Natural selection hampers divergence of reproductive traits in a seed beetle

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Abstract

Speciation is thought to often result from indirect selection for reproductive isolation. This will occur when reproductive traits that cause reproductive isolation evolve (i) as a by-product of natural selection on traits with which they are genetically correlated or (ii) as an indirect result of diversifying sexual selection. Here, we use experimental evolution to study the degree of divergent evolution of reproductive traits by manipulating the intensity of natural and sexual selection in replicated selection lines of seed beetles. Following 40 generations of selection, we assayed the degree of divergent evolution of reproductive traits between replicate selection lines experiencing the same selection regime. The evolution of reproductive traits was significantly divergent across selection lines within treatments. The evolution of reproductive traits was both slower and, more importantly, significantly less divergent among lines experiencing stronger directional natural selection. This suggests that reproductive traits did not evolve as an indirect by-product of adaptation. We discuss several ways in which natural selection may hamper divergent evolution among allopatric populations.

Ever since Darwin (1859) made his case for the diversifying nature of natural selection, evolutionary biologists have recognized the crucial role that adaptive evolution may play in population divergence and speciation. Although genetic drift can clearly contribute to the diversification of traits and ultimately the evolution of reproductive isolation, empirical work during the last few decades suggests that selection generally plays the larger role (Schluter, 2000; Coyne & Orr, 2004). In theory, selection can cause evolutionary diversification in reproductive traits that result in reproductive isolation by either direct or indirect selection (Coyne & Orr, 2004). Direct selection on reproductive traits may occur in sympatric or parapatric populations and will occur whenever individuals showing greater discriminatory behaviour in their mate choice will enjoy higher fitness

as a direct result of their choice, avoiding the often negative effects of limited reproductive compatibility with genetically divergent individuals. Examples of direct selection include selection for assortative mating under sympatry (Johnson *et al.*, 1996; Via, 2001; Gavrilets, 2004; van Doorn *et al.*, 2009) and reinforcement in parapatric populations (Alonzo & Warner, 2000; Schluter, 2000, 2001; Rundle & Nosil, 2005). Direct selection for reproductive isolation will, obviously, not occur under allopatry. Here, divergence in reproductive traits that result in reproductive isolation may instead emerge as a by-product of selection that is unrelated to isolation *per se* (Coyne & Orr, 2004). For example, a classic scenario suggests that natural selection on ecological traits in diverging allopatric populations generates indirect selection for reproductive diversification (Dobzhansky, 1937; Mayr, 1970; Schluter, 2000; Rundle & Nosil, 2005). Here, divergence in reproductive traits evolves as a correlated response to selection on ecological traits with which they are genetically correlated (by pleiotropy or linkage) (Coyne & Orr, 2004). Artificial selection experiments have shown that partial sexual isolation can indeed evolve between populations

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experiencing divergent natural selection regimes (Rice & Hostert, 1993; Coyne & Orr, 2004) and comparative work has revealed positive associations between ecological divergence and reproductive isolation across taxa (Funk *et al.*, 2006).

Another possibility is that the traits causing reproductive isolation have themselves been the targets of selection within diverging allopatric populations, although reproductive isolation *per se* is an indirect outcome of selection (Coyne & Orr, 2004). Recent theory and comparative work have suggested that sexual selection may play a key role here (West-Eberhard, 1983; Panhuis *et al.*, 2001; Coyne & Orr, 2004). At least three facets of sexual selection make it potentially important in this regard. First, reproductive isolation is a result of the evolution of reproductive traits. Because sexual selection acts directly on reproductive traits while natural selection acts only indirectly, sexual selection may generally be a more potent generator of the evolution of reproductive traits (Panhuis *et al.*, 2001). Second, sexual selection may be more powerful than natural selection in causing genetic fission, because it tends to be more efficient in generating linkage disequilibrium across loci (Kirkpatrick & Ravigné, 2002). Third, sexual selection is expected to be more arbitrary, in the sense that there may be a great number of possible evolutionary trajectories under a given sexual selection regime (Lande, 1981; Arak & Enquist, 1993, 1995; Schluter & Price, 1993; Fricke & Arnqvist, 2004; Hotzy & Arnqvist, 2009). Theory predicts that sexual selection that results from sexually antagonistic coevolution should be particularly efficient in generating population divergence in reproductive characters (Rice, 1998; Gavrilets, 2000; Gavrilets *et al.*, 2001; Gavrilets & Waxman, 2002; Arnqvist & Rowe, 2005) and comparative and empirical studies have provided some support for this prediction (Arnqvist *et al.*, 2000; Martin & Hosken, 2003).

