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The effect of inbreeding and outcrossing of *Tribolium castaneum* on resistance to the parasite *Nosema whitei*

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ABSTRACT

Background: The microsporidian *Nosema whitei* is a natural parasite of the red flour beetle, *Tribolium castaneum*. The results of a previous study showed that, during co-evolution of the two species in the laboratory, host populations maintained elevated levels of heterozygosity.

Hypothesis: Heterozygote advantage accounts for the maintenance of high levels of host heterozygosity during co-evolution with the parasite. Reduced heterozygosity of the beetle will lead to a decrease in resistance against parasite infection.

Methods: In two experiments, we tested for (a) the effect of inbreeding and (b) the effect of inbreeding and outcrossing on the beetle's resistance to infection, in relation to effects on egg hatching success, development time, and reproductive success.

Results: Inbreeding reduced egg hatching success, prolonged development time, and resulted in lower reproductive success. Outcrossing shortened development time, while we did not find evidence for heterosis for reproductive success. We were unable to detect an effect of changes in heterozygosity on overall resistance to parasitism. The effect of inbreeding on development time did, however, influence parasite-induced mortality profiles: the prolonged development time, resulting from inbreeding, led to higher mortality in earlier developmental stages, but left the overall mortality rate unchanged. Hence, we conclude that heterozygosity is not a principal determinant of the beetle's resistance to infection by *N. whitei*.

Keywords: genetic diversity, genetic variation, homozygosity, host–parasite co-evolution, negative frequency-dependent selection.

INTRODUCTION

Inbreeding, sexual reproduction between related individuals, is inevitable in small, isolated populations. Inbreeding usually does not come without a cost, as inbred progeny often suffer from reduced fitness relative to outbred progeny. This phenomenon is called

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'inbreeding depression' (Charlesworth and Charlesworth, 1987). There are two main hypotheses for inbreeding depression, both of which emphasize the potentially detrimental effects of increased homozygosity, which is a consequence of inbreeding. The dominance hypothesis states that inbreeding increases the expression of deleterious recessive alleles (Roff, 2002). In contrast, the overdominance hypothesis states that inbreeding decreases the chances of expressing overdominance, the situation in which the heterozygote is superior to both homozygotes (Ziehe and Roberds, 1989). Whatever the mechanism is, inbreeding depression has been observed for a wide range of traits (see review in Keller and Waller, 2002). In insects, inbreeding has been shown to have a negative effect on, for example, egg hatching success (van Oosterhout *et al.*, 2000; Haikola, 2003; Fox and Scheibly, 2006), juvenile survival (Armbruster *et al.*, 2000), development time (Roff, 1998; Fox and Scheibly, 2006), juvenile size (Roff, 2002), female fecundity (Roff, 1998; van Oosterhout *et al.*, 2000), and adult lifespan (van Oosterhout *et al.*, 2000).

Several studies have suggested that reduced individual genetic diversity also increases susceptibility to parasites in insects. For instance, inbred fruit flies (*Drosophila nigrospiracula*) showed a higher susceptibility to ectoparasitism by a mite (*Macrocheles subbadius*) (Luong *et al.*, 2007). Similarly, inbreeding in *Drosophila melanogaster* populations lowered the resistance to the bacterial pathogen *Serratia marcescens* (Spielman *et al.*, 2004). Other studies, however, have shown a different picture, and in insects it often proves hard to find a relationship between heterozygosity and resistance. Inbreeding in the red flour beetle *Tribolium castaneum* did not result in an overall decrease in resistance to infections by the rat tapeworm, *Hymenolepis diminuta* (Stevens *et al.*, 1997), and inbreeding depression could not be detected for resistance of the Asian tiger mosquito, *Aedes albopictus*, to infection with *Plasmodium gallinaceum* (O'Donnell and Armbruster, 2010). On a more mechanistic level, inbreeding also seemed to have little or no effect on immune function in the sand cricket, *Gryllus firmus* (Rantala and Roff, 2006). In other cases, effects of inbreeding only indirectly influenced resistance. For example, decreased heterozygosity increased disease susceptibility in termites, *Zooptermopsis angusticollis*, indirectly by affecting social behaviour or other group-level processes, but not directly by affecting the actual immune response (Calleri *et al.*, 2006).

In contrast to inbreeding, outcrossing increases heterozygosity, and it is therefore hypothesized to increase resistance to parasitism (Stevens *et al.*, 1997). In line with the proposed mechanisms underlying inbreeding depression, the key mechanisms explaining heterozygote advantage are dominance – recessive deleterious alleles are complemented in the hybrid – and increased overdominance (Birchler *et al.*, 2010). There is some empirical evidence to support the idea that outcrossing can result in a short-term advantage for hosts when parasites are involved. In an experiment with *Daphnia magna* and its microsporidian parasite *Octosporea bayeri*, it was shown that offspring from outbreeding parents were infected less than offspring that resulted from selfing (Ebert *et al.*, 2007). Because the set-up of the experiment was such that the parasite had to be transmitted to the offspring vertically, from the infected mother to the offspring, this result can be explained by the fact that outcrossed offspring are genetically more different from their parents than selfed offspring. When parasites are adapted to hosts that are common in the current generation, hosts in the next generation will benefit from being different than their predecessors, which represents the core of the Red Queen Hypothesis (Hamilton, 1980). Thus, it is possible that the benefit of outcrossing did not arise directly from an increase in heterozygosity, but rather from dissimilarity between parents and offspring [i.e. similarity selection (Agrawal, 2006)]. Positive correlations between heterozygosity and resistance to parasites as observed in nature could possibly also stem from such Red Queen-like mechanisms. This is especially true when parasites adapt to

locally common host genotypes (Lively and Dybdahl, 2000). Overall, the association between heterozygosity and resistance to parasites remains unclear, and it has been suggested that there might not be a direct link between the two at all (Caughley, 1994; Stevens *et al.*, 1997).

