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Experimental elimination of parasites in nature leads to the evolution of increased resistance in hosts

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A reduction in the strength of selection is expected to cause the evolution of reduced trait expression. Elimination of a parasite should thus cause the evolution of reduced resistance to that parasite. To test this prediction in nature, we studied the fourth- and eighth-generation descendants of guppies (*Poecilia reticulata*) introduced into four natural streams following experimental elimination of a common and deleterious parasite (*Gyrodactylus* spp.). After two generations of laboratory rearing to control for plasticity and maternal effects, we infected individual fish to assess their resistance to the parasite. Contrary to theoretical expectations, the introduced guppy populations had rapidly and repeatedly evolved increased resistance to the now-absent parasite. This evolution was not owing to a resistance-tolerance trade-off, nor to differences in productivity among the sites. Instead, a leading candidate hypothesis is that the rapid life-history evolution typical in such introductions pleiotropically increases parasite resistance. Our study adds a new dimension to the growing evidence for contemporary evolution in the wild, and also points to the need for a re-consideration of simple expectations from host–parasite theory. In particular, our results highlight the need for increased consideration of multiple sources of selection and pleiotropy when studying evolution in natural contexts.

1. Introduction

Natural selection is the driving force behind adaptation in the wild [1]. As such, environmental changes that alter the direction or strength of selection should immediately initiate adaptive evolution—and a number of studies have confirmed that such 'contemporary evolution' can indeed occur in very short time frames [2]. In a number of instances, environmental change can be so dramatic as to cause the emergence of a new selective factor or the removal of an existing selective factor. The first situation (emergence) would be expected to cause an evolutionary increase in the ability of affected populations to cope with the new challenge. For instance, several studies have documented evolutionary increases in the ability of formerly naive populations to cope with new contaminants [3], new prey [4], new competitors [5] or new parasites [6]. The second situation (removal) would be expected to cause an evolutionary decrease in the ability of populations to cope with the now-absent challenge—at least when that ability trades off with another fitness component or is sensitive to mutation accumulation [7]. Although the loss of a selective factor is less often studied than the gain of a selective factor, cases have been documented of evolutionary decreases in the ability of populations to cope with recently removed contaminants [8] or predators [9]. In this study, we provide a counter-example from a host–parasite system, where removal of a selective pressure caused a rapid evolutionary *increase* in the ability to cope

with a now-absent pressure. This finding invites a re-examination of the above tenets and points to the need for new theory and experiments.

Evolutionary models of resistance (the host's ability to reduce its parasite load) predict that increases in parasite-induced mortality should drive the evolution of increased resistance in the host population—because individual hosts that are better able to avoid, control or clear parasites gain higher lifetime reproductive success [10,11]. This expectation has been supported in laboratory studies on bacteria [12] and non-vertebrate organisms [13], as well as in comparative field studies [14]. On the flip-side, theoretical studies suggest that decreases in parasite-induced mortality and morbidity should drive the evolution of decreased resistance—because investing resources in resistance comes at the expense of investment in other fitness-related traits [10,11]. Supporting these ideas, resistance–fecundity trade-offs have been documented in many organisms [15]. The handful of studies that have directly tested for the evolution of resistance under relaxed selection, all laboratory-based, have found that removal of parasites led to no evolutionary change or the evolution of decreased resistance ([16,17] and references within). However, planned experimental evolution studies that reduce or remove parasite loads have not been performed in nature—and yet this is the context where inference is most critical because other environmental factors could modify evolutionary responses to changes in parasitism.

We used Trinidadian guppies to investigate the evolution of resistance to an ectoparasite (*Gyrodactylus* spp.) after that parasite had been eliminated in nature. *Gyrodactylus* spp. are directly transmitted parasites that reproduce and browse on the skin of guppies [18]. Furthermore, gyrodactylids have important fitness consequences for their guppy hosts—they cause high levels of mortality in both laboratory [19] and field [20], are the most prevalent macroparasite in the wild [21], affect mate choice [22] and cause lesions that can serve as entry points for secondary fungal and bacterial infections [23]. Although the mechanisms of resistance to *Gyrodactylus* are still not fully characterized [24], involvement of the immune system is inferred from experiments on salmonids where macrophages produce Interleukin 1β which stimulates mucus production and Complement factor C3 which binds to and kills the parasite [24]. Guppies show heritable [25] as well as a non-heritable [26] components of resistance, and those individuals that survive infection express acquired resistance upon challenge infection [27]. As *Gyrodactylus* are easily visible using a dissecting scope, repeated parasite counts can provide appropriate quantitative data throughout the course of an infection [24], and this is the most direct method to assess the host ability to control parasite numbers. *Gyrodactylus* load on individual guppies is known to vary both within and among populations, and this variation is attributed to variation in host resistance [19], particularly when infections occur in isolated hosts raised in common garden. In this sense, fish with fewer parasites or fish on which the parasites have a slower growth rate are taken to be more resistant.