It is clear that both natural and sexual selection in isolation can contribute to the evolution of diversification in reproductive traits and can ultimately to the evolution of reproductive isolation (Rice & Hostert, 1993; Schluter, 2000, 2001; Panhuis *et al.*, 2001; Via, 2001; Coyne & Orr, 2004; Rundle & Nosil, 2005). However, we often expect natural and sexual selection to act simultaneously and it is much less clear how these processes interact in their effects on the evolution of reproductive traits in allopatric populations (Blows, 2002; Svedin *et al.*, 2008; Rundle *et al.*, 2009; Chenoweth *et al.*, 2010). The fact that natural selection acts indirectly and sexual selection directly on reproductive traits may further complicate matters. We see two contrasting possibilities. First, sexual selection can reinforce natural selection if the two types of selection are concordant. This would be the case, for example, if traits under direct natural selection are the same as, or are positively genetically correlated with, those that cause high reproductive success among individuals (Schluter, 2000, 2001; Via, 2001; Rundle & Nosil,

2005; van Doorn *et al.*, 2009). Second, natural selection can constrain sexual selection and thus hamper divergent evolution of reproductive traits across allopatric populations. Multivariate selection theory predicts that this would occur, for example, (i) if natural and sexual selection are antagonistic (i.e. different in sign) either for specific traits or for traits that are positively genetically correlated or (ii) if traits under positive natural and sexual selection are negatively genetically correlated (Dickerson, 1955; Lande, 1979; Arnold, 1992; Chenoweth *et al.*, 2010). Artificial selection experiments have confirmed that conflicting selection on genetically correlated characters does tend to reduce their rate of evolution (Rutledge *et al.*, 1973; Zijlstra *et al.*, 2003; Frankino *et al.*, 2005), in line with such general predictions (Houle, 1991; Schluter, 1996; Hansen *et al.*, 2003). Despite these contrasting possibilities, very few studies of experimental evolution have addressed how natural selection affects the rate of evolution of reproductive traits that are under sexual selection (Rundle *et al.*, 2009) and none has focussed explicitly on divergent evolution of reproductive traits within selection regimes.

The foundation of the experiments reported here is the observation that continual postmating sexual selection in our model system, the seed beetle *Callosobruchus maculatus*, generates divergent evolution of reproductive characters across allopatric populations even in the absence of differences in selection regimes (Fricke & Arnqvist, 2004). Our aim here was to assess the indirect effects of natural selection on the rate of divergent evolution of reproductive traits that are under direct sexual selection. We achieved this goal by employing experimental evolution in a seed beetle model system in which we manipulated the intensity of both natural and sexual selection in replicated selection lines. We then assessed the resulting evolution of a large number of reproductive traits. Our analyses focus on composite measures of reproductive traits because we wished to assess the global pattern of evolution, to establish the extent of overall divergence that occurred between lines within selection regimes, rather than the evolution of particular reproductive traits. Given that sexual selection generates arbitrary diversification of reproductive traits across replicate lines within selection regimes (Fricke & Arnqvist, 2004), we test whether natural selection reinforces or hampers such diversification.

Material and methods

For details on the biology of our study species (Bruchidae; *Callosobruchus maculatus*) and for a full account of the selection regimes implemented, we refer to Fricke & Arnqvist (2007). Briefly, *C. maculatus* females mate multiply and the mating system is best characterized as scramble competition polygyny, with ample opportunity for both postmating (Wilson *et al.*, 1997) and premating (Savalli & Fox, 1999) sexual selection. We used a single,

large and outbred base population to establish replicated selection lines, in which we manipulated the intensity of both natural selection and sexual selection using a fully crossed 2×2 factorial experimental design and assessed the resulting evolution of a large number of reproductive traits. Seed beetle populations were kept either on their natural host to which they are adapted (B lines; black-eyed beans [*Vigna unguiculata*] – weak natural selection) or on a novel host (C lines; chick peas [*Cicer arietinum*] – strong natural selection). Further, sexual selection was either completely removed (by rearing lines under strict genetic monogamy; M lines) or merely reduced in intensity (by rearing lines under limited polygamy; P lines) compared to the base population, which in this sense provides a control (high level of multiple mating by both sexes) (Holland & Rice, 1999; Rice & Holland, 2005). Females in the P lines encountered two males sequentially, allowing for both intersexual selection (overt and cryptic female choice) and intrasexual selection (sperm competition). Males in the polygamy treatment were rotated across females within lines, such that males and females encountered two mates each. The effective population size was kept the same in all replicate lines ($N_e = 80$), by seeding each generation with 50 randomly selected pairs in each of the M lines and 57 randomly selected pairs in each of the P lines to compensate for differences in variance in family size in the two mating system treatments (for a detailed account see Fricke & Arnqvist, 2007). There were four replicate populations of each treatment combination ($N = 16$ lines).

