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REVERSE EVOLUTION: SELECTION AGAINST COSTLY RESISTANCE IN DISEASE-FREE MICROCOSM POPULATIONS OF *PARAMECIUM CAUDATUM*

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Evolutionary costs of parasite resistance arise if genes conferring resistance reduce fitness in the absence of parasites. Thus, parasite-mediated selection may lead to increased resistance and a correlated decrease in fitness, whereas relaxed parasite-mediated selection may lead to reverse evolution of increased fitness and a correlated decrease in resistance. We tested this idea in experimental populations of the protozoan *Paramecium caudatum* and the parasitic bacterium *Holospora undulata*. After eight years, resistance to infection and asexual reproduction were compared among paramecia from (1) "infected" populations, (2) uninfected "naive" populations, and (3) previously infected, parasite-free "recovered" populations. Paramecia from "infected" populations were more resistant (+12%), but had lower reproduction (–15%) than "naive" paramecia, indicating an evolutionary trade-off between resistance and fitness. Recovered populations showed similar reproduction to naive populations; however, resistance of recently (<3 years) recovered populations was similar to paramecia from infected populations, whereas longer (>3 years) recovered populations were as susceptible as naive populations. This suggests a weak, convex trade-off between resistance and fitness, allowing recovery of fitness, without complete loss of resistance, favoring the maintenance of a generalist strategy of intermediate fitness and resistance. Our results indicate that (co)evolution with parasites can leave a genetic signature in disease-free populations.

KEY WORDS: ***Holospora undulata*, life-history, parasite, selection, trade-off.**

Evolutionary trade-offs occur if selection on one trait reduces the value of another (Stearns 1992). Such a cost of adaptation can lead to negative genetic correlations between fitness components, thereby maintaining genetic diversity and preventing the evolution of omnipotent generalists (Kassen 2002). This universal principle of adaptation costs may also play an important role in coevolutionary interactions between hosts and parasites. Parasites are generally expected to select for higher resistance in the host. However, an increase in resistance can be accompanied by a reduction in fitness-relevant life-history traits, such as competitive ability, fecundity, seed production, etc. Costs of resistance are then defined (and measured) as a reduction of fitness in the absence of parasites (Simms and Rausher 1987; Kraaijeveld et al. 2002; Strauss et al. 2002). Associated costs are predicted to select for optimal levels of resistance balanced against other important fitness related life-history traits. Theoretical models show that trade-offs between resistance and other fitness components can help maintain resistance polymorphism within populations (e.g., Gillespie 1975; Parker 1992; Antonovics and Thrall 1994; Bowers et al. 1994; Agrawal and Lively 2002), but also promote

genetic divergence between populations that vary in their exposure to parasite-mediated selection (Elmqvist et al. 1993; Hasu et al. 2009) or that live in environments where costs of resistance are expressed differently (Jessup and Bohannan 2008).

Generally, costs of resistance are thought to arise from a conflict between allocation of limited resources to the defense machinery and to other fitness-relevant functions (Simms and Rausher 1987; Coustau et al. 2000; Labbé et al. 2010). In various plant, invertebrate, or microbial systems, costs of resistance have been demonstrated among naturally occurring genotypes (Biere and Antonovics 1996; Strauss et al. 2002; Tian et al. 2003; Carton et al. 2005; Gwynn et al. 2005; Jessup and Bohannan 2008); over the past few years, an increasing number of studies has also addressed this issue in laboratory selection experiments (Table 1). It is commonly assumed that these trade-offs result from antagonistic pleiotropy of genes conferring resistance, but impairing other fitness functions (Lenski 1988; Tian et al. 2003). The underlying functional basis may vary from system to system. Overproduction of defense structures or molecules may have energetic costs, interfere with other biochemical pathways, or even be immunopathogenic (Coustau et al. 2000; Kraaijeveld et al. 2001; Brown 2003).

The form of the relationship between parasite resistance and other fitness traits is important for the evolution of resistance characteristics in host populations. In particular the shape of the relationship is important regarding whether parasite-mediated selection will maintain resistance polymorphism or not. An increasingly costly, or convex, relationship between parasite resistance and other fitness traits may select for one evolutionary stable, generalist strategy in a host population. Conversely, for a decreasingly costly relationship, coexistence of highly resistant and highly susceptible types is possible (Boots and Haraguchi 1999).

Given the above genetic and functional constraints, we can establish two main predictions about the evolution of costs of resistance. First, parasite-mediated selection for increased resistance should lead to a correlated decrease in fitness in the absence of the parasite. This can be tested by artificial selection or experimental evolution, comparing the direct response to selection for resistance and the correlated response for fitness (Table 1A). These types of experiments can reveal very strong evolutionary trade-offs between resistance and fitness; often, however, these costs are only detectable for certain fitness components and under certain environmental conditions (mostly stressful). In a few cases, resistance benefits were detected for certain traits (Table 1A).