Guppies from a naturally infected population in the Guanapo River ('source') were collected, treated to remove their gyrodactylids and introduced into four previously guppy-free tributary streams in the same river. After 1 and 2 years, fish were collected from the source and introduction populations, bred to a second generation under common
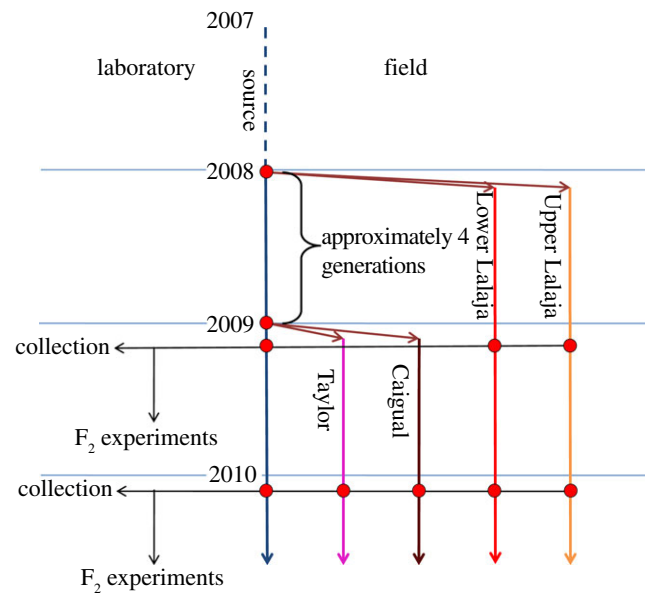

Figure 1. Experimental design overview. Guppy introductions were made from a source population in the Guanapo River in 2008 to the guppy-free Lower Lalaja and Upper Lalaja sites and in 2009 to the guppy-free Taylor and Caigual sites. In 2009 and 2010, guppies collected from each site (red dots) were bred for two generations in the laboratory and the F$_2$ fish were used in experiments. See Material and methods for details.

garden in the laboratory, infected with *Gyrodactylus turnbulli* and the exponential increase (and decline) of the parasite population on the skin of each isolated fish was monitored for 24 days to assess their resistance to the parasite. We predicted that removal of *Gyrodactylus* spp. in the field would lead to the evolution of decreased resistance to that parasite. Contrary to expectations from theory [10,11] and laboratory-based experimental evolution studies [16,17], the introduced guppy populations were found to have rapidly and repeatably evolved increased resistance to the now-absent parasite.

2. Material and methods

(a) Field introductions

The guppy introductions were carried out as part of a United States National Science Foundation Frontiers in Integrative Biological Research (FIBR) project led by D. Reznick. Guppies were captured as juveniles from the Guanapo 'source' population (10°38′23″ N, 61°14′54″ W and 10°39′14″ N, 61°15′18″ W) and held in a laboratory in Trinidad. They were quarantined in aquaria and treated with Fungus Eliminator (Jungle Laboratories, Cibolo, TX, USA), Clout (Sentry AQ Mardel, Omaha, NE, USA) and commercial forms of erythromycin and monocyclene (Maracyn and Maracyn Two–Sentry AQ Mardel). These treatments remove parasites, including *Gyrodactylus*, and all fish were monitored to ensure that they were in good health. Approximately 40 males and 40 females were then released into each 'introduction' site. In March 2008, fish were introduced into two Guanapo tributaries (Lower Lalaja and Upper Lalaja; figure 1). In April 2009, fish were introduced into two additional Guanapo tributaries (Taylor and Caigual; figure 1). Owing to physical barriers such as waterfalls, all introduction sites lacked dangerous predatory fishes (they were considered low predation) and did not have resident guppy populations. These sites also lacked *Gyrodactylus* parasites at the time of the introduction (owing to the absence of their guppy hosts) and throughout the course of this study—no parasites have been observed on

the fish captured from these sites (F. Dargent 2011, personal observation and D. Reznick 2012, personal communication). In addition, the introduction sites also differed from the source by having more closed canopies. All introduction sites are reasonably similar in the above mentioned properties, except that two introductions sites (Upper Lalaja and Caigual) have more open canopies, therefore higher productivity, owing to experimental canopy thinning (approx. 4% thinning) [28].

(b) Laboratory breeding

To assess the evolution of resistance to *Gyrodactylus* parasites following removal from parasitism, guppies were collected by the FIBR team from the Guanapo source population and from the introduction sites in April 2009 (the first two introduction sites—Lower and Upper Lalaja) and April 2010 (all four introduction sites; figure 1). The 2009 collection was thus of fish that had been evolving at the introduction sites without parasites for 1 year, which corresponds to approximately four guppy generations [29]. The 2010 collection was of fish that had been evolving at the introduction sites without parasites for 1 year (Taylor and Caigual—four guppy generations) and 2 years (Lower Lalaja and Upper Lalaja—eight guppy generations).