We have previously shown that our mating system treatment affected the rate of adaptation to the novel host in these lines, e.g. by increasing female acceptance of chickpeas as a novel oviposition substrate and by increasing larval survival (Fricke & Arnqvist, 2007). Here, we report assays of the evolution of reproductive traits in our selection lines relative to the base population. We focused on postmating processes, as postmating sexual selection has been implicated in incipient reproductive isolation in this group of insects (Brown & Eady, 2001; Fricke & Arnqvist, 2004; Rugman-Jones & Eady, 2007; Gay *et al.*, 2009) and was likely the major source of sexual selection acting in these lines. Following 40 generations of selection, we performed two types of tests. We either crossed males and females from all replicate lines to (i) mates from their own population or (ii) mates from the original base population. We recorded the differences in reproductive responses between these two types of matings over 14 distinct reproductive response variables. A common problem in these types of experiments is that within-line coevolution between males and females may effectively mask and/or confound the measurement of evolution of reproductive traits (Rice & Holland, 1997; Holland & Rice, 1998; Long *et al.*, 2006). We evade this problem by using the base population as a common background

control and expressing all traits relative to matings with base line mates. Because we thus focus on differences in reproductive response to own vs. base population mates, our response variables collectively quantify the amount and type of effective evolution that occurred during selection in each line. Our focal measure of divergence, however, quantifies divergence across selection lines within a given selection regime and is thus independent of the divergence that has occurred from the base population (see inferential rationale below for details). All experiments were performed under adult aphagy (Moller *et al.*, 1989; Fox, 1993; Fox & Dingle, 1994).

Cost of mating

Virgin or singly mated females were kept individually in small petri dishes (ø 3 cm) without any oviposition substrate, to preclude egg laying and their lifespan was recorded. In generation 40, we collected virgin females and assigned 60 random females from each selection line to one of three treatments, the same day as they eclosed. Females were (i) kept as virgins, or were mated once either (ii) to a male from their own selection line or (iii) to a male from the base line (all males were virgin and 0–3 days post-eclosion). Additionally, we collected virgin base females in the same manner and assigned them to the following three treatments: (i) kept as virgins, mated once to (ii) a base male or (iii) a selection line male. We aimed at collecting 20 replicates for each treatment combination and line. All females were then placed in an incubator under standard conditions and were checked daily until their natural death occurred.

Effects of mating on early fecundity

In generation 41, we assessed female fecundity early in life. We collected 40 virgin females from each selection line as well as virgin females from the base population. For each line, line females were mated once to either a virgin male from their own selection line ($N = 20$) or a virgin base population male ($N = 20$). Base females were also mated once to either a virgin line male ($N = 20$ for each line) or a base male ($N = 20$). Following matings, females were placed individually in a petri dish provided with 4 g of beans of the appropriate host type (black-eyed beans for base and B line females and chick-peas for C line females) and were allowed to oviposit for 24 h. Females were removed the following day and preserved in ethanol, to allow subsequent measures of female body size. Oviposition vials were stored in an incubator for seven days under standard conditions. After this period, the number of eggs laid was counted.

Sperm competition

Sperm competition assays quantify a male's ability to compete for the fertilization of a given set of female ova

against rivalling male ejaculates (Parker, 1970). We measured sperm offense ability (P2; i.e. a male's ability to outcompete the ejaculate of the previous mate of a twice-mated female) of line males in generations 36 and 37. We performed a sperm competition experiment where selection line males competed for fertilization with sterilized base males in a common sperm competitive background (base population females). Beetles were collected from the base population and the selection lines, and all beetles used were virgin and females were one day, first males were one-two days and second males were three-four days post-eclosion when used in these experiments. Base males were sterilized by irradiation one-four hours prior to mating, in a standard Caesium source to a total dose of 70 Gy. This dose effectively rendered males sterile (99.3% sterility see Dowling *et al.*, 2007) as ova fertilized with sperm from irradiated males do not hatch (Eady, 1991). Virgin base females were first allowed to mate with an irradiated base male. Thus once-mated females were then housed in a small petri dish (ø 3 cm) in an incubator at standard conditions for 24 h. They were provided with a single black-eyed bean, to elicit oviposition which elevates subsequent remating (Eady *et al.*, 2004). Following these 24 h, females were offered a selection line male for a second mating, after which they were transferred to an oviposition vial with 8 g of black-eyed beans and were allowed to oviposit for three days. Females who did not mate a second time were discarded. All successful selection line males and all females were preserved in ethanol to allow body size measurements. Following 7 days of storage of the oviposition vials in an incubator under standard conditions, we recorded the number of hatched and unhatched eggs. Hatched eggs were considered fertilized by selection line males and unhatched eggs by sterilized base males. We note that the hatching rate of eggs in this species is normally very high (Fricke & Arnqvist, 2004).