Nevertheless, parasites are still thought to be one of the main selective factors maintaining genetic diversity (Haldane, 1949). For example, *Tribolium castaneum* populations that co-evolved with their microsporidian parasite *Nosema whitei* maintained higher levels of genetic diversity, and consequently also heterozygosity, than control populations (Bérénos *et al.*, 2011). Co-evolution increased recombination rate of the host in the same experimental populations (Kerstes *et al.*, 2012), yet the selective mechanisms behind the maintenance of variation and the increase in recombination rate remain unclear. On the one hand, such an increase could be the result of balancing selection, as recombination relaxes the conflict between loci under strong balancing selection and genes linked to these loci (Beye *et al.*, 1999; Hasselmann and Beye, 2006). On the other hand, Red Queen dynamics can also lead to higher recombination rates (Peters and Lively, 1999) and the maintenance of genetic diversity (Brockhurst *et al.*, 2004; Schulte *et al.*, 2010). Here, genetic diversity will be maintained because negative frequency-dependent selection prevents the fixation of alleles (Hamilton *et al.*, 1990). To determine whether the observed maintenance of genetic diversity during co-evolution (Bérénos *et al.*, 2011) is a result of selection against increased homozygosity, we set up two experiments investigating the effects of inbreeding and outcrossing of *Tribolium castaneum* on resistance to *Nosema whitei*, and on egg hatching success, development time, and reproductive success. By investigating several life-history traits in parallel with parasite resistance allowed us to further discriminate whether any observed effects on resistance are direct, or mitigated by indirect effects of inbreeding on other traits (Calleri *et al.*, 2006). Expectations prior to the experiments were that a reduction in heterozygosity would not negatively influence resistance, while it was expected to have detrimental effects on the other measured traits. Our results will provide mechanistic insights into the maintenance of genetic diversity throughout a host–parasite co-evolution experiment (Bérénos *et al.*, 2011).

METHODS AND MATERIALS

All beetles were kept on standard medium (organic flour containing 5% dried yeast) and under our standard environmental conditions (24 h dark, 32°C, 70% humidity). For the initial experimental study of host–parasite co-evolution (Bérénos *et al.*, 2009), we created eight beetle lines by crossing unique pairs of stock populations to increase genetic diversity. All eight lines were maintained at large population sizes (500 unsexed individuals were used to start each new generation, in 200 g of medium) for 11 generations (for further details, see Bérénos *et al.*, 2009). After 11 generations, we used the eight lines that were kept under control (i.e. parasite-free) conditions as the mass-bred base populations of our inbred lines. We derived 10–15 inbred lines from each of these base populations, while maintaining all outbred base populations under the same conditions as before (500 beetles every generation, 200 g of flour) during the creation of the inbred lines. Inbred lines were generated by five generations of full-sib matings, leading to an inbreeding coefficient (F) of 0.67 (Wright, 1922). Every mating, a single virgin male and a single virgin female were placed together in a plastic vial with 3 g of standard medium. After 7 days, we removed the parents from the flour. Next, up to five males and five females from the offspring produced per mating were collected as pupae. About 2 weeks after the collection of the pupae, two pairs of full siblings were allowed to mate (two matings, a single pair per mating). Full-sib matings were

replicated to reduce the chance of a line going extinct due to pairs not producing offspring. We used offspring from only one of the two pairs to initiate the next generation of inbreeding. After repeating this inbreeding regime for five generations, all surviving inbred lines were maintained at the same density, although at a lower population size, as the mass-bred lines they were derived from (every generation 40 unsexed adults were transferred to 16 g of medium). In seven of eight lines, we successfully created a sufficient number of inbred lines, which were used for further experiments. Our two experimental set-ups tested the effects of (a) inbreeding and (b) inbreeding versus outcrossing on parasite resistance in addition to other life-history traits.

Experiment 1: Inbreeding and its effect on egg hatching success, development time, and parasite resistance

Fifty unsexed beetles were collected from the seven mass-bred base populations from which inbred lines were successfully produced (17th generation, six generations since the inbred lines were derived from these populations), and from 30 inbred lines derived from those seven base populations (7th generation after initiation of inbreeding regime, 3–5 inbred lines per base population). The 50 beetles were placed on 20 g of flour without yeast for 6 days, after which eggs were collected from the flour. Per line, 48 eggs were put singly in sterile glass vials (40 × 13 mm; VWR, Dietikon, Switzerland). Half of the 48 vials contained 0.1 g of parasite-free standard medium (the control treatment), the other half 0.1 g of standard medium inoculated with *Nosema whitei* spores (the parasite treatment). The *N. whitei* inoculate consisted of a mixture of equal spore numbers from eight different *N. whitei* laboratory isolates (Blaser and Schmid-Hempel, 2005; Bérénos *et al.*, 2009), and was applied at a concentration of 5×10^4 spores per gram of medium. We scored all vials for hatching success, developmental status was recorded every fourth day in the control vials, and mortality was checked after 50 days. A total of 1824 eggs were distributed for this experiment; 836 of these eggs hatched and were used to score mortality.