All collected fish were treated with Fungus Eliminator, Clout, Maracyn and Maracyn Two, transferred to Colorado State University (C. Ghalambor laboratory) and raised to maturity following standard protocols [30] (high food treatment only). To eliminate differences driven by phenotypic plasticity and maternal effects, the collected guppies were bred without parasites under common-garden conditions in the laboratory to generate F_2 guppies [31]. For the 2009 collections, offspring from field-collected mothers were used to create 15 Guanapo (source) families, 15 Lower Lalaja (introduction) families and 14 Upper Lalaja (introduction) families (see the electronic supplementary material, appendix S1). For the 2010 collections, offspring from field-collected mothers were used to create 14 Guanapo (source) families, 10 Lower Lalaja (introduction) families, five Upper Lalaja (introduction) families, 10 Caigual (introduction) families and 11 Taylor (introduction) families (see the electronic supplementary material, appendix S1). F_1 females were mated with multiple F_1 males from different families descended from the same collection to generate F_2 offspring. Fish from the 2009 collections were transferred to McGill University in the F_2 generation, whereas fish from the 2010 collections were transferred to McGill University in the F_1 generation and were there bred to form the F_2 generation.

(c) Laboratory infections

All infection trials were performed at McGill University in flow-through systems (Aquaneering Inc., San Diego, CA, USA) that standardized water quality and temperature (26°C). Filters prevented movement of parasites between tanks. Females rather than males were used for this study because: (i) females have a broader range of peak *Gyrodactylus* loads [19] that allows better detection of statistical differences, (ii) inferences about the impact of parasites on female fitness are more straightforward (male guppies can continue to sire offspring after their death—owing to sperm storage [32]), and (iii) the consequences of females for population dynamics are direct (population growth is limited by female—not male—reproductive output). For the 2009 collections, F_2 fish were infected at 12 weeks of age or older and all females derived from each population were from different family lines. For the 2010 collections, F_2 fish were infected at 12 weeks of age and up to three females were used per family line (see the electronic supplementary material, appendix S1). These small differences in protocol between the 2009 and 2010 collections were the result of the different transfers (F_2 fish versus F_1 fish) from Colorado State University. Therefore,

our inferences are based on comparisons between populations for a given collection year rather than between years.

Each fish was isolated in a 1.8 l tank one week prior to being infected and was fed 10 µl d^{-1} of fish food paste (Tetramin Tropical Flakes) until the end of the experiment. On the day of infection, each fish ($n = 173$) was anaesthetized in MS-222 (buffered to a neutral pH with NaHCO$_3$), weighed twice to the nearest 0.0001 g in a container with 20 ml of water, photographed (for size measurements) and manually infected. To initiate an infection, we removed a scale with *G. turnbulli* from a donor fish and allowed the parasite to transfer to the recipient host's caudal fin [18] until two parasites were attached. We used *G. turnbulli* from an isogenic strain initially isolated from guppies obtained from a Montréal pet store in 2008. The use of this strain, and the fact that we maintained it on feeder guppies (descendants of pet store guppies held in our laboratory), ensured that all fish were infected with the same strain of parasites and that none of the guppy populations had an evolved history with the strain. Although testing with additional parasite strains would be interesting and potentially informative, we did not do so because previous work suggests that population differences in resistance are maintained across diverse parasite strains [19]. Every 2 days for 24 days, or until death, each fish was anaesthetized in MS222 and its parasites were counted using a dissecting scope at 18× magnification, a standard procedure in guppy–*Gyrodactylus* studies. We also performed control trials with sham-infected fish ($n = 66$) that provided baseline values for parasite-induced mortality (see the electronic supplementary material, appendix S1) and for initial size–weight regressions.

(d) Resistance and tolerance

As a measure of guppy size, we used standard length (SL)—the distance from the mouth of the fish to the end of the caudal peduncle. SL was measured to the nearest 0.1 mm from photographs taken with a Nikon D90 camera at the start of infection. On day 10 of infection, we twice weighed all fish to the nearest 0.0001 g inside a container with water to avoid desiccation of the parasites, but we did not photograph the fish for size measurements, as this would have involved taking the fish out of the water. Individual SL and average mass were used to calculate relative condition index (K_n) at the start and on day 10 of infection, following Le Cren [33]. K_n estimates condition based on a log-mass to log-SL least-squares regression of all fish (infected and sham-infected), where residuals indicate the amount by which an individual is above (greater than 1) or below (less than 1) average condition. Therefore, K_n calculations for day 10 are based on the log-SL of the fish at day 0, under the assumption that change in SL would be minor during this period. In addition, no differences in guppy relative condition index [33] were evident among the populations immediately before infection: ANOVA 2009 ($F_{2,41} = 0.137$, $p = 0.872$) and ANOVA 2010 ($F_{4,124} = 1.92$, $p = 0.111$).

