THE WILD SIDE: ASSESSING EVOLUTIONARY ECOLOGY OF DEFENCE AGAINST PARASITES IN NATURE

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DEDICATION

To Gabriela,

my love

And to Julius,

with the hope that you too find greatness and awe in all living things.

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ABSTRACT

Interactions with enemies are an important source of extrinsic mortality in nature. As such, traits that alleviate the detrimental effects of enemies are expected to be under strong selection and to evolve rapidly following shifts in the level of enemy-induced mortality. The evolutionary effects of increasing enemy pressure on defensive traits against those enemies are straight forward and well supported both theoretically and empirically – increased enemy pressure should lead to increased investment in defences and promote the evolution of defences. The consequences of relaxing enemy pressure, on the flip-side, are more complex to predict given that the evolution of defensive traits are now more strongly influenced by their trade-offs with other fitness enhancing traits. In this dissertation I assessed, in nature, the eco-evolutionary implications of enemy release on defence against parasites and on host speciation. Through laboratory and field assays I used a common and deleterious parasite (*Gyrodactylus* spp.) and its poeciliid host (*Poecilia reticulata* - guppies) as a model system.

First, I assessed whether removal of this parasite in replicate introductions in the wild led to the rapid evolutionary loss of resistance to *Gyrodactylus*, thus conforming to theoretical and laboratory-informed expectations, and whether this evolution occurred in parallel between the sexes. After two generations of laboratory rearing to control for plasticity and maternal effects I found that, contrary to theoretical expectations, female guppies that had evolved for four and eight generations released from *Gyrodactylus* had rapidly and repeatably evolved increased resistance to the parasite. After consideration of alternative explanations I concluded that this evolution is likely caused by rapid life-history evolution in response to release from predators. In addition, I found non-parallel evolution of the sexes. Male guppies in the ancestral population had higher resistance to *Gyrodactylus* than females, and parasite release in the introduced

populations did not lead to male evolution of resistance, which –in turn - led to the evolutionary reduction of sexual dimorphism. I argue that the non-parallel evolution of resistance is caused by previous selection for high resistance in males that constrains further evolution of the trait.

Given that the results indicate that simultaneous relaxation in predation pressure may have accounted for the findings, I then evaluated in a multidimensional selection context (i.e., changes in parasitism, predation, diet and productivity) whether evolution under enemy release, led to the formation of reproductive barriers (ecological speciation) through the evolution of mate choice and signaling traits. Despite theoretical expectations that multidimensional divergent selection would promote ecological speciation and observed rapid evolution of male signaling traits, enemy release in this system does not promote the evolution of assortative mating. I conclude that other aspects of sexual selection – conserved preference for novel males – counteracts the influence of strong multifarious divergent selection.

Finally, I tested whether inter-specific social interactions (mixed-species group formation) can provide a degree of enemy release against contagious parasites, a rarely considered but potential advantage of mixed-species group formation. I found that the presence and abundance of *Gyrodactylus* is lower in poeciliids forming mixed-species groups than those forming single species groups, which is consistent with the hypothesis that mixed-species groups provide a level of protection against contagious parasites.

These studies add a new dimension to the growing evidence of contemporary evolution in the wild and point to the need for the re-consideration of simple expectations from host-parasite theory, and more broadly relaxed selection. In particular, the results highlight the need for increased consideration of multiple sources of selection and pleiotropy when studying evolution in natural contexts.

RÉSUMÉ

Les interactions avec les ennemis sont une source importante de mortalité dans les milieux naturels. De ce fait, on doit s'attendre à ce que les traits qui réduisent les effets néfastes des ennemis soient soumis à une forte sélection et qu'ils évoluent rapidement suivant des changements au niveau de la mortalité induite par l'ennemi. Les effets évolutifs de l'accroissement de la pression due aux ennemis sur les traits défensifs contre ces ennemis sont évidents et bien soutenus à la fois théoriquement et empiriquement – l'accroissement de la pression due à l'ennemi doit conduire à un accroissement de l'investissement dans les défenses et promouvoir l'évolution des défenses. Les conséquences du relâchement de la pression due aux ennemis, en revanche, sont plus complexes à prévoir étant donné que l'évolution des traits défensifs est à présent plus fortement influencée par leurs "trade-offs" avec d'autres traits augmentant la fitness. Dans cette dissertation, j'évalue, en milieu naturel, les implications eco-évolutives du relâchement de la pression de l'ennemi sur la défense contre les parasites et sur la spéciation. Au travers d'expériences en laboratoire et sur le terrain j'utilise un parasite commun et délétère (*Gyrodactylus* spp.) et son hôte poecilidé (*Poecilia reticulata* – guppies) comme un système modèle.

Tout d'abord, je teste si le retrait de ce parasite dans des introductions répliquées en milieu naturel entraîne une rapide perte évolutive de la résistance à *Gyrodactylus*, en conformité avec les prévisions théoriques et celles issues de tests en laboratoires, et si cette évolution a lieu en parallèle entre les sexes. Après deux générations de surveillance en laboratoire pour contrôler pour la plasticité et les effets maternels, j'ai trouvé que, contrairement aux prédictions théoriques, les femelles guppies qui avaient évolué depuis quatre et huit générations en l'absence

de *Gyrodactylus* avaient rapidement et de manière répétée évolué vers un accroissement de la résistance envers le parasite. Après avoir considéré des explications alternatives, j'en ai conclu que cette évolution est probablement due à une évolution rapide d'histoire de vie en réponse à la diminution de pression par les prédateurs. De plus, j'ai trouvé une évolution non-parallèle des sexes. Les mâles guppies de la population ancestrale avaient une résistance plus forte au *Gyrodactylus* que les femelles, et le relâchement de la pression parasitaire dans les populations introduites n'a pas conduit à l'évolution de la résistance des mâles, ce qui –en retour – conduit à une réduction évolutive du dimorphisme sexuel. Je soutiens que l'évolution non-parallèle de la résistance est causée par la sélection passée pour une forte résistance chez les mâles qui contraint l'évolution future de ce trait.

Etant donné que les résultats indiquent que le relâchement simultané de la pression de prédation pourrait expliquer les résultats, j'ai testé ensuite, dans un contexte de sélection multidimensionnelle (i.e., changements en termes de parasitisme, prédation, alimentation et productivité), si l'évolution en conditions de relâchement de la pression due à l'ennemi, conduit à la formation de barrières reproductives (spéciation écologique) au travers de l'évolution du choix du partenaire et des traits de signalisation. Malgré les prédictions théoriques que la sélection divergente multidimensionnelle devrait promouvoir la spéciation écologique et l'évolution rapide observée des traits de signalement des mâles, la diminution de pression due à l'ennemi dans ce système ne promeut pas l'évolution de l'accouplement entre semblables. Je conclus que d'autres aspects de la sélection sexuelle –préférence conservée pour les nouveaux mâles- contrebalance l'influence de la sélection divergente forte et très diverse.

Enfin, je teste si les interactions sociales inter-spécifiques (formation de groupes mixtes d'espèces) peut fournir un certain degré de relâchement de la pression due à l'ennemi contre les

parasites dangereux, un avantage potentiel mais rarement considéré de la formation des groupes mixtes d'espèces. J'ai trouvé que la présence et l'abondance des *Gyrodactylus* est plus faible dans les poecilidés formant des groupes mixtes d'espèces que dans ceux formant des groupes d'une seule espèce, ce qui est en accord avec l'hypothèse selon laquelle les groupes mixtes d'espèces fournissent un niveau de protection contre les parasites contagieux.

Ces études ajoutent une nouvelle dimension aux preuves grandissantes d'une évolution contemporaine en milieu naturel et établit la nécessité de reconsidérer les prédictions simples de la théorie hôte-parasite, et plus largement de la sélection en contexte de relâchement de contraintes. En particulier, les résultats de ces expériences mettent en lumière la nécessité de considérer davantage les multiples sources de sélection et la pléiotropie quand on étudie l'évolution en contexte naturel.

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CONTRIBUTION OF AUTHORS

The research contained in this thesis is predominantly my own. I primarily developed the research questions; designed, implemented and conducted the experiments; analyzed and interpreted the data; and wrote the documents contained in this thesis. My co-authors were involved in all or some steps of the research and manuscript writing.

In Chapter 3, Gregor Rolshausen was instrumental in implementing the phenotypic change vector analysis (PCVA). In Chapter 4, Lisa Chen, shouldered most of the experimental data collection. In Chapter 5, Julian Torres-Dowdall had a crucial role by introducing me to a scarcely studied system, exploration of field sites, collection of data, development of the research questions and manuscript editing. Indar Ramnarine provided resources and guidance. Gregor Fussmann, Marilyn Scott and Andrew Hendry participated in all stages of most chapters. My co-authors have given me their permission to include the chapters associated with their names as part of my thesis.

STATEMENT OF ORIGINALITY

The manuscripts contained as chapters in this thesis (Chapters 2-5) are novel contributions to scientific knowledge that advance our understanding of the evolution and ecology of host-parasite interactions. Chapter 2 expands the understanding of the evolution of defence against parasites, specifically under relaxed selection, by showing that a common theoretical and empirical expectation – that release from selection by parasites should lead to the evolutionary loss of resistance – does not hold in nature, under multifarious selection. Chapter 3 broadens this knowledge by showing that, when subject to a similar environmental divergence (removal of parasites), the sexes do not evolve in parallel, and as a result sexual dimorphism in resistance – a common occurrence in nature – decreases and in some cases disappears. In Chapter 4 we extend the understanding of speciation by showing that, although parasitism is expected to be a driver of ecological speciation, strong divergence in parasitism under multifarious selection does not lead to assortative mating in the guppy system. Furthermore, through preference for novel males, Chapter 4 reconciles the common observation that guppies show strong and rapid divergent adaptive evolution and yet often show no signs of assortative mating. Chapter 5 empirically explores a novel mechanism for mixed-species group formation and shows that when two different fish species form interspecific groups their parasite loads are lower than when they form single-species groups.

Chapter 2 was published in Proceedings of the Royal Society B, Chapter 5 was published in PLOS One, Chapter 3 is under review at the Journal of Evolutionary Biology, and Chapter 4 will soon be submitted to Animal Behaviour.

CHAPTER 1

Introduction

The effects of ecology and evolution on enemy-victim interactions in nature are the focus of my dissertation. I explore enemy-victim interactions in the context of enemy release, a form of relaxed selection, where the strength of a previously important source of selection is decreased or eliminated. Throughout my thesis I first assess the implications of enemy release, in nature, on the evolution of defence against enemies and how this process differs between the sexes. I then expand the focus to a broader scale and ask whether enemy release and multifarious selection can lead to rapid speciation through the evolution of assortative mating. Finally I assess whether interspecific social interactions can provide a level of enemy release when victims cannot fully evade but have to co-exist with their enemies.

1.1 Relaxed selection and enemy release:

Relaxed selection

Natural selection is the main cause of adaptive evolution in the wild (1, 2) and commonly occurs when populations are exposed to changes in their biotic or abiotic environment. Environmental change can introduce a number of alterations that can modify the selective regime experienced by a population and lead to rapid initiation of adaptive evolution (3, 4). These changes can be broadly categorised along a continuum from increased to decreased action of a source of selection. In the first case, there is an increase in the presence of a previously existent source of selection or, in an extreme case, the appearance of a novel source of selection (increased selection or emergence); the second case is known as relaxed selection (5, 6), a decrease in the presence of a source of selection where its most extreme expression would be the complete loss of that previously important source (5). Although increased selection and emergence are more often studied than relaxed selection, both in nature and in the laboratory,

there is no *a priori* reason to expect it to be a more common process than relaxed selection in nature. Therefore, there is a major gap in our understanding of evolutionary processes.

The distinction between increased versus relaxed selection is important given that traits are expected to evolve differently under these two situations (5, 6). Increased selection and emergence are well studied phenomena with straight forward expectations: increased response of the affected trait, and evidence from both field and laboratory studies often support this hypothesis (7-12). However, under relaxed selection the evolution of a trait is no longer under the direct effects of a previously important source of selection and therefore the trait's contribution to fitness now depends on its interactions with other fitness enhancing traits (i.e., trade-offs) (13, 14). Although most studies have been performed in the laboratory, there is accumulating evidence showing how relaxed selection affects traits in nature (15-17). In both lab and field studies the reported outcome is either no change in trait expression or loss of the trait (5, 6, 13). The evolution of a trait under relaxed selection is thus determined by the type of correlation between that trait and those traits with which it co-varies (5). Furthermore, the degree to which the trait under relaxed selection is costly or prone to mutation accumulation is expected to determine the rate at which the trait will be lost (5, 6).

Enemy release

A special case of relaxed selection is enemy release, which is the focus of this dissertation. I define enemy release as the increased opportunity of victims to reduce the direct detrimental effects on their fitness caused by another species (i.e., the enemy). In that sense, parasites and predators are the most common enemies that victims face. Enemy release can occur in many instances, perhaps the most immediate concept that comes to mind is enemy release

following a species translocation to a new environment, specifically, out of its natural range (18, 19). Nonetheless, migration within the species range, following changes in the biotic and abiotic environment can also lead to release from enemies (20). Yet, not all cases of enemy release need to be as drastic as to involve a complete disappearance of the enemy, victims can also achieve a level of reduction of the detrimental effects caused by their enemies through behavioural (21-23), developmental (24-26) or evolutionary (27-30) changes. In this thesis I assess in nature, through laboratory and field assays, the evolutionary impacts of enemy release on victims and a novel behavioural mechanism that can lead to enemy release.

1.2 What is a parasite?

The type of enemy I focus on in this dissertation are parasites. Parasites are classically defined as organisms that cause harm (i.e., have a negative impact on the host's fitness) and which possess adaptations that allow them to [1] feed on, and [2] live on (ectoparasites) or in (endoparasites), another organism, the host (31-33). These activities do not necessarily or immediately lead to the host's death (a key distinction from parasitoids and predators). There are more restrictive definitions of parasites which limit the group to metazoans and some protozoans (31), thus excluding other important pathogens such as viruses, bacteria and fungi. Although the core of my experiments relates to the effects of a metazoan ectoparasite (*Gyrodactylus* spp.) from the taxon Platyhelminthes, I will use the term parasite in its more general and broadest connotation, that is, to include all pathogens.

Parasites are a pervasive and influential driver of ecological processes (34). Parasites account for more than one third of animal diversity (32, 35) and can represent a larger biomass than that of predator within ecosystems (36). Parasitism is a common trophic strategy (35, 37),

an important source of extrinsic mortality (38), can modify the strength of interspecific interactions (39) and can shift the structure and energy flow of foodwebs (37). However, until recently parasites have often been neglected in ecological and evolutionary studies, yet understanding their effects on ecological and evolutionary dynamics is essential to improve our conservation programmes (40, 41), food security (42), disease control strategies (43), invasive species management (19) and possibly poverty alleviation (44). Finally, a more personal reason for the study of these questions is that parasites and their eco-evolutionary interactions with their hosts are simply fascinating.