The second prediction holds that costly resistance should be selected against in the absence of parasites. That is, relaxing parasite-mediated selection should re-establish fitness and lead to a correlated decrease in resistance. Only very few studies have properly tested this second prediction. A number of studies that initially set out to test the first prediction tentatively explored the consequence of subsequent relaxed parasite-mediated selection for a relatively limited number of selection lines and generations (Table 1A). Among these studies, one identified an increase in fitness after two to four generations of relaxed parasite-mediated selection in one population (Boots and Begon 1993), and three studies a reduction in resistance (Fuxa and Richter 1998; Luong and Polak 2007; Ye et al. 2009), but they do not report the corresponding changes in resistance or fitness, respectively. Other studies relaxed parasite-mediated selection for cost-free resistance and, not surprisingly, no change in resistance was later observed (Milks et al. 2002; Kolss et al. 2006; Meyer et al. 2010). To our knowledge only one study has explored the effect of long-term relaxed parasite-mediated selection for populations where costs of resistance were identified. In *Escherichia coli* populations, the cost of resistance against a bacteriophage declined by 50% over 400 generations in the absence of phage, and this without loss of resistance (Lenski 1988). This was explained by the action of compensatory mutations restoring fitness functions, without compromising resistance. Similarly, when retracting the bacterial biopesticide *Bacillus thuringiensis*, resistant diamondback moth populations showed reversal toward susceptibility and an increase in fitness within several generations, although not necessarily to ancestral levels (Tabashnik et al. 1994). Thus relaxed selection does not necessarily lead to the evolutionary return to the ancestral state, raising the general question of the reversibility of evolutionary trajectories (Teotonio and Rose 2000).

We investigated reverse evolution of costs of resistance in experimental long-term populations of the protozoan *Paramecium caudatum* and the bacterial parasite *Holospora undulata*. For these populations, a previous study had indicated parasite-mediated selection and costs of resistance: paramecia from populations coevolving with the parasite had higher levels of resistance, but lower growth rates than paramecia from naive populations, never exposed to the parasite (Lohse et al. 2006). The present study was motivated by the occurrence of a third population type: Over the course of the long-term experiment, some initially infected populations lost infection and became disease-free. We compared resistance and fitness (reproductive rate) of these "recovered" populations with still "infected" populations, and "naive" populations that had never been exposed to the parasite. We predicted selection for increased fitness and a concomitant decrease in resistance in the "recovered" populations, after extinction of the parasite. If selection occurs along a trade-off function, we expected fitness and resistance of the "recovered" populations to be identical to that of naive populations (full reversal) or intermediate between those of still "infected" and naive populations (partial reversal). The position of the recovered populations along this trade-off function would then depend on time required for acquisition of

Table 1. **Studies on the evolution of costs of resistance under parasite-mediated selection (A) and under relaxed parasite-mediated selection (B). "Artificial selection" refers to multigenerational studies where the experimenters selected parasite-free individuals and/or individuals surviving infection to initiate and infect a new cohort at each generation.**

(A) Studies testing effects of parasite-mediated selection

Reference	Host	Type of experiment[1]	Selection for resistanceagainst	Cost (bold type = significant fitnesscost identified)	Environment costs tested	Additional observations	Subsequent relaxed selection[2]
Insects							
(Hurd et al. 2005)	*Anopheles gambiae*	Artificial selection	*Plasmodium yoelii nigeriensis*	**Hatch rate (–18%)**; Longevity, mating success, size, bloodmeal size, egg production	Temperature stress, starvation, flight activity	Parallel selection for susceptibility	
(Fuxa and Richter 1998)	*Anticarsia gemmatalis*	Artificial selection	Nucleopolyhedro-virus	**Fertility (–39% viable offspring), pupal weight (–10%), neonate survival** (–7%); Longevity	Standard laboratory conditions	Resistant insects lived longer	Repeated (3x) loss of resistance within 3 generations
(Kraaijeveld and Godfray 1997)	*Drosophila melanogaster*	Artificial selection	Endoparasitoid *Asobara tabida*	**Competitive ability (–50%, low food)**; Larval and pupal sur-vival/development, adult longevity/size, early fecundity, fluctuating asymmetry	Different food levels		
(Fellowes et al. 1998)	*Drosophila melanogaster*	Artificial selection	Endoparasitoid *Leptopilina boulardi*	**Competitive ability (–45%, low food)**; Fecundity, egg viability, starvation tolerance, size and development rate	Different food levels		
(Kolss et al. 2006)	*Drosophila melanogaster*	Experimental evolution	Endoparasitoid *Asobara tabida*	Learning ability to avert shock	Standard laboratory conditions		No loss of resistance after 50 generations

Continued.