We derived three metrics of resistance for each infected individual from the parasite data collected on alternating days: peak load, load on day 10 and *Gyrodactylus* intrinsic rate of increase (r). These measures are commonly used in studies involving *Gyrodactylus* [27,34] and provide a summary of the infection dynamics and the host ability to fight infection. Peak load was calculated as the highest number of parasites an individual carried at a given day throughout infection or before they died, but since the day at which peak load is reached varied from one individual to the other, we included a measure of parasite load at the same point in time for all individuals. Load on day 10 is the number of parasites each individual carried 10 days after being infected with two *G. turnbulli* flukes. Day 10 was chosen as the reference day because fish mortality started to increase after this point (see the electronic supplementary material, appendix S1). We estimated

Table 1. Guppy survival as a measure of tolerance. (Cox proportional hazards results for survival until end of experiment with 'day of death' as a response variable, and 'population of origin' and 'parasite peak load' as explanatory variables. Values are for individuals of a given introduction site relative to the source population. Higher tolerance is indicated by higher survival for a given parasite load at peak (i.e. significant interaction coefficient).)

collection	coefficient	estimate	s.e.m.	Z-value	$p\,(>|z|)$
2009, 1 year after introduction	Lower Lalaja	−1.31	1.38	−0.95	0.34
	Upper Lalaja	−1.19	1.63	−0.73	0.47
	load at peak	0.01	0.003	2.56	0.01
	peak × Lower	0.002	0.003	0.5	0.62
	peak × Upper	0.01	0.01	1.08	0.28
2010, 2 years after introduction	Lower Lalaja	−1.32	0.59	−2.24	0.03
	Upper Lalaja	−0.4	0.65	−0.61	0.54
	load at peak	−0.001	0.001	−0.92	0.36
	peak × Lower	0.003	0.003	1.23	0.22
	peak × Upper	<0.001	0.004	0.12	0.91
2010, 1 year after introduction	Caigual	−1.02	0.51	−1.99	0.046
	Taylor	−0.57	0.57	−1.01	0.31
	load at peak	<−0.001	0.001	−0.79	0.43
	peak × Caigual	0.002	0.002	1.11	0.27
	peak × Taylor	0.001	0.003	0.42	0.68

r as the slope of the regression of ln-transformed *G. turnbulli* load versus time, measured every other day from the start of infection to day 10. As a measure, r integrates over time the demographic response of the parasite population to its host-environment, where higher r represents lower host resistance.

To measure tolerance (the host's ability to reduce the damage caused by a given load of parasites), we used two metrics that represent how populations differ in the relationship between *Gyrodactylus* numbers and the effect they have on guppy fitness. We compared whether (i) the effect of peak parasite load on guppy survival throughout the experiment and (ii) the effect of the cumulative *Gyrodactylus* load for the first 10 days of infection on the change in relative condition index of guppies on those 10 days, was different between populations (statistical interaction between (i) population and peak load, and (ii) population and cumulative load). As guppy deaths often happened at times when there was no personnel at the laboratory, deaths that occurred between alternating count days were recorded as the day of last parasite count.

(e) Analysis

Although guppy size differed between the source and introduced populations at the start of experiment in the 2009 sample (ANOVA: $F_{2,41} = 3.873$, $p = 0.0288$), these differences were minor and no differences were evident in the 2010 sample (ANOVA: $F_{4,124} = 1.996$, $p = 0.0992$; see the electronic supplementary material, appendix S2). For these reasons, and to maximize degrees of freedom, body size is not included in the statistical models we report here, although redoing the analysis with SL as a covariate (not shown) did not alter our interpretation.

To test whether the population from which the fish had been collected had an effect on day of peak parasite load and parasite load on day 10 of infection, we used generalized linear models (GLMs) with negative binomial distributions and a log link function as data were over-dispersed and did not fit assumptions of normality (Shapiro–Wilk normality test). Similar results (not shown) to those of load on day 10 were obtained when we analysed load on day 8 and on day 12. Given our interest in whether

or not each introduction population differed from the source population, planned contrasts were performed separately for each source-introduction pair in each collection. Alpha levels for multiple comparisons for each response variable were evaluated using false discovery rate corrections. We used ANOVAs to test whether parasite intrinsic rate of increase (r) differed among populations; Tukey HSDs were used for post hoc tests. Finally, we used a Cox proportional hazard model to determine whether the effect of peak load on fish survival was affected by population, and an ANCOVA to test whether the effect of cumulative load on change in K_n was affected by population. All analyses were performed using R Language and Environment for Statistical Computing (http://www.r-project.org/). Level of significance was set at $p < 0.05$.

