentheses is reported.

Table 2: Estimates of sexual isolation (I_{PSI} parameter) and G values for mating propensity (GS) between pair population combinations and total estimates in two selection treatments – for early reproduction (E populations) and late reproduction (L populations)

populations (no significant difference in mating propensity; GS in Table 2).

Mating Speed and Copulation Duration

Mating speed, the time to initiation of mating, was faster between females and males from the L than the E selection treatment (unpaired *t*-tests for the pooled homotypic and heterotypic crosses were 2.92, *df* = 367, *p* < 0.01, and 4.23, *df* = 915, *p* < 0.001, respectively; Fig. 1a). The results of the nested ANOVA, however, showed that mating type had no significant effect on mating speed in either the E ($F_{1,404} = 0.46$, *p* > 0.05) or the L treatment ($F_{1,866} = 0.78$, *p* > 0.05).

Copulation duration was significantly longer in both mating types for beetles originating from the E treatment than from the L treatment ($t_{367} = 5.23$ and $t_{915} = 5.04$, both at the 0.001 level, for pooled homotypic and heterotypic data, respectively;

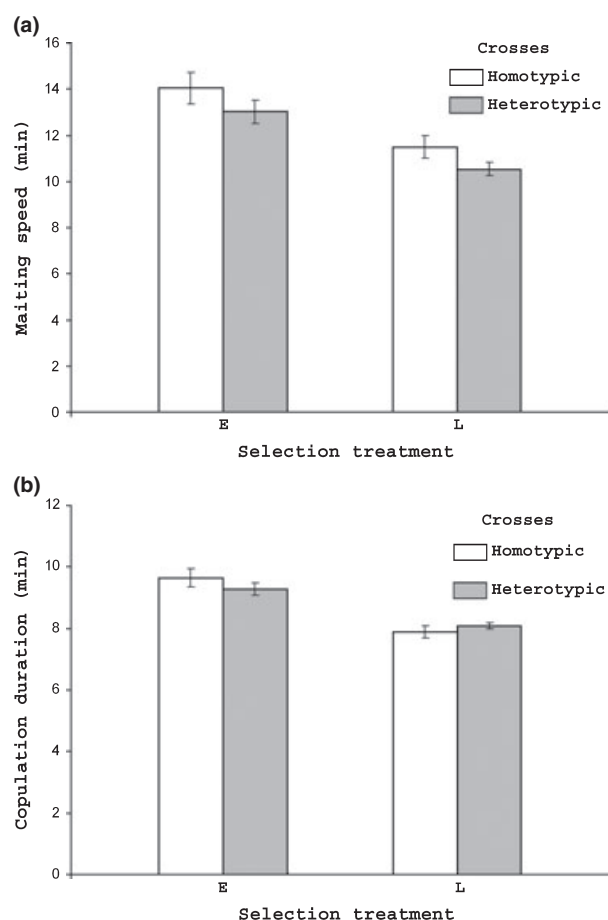


Fig. 1: Mean (±SE) of mating speed (a) and copulation duration (b) in homotypic (within replicate populations) and heterotypic (between replicate populations) crosses within the E and L selection treatment.

Fig. 1b). Again, we did not detect a significant difference in copulation duration between homotypic and heterotypic crosses either in the E ($F_{1,404} = 0.38$, *p* > 0.05) or in the L treatment ($F_{1,866} = 0.39$, *p* > 0.05).

Discussion

Although many studies have tested pre-zygotic RI by examining the relative frequencies of mating within and between populations (e.g., Hollocher et al. 1997; Martin & Hosken 2003; Wigby & Chapman 2006; Bacigalupe et al. 2007; for earlier surveys, see the review in Pérez-Figueroa et al. 2005), using this measure, we found no evidence for sexual isolation (i.e., a higher proportion of successful mating in homotypic compared with heterotypic pairs) in either selection treatment. On the contrary, when the evaluation of RI was based mainly on mate choice (Rolan-Alvarez & Caballero 2000), a signature of incipient pre-zygotic isolation between populations (i.e., significant I_{PSI}) was detected under selection for early reproduction and was absent from the L selection treatment. Our results imply that simple copulation frequency measures can blur the separate effects of distinct components of mating behavior on RI, as was suggested by Pérez-Figueroa et al. (2005), because differences in numbers of matings between homo- and heterotypic crosses may be found only at the level of successfully copulated pairs and not in the whole sample of pairs (see Methods). These results also indicate that mate choice can evolve independently or prior to eventual mating propensity within populations.