1.3 Evolution in the lab and in nature:

The empirical evidence for evolution under enemy release is derived from two main approaches: [1] laboratory-based experimental evolution and [2] field surveys. The first approach, experimental evolution in the laboratory, manipulates the presence of enemies in a repeatable way while controlling for or removing variation in other selective factors (45). Yet, evolution in nature occurs in the context of these other factors (i.e., multidimensional selection), which could strongly modify the evolutionary response to divergence in enemy levels and thus influence our ability to predict and understand the process. These other factors become of particular importance when studying enemy release given that trait evolution following the removal of a previously important source of selection becomes subject to trade-offs with other traits and therefore to effects of other sources of selection (5).

The second approach, field surveys, incorporates the effects of multiple interacting selective pressures, and therefore informs on the effects of multidimensional selection and the relative importance of the selective factor of interest. Nonetheless, this approach is rarely able to

discriminate cause and effect or the rate at which evolution proceeded. Furthermore, field surveys rarely allow to disentangle the ecological causes of the phenotype (i.e., phenotypic plasticity and maternal effects) from the genetic component of the phenotype (the one which is subject to evolution).

A way to link these two approaches is to experimentally manipulate enemy levels in nature which then allows assessing the rate and nature of the response to changes in enemy levels in the context of natural variation in other selective factors (46). This third approach is more powerful when informed by data gathered from the previous two approaches, such that there is a clear understanding of how evolution is expected to proceed when only the focal source of selection varies and there is substantial understanding of the other relevant sources of selection and trait variation under natural conditions. Therefore, in order to assess evolution under enemy release I use experimental manipulations of enemy levels in nature.

1.4 Evolution of defence:

The impacts of parasites on host fitness can be partitioned into different components, for example, parasite infection has been shown to reduce the host's reproductive output, growth rate, mating success, and survivorship (34, 47). Given that parasites can cause intense negative effects (directly and indirectly) on host fitness and that they are ubiquitous in nature (32, 34, 37) it is generally expected that parasites impose strong selection on a host's defence (i.e., mechanisms and processes that reduce fitness loss when parasites are present (33)). Defence against parasites can be expressed by hosts in multiple ways (48, 49) which can include physiological/immunological (49, 50) and behavioural (51-53) responses. The magnitude of the detrimental effects caused to the host by parasites is determined by [1] the parasite's ability to

take away resources from the host, [2] the number of parasites carried by the host (54), [3] the likelihood that infection facilitates the establishment of other pathogens (55), [4] the amount of resources and energy the host invests in its response to the parasite (49, 50, 56), [5] the duration of the infection, and [6] the host's ability to reduce the number of parasites (resistance) or the damage caused by a given number of parasites (tolerance) (57-59).

Investment in defence against parasites has associated benefits and costs which affect the evolution of this trait. Evolutionary models of host resistance to parasites (60, 61) predict that increased parasite-induced mortality, which correlates with increased parasite abundance and prevalence, selects for hosts that better avoid, control or clear parasites, leading to the evolution of increased resistance in the host population - since these individuals have higher lifetime reproductive success. Laboratory studies with bacteria (62-65), protozoans (12) and invertebrates (29, 66-68) as well as comparative field studies (30, 69, 70) strongly support this pattern. Similarly, theory predicts that a decrease in parasite-induced mortality and morbidity, which correlates with decreased parasite abundance and prevalence, will select for individuals that invest less in resistance and more in other fitness-enhancing traits, leading to the evolution of decreased resistance (60, 61). Only laboratory-based studies on bacteria and invertebrates have directly tested for the evolution of resistance under relaxed selection; in all cases the result has been no evolutionary change or the evolution of decreased resistance (13, 71-76), as would be predicted under relaxed selection (5).

Resistance against parasites increases the fitness of exposed individuals, and yet the degree of resistance varies among populations of the same host species and among individuals within those populations (i.e., high resistance it is not a fixed trait), which suggests that resistance has associated costs (33, 77). These evolutionary costs of resistance are often assessed

as trade-offs with life-history traits (13, 29); one such trait that often trades-off with resistance is fecundity/fertility (78, 79, but see 80). When multiple traits are measured as potential costs of resistance, the most frequent outcome is that a few, but not all, of the evaluated traits show a negative correlation with resistance. Therefore, the fact that some studies (e.g., 59, 81) do not detect a trade-off between resistance and some specific life-history traits should not be taken as evidence of lack of costs of resistance; it seems more likely that resistance either trades off more strongly with other unmeasured life-history traits, or alternatively the costs are expressed differently (e.g. physiological costs).

One further consequence of divergent exposure to enemies among victim populations is ecological speciation (82) (i.e., the build-up of reproductive barriers owing to divergent natural selection between different environments). On the one hand, divergent predation levels have been shown to drive reproductive isolation (83, 84), where individuals from different populations preferentially mate with individuals from similar environments and similar adaptive traits rather than with more divergent, but more closely related, ecotypes. Parasitism, on the other hand, is increasingly suggested as an important driver of ecological speciation (85-87) yet empirical support for parasites as drivers of ecological speciation is weak (86). Additionally, natural divergence in enemy levels is often accompanied by divergence in other biotic and abiotic components of the environment (86) (i.e., high dimensionality) which should increase the number of independent traits under selection (i.e., multifarious selection), and lead to increased progress towards ecological speciation (88, 89).

1.5 Various ways to measure defence:

Hosts can defend themselves from parasites in many ways. These include behavioural and physiological (including immunological) mechanisms that reduce the probability of becoming infected or reduce parasite loads once infection has occurred (33, 48, 90). Behavioural components of defence include, for example, active avoidance of places associated with higher parasite abundances (52), modifying grouping behaviour to reduce attack or transmission of parasites (91-93), engaging in cleaning behaviours such as grooming and allogrooming (51, 94) or the ingestion of anti-parasitic substances (95).

Physiological mechanisms of defence are varied and include, among others, changes in body temperature (96, 97) and the various molecules and pathways that compose the recognition, signaling and effector branches of the immune system (33). These components can be measured in various ways, for example by targeting direct measures of the amount of swelling in response to antigen injections (e.g., PHA) (98), the degree and rate of encapsulation of foreign objects (99) and antibody titers after exposure to an antigen (100). These measures are useful when variation in parasite loads does not correlate with damage experienced by hosts or when direct counts of parasite numbers are difficult. However, given the multitude of possible behavioural and physiological mechanisms that need to be accounted for, the most direct and integrative measure of the host ability to control parasite numbers is to directly assess parasite numbers under controlled infection. Under certain circumstances (e.g., when using ectoparasites that reproduce on their hosts) the whole infection dynamic can be monitored at the individual host level and measures such as establishment success, peak load, parasite growth rate and infection clearance rate can be determined (101). In systems where parasite loads cannot be directly counted or where the main interest lies in host population dynamics, resistance can be measured

as the proportion of individuals in a group that become infected or survive infection (12, 67, 68, 102), or as the concentration of parasites needed to kill a certain proportion of the host populations (71, 76).

One common problem that the above metrics share when interpreted alone is the assumption that a stronger response or a lower parasite load necessarily implies higher host fitness (103). Although this is partially correct, given that lower loads correlate with lower damage, overinvesting in resistance is done at the cost of investment in other fitness enhancing traits (56) and high resistance can lead to self-reactivity (e.g. autoimmune responses) (104). Furthermore, defence can be conceptualised as not only being constituted by resistance mechanisms (the ability of hosts to reduce their parasite loads) but also as being constituted by tolerance mechanisms (the ability of hosts to reduce the negative impacts of a given parasite load) (57-59, 61, 105). Investment in these components is expected to have a negative correlation (57, 61) because reducing the damage caused by parasites that are cleared before they can cause damage, or investing resources in eliminating parasites that cause no damage, would both be an inefficient use of resources. Therefore, a comprehensive understanding of defence and its evolution under enemy release should include not only measures of resistance but of tolerance too.

1.6 Group formation as a mechanism of defence against enemies:

As mentioned above, hosts have non-physiological ways of defending themselves against enemies, and behavioural strategies might play a crucial role in defence when victims cannot fully evade their enemies. One such strategy is group formation (106). Group formation is a common adaptive strategy by which individuals that maintain a close spatial association have a

higher relative fitness than those that are by themselves (106). Although members of a group benefit from a series of advantages (such as increased foraging success (107, 108), reduced thermal stress (109), increased locomotion efficiency (110) and enhanced mating opportunities (111)), reduced predation risk (21, 112-115) is an important and well-studied mechanism of group formation. As group size increases, victims (i.e., prey-species) are more likely to detect (113), reduce their exposure to (112) and decrease their probability of being attacked by (21) enemies (i.e., predators) as well as reduce the ability of predators to make effective attacks (115). In this sense, group formation is an effective enemy release mechanism when victims cannot fully escape their enemies.

Parasites are another type of enemy that is affected by groups, yet the effect of group size is more nuanced than for predators. On the one hand, when parasites transmit through free-living stages or vectors (i.e., actively or passively search for their hosts) increasing group size may confer similar benefits as those obtained against predators (116). On the other hand, parasites that are transmitted through host to host contact (i.e., contagious parasites (116)) benefit from increased contact among suitable hosts as group size increases. Therefore, as group size increases there is a higher mean number of parasites per individual victim (i.e., host) and larger percentage of infected individuals in the group (116-118). In this sense, increased contagious parasite transmission is a common cost of group living (119).

One way in which in which victims may be able to reduce the costs of forming groups (i.e., constagious parasite transmission) while keeping some of the advantages of larger groups (i.e., antipredator advantages) is by forming mixed-species groups (119). Mixed-species groups are frequent in nature (120-122) and occur when members of two or more species overlap spatially and temporally (106, 120). These groups are commonly explained as providing

foraging, vigilance and anti-predator benefits to members (122, 123), but the effect of parasitism as a possible driver of mixed-species groups has been mostly overlooked. Mixed-species group formation can serve as a strategy to avoid mobile and vector transmitted parasites, and has been reported to occur when the numbers of conspecific host individuals are low (93).While a heterospecific association provides the same benefits as a monospecific group in terms of the numerical advantage (i.e., larger groups lead to decreased individual risk and exposure), the heterospecific nature of mixed-species groups may play a crucial role as a mechanism against contagious parasites (119). For example, when contagious parasites are highly host-specific, an equally-sized single-species group provides twice as many opportunities for transmission and establishment as a mixed-species group with an evenly-balanced host-species composition, thus leading to higher parasite loads (mean abundance and prevalence) in the single-species groups. In my thesis, I conducted a first empirical test of mixed-species group formation as a potentially important mechanism providing enemy release to parasitised fish.

1.7 Guppies and *Gyrodactylus*: The study system:

In order to assess the ecological and evolutionary effects of enemies on their victims I use a *Poecilia reticulata – Gyrodactylus* spp. host-parasite system. The host species, guppies (*Poecilia reticulata*), are live-bearing poeciliid fish native to north-eastern South America (i.e., Guyana, Venezuela and, Trinidad and Tobago) (124) and are a well-established microevolution model species (124). *Gyrodactylus* is a diverse genus of teleost skin ectoparasites (>400 species) that have important ecological and economic consequences (125). Furthermore, they are a well-studied model species for host parasite dynamics and there has been recent increasing interest in their importance as an ecological driver in the guppy system (126, 127).

Guppies: Poecilia reticulata

Guppies occur in first to fourth order tropical streams in Trinidad, and occur under a wide range of physiochemical conditions, ranging from pristine freshwater streams, brackish anoxic streams, naturally occurring high-temperature oily streams (>26 ºC), highly human-modified streams, as well as farm, urban and industrial polluted reaches (*Pers. Obs.*).

A key feature of the Trinidadian guppy system, in the northern range mountains, is that guppy populations within watersheds are separated by waterfalls. Each population located above a waterfall represents an independent colonization event from the adjacent below-waterfall population – genetic divergence between watersheds is greater than between populations above and below waterfalls (128, 129). Therefore above waterfall populations represent multiple independent and repeatable events of local adaptation. Below and above waterfall environments diverge in many biotic and abiotic characteristics (124) that lead to divergent selection between these environments. For example, below waterfall environments are characterised by higher predator pressure (130, 131), higher primary productivity (132), wider streams and lower guppy-densities (20, 133), whereas above waterfall environments have the opposite characteristics. This environmental divergence has been shown to correlate with divergence in life-history (134) as well as in morphological (135, 136) and behavioural (137-140) traits in guppies. Moreover, guppy experimental translocations in Trinidad - from below to above waterfall sites - have been effectively used to demonstrate the role of environmental divergence (i.e., divergent selection) on trait evolution, and have identified chiefly predation as the main cause of guppy trait divergence (life-history (141-143), morphological (144) and behavioural (145)).

Traditionally, strong variation in guppy adaptive traits has been explained by the binomial division between high predation and low predation environments (130). Specifically,

high predation environments are characterised by a diverse community of piscivorous fish that include *Chrenicichla* spp. *Aequidens pulcher*, *Cichlasoma bimaculatum* and *Hoplias malabaricus*, whereas in low predation environments only a gape limited fish is present (*Rivulus hartii*) (124, 131). In high predation communities mortality tends to be higher for adult guppies, and higher for males than females (146), while in low predation sites juvenile guppies may be preyed upon by *R. hartii* (147). Yet, the reality is that guppies show fine-scale gradual variation in life-history traits in response to more subtle gradual changes in the predator community richness (148), and there is increasing awareness of the importance of other biotic and abiotic factors that co-vary along the predation divide. Some of these covarying factors that can be relevant for guppy evolution are divergent diets (149, 150), productivity (132), and density effects (151, 152). Perhaps the strongest evidence in favour of a predominant role of predation over other axes of variation comes from the observation that populations a few meters apart above and below waterfalls differ strongly in their traits and exposure to predators but the abiotic conditions of their environment are almost identical (20), suggesting that predation is the most likely cause for the observed differences.

One possible source of variation among guppy populations that has received relatively less attention until recently is parasitism. Nonetheless, the effects of environmental divergence on parasite loads in the wild has gained increasing interest during the last decade and shows increased parasitism in high predation reaches (126, 130). Yet the understanding of the evolutionary dynamics of defence against parasites is scarce and derived from correlational evidence. In nature, guppies are infected by a diverse array of pathogens such as *Trichodina* spp., *Camallanus* spp. and *Ichtyophthirius* spp. (*Pers. Obs., Gheorghiu et al unpublished data*).

Among these pathogens, the most prevalent macroparasite of wild guppies is *Gyrodactylus* spp. (153).

Gyrodactylus spp.

Two species of *Gyrodactylus* infect guppies in the wild – *G. turnbulli* and *G. bullatarudis* (153). Although these two species have similar requirement niches, life-histories and morphologies (154), they can coexist in the same host populations and even on the same individual hosts (153), presumably because of a low degree of resource partitioning (i.e. microhabitat specialization - 153, 155). *G. turnbulli* has been reported to have a higher degree of host specificity than *G. bullatarudis* under experimental infections in the laboratory (156, 157), yet there is no supporting evidence for these claims from wild populations; it seems unlikely that given the opportunity to infect their specific hosts these parasites will establish on non-specific hosts.