Table 1. Continued

(A) Studies testing effects of parasite-mediated selection							
Reference	Host	Type of experiment[1]	Selection for resistanceagainst	Cost (bold type = significant fitnesscost identified)	Environment costs tested	Additional observations	Subsequent relaxed selection[2]
(Vijendravarma et al. 2009)	*Drosophila melanogaster*	Artificial selection	Microsporidian *Tubulinosema kingi*	**Fecundity (–38%)**, **Competitive ability (–78%, low food)**; Survival	High and low food		
(Ye et al. 2009)	*Drosophila melanogaster*	Artificial selection	Bacterium *Pseudomonas aeruginosa*	**Longevity (–11%, females only), Egg viability (–30%);** Development time, body mass, attractiveness, offspring produced	Standard laboratory conditions	~6% faster development time for resistance selected lines	Resistance lost within 5 generations
(Luong and Polak 2007)	*Drosophila nigrospiracula*	Experimental evolution	Ectoparasitic mite *Macrocheles subbadius*	**Fecundity (–30%);** Longevity	Low and high temperature		Reduced resistance within 5 generations, maintained further 20 generations (Polak 2003)
(Boots and Begon 1993)	*Plodia interpunctella*	Experimental evolution	Granulosis virus	**Development time (24% slower), Egg hatch (–3%);** Egg production, pupal weight	Standard laboratory conditions	Resistant moths 5% larger	After 2 generations reduction in fitness 8%
(Milks et al. 2002)	*Trichoplusia ni*	Artificial selection	Nucleopolyhedro-virus	Pupal weight, development time, survival, fecundity	Standard laboratory conditions	Parasite allowed to coe-volve;Resistant selected pupae heavier and develop faster	Resistance retained after seven generations of relaxed selection

Continued.

Table 1. Continued

(A) Studies testing effects of parasite-mediated selection							
Reference	Host	Type of experiment[1]	Selection for resistanceagainst	Cost (bold type = significant fitnesscost identified)	Environment costs tested	Additional observations	Subsequent relaxed selection[2]
Snail							
(Webster and Woolhouse 1999)	*Biomphalaria glabrata*	Artificial selection	*Schistosoma mansoni*	**Fertility (–75% egg production);** Longevity	Standard laboratory conditions	Parallel selection for higher susceptibility	
Crustacean							
(Zbinden et al. 2008)	*Daphnia magna*	Experimental evolution	Microsporidian *Octosporea bayeri*	**Clonal growth rate (–40%);** Competitive ability, survival	High and low food, low density	Resistant selected *Daphnia* superior competitors	
Bacteria							
(Chao et al. 1977)	*Escherichia coli*	Experimental evolution	Phage T7	**Competitive ability (–20%)**	Standard laboratory conditions	2 outcomes, with different coexisting mutants	
(Lenski and Levin 1985)	*Escherichia coli*	Experimental evolution	Phage T2, T4, T5, T7 (all separately)	**Competitive ability (–50% T4)**	Standard laboratory conditions	No costs of resistance against T5	
(Brockhurst et al. 2004)	*Pseudomonas fluorescens*	Experimental evolution	Phage SBW25Φ2	**Competitive ability (–19%)**	Standard laboratory conditions		
(Buckling et al. 2006)	*Pseudomonas fluorescens*	Experimental evolution	Phage SBW25Φ2 for lines with high and low mutation loads	**Competitive ability (–5%, high mutation load only)**	Standard laboratory conditions		
(Lopez-Pascua and Buckling 2008)	*Pseudomonas fluorescens*	Experimental evolution	Phage SBW25Φ2	**Competitive ability (–5% in rich, –25% in poor environment)**	High and low resources		
(Morgan et al. 2009)	*Pseudomonas fluorescens*	Experimental evolution	Phage SBW25Φ2	**Growth**	Standard laboratory conditions		

Continued.

Table 1. Continued

(A) Studies testing effects of parasite-mediated selection

Reference	Host	Type of experiment[1]	Selection for resistanceagainst	Cost (bold type = significant fitnesscost identified)	Environment costs tested	Additional observations	Subsequent relaxed selection[2]
(Gallet et al. 2009)	*Pseudomonas fluorescens*	Experimental evolution	Predatory bacterium *Bdellovibrio bacteriovorus*	**Carrying capacity (–75%, for FM morph at end of experi-ment);Growth**	Standard laboratory conditions		
Protozoan							
(Lohse et al. 2006)	*Paramecium caudatum*	Experimental evolution	Bacterium *Holospora undulata*	**Clonal growth (–25%)**	Standard laboratory conditions		
(Schulte et al. 2010)	*Caenorhabditis elegans*	Artificial selection	Bacterium *Bacillus thuringiensis*	**Adult size, Population growth**	Standard laboratory conditions		