Several experimental factors could be invoked as potential explanations for the observed patterns of pre-zygotic RI within the E and L selection regimes. For example, it can be argued that the E populations, which were selected for 222 generations, had more opportunity to accumulate genetic differences affecting mating behavior than the L populations during their 155 generations of selection. Such a bias in generation numbers is unavoidable in long-term experiments involving age-specific selection. However, we believe that the lengths of both selection regimes with respect to producing new mutations are comparable because of the parental age effect observed in the L populations (Tucić et al. 2004).

Another possibility is that some of the observed changes in mating behavior could be the result of correlated responses to selection on age at reproduction because 'genes regulating mating behavior are as likely to be genes involved in neurogenesis,

metabolism, development, and general cellular processes as genes with specific effects on behavior' (Mackay et al. 2005, p. 6628). For example, MacKay and associates have shown that divergent artificial selection for mating speed in *D. melanogaster* produces transcription differences in approx. 20% of genes between selected lines. Because we have also found a significantly higher mating speed in the L than in the E populations, it is very likely that these phenotypes were among the correlated responses to the imposed selection regimes. In addition, numerous examples show that the link between life-history and behavioral traits in insects may indeed reflect their joint dependence on hormonal and other metabolic changes (e.g., Cordts & Partridge 1996; Beaver & Giebultowicz 2004; McBrayer et al. 2007). Although there is no doubt that some of the observed changes in mating activity between selection regimes can be linked with evolved life-history traits, the question is to what degree sexual behavior can evolve independently from those ecological traits. In contrast to post-zygotic RI, which usually evolves as a byproduct of genetic differentiation between allopatric populations, several laboratory evolution experiments suggest that pre-zygotic RI may be directly selected via sexual and sexually antagonistic selection in populations that experience otherwise uniform natural selection (Prout 1971; Rice 1992; Martin & Hosken 2003; Fricke & Arnqvist 2004). However, two experiments on *Drosophila melanogaster* (Wigby & Chapman 2006) and *D. pseudoobscura* (Bacigalupe et al. 2007) found no evidence for stronger RI among populations experiencing greater sexual antagonistic selection. Thus, our understanding of conditions that favor or hamper pre-zygotic RI under uniform natural selection is still very limited, and the results are variable. The same is true for several attempts to conduct experiments in which both natural and sexual selection are manipulated simultaneously to disentangle the relative effects of these two forces on trait evolution (Rundle et al. 2006; Fricke & Arnqvist 2007; Maklakov et al. 2009, 2010).

Maklakov et al. (2010) have investigated the evolution of mating behavior in replicate populations of *C. maculatus* subjected to age-specific selection under contrasting regimes of sexual selection ('monogamy' vs. 'polygamy') and found no significant effect of the mating system on the degree of RI. Moreover, they showed that neither sexual selection nor interaction between sexual and age-specific selection contributed substantially to the evolution of mating behavior in this seed beetle. It seems, therefore, that the level of sexual activity in *C. maculatus* is a byproduct

of the imposed age-specific selection, i.e., ecological conditions. Our experimental procedure is not specifically designed to address the divergent effects of age-specific and sexual selection on mating behavior. However, Šešljija et al. (2009) convincingly showed that the observed changes in mating behavior were not a byproduct of selection on age at reproduction in *A. obtectus* populations. In contrast to the study performed by Maklakov et al. (2010), we revealed that early-reproducing populations exhibited lower levels of sexual activity.

There are at least two explanations for the observed opposite directions of the evolution of sexual behavior and predicted life-history outcomes in the two bruchid species. First, *A. obtectus* females, selected without egg-laying substrates, are expected to benefit more from water and/or nutrients received from male ejaculates (Tucić et al. 1996; Edvardsson 2007; Ursprung et al. 2009) compared with *C. maculatus* females that are selected in the presence of host seeds (Maklakov et al. 2010). In the absence of beans, *A. obtectus* females lay fewer eggs and can use males' nuptial gifts for somatic maintenance. On the other hand, it is known that the ejaculates of *A. obtectus* males contain some toxic accessory gland products, which enhance males' fertilization success (perhaps by destroying or disabling the sperm of previous mates) and expectedly decrease longevity in females (Das et al. 1980). Therefore, the toxicity of seminal fluids may be considered a sexually antagonistic trait (Holland & Rice 1999). However, the L females, kept under starvation and without a laying substrate, have evolved relatively high mating and re-mating abilities (Šešljija et al. 2009). Such elevated sexual activity of females could additionally increase both competition and the sexual activity of L males.