Resistance to *Gyrodactylus* spp. in the guppy can be partially decomposed into innate and acquired resistance (155, 158-160), where innate resistance can limit the initial establishment and growth rate of the parasite population and acquired resistance affects the later stages of the primary infection and the establishment and development upon re-infection. The initial host response to *G. turnbulli* and *G. bullatarudis* in guppies is not species specific to either one of these parasites (155), yet guppies vary in their ability to resist both parasites species (161) and even different strains within a given species (161; Perez-Jvostov et al. unpublished). Nonetheless, overall guppy differences in ranking of resistance among populations are maintained independently of the parasite species/strain used (161). Although mechanisms involved in guppy resistance to *Gyrodactylus* have not been fully characterised, the involvement

of the immune system is inferred from experiments on salmonids which have shown that Interleukin 1β stimulates mucus production, thus impairing the parasite's ability to move and feed, and Complement factor C3 binds to and kills the parasite (125). Additionally, although it is clear that guppy resistance to *Gyrodactylus* spp. has a heritable component (162, 163), it is also influenced by multiple non-genetic components, such as temperature (164), salinity (165, 166), contaminants (167-169), diet (170, 171), host condition (171), predation (126, but see 127), and social interactions (172, 173). Furthermore, resistance to *Gyrodactylus* spp. in other systems has been shown to respond to host stress (174), seasonality (175-177) and testosterone (178).

Overall, the effects of the non-genetic components appear strong enough to confound the heritable component if phenotypic plasticity (and perhaps maternal effects) between individuals is not controlled for, which could therefore compromise the conclusions of studies that make inferences about variation on individual resistance. I find particularly concerning the effect of acquired resistance, since contrary to Scott (179), who found total loss of acquired resistance on pet-store guppies after 4 to 6 weeks post exposure, Cable and Van Oosterhout (160) report that wild guppies remain refractory to infection for at least 53 days after the end of a primary infection; perhaps due to unidentified infection by multiple parasites in wild-caught guppies or perhaps due to more than five-fold variation in MHC diversity between pet-store and wild guppies (160, 180). Moreover, although there is support for a role of MHC Class IIB alleles explaining some of the of guppy variation in resistance to *Gyrodactylus* (163, 181, 182), our understanding of the mechanisms and contributions of MHC to guppy defence is limited. It is therefore most appropriate to use wild-caught, laboratory-reared F2 guppies to assess the evolution of resistance against *Gyrodactylus*; this approach controls for the confounding effects of phenotypic plasticity and maternal effects.

Advantages of the model system:

The guppy-*Gyrodactylus* host-parasite model is a convenient and appropriate system to explore questions on enemy release and evolution in nature. It is convenient because both *Gyrodactylus* and guppies are amenable to laboratory breeding over multiple generations, have relatively short generation times, are well studied model species both in the laboratory and field, and because the whole infection dynamic of the parasite can be tracked without killing the host. It is an appropriate system because resistance shows potential for rapid evolution through natural selection and *Gyrodactylus* can be an important source of selection. Previous research in wild guppies has shown rapid evolution of life-history, behavioural and morphological traits in response to divergence in predation (141, 142) and productivity (132) suggesting the potential for rapid evolution of resistance. Resistance shows all the components for evolution through natural selection, [1] guppies show variation in their resistance to *Gyrodactylus* (126, 127, 160, 161), [2] this resistance is heritable (162, 163) and [3] defence against this parasite influences individual survival (161, 183) and reproduction (184-187). *Gyrodactylus* is highly likely to be an important agent of selection because [1] it is the most prevalent macroparasite of wild guppies (188), [2] causes high levels of mortality in both the laboratory (161) and field (183), [3] the lesions it causes on the skin serve as entry point for secondary bacterial and fungal infections (55, 189) [4] and infection influences mate choice in both sexes (159, 184-187). Therefore, the system has the potential to allow the evaluation of contemporary evolution of defence under enemy release in nature.

Furthermore, guppies are used as a sexual selection and behavioural ecology model species, (124) two subject areas which provide further insights into the mechanisms and process of defence evolution against parasites and into the ecological drivers of variation in infection. In

general, guppies show adaptively divergent preferences for male colouration in response to environmental divergence, specifically the presence of predators (see 190 for a review). Yet guppy size (191), nutritional status (192) and infection history (159, 184-187) also affect female and male mate preferences. In a similar way, guppies also show divergent social behaviour in response to changes in predation (137, 139, 140) and parasitism (193). Additionally, guppy distribution overlaps with a closely related poeciliid fish - *Poecilia picta* (194, 195) – and when these two species co-occur they form mixed-species groups (196, 197). Thus, the system also has the potential to allow the assessment of the effects of enemy release on ecological speciation, and the effects of social interactions on enemy release.

1.8 Chapter overview:

Although the guppy-*Gyrodactylus* system has provided insight into some aspects of the ecology of host-parasite dynamics there has been no assessment of the evolutionary dynamics of defence against parasites in the system, nor of its contribution to speciation or how direct interactions with other species affect parasitism. Additionally, previous research has rarely disentangled individual defence against parasites from the influence of stochastic events of transmission caused by interactions within groups (i.e., infection dynamics with contagious parasites in groups are strongly influenced by inter-individual interactions). Furthermore, previous research has not isolated maternal effects or phenotypic plasticity from the genetic component of antiparasite defence. Therefore, throughout this thesis I assess, through laboratory and field assays, the evolutionary impacts of enemy release on victims in nature and a novel behavioural mechanism that can lead to enemy release. Specifically I assess the evolution of

defence against parasites (Chapters 2 and 3), its implications for speciation (Chapter 4), and whether social interactions among species affect defence against parasites (Chapter 5).

In Chapter 2, I assess the evolution of resistance against a common and deleterious parasite, *Gyrodactylus* spp, after the experimental release from this parasite in four replicate introductions of guppy (host-)populations in nature. Although a reduction in the strength of selection is expected to cause the evolution of reduced trait expression or no change in trait value, we found that, contrary to theoretical expectations, the fourth- and eighth-generation descendant female guppies had rapidly and repeatably evolved increased resistance to the now absent parasite. This change in resistance is not owing to plastic or maternal effects (removed by common garden laboratory rearing to F_2 (141)), nor to resistance-tolerance trade-offs or differences in productivity among the sites. I suggest as a leading mechanism that rapid life-history evolution, common to such introductions (141-143), in response to release from predators pleiotropically drives increased resistance to *Gyrodactylus*. In Appendix A, I discuss further and reject some alternative explanations - artificial selection and density effects – while highlighting the importance of experimental evolution in nature.

Females and males often show stable trait dimorphisms, even when they constantly exchange genetic material within a population, and would thus be incorrect to assess the trait as a character of the population and not of each sex independently. One trait that is often sexually dimorphic is defence against parasites, yet the implications of sexual dimorphism are rarely considered when assessing defence evolution. Therefore in Chapter 3, I expand on the previous work done on females to assess whether both male and female guppies evolve resistance in parallel in response to enemy release. This question results from the increasing attention given to the reality that populations in different environments often differ substantially in adaptive traits,

suggesting a non-parallel component of evolution, and the fact that my co-authors and I (126) previously found that wild guppies are sexually dimorphic in resistance but were unable to tease apart the genetic component of resistance from phenotypic plasticity, maternal effects and social interactions. To address these questions I used fourth- and eight-generation descendant guppies released from *Gyrodactylus* spp. from the four replicate introductions. I found that in the ancestral source population male guppies had higher resistance to *Gyrodactylus* than females, and that parasite release in the derived populations led to non-parallel evolution of the sexes. Male guppies did not evolve in resistance, as expected by enemy release theory, whereas the previously reported increased resistance of females led to the evolutionary reduction of sexual dimorphism in resistance.

In Chapter 4, I continue assessing the evolutionary effects of enemy release in two of the introduced populations and use the source population as a reference. I evaluate if enemy release, within a broader multifarious selection framework, leads to ecological speciation by assessing mate-choice preferences among the source and introduced populations. This chapter is not exclusively concerned with the evolution of defence against parasites (which is expected to be most strongly influenced by parasitism) but also with speciation. Here, parasitism is one of many axes of environmental change that could lead to reproductive isolation. I therefore focus on the multifarious selective forces to which the introduced populations where subjected. I used the eighth- and twelfth-generation of the introduced guppies to assess the preference of females from each population towards males from each population in a paired design. I found that despite the rapid evolution of male signaling traits, positive assortative mating did not evolve. In its place, dissasortative mating occurred due to the maintenance of the ancestral preference for novel

males. I conclude that strong multifarious divergence in this system did not promote the rapid evolution of assortative mate choice.

Finally, in Chapter 5, I assess a social mechanism of enemy release when victims cannot fully escape their enemies - as in previous chapters - but have to coexist with them. Predation and parasitism are two of the most important sources of extrinsic mortality in nature (38), by forming groups, victims can gain some protection against predators but may increase their risk of being infected by contagious parasites (119). A way of resolving this conflict might be by forming mixed-species groups, thereby reducing the number of available hosts to host-specific contagious parasites. To test this hypothesis I move away from the introduction experiments into the lower reaches of Trinidadian rivers, and into the transition zone between fresh to brackish water, to explore how the *Gyrodactylus* load of guppies and their sister species (*Poecilia picta* (194) – which is infected by their own species of *Gyrodactylus*: *Gyrodactylus pictae* (198)) – changes between single-species and mixed-species groups. Fish were collected from sites were single- and mixed-species groups occur in three different basins and assessed for their *Gyrodactylus* loads. The sites of collection were determined based on whole island exploration as part of a project with my co-authors to determine the ecological correlates of the distribution limits of these two poeciliid species (195). I found that the presence and abundance of *Gyrodactylus* was lower when fish of both species were part of mixed species groups relative to single-species –groups, which is consistent with the hypothesis that mixed-species groups provide a level of protection against contagious parasites.

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CHAPTER 2

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

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2.1 Abstract:

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 repeatably evolved increased resistance to the now-absent parasite. This evolution was not due 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.

Keywords: Relaxed selection, experimental evolution, rapid evolution, resistance, tolerance,

2.2 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 on 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-naïve 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 the present 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 (e.g. 12) and non-vertebrate organisms (e.g. 13), as well as in comparative field studies (e.g. 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 (e.g. 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 characterised (24), involvement of the immune system is inferred from experiments on salmonids where macrophages produce Interleukin 1β which stimulates mucus production and Complement factor 3 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). Since *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 one and two years, fish were collected from the source and introduction populations, bred to a second generation under common 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.

2.3 Materials and methods:

Field introductions

The guppy introductions were carried out as part of a US 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 Labs, Cibolo, USA), Clout (Sentry AQ Mardel, Omaha, USA), and commercial forms of erythromycin and monocyclene (Maracyn and Maracyn Two –Sentry AQ Mardel, Omaha, USA). 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) (Fig.2.1). In April 2009, fish were introduced into two additional Guanapo tributaries (Taylor and Caigual) (Fig.2.1). 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.D. Pers. Obs. and D. Reznick Pers. Comm.). 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, due to experimental canopy thinning (approximately 4% thinning) (28).

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) (Fig.2.1). The 2009 collection was thus of fish that had been evolving at the introduction sites without parasites for one 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 one year (Taylor and Caigual – four guppy generations) and two 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 F2 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 Appendix 2.A1 in Electronic Supplementary Materials). For the 2010 collections, offspring from field-collected mothers were used to create 14 Guanapo (source) families, 10 Lower Lalaja (introduction) families, 5 Upper Lalaja (introduction) families, 10 Caigual (introduction) families, and 11 Taylor (introduction) families (Appendix 2.A1). F1 females were mated with multiple F1 males from different families descended from the same collection to generate F2 offspring. Fish from the 2009 collections were transferred to McGill University in the F2

generation, whereas fish from the 2010 collections were transferred to McGill University in the F1 generation and were there bred to form the F2 generation.

Laboratory infections

All infection trials were performed at McGill University in flow-through systems (Aquaneering Inc., San Diego, USA) that standardized water quality and temperature (26°C). Filters prevented movement of parasites between tanks. Females rather than males were used for the present study because [1] females have a broader range of peak *Gyrodactylus* loads (19) that allows better detection of statistical differences, [2] 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 [3] the consequences of females for population dynamics are direct (population growth is limited by female – not male – reproductive output). For the 2009 collections, F2 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, F2 fish were infected at 12 weeks of age and up to three females were used per family line (Appendix 2.A1). These small differences in protocol between the 2009 and 2010 collections were the result of the different transfers (F2 fish versus F1 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 1.8L tank one week prior to being infected and was fed 10 µl/day 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 0.0001g 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 lab), 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 two days for 24 days, or until death, each fish was anaesthetized in MS222 and its parasites were counted using a dissecting scope at 18x 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 (Appendix 2.A1) and for initial size-weight regressions.

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 outside of 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 (>1) or below (<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 ten 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 (Appendix 2.A1). We estimated *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 if [1] the effect

of peak parasite load on guppy survival throughout the experiment, and [2] the effect of the cumulative *Gyrodactylus* load for the first ten days of infection on the change in relative condition index of guppies on those ten days, was different between populations (statistical interaction between [1] population and peak load, and [2] population and cumulative load). Since 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.

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 Appendix 2.A2). 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 where 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 $p < 0.05$.

2.4 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 2.1). For the 2010 field collections, guppy survival was significantly higher for two of the four introduced populations (Table 2.1–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 2.1).

For all of the guppy populations, *Gyrodactylus turnbulli* infection showed a typical phase of rapid increase that decelerated until reaching a peak and finally crashed (Fig.2.2A, 2.2C, 2.2E). Starting at day 8 of infection, average parasite load began to differ noticeably 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 [1] two of the four introduced populations (Upper Lalaja and Taylor) studied after one year of evolution in nature, and [2] both introduced populations (Upper Lalaja and Lower Lalaja) studied after two years of evolution in nature (Table 2.2; Fig.2.2). 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 [1] one of the four introduced populations (Taylor) after one year of evolution in nature (Fig.2.2F; Table 2.3), and [2] both of the introduced populations after two years of evolution in nature (Fig.2.2D; Table 2.3). 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 2.1) or lower condition (see Appendix 2.A3). If anything, the trend was toward increased tolerance in the introduced populations (Fig.2.3A). Furthermore, we found no evidence of a negative association between measures of resistance and measures of tolerance (Fig.2.3A and 2.3B) – as would have

been expected if the evolution of decreased tolerance caused a correlated response toward increased resistance.

2.5 Discussion:

We found that guppies rapidly and repeatably 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 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 2.1 and Appendix 2.A1), 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 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 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, since 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 Appendix 2.A4).

A third possible biological explanation relates to potential interactions between predation and parasitism. This hypothesis deserves special consideration because [1] interactions between selection by predators and selection by parasites have been reported in other systems (39, 40), [2] the introduced populations were not only removed from parasites but also underwent a dramatic shift in predation intensity (from high to low), [3] guppies show many adaptive responses to different predation regimes (41), that can evolve rapidly in experimental introductions (31), and [4] 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 defense, 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 defense 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 ten 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.