(B) Studies specifically testing effects of relaxed parasite-mediated selection

Reference	System	Type of experiment	Relaxed selection for resistance against	Cost (bold type = trait for which fitness cost was still identified)	Environment costs tested	Additional observations	Number of generations selection relaxed
(Lenski 1988)	*Escherichia coli*	Experimental evolution	Phage T4	**Competitive ability (Compensatory mutation reduced fitness cost from 43% to 22%)**	Standard laboratory conditions	No loss of resistance	400 generations
(Meyer et al. 2010)	*Escherichia coli*	Experimental evolution	Phage T6	Competitive ability	Standard laboratory conditions	No loss of resistance; No cost of resistance for ancestral lines; correlated responses to other phages	~44 500 generations

[1] "Experimental" evolution refers to studies where experimenters did not interfere with selective and demographic processes occurring in the long-term experimental lines.

[2] A cost of resistance represents a reduction in trait value relative to unselected control. Certain studies in (A) report supplementary results on short-term evolutionary change of resistance when parasite-mediated selection was subsequently relaxed.

compensatory or back mutations. Our experimental design also allowed us to investigate the shape of the trade-off function, between resistance and fitness in the absence of the parasite, using multiple genotypes and populations.

Material and Methods

LITERATURE SEARCH

We compiled laboratory selection experiments investigating costs of resistance. Studies were identified using Web of Science with search terms "costs of resistance," "parasite," "experimental evolution," and "artificial selection." We only included studies investigating evolutionary responses to parasites where costs of resistance were assayed in the absence of the parasite. In Table 1A, we specify whether studies used artificial selection for resistance, or experimental evolution in the presence of the parasite. We report life-history traits for which correlated costs of resistance were found, and how strong the costs were (reduction in trait value relative to unselected control). We also provide relevant additional information, such as the environment, the cost was assayed, or whether supplementary data on effects of relaxed parasite-mediated selection were available. Two studies specifically studied the relationship between relaxed selection for resistance and the correlated response in life-history traits (Table 1B).

STUDY ORGANISMS

Paramecium caudatum is a freshwater ciliate found in still water bodies in the northern hemisphere that feeds on bacteria and detritus within the water column (Wichtermann 1986). Reproduction is predominantly asexual through mitotic division, with most gene expression occurring in the polyploid macronucleus; sexual reproduction occurs by conjugation between different mating types. Our long-term selection lines were kept as asexual clones and thus responses to selection likely occurred in the macronucleus.

The micronucleus-specific *Holospora undulata* is a gram-negative alpha-proteobacterium (Fujishima 2009a). While feeding, paramecia ingest immobile infectious forms (15–20 μm), which then mediate their transport from the digestive vacuole to the micronucleus. In the micronucleus, the infectious form differentiates into the short reproductive forms. Reproductive forms multiply for 7–10 days, until they begin to differentiate into infectious forms, which are released into the medium upon cell division of the host or when the host dies. Thus, infectious forms are horizontally transmitted, whereas reproductive forms are vertically transmitted at each mitotic division of the host. Fully established infections usually carry a mix of reproductive and infectious forms. Infection reduces host division and survival, in particular when infectious forms accumulate in the micronucleus (Restif and Kaltz 2006).

LONG-TERM EXPERIMENT AND PREPARATION OF LINES

Infected and uninfected long-term replicate populations of different *Paramecium* clones ("clonal backgrounds," hereafter) were established in 2002 (Adiba et al. 2010). Initial infections were established by adding an inoculum prepared from an infected clonal mass culture, provided by H.-D. Görtz (Universität Stuttgart, Germany). Populations were kept in 50 mL culture medium in 50-mL plastic tubes at 23°C. The culture medium is based on organically grown lettuce, inoculated with the bacterium *Serratia marcescens* (Strain A173; Institut Pasteur, Paris, France) as a food resource. In two- to three-week intervals, we replaced 10 mL from each tube (medium and paramecia) with fresh medium. This protocol maintains cultures at densities of 50–150 individuals per mL. Although generation time is difficult to estimate for populations at carrying capacity, we estimate that the 8 years of the experiment spanned approximately 800 generations.

Infection prevalences reached up to 90% in the first year (2003), then declined over subsequent years and certain populations lost the parasite. Between 2005 and 2007, several experiments investigated patterns of reciprocal selection and costs of resistance in these populations (Lohse et al. 2006; Nidelet and Kaltz 2007; Adiba et al. 2010). For the present experiment, in early 2010, we used 18 infected replicate populations (mean prevalence: SD 24% ± 20), 25 naive populations, and 14 recovered populations. Thirteen of the recovered populations were still infected in 2003, but due to incomplete yearly sampling, we can only distinguish between populations that recovered from the parasite before or after 2007. The 57 replicate populations represented six clonal backgrounds, initially derived from independent matings between two Japanese parental strains (Adiba et al. 2010). Each clonal background was represented in each of the three population types, although with variable numbers of replicate populations (average: SD 3.2 ± 1.6 per clone and type).