3. Results

Gyrodactylus turnbulli successfully established and increased in abundance on all laboratory fish exposed to the parasite, with the exception of two Guanapo source fish from the 2009 collection and one Lower Lalaja fish from the 2010 collection (not included in analyses). Subsequent *Gyrodactylus*-induced mortality was high: 70% of all infected fish died before the end of the experiment whereas sham-infected control fish experienced only 3% mortality. For the 2009 field collections, guppy survival was not different between the source population and the introduced populations (table 1). For the 2010 field collections, guppy survival was significantly higher for two of the four introduced populations (table 1, Lower Lalaja and Caigual) than for the source population. Across individuals, guppy survival was negatively associated with parasite load in 2009 but not 2010 (table 1).

For all of the guppy populations, *G. turnbulli* infection showed a typical phase of rapid increase that decelerated until reaching a peak and finally crashed (figure 2a,c,e). Starting at day 8 of infection, average parasite load began to differ
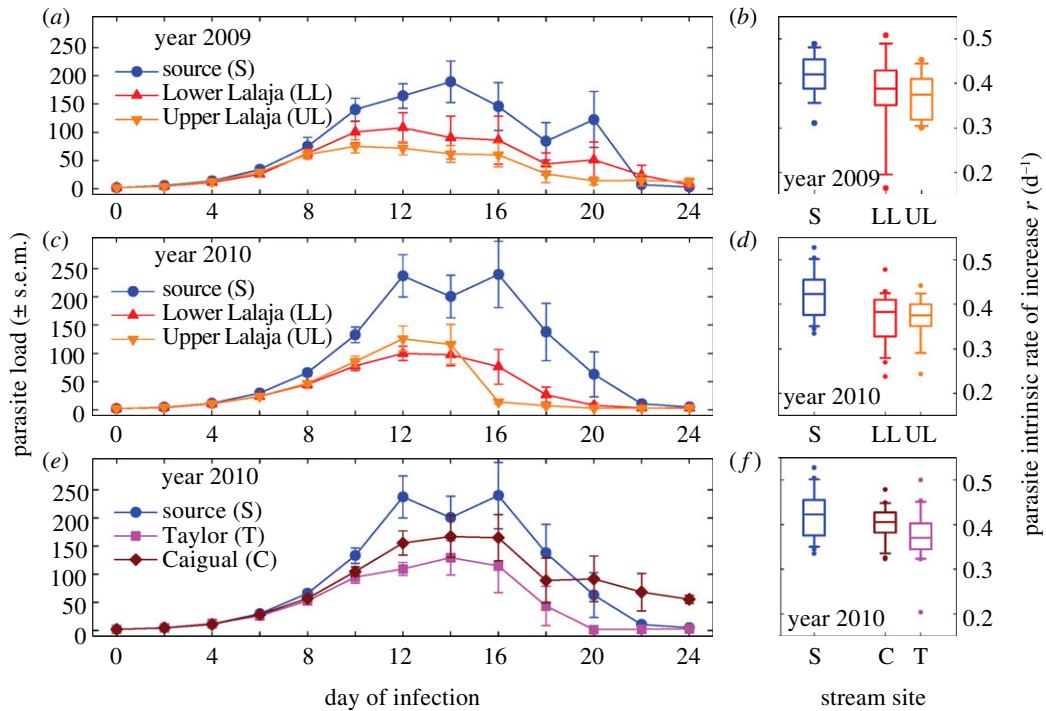
Figure 2. Resistance of female guppies (*P. reticulata*) to parasites (*G. turnbulli*). Resistance measures based on parasite load on each experimental day (*a,c,e*) and regression-based estimates of parasite intrinsic rate of increase (*r*) covering experimental days 0 through to 10 (*b,d,f*). High parasite load is indicative of low resistance. The data (means ± s.e.m.—*a,c,e*; median and 10, 25, 75, 90 percentiles—*b,d,f*) are for (*a,b*) Guanapo source, Lower Lalaja and Upper Lalaja fish collected in 2009, 1 year after introduction; (*c,d*) Guanapo source, Lower Lalaja and Upper Lalaja fish collected in 2010, 2 years after introduction; and (*e,f*) Guanapo, Caigual and Taylor fish collected in 2010, 1 year after introduction.

notably among the populations. We used three common and robust metrics to quantify this variation in guppy resistance to *G. turnbulli*: peak load, load on day 10 and the parasite intrinsic rate of increase (*r*). Relative to the source population, both peak load and load on day 10 were significantly lower (and resistance therefore higher) for (i) two of the four introduced populations (Upper Lalaja and Taylor) studied after 1 year of evolution in nature, and (ii) both introduced populations (Upper Lalaja and Lower Lalaja) studied after 2 years of evolution in nature (table 2 and figure 2). In the first case, another of the introduced populations (Caigual) also showed evidence of increased resistance based on load on day 10. Relative to the source population, *r* was significantly lower (and resistance therefore higher) for (i) one of the four introduced populations (Taylor) after 1 year of evolution in nature (figure 2*f* and table 3), and (ii) both of the introduced populations after 2 years of evolution in nature (figure 2*d* and table 3). In summary, none of the guppy populations released from parasite (*Gyrodactylus*) pressure evolved decreased resistance to that parasite: instead, most evidence pointed toward a rapid evolution of *increased* resistance.