2.6 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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Data Accessibility: All data are publicly available as an electronic supplementary material.

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Table 2.1: Guppy survival as a measure of tolerance.

Collection	Coefficient	Estimate	SEM	Z-value	P (>|z|)
2009, One 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, Two 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, One 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

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).

Table 2.2: GLMs for *Gyrodactylus turnbulli* load on *Poecilia reticulata.*

Collection	Response variable	Coefficients	Estimate	SEM	Z value	P (>|z|)
2009, One 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, Two 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, One 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

Generalized linear model results for parasite load at the tenth day of experimental infection and for peak load (integer variables with a negative binomial distribution) of *Gyrodactylus turnbulli* on *Poecilia 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.

Table 2.3: ANOVAs on *Gyrodactylus turnbulli* intrinsic rate of increase (*r*).

Collection	df	SS	Mean Sq	F	P-value	Difference§
2009 – One year*	2	0.01451	0.007256	1.798	0.179	-
2010 – Two years†	2	0.04885	0.024427	8.984	<0.001	LL&UL<S
2010 – One year‡	2	0.02656	0.013281	5.171	0.008	T<S

ANOVA results for parasite intrinsic rate of increase (*r*) between experimental day 0 and10 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.* S, LL and UL populations derived from field in 2009.

† S, LL and UL populations derived from field in 2010.

‡ S, T and C populations derived from field in 2010.

§ Differences between populations in significant models were detected through a Tukey HSD *post-hoc* test.

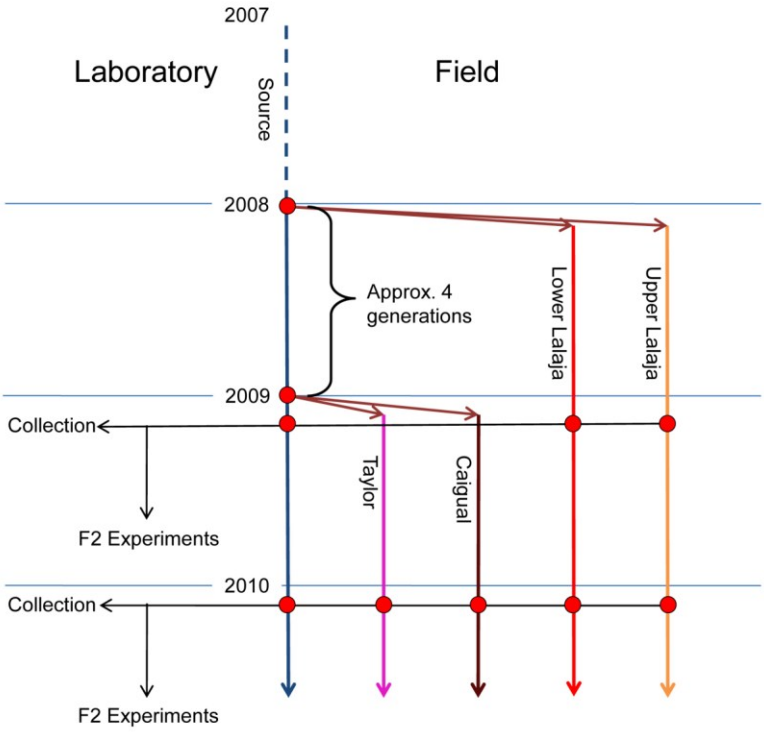


Figure 2.1: Experimental design overview. Guppy introductions were made from a source population in the Guanapo River in 2008 to the guppy-free Lower Lalaja and Upper Lalaja sites and in 2009 to the guppy-free Taylor and Caigual sites. In 2009 and 2010 guppies collected from each site (red dots), were bred for two generations in the laboratory and the F2 fish were used in experiments. See methods for details.

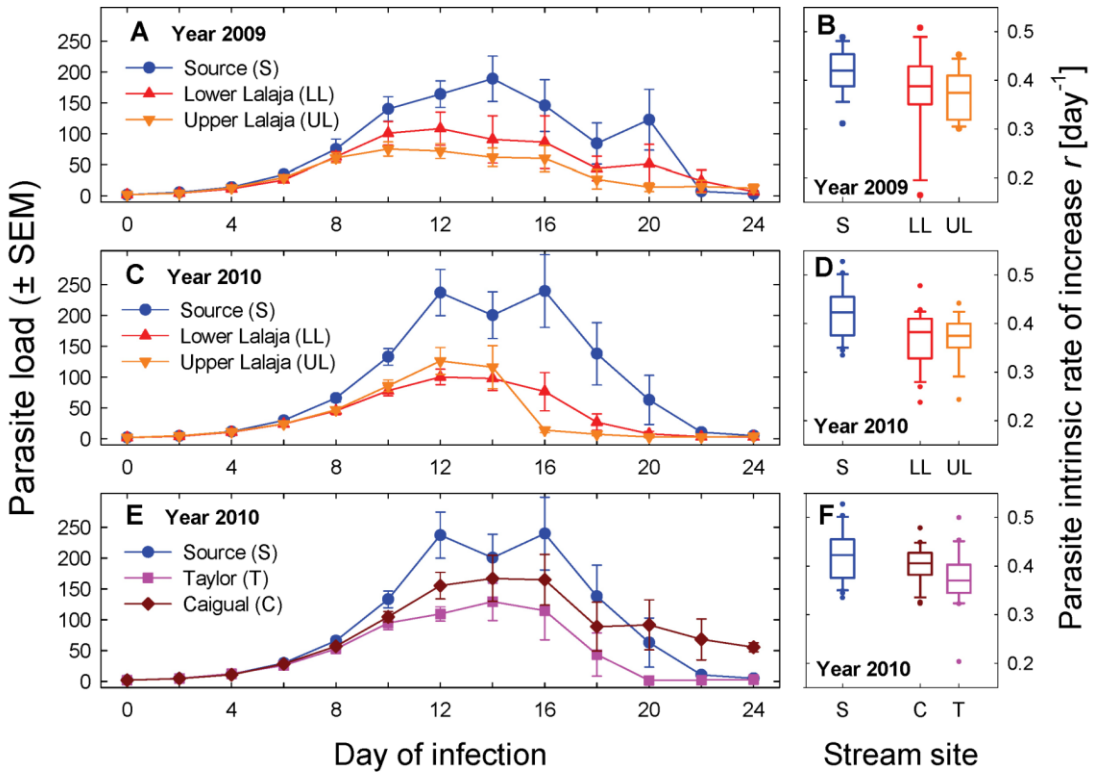


Figure 2.2: **Resistance of female guppies (*Poecilia reticulata*) to parasites (*Gyrodactylus turnbulli*).** Resistance measures based on parasite load on each experimental day (A, C, E) and regression-based estimates of parasite intrinsic rate of increase (*r*) covering experimental days 0 through 10 (B, D, F). High parasite load is indicative of low resistance. The data (means ±SEM – 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, one year after introduction ; (C, D) Guanapo source, Lower Lalaja, and Upper Lalaja fish collected in 2010, two years after introduction ; and (E, F) Guanapo, Caigual, and Taylor fish collected in 2010, one year after introduction.

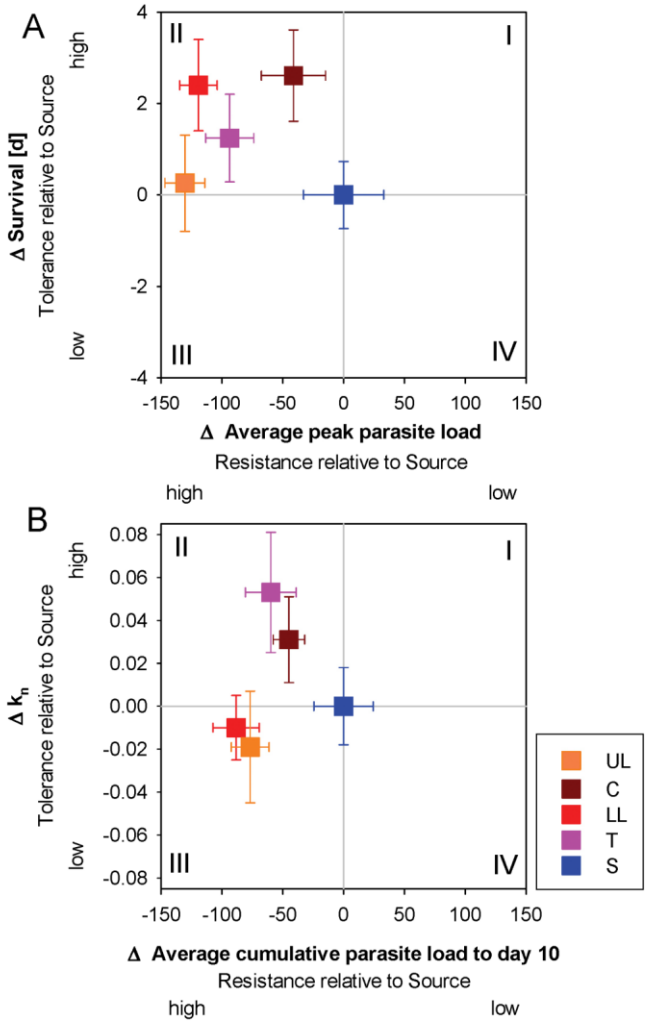


Figure 2.3: Evolution of guppies' resistance and tolerance to their *Gyrodactylus* parasites. (A) Difference in average *G. turnbulli* peak load (a measure of resistance) vs. average difference in survival (day of death – a measure of tolerance) and (B) average cumulative parasite load from day 0 to 10 (resistance) vs. change in relative condition index (K_n) between day 0 to 10 of infection (tolerance). All differences (Δparasite load, Δsurvival, 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 SEM.

Appendix 2.A

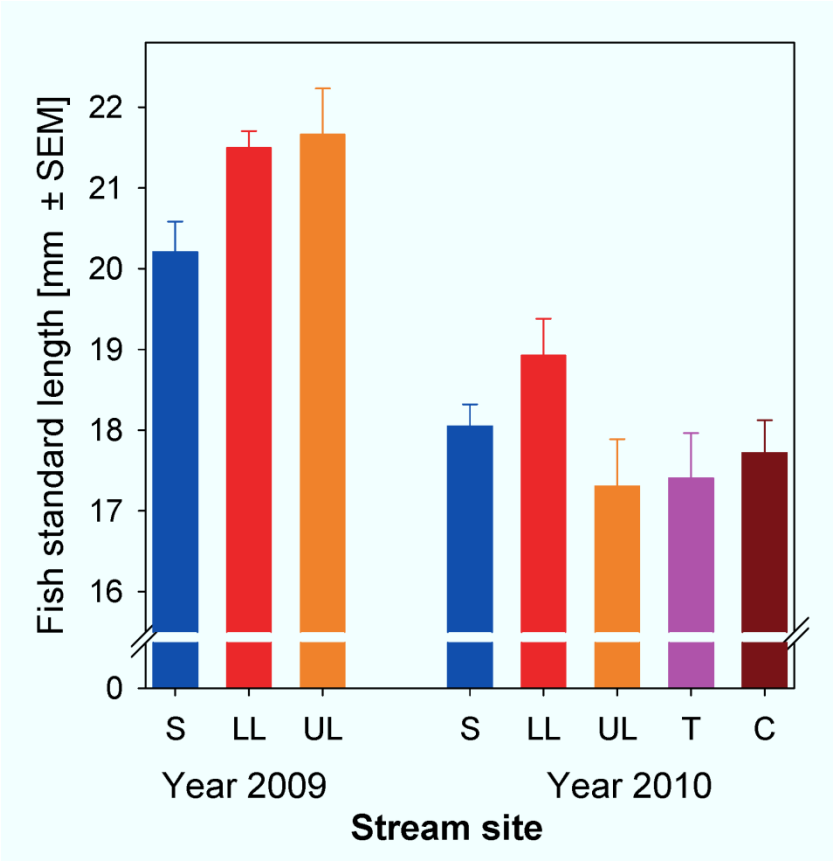
Experimental elimination of parasites in nature leads to the evolution of increased resistance in hosts

Felipe Dargent, Marilyn E. Scott, Andrew P. Hendry and Gregor F. Fussmann **(**2013). Experimental elimination of parasites in nature leads to the evolution of increased resistance in hosts. Proceedings of the Royal Society B. (280) 1773. DOI: 10.1098/rspb.2013.2371

Appendix 2.A1: Summary table of results and environmental characteristics (see Text, 2.3 Materials and methods).

	2009 Collection			2010 Collection				
Population	Guanapo (Source)	Lower Lalaja (Introduction)	Upper Lalaja (Introduction)	Guanapo (Source)	Lower Lalaja (Introduction)	Upper Lalaja (Introduction)	Taylor (Introduction)	Caigual (Introduction)
Generation at time of collection	-	4	4	-	8	8	4	4
n	15	15	14	34	24	18	24	29
n family	15	15	14	14	10	6	11	10
SL (SEM) in mm	20.2 (0.4)	21.5 (0.2)	21.7 (0.6)	18.1 (0.3)	18.9 (0.5)	17.3 (0.6)	17.4 (0.6)	17.7 (0.4)
Initial K_n (SEM)	0.984(0.03)	1.002(0.02)	0.999(0.02)	1.034 (0.02)	0.991 (0.02)	0.995 (0.02)	1.022 (0.01)	0.988 (0.02)
Mean peak load (SEM)	248.6 (34)	172.7 (41.7)	110.5 (15.3)	247.6 (33)	128.3 (15.4)	117.2 (16.4)	154 (19.8)	206.4 (26.5)
Survival n (%)	6 (40)	12 (80)	10 (71.4)	2 (5.9)	7 (29.2)	4 (22.2)	6 (25)	5 (17.2)
Sham n (% Survival)	5 (100)	7 (100)	7 (100)	14 (92.9)	8 (100)	6 (100)	9 (100)	10 (90)
Change in K_n to day 10	-	-	-	0.037 (0.02)	0.027 (0.02)	0.018 (0.03)	0.09 (0.03)	0.068 (0.02)
	Environmental Characteristics							
Predation	High	Low	Low	High	Low	Low	Low	Low
Productivity	High	Low	Low-Med	High	Low	Low-Med	Low	Low-Med
	Individuals alive							
Day 8 (%)	15 (100)	15 (100)	14 (100)	34 (100)	24 (100)	18 (100)	24 (100)	29 (100)
Day 10 (%)	15 (100)	15 (100)	14 (100)	30 (88.2)	22 (91.7)	17 (94.4)	22 (91.7)	26(89.7)
Day 12 (%)	15 (100)	15 (100)	14 (100)	21 (61.8)	21 (87.5)	13 (72.2)	18 (75)	23 (79.3)
Day 14 (%)	15 (100)	14 (93.3)	13 (92.9)	13 (38.2)	15 (62.5)	9 (50)	13(54.2)	18 (62.1)

SL = Fish size measured as standard length. K_n = Relative condition index (Le Cren 1951). Survival = individuals alive at end of experiment. For Sham n = number of sham-infected fish, each individual was derived from an independent family. Change in K_n to day 10 = change in K_n from the start of infection until day 10.