Experiment 2: Inbreeding, outcrossing, and their effects on development time, reproductive success, and resistance

In contrast to the first experiment, Experiment 2 was devised to allow simultaneous analysis of the effects of inbreeding and outcrossing on our traits of interest. We collected and sexed pupae from seven mass-bred base populations and 14 inbred lines (two per base population). The base populations were in their 19th generation (8th generation since initiation of the inbred lines), the inbred lines in their 9th generation since their initiation. Four weeks after collecting the pupae, we set up mass matings between three virgin males and three virgin females, according to the crossing scheme as shown in Fig. 1. Crosses within lines were replicated twice, and for the crosses between lines each reciprocal cross was seen as a replicate. Crosses were made in sterile plastic vials. Initially, the beetles were kept without medium for one day to facilitate mating. The next day, 3 g of flour without yeast was added to each vial. One day later, all beetles were collected and transferred to a larger vial containing 10 g of standard medium. They were kept on this flour for 3 days, and then transferred to vials containing 10 g of flour without yeast. The vials with standard medium were frozen after 4 weeks, and the number of offspring was counted (as a measurement of reproductive success). After 7 days on the yeast-free flour, larvae were collected and

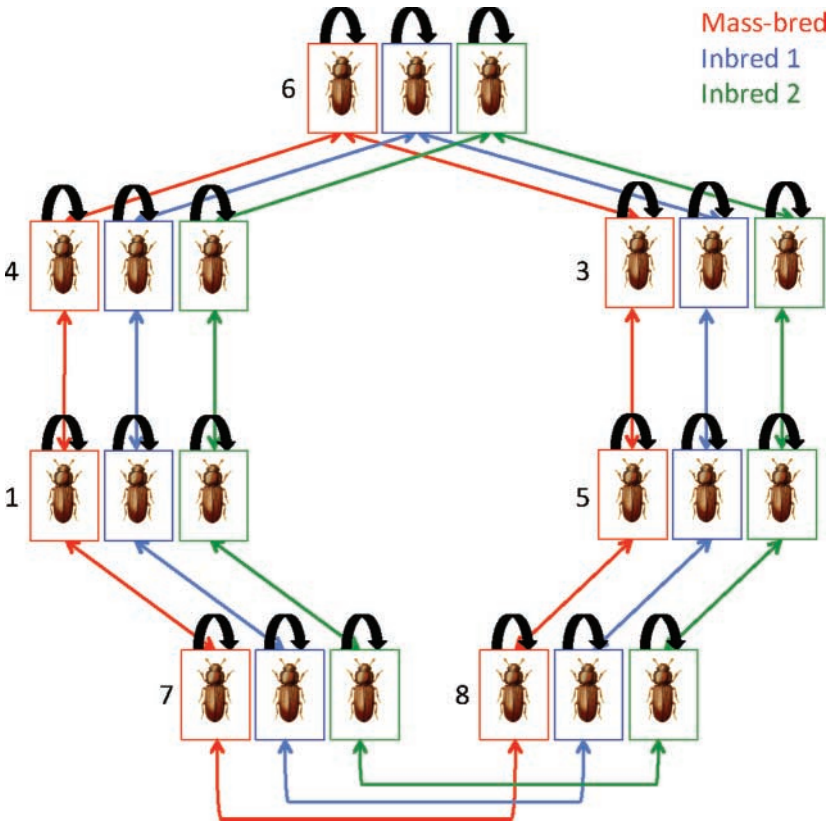

Fig. 1. The crossing scheme of Experiment 2. We used seven mass-bred base populations (red boxes), and two inbred lines derived from each mass-bred population (blue and green boxes). Each line, inbred or mass-bred, was crossed within itself (inbred – black arrows) and with two other lines, in three separate rounds of crosses (outcrossed – red, blue, and green arrows). The final analyses for the effect of outcrossing were done within each round, and with the three rounds combined.

distributed singly over glass vials containing 0.1 g of standard medium (control treatment, 6 per line) or 0.1 g of standard medium inoculated with *N. whitei* spores (parasite treatment, 18 per line, spore concentration of 5×10^3 spores per gram of medium). We checked developmental status on a daily basis in the control treatment, and mortality was checked after 50 days. A total of 2016 larvae were distributed, and 1721 were scored for mortality.

Data analysis

Mortality

Control mortality in many cases was larger than zero, and therefore we had to correct for it (total control mortality was around 10% in Experiment 1 and 3% in Experiment 2). Parasite-induced mortality was calculated as:

$$M_{pi} = \frac{S_c - S_p}{S_c} \quad (1)$$

where M_{pi} represents parasite-induced mortality, S_c is survival in the control treatment, and S_p is survival in the parasite treatment. Parasite-induced mortality was calculated in total and for each developmental stage (larva, pupa, adult) separately. Mortality of a certain developmental stage is the number of beetles that died in that stage divided by the total number of beetles.

Effect of inbreeding on life-history traits

To determine the effect of inbreeding on each trait, the difference between the mean of each inbred line and the mean of its mass-bred base population was calculated. Next, for each base population the average of these differences to their inbred lines was determined, to obtain the average effect of inbreeding on each base population. This resulted in seven 'difference' values for each trait in both experiments (as there were seven base populations). As normality cannot be reliably tested here, we decided to use a non-parametric test (Wilcoxon signed rank test, SPSS v.19 for MacOS X, IBM) to determine whether the median of the differences significantly deviated from zero, thus indicating a unidirectional effect of inbreeding.

To assess the influence of the original trait values of the mass-bred base population on the effect of inbreeding, the trait value after inbreeding was plotted as a function of the original value of the base population (Linear regression, SPSS v.19 for MacOS X).