The evolution of increased resistance in the absence of a parasite might reflect a correlated response to the evolution of decreased tolerance (the host's ability to reduce the damage caused by a given number of parasites). We measured tolerance as a population's slope of survival in relation to peak parasite load (higher survival for a given parasite load would mean increased tolerance) and as change in relative condition index in relation to cumulative parasite load in the first 10 days of infection (higher condition for a given parasite load would mean increased tolerance). Using these metrics, no evidence was found of the evolution of decreased tolerance: relative to guppies from the source population, guppies from the

introduced populations did not have lower survival rates for a given parasite load (table 1) or lower condition (see the electronic supplementary material, appendix S3). If anything, the trend was toward increased tolerance in the introduced populations (figure 3*a*). Furthermore, we found no evidence of a negative association between measures of resistance and measures of tolerance (figure 3*a,b*)—as would have been expected if the evolution of decreased tolerance caused a correlated response toward increased resistance.

4. Discussion

We found that guppies rapidly and repeatedly evolved increased resistance to a common [21] and deleterious parasite [19,20] (*Gyrodactylus* spp.) after that parasite's experimental removal in nature. This robust result runs counter to theoretical expectations [10,11] and to a number of laboratory-based experimental evolution studies [16,17]. It might at first be tempting to think that our results could be explained quite simply if resistance was not costly. If this was the case, however, we would expect no evolution of resistance when the parasite was removed—rather than an increase in resistance. We therefore here ask what mechanisms could explain why the removal of a parasite from populations in nature led to the evolution of increased rather than decreased resistance. Consideration of these mechanisms leads to new insights into how host–parasite relationships evolve and how these interactions can best be uncovered.

We start by considering possible methodological artefacts. First, perhaps decreased resistance did evolve in the introduced populations, which led to increased mortality of the least resistant fish when exposed to parasites in the

Table 2. GLMs for *G. turnbulli* load on *P. reticulata*. GLM results for parasite load at the 10th day of experimental infection and for peak load (integer variables with a negative binomial distribution) of *G. turnbulli* on *P. reticulata* for each year of collection. (Coefficient estimates are for individuals derived from each introduction site relative to the individuals derived from the source population.)

collection	response variable	coefficients	estimate	s.e.m.	Z-value	p (>|z|)
2009, 1 year after introduction	load day 10	intercept (source)	4.94	0.15	32.3	<0.001
		Lower Lalaja	−0.33	0.22	−1.5	0.13
		Upper Lalaja	−0.62	0.22	−2.8	0.005
	peak load	intercept (source)	5.52	0.16	34.7	<0.001
		Lower Lalaja	−0.36	0.23	−1.6	0.106
		Upper Lalaja	−0.81	0.23	−3.5	<0.001
2010, 2 years after introduction	load day 10	intercept (source)	4.91	0.09	53.4	<0.001
		Lower Lalaja	−0.55	0.14	−3.9	<0.001
		Upper Lalaja	−0.46	0.15	−3	0.003
	peak load	intercept (source)	5.51	0.11	49.5	<0.001
		Lower Lalaja	−0.66	0.17	−3.8	<0.001
		Upper Lalaja	−0.75	0.19	−3.9	<0.001
2010, 1 year after introduction	load day 10	intercept (source)	4.91	0.09	55.8	<0.001
		Caigual	−0.26	0.13	−2	0.047
		Taylor	−0.36	0.14	−2.6	0.009
	peak load	intercept (source)	5.51	0.12	46.6	<0.001
		Caigual	−0.18	0.17	−1.04	0.3
		Taylor	−0.48	0.18	−2.58	0.01

Table 3. ANOVAs on *G. turnbulli* intrinsic rate of increase (*r*). ANOVA results for parasite intrinsic rate of increase (*r*) between experimental day 0 and 10 with population (source and introductions) as factor. (Abbreviations for population names: Guanapo source (S) population, and Lower Lalaja (LL), Upper Lalaja (UL), Caigual (C) and Taylor (T) introduction populations.)

collection	d.f.	SS	mean square	F	p-value	difference[d]
2009—1 year[a]	2	0.01451	0.007256	1.798	0.179	–
2010—2 years[b]	2	0.04885	0.024427	8.984	<0.001	LL and UL < S
2010—1 year[c]	2	0.02656	0.013281	5.171	0.008	T < S