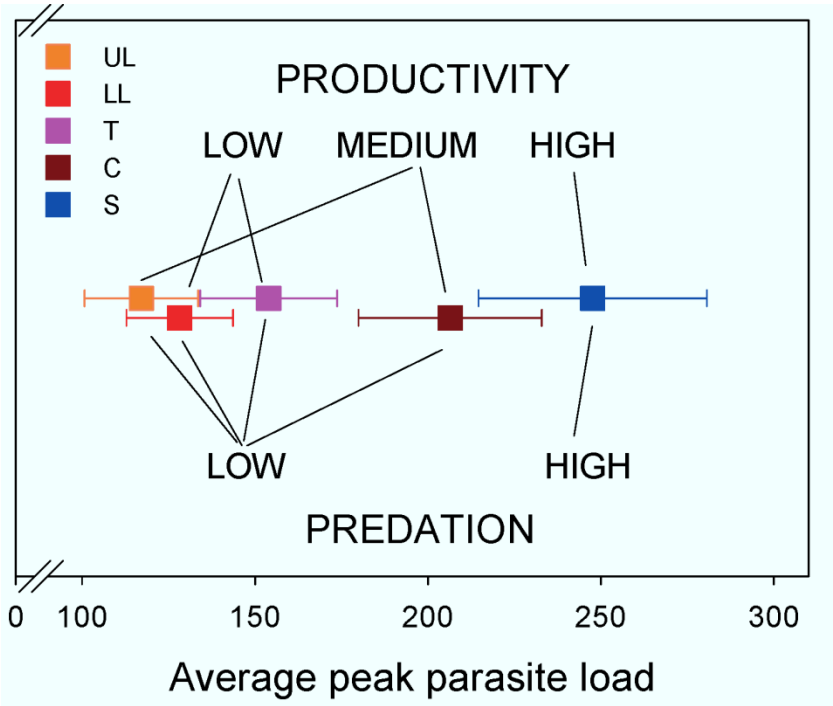


Appendix 2.A2: F2 fish size (mm) at the start of experimental infections. We measured the standard length SL (in mm) of experimentally infected individuals. At the time of experimental infection, F2 fish derived from the 2009 collection were of various ages and generally older than F2 fish derived from the 2010 collection, which explains the difference in SL between years. Fish derived from the 2010 collection were all measured and infected at 12 weeks of age. This explains why there is less variation in SL; differences in size are expected to be genetic. Site abbreviations: Lower Lalaja (LL), Upper Lalaja (UL), Taylor (T) and Caigual (C) introductions, and the Guanapo source population (S).

Appendix 2.A3: Tolerance measured as change in relative condition index.

Collection	Independent variable	df	SS	Mean Sq	F	Pr (>F)
2010, Two Years After Introduction	Population	2	0.0008	0.0004	0.046	0.96
	Cum Load at Day 10	1	0.0072	0.0072	0.862	0.36
	Population × Cum Load at Day10	2	0.0187	0.0094	1.123	0.33
	Residuals	63	0.5250	0.0083		
2010, One Year After Introduction	Population	2	0.0234	0.0116	1.374	0.25
	Cum Load at Day 10	1	0.0361	0.0361	4.249	0.04
	Population × Cum Load at Day10	2	0.0462	0.0231	2.714	0.07
	Residuals	71	0.6038	0.0085		

ANCOVA results for change in relative condition index (K_n) between day 0 and 10 as the response variable, "population of origin" as factor and "cumulative parasite load on day 10" (Cum Load at Day 10) as a covariate. Base data for the change-in-K_n analysis were not measured for the 2009 collection. Higher tolerance is indicated by a lower decrease in relative condition index after ten days of infection for a given cumulative parasite load during the same time frame (i.e. significant interaction coefficient).



Appendix 2.A4: The relation of resistance to environmental characteristics (productivity, predation). Average peak parasite load (±SEM), population productivity and predation characteristics for the Lower Lalaja (LL), Upper Lalaja (UL), Taylor (T) and Caigual (C) introductions, and the Guanapo source population (S), from the 2010 collection.

CONNECTING STATEMENT

Thus far I have shown that females have rapidly and repeatably evolved increased resistance to *Gyrodactylus* after experimental removal of the parasite in nature. Evolutionary biologists and ecologists often tend to think that traits shared by both sexes should diverge in a similar manner from the ancestral population traits when faced with a new environment. This line of thought perhaps has its basis in the common observation that it is difficult to imagine two organisms more genetically similar than males and females within a population. After being released from *Gyrodactylus*, the simplest expectation, all else being equal, is that male and female guppy resistance will diverge in parallel from the ancestral Guanapo source population. Yet, all else is not equal. Male and female guppies are dimorphic in a series of secondary sexual traits that could influence resistance to *Gyrodactylus*. In chapter 3 I use the guppy-*Gyrodactylus* system to quantify the difference in evolutionary response of males and females from the same species to a common selective environment.

CHAPTER 3

Parting ways: Parasite release in nature leads to non-parallel evolution of the sexes

Felipe Dargent, Gregor Rolshausen, Andrew Hendry, Marilyn Scott and Gregor Fussmann. (in review). Parting ways: Parasite release in nature leads to non-parallel evolution of the sexes. *Journal of Evolutionary Biology.*

3.1 Abstract:

Parallel evolution is considered to represent strong evidence for the deterministic action of natural selection. Recently, increased attention has been brought to the reality that populations in similar environments often differ substantially in adaptive traits (i.e., non-parallel evolution). We considered a related problem, the extent to which the sexes evolve similarly in response to the same shift in environmental conditions. Using replicate introductions in nature, we evaluated whether release from a key parasite (*Gyrodactylus*) produced a parallel evolutionary response in male and female guppies. After 4-8 generations of relaxed selection, guppies were collected from the ancestral and derived populations and bred in the laboratory to remove non-genetic effects. Guppies were infected with *Gyrodactylus* and infection dynamics were monitored. We found that, in the ancestral population, male guppies had higher resistance to *Gyrodactylus* than females, and that parasite release in the derived populations led to non-parallel evolution of the sexes: Males did not show much evolution of resistance, whereas females showed increased resistance. The end result was an evolutionary reduction of sexual dimorphism in resistance. We argue that previous selection for high resistance in males constrained further evolution of the trait (relative to females).

3.2 Introduction:

Spatially segregated populations often experience distinct selective environments, which causes the adaptive divergence of traits that influence survival and reproduction (1, 2). When this divergence occurs in a similar fashion within independent evolutionary lineages, the outcome is often called "parallel" or "convergent" evolution and is generally taken as strong evidence that natural selection has deterministically caused divergence (3-5). Recently, however, increasing emphasis has been placed on the reality that populations and species in similar environments often differ substantially in adaptive traits, suggesting a strong "non-parallel" or "non-convergent" element of evolution in response to similar selection pressures (3, 6, 7). Potential reasons for these deviations from determinism include unrecognized differences in natural selection between seemingly similar environments, variation in sexual selection, and differences in genetic variation among populations (7).

Although parallel and convergent evolution are typically considered in the context of populations or species, related questions surround adaptation of the two sexes (8, 9). On the one hand, males and females in a given population should experience a similar environment and share most of their genetic background – suggesting they might adapt in a similar fashion. On the other hand, males and females can experience a given environment in different ways and do not share their entire genome – suggesting they might adapt in different ways. Moreover, males and females express differences in a broad array of behavioral, morphological, and physiological traits – some of which can be extreme (10-13). It thus seems reasonable that males and females will experience different selective pressures even when they share the same spatial location. Fitting this expectation, studies that have estimated selection separately for males and females often find large differences between the sexes (e.g., 14, 15). Given the wide range of possible

outcomes, we here sought to determine the extent to which males and females evolve similarly (parallel) or differently (non-parallel) in response to a similar shift in environmental conditions.

The environmental shift we here consider is parasitism. Parasites are an important selective agent known to cause adaptive divergence between populations and species (16). For instance, populations that are more heavily parasitized often evolve towards increased resistance to parasites (17-19) – presumably because parasites influence fitness by reducing survival (20), fecundity (21), or mating success (22). By contrast, populations that are less heavily parasitized often evolve towards reduced resistance (23) – presumably because resistance mechanisms in the absence of parasites can reduce fitness through immunopathology (24, 25), physiological costs of maintenance of immune responses (26), or antagonistic pleiotropy such as trade-offs with fecundity (27). Of course, these examples of the evolution of resistance under different parasite pressures are generalizations, and other studies also report unexpected evolutionary trajectories in response to a shift in parasite pressure (28). Given all this variation, parasites are an appropriate selective force for considering the differential responses of males and females to an environmental shift.

Our main goal is to consider whether males and females from a common ancestral population evolve similarly or differently in response to a similar shift in parasite pressure. The answer is not obvious given that – as introduced above – a number of reasons exist why males and females might respond differently. On the one hand, males and females can be similarly influenced by parasites and the genes that influence resistance are often found on autosomes (29). These shared properties suggest that a shift in parasite pressure might lead to parallel evolution of the sexes. On the other hand, males and females often experience different parasite levels, have different costs of infection, and can have different costs of defense. For instance,

males and females often differ in their resistance to parasites (16, 30, 31), perhaps owing to [1] differences in body size (larger individuals have more parasites (32, 33)), [2] different exposure to parasites (34), [3] functional trade-offs between testosterone production and immune system function (35), or [4] differences in time and energy allocation budgets between sexual activities (i.e., courting, fighting) and resource acquisition (36). Any of these differences could mean that a similar shift in parasite pressure will lead to non-parallel evolution of the sexes.

The guppy-Gyrodactylus host-parasite system

An excellent context to study sexual differences in evolutionary responses to parasites is represented by the Trinidadian guppy (*Poecilia reticulata*) –*Gyrodactylus* host-parasite system. The guppy is a sexually dimorphic poeciliid fish that is an important model for evolutionary studies. In particular, experimental introductions to novel environments in nature have frequently revealed the rapid parallel evolution of behavioral, morphological, and life-history traits (37, 38). The (mostly) host-specific monogenean worms *Gyrodactylus turnbulli* and *Gyrodactylus bullatarudis* are the most prevalent macroparasites of wild guppies (39). They attach to the scales of their host, where they give birth to flukes with fully developed embryos "in-utero", and they are transmitted during host to host contact (40). These characteristics lead to exponential parasite population increases on individual guppies and epidemics in guppy populations (41)..

The necessary elements for adaptation by guppies to *Gyrodactylus* have been demonstrated in previous work. First, *Gyrodactylus* are an important selective pressure: [1] they cause high mortality in the field (42) and the laboratory (43), [2] they cause lesions that can serve as entry points for bacterial and fungal secondary infections (44, 45), [3] they affect guppy mate choice (46-48), and [4] they influence the ability of guppies to maintain their position during

floods (42). Second, individual guppies differ in their ability to reduce or eliminate gyrodactylid infections (resistance) and this variation has genetic (49, 50) and non-genetic (51-53) components. As would be expected in the presence of these elements, guppy populations differ in their resistance to *Gyrodactylus* infection (54, 55) and in the MHC genes that influence this resistance (49, 56). However, although evidence exists that male and female guppies differ in their resistance to *Gyrodactylus* (55), it is not known how these differences might influence adaptive divergence between populations and the sexes.

Our study

Inferences regarding adaptive divergence in parasite resistance are usually based on field surveys (57, 58) or laboratory experiments (23, 59, 60). Although very informative, these approaches are limited in several respects. Field surveys are limited because [1] it is difficult to infer causation when the selective factor of interest is not experimentally manipulated, and [2] it is difficult to infer rates and directions of evolution when the ancestral state is uncertain. Insights from laboratory studies are limited because organisms are removed from the natural context, which might mean that the observed evolution will not reflect evolution in nature. The solution is to combine these two approaches through experimental manipulations in nature, which are rare in general (61) and apparently absent for evolutionary response to parasite pressure. Our study fills this gap by comparing guppies from a *Gyrodactylus*-present source population to their descendants from four independent *Gyrodactylus*-absent experimental field introduction sites.

After being subjected to the selective pressures of the new parasite-free environment for one and two years (i.e., approximately four and eight guppy generations (62)), juvenile guppies were collected from the four derived *Gyrodactylus*-absent populations and from the source

Gyrodactylus-present population. These guppies were then bred for two generations under parasite-absent common garden conditions in the laboratory, thus controlling for plastic and maternal effects. We then performed individual-based laboratory infections to assess resistance to *Gyrodactylus turnbulli*. We previously analyzed data for the females in this experiment, and showed that they rapidly and repeatably evolved increased resistance to *Gyrodactylus* after this parasite is removed, a result that did not match theoretical expectations (28). In the present paper, we ask to what extent males show similar evolutionary responses in resistance, by specifically addressing five key questions related to parallel evolution of the sexes (Table 3.1).

3.3 Methods:

Experimental Introductions

In 2008, juvenile guppies were collected with butterfly nets from a site in the Guanapo River ("Guanapo source" -10° 38′ 23″N, 61°14′54″W and 10° 39′ 14″N, 61°15′ 18″W) in Trinidad, as part of a United States National Science Foundation Frontiers in Integrative Biological Research project led by D. Reznick (63). All guppies were quarantined and treated for a wide spectrum of pathogens by means of Fungus Eliminator (Jungle Labs, Cibolo, USA), Clout (Sentry AQ Mardel, Omaha, USA), and commercial versions of erythromycin and monocyclene (Maracyn and Maracyn Two – Sentry AQ Mardel, Omaha, USA). The guppies were reared separated by sex so that they remained virgin and, 3 weeks prior to the introductions when they were already mature, they were mated in tanks of 5 males and 5 females. In March (dry season), these guppies were introduced into two previously guppy-free upstream tributaries (Lower Lalaja and Upper Lalaja) of the Guanapo River, and the sexes of a given mating group were introduced into separate streams to maximize effective population size. Of these individuals, 37 and 38 of

each sex were introduced into the Lower Lalaja and Upper Lalaja sites, respectively. In 2009, this process was repeated with newly-collected Guanapo source guppies that were introduced into two additional guppy-free upstream tributaries (Taylor and Caigual) of the Guanapo River. On this occasion, 51 and 64 guppies of each sex where introduced into the Taylor and Caigual sites, respectively.

The four tributaries used for the introductions differed from the Guanapo source environment in several ways. First, the Guanapo source site had a diversity of parasites (including *Gyrodactylus* spp.), whereas the introduction sites were free of guppies and therefore of their host-specific parasites (including *Gyrodactylus* spp.). Second, the Guanapo source site had several important predatory fishes (including the pike cichlid, *Crenicichla frenata*), whereas the introduction sites had only the weak gape-limited fish *Rivulus hartii* (64). Third, the Guanapo source site had a more open forest canopy and possibly higher resource availability for guppies than did the introduction sites (65, 66). The different introduction sites were reasonably similar in these properties, except that two (Upper Lalaja and Taylor) had more open canopies, and therefore higher productivity, as a result of experimental canopy thinning (66). More details on the experimental introductions and sites can be found in (28, 62, 65, 66, 67, 68).