We have no measurements of the resistance of recovered populations before they lost the parasite. These populations may have lost the parasite by chance, because they were more resistant, or for some other unknown reasons. By the end of 2003, the future recovered populations showed lower prevalences (SE 48 ± 7%) than populations that remained infected (61 ± 5%), although this difference was not statistically significant ($F_{1,25} = 1.3, P = 0.2639$). We have previously shown that population-level prevalences can be positively correlated with infection success in inoculation experiments (Lohse et al. 2006). This suggests that recovered populations originally were at least as, if not more, resistant than still infected populations. The 2003 assay showed no

significant difference in population density ($F_{1,20} = 0.17$, $P > 0.6$) between later-recovered and still-infected populations.

FITNESS ASSAY

As a proxy of fitness with asexual reproduction, we measured the clonal reproductive rate of the paramecia, starting from single individuals. From each replicate population, we randomly selected up to six uninfected individuals (average: SD 5.7 ± 0.8; total $n = 330$). Each individual was washed twice in 150 µl of fresh growth medium, then placed individually in 500-µl plastic tubes, containing 100 µl of medium. The individuals were allowed to divide for 10 days, with 100 µl of medium added on day 2, 5, and 8.

Final density was measured by determining the number of individuals in two 20-µl samples, under a dissecting microscope. The resulting small clonal populations (<200 individuals) are referred to as "subclones" hereafter. All subclones derived from infected populations were double-checked for infection to ensure that only uninfected subclones were used for the tests. This assay protocol produces continuous clonal replication (Adiba et al. 2010) and final density is generally well correlated with maximum daily population growth rate (C. Boëte and O. Kaltz, unpubl. data).

RESISTANCE ASSAY

Up to four subclones (average: SD 3.4 ± 1; total $n = 188$) from each of the 55 long-term populations were available for the resistance assay. An aliquot of 150 µl from each subclone was transferred to a new tube with 100 µl of fresh medium. To each tube, we added 2.5×10^4 infectious forms, extracted from a mix of equal volumes of all infected long-term populations (for details of the protocol, see [Adiba et al. 2010]). This dose chosen was based on previous experience to achieve sufficiently high levels of infection for statistical comparison among the different host origins. After 48 h, approximately 50 individuals from each tube were fixed with lacto-aceto-orcein to determine infection success (= proportion of infected individuals, at 1000× magnification, phase contrast).

STATISTICAL ANALYSIS

To analyze variation in resistance (arcsine-transformed proportion infection) and growth (log-transformed final density), we fitted initial full models containing the fixed effect of population type (infected, recovered, naive) and the random effects of clonal genetic background and identity of long-term replicate population, using the REML procedure in the JMP statistical package (SAS 2009). In a second analysis, measures of resistance and growth were averaged over subclones to obtain means per replicate population. An analysis of covariance, with population type and clonal background as cofactors, tested for linear and nonlinear (second-order polynomial) relationships between mean resistance and mean growth. To distinguish between recovered populations that lost the parasite before or after 2007, we also ran these analyses with population type having four levels: infected, recovered before 2007, recovered after 2007, and naive. In all analyses, clonal background and its interaction with population type were statistically nonsignificant, and sequential backward model simplification showed that these effects could be removed from the statistical models without significant loss of variance explained (all $P > 0.2$). Therefore, in minimal adequate models, the effect of population type was tested over the replicate population(type) term.

Results

REPRODUCTIVE RATE

At the end of the one-week assay period, a larger fraction of subclones from infected populations were found to be extinct (20%) than subclones from recovered (4%) or naive (8%) populations. As infection status could not be double-checked for extinct subclones, we only present the analysis for subclones surviving until the end of the assay ($n = 292$ subclones from all 57 replicate populations). However, including the 38 extinct subclones in the analysis (with a density of "0") leads to identical results (not shown).

On average, we obtained six to seven population doublings for the surviving subclones (SE: 6.6 ± 0.1). We observed a significant effect of population type ($F_{2,49} = 6.67$, $P = 0.0028$) on reproductive rate. Multiple comparisons (Student's SNK) revealed that paramecia from infected populations grew less well than those from naive and recovered populations. In turn, mean final densities of naive and recovered populations were nearly identical (Fig. 1). More detailed analysis showed no significant difference in growth between recovered replicate populations that had lost the parasite before or after 2007 ($t_{45} = 0.18$, $P > 0.8$; Fig. 1). This indicates that additional years spent in disease-free conditions did not significantly influence growth performance in 2010.

RESISTANCE

Infection was detected in 107 of the 188 inoculated assay tubes (57%); the mean percentage of infected individuals per assay tube was 18 ± 1% (max = 80%). There was a significant effect of population type ($F_{2,54} = 8.48$, $P = 0.0006$) on levels of infection observed. Multiple comparisons showed that, on average, paramecia from infected populations were most resistant and those from naive populations least resistant (Fig. 2).