[a]S, LL and UL populations derived from field in 2009.
[b]S, LL and UL populations derived from field in 2010.
[c]S, T and C populations derived from field in 2010.
[d]Differences between populations in significant models were detected through a Tukey HSD post hoc test.

laboratory, and as a consequence high parasite loads could not build up, which made it appear as if resistance was high. In the laboratory experiments, however, survival was not lower for the introduced populations relative to the source population (table 1 and electronic supplementary material, appendix S1), and so lower parasite loads in the former cannot be explained by increased mortality. Second, perhaps fish from the introduced populations were smaller or in worse condition [33] than those from the source population, which could constrain the spread of parasites on the former. At the start of our laboratory experiments, however, guppies from the introduced populations were not smaller or in lower condition than those from the source population (see Material and methods). Exclusion of these potential methodological artefacts led us to consider biological mechanisms for why parasite removal in nature leads to the evolution of increased resistance to that parasite.

One possible biological mechanism is that parasite removal selects more strongly for decreased tolerance (the host's ability to reduce the damage caused by a given number of parasites [35]) than it does for decreased resistance, with the resulting evolution of reduced tolerance then driving the evolution of increased resistance as a pleiotropic or functional by-product. This idea seems reasonable on the surface given that evolutionary models [10,11] and empirical evidence [35] point to a trade-off between resistance and tolerance. However, our analyses yielded no evidence of the evolution of decreased tolerance: relative to the source population, guppies from the introduced populations did not have higher mortality or lower condition for a given parasite load. Furthermore, we did not detect a negative relationship between resistance and tolerance, suggesting the absence of a trade-off, at least at the phenotypic level, that would lead to pleiotropic effects. Our finding of the evolution of increased
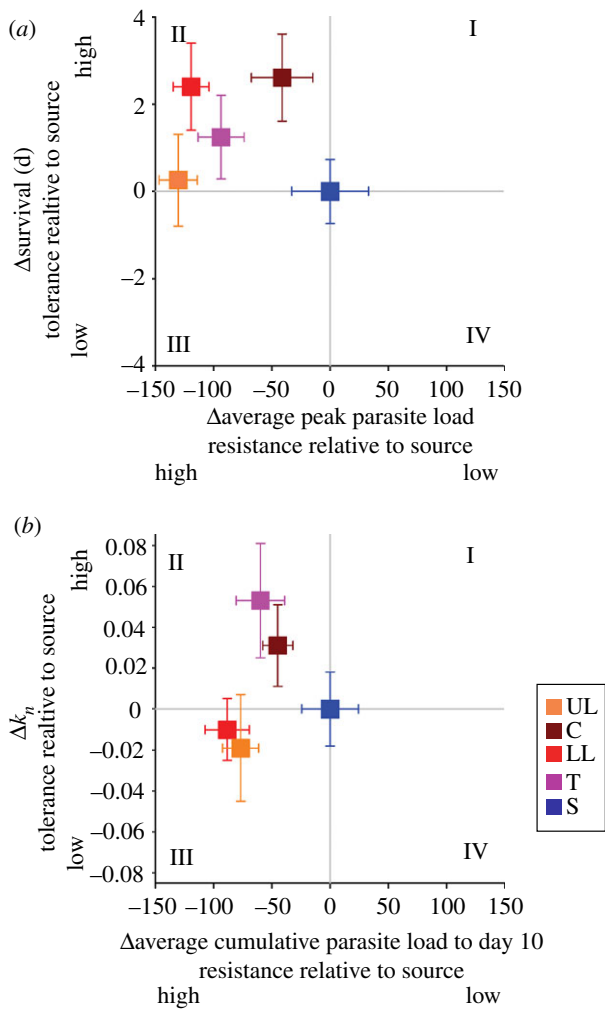
Figure 3. Evolution of guppies' resistance and tolerance to their *Gyrodactylus* parasites. (*a*) Difference in average *G. turnbulli* peak load (a measure of resistance) versus average difference in survival (day of death—a measure of tolerance), and (*b*) average cumulative parasite load from day 0 to 10 (resistance) versus change in relative condition index (K_n) between day 0 and 10 of infection (tolerance). All differences (Δparasite load, Δsurvival and ΔK_n) are relative differences between the Lower Lalaja (LL), Upper Lalaja (UL), Taylor (T) and Caigual (C) introductions, and the Guanapo source population (S), from the 2010 collection. Non-zero values suggest evolution in a given direction. Quadrants I and III indicate a negative correlation (trade-off) between resistance and tolerance. Error bars are ± 1 s.e.m.

resistance in the introduced parasite-free populations is therefore not due to the evolution of decreased tolerance.