Heterosis

We define heterosis as 'the amount by which the mean of an F1 family exceeds its better parent' (Mather and Jinks, 1982). The effect of outcrossing was therefore calculated by subtracting the mean of the F1s from the original mean value of the better parent. 'Better' was defined as having produced the most offspring, the shortest development time, or the lowest mortality. Faster growth is generally assumed to be associated with higher fitness, and has been linked to higher fecundity and better survival (Moller, 1997). We again used Wilcoxon signed rank tests to determine whether the median of the differences deviated significantly from zero. As the Wilcoxon signed rank test requires independent differences, and because each parent of each outcrossed combination is shared with one other combination, we tested whether the effect of outcrossing in one cross with a certain parent correlated with the effect of outcrossing in the other cross with that parent (Spearman's rank correlation coefficient, SPSS v.19 for MacOS X).

RESULTS

Experiment 1

Inbreeding significantly decreased egg hatching success (Wilcoxon signed rank test, $n = 7$, $T = 0$, $P = 0.018$; Fig. 2A) and increased development time (time to adulthood: Wilcoxon signed rank test, $n = 7$, $T = 27$, $P = 0.028$; Fig. 2A). Total control mortality was not significantly different in the inbred lines, although there was a trend towards higher control mortality in inbred lines (Wilcoxon signed rank test, $n = 7$, $T = 24$, $P = 0.091$), which was

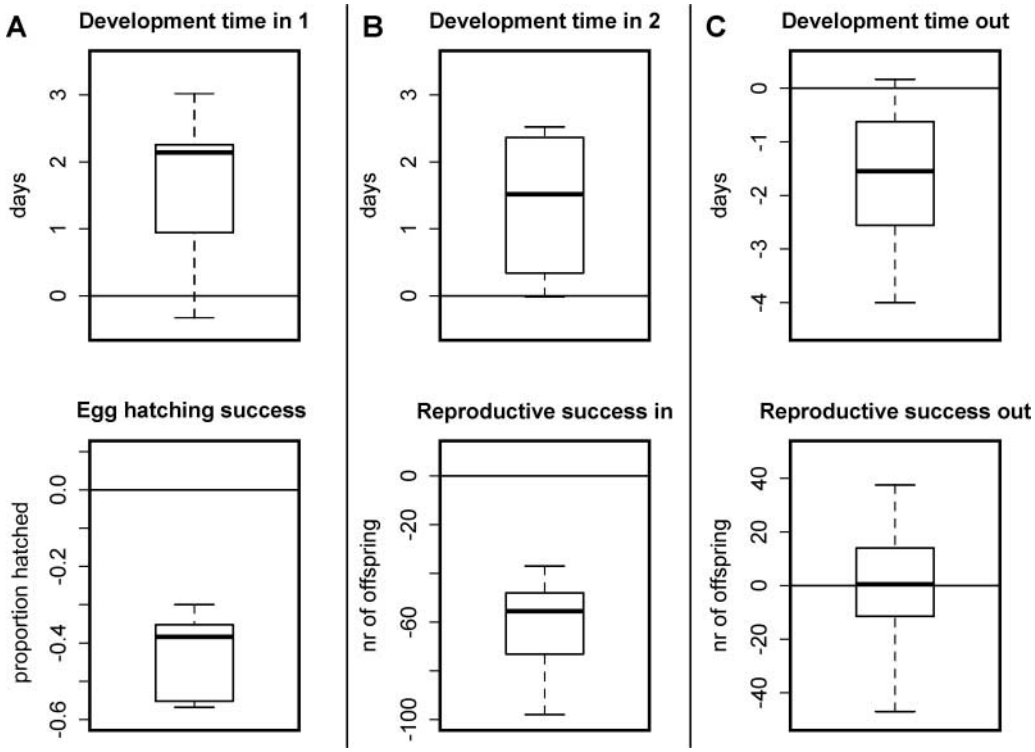

Fig. 2. Box plots showing the effect (change in value) of inbreeding (A and B) and outcrossing (C) on development time, egg hatching success, and reproductive success. The mean of the mass-bred base population was subtracted from the mean of each inbred line, such that positive median values indicate that inbreeding increased the trait value. Results from Experiment 1 are shown in (A), results from Experiment 2 in (B) and (C). Inbreeding prolonged development time and decreased egg hatching success and reproductive success, whereas outcrossing shortened development time but had no effect on reproductive success.

mainly caused by higher adult control mortality in inbred lines ($T = 21$, $P = 0.028$). Neither larval ($T = 7$, $P = 0.465$) nor pupal ($T = 6$, $P = 0.715$) control mortality was significantly altered by inbreeding.

For total parasite-induced mortality after 50 days, we found no significant effect of inbreeding when all life stages were included ($P = 0.866$; Fig. 3A, Table 1). Inbreeding seems to have increased parasite-induced larval mortality, although the difference was marginally non-significant ($P = 0.063$). Larval and pupal mortality combined did not change significantly due to inbreeding ($P = 0.310$), leading to the conclusion that there was no significant change in survival to adulthood. During adulthood, on the other hand, parasite-induced mortality was significantly lower in the inbred lines ($P = 0.043$).