The average level of resistance of recovered populations fell in-between those of infected and naive populations. There was, however, heterogeneity within the recovered group: populations that lost the parasite before 2007 were less resistant to infection

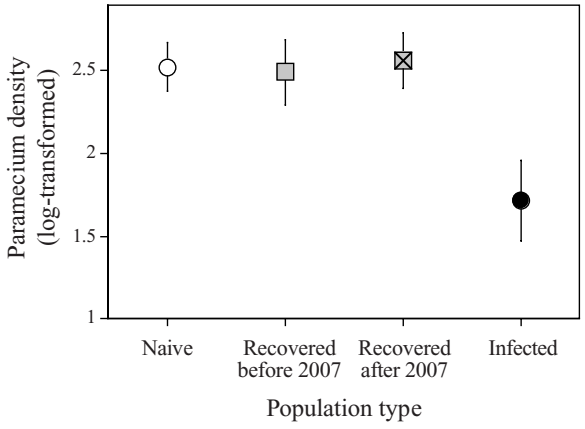

Figure 1. Mean final density (log-transformed) in the fitness assay testing paramecia from naive, recovered, and infected population types. The recovered type is divided into populations that have lost infection before or after 2007. Means and standard errors calculated from replicate population means.

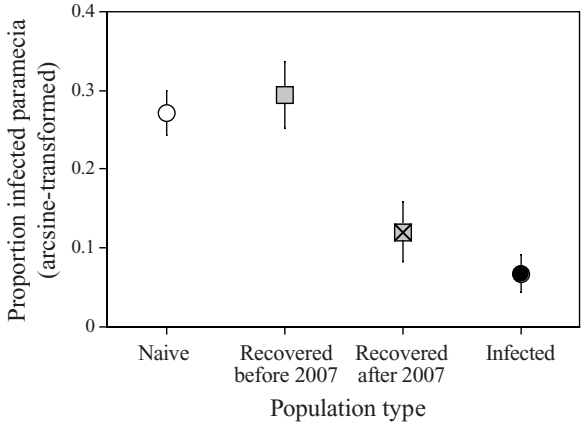

Figure 2. Mean proportion of infected individuals (arcsine-transformed) in the resistance assay testing paramecia from naive, recovered, and infected population types. The recovered type is divided into populations that have lost infection before or after 2007. Means and standard errors calculated from replicate population means.

(mean% infection: SE: 9.4 ± 2.5) than populations that have lost the parasite more recently (2.1 ± 1.1). Thus, more time spent in the absence of the parasite was associated with higher susceptibility to infection. When specifying these two types of recovered populations in the analysis, we found that the long-time recovered populations grouped with the naive populations, whereas the resistance of more recently recovered populations was similar to that of paramecia from infected populations (Fig. 2).

CORRELATION BETWEEN REPRODUCTIVE RATE AND RESISTANCE

The ANCOVA showed that the relationship between resistance and reproductive rate had significant linear ($F_{1,52} = 13.90$, $P = 0.0005$) and nonlinear (second-order polynomial: $F_{1,52} = 8.81$, $P = 0.0045$) components. These relationships did not significantly vary with clonal background or with population type (all interactions of these two factors with the first- and second-order resistance covariate: $P > 0.3$). Thus, the overall pattern emerging is that, when resistance decreases, reproductive rate first steeply increases, but then reaches a plateau for lower values of resistance. Figure 3A shows this nonlinear, convex relationship between resistance and reproductive rate across replicate populations, Figure 3B for the means of the different population types.

Discussion

Costs of resistance arise if genes conferring higher resistance also cause reductions in fitness-related traits, such as fecundity or growth. Thus parasite-mediated selection should lead to increased resistance and a correlated decrease in fitness, whereas relaxed parasite-mediated selection should lead to reverse evolution of increased fitness and a correlated decrease in resistance. Consistent with the first prediction, paramecia from infected populations were more resistant, but grew less well than paramecia from naive populations, indicating an evolutionary trade-off between resistance and fitness. Paramecia from recovered populations, where parasite-mediated selection had been relaxed, had the same average reproductive rate as naive paramecia, but levels of resistance were more variable. Namely, more recently recovered populations (>2007) showed high levels of both reproductive rate and resistance, suggesting that evolutionary reversal toward higher fitness is not necessarily coupled with complete loss of resistance.

EVOLVING RESISTANCE BEARS FITNESS COSTS

The contrasting patterns of reproductive rate and resistance between paramecia from naive and infected populations indicate that evolving resistance against *H. undulata* trades off with fitness, a trend already observed for a small subset of our experimental populations at an earlier time point during long-term evolution (Lohse et al. 2006). It has been argued that artificial selection may misrepresent the pervasiveness of costs in natural settings (Lazzaro and Little 2009). Our experiment, however, shows that substantial costs can build up among freely (co)evolving microcosm populations, not submitted to artificial selection. The same conclusion can be drawn from several other studies, using the same approach of experimental evolution (Table 1).