We also measured male sperm defence ability (P1; i.e. a male's ability to withstand competition from a second ejaculate within a twice-mated female). This test was performed for all selection lines during generation 41, using the same experimental protocol as described earlier with the following two exceptions. First, beetles used were not collected directly from selection conditions. In generation 40, 250–300 beetles were randomly collected from each selection line and placed on 120 g of the respective host. The offspring of these beetles were then used in the sperm competition experiment. Second, the mating order of the two males was reversed such that virgin base population females first mated with a line male and subsequently with a sterilized base population male. For both P1 and P2, we then used the square root arcsine transformed ratio of the number of hatched eggs to the total number of eggs laid by each female as our measure of sperm competition success of selection line males.

Female remating rate

We tested for male and female influence on female remating behaviour by employing the following basic design on four different mating combinations ($N = 20$ per line and mating combination). In generation 41, we collected 200–250 beetles randomly from each selection line and placed them on 120 g of the respective host. The offspring of these beetles were then used to perform the following assays. Virgin females, aged one day post-eclosion, were placed individually with a single virgin male each and allowed to mate. Males were removed after mating and discarded, whereas females were provided with a single bean and held individually in a small petri dish (ø 3 cm) at 22.5 ± 1 °C. After 24 h, the bean was removed from each petri dish and females were presented with a second virgin male (aged 1–2 days post-eclosion) and remating behaviour was recorded during a 30 min period. Our four mating combinations were as follows: (1) females were presented with a male from her own selection line (or base population for base females) as both first and second males. This allowed us to estimate the remating rate for each population. (2) Selection line females were presented with base population males as both first and second males. (3) Base population females were first mated to a selection line male and subsequently presented a base population male. (4) Base population females were first mated to a base population male and then offered a selection line male.

Reproductive response variables

From the experiments described earlier, we extracted the following 14 reproductive response variables. For all variables, we used the mean value for a given line when assessing evolutionary response to selection. We note here that our rationale assumes that differences in reproductive response variables are caused by differences in the underlying reproductive traits that affect such responses, such as accessory seminal fluid substances of the ejaculate or genital morphology. We note that both ejaculate composition (Savalli & Fox, 1998; Savalli *et al.*, 2000; Bilde *et al.*, 2008) and male genital morphology (Hotzy & Arnqvist, 2009) show genetic variation and are known to affect female reproductive responses (Rönn *et al.*, 2008) in this model system.

These following 14 reproductive response variables were extracted for further analysis:

(1) Female resistance: female resistance to mating costs induced by own males; lifespan of line females mated once to base males minus lifespan of line females mated once to their own males.

(2) Male harmfulness: male induction of mating costs in base females; lifespan of virgin base females minus lifespan of base females mated once to line males.

(3) Coevolved fecundity effects: male gonadotropic effects on own females; the number of eggs laid after mating with own males minus number of eggs laid after mating with base males.

(4) Male fecundity effects: male gonadotropic effect on base females; the number of eggs laid after mating with line males minus number of eggs produced after mating with base males.

(5) Remating rate: female remating rate with own males; the rate of remating when both 1st and 2nd males were own males.

(6) Remating inhibition: time until remating with own males; the time until remating when both 1st and 2nd males were own males.

(7) Female remating rate: female remating rate with base males; the rate of remating when both 1st and 2nd males were base males.

(8) Female remating inhibition: time until remating with base males; the time until remating when both 1st and 2nd males were base males.

(9) Female remating resistance (rate): female net resistance to remating (rate); remating rate for line females when both 1st and 2nd males were base males minus that when both 1st and 2nd males were own males.

(10) Female remating resistance (time): female net resistance to remating (time); the time until remating for line females when both 1st and 2nd males were base males minus that when both 1st and 2nd males were own males.

(11) Male defence ability (remating): remating rate of base females with base males, after females first mated to a line male.

(12) Male mating success: remating rate of base females with line males, after females first mated to a base male.

(13) Male defence ability (P1): proportion of offspring sired by line males when base females first mated a line male and then a base male.

(14) Male offense ability (P2): proportion of offspring sired by line males when base females first mated a base male and then a line male.

Inferential rationale

To reiterate, our chief goal was to compare overall divergence in reproductive traits between replicate lines within selection regimes. However, the global quantification of net divergence represents a nontrivial analytical challenge. Our analytical strategy follows a geometric approach (Arnqvist, 1998). In total, we measured 14 different reproductive response variables, which collectively reflect the amount and type of evolution of reproductive traits in selection lines relative to the common base line control. Following standardization of all 14 response variables (by subtraction of the mean and division with the standard deviation), we ordinated each

replicate line in a 14-dimensional space formed by our reproductive response variables. We then measured the Euclidean distance from the position of each selection line within the 14-dimensional space to the mean of its treatment level within that space (i.e. the length of the vector connecting the two points). We then asked, for example, if lines evolving under monandry are more or less different from one another (i.e. have evolved more or less divergently) than lines evolving under polyandry are from one another (see Fig. 1 for a two-dimensional representation of the inferential logic). These Euclidian distances were then used as a response variable in an analysis of variance.