Also, the unexpectedly high level of mating activities in populations selected for late reproduction may be the consequence of long-term experimental procedure on age-specific selection, where we inadvertently created conditions for the evolution of polygamous (L populations) and monogamous (E populations) mating systems in *A. obtectus* (Šešljija et al. 2009; Stojković et al. 2010). Here, the L experimental regime can be considered a treatment with a higher level of sexual conflict because allowing individuals to mate freely for a long time (see Methods) increases the frequency of sexual interactions, as well as post-copulatory sperm competition in males, and ultimately elevates the sexual activity of both sexes.

As indicated earlier, the patterns of mating behavior evolution under age-specific selection in

A. obtectus are completely opposite to those obtained in *C. maculatus*. However, both seed beetle species reveal similar trends if the effects of sexual activity on potential RI are compared. Higher mating activity (early reproducing in *C. maculatus* and L populations in *A. obtectus*) was associated with increased success in heterotypic crosses, i.e., the absence of pre-zygotic RI. The study of *C. maculatus* was not specifically focused on the investigation of RI, and the only RI parameter employed was based on simple mating frequencies (*I* index; Powell 1997). Therefore, it could not be inferred whether the evolution of lower sexual activity in late-reproducing lines was followed by the increase of intrapopulation assortative mating (based on mate choice), as was detected in E populations of *A. obtectus*.

Our results indicate that elevated sexual activity may hinder the evolution of pre-zygotic RI. The correlation between the levels of hetero- and homosexual activity found in *A. obtectus* (Stojković et al. 2010) suggests that beetles in late-reproducing populations exhibit a low degree of sexual discrimination. It is reasonable to hypothesize that indiscriminate sexual behavior is associated with low levels of specific mating preferences; these are the circumstances in which RI is hampered (Parker & Partridge 1998). On the other hand, if lower sexual activity is related with significant pre-zygotic RI between E populations, we have to presume that such mating behavior is connected with elevated 'choosiness' of beetles. Finally, one could ask 'who is choosing whom?' in this selection treatment. Because E beetles usually have only one opportunity to copulate, the fitness of both sexes depends on the quality of their single mate. Thus, we can expect both females and males to be fastidious. If we suppose that in this treatment 'choosiness' is correlated with mating activity, then it is more probable that females control reproduction in E populations. Namely, our previous study revealed that E females were more sexually active than E males (E males were characterized as 'lazy'; Stojković et al. 2010). As predicted by Parker (2006), under these circumstances, isolation barriers increase, and the first step in speciation is favored.

The scenario outlined earlier, which is very similar to those proposed by Rowe & Day (2006), implies that a detailed understanding of the ways in which mating is affected by female and male behavior may be crucial for inferring which processes underlie the first steps in the pre-zygotic isolation between allopatric populations subjected to uniform natural selection.

Acknowledgements

We gratefully acknowledge Professor Tom Tregenza and two anonymous referees for helpful comments on the manuscript. This study was supported by the Ministry of Education and Science of Serbia, project No. 173007.

Literature Cited

- Bacigalupe, L. D., Crudington, H. S., Hunter, F., Moore, A. J. & Snook, R. R. 2007: Sexual conflict does not drive reproductive isolation in experimental populations of *Drosophila pseudoobscura*. *J. Evol. Biol.* **20**, 1763–1771.
- Beaver, M. & Giebertowicz, J. M. 2004: Regulation of copulation duration by period and timeless in *Drosophila melanogaster*. *Curr. Biol.* **14**, 1492–1498.
- Boake, C. R. B., McDonald, K., Maitra, S. & Ganguly, R. 2003: Forty years of solitude: life-history divergence and behavioural isolation between laboratory line of *Drosophila melanogaster*. *J. Evol. Biol.* **16**, 83–90.
- Carvajal-Rodriguez, A. & Rolan-Alvarez, E. 2006: JMATING: a software for analysis of sexual selection and sexual isolation effects from mating frequency data. *BMC Evol. Biol.* **6**, 40.
- Cohan, F. M. 1984: Can uniform selection retard random genetic divergence between isolated conspecific populations. *Evolution* **38**, 495–504.
- Cohan, F. M. & Hoffmann, A. A. 1989: Uniform selection as a diversifying force in evolution: evidence from *Drosophila*. *Am. Nat.* **134**, 613–637.
- Cordts, R. & Partridge, L. 1996: Courtship reduces longevity of male *Drosophila melanogaster*. *Anim. Behav.* **52**, 269–278.
- Coyne, J. A. & Orr, H. A. 2004: Speciation. Sinauer Associates, Sunderland, MA.
- Coyne, J., Elwyn, S. & Rolan-Alvarez, E. 2005: Impact of experimental design on *Drosophila* sexual isolation studies: direct effects and comparison to field hybridization data. *Evolution* **59**, 2588–2601.
- Das, A. K., Huignard, J., Barbier, M. & Quesneau-Thierry, A. 1980: Isolation of two paragonial substances deposited into the spermatophores of *Acanthoscelides obtectus* (Coleoptera, Bruchidae). *Experientia* **36**, 918–920.
- Edvardsson, M. 2007: Female *Callosobruchus maculatus* mate when they are thirsty: resource rich ejaculates as mating effort in a beetle. *Anim. Behav.* **74**, 183–188.
- Fricke, C. & Arnqvist, G. 2004: Divergence in replicate phylogenies: the evolution of partial post-mating prezygotic isolation in bean weevils. *J. Evol. Biol.* **17**, 1345–1354.
- Fricke, C. & Arnqvist, G. 2007: Rapid adaptation to a novel host in a seed beetle (*Callosobruchus maculatus*): the role of sexual selection. *Evolution* **61**, 440–454.