Furthermore, it has been shown that trade-offs between resistance and fitness can vary with ecological or genetic factors, such as genetic background (Bergelson 1994; Raymond et al. 2005; Buckling et al. 2006). We found that clonal origin of the paramecia did not significantly affect variation or covariation in resistance and growth, indicating generality of costs of resistance, across genetic backgrounds. This is perhaps not surprising because the

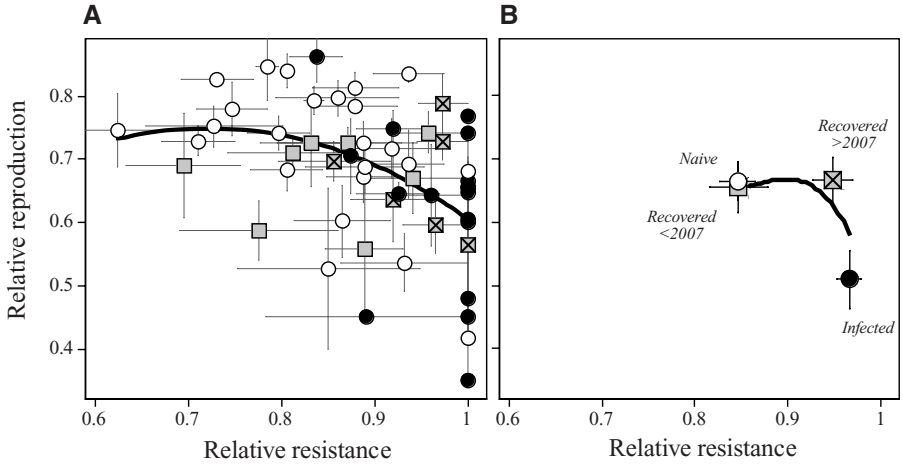

Figure 3. Relationship between resistance and fitness (reproductive rate), based on replicate population means (A) or the means per population type (B). To make ranking on the two axes comparable, values were standardized by dividing them by the maximum levels of resistance and reproductive rate, respectively. Means and standard errors were calculated over paramecium subclones (A) or replicate populations (B; means taken from Fig. 1 and 2). Different symbols represent the different population types, as indicated in (B). To illustrate nonlinearity, we fitted a second-order polynomial function in (A) and a smoothing spline in (B).

different clones all share the same parental strains. Similarity of (co)evolutionary trajectories was also indicated by a recent study demonstrating local parasite maladaptation to these *Paramecium* clones (Adiba et al. 2010).

The mechanistic and genetic basis of resistance in our system is still not well understood. Several defense barriers may exist before the parasite reaches its target site, the micronucleus (Fujishima 2009b). Infection occurs during food uptake, when paramecia ingest infectious forms from the medium. Thus, behavioral resistance may be acquired by reducing parasite contact rate through reduced filtering. This, in turn, may reduce the rate of uptake of resources necessary for growth and reproduction. Indeed, it appears that paramecia from infected populations have constitutively lower feeding rates than paramecia from naive populations (C. Bénédet and O. Kaltz, unpubl. data). This would provide a simple mechanistic explanation for the trade-off between resistance and fitness in the absence of infection observed in our experiments. Costs of behavioral resistance against ectoparasitic mites have also been established in artificial selection experiments with *Drosophila* (Luong and Polak 2007). A functional link between food intake rate and resistance may also cause a trade-off in a *Drosophila*-parasitoid system (Kraaijeveld et al. 2001).

THE SHAPE OF THE RESISTANCE-FITNESS TRADE-OFF

The few existing studies on the evolutionary reversal of costly parasite resistance often follow changes in very few replicate populations and over relatively short time scales (Table 1A, but see Lenski 1988). Our experiment aimed at inferring a general picture of reverse evolution and of the shape of the resistance-fitness trade-off from a large number of independently evolving replicate populations assayed at a single point in time. One key finding of this multipopulation approach is a convex relationship between resistance and fitness, both at the level of the replicate population and population type (Fig 3), and also at the subclone level (not shown). This pattern suggests a weak trade-off function, with a flat part where it is possible to acquire a certain level of resistance, without compromising fitness. Strong fitness loss then occurs only for higher resistance. Although rarely measured, nonlinear trade-offs may not be uncommon and are probably a more realistic description than linear relationships between traits. Convex relationships have also been demonstrated in plants (Biere and Antonovics 1996; Mauricio and Rausher 1997), bacteria (Jessup and Bohannan 2008), and insects (Janmaat and Myers 2003).