The assays described earlier were conducted with individuals reared under selection conditions, rather than under common garden conditions, a design that can be susceptible to the influence of direct maternal environmental effects. However, several facts collectively suggest that such effects do not significantly influence our results. First, our B lines shared natural selection conditions with the base population. Thus, any differences between these cannot result from maternal environmental effects because of different food resources. Second, we have shown elsewhere that evolution of key fitness traits in this experiment, including female
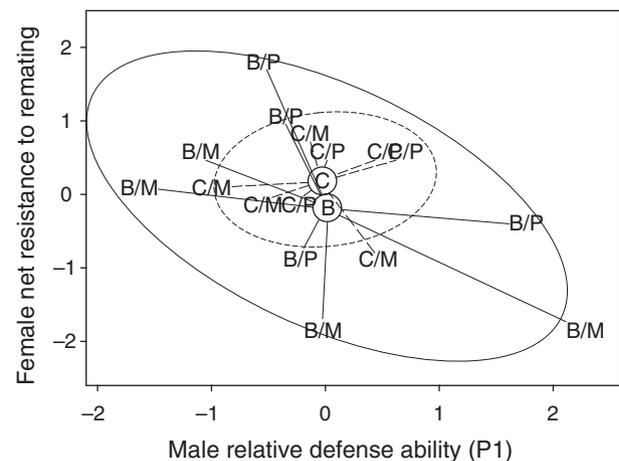

Fig. 1 Illustration of the strategy used to analyse reproductive divergence. The figure shows ordination of all replicated selection lines in a two-dimensional space described by two reproductive variables (C: chick peas, B: black-eyed beans, M: monogamy, P: polygamy). The Euclidean distances between selection lines and their treatment level mean (exemplified here with food treatment, denoted by encircled letters) quantify evolutionary divergence within that treatment level. Thus, for a given food treatment level, the eight distances collectively describe the degree of evolutionary divergence within that treatment level. Our analysis shows that evolutionary divergence across lines within treatment level, in 14 dimensions simultaneously, was significantly larger when beetles were kept on black-eyed beans (solid lines) compared to chick peas (hatched lines). Ellipses represent Gaussian bivariate confidence ($P = 0.6$) ellipses.

reproductive traits, was gradual and monotonic over the course of the 40 generations of selection used (Fricke & Arnqvist, 2007). This is inconsistent with an important general influence of maternal environmental effects. Third, female body size did not differ across selection regimes in our experiment (Fricke & Arnqvist, 2007), which is inconsistent with a general role of maternal environmental effects. Fourth, and most importantly, our primary inferences concerns differences between replicate lines within treatments. As the lines being compared thus share the same selection conditions, simple differences in maternal environmental effects are very unlikely to affect our main results. In addition, we note that reproductive traits that affect postmating reproductive responses are known to evolve rapidly and show genetic differentiation even across closely related laboratory populations in this model system (Savalli *et al.*, 2000; Brown & Eady, 2001; Fricke & Arnqvist, 2004; Fricke *et al.*, 2006).

Results

Our main goal was to compare the degree to which evolution of reproductive traits within treatments was divergent. Such arbitrary evolutionary divergence is at the heart of incipient speciation, although very rarely quantified (Funk *et al.*, 2006). However, we first assessed overall evolution of reproductive traits relative to the base population in our selection lines. Two of our 14 reproductive response variables (P1 and P2, standard key traits in studies of postmating sexual selection) offered a direct comparison between the base population on one hand and the selection lines, collectively, on the other, by planned post-contrasts following one-way ANOVAS. This analysis showed that our selection lines differed significantly from the base line in P1 ($F_{1,316} = 10.96$, $P = 0.001$) but not in P2 ($F_{1,343} = 0.29$, $P = 0.59$). A similar comparison between the base line and the subset of eight selection lines that shared the natural selection regime with the base line again showed significant differences in P1 ($F_{1,169} = 10.27$, $P = 0.002$) but not P2 ($F_{1,184} = 0.28$, $P = 0.59$). Although these analyses suggest that the altered sexual selection regime in our selection lines compared to the base population led to directional selection on reproductive traits that determine P1, we note that this difference could also have resulted from differences in effective population size between the base population vs. the selection lines (N_e was larger in the base population). When restricting the analysis to only include all selection lines, there were highly significant differences across the 16 selection lines in both P1 and P2 (one-way ANOVAS; P1: $F_{15,296} = 2.85$, $P < 0.001$; P2: $F_{15,323} = 2.42$, $P = 0.002$). Further, there were significant differences across replicate B lines in both P1 and P2 (one-way ANOVAS; P1: $F_{7,149} = 5.70$, $P < 0.001$; P2: $F_{7,164} = 3.02$, $P = 0.005$), whereas replicate C lines differed in P2 but not in P1 (one-way ANOVAS; P1: $F_{7,147} = 0.80$, $P = 0.59$; P2: $F_{7,159} = 2.11$, $P = 0.045$). Thus, we conclude that at least some key reproductive traits in our selection lines evolved away from the base population and, more importantly, did so to different extents across replicate selection lines. This finding forms a prerequisite for the focal analyses of selection line means which follows below.