Correlated responses to inbreeding of different traits could provide insight into mechanisms behind changes in mortality caused by inbreeding. When considering correlations between mortality and development time or egg hatching rate, we found a significant positive relationship between the relative change in development time caused

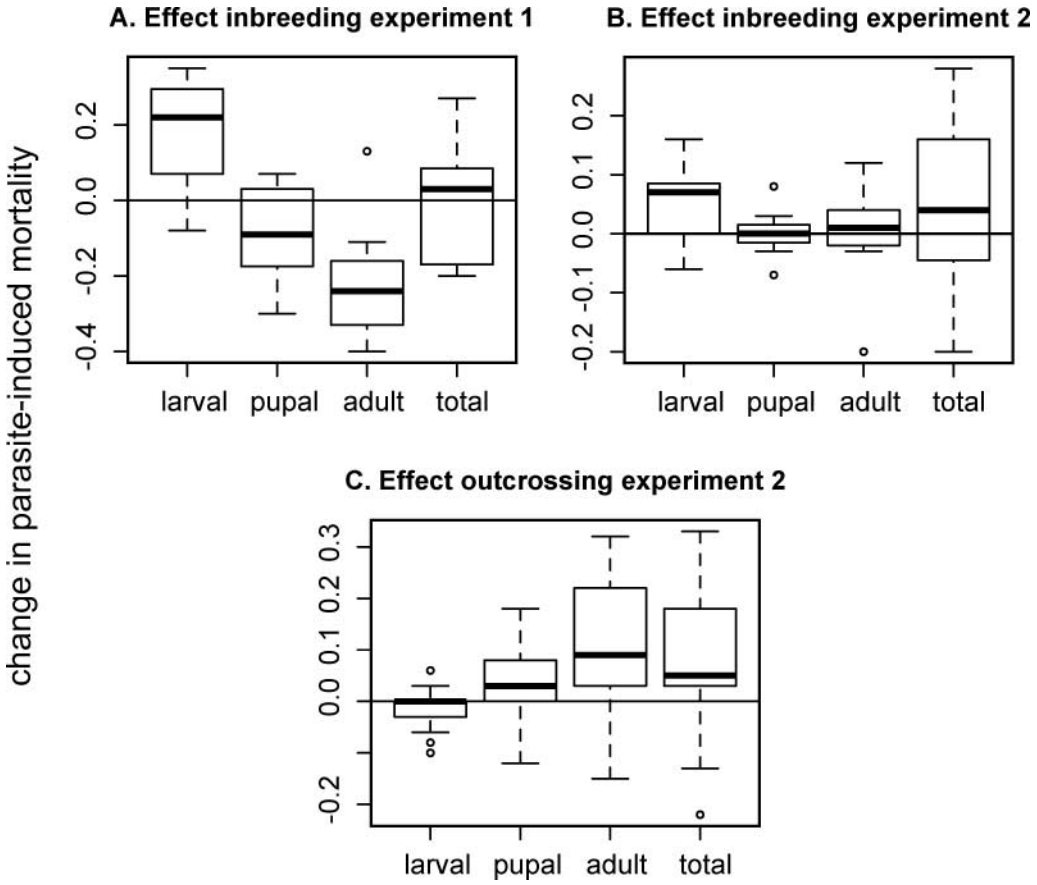

Fig. 3. The effect of inbreeding on parasite-induced mortality in Experiment 1 (A) and Experiment 2 (B), and the effect of outcrossing on parasite-induced mortality in Experiment 2 (C; the three rounds – base populations, inbred lines – combined). To analyse the effect of inbreeding, the mean of the original mass-bred base population was subtracted from the mean of each inbred line. The effect of outcrossing was calculated by subtracting the mean of the F1s from the original mean value of the better parent (i.e. the one with the lowest parasite-induced mortality). Positive values therefore indicate that inbreeding/outcrossing increased parasite-induced mortality. Parasite spore doses were higher in Experiment 1, and consequently so was total parasite-induced mortality. In Experiment 1, inbreeding increased larval mortality, but decreased mortality in later stages, and had no net effect on total mortality (A). In Experiment 2, inbreeding had no significant effect on mortality in any stage (B). Outcrossing increased adult and total mortality compared with the better parent (C).

by inbreeding and the relative change in larval mortality caused by inbreeding (linear regression, $n = 30$, $F_{1,28} = 20.348$, $P < 0.001$, $R^2 = 0.421$; Fig. 4). Relative change in egg hatching success did not correlate with relative change in total parasite-induced mortality (Pearson correlation, $n = 30$, $r = 0.086$, $P = 0.652$) or relative change in development time ($n = 30$, $r = -0.099$, $P = 0.603$).

Table 1. The effect of inbreeding on parasite-induced mortality in the two experiments

	Experiment 1			Experiment 2		
Stage	median	*T*	*P*	median	*T*	*P*
Larval	0.22	25	0.063	0.07	23.5	0.108
Pupal	–0.09	6	0.176	0.0	11	0.917
Adult	–0.24	2	0.043	0.01	16	0.735
Total	0.03	13	0.866	0.04	18	0.499

Note: The mean of the original mass-bred base population was subtracted from the mean of each inbred line, such that positive median values indicate an increase in parasite-induced mortality caused by inbreeding. The test statistic *T* is defined as the sum of positive ranks.