Field Collections

Field collections were performed in 2010, two years (approximately eight guppy generations) after the introductions into the Upper and Lower Lalaja and one year (approximately four guppy generations) after the introductions into the Taylor and Caigual. Guppies were also collected at these times from the Guanapo source population, which continued to be infected with *Gyrodactylus* spp (55; Dargent pers. obs.)). All collections were made with

butterfly nets, and the fish were immediately treated with Fungus Eliminator, Clout, Maracyn, and Maracyn Two. The guppies were held for about three months in Trinidad in population- and sex-specific tanks, before being transported in plastic bottles by airplane to the C. Ghalambor laboratory at Colorado State University. In the laboratory, the guppies were housed in population- and sex-specific tanks following Reznick (69) with two modifications, [1] flow-through systems were used to standardize water quality, and [2] the tanks were smaller (1.8 l).

When the field-collected guppies reached sexual maturity, each female was randomly mated to a field-collected male from the same site (males were not reused) to produce a first laboratory generation (F1). This F1 generation was then raised as described above before being transferred at 84-140 days of age to our laboratory at McGill University. At McGill, the guppies were mated as described above to produce a second (F2) laboratory generation. All rearing procedures at McGill were the same as those at Colorado State except for a minor change of diet. At Colorado State, the guppies were fed brine shrimp and paste made from Tetramin Tropical Flakes (Tetra, Melle, Germany) [following Reznick (69)]. At McGill, we replaced the brine shrimp component with the same volume of paste.

Experimental Infections

Infection trials for the F2 guppies took place after they were at least 12 weeks old. For each trial, experimental guppies were individually isolated for 7 days prior to infection and for 24 days after initial infection. This isolation took place in 1.8 l containers in an Aquaneering Inc. flow-through system, with filters to prevent any movement of parasites, food, or waste products between tanks. During isolation each guppy was fed a daily diet of 10 µl paste of Tetramin Tropical Flakes. For infection, each individual was first anaesthetized in 0.02% MS222 (Tricaine

Methanesulfonate – Argent Chemical Laboratories, Redmond, USA) buffered to a neutral pH with $NaCO_3$. Each guppy was then weighed to the nearest 0.0001 g, photographed for size (standard length) measurements in ImageJ, and infected with two *Gyrodactylus turnbulli* (details below). We infected a total of 63 male and 129 female guppies (Appendix 3.A1). To avoid the possibility that host-parasite co-evolutionary dynamics influenced our results, we used a *G. turnbulli* strain that was novel to all the populations. These test parasites were an isogenic strain isolated from – and propagated on – local (Montreal, Canada) pet store guppies.

All guppies were mature and un-mated at the time of the experiments. The infections were started by removing scales carrying the parasite from a donor guppy held in a separate container (70), and then placing this scale next to the anaesthetized recipient's caudal fin. This procedure routinely succeeded in transferring two parasites within 5 minutes. Afterward, each guppy was allowed to recover and was then returned to its isolation tank. We then monitored parasite population growth on each isolated guppy for 24 days or until the guppy died. This monitoring was achieved by – every two days – anaesthetizing each guppy and counting its parasites using a dissecting scope at 18x magnification.

Analysis

We first analyzed whether the populations or sexes differed in body size or mortality. For body size, we used ANOVA with population and sex as factors and standard length (SL - distance from the tip of the snout to the end of the caudal peduncle) as the measure of size. For mortality, we fit a generalized linear model (GLM) with a binomial response variable (died vs. survived) and a logit link function, with population and sex as predictors. We also tested whether

guppies that died differed in their day of death by fitting a GLM with ln-transformed day of death as a response variable and population and sex as predictors.

We next analyzed two measures of parasite resistance that are used commonly in studies of host-*Gyrodactylus* dynamics (28, 43, 71). "Peak load" is the maximum number of parasites counted on an individual guppy on any day during the experiment. Analyses based on this measure included individuals that died before the end of the experiment because mortality generally occurred when parasite load had already stabilized or started to decrease. "Intrinsic rate of increase" (r) of the parasite population is the slope of the regression of ln-transformed *Gyrodactylus* counts between day 0 and day 10 on an individual guppy. Analyses based on this measure included only individuals that survived to at least day 10. Within this framework, lower peak load and lower r values represent higher resistance as they indicate a higher capability of the guppies to limit infection.

To test whether population and sex influenced parasite peak load and r, we used GLMs. Models included population and sex as factors, size as a covariate and interactions between population and both sex and size. Peak load was modeled with GLMs using a negative binomial error distribution and the log link function. All other data were modeled with GLMs using the identity link function. Analysis of each model was followed by a series of planned contrasts that specifically informed our questions (Table 3.1).

The degree of parallelism in resistance evolution between the sexes and populations was assessed by [1] graphical interpretation of divergence in sex trait-space and [2] phenotypic change vector analysis (PCVA) (72, 73) of evolutionary trajectories in multivariate resistance trait-space (i.e., orientation of divergence). For the graphical interpretation, relative change in resistance by sex was represented as trait divergence between the source and the introduced

populations with change for each sex on a different axis. The source population values were centered to a mean of 0 and the values of the introduced populations were recalculated relative to the new source mean. A 1:1 line was used to depict the trajectory of equal amount of change in trait value for both sexes (i.e., parallel evolution). For the PCVA, the sex-specific population centroids used in this analysis were multivariate least squares means derived from MANCOVA models with the predictor variable being population contrasts (source vs. each introduced population) and the response variables being *r* and ln peak load. The respective change vectors (connecting each introduced population to the source) were then compared to each other with respect to their orientation (angle between vectors). Statistical significance for these comparisons was based on permutation of model residuals to obtain n=1000 randomized datasets (72, 73). All statistical procedures were conducted in R 3.0.2 (R Development Core Team 2013).

3.4 Results:

General effects

Body size (SL) of the experimental guppies did not differ among populations, except that Lower Lalaja guppies were larger than Upper Lalaja guppies (Appendices 3.A1, 3.A2 and 3.A3). Female guppies were larger than male guppies – and this sexual dimorphism was similar among populations (non-significant interaction between population and sex – Table 3.A2). Infection with *Gyrodactylus* induced higher mortality in female than male guppies (Appendix 3.A4). Although the maximal model also included population (results not shown), its effects were not significant and so population was removed from the final model. Among the guppies that died, day of death was not explained by either population, sex, or the interaction (Appendix 3.A5). A survival analysis using Cox proportional hazards lead to the same conclusion (Appendix 3.A6).

Note that similarity among populations in overall parasite-induced mortality aided our subsequent ability to uncover differences in parasite load because differences in parasite peak load were not then due to a greater accumulation of parasites on individuals that survived for longer periods of time.

Resistance (Questions 1-4)

Population, sex, and their interaction all had strong effects on *Gyrodactylus* peak load and intrinsic rate of increase (*r*) (Table 3.2). We therefore performed planned comparisons that specifically answered our initial questions about parallel evolution of the sexes (Table 3.1). These comparisons were based on simplified models that excluded body size because [1] body size effects were inconsistent (Table 3.2, Appendices 3.A1, 3.A7 and 3.A3), and [2] model simplification through stepwise deletion tended to drop size from all models (except for female peak load). Nonetheless, including body size as a covariate generally did not qualitatively change the results (Appendix 3.A8).

Answer 1 – On sexual dimorphism in the source population: Guppies from the Guanapo source population were sexually dimorphic in resistance – males had higher resistance (lower peak load and lower *r*) than females (Table 3.3). Answer 2 – On female evolution: Females from three of the four introduced populations had higher resistance (lower peak loads and lower *r*) than females from the Guanapo source population (Table 3.3), indicating the evolution of increased resistance in the absence of parasite pressure (as earlier reported by 28). Answer 3 – On male evolution: In contrast to the higher resistance that had evolved in females from three of the four introduced populations (28), male resistance to parasites did not differ between the introduced populations and the Guanapo source population for *r*, and only differed in one case

for peak load (Table 3.3). The exception was that Taylor males had a higher peak load (i.e. decreased resistance) than Guanapo source males. Together these results indicate lack of evolution of resistance in male guppies in the absence of parasites. Answer 4 – On sexual dimorphism in the introduced populations: Of the three populations in which females evolved increased resistance, only one (Lower Lalaja) showed a difference in peak load between males and females (Table 3.3), and none of the three populations showed a difference between males and females in *r* (Table 3.3). In the fourth introduced population (Caigual) neither females nor males evolved and sexual dimorphism was maintained in both *r* and peak load. Overall, sexual dimorphism in resistance decreased in the introduced populations as females evolved to become more similar to the ancestrally more-resistant males (Figure 3.1A), which did not themselves show any (except Taylor for peak load) post-introduction evolution of resistance

Parallelism (Question 5 – On the sex-specific trajectories)

To more formally assess the degree of parallelism in the evolution of resistance between the sexes and populations, we performed two analyses. The first analysis considered trait divergence between the source and introduction populations in male (y-axis) versus female (x-axis) trait-space. This analysis most directly evaluates the relative amount of change in males versus females within populations (Figure 3.2), which thus informs the extent to which the sexes evolved in parallel. In this analysis, all of the four introduced populations showed resistance trait changes that did not overlap with the 1:1 line for peak load and two out of four populations overlapped (for male traits only) with the 1:1 for intrinsic rate of increase, thus confirming non-parallel evolution.

The second analysis considers evolutionary trajectories in resistance trait-space (Figure 3.1), which assesses both parallelism between the sexes (as above) and between the populations within sexes. In this analysis, visual inspection showed that females from the four introduced populations shared the same orientation of phenotypic change vectors, indicating a clear pattern of parallel evolution among female populations, towards higher resistance than the Guanapo source population and towards the ancestral male position (Figure 3.1A). Consistent with the visual inspection, the angles of female trajectories in trait space did not differ among the introduced populations in the PCVA (Table 3.4A). Furthermore, variation around the mean female phenotypic trait spaces only overlapped between the Caigual introduced population and the Guanapo source population, coherent with the lack of resistance evolution in these females (Figure 3.1B). Males showed a very different pattern, with visual inspection implying non-parallel evolution among populations (i.e., different orientations of phenotypic change vectors). However, only 2 of 6 paired comparisons of male phenotypic vector orientations among populations were significant (Table 3.4A), presumably owing to high variation in resistance traits among males (Figure 3.1C). Visual inspection (and the first analysis above) indicated differences between males and females in the orientation of phenotypic trait vectors, although this was statistically significant only for guppies from the Taylor introduced population (Table 3.4B)– for the same reason (high variation in males). Thus, the vector analysis had sufficient power to detect non-parallel evolution when substantial evolution had occurred but not when little evolution had occurred).

3.5 Discussion:

In the ancestral population (Guanapo source), resistance to infection by *Gyrodactylus* parasites was greater for male than for female guppies (Question 1 in Table 3.1). After release from parasite pressure through replicate experimental introductions, the evolution of resistance did not proceed in parallel (Question 5). In particular, males did not show much (if any) evolution of resistance (Question 3), whereas females generally evolved increased resistance (Question 2). The end result was an evolutionary reduction of sexual dimorphism in resistance to parasite infection (Question 4). Our main focus now will be on potential causal explanations for the observed non-parallel evolution, but we must first deal with the possibility that observed sexual dimorphism in resistance could be simply a correlated effect of other differences between the sexes.

Male and female guppies differ in a number of ways that might influence parasite loads, which could thereby confound inferences about sexual differences in resistance *per se*. First, female guppies might owe their higher *Gyrodactylus* numbers in nature (55) to being more gregarious than males (74), because this might promote higher rates of parasite transmission. Second, lower *Gyrodactylus* loads on males in nature might result from greater (relative to females) infection-induced susceptibility to predation or flooding (42), which would truncate the accumulation of parasites. Third, studies in other systems have suggested that ecological differences between the sexes, such as different diets or microhabitats, could lead to sexual dimorphism in parasite loads (75). While these effects could potentially have altered the selective environment of our field guppy populations (see below "selective explanations"), none of them are relevant to the laboratory trial part of our study because (1) the fish were isolated and so parasite transmission was not possible, (2) predators and flooding were absent, and (3) the

sexes had similar diets and habitats. Finally, differences in body size can influence parasite loads (43), but the larger size of females did not explain their lower resistance in our study (Table 3.2, Appendices 3.A7 and 3.A8). All of these observations suggest that the sexual dimorphism in resistance observed in guppies from the Guanapo source, and the Lower Lalaja and Caigual introductions is not simply a correlated effect of other differences between the sexes.

Selective explanations

The key question that our results bring to the forefront is: why should resistance evolve differently for males than for females when both sexes are similarly removed from parasite pressure? We can see three basic possibilities. First, selection imposed by *Gyrodactylus* might have shifted differently for males versus females when the parasite was eliminated through the experimental introductions. Second, selection imposed by other environmental factors might have shifted differently for males versus females, and the resulting non-parallel evolution of other traits might then have driven correlated non-parallel changes in resistance. Third, even if selection shifted similarly for males versus females, they might have responded differently owing to different genetic or functional constraints. We now consider each of these possibilities in turn.

Males and females in the ancestral population were sexually dimorphic for resistance (Figure 3.1), which might imply that they were – in that environment – under different intensities of selection for resistance. In particular, the higher resistance of males than females suggests a history of stronger selection on the former than the latter – perhaps because the negative effects of *Gyrodactylus* infection are stronger for males than for females (42). Stated another way, males might be under stronger selection for resistance to infection because – once infected – they are

less tolerant than females (sensu 76), or more susceptible to predators or flooding. Indeed, this expectation makes sense given that (1) males invest much more of their time and energy into sexual activities – as opposed to feeding – than do females (77, 78), (2) males are more likely to be preyed upon than females (79), (3) infected males are more likely than females to be displaced downstream during flooding events (42), and (4) infected males (but presumably not females) have reduced mating opportunities (46). Starting from these ancestral conditions (stronger selection for resistance in males than females), parasite removal would cause a greater shift in selection for resistance in males (from very strong to absent) than females (from modest to absent). However, it is not clear how these differences could explain our results: according to theory (17), males would be expected to evolve decreased resistance (assuming resistance is costly) and females would be expected to evolve in the same direction but less rapidly. This result clearly was not obtained in our study: males did not evolve resistance (except Taylor males for peak load) and females evolved increased resistance (Figure 3.1).

Alternatively, empirical evidence (80) and theory (18) suggest that hosts could invest in reducing the damage caused by a given parasite load (i.e., tolerance) rather than in mechanisms to reduce parasite numbers (i.e., resistance). It is therefore conceivable that the lack of male resistance evolution (except for Taylor peak load) could be explained if males were instead evolving along the tolerance axis of defense. Yet we did not find evidence to support the evolution of tolerance in males (Appendices 3.A9 and 3.A10) or in females (as reported in 28) for any of the populations. Finally, females in the introduced populations have lower mortality rates and longer life expectancy than males (68), and might thus benefit more from increased resistance (26, see below - 81). Nonetheless, this possibility seems unlikely to explain the differential responses of the two sexes in the introduction given that higher male than female

mortality is a general feature of all guppy populations (79, 82). These results suggest that we must look beyond parasites themselves if we are to explain the non-parallel responses of males and females.

