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Host–parasite local adaptation after experimental coevolution of *Caenorhabditis elegans* and its microparasite *Bacillus thuringiensis*

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Coevolving hosts and parasites can adapt to their local antagonist. In studies on natural populations, the observation of local adaptation patterns is thus often taken as indirect evidence for coevolution. Based on this approach, coevolution was previously inferred from an overall pattern of either parasite or host local adaptation. Many studies, however, failed to detect such a pattern. One explanation is that the studied system was not subject to coevolution. Alternatively, coevolution occurred, but remained undetected because it took different routes in different populations. In some populations, it is the host that is locally adapted, whereas in others it is the parasite, leading to the absence of an overall local adaptation pattern. Here, we test for overall as well as population-specific patterns of local adaptation using experimentally coevolved populations of the nematode *Caenorhabditis elegans* and its bacterial microparasite *Bacillus thuringiensis*. Furthermore, we assessed the importance of random interaction effects using control populations that evolved in the absence of the respective antagonist. Our results demonstrate that experimental coevolution produces distinct local adaptation patterns in different replicate populations, including host, parasite or absence of local adaptation. Our study thus provides experimental evidence of the predictions of the geographical mosaic theory of coevolution, i.e. that the interaction between parasite and host varies across populations.

Keywords: local adaptation; host–parasite coevolution; *Caenorhabditis elegans*; *Bacillus thuringiensis*; experimental evolution

1. INTRODUCTION

Host–parasite coevolution is one of the most powerful selective forces in evolution [1]. In a spatially structured environment, it can result in local adaptation of the parasite to the host and vice versa. Local adaptation has been studied extensively in cross-infection experiments by comparing sympatric (from the same location) and allopatric (from different locations) host–parasite combinations. Local adaptation of the parasite is inferred from such comparisons if parasites show higher fitness in local hosts than in hosts from a different location. An analogous observation is required for host local adaptation (also called local maladaptation of the parasite; for simplicity, we will only use the term host local adaptation throughout our manuscript). If local adaptation is found for only one of the antagonists, then it is believed to be ahead in the 'arms race' [2,3].

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Previous studies revealed all possible scenarios: local adaptation of the parasite, the host, and no local adaptation [4–8]. However, the latter group might encompass local adaptation for the parasite in some populations and for the host in other populations, consequently yielding absence of an overall effect. The correct interpretation of such inferences is thus not straightforward and it additionally requires consideration of the following factors. First, the relative evolutionary potential of each antagonist (i.e. their ability to generate new genetic variants) influences the speed of adaptation and determines which antagonist will be locally adapted [9–12]. The evolutionary potential may also be affected by the antagonists' migration behaviour [3,13–15]. Second, coevolutionary responses occur with a time lag, and therefore, either of the coevolving antagonists will sometimes appear non- or even maladapted [4,16,17]. Third, the specific trait used to study local adaptation (e.g. host infection load) may not be under selection and therefore it may be non-informative [18–20]. Fourth, the interaction between parasite and host may be influenced by the ecological environment and therefore differ between regions (geographical

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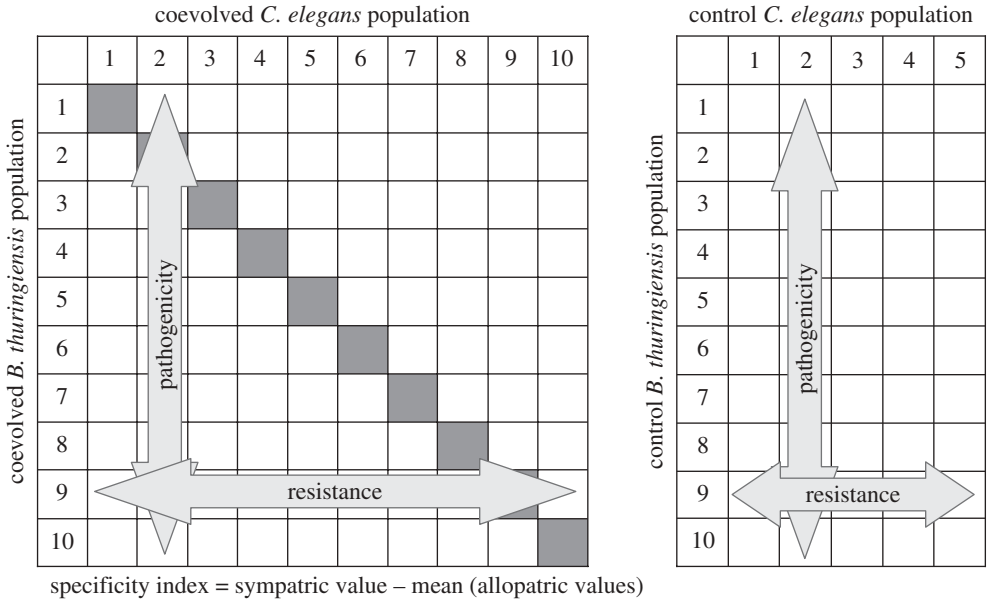