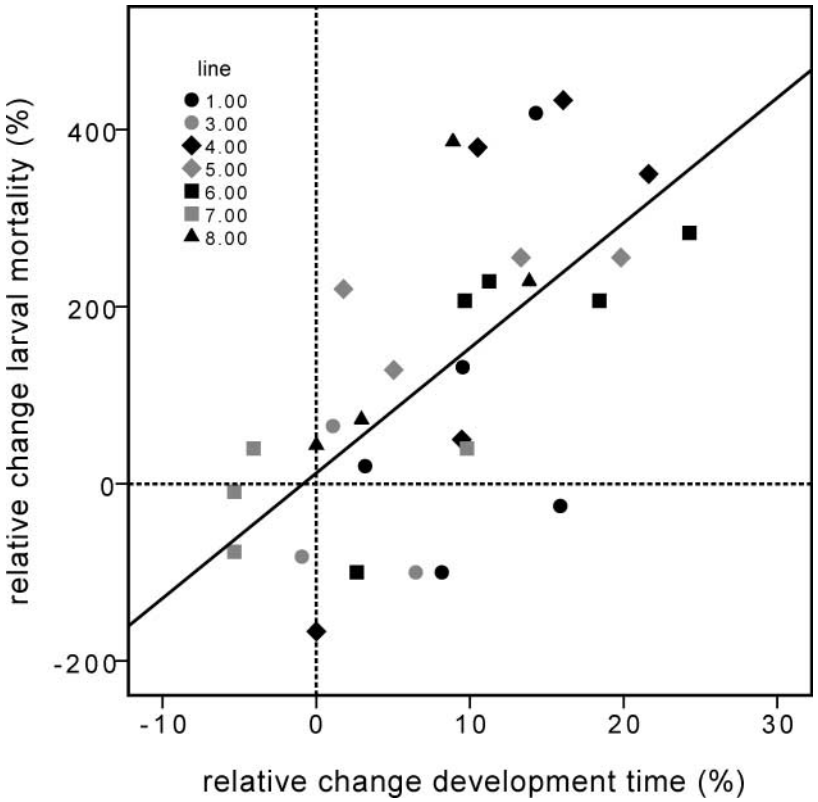

Fig. 4. Correlation between the relative change in parasite-induced larval mortality and the relative change in development time (time to adulthood), as a result of inbreeding (Experiment 1). The larger the relative increase in development time, the larger the relative increase in larval mortality. The different symbols indicate the different lines from which each data point originates.

Experiment 2

Similar to Experiment 1, in Experiment 2 we found that inbreeding significantly increased the development time to adulthood (Wilcoxon signed rank test, $n = 7$, $T = 27$, $P = 0.028$; Fig. 2B). There was a strong negative effect of inbreeding on reproductive success (Wilcoxon signed rank test, $n = 7$, $T = 0$, $P = 0.018$; Fig. 2B). All inbred lines produced less offspring than the mass-bred populations they were derived from.

Inbreeding did not have a significant effect on control mortality. We did not observe an effect of inbreeding on total parasite-induced mortality (Wilcoxon signed rank test, $n = 7$, $T = 18$, $P = 0.499$; Fig. 3B); even when considering stage-specific mortality, no significant effects were observed (see Table 1).

We found heterosis for development time (see Table 2, Fig. 2C), suggesting that the average development time of the hybrids was shorter than the development time of the fastest parent. In contrast to the negative effect of inbreeding on reproductive success, outcrossing did not show heterosis for reproductive success (Table 2, Fig. 2C). We also found no evidence for heterosis for control mortality (Table 2). In addition, while there was some indication that larval mortality might be decreased due to outcrossing, there was no evidence for heterosis for total parasite-induced mortality (Fig. 3C, Table 2). In general, outcrossed F1s were not more resistant than their most resistant parent. Total parasite-induced mortality in the mass-bred base populations (red in Fig. 1) was significantly higher in the F1s than in the better parent after outcrossing, indicating that outcrossing can be disadvantageous for relatively resistant lines.

Table 2. The effect of outcrossing on development time, reproductive success, and control and parasite-induced mortality

	Test for independence				Effect of outcrossing	
Trait	Round	ρ	P	Observed median	T	P
Development time	1	0.536	0.215	**–1.310**	**0.0**	**0.018**
	2	0.373	0.410	**–2.870**	**0.0**	**0.018**
	3	–0.282	0.540	**–0.630**	**1.0**	**0.028**
	total	**0.479**	**0.028**	**–1.550**	**1.0**	**<0.001**
Reproductive success	1	–0.627	0.132	0.500	11.0	0.612
	2	–0.264	0.568	1.500	17.0	0.612
	3	0.209	0.653	–6.000	15.0	0.866
	total	0.021	0.928	0.500	113.5	0.945
Control mortality						
Total	1	0.047	0.921	0.000	6.0	0.109
	2	–0.457	0.303	0.000	3.5	0.785
	3	NA	NA	0.000	0.0	1.000
	total	–0.093	0.687	0.000	17.5	0.140
Larvae	1	NA	NA	0.000	0.0	1.000
	2	NA	NA	0.000	0.0	1.000
	3	NA	NA	0.000	0.0	1.000
	total	NA	NA	0.000	0.0	1.000

Table 2.—*continued*

	Test for independence				Effect of outcrossing	
Trait	Round	ρ	P	Observed median	T	P
Pupae	1	NA	NA	0.000	1.0	0.317
	2	NA	NA	0.000	0.0	1.000
	3	NA	NA	0.000	0.0	1.000
	total	NA	NA	0.000	1.0	0.317
Adults	1	0.278	0.546	0.000	3.0	0.180
	2	–0.389	0.389	0.000	3.0	0.180
	3	NA	NA	0.000	0.0	1.000
	total	0.083	0.721	0.000	10.0	0.066
Parasite-induced mortality						
Total	1	–0.518	0.233	0.040	19.0	0.398
	2	0.355	0.435	0.040	24.0	0.091
	3	0.136	0.771	**0.090**	**28.0**	**0.018**
	total	–0.111	0.631	**0.040**	**14.5**	**0.009**
Larvae	1	0.322	0.481	0.000	3.0	1.000
	2	0.311	0.497	**–0.060**	**0.0**	**0.042**
	3	–0.342	0.453	0.000	3.0	0.180
	total	**0.547**	**0.010**	0.000	132.0	0.181
Pupae	1	0.464	0.295	0.040	15.0	0.345
	2	–0.019	0.969	**0.040**	**21.0**	**0.027**
	3	0.167	0.721	0.000	12.0	0.225
	total	0.134	0.563	**0.030**	**165.0**	**0.009**
Adults	1	0.370	0.413	0.000	11.0	0.345
	2	0.336	0.461	**0.200**	**26.0**	**0.043**
	3	0.209	0.653	**0.070**	**28.0**	**0.018**
	total	0.389	0.082	**0.090**	**191.0**	**0.005**

Note: Also shown are the results of the tests for independence of the different crosses within a round. Round 1 and 2 represent the two inbred rounds (blue and green in Fig. 1), while round 3 represents the mass-bred round (red in Fig. 1). Significant correlations or deviations from a median of zero are shown in **bold**.

Development time after inbreeding was related to the original development time of the mass-bred base population (linear regression, $n = 7$, $F_{1,5} = 49.225$, $R^2 = 0.908$, $P = 0.001$; Fig. 5). Development time after outcrossing, however, did not relate to the original development time of the better parent ($n = 21$, $F_{1,19} = 0.494$, $P = 0.490$). Both slopes of the regression lines were significantly different from a slope of 1 (inbreeding: $\beta_1 = 0.441$, $t_5 = -8.873$, $P < 0.001$; outcrossing: $\beta_1 = 0.102$, $t_{19} = -6.193$, $P < 0.001$). These observations illustrate that the effect of inbreeding depends on the original value of the base population, such that inbreeding prolongs development time more in faster base populations. There appeared to be constraints to development time, which is illustrated by the fact that outcrossed beetles all had relatively similar development times, independent of the development time of the better parent.

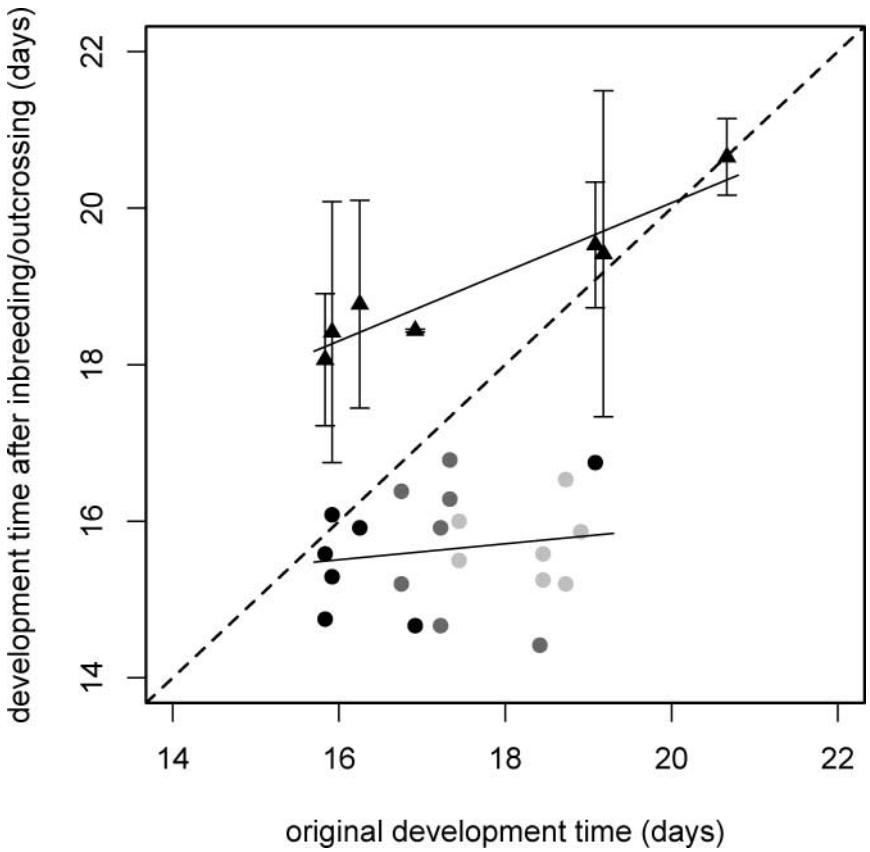

Fig. 5. Development time after inbreeding (triangles) and outcrossing (circles) versus the original development time. For the inbreeding data, the x-axis shows the development time of the mass-bred base population. For the outcrossing data, the x-axis shows the development time of the better parent. The black circles indicate the mass-bred round of crosses (red in Fig. 1), dark grey circles indicate the first inbred round (blue in Fig. 1), and light grey circles indicate the second inbred round (green in Fig. 1). The dashed line represents y = x. Development time after inbreeding is significantly related to the original development time, but development time after outcrossing is not. The slopes of both regression lines deviate significantly from a slope of 1.

DISCUSSION

Similar to previous work that shows that inbreeding of *T. castaneum* does not increase the beetle's susceptibility to rat tapeworm infection (Stevens *et al.*, 1997), we found no evidence for a direct, unidirectional effect of inbreeding or outcrossing on overall resistance to infection with *N. whitei* (Fig. 3). Larval parasite-induced mortality seemed to be increased due to inbreeding (Experiment 1), or decreased due to outcrossing (Experiment 2), but we cannot disentangle these effects from the much stronger effects of shortened versus elongated development time (Fig. 4). The change in mortality in the larval stage was compensated in later developmental stages, suggesting that changes in heterozygosity did not directly affect resistance.

Although our statistical methods are conservative, they had sufficient power to detect strong effects on development time, egg hatching success, and reproductive success (Fig. 2). Therefore, it is likely that we would have been equally able to detect strong unidirectional effects on resistance if they were present. A benefit of our analytical strategy is that it allowed us to examine the results from the two different experiments, with different traits and different treatments (inbreeding and outcrossing), in exactly the same way. The inbreeding coefficient of our beetles was fairly high ($F = 0.67$), and as no negative effect of inbreeding could be detected with such high values, it is questionable how relevant a potentially overlooked effect would be under more natural conditions. Because other research has confirmed the absence of general heterosis for resistance in *T. castaneum* (Wegner *et al.*, 2008; N.A.G. Kerstes and K.M. Wegner, unpublished), genome-wide heterozygosity is probably not one of the main determinants of *T. castaneum* resistance to infection with *N. whitei*.

Inbreeding can lead to the loss of good alleles, and outcrossing can bring together good alleles from different backgrounds. Consequently, we found that inbreeding decreased egg hatching success and reproductive success (Fig. 2). At the same time, it increased development time while outcrossing decreased it, confirming earlier findings in insects (Pray and Goodnight, 1995; Roff, 1998). The effect of inbreeding on development time depends on the original value of the outbred base population (Fig. 5). This observation indicates that one has to be careful about drawing conclusions about the effect of inbreeding in studies that only use inbred lines derived from one single base population. Variation in genetic architecture can influence the extent of inbreeding depression considerably, as was shown in *T. castaneum* (Pray and Goodnight, 1995), and the effect of inbreeding in one population can therefore be very different from the effect of inbreeding in an unrelated population of the same species. Correspondingly, we found variation in the effect of inbreeding on resistance to parasite infection. As shown in Fig. 3, the effect of inbreeding on total parasite-induced mortality varied between the seven mass-bred base populations, as mortality decreased in some and increased in others. This is to be expected, as it has been shown that there is considerable variation in parasite resistance within and between beetle populations (Wegner *et al.*, 2008, 2009).

Although we found a negative effect of inbreeding on larval parasite mortality (Fig. 3A), the observation that heterozygosity was higher in co-evolved *T. castaneum* lines than in control lines (Bérénos *et al.*, 2011) can probably not only be explained by heterotic balancing selection. In the co-evolution experiment (Bérénos *et al.*, 2009), parasite spores from dead larvae from the previous generation were used to infect beetles in the next generation. Being more heterozygous, and therefore having shorter development time, could confer a clear advantage, as parasites that killed the host after the larval stage did not get transferred to the next generation, and were thus unable to adapt to their hosts. Under such a scenario, one would expect that the development time of the host decreased during co-evolution. Such a response to selection, however, was not observed, as no significant difference between the time to pupation of co-evolved and control lines was found (C. Bérénos *et al.*, in prep.).

The relationship between heterozygosity and larval mortality was most apparent in Experiment 1, where parasite spore concentration and, as a consequence, total parasite-induced mortality (around 65%) were highest. In Experiment 2, where average parasite-induced mortality was lower (around 20%), no significant effect of inbreeding on larval mortality was found. Outcrossing did decrease larval mortality in only one of the three rounds of crosses and did not have an effect on larval mortality of the mass-bred base populations (Table 2). These findings again support the idea that the maintenance of genetic diversity during host–parasite co-evolution cannot be attributed to heterozygote advantage.

The present study is not the first to find no overall effect of heterozygosity on resistance to parasitism in insects (Rantala and Roff, 2006; O'Donnell and Armbruster, 2010), or in *T. castaneum* in particular (Stevens *et al.*, 1997). Several studies that did find a negative effect of inbreeding on heterozygosity reported an indirect effect, by illustrating that resistance is compromised by inbreeding via a trait that is not directly linked to immune physiology, such as social (Calleri *et al.*, 2006) or defensive (Luong *et al.*, 2007) behaviours. Inbreeding of the mealworm *Tenebrio molitor* did significantly reduce the realized immune response (resistance against an entomopathogenic fungi), but did not affect the potential immune (encapsulation) response (Rantala *et al.*, 2011). Our data suggest that the observed negative effect of inbreeding on larval mortality was linked to an increase in development time (Fig. 4), again a trait that is not directly related to immune physiology. Inbreeding was found to negatively affect resistance to *S. marcescens* in *D. melanogaster*, but the lines used were highly inbred ($F = 0.986$) and more than half of the inbred lines were as resistant as their outbred base population (Spielman *et al.*, 2004). The same study also reported that inbreeding reduced resistance to *Bacillus thuringiensis*, but again this effect was mainly found at extremely high inbreeding coefficients ($F = 0.999$). Overall, there is only limited evidence for a direct link between heterozygosity and the strength of the immune response in insects. In many cases, a reduction of parasite resistance by inbreeding seems to be mediated by indirect consequences of inbreeding on related traits that are not exclusively involved in the immune response.

CONCLUSIONS

Our experiments show that increasing homozygosity in *T. castaneum* negatively affects important life-history and fitness traits such as egg hatching success, development time, and reproductive success. No overall effect of inbreeding on resistance to infection with *N. whitei* was found, although mortality appeared to be shifted to earlier developmental stages. This represents an indirect effect of inbreeding on resistance, causing beetles to die in an earlier stage due to prolonged development time. In a similar fashion, outcrossing decreased development time, but it did not increase the beetle's overall resistance to infection. Our results support the view that empirical evidence for a direct link between heterozygosity and resistance to parasites in insects in general is weak. Therefore, host genetic diversity that was maintained during co-evolution of *T. castaneum* with *N. whitei* (Bérénos *et al.*, 2011) cannot be explained by dominant or overdominant selection alone. Hence, other explanations, such as negative frequency-dependent selection, are more likely to explain the maintenance of genetic diversity, especially when new genotypic variants are created at a higher rate during host–parasite co-evolution (Kerstes *et al.*, 2012).

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