The shape of resistance-fitness trade-offs likely depends on the functional relationship between the two traits or nonadditive effects of multiple genes/alleles involved in their expression (Boots and Haraguchi 1999; Brown 2003). Nonlinearity may also result from the existence of different resistance components that vary in their fitness costs (Rigby et al. 2002). Indeed, certain defense mechanisms may only be activated upon contact with the parasite and therefore be less costly in the absence of parasitism. For example, in our system, rather than constitutively reduce feeding rate, paramecia may evolve the capacity to filter selectively, thereby avoiding ingestion of infectious forms (C. Bénédet and O. Kaltz, unpubl. data). Hence, high levels of resistance may require the full, but costly defense arsenal, whereas intermediate resistance may be based on fewer and less costly components.

How different shapes of trade-offs influence the evolutionary maintenance of resistance polymorphism has been investigated by

Boots and Haraguchi (1999). Namely, a weak trade-off with accelerating costs at high levels of resistance favors a generalist resistance strategy in a host population, with some intermediate level of resistance and fitness, instead of polymorphic resistance types. Conversely, a strong trade-off with decelerating costs facilitates coexistence of specialist strategies, that is, very resistant and highly susceptible types (Bowers et al. 1994; Boots and Haraguchi 1999). Here, we were focusing on patterns "across" populations with different (co)evolutionary histories. This limits the extent we can link our study to theoretical predictions as model assumptions are not completely congruent with our study or system. For example, concerning the maintenance of polymorphism, it would be interesting to test for trade-offs among genotypes "within" populations in more detail as a more rigorous test of theoretical predictions.

TRAJECTORIES OF REVERSE EVOLUTION

We found that recovered populations were mostly located on the flat part of the trade-off, with resistance levels more spread out between susceptible and resistant (Fig. 3A). One interpretation of this finding is that, because the loss of the parasite, recovered populations have already regained high levels of fitness, but not (yet) entirely reverted to the level of susceptibility observed in naive populations. In addition, more recently recovered populations were more resistant than populations that have been parasite-free for a longer period ($\geq$3 years). This is coherent with the idea of populations moving along the trade-off toward higher fitness in absence of parasite and lower resistance. Similar patterns of increase in mean population fitness and decrease in mean resistance have been reported for experimental insect populations after the retraction of treatment with the bacterial biopesticide *B. thuringiensis* (Tabashnik et al. 1994).

Inferring evolutionary trajectories from a single snapshot in time has obvious limits, in particular if the precise evolutionary starting point of the recovered populations is unknown (see Material and Methods). Furthermore, different types of mutational events may occur. For example, in the absence of the parasite, deleterious mutations may accumulate in now unnecessary resistance genes and therefore populations continue to lose resistance even after regaining original fitness levels. It is also possible that reverse evolution involved compensatory mutations at loci unrelated to resistance that ameliorate fitness in the absence of parasitism, without compromising resistance. Thus, unlike in a simple antagonistic-pleiotropy scenario, resistance levels in recovered populations may not necessarily converge toward those in naive populations as an evolutionary endpoint. For example, compensatory mutations reduced fitness costs of resistance of *E. coli* against bacteriophage *T4*: After 400 generations of parallel evolution in the absence of phage, sensitive and resistant bacterial populations had reached different genetic states: competitive fitness of resistant populations had increased to levels comparable to sensitive populations, but resistance to phage was fully maintained (Lenski 1988).

Detailed analysis of underlying genes is required to distinguish between the relative contributions of antagonistic pleiotropy and compensatory mutation, a question beyond the scope of our experiment. More generally, however, experimental evolution approaches offer the unique possibility to address this issue more pragmatically, by tracking the trajectories of resistance and fitness in experimental populations in real time. As we have shown here, this allows to identify the shape of the trade-off; with repeated sampling, it would further be possible to precisely compare forward and reverse evolutionary dynamics among independent replicate populations or in different environments (Teotonio and Rose 2001). Ideally, this approach would be complemented by genetic analyses. Tracking the actual dynamics of reverse evolution and fitness compensation is also highly informative in the context of disease management, because the spread of pathogen mutants resistant against (bio)pesticides or antibiotics may critically depend on their capacity to regain competitive ability in the absence of treatment (Janmaat and Myers 2003; Schulz zur Wiesch et al. 2010).

Conclusions

This study adds to a very small body of literature dealing with reverse evolution in host–parasite systems. Our results show that host populations, once recovered from parasitic infection, can regain levels of fitness matching those of naive populations, without however completely losing resistance. This observation can be explained by a weak pleiotropic trade-off function between resistance and fitness, by compensatory mutations ameliorating costs of resistance, or a combination of the two. Regardless of the underlying mechanisms, our study demonstrates that (co)evolution with parasites can leave a signature in the genetic constitution of populations, even when the parasite has disappeared for many generations. This highlights the importance to consider the evolutionary past when analyzing the features of contemporary populations or genotypes.

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