Divergent evolution between lines within treatments, which was the focus of our efforts, was analysed by ANOVA of the Euclidean distances between each line and its group mean. Effects were evaluated both by conventional F-tests and by the randomization test suggested by Manly (1997), based on 100 000 random pertubations of our data (denoted P_{rand} below). These analysis revealed that evolution of reproductive traits was less divergent across replicated selection lines evolving under more, compared to less, intense natural selection (effect of food treatment: $F_{1,14} = 6.867$; $P = 0.020$; $P_{rand} = 0.023$; Levene's test of homogeneity of variance: $P = 0.779$) (mean Euclidean distances; B-lines: 3.654, SE = 0.155; C-lines: 3.084, SE = 0.153; see Fig. 1). However, divergence did not differ significantly between lines experiencing different mating systems (effect of mating system: $F_{1,14} = 0.071$; $P = 0.794$; $P_{rand} = 0.799$; Levene's test of homogeneity of variance: $P = 0.543$). Nor did our food and mating system treatments interact significantly in their effects on evolutionary divergence (effect of interaction: $F_{3,12} = 2.373$; $P = 0.121$; $P_{rand} = 0.129$; Levene's test of homogeneity of variance: $P = 0.226$). Thus, our results show that strong natural selection was associated with a lower diversification of reproductive traits.

Although our main goal was to assess divergence between lines within treatments, we wished to test for effects of our treatments on the overall magnitude of evolution in reproductive traits. Our limited sample size relative to the large number of response variables (N per cell = 4 for 14 response variables) precluded the use of MANOVA to this end. We instead first reduced the number of reproductive response variables in a standard principal component analysis (PCA) and then assessed treatment effects on evolution along the resultant multivariate vectors. Our principal component analysis of the reproductive response variables yielded five PCs with an eigenvalue higher than one. The per cent of total variance explained by these PCs were 26.8%, 26.1%, 13.9%, 9.5% and 8.6%, respectively.

We used two-way ANOVA of the five PCs of our response variable matrix to assess treatment effects on the magnitude of evolution in reproductive traits. In no case were variances significantly nonhomogenous across treatment levels (Levene's test; $P > 0.119$ in all cases) and the residuals of these models did not deviate significantly from normality (Shapiro-Wilk's test; $P > 0.073$ in all cases). Yet all inferential ANOVAS were also tested using randomization tests (Manly, 1997) but in no case did these tests differ from the conventional F-tests in terms of our ability or inability to reject null

hypotheses at α. We failed to find any significant differences in the mean of reproductive traits following a removal vs. a reduced intensity of sexual selection, as our models revealed no significant effects of either mating system or the interaction between food and mating system on any of our five PCs (e.g. for PC1: $F_{1,12} = 0.08$, $P = 0.78$; and $F_{1,12} = 0.06$, $P = 0.81$; respectively) (Fig. 2). In contrast, we found significant effects of our food treatment along PC1 ($F_{1,12} = 6.18$, $P = 0.029$; $P > 0.05$ for all lower order PCs) (Fig. 2 and Table 1). This effect was because of lines reared on chick peas generally having lower absolute values for those reproductive response variables (which contrasts the focal line with the base population) that showed the strongest loading on PC1. Thus, reproductive traits in lines reared on the novel host (chick peas) tended to resemble those in the base population more than did those in lines reared on black-eyed beans. Previous analyses have shown that directional natural selection for adaptation to the novel host occurred in lines reared on chick peas, mostly manifested as increased larval survival and higher acceptance of chick peas as a host during oviposition, and C lines experienced stronger natural selection than B lines (e.g. standardized linear selection gradient for host acceptance; $s' \approx 0.27$) (see Fricke & Arnqvist, 2007). In summary, our analyses showed that lines evolving under more intense natural selection showed both a less divergent and a less rapid evolution of reproductive traits.

Discussion

We found that natural selection had a significant effect on the evolution of reproductive traits in our allopatric selection lines. While lines held on the novel host showed more evolution in terms of traits under natural selection (e.g. host acceptance during oviposition, juvenile survival and juvenile growth: see Fricke & Arnqvist, 2007), we found that they showed a less divergent and a less rapid evolution of reproductive traits compared to lines held on the ancestral host. Below, we first discuss the potential causes for our results and then briefly consider some of their implications.

Although it is often predicted that evolution of incipient speciation is more likely under strong natural selection (Dobzhansky, 1937; Mayr, 1970; Schluter, 2000), the opposite may also hold true in some situations and the generality of the classic prediction may be somewhat restricted when considering the simultaneous effects of sexual and natural selection. At least three distinct effects associated with natural selection may in theory act to hamper evolutionary diversification of reproductive traits that are under diversifying sexual selection. First, the reduction in effective population size that follows from more intense natural selection can, in itself, act to slow down the response to sexual selection (Falconer & Mackay, 1996). However, our selection lines
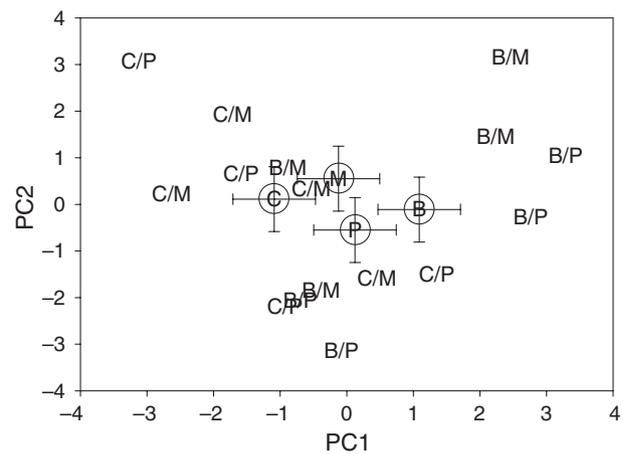

Fig. 2 Bivariate plot, showing ordination of all selection lines along the two first principal components of the matrix describing net evolution of reproductive characters (C: chick peas, B: black-eyed beans, M: monogamy, P: polygamy) across selection lines. Shown are also treatment level least-squares means (± SE) for the two selection factors (denoted by encircled letters). Note that a positive score along PC1 is associated with the evolution of reproductive traits that are more different from those in the base population.

Table 1 Loadings of each reproductive response variable on the first principal component. Reported are also *F*-values from analyses of variance for each of the 14 variables separately, to illustrate the response to selection for each variable in isolation.

Variable†	Loading (PC1)	Univariate ANOVAS ($F_{1,12}$)		
		Food	Mating	Food × Mating
Female resistance	0.698	1.819	1.440	0.930
Male harmfulness	−0.738	5.251**	0.002	0.150
Coevolved fecundity effects	−0.389	0.904	0.002	0.617
Male fecundity effects	0.413	0.002	0.586	1.751
Remating rate	0.615	4.313*	1.806	0.373
Remating inhibition	−0.501	2.924	0.217	0.006
Female remating rate	0.335	5.378**	0.178	0.044
Female remating inhibition	0.641	0.923	0.879	0.645
Female remating resistance (rate)	−0.471	0.558	2.620	0.558
Female remating resistance (time)	0.804	4.611*	0.036	0.346
Male defense ability (remating)	−0.183	0.059	0.059	0.322
Male mating success	−0.219	0.581	2.701	0.398
Male defence ability (P1)	0.536	0.008	0.593	0.065
Male offense ability (P2)	−0.088	0.085	0.172	0.332

†See Materials and Methods for a description of reproductive response variables.
*$P < 0.10$; **$P < 0.05$.

were never bottlenecked and the effective population size was large ($N_e \sim 80$), constant over time and efforts were made to achieve the same effective population size in the two food treatments (Fricke & Arnqvist, 2007). Hence, this effect is very unlikely to alone have caused

our results (Ödeen & Florin, 2000). We also note that differential genetic drift is incompatible with the observation that evolution was more rapid and divergent among those of our lines that experienced less intense natural selection: such lines should, if anything, have a higher effective population size and thus be less subject to drift. Second, because natural selection tends to erode genetic variation (Fisher, 1930), the response to sexual selection may decelerate owing to a general depletion of additive genetic variance under strong natural selection (Blows & Hoffmann, 2005). Assuming that all evolving genes have predominantly additive effects, we expect the number of generations required to reach half-way to the selection limit (i.e. the half-life) to be approximately 1.4 N_e, in our case 112, generations (Falconer & Mackay, 1996). Because we assessed effects after 40 generations, this effect is also unlikely to alone have caused our results.

Third, populations under natural selection may generally be less able to respond to sexual selection because the two types of selection may exert opposing effects through selection on traits/loci that are genetically correlated (Dickerson, 1955; Hansen *et al.*, 2003). Most importantly, reproductive traits under sexual selection may evolve less divergently if natural selection, through selection on genetically correlated traits, dictates the evolutionary paths that reproductive traits can evolve along (Arnold, 1992; Schluter, 1996; Chenoweth *et al.*, 2010). Such genetic coupling across traits could quite generally be brought about by either pleiotropy or linkage disequilibrium. It is worth noting that genetic drift in finite populations is predicted to generate negative linkage disequilibrium between distinct loci that are simultaneously under directional selection. Such 'Hill–Robertson' effects (Hill & Robertson, 1966; Felsenstein, 1974) reduce the rate of incorporation of advantageous alleles and limit the rate of evolution (Barton, 1995). These considerations suggest that natural selection can sometimes exert a hampering effect on the divergent evolution of reproductive traits caused by sexual selection, thus constraining incipient population divergence, by effectively impeding the response to sexual selection (Rundle *et al.*, 2009). To the extent that traits that directly influence reproductive isolation are under sexual selection (Panhuis *et al.*, 2001; Coyne & Orr, 2004), these effects may be quite general. In seed beetles, traits under postmating sexual selection are known to be genetically correlated with general traits, such as body size (Czesak & Fox, 2003) and viability (Bilde *et al.*, 2009). This supports the possibility that natural selection may have hampered evolutionary divergence of reproductive traits in our selection lines by selection acting on genetically correlated characters.

We suggest that the type of experimental evolution created in our selection lines is a reasonable representation of incipient speciation scenarios in this group of insects, which adds some validity to our findings. Host shifts have occurred repeatedly in this genus and have been associated with other types of adaptations (Tuda *et al.*, 2006). Further, postmating sexual selection has been identified as a potent form of selection, both in comparative studies of mating system evolution in the genus (Rönn *et al.*, 2006, 2008; Katvala *et al.*, 2008) and in experimental studies of reproductive divergence (Brown & Eady, 2001; Fricke & Arnqvist, 2004; Fricke *et al.*, 2006; Rugman-Jones & Eady, 2007; Gay *et al.*, 2009). Gametic isolation, such as conspecific sperm precedence resulting from postmating sexual selection, can effectively reduce gene flow between diverging populations in the face of incomplete barriers to premating isolation both in general (Howard, 1999; Eady, 2001) and in this genus (Rugman-Jones & Eady, 2007). Previous empirical work in this group (see references above) have shown that reproductive traits underlying postmating responses show rapid evolution, and the current results underpin this general observation. In part, this is likely because male and female postmating traits are subjected to direct selection resulting from sexually antagonistic coevolution (Arnqvist & Rowe, 2005; Rönn *et al.*, 2007; Hotzy & Arnqvist, 2009).

We expected the altered sexual selection regimes in our selection lines (i.e. either reduced or removed, compared to base population conditions) to result in significant differences in the evolution of reproductive traits (Blows, 2002; Martin & Hosken, 2003; Crudgington *et al.*, 2005) but we failed to find any such effects. We note that our mating system treatment did have a significant effect on the rate of adaptation *per se* in these lines (Fricke & Arnqvist, 2007). In one sense, our data suggest that the removal/reduction of sexual selection led to more divergence in reproductive traits across lines when unchained from strong natural selection (Fig. 1). This is generally in line with theory suggesting that there are a great number of possible evolutionary paths that can result from a given alteration of the sexual selection regime (Lande, 1981; Arak & Enquist, 1993, 1995; Schluter & Price, 1993; Fricke & Arnqvist, 2004). Yet because sexual selection is, at least in part, generated by sexual conflict in seed beetles (Crudgington & Siva-Jothy, 2000; Rönn *et al.*, 2006, 2007; Gay *et al.*, 2009; Hotzy & Arnqvist, 2009; Maklakov & Arnqvist, 2009), the fact that we failed to find differences between a removal and a reduction of sexual conflict is not congruent with theoretical predictions (Rice, 1998; Arnqvist *et al.*, 2000; Gavrilets, 2000; Arnqvist & Rowe, 2005). The difference in sexual selection regime between our mating system treatment levels was rather small when compared to the highly promiscuous base population conditions, and we suggest that the lack of a significant effect seen here might have been because of this fact.

Our explicit focus on evolutionary divergence yielded distinct insights. Both natural selection and sexual selection in isolation can contribute to reproductive incompatibilities and, ultimately, to reproductive

isolation. However, we show that the diversifying effect of an altered sexual selection regime can apparently be hampered by strong natural selection (Chenoweth *et al.*, 2010). We suggest that the deceleration of the rate of divergent evolution of reproductive traits seen in *C. maculatus* was because of selection on correlated characters. While sexual selection reinforced the effects of natural selection in terms of the rate of adaptation in our selection lines (Fricke & Arnqvist, 2007), presumably by accelerating the rate of spread of alleles beneficial when adapting to a novel environment, natural selection apparently weakened the effects of altered sexual selection on reproductive traits. Our results illustrate that the analyses of the joint effects of natural and sexual selection can lead to important new insights into the divergent evolution of reproductive traits. Studies of experimental evolution, such as the one reported here, can clearly shed light on how indirect selection for reproductive isolation in allopatric populations can generate incipient speciation and can help entangle complex interactions between multiple forms of selection (Blows, 2002; Houle & Rowe, 2003; Fuller *et al.*, 2005; Rundle *et al.*, 2009; Chenoweth *et al.*, 2010).

Acknowledgments

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