The introduced populations experienced a dramatic shift not only in parasitism but also in several other potential selective forces. Principal among these forces is predation, which is known from many studies to exert a very large effect on guppy evolution (37, 38). Specifically, the ancestral population is subject to high predation whereas the introduced populations are subject to low predation (65). Perhaps the shift in predation differentially influenced selection on males versus females, which then differentially influenced – through a correlated evolutionary response – the evolution of resistance. Indeed, we previously argued that the evolution of *increased* resistance in females following parasite removal might be a pleiotropic by-product of evolution in response to release from predation (28). Under this scenario, strong predator-based selection on, and evolution of, a trait that correlates with resistance drives the evolution of increased resistance despite release from parasitism. For example, the selective shift from a shorter to a longer life expectancy has been shown to cause the evolution of a slower life history in guppies (e.g., maturation at a later age and lower reproductive effort) (83, 84), and this in turn is expected to lead to increased investment in resistance (26, 81). We previously invoked this explanation for the evolution of *increased* resistance in females but it doesn't seem likely to explain why resistance did not evolve in males. The main reason is that males experience stronger predation pressure than females in the ancestral environment (79), and so release from predation should have an stronger effect on males than on females. Thus, we will now assume that males were – like females – under selection for increased resistance following parasite removal.

In the absence of a clear *selective* reason for non-parallel resistance evolution by the sexes, we are drawn to consider explanations not based on the immediate shift in selection. One explanation that emerged naturally from the data and from the above considerations was the presence of a stronger evolutionary constraint on males than on females. First, if historical selection for resistance was stronger on males than on females (see above), males might retain lower available genetic variance for resistance, leading to a weaker response even under an equal shift in selection (85). (Note that, despite sharing their autosomes, males and females often show different genetic variation for the same traits (86)). Supporting this idea, studies of other systems have shown that evolutionary responses to shifts in predation pressure can be limited owing to a lack of variation in defense traits (87). Second, the costs of increasing resistance might not be linear, making progress towards ever higher resistance progressively more costly and likely to occur at a decelerating rate. In our study, this constraint would be greater for males than for females given the former's initially higher resistance (i.e., males are closer to the absolute limit of having no parasites). In either case, it is conceivable that males could be experiencing selection for increased resistance (perhaps for the same reason as females) but they might not increase further in resistance, or they might do so more slowly. Thus, we suggest that sexual dimorphism in the source populations (males more resistant than females) might be constraining further increases in resistance more strongly for males than for females. That is, sexual dimorphism in resistance can constrain parallel evolution of the sexes in response to a similar shift in selection.

Conclusions

Our study was conducted in the growing tradition (e.g., 6, 7) of not just assessing whether evolution is parallel (or convergent) but also of *quantifying* the degree to which evolution is (non)parallel. We here merged this quantitative perspective with the idea of considering parallelism between the sexes (8, 9, 88) rather than only among populations or species. Our most striking result was that males and females showed considerable differences in their evolution of resistance in response to parasite removal. This outcome could arise because the same environmental shift has different consequences for selection on males versus females, as might often be the case. However, we here suggest that the same result can emerge owing to constraints even when the shift in selection is the same. As one type of constraint, the sex that has ancestrally experienced stronger selection might harbor less genetic variation in the trait. As another, the sex with higher trait values might have more difficulty evolving ever higher trait values if the costs of increased trait expression are non-linear. Specifically, males were – in our study –ancestrally more resistant to parasites than were females, and so the dramatic increase in resistance for females was probably unattainable for males. Studies of parallel evolution would benefit from increasing attention to sexual differences and their potential causes and consequences.

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Ethics statement:

All field procedures were approved by the Trinidad and Tobago Ministry of Fisheries. All laboratory procedures were in accordance with animal care guidelines of McGill University (AUP-5759).

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Table 3.1: Planned comparisons representing specific hypotheses to test for parallel evolution of the sexes

Question	Description	Comparison
Q1	Is resistance sexually dimorphic in the source population?	Males vs. females of the Guanapo source
Q2	Did females evolve resistance in the introduced populations?	Introduced females by population vs. Guanapo source females
Q3	Did males evolve resistance in the introduced populations?	Introduced males by population vs. Guanapo source males
Q4	Is resistance sexually dimorphic in the introduced populations?	Males vs. females within introduced populations
Q5	Did the sexes evolve resistance in a similar way?	Change in males vs. change in females from the introduced populations

The set of question addresses the parallel evolution of the sexes under our experimental scenario where we removed parasites and introduced guppies from an ancestral population [Guanapo source (S)] into four tributary streams [Lower Lalaja (L), Upper Lalaja (U), Taylor (T) and Caigual (C)]. The right column lists the planned comparisons of resistance levels between the sexes or populations that need to be conducted to answer the question.

Table 3.2: GLM for *Gyrodactylus* peak load and intrinsic rate of increase (*r*) on *Poecilia reticulata*

Response	Independent Variables	df	Mean Sq	F	P-value
Peak load	Population	4	87392	5.38	<0.001
	Sex	1	107815	6.64	0.011
	Size	1	294972	18.15	<0.001
	Population:Sex	4	59945	3.69	0.007
	Population:Size	4	31981	1.97	0.101
	Residuals	177	16249		
r	Population	4	0.015	4.36	0.002
	Sex	1	0.13	37.22	<0.001
	Size	1	0.002	0.66	0.418
	Population:Sex	4	0.009	2.69	0.033
	Population:Size	4	<0.001	0.17	0.954
	Residuals	159	0.003		

Generalized linear model results for peak load (integer variable with a negative binomial distribution) and intrinsic rate of increase (continuous variable with a normal distribution) of *Gyrodactylus turnbulli* on *Poecilia reticulata* with population and sex as factors and guppy standard length as a covariate.

Table 3.3: Planned contrasts of *Gyrodactylus* peak load and intrinsic rate of increase (*r*) by population and sex

Question	Comparison	Peak load[a]	*r*[b]
Q1	S♀ vs S♂	-3.84***	-4.42***
Q2	S♀ vs C♀	-1.11	-1.43
	S♀ vs L♀	-3.8***	-3.83***
	S♀ vs T♀	-2.75**	-3.19**
	S♀ vs U♀	-3.95***	-3.36**
Q3	S♂ vs C♂	0.22	-1.32
	S♂ vs L♂	-1.91	-0.86
	S♂ vs T♂	2.98**	0.65
	S♂ vs U♂	-0.25	-0.22
Q4	C♀ vs C♂	-2.42*	-4.93***
	L♀ vs L♂	-3.78***	-2.27[c]
	T♀ vs T♂	1.81	-0.83
	U♀ vs U♂	-1.08	-1.88

Planned comparisons with generalized linear models for peak load (integer variable with a negative binomial distribution) and intrinsic rate of increase (*r* -continuous variable with a normal distribution) of *Gyrodactylus turnbulli* on *Poecilia reticulata.* Comparisons test whether peak load or *r*: [Q1] was sexually dimorphic in the ancestral population; [Q2] evolved in the introduced females; [Q3] evolved in the introduced males; and [Q4] was sexually dimorphic in the introduced populations. [a]z-values reported for peak load and [b]t-values for *r*. Abbreviations for population names: Guanapo source (S) population, and Lower Lalaja (L), Upper Lalaja (U), Caigual (C) and Taylor (T) introduced populations. *p<0.05, **p<0.01, ***p<0.001, [c] Non-significant after FDR correction.

Table 3.4: Vector angles of resistance evolutionary trajectories

(A)
Between populations

	Comparison	Angle (°)
Males	Caigual vs. Taylor	164.5
	Lower Lalaja vs. Caigual	13.6
	Lower Lalaja vs. Taylor	**178.1****
	Lower Lalaja vs. Upper Lalaja	1.3
	Upper Lalaja vs. Caigual	14.9
	Upper Lalaja vs. Taylor	**179.4****
Females	Caigual vs. Taylor	3.1
	Lower Lalaja vs. Caigual	1.6
	Lower Lalaja vs. Taylor	4.7
	Lower Lalaja vs. Upper Lalaja	4.1
	Upper Lalaja vs. Caigual	5.7
	Upper Lalaja vs. Taylor	8.8

(B)
Within populations (females vs. males)

	Population	Angle (°)
	Caigual	1.8
	Taylor	**165.2***
	Upper Lalaja	14.9
	Lower Lalaja	17

Quantification of evolutionary trajectories in resistance trait space based on measures of orientation (vector angles in degrees) derived from PCVA. (A) Comparisons of female and male trajectories within each of the introductions relative to the source populations. (B) Comparisons between populations within the sexes. Observed significance levels are based on empirically generated (n=1000) residual permutations (72,73). *$p<0.05$, **$p<0.01$.

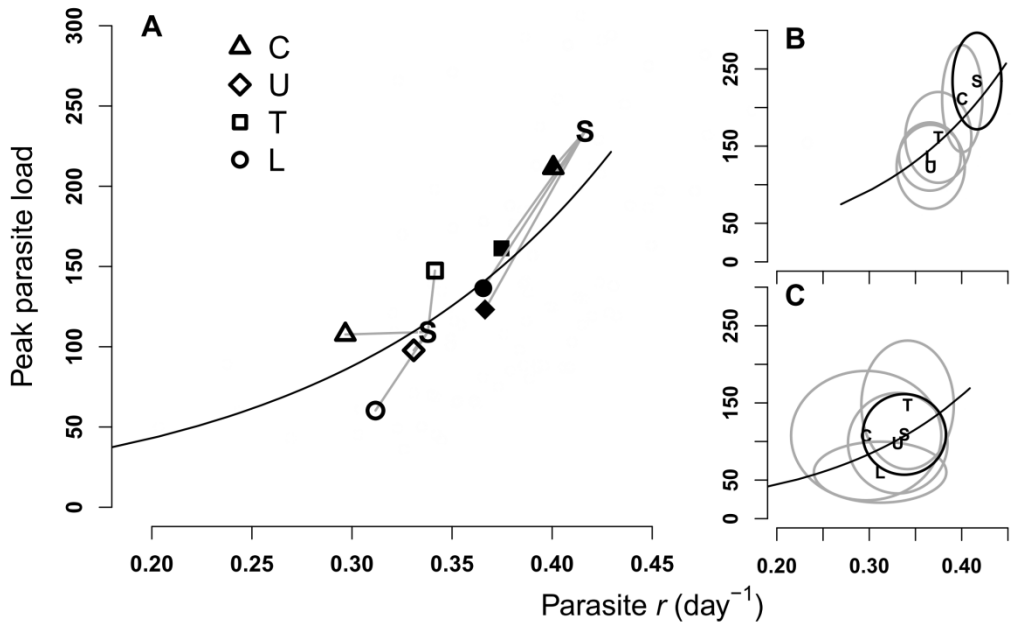

Figure 3.1: Evolutionary divergence for males and females in resistance trait-space

(A) For each of the four introduced populations (L, U, T, and C), the evolutionary trajectory within the sexes (males: empty symbols, females: filled symbols) is depicted as the line connecting the source population centroid (S) to the introduced population centroids. Panels B (females) and C (males) show the same data for each sex separately, with letters depicting population centroids and ellipses representing the 75% data spread. Curved trajectories in each of the three panels depict the negative binomial GLM fit to the raw data to further illustrate the evolutionary trajectories overall (A), for females (B), and for males (C).

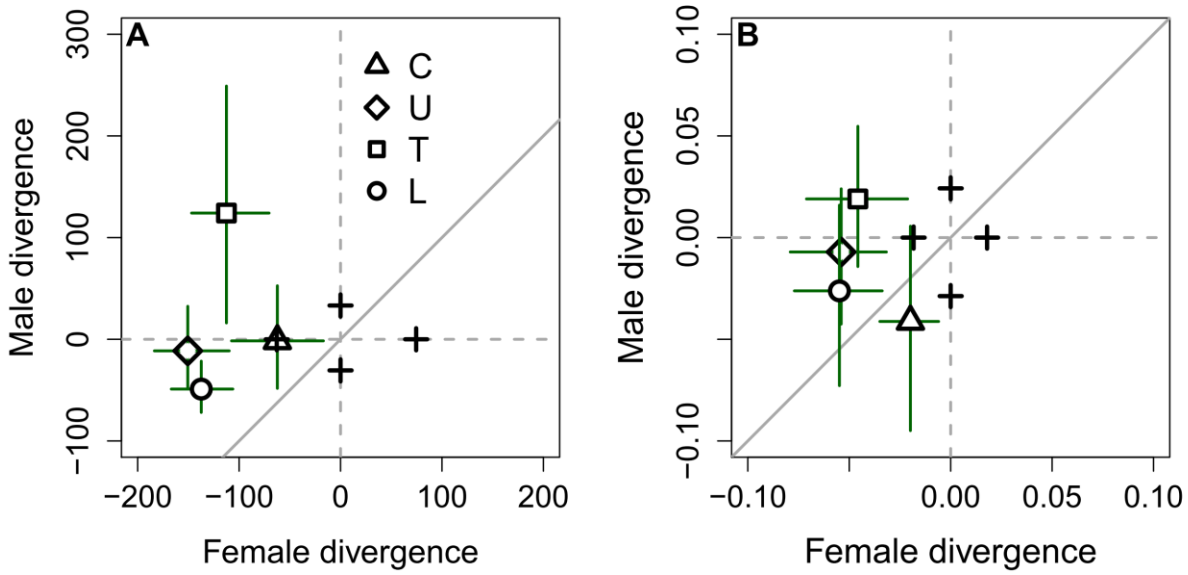

Figure 3.2: Symmetry plots of the evolution of resistance by sex

Evolution of parasite resistance for females (x-axis) and males (y-axis) in the four introduced populations (L, U, T and C) based (A) on the mean number of *Gyrodactylus* at peak load, and (B) on the mean intrinsic rate of *Gyrodactylus* increase from the start of infection to day 10 of infection. In each case, population means are shown relative to the Guanapo source population mean (the origin in each graph); e.g., intrinsic rates of increase of parasites on female Taylor guppies were on average 0.046 day^{-1} lower than those on Guanapo Source females but 0.019 day^{-1} higher when the respective male populations were compared with one another. Points depict population means with 95% CIs and crosses depict 95% CIs for the source mean (CIs based on n=1000 bootstrap samples). The 1:1 line represents parallel evolution, i.e., where both males and females would have experienced identical changes. Non-overlap of the introduced population CIs with the 1:1 line suggests that the sexes in a given population did not evolve resistance in a parallel manner.