A second possible biological mechanism is that a difference in food resources influenced selection on resistance. In particular, the introduction sites had less open forest canopies—and therefore lower resource levels—than did the source site, and previous research on guppies has suggested that resource levels contribute to the evolution of several guppy traits [36]. Moreover, a number of studies have shown that resource levels can influence the evolution of parasite resistance [37] and can directly influence guppy resistance [26]. In our study, however, two lines of evidence suggest that different resource levels cannot explain the evolution of increased resistance in the introduced populations. First, one would expect lower resource levels to select for decreased resistance [37], not the increased resistance that we observed, because selection on more efficient use of resources should be stronger. Second, the introduction sites differed from each other in canopy openness owing to

experimental canopy thinning at two of the sites (Upper Lalaja and Caigual) [28,38], but we did not find consistent differences in resistance between the sites with and without thinning (see the electronic supplementary material, appendix S4).

A third possible biological explanation relates to potential interactions between predation and parasitism. This hypothesis deserves special consideration because: (i) interactions between selection by predators and selection by parasites have been reported in other systems [39,40], (ii) the introduced populations were not only removed from parasites but also underwent a dramatic shift in predation intensity (from high to low), (iii) guppies show many adaptive responses to different predation regimes [41], that can evolve rapidly in experimental introductions [31], and (iv) guppies from the introduced populations show rapid evolution of certain life-history traits [38]. In particular, when guppies from high-predation environments are introduced into low-predation environments, the resulting increase in life expectancy causes the rapid evolution of life-history traits that convert a formerly 'fast' lifestyle into a 'slow' lifestyle [31]. Increased life expectancy should also provide benefits for increased investment into parasite defence, as has been reported in a number of studies [42,43]. Although this logic is normally applied to situations where a source of extrinsic mortality changes but the parasite remains present, we suggest that it can be modified to consider the situation when parasites are removed. This expanded argument starts from the common observation that reproductive effort and parasite defence are negatively correlated [44], such that the evolution of one should cause a correlated response in the other [45]. In this situation, the evolution of decreased allocation to reproduction that occurs when guppies are introduced from a high-predation to a low-predation environment [31] could cause increased investment into parasite defence. Stated another way, relaxed selection for defence against parasites (owing to parasite removal) can be overpowered by the evolution of a slower life history (owing to predator removal) that, through pleiotropic or functional associations, leads to increased resistance. We advance this hypothesis as the most plausible explanation for our observations as it is the only one standing after a critical confrontation with our own data. To positively establish a causal relationship between life history and resistance evolution in our study system, further experimentation will be required.

As surprising as our results might initially seem, we can find additional support for them in several observations from previous studies. First, many low-predation guppy populations are not parasitized by *Gyrodactylus* or, if they are parasitized, have low parasite loads [46]. Second, guppies from *Gyrodactylus*-free sites often have high frequencies of alleles [47] that are associated with elevated *Gyrodactylus* resistance in laboratory trials (as measured by load over the first 10 days of infection) [25]. Third, high-predation populations, which tend to show higher parasite loads, do not show greater resistance to *Gyrodactylus* when tested in mesocosms [48]. In aggregate, these observations suggest that low-predation populations, and populations with few or no parasites, can still mount effective parasite defence and can perhaps even resist colonization by parasites. In summary, our study provides clear experimental evidence for a pattern of evolution unpredicted by theoretical models and laboratory studies that is nevertheless consistent with observations from

natural populations and for which a plausible biological mechanism can be advanced.

5. Implications

Current empirical understanding of the evolution of defence against parasites is based mainly on two approaches. First, experimental evolution studies in the laboratory manipulate parasite presence while intentionally controlling or removing variation in other environmental factors. Evolution in nature, however, occurs in the context of these other factors, which might strongly modify evolutionary responses to parasitism [49]. Second, field surveys intentionally incorporate this natural complexity but are rarely able to unambiguously disentangle cause and effect [31]. The bridge between these two approaches is to experimentally manipulate infection levels in nature, which thus informs how changes in parasitism influence defence within the context of natural variation in other environmental factors. Using this bridge, we documented evolutionary patterns inconsistent with theoretical models and laboratory experiments: that is, the removal of an important parasite led to the rapid evolution of increased resistance to that same parasite. This result suggests that existing theory and laboratory experiments might need to be modified—and we propose that a good starting point is to include a consideration of selection acting on life-history traits that are correlated with resistance.

Our results could have broad implications. As one example, humans have created many situations in which selection by parasites has been reduced through use of antibiotics, antivirals, pesticides and herbicides, as well as through improved hygiene. As another example, many cultured organisms and endangered species are intentionally raised in the absence of a number of their natural pathogens. Finally, invasive species are often released from a number of their natural enemies [50]. In the rare instances when consideration has been given to how resistance to pathogens might evolve following such changes, expectations have followed the classical interpretation that resistance should decrease. Our results challenge this simple and standard interpretation and could therefore have fundamental implications for disease control, conservation and invasion biology. More replicated experimental manipulations of parasite pressure in nature are urgently needed.

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