Figure 1. Experimental set-up. Shaded squares indicate sympatric combinations. In the control matrix, 50 different sympatric combinations were simulated by calculating a specificity index for each combination. Comparisons within columns indicate pathogenicity data, and comparisons within rows resistance data following the local versus foreign criterion.

mosaic of coevolution, [21]). This selection mosaic leads to differentiation between populations, possibly resulting in contrasting patterns in different populations at a particular time point (i.e. parasite, host or no local adaptation). Thus, the geographical mosaic may obscure the presence of local adaptation if it is deduced from an overall effect in a cross-infection experiment that includes several populations. Furthermore, environmental factors may dramatically influence the interaction between parasite and host [15,22–26], and thus the experimental design can be crucial for the ability to detect local adaptation [27,28].

Here, we specifically tested for local adaptation as an outcome of coevolution. We compared experimentally evolved populations of the nematode *Caenorhabditis elegans* and its bacterial micro-parasite *Bacillus thuringiensis*. These two species most probably coexist and coevolve in nature [29,30], and they have the potential to coevolve in the laboratory [31]. We studied local adaptation using material from a laboratory-based evolution experiment [31] including two treatments. In one case, we combined host and parasite populations, which were forced to co-adapt to each other for approximately 48 host generations (the coevolution matrix). In the other case, we examined combinations of control parasite and control host populations, which both evolved in the absence of the respective antagonist (the control matrix). Each matrix was examined in a full-factorial cross-infection experiment (figure 1), comparable to former studies on natural or experimentally evolved populations. This experimental set-up enabled us to test for local adaptation in the coevolution matrix by comparing sympatric and allopatric combinations. Furthermore, the control matrix was used to generate a biological null distribution, which exclusively consisted of host–parasite combinations that are known to not have coevolved with each other in the past. This null distribution gives us the opportunity to evaluate whether local adaptation in the coevolution matrix differs from random associations and whether local adaptation might take different directions (i.e. parasite or host local adaptation) in the various replicate populations.

2. MATERIAL AND METHODS

(a) *Study system and coevolution*

The bacterial micro-parasite *B. thuringiensis* causes persistent gut infections in the nematode *C. elegans* that potentially lead to death [29,32,33]. Both antagonists have revealed potential for specific interactions: *B. thuringiensis* strains show high specificity towards nematodes, including *C. elegans* [29–31,34], and *C. elegans* expresses specific immune reactions towards different pathogens (e.g. [35–37]).

The exact protocol of experimental evolution is described elsewhere [31]. Importantly, we used mixtures of three different *B. thuringiensis* strains (NRRL B-18246, NRRL B-18247, NRRL B-18679; provided by the Agricultural Research Service Patent Culture Collection (US Department of Agriculture, Peoria, IL, USA)) and populations derived from three different natural *C. elegans* isolates (MY8, MY15, MY18 [37]). The parasite strains differ in genotype and in crystal toxin production [31,38–40]. Crystal toxins are most probably of prime importance for the interaction with *C. elegans* [41]. The host strains are genetically diverse, encompassing a large part of the genetic diversity present worldwide [42] and differ in the interaction with various pathogens, including *B. thuringiensis* [31,43,44]. The three *C. elegans* isolates were reciprocally crossed over several generations before beginning the evolution experiment in order to obtain a genetically diverse starting population.

In the coevolution treatment ($n = 20$ replicate populations), we simultaneously selected for nematode resistance and parasite infectivity. The control parasites ($n = 10$ replicate populations) could adapt to the general environmental conditions of the experiment without presence of hosts. In the host control ($n = 20$ replicate populations), worms could similarly adapt to the conditions of the experiment. During selection, we regularly added the original genotypes at low concentration (5% every fourth worm generation) to

the evolving host and parasite populations, in order to simulate immigration and thus to prevent random loss of genetic diversity. After selecting for 48 host generations, the evolved *B. thuringiensis* and *C. elegans* were frozen in glycerol for later analysis. In particular, as for *C. elegans* mainly young larvae survive the freezing procedure [45], we first isolated for each replicate population 20 adult hermaphrodites, which were then allowed to reproduce either by self-fertilization or by outcrossing if they mated before isolation. The resulting genetically variable offspring were frozen as separate family lines, i.e. 20 family lines per replicate population, to prevent accidental loss of genotypes. For the local adaptation experiment (see below), the frozen family lines were thawed and for each replicate population, eight or more of these lines were mixed in equal proportions to simulate the genotype composition of each replicate population after experimental evolution and before freezing. As *B. thuringiensis* spores survive freezing [46], we froze complete, spore-enriched populations and thawed these for the local adaptation experiment.

(b) *Local adaptation experiment*

For the local adaptation experiment, we tested in total 10×10 combinations of *B. thuringiensis* and *C. elegans* replicate populations in the coevolution matrix and 10×5 respective combinations in the control matrix (figure 1), which were randomly chosen from all replicates of experimental evolution. Within both matrices, every combination was tested once. The experimental set-up was similar to the conditions during selection and as described for the final experiment in Schulte *et al.* [31]. In short, experimental temperature was 18°C. For each combination, 20 hermaphroditic worms of the last larval stage (L4) were transferred onto peptone-free nematode growth medium inoculated with the respective bacteria inside a 'wormball' [44]. We used a total of 3.5×10^7 *B. thuringiensis* particles and ad libitum *Escherichia coli* OP50 as a food source. After 3 days, the survival rate of worms was measured (surviving worms/(surviving + dead worms)) in order to characterize host resistance and parasite pathogenicity. This trait was directly selected during experimental evolution, i.e. surviving worms and killing parasites were transferred to the next round of selection, and it was previously shown to provide an informative measure for both resistance and pathogenicity [31]. Furthermore, we used log-transformed ($\log_{10}(x + 1)$) worm offspring number of these 20 worms after 3 days (i.e. host reproduction) as an additional measure for both resistance and pathogenicity. To enhance clarity when focusing on parasite pathogenicity, survival rate was translated into killing rate (-1*survival rate) and host reproduction into reduction in host reproduction (-1*host reproduction).

(c) *Data analysis*

Local adaptation was assessed from either the parasite or the host perspective. We focus on the 'local versus foreign' criterion following Kawecki & Ebert [47]. Thus, we assess, for example, if the level of pathogenicity to a particular host population is higher for the sympatric parasite than for the allopatric parasites (columns in figure 1). Consequently, comparisons of parasites within a particular host population are used as pathogenicity data (the columns in figure 1) and comparisons of hosts within a particular parasite population serve as resistance data (rows in figure 1).

To compare sympatric with allopatric combinations, we calculated a specificity index as the difference between the sympatric value and the allopatric mean of each column/row (figure 1), resulting in 10 indices for pathogenicity and resistance each. If the index differs from zero, then the sympatric value deviates from the allopatric mean, i.e. one of the antagonists is locally adapted, depending on the sign of the indices. For the control matrix, we calculated all possible indices (i.e. 50 indices for pathogenicity and resistance each) by taking one combination as sympatric and all other combinations of each column/row as allopatric. These values served as null distribution for either pathogenicity or resistance. For host reproduction, one replicate was excluded from analysis. For this replicate, reproduction was zero on the control pathogen, which we consider an artefact, because in all other cases and also in our previous experiences, control pathogens only slightly reduced reproduction compared with non-pathogenic conditions and because this particular host population did even produce offspring on the more pathogenic coevolved *B. thuringiensis* (R. D. Schulte & H. Schulenburg 2006, unpublished data, this study and [31]). As a consequence, we calculated 49 control indices for host reproduction and reduction in host reproduction.

We considered the following alternatives to test for local adaptation.

(i) We evaluated the overall distribution of the actual-value specificity indices (i.e. unsigned indices in contrast to absolute-value indices, as used below). If this distribution differed from zero (tested with a one-sample *t*-test), sympatric values varied from allopatric values and thus, either hosts or parasites were locally adapted. Therefore, this analysis should yield insight into the general occurrence of local adaptation.

(ii) We also tested whether the distribution of actual-value specificity indices from the coevolution matrix is significantly different from that of the control matrix (Mann–Whitney *U*-test), which may reveal whether the inferred local adaptation pattern could have been produced by chance (as in the control matrix).

(iii) We examined the overall distribution of absolute-value specificity indices. This assessment should allow identification of all those cases, where in some replicates the parasite and in others the host are locally adapted and which would produce an insignificant overall pattern in the analysis of the signed specificity indices. For this analysis, we compared the absolute-value specificity index distributions of the coevolution and the control matrices (Mann–Whitney *U*-test).

(iv) We determined the significance of individual specificity indices for every single replicate via comparison with the distribution of the control matrix and the help of standard deviation scores (*z*-scores), following standard procedures [48]. In particular, for a standardized normal distribution, a certain percentage of the values fall within a defined range of the values. For example, a range of ± 2 s.d. includes 95 per cent of the values and thus the probability of observing a value that falls out of this range is $p \leq 0.05$. *z*-scores and *z*-tables allow the application of these rules for any normally distributed data. Thus, *z*-scores can be used to evaluate in how far a particular observed value falls within a particular range of a corresponding control distribution. *z*-tables contain the *p*-values for each of these *z*-scores. In the above example, the

z-score for a significance level of $p \leq 0.05$ is ± 2, and every value, which falls out of the range of 2 s.d. around the mean, can be considered significantly different [48,49]. Vice versa, for any given value, one can calculate the probability that it belongs to a certain distribution of values by estimating its z-score and the corresponding p-value. Thus, we were able to estimate the z-score for each single value (i.e. the distance to the mean in x-fold standard deviations) and thus the probability that the single value belongs to the control distribution.

The control indices for killing rate and survival rate were transformed to ensure normal distribution of the data (killing rate: $\sqrt[4]{(x + 0.5)}$; survival rate: $(x + 1.5)^2$). The control indices for the other two measures were normally distributed (host reproduction and reduction in host reproduction). The sign of the significant untransformed values was taken as an indication of either parasite or host local adaptation.

To account for multiple testing and thus an increased type I error, we adjusted the significance level according to the false discovery rate [50], taking into account $K = 13$ related tests per trait measure (three types of overall analyses, points (i)–(iii), plus 10 tests for the analysis of individual specificity indices, point (iv)). For this adjustment procedure, the p-values of K-related tests were ranked in ascending order, so that the largest p-value had the highest rank i. We then identified the largest rank i that fulfils the condition of $p \leq 0.05*i/K$. All hypotheses with a larger rank were rejected, all those with a smaller rank accepted [50]. Thus, the corrected critical significance level for killing rate was 0.007, for reduction in host reproduction 0.0128 and for host reproduction less than 2×10^{-6}. For survival rate, no value was significant after applying the correction procedure.

Statistical analysis was performed with SPSS 15.0.0 (SPSS Inc., Chicago, IL, USA).

3. RESULTS

The analysis of actual-value specificity indices did not reveal any significant differences, neither when the coevolution values were compared against zero (t-tests, $t_9 \leq |-1.906|$, $p \geq 0.089$) nor when they were tested against the control distribution (Mann–Whitney U: $U_{10,49 \text{ or } 50} \geq 137$, $p \geq 0.028$). By contrast, the analysis of absolute specificity indices showed that the coevolution values for the two indices of pathogenicity (killing rate and reduction in host reproduction) differed significantly from the control distribution (Mann–Whitney U, killing rate: $U_{10,50} = 91$, $p = 0.001$; reduction in host reproduction: $U_{10,49} = 106$, $p = 0.004$). The remaining two indices (for host resistance) did not produce any significant differences (Mann–Whitney U: $U_{10,49 \text{ or } 50} \geq 193$, $p \geq 0.265$).

The control distribution was also used to evaluate the significance of individual specificity indices. This approach revealed several significant cases of local adaptation, which referred to a total of four replicate populations (figure 2 and table 1). In particular, six indices for pathogenicity were significant (two for killing rate, four for reduction in host reproduction), belonging to a total of four replicates. Of these significant indices, two had larger values and four lower values for the sympatric combinations. Furthermore, two host resistance indices were significant (both for host reproduction), which referred to different replicates and which both produced lower values for the sympatric combinations.

4. DISCUSSION

Studies on local adaptation are essentially concerned with situations in which a population adapts to its environment [51]. In host–parasite coevolution, the host represents the environment for the parasite and vice versa [47]. Therefore, the environment changes depending on the evolution of the focal species. Obviously, both antagonists should evolve local adaptation: the parasite should increase its pathogenicity to optimally exploit the host, while the host has to evolve resistance to minimize damage caused by the parasite. It is conceivable that the direction of host–parasite local adaptation will differ between populations [21]. If either only the parasite or only the host is locally adapted, then this locally adapted antagonist is usually assumed to have had a higher evolutionary potential [4–7]. The latter is the focus of the 'classical' approach for the study of local adaptation, which looks at an overall pattern and thus only reveals local adaptation if the pattern is more or less uniform across the compared populations. Such an overall pattern would also be expected from directional adaptive evolution, where only one of the antagonists adapts, while the other does not show any reciprocal coevolutionary change.

For enhanced clarity in these studies, we propose to extend the available concepts and suggest usage of the following two terms:

— 'local adaptation', which refers to situations in which a significant overall pattern can be detected based for example on the classical study approach; and
— 'mosaic adaptation', which describes situations where in some populations it is the parasite and in others it is the host that is locally adapted.

The calculation of specificity indices combined with a controlled experimental set-up, as used in our study, allows disentangling local adaptation from mosaic adaption: an indication for local adaptation is given if the actual-value indices differ significantly from zero and a control distribution (describing random associations). Mosaic adaptation is suggested if the actual-value indices do not differ from zero, while at the same time the absolute indices show significant deviation from a control distribution, for example, owing to the presence of more extreme values and thus higher variance.

Based on these criteria, we only found support for mosaic adaptation: in case of the two measures for parasite pathogenicity, significant results were obtained using the absolute but not the actual-value indices. Thus, in some cases, hosts survive and reproduce better with sympatric than with allopatric parasites, indicating host local adaptation, while in other cases, hosts survive and reproduce worse with sympatric parasites, suggesting parasite local adaptation.

These results were confirmed through our evaluation of the individual populations. In this context, the control distribution is of particular importance, because it highlights the extent of local adaptation patterns that may

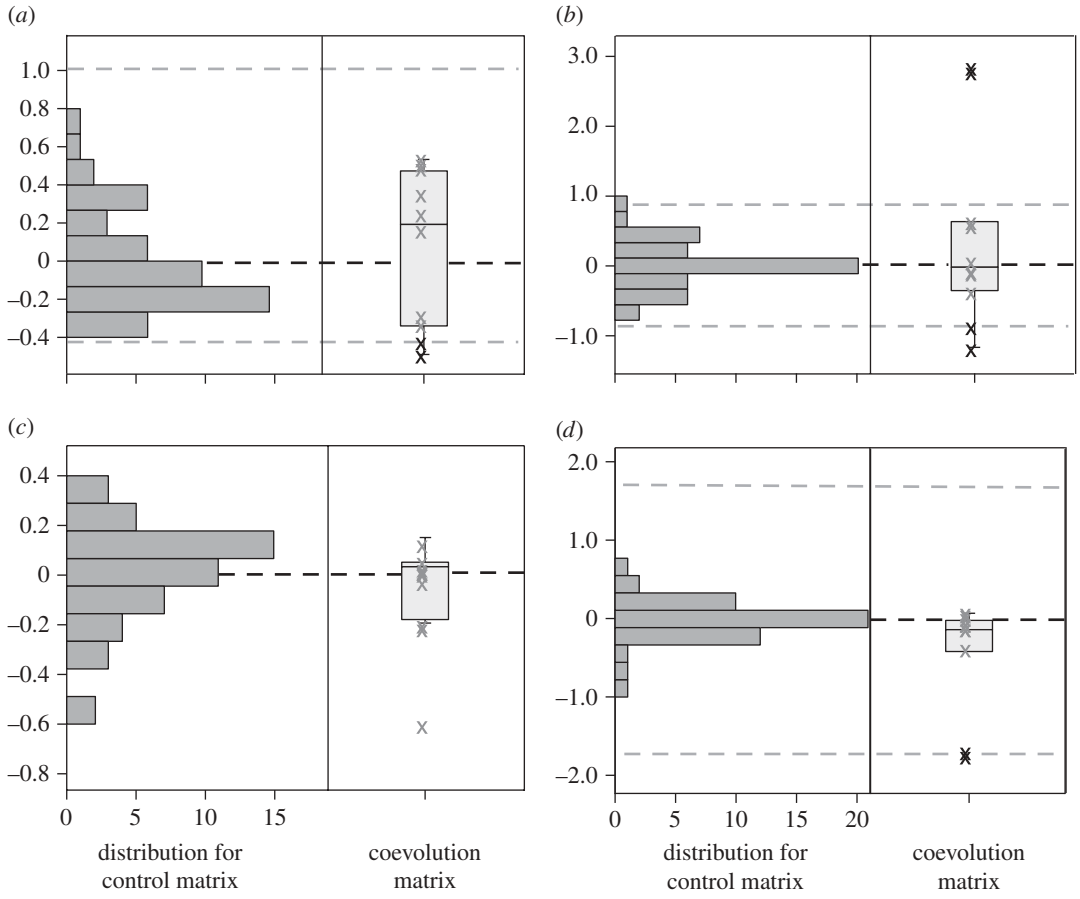

Figure 2. Distribution of specificity indices (actual values). On the left side of each graph, the distribution of all possible control indices is displayed. On the right, boxplots indicate the distribution and stars indicate the individual values of the coevolution specificity indices. The black dashed lines show the mean (always zero); the grey dashed lines indicate the critical significance level, following analysis of *z*-scores and adjustment of the significance level according to the false discovery rate. Black stars highlight significant individual values. For boxplots, the horizontal line indicates the median, the box the 25% quartile above and below the median and the whiskers the data range. All graphs show original values, even if they were transformed for statistical analysis (see details in §2). (*a*) Parasite pathogenicity measured as host killing rate. (*b*) Parasite pathogenicity inferred from the reduction in host reproduction. (*c*) Host resistance as indicated by host survival rate. (*d*) Host resistance measured as host reproduction.

Table 1. Analysis of local adaptation for each individual replicate. (If significant, then the table indicates whether sympatric or allopatric populations produce the larger values (i.e. the specificity index is positive or negative, respectively). Italic font indicates host, and bold font parasite local adaptation. The distribution of the individual values is illustrated in figure 2.)

	pathogenicity		resistance		
replicate	killing rate	reduction of host reproduction	survival rate	host reproduction	conclusion
1	*allopatric > sympatric*	*allopatric > sympatric*	*n.s.*	*n.s.*	*host local adaptation*
2	n.s.	n.s.	n.s.	n.s.	no local adaptation
3	n.s.	n.s.	n.s.	n.s.	no local adaptation
4	**n.s.**	**sympatric > allopatric**	**n.s.**	**allopatric > sympatric**	**parasite local adaptation**
5	n.s.	n.s.	n.s.	n.s.	no local adaptation
6	n.s.	n.s.	n.s.	n.s.	no local adaptation
7	n.s.	n.s.	n.s.	n.s.	no local adaptation
8	**n.s.**	**sympatric > allopatric**	**n.s.**	**allopatric > sympatric**	**parasite local adaptation**
9	*allopatric > sympatric*	*allopatric > sympatric*	*n.s.*	*n.s.*	*host local adaptation*
10	n.s.	n.s.	n.s.	n.s.	no local adaptation

result from random associations, as visible in the left parts of figure 2*a–d*. Thus, it helps to distinguish significant cases of local adaptation from chance effects and to evaluate the significance of individual specificity indices for the different replicates. Based on these comparisons, we identified the following patterns (table 1).

— Allopatric parasites reduce host fitness more strongly than sympatric parasites as measured with both killing rate and reduction of host reproduction (two replicates). This pattern is consistent with host local adaptation.
— Sympatric parasites reduce reproduction of hosts more strongly than allopatric parasites and allopatric hosts produce more offspring in the presence of a parasite than the sympatric hosts (two replicates). This pattern indicates parasite local adaptation.
— No evidence for adaptation (six replicates).

How can we explain such a 'geographical' diversification between our replicates, although they all evolved under the same environmental conditions (i.e. controlled laboratory conditions)?

On the one hand, the observed pattern may be owing to the time lag of coevolution [4,16], so that at a different time point, we could have obtained a different pattern of local adaptation. In future studies, the importance of such time lags could be revealed by examining changes in local adaptation patterns over time [52]. On the other hand, our replicate populations do represent a selection mosaic, since they are likely to vary in several random factors, which cannot be controlled entirely under laboratory conditions, including small temperature variations or random differences in the genetic composition of replicate populations [31].

The fact that we could not detect local adaptation in six out of 10 replicate populations may have different reasons.

First, these replicate populations may be coevolutionary coldspots, i.e. there is no reciprocal coevolution between parasite and host. This is generally consistent with the geographical mosaic of coevolution [21]. At the same time, the results from our previous study [31] may suggest that the finding of six coldspots out of 10 possibilities (and thus more than half of the sample) is too high. In that study, significant changes in multiple phenotypic and genetic traits were found for the same experimentally evolved material. It was of no relevance that the experimental populations were initiated with only three genotypes for the parasite or derived from the offspring of repeated reciprocal crosses among three natural isolates for the host, which could have led to low genetic variation and thus low evolutionary potential in the two antagonists. However, our previous results demonstrated that experimental coevolution did lead to reciprocal phenotypic adaptations among the two antagonists and to an increase in genetic diversity for parasite toxin gene loci and several host loci. Furthermore, coevolution was associated with an increased change in allele frequencies over time, consistent with constant adaptation to the coevolving antagonist, which in turn should result in local adaptation patterns.

Second, it is possible that the antagonists might have evolved non-specific responses, like general resistance, which would then also be efficient towards non-coevolving pathogens.

Third, the ability to detect local adaptation depends on the exact time point within the coevolutionary cycles. If coevolution between parasite and host is currently balanced, neither is expected to be locally adapted.

Fourth, coevolution may act on different traits in the different populations [20,53,54]. Since we observed genetic diversification between replicate populations [31], it is likely that the antagonists evolved various strategies. Because we only evaluated a limited set of traits and because it is well known that the choice of traits can be crucial for the detection of local adaptation [18–20], we may have missed some of these strategies and thus failed to identify local adaptation in some replicate populations. In detail, we focused on killing rate as a measure for pathogenicity, because it should directly associate with parasite fitness during experimental evolution, where we specifically selected for killing pathogens [31]. Nonetheless, other traits like infection rate or intensity, which also correlate directly with parasite fitness, may have allowed identification of additional cases of local adaptation and should thus be included in further studies in this system.

The comparison of individual specificity indices to a control distribution provides a powerful tool to characterize the geographical mosaic of coevolution. The approach is particularly valuable, because it allows inference of local adaptation in some populations even if an overall pattern is absent. It however requires the availability of a control distribution that might not be straightforward in nature. Natural populations are furthermore problematic as the environment is known to influence local adaptation. Laine [27], for example, could not verify local adaptation in a field transplant experiment, although she revealed local adaptation for exactly the same populations in a laboratory-based cross-infection study. As the abiotic environment crucially influences the interaction between host and parasite [55], but may also obscure local adaptation, experimental designs to study local adaptation in natural populations become rather complicated [28].

Thus, experimental evolution under controlled laboratory conditions offers a promising alternative, as it allows for testing of the importance of specific environmental factors. This approach was previously used to study, for example, the effect of the genetic background [19], migration or gene flow [9,13,56] on local adaptation or whether an adaptation cost exists [57,58]. However, with one exception, the previous studies did not include a control evolution treatment. To our knowledge, the only exception is the evolution experiment by Poullain and co-workers [59]. In this study, two selection regimes were imposed: either coevolution or adaptive evolution of phages with their bacterial hosts. The two treatments produced different results: the evolved phages were better at infecting their 'sympatric' hosts (ancestral host for coevolution) when compared with the coevolved phages. By contrast, the coevolved phages were not locally adapted. These results highlighted that overall local adaptation may be produced by directional adaptive evolution but not necessarily coevolution. This study and our study consistently demonstrate that experimental evolution approaches provide new insights into local adaptation, because coevolutionary scenarios can be compared with control evolution treatments, which then help to reveal the exact consequences of coevolution versus other types of selective dynamics.

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