- Fricke, C., Andersson, C. & Arnqvist, G. 2010: Natural selection hampers divergence of reproductive traits in a seed beetle. *J. Evol. Biol.* **23**, 1857–1867.
- Gay, L., Eady, P. E., Vasudev, R., Hosken, D. J. & Tregenza, T. 2009: Does reproductive isolation evolve faster in larger population via sexually antagonistic coevolution? *Biol. Lett.* **5**, 693–698.
- Hartl, D. L. & Clark, A. G. 1989: *Principles of Population Genetics*, 2nd edn. Sinauer, Sunderland, MA.
- Holland, B. & Rice, W. R. 1999: Experimental removal of sexual selection reverses antagonistic coevolution and removes a reproductive load. *Proc. Natl Acad. Sci. USA* **96**, 5083–5088.
- Hollocher, H., Ting, C.-T., Pollack, F. & Wu, C.-I. 1997: Incipient speciation by sexual selection in *Drosophila melanogaster*: variation in mating preference and correlation between sexes. *Evolution* **51**, 1175–1181.
- Mackay, T. F. C., Heinsohn, S. L., Lyman, R. F., Moehring, A. J., Morgan, T. J. & Rollmann, S. M. 2005: Genetics and genomics of *Drosophila* mating behaviour. *Proc. Natl Acad. Sci. USA* **102**, 6622–6629.
- Maklakov, A. A., Kremer, N. & G. Arnqvist, G. 2006: Ageing and the evolution of female resistance to remating in seed beetle. *Biol. Lett.* **2**, 62–64.
- Maklakov, A. A., Kremer, N. & Arnqvist, G. 2007: The effects of age at mating on female life-history traits in a seed beetle. *Behav. Ecol.* **18**, 551–555.
- Maklakov, A. A., Bonduriansky, R. & Brooks, R. C. 2009: Sex difference, sexual selection, and ageing: an experimental evolution approach. *Evolution* **63**, 2491–2503.
- Maklakov, A. A., Cayetano, L., Brooks, R. C. & Bonduriansky, R. 2010: The roles of life-history selection and sexual selection in the adaptive evolution of mating behaviour in a beetle. *Evolution* **64**, 1273–1282.
- Mani, G. S. & Clarke, B. C. 1990: Mutation order: a major stochastic process in evolution. *Proc. R. Soc. Lond. B* **240**, 29–37.
- Martin, O. Y. & Hosken, D. J. 2003: The evolution of reproductive isolation through sexual conflict. *Nature* **423**, 979–982.
- McBrayer, Z., Ono, H., Shimell, M.-J., Parvy, J.-P., Beckstead, R. B., Warren, J. T., Thummel, C. S., Dauphin-Villeman, C. & Gilbert, L. I. 2007: Prothoracicotropic hormone regulates developmental timing and body size in *Drosophila*. *Dev. Cell.* **13**, 857–871.
- Merrell, D. J. 1949: Selective mating in *Drosophila melanogaster*. *Genetics* **34**, 370–389.
- Parker, G. A. 2006: Sexual conflict over mating and fertilization: an overview. *Philos. Trans. R Soc. Lond. B Biol. Sci.* **361**, 235–259.
- Parker, G. A. & Partridge, L. 1998: Sexual conflict and speciation. *Philos. Trans. R Soc. Lond. B Biol. Sci.* **353**, 261–274.
- Pérez-Figueroa, A., Caballero, A. & Rolan-Alvarez, E. 2005: Comparing the estimation properties of different statistics for measuring sexual isolation from mating frequencies. *Biol. J. Linn. Soc.* **85**, 307–318.
- Powell, J. R. 1997: *Progress and Prospects in Evolutionary Biology: The Drosophila Model*. Oxford Univ. Press, Oxford, UK.
- Prout, T. 1971: The relation between fitness components and population prediction in *Drosophila*. I. The estimation of fitness components. *Genetics* **8**, 127–149.
- Rice, W. R. 1992: Sexually antagonistic male adaptation triggered by experimental arrest of female evolution. *Science* **256**, 1436–1439.
- Rice, W. R. & Hostert, E. E. 1993: Laboratory experiments on speciation – what have learned in 40 years. *Evolution* **47**, 1637–1653.
- Rolan-Alvarez, E. & Caballero, A. 2000: Estimating sexual selection and sexual isolation effects from mating frequencies. *Evolution* **54**, 30–36.
- Rowe, L. & Day, T. 2006: Detecting sexual conflict and sexually antagonistic coevolution. *Philos. Trans. R Soc. Lond. B Biol. Sci.* **361**, 277–285.
- Rundle, H. D., Chenoweth, S. F., Doughty, P. & Blows, M. W. 2005: Divergent selection and the evolution of signal traits and mating preference. *PLoS Biol.* **3**, 1988–1995.
- Rundle, H. D., Chenoweth, S. F. & Blows, M. W. 2006: The roles of natural and sexual selection during adaptation to a novel environment. *Evolution* **60**, 2218–2225.
- SAS Institute, Inc. 2003: *The SAS System for Windows*. Release 9.1. SAS Institute, Cary, NC.
- Šešlija, D., Lazarević, J., Janković, B. & Tucić, N. 2009: Mating behavior on the seed beetle *Acanthoscelides obtectus* selected for early and late reproduction. *Behav. Ecol.* **20**, 547–552.
- Snook, R. R., Brustle, L. & Slate, J. 2009: A test and review of the role of effective population size on experimental sexual selection patterns. *Evolution* **63**, 1923–1933.
- Sokal, R. R. & Rohlf, F. J. 1995: *Biometry: The Principles and Practice of Statistics in Biological Research*, 3rd edn. Freeman, New York.
- Spieth, H. T. & Ringo, J. M. 1983: Mating behaviour and sexual isolation in *Drosophila*. In: *The Genetic and Biology of Drosophila* (Thompson, J., ed.). Academic Press, New York, pp. 223–284.
- Stojković, B., Šešlija-Jovanović, D., Tucić, B. & Tucić, N. 2010: Homosexual behaviour and its longevity cost in females and males of the seed beetle *Acanthoscelides obtectus*. *Physiol. Entomol.* **35**, 308–316.
- Thornhill, R. & Alcock, J. 1983: *The Evolution of Insect Mating Systems*. Harvard Univ. Press, Cambridge.
- Tucić, N., Gliksman, I., Šešlija, D., Milanović, D., Mikuljanac, S. & Stojković, O. 1996: Laboratory evolution of longevity in the bean weevil (*Acanthoscelides obtectus*). *J. Evol. Biol.* **9**, 485–503.

- Tucić, N., Šešlija, D. & Stanković, V. 2004: The short-term and long-term effects of parental age in the bean weevil (*Acanthoscelides obtectus*). *Evol. Ecol.* **18**, 187—201.
- Ursprung, C. M., den Hollander, M. & Gwyne, D. T. 2009: Females seed beetles, *Callosobruchus maculatus*, remate for mate-supplied rather than ejaculate nutrition. *Behav. Ecol. Sociobiol.* **63**, 781—788.
- Wigby, S. & Chapman, T. 2006: No evidence that experimental manipulation of sexual conflict drives premating reproductive isolation on *Drosophila melanogaster*. *J. Evol. Biol.* **19**, 1033—1039.
- Williams, G. G. 1957: Pleiotropy, natural selection, and the evolution of senescence. *Evolution* **11**, 398—411.