APPENDIX 3.A

Parting ways: Parasite release in nature leads to non-parallel evolution of the sexes

Felipe Dargent, Gregor Rolshausen, Andrew Hendry, Marilyn Scott and Gregor Fussmann. (in review). Parting ways: Parasite release in nature leads to non-parallel evolution of the sexes. *The American Naturalist.*

Table 3.A1: Experimental population characteristics and summary results

Variable	Guanapo source		Lower Lalaja		Upper Lalaja		Taylor		Caigual	
Sex	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male
Sample size	34	12	24	13	18	12	24	15	29	11
SL (SEM)	18.1 (0.3)	16.2 (0.3)	18.9 (0.5)	16 (0.3)	17.3 (0.6)	15.6 (0.2)	17.4 (0.6)	15.9(0.2)	17.7 (0.4)	15.7 (0.2)
Survival n (%)	2 (6)	4 (31)	7 (29)	6 (46)	4 (22)	3 (25)	6 (25)	4 (25)	5 (17)	6 (50)
Alive at day 10 n (%)	30 (88)	10 (83)	22 (92)	12 (92)	17 (94)	11 (92)	22 (92)	15 (100)	26 (90)	11 (100)
Site Characteristics										
Gyrodactylus presence	Present		Absent		Absent		Absent		Absent	
Predation	High		Low		Low		Low		Low	

Characteristics of the populations and individuals used for this study by sex.

Table 3.A2: ANOVA for guppy standard length by population and sex

	df	Mean Sq	F	Pr (>F)	Differences
Population	4	9.87	2.77	0.03	L>U
Sex	1	166.77	46.8	<0.001	F>M
Population:Sex	4	2.46	0.7	0.6	
Residuals	182	3.57			

ANOVA model with guppy standard length (SL) as response variable and population of origin (S, L, U, T or C) and sex (male or female) as factors.

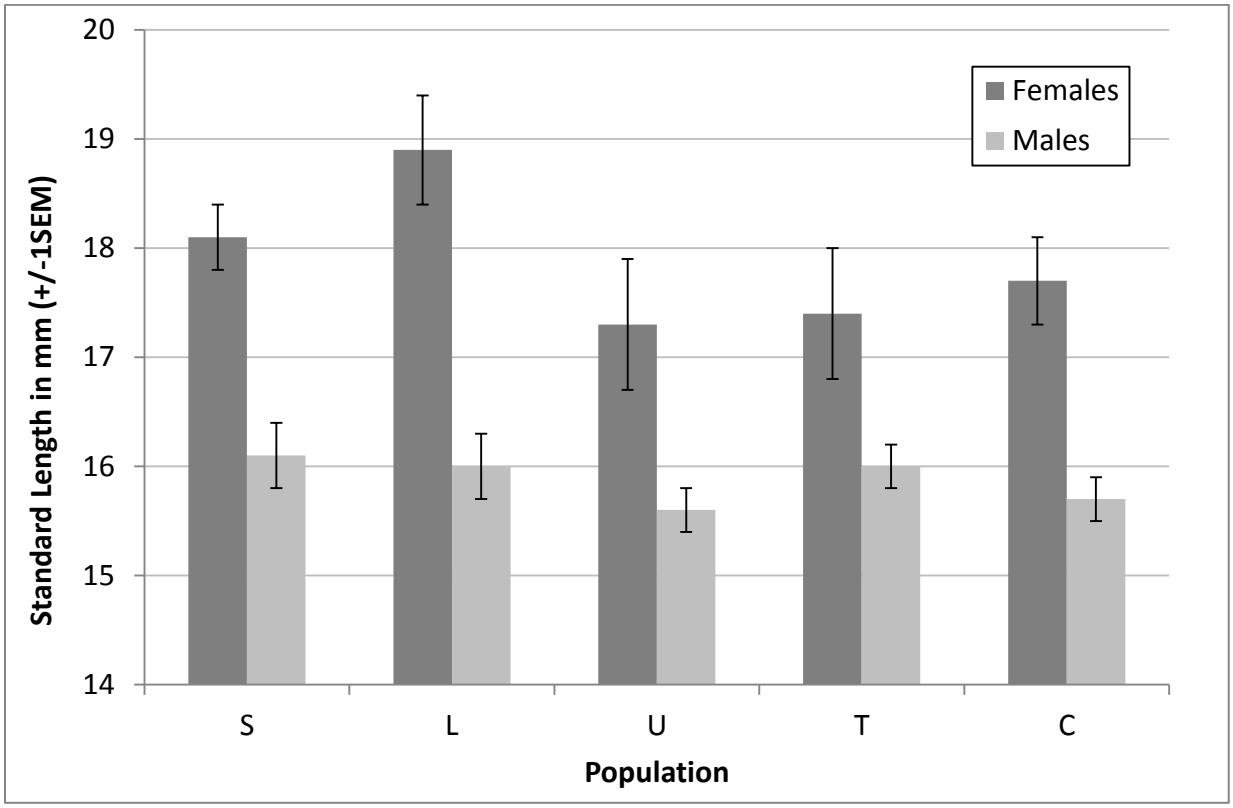

Figure3.A3: Guppy size (mm) at start of infection by population and sex

Mean standard length of infected guppies on the first day of infection (±1SEM). Abbreviations for population names: Guanapo source (S) population, and Lower Lalaja (L), Upper Lalaja (U), Caigual (C) and Taylor (T) introduced populations.

Table 3.A4: GLM for death during experiment by sex

	Estimate	SE	z-value	p > |z|
Intercept (females)	1.53	0.23	6.64	<0.001
Males	-0.97	0.35	-2.8	0.005

Generalized linear model results for death (nominal variable with two levels, died or survived, with a binomial distribution) of *Poecilia reticulata* with sex and population as factors. Maximal model including population and its interactions with sex was simplified through stepwise AIC deletions. Values are presented relative to females.

Table 3.A5: GLM for day of death by population and sex

variable	df	Mean Sq	F	p-value
Population	4	0.07	0.91	0.46
Sex	1	0.14	1.98	0.16
Population:Sex	4	0.06	0.88	0.48
Residuals	136	0.07		

Generalized linear model results for ln-transformed day of death (continuous variable with a normal distribution) of *Poecilia reticulata* individuals that died during the experiment. Sex and population, and their interaction were included as factors.

Table 3.A6: Survival analysis using Cox proportional hazards

variable	df	χ^2	p>|Chi|
Population	4	8.31	0.081
Sex	1	10.13	0.001
Population:Sex	4	1.46	0.834

Survival analysis with Cox proportional hazards for survival until the end of the experiment (day 24) using day of death as response variable, and population of origin and sex as explanatory variables.

Table 3.A7: GLMs for *Gyrodactylus turnbulli* peak load and intrinsic rate of increase (*r*) on *Poecilia reticulata* by sex

Response	Sex	variable	df	Mean Sq	F	p-value
Peak load	Males	Population	4	64200	3.278	0.018
		Size	1	2531	0.129	0.721
		Population:Size	4	3577	0.183	0.946
		Residuals	53	19587		
	Females	Population	4	81816	5.488	<0.001
		Size	1	302092	20.263	<0.001
		Population:Size	4	35616	2.389	0.055
		Residuals	119	14908		
r	Males	Population	4	0.007	1.36	0.261
		Size	1	0.018	3.60	0.064
		Population:Size	4	0.002	0.42	0.796
		Residuals	48	0.005		
	Females	Population	4	0.014	5.34	<0.001
		Size	1	0.007	2.51	0.116
		Population:Size	4	<0.001	0.12	0.976
		Residuals	106	0.003		

Generalized linear model results for peak load (integer variable with a negative binomial distribution) and intrinsic rate of increase (continuous variable with a normal distribution) of *Gyrodactylus turnbulli* on *Poecilia reticulata* by sex, with population as factor. Abbreviations for population names: Guanapo source (S) population, and Lower Lalaja (L), Upper Lalaja (U), Caigual (C) and Taylor (T) introduced populations.

Table 3.A8: Planned contrasts of *Gyrodactylus* peak load on *Poecilia reticulata* with size as a covariate

Question	Comparison	Peak load[a]
Q1	S♀ vs S♂	-1.41[b]
Q2	S♀ vs C♀	-0.94
	S♀ vs L♀	-4.39***
	S♀ vs T♀	-2.44*
	S♀ vs U♀	-3.53***
Q3	S♂ vs C♂	-0.15
	S♂ vs L♂	-1.94
	S♂ vs T♂	2.91**
	S♂ vs U♂	-0.32
Q4	C♀ vs C♂	-1.09[b]
	L♀ vs L♂	-2.42*
	T♀ vs T♂	2.63**[b]
	U♀ vs U♂	-0.42

Planned comparisons with generalized linear models for peak load (integer variable with a negative binomial distribution) of *Gyrodactylus turnbulli* on *Poecilia reticulata.* Models included standard length (SL) as a covariate. Comparisons test whether peak load: [Q1] was sexually dimorphic in the ancestral population; [Q2] evolved in the introduced females; [Q3] evolved in the introduced males; and [Q4] was sexually dimorphic in the introduced populations. [a]z-values reported for peak load. Abbreviations for population names: Guanapo source (S) population, and Lower Lalaja (L), Upper Lalaja (U), Caigual (C) and Taylor (T) introduced populations. *p<0.05, **p<0.01, ***p<0.001. [b]tests which differ qualitatively from Table 3.3 (for Q1 SL was significant z=3.87, p<0.001; for Q4, Caigual: SL was significant z=2.26, p=0.02; Taylor: SL was non-significant z=1.96, p=0.05).

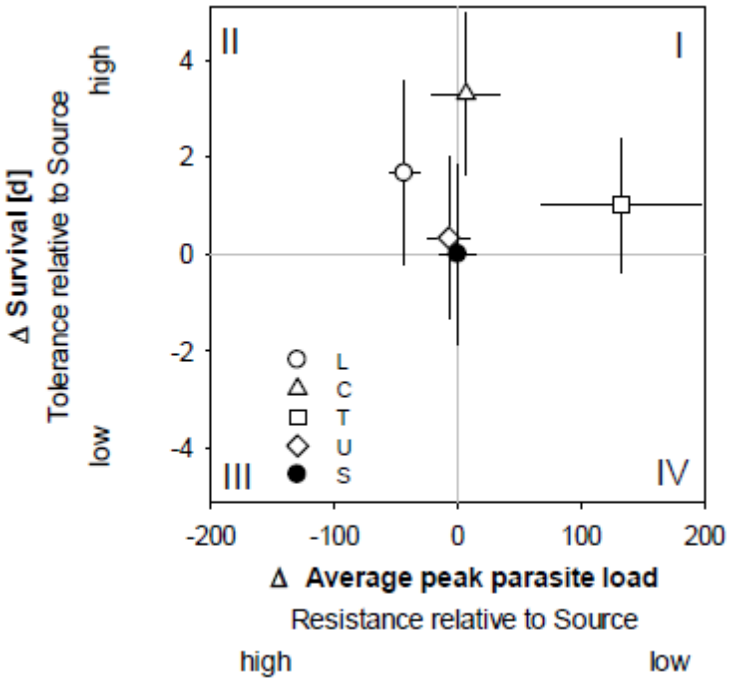

Figure 3.A9: Evolution of male guppy resistance and tolerance to *Gyrodactylus* parasites

Difference in average *G. turnbulli* (a measure of resistance) versus average difference in survival (day of death – a measure of tolerance) between each of the introduced populations and the Guanapo source population (centered at the origin). Non-zero differences suggest evolution in a given direction. Quadrants I and III indicate a negative correlation (trade-off) between resistance and tolerance. Abbreviations for population names: Guanapo source (S) population, and Lower Lalaja (L), Upper Lalaja (U), Caigual (C) and Taylor (T) introduced populations. Error bars represent 1 SEM.

Table 3.A10: Male guppy survival as a measure of tolerance

Coefficient	Estimate	SEM	Z-value	p-value
Caigual	-2.21	1.17	-1.89	0.058
Lower Lalaja	-1.72	1.04	-1.65	0.098
Taylor	-0.53	0.86	-0.62	0.538
Upper Lalaja	-0.8	0.97	-0.83	0.407
Peak load	-0.003	0.01	-0.48	0.634
Caigual:Peak load	0.01	0.01	1.53	0.125
Lower:Peak load	0.02	0.01	1.91	0.056
Taylor:Peak load	0.004	0.01	0.57	0.572
Upper:Peak load	0.01	0.01	1.06	0.291

Cox proportional hazards results for survival until the end of the experiment (day 24) with "day of death" as a response variable, and "population of origin" and "parasite peak load" as explanatory variables. Values are for males of a given introduction relative to males from the Guanapo source population. Higher tolerance is indicated by higher population survival for a given parasite load at peak (i.e. significant interaction coefficient).

CONNECTING STATEMENT

In chapters 2 and 3 I explored the direct effects of enemy release on the evolution of resistance. A population of guppies that was initially sexually dimorphic in resistance to a common and deleterious parasite (*Gyrodactylus* spp) showed non-parallel evolution of the sexes after experimental removal of the parasite in the wild: females evolved increased resistance while males did not evolve. Rapid divergence in resistance, as well as in other traits (i.e. life-history), between the source and introduced populations indicates the potential for speciation.

Temporally stable divergent parasite loads that impose fitness costs to their hosts are a potential, and scarcely explored, mechanism of speciation. Host evolution in response to divergent parasitism could lead to reproductive isolation through immigrant or hybrid inviability as well as through differences in mate choice. Given that the traits assessed in previous chapters – defence against parasites – are expected to be most strongly influenced by interactions with parasites, I place special emphasis on parasitism as an important axis of environmental change. Nonetheless, as a driver of speciation, parasites are one among many axes of environmental change that could lead to reproductive isolation.

In the following chapter I continue to assess the evolutionary implications of enemy release. I test whether strong multidimensional divergent selection leads to ecological speciation using the guppy introductions described in previous chapters. This shift to multidimensionality, which is expected to enhance the strength of selection and its multifariousness (i.e., the existence of independent trait dimensions on which selection occurs), occurs due to divergence in enemy levels (both in parasitism and predation), as well as divergent diets and resource availabilities. Since there has been no contact in nature between the introduced and source populations I do not explore immigrant or hybrid inviability; instead, I assess the evolution of mate choice.

CHAPTER 4

An experimental manipulation in nature testing whether multifarious divergent selection promotes the contemporary evolution of assortative mating

Felipe Dargent*, Lisa Chen*, Gregor Fussmann and Andrew Hendry. (in prep.). An experimental manipulation in nature testing whether multifarious divergent selection promotes the contemporary evolution of assortative mating.

*Equally contributing authors.

4.1 Abstract:

Progress toward ecological speciation is expected to be enhanced when divergent selection is multi-dimensional, because this property increases the total strength of selection and enhances its multifariousness. We used replicate experimental introductions of guppies (*Poecilia reticulata*) in nature to test whether an abrupt and dramatic shift in at least four important ecological dimensions (diet, resource levels, predation, and parasitism) promoted the contemporary evolution of assortative mating. After 8-12 guppy generations of evolution in the wild, we bred each population in a common-garden laboratory environment for two generations, after which we recorded the preferences of females from each population for males from each population in a paired design. Despite the rapid evolution of male signaling traits, positive assortative mating did not evolve. Instead, females tended to prefer novel males, which generated dissassortative mating. In our system, strong multifarious divergent selection did not promