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Antagonistic coevolution with parasites maintains host genetic diversity: an experimental test

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Genetic variation in natural populations is a prime prerequisite allowing populations to respond to selection, but is under constant threat from forces that tend to reduce it, such as genetic drift and many types of selection. Haldane emphasized the potential importance of parasites as a driving force of genetic diversity. His theory has been taken for granted ever since, but despite numerous studies showing correlations between genetic diversity and parasitism, Haldane's hypothesis has rarely been tested experimentally for unambiguous support. We experimentally staged antagonistic coevolution between the host *Tribolium castaneum* and its natural microsporidian parasite, *Nosema whitei*, to test for the relative importance of two separate evolutionary forces (drift and parasite-induced selection) on the maintenance of genetic variation. Our results demonstrate that coevolution with parasites indeed counteracts drift as coevolving populations had significantly higher levels of heterozygosity and allelic diversity. Genetic drift remained a strong force, strongly reducing genetic variation and increasing genetic differentiation in small populations. To our surprise, differentiation between the evolving populations was smaller when they coevolved with parasites, suggesting parallel balancing selection. Hence, our results experimentally vindicate Haldane's original hypothesis 60 years after its conception.

Keywords: host–parasite coevolution; genetic variation; Red Queen hypothesis; natural selection

1. INTRODUCTION

The persistence of high genetic variability in natural populations is a classical evolutionary puzzle because most evolutionary forces, such as drift [1,2] and directional selection [3–5], reduce genetic variability. Haldane [6] suggested that selection by pathogens might be important in maintaining genetic variation in populations. Theoretical support for this hypothesis comes from models of antagonistic host–parasite coevolution, where a host population is kept in a genotypically diverse state through the effects of time-lagged negative-frequency-dependent selection [7,8]. If this occurs in spatially separated populations, differences in local selection patterns can potentially lead to rapid host population divergence, while maintaining allelic diversity on a metapopulation level [9–11]. In this spirit, Haldane also suggested that parasites facilitate the speciation of their hosts [6].

Whereas theory is well developed, direct experimental evidence for the hypothesis that antagonistic coevolution can maintain genotypic diversity in populations is virtually absent [10]. On the other hand, there is ample evidence for the importance of genetic variation in the defence against parasites [12–19]. For example, it has been shown that the frequency of sexuals correlates

positively with infection prevalence [20], that social insect colonies with a low genetic diversity showed a higher infection intensity than colonies with a high diversity [21] and that infected *Daphnia* populations show a higher clonal diversity than non-parasitized populations [22]. Furthermore, host resistance is generally based on a few loci [23], but there is also good evidence for a complex genetic architecture of resistance, with strong effects of epistasis between loci, primarily those on different chromosomes [24,25]. Hence, it is expected that the effects of parasite-mediated selection on host genetics may act on genome-wide diversity and are not restricted to confined parts of the genome [23,26–28].

To experimentally test Haldane's hypothesis that selection by parasites maintains genotypic diversity in host populations, we set up a coevolution experiment using the Red Flour Beetle (*Tribolium castaneum*) and its natural specific microsporidian parasite *Nosema whitei* [29,30]. To assess whether selection by coevolving parasites might override the effects of genetic drift, we included population size as an additional factor, and we report the results after 12 discrete generations of coevolution. We have previously shown that under these conditions, both host and parasite populations coevolve with one another [31]. Here, we specifically asked: (i) is genetic diversity of host populations higher when coevolving with parasites than under control conditions? (ii) How strong is this effect relative to genetic drift? (iii) Does selection by parasites lead to divergent evolution as expected from the postulate of local adaptation [11,32]? That is, do coevolving populations diverge more from each other than control populations?

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2. MATERIAL AND METHODS

(a) *Experimental evolution regime*

To increase genetic variability, we first crossed different pairs of stock lines that have been kept under standard conditions for over 50 generations [24,31]. Population crosses were done as described in [31] and the resulting fully hybrid F_1 adults were pooled as the starting breeder population of the line. A total of eight such populations were used to form eight experimental lines [31].

Each experimental line was subsequently divided into two lines of small population size ($n = 50$) and two lines of large population size ($n = 500$). One of these two lines for each population size was assigned to the coevolution treatment and subjected to selection by coevolving *N. whitei*. The remaining lines (for both small and large population size) were assigned to the respective control treatment, i.e. were kept on standard medium free of parasites. Thus, the total of eight lines \times two population sizes \times two treatments = 32 populations represented eight different genetic backgrounds, such that each genetic background was present in each treatment and population size. Host population size for the large and small population size was kept constant by always collecting, respectively, 500 or 50 adult (unsexed) beetles from the previous generation as breeders for the next generation. Host density per available unit of food was kept constant by using 200 g of flour for the large population size and 20 g of flour for the small population size, so that the amount of medium scaled with population size [31]. Average host mortality in the coevolution selection regime differed between host lines, but was generally between 10 and 40 per cent [31].

(b) *DNA extraction and marker amplification*

A total of 24 surviving individuals per experimental unit (line \times size \times treatment) were randomly collected for genetic analysis in generations 4, 8 and 12. In the starting populations from generation 0, we only used a total of 24 individuals per line. Thus, genomic DNA was extracted from a total of 2376 whole beetles using Qiagen DNeasy 96 well plate extraction kit (Qiagen, Basel, Switzerland). Individuals were genotyped for 10 microsatellite loci (electronic supplementary material, appendix A; [33]) spread over six linkage groups. Loci were amplified with polymerase chain reaction (PCR) on a 96-Well GeneAmp PCR System 9700 thermocycler (Applied Biosystems). Each 10 μ l reaction contained the following components: 1 \times Reaction Buffer (Promega, Switzerland), 0.8 mM of dNTP mix, 0.125 μ M of each dye-labelled forward primer (either FAM, TAMRA or HEX), 0.125 μ M of unlabelled reverse primer and 1 μ l of genomic DNA. PCR conditions included an initial denaturation step of 3 min at 94°C, followed by 28 cycles consisting of 30 s denaturation at 94°C, 30 s annealing at 58°C and 30 s extension at 72°C. Final extension was at 72°C for 7 min. PCR products were run on a MegaBACE 750 sequencer and genotypes were scored using the software FRAGMENT PROFILER (General Electric, Switzerland).

(c) *Genetic data analysis*

GENALEX 6.2 [34] was used to calculate observed heterozygosity, expected heterozygosity, fixation index, number of alleles, the Shannon index of allelic diversity and pairwise F -statistics between populations. Before statistical analysis, means of response variables (e.g. heterozygosity, averaged

over all 10 loci) were calculated within each experimental block to avoid pseudo-replication. All response variables were subsequently analysed as mixed-model ANOVA with treatment and population size as fixed effects, generation as repeated measures and all possible interactions between the fixed effects and line as random effects. Pairwise F -statistics, a measure of population differentiation, was first analysed for all pairwise combinations within generation nested within selection regime nested within population size. We used ANOVA with generation, selection regime, population size and the interactions between all factors as fixed factors. For the pairwise F_{ST} within each generation, the datafile consisted of 28 pairwise F_{ST} s per generation per selection regime, meaning $28 \times 4 \times 3 = 336$ data points. As these are not all independent measurements (given that for each line we have seven pairwise F_{ST} values), we decided to test significance with fewer degrees of freedom in the denominator. Given that there are eight lines, four selection regimes and three time points, we used a total of 96 degrees of freedom in our F -test to prevent type I errors in the analysis because of multiple pairwise comparisons. Then we analysed pairwise F -statistics between the ancestral lines and the evolved lines within the same selection regime, population size and line to test for differences in intergenerational population differentiation. For this, we used mixed model ANOVA with interval (lag between generations used in the analysis, i.e. between G0 and G4, G0–G8 and G0–G12), population sizes, selection regime and the interactions between all factors as fixed factors. Host line was treated as a random factor in the model. All statistical analyses were conducted with the statistical package implemented in R [35].

3. RESULTS

The experiment was started with outcrossed hybrid populations [31], which led to an expected decrease in the number of alleles during the experiment (table 1 and electronic supplementary material, appendix B). As expected from the effects of genetic drift, the rate at which alleles were lost was higher for the small than for the large populations (see the significant interaction term generation \times size using the Shannon index of allelic diversity as the response variable, tables 1 and 2). The number of alleles did not differ significantly between coevolved and control lines (table 1 and figure 1*a*), but the index of allelic diversity was significantly higher in the coevolved lines than in the control lines, and in large populations when compared with small populations (table 2, electronic supplementary material, appendix B and figure 1*b*). Allelic diversity decreased during the course of the experiment, but small populations lost allelic diversity faster than large populations (tables 1 and 2). Similarly, the number of alleles was higher in large population sizes than in small populations (table 1).

As expected, both observed (least-square regression $R^2 = 0.53$, $F_{1,94} = 103.34$, $p < 0.001$) and expected heterozygosity (least square regression $R^2 = 0.68$, $F_{1,94} = 200.17$, $p < 0.001$) correlated positively with the number of alleles, and observed heterozygosity correlated positively with expected heterozygosity ($R^2 = 0.87$, $F_{1,94} = 615.91$, $p < 0.001$). Consequently, both measures of heterozygosity showed similar results. Coevolved lines had higher levels of heterozygosity than control lines

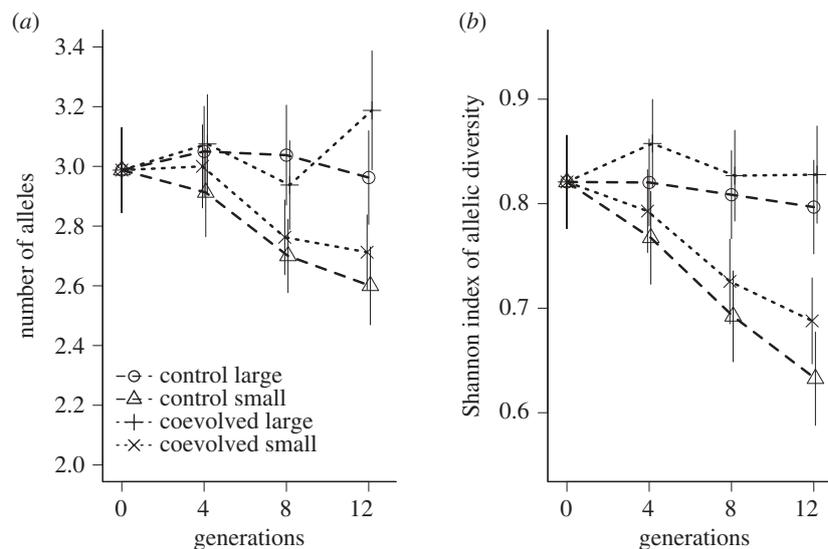


Figure 1. Dynamics of allelic diversity during the selection experiment. (a) The average number of alleles (\pm s.e.m.). There was a strong effect of drift, as in small populations allelic number decreased over time, whereas numbers were equal in coevolved and control populations. For statistics, see table 1. (b) Shannon index of allelic diversity (\pm s.e.m.). Allelic diversity similarly showed strong effects of drift. Furthermore, allelic diversity was higher in coevolved than in control populations. For statistics, see table 2.

Table 1. Analysis of variance (ANOVA) table of the number of alleles.

	d.f.	sum of square	mean square	<i>F</i> -value	Pr(> <i>F</i>)
treatment	1	0.113	0.113	2.409	0.125
size	1	1.627	1.627	34.559	<0.001
generation	2	0.461	0.230	4.893	0.009
treatment \times size	1	0.008	0.008	0.179	0.673
treatment \times generation	2	0.143	0.071	1.513	0.227
size \times generation	2	0.391	0.195	4.149	0.019
treatment \times size \times generation	2	0.077	0.039	0.823	0.44
residuals	77	3.626	0.047		

Table 2. ANOVA table of the Shannon index of allelic diversity.

	d.f.	sum of square	mean square	<i>F</i> -value	Pr(> <i>F</i>)
treatment	1	0.027	0.027	10.297	0.002
size	1	0.273	0.272	104.412	<0.001
generation	2	0.088	0.044	16.835	<0.001
treatment \times size	1	0.001	0.001	0.196	0.659
treatment \times generation	2	0.001	0.001	0.240	0.787
size \times generation	2	0.035	0.017	6.676	<0.002
treatment \times size \times generation	2	0.001	0.001	0.268	0.766
residuals	77	0.201	0.002		

(observed heterozygosity: table 3 and figure 2a; expected heterozygosity: table 3 and figure 2b), a pattern that was observed irrespective of population size. Population size mattered, of course, as large populations showed higher heterozygosity than small populations (table 3 and electronic supplementary material, appendix B). In line with the loss of alleles, heterozygosity also decreased rapidly during the course of the experiment, and small populations showed a faster decrease than large populations—at least for expected but not for observed heterozygosity (table 3). Over the course of the experiment, both coevolved ($t = 3.305$, d.f. = 47, $p = 0.002$)

and control lines ($t = 5.361$, d.f. = 47, $p < 0.001$) showed a significant excess of homozygotes, but there was no difference in F_{IS} -values between either of the population sizes, selection regimes and there was no sign of any trend in time (table 4 and electronic supplementary material, appendix B).

Pairwise F_{ST} -values between lines were smaller within the coevolved host populations than in the control populations, increased in time and were higher within the small population sizes than in the large populations (table 5, electronic supplementary material, appendix C and figure 3a). There was no significant treatment \times size

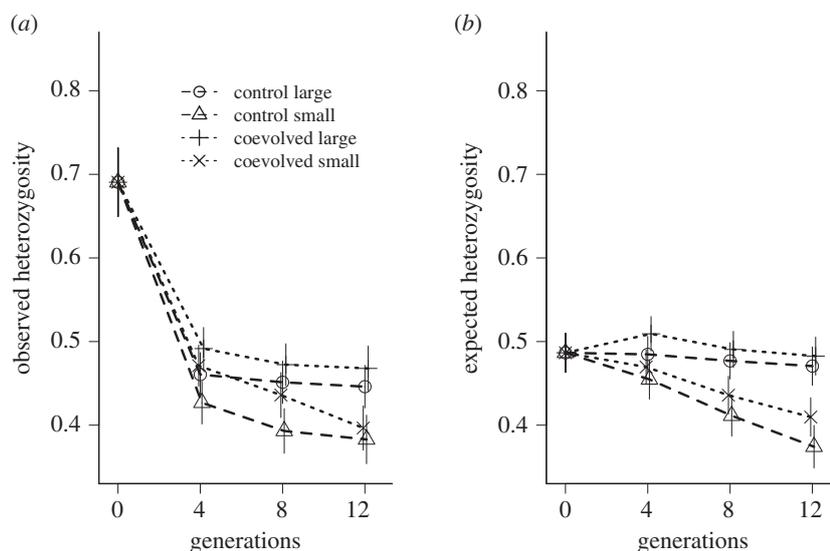


Figure 2. Temporal dynamics of heterozygosity. (a) Observed heterozygosity (\pm s.e.m.) and (b) expected heterozygosity (\pm s.e.m.). Both indices of heterozygosity show qualitatively similar results (for statistics, see table 3), with drift reducing heterozygosity and coevolution maintaining heterozygosity.

Table 3. Analysis of variance (ANOVA) table of observed heterozygosity and expected heterozygosity during the selection experiment.

	d.f.	sum of square	mean square	<i>F</i> -value	Pr(> <i>F</i>)
observed heterozygosity					
treatment	1	0.204	0.204	12.465	<0.001
size	1	0.535	0.535	32.7039	<0.001
generation	2	0.250	0.125	7.645	<0.001
treatment \times size	1	0.005	0.005	0.298	0.586
treatment \times generation	2	0.017	0.009	0.517	0.598
size \times generation	2	0.064	0.032	1.968	0.146
treatment \times size \times generation	2	0.010	0.005	0.297	0.746
residuals	77	0.125	0.002		
expected heterozygosity					
treatment	1	0.104	0.104	12.364	<0.001
size	1	0.861	0.861	102.169	<0.001
generation	2	0.330	0.165	19.589	<0.001
treatment \times size	1	0.004	0.004	0.477	0.492
treatment \times generation	2	0.001	0.001	0.06	0.943
size \times generation	2	0.100	0.050	5.906	0.004
treatment \times size \times generation	2	0.011	0.006	0.674	0.513
residuals	77	0.065	0.001		

Table 4. ANOVA table of F_{IS} (inbreeding coefficient).

	d.f.	sum of square	mean square	<i>F</i> -value	Pr(> <i>F</i>)
treatment	1	0.008	0.008	2.386	0.127
size	1	0.004	0.004	1.284	0.261
generation	2	0.004	0.002	0.683	0.508
treatment \times size	1	<0.001	<0.001	0.002	0.961
treatment \times generation	2	0.009	0.005	1.418	0.248
size \times generation	2	<0.001	<0.001	0.022	0.978
treatment \times size \times generation	2	0.006	0.003	0.898	0.412
residuals	77	0.264	0.003		

interaction, meaning that selection regime had the same effect irrespective of population size (table 5). There was no difference between selection regimes in genetic differentiation between time points (table 5, electronic

supplementary material, appendix C, and figure 3b), indicating that allele compositions are not changing faster in the coevolved lines than in the control lines, but F_{ST} -values were higher for small population sizes than

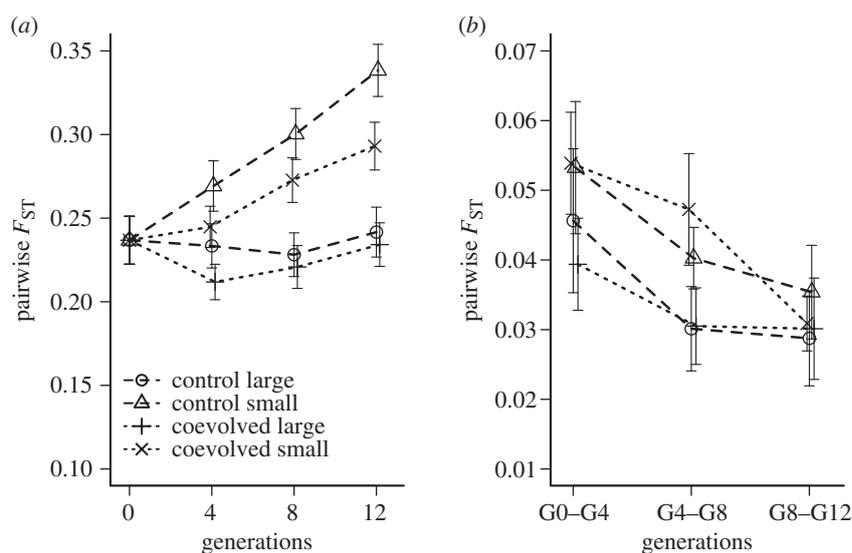


Figure 3. Trend of divergence during the selection experiment. (a) Pairwise F_{ST} (\pm s.e.m.) between all lines in an experimental block and analysed per generation. The graph shows the counteracting forces of overall host–parasite coevolution (i.e. keeping populations genetically similar) and genetic drift (small populations diverge faster). (b) Pairwise F_{ST} (\pm s.e.m.) between each of the evolved lines after 4, 8 or 12 generations and their respective replicate line immediately preceding time points. Coevolution seems to have no effect on the change in allelic composition over time, while populations that are experiencing stronger drift, diverge faster. Statistical details can be found in table 5.

Table 5. Analysis of variance (ANOVA) table of population pairwise F_{ST} during the selection experiment.

	d.f.	sum of square	mean square	<i>F</i> -value	Pr(> <i>F</i>)
pairwise F_{ST} between all lines within each experimental block, analysed per generation					
treatment	1	0.042	0.042	7.954	0.006
size	1	0.283	0.283	53.992	<0.001
generation	2	0.077	0.039	7.368	<0.001
treatment \times size	1	0.009	0.009	1.653	0.202
treatment \times generation	2	0.001	<0.001	0.108	0.898
size \times generation	2	0.027	0.014	2.579	0.081
treat \times size \times generation	2	0.004	0.002	0.407	0.667
residuals	85	1.700	0.020		
pairwise F_{ST} between consecutive time points within replicate evolved lines					
treatment	1	<0.001	<0.001	0.004	0.951
size	1	0.002	0.002	6.446	0.013
generation	2	0.005	0.002	7.052	0.002
treatment \times size	1	<0.001	<0.001	0.118	0.732
treatment \times generation	2	<0.001	<0.001	0.290	0.749
size \times generation	2	<0.001	<0.001	0.629	0.536
treatment \times size \times generation	2	<0.001	<0.001	0.323	0.725
residuals	77	0.025	<0.001		

for large population sizes, and the speed with which allelic compositions changed slowed down during the experiment (table 5, electronic supplementary material, appendix C and figure 3b).

4. DISCUSSION

Numerous studies showed a putative selective advantage of heterozygous individuals in wild populations when exposed to parasites [12–14], but this seems to be the first to experimentally show that ongoing antagonistic coevolution with parasites can lead to the maintenance of genetic polymorphism in strictly outbreeding and obligatory sexual host populations. This approach is fundamentally different from studies of a correlational

nature, as these do not take into account whether, and how, temporal dynamics in host–parasite interactions can shape genetic diversity. Our finding supports comparable results from a recent study where facultative sexual host populations of *Caenorhabditis elegans* that were coevolving with *Bacillus thuringiensis* showed higher levels of genetic diversity than host populations that were kept in the absence of parasites [10].

The experiment primarily tested the effect of coevolution, whereas the exact genetic mechanisms underlying the results have not yet been a major focus. Nevertheless, it appears that genetic drift had a strong effect in our experiment, as population size generally explained more of the variation of all the indices used to quantify genetic diversity than the selection regime (see sum of squares in

tables 1–3). However, within both the large and small population sizes, we observed the same pattern, that is, there was higher genetic diversity in the coevolved hosts than in the controls. A parsimonious explanation for the higher genetic diversity in coevolved populations at the level of genetic processes could be overdominance (heterozygote advantage; [36]), which is a general phenomenon in host–parasite systems [17]. However, there was no significant difference in F_{IS} -values between coevolved and control lines, which suggests that overdominance alone might not explain the observed pattern. We intentionally started our experiment with hybrid lines showing high levels of heterozygosity in generation zero (figure 2a) to simulate non-equilibrium starting conditions and observe evolutionary changes within these populations on their trajectory towards equilibrium. It is uncertain whether findings would be similar if the experiment were initiated with equilibrium populations, but pure hybrid overdominance seems unlikely, as an earlier study showed that F_1 or F_2 hybrids are rarely more resistant to *N. whitei* than the most resistant parental line [24].

When testing for genetic divergence between the replicate experimental populations, we found that the divergence between populations was smaller among the coevolved host populations than among the control populations. The divergence increased over time and was more pronounced among the small than among the large populations (table 5 and figure 3a). The results therefore suggested that genetic drift leads to divergence over time and that coevolution with parasites tends to reduce it. Additionally, when testing for longitudinal genetic divergence within lines, allele compositions were not changing faster in the coevolved lines than in the control lines. On the other hand, stochastic events played a large role in changing allele compositions, as temporal genetic divergence was larger in the smaller population sizes than in the large population sizes (table 5 and figure 3b). Temporal divergence was more pronounced the beginning of the experiment, which may be due to the non-equilibrium starting conditions (figure 3b). We were surprised to find a lower divergence between coevolved lines, as it is often assumed that coevolution with parasites leads to rapid local adaptation [11] and, therefore, to more differentiation among populations [6], as was indeed recently found in facultative sexual species [10]. By contrast, our experimental data suggest that parallel balancing selection might occur during coevolution, which leads to less divergence when compared with controls. If so, antagonistic coevolution with parasite thus counteracts the drift effect on interpopulation divergence by maintaining a larger allelic diversity and thus a larger proportion of shared ancestry.

In conclusion, we experimentally show that parasites can maintain genetic diversity in host populations and thereby reduce divergence against the effects of genetic drift. Previously, there have been a number of studies showing that phenomena creating genetic diversity are associated with parasitism. For instance it has been shown that the frequency of sexuals correlates positively with infection prevalence [20], that recombination is selected for under parasite pressure [37], and that infected *Daphnia* populations show a higher clonal diversity than non-parasitized populations [22]. With our

results we support these studies, and in contrast with field studies, we can attribute our results to the factors we have experimentally manipulated, i.e. drift and selection by parasites. We showed that parasites keep host populations in a genetically diverse state, be it either by overdominance or by rare allele advantage. Hence, our results experimentally vindicate Haldane's [6] suggestions for the maintenance of genetic diversity but not necessarily with respect to speciation, 60 years after these ideas had been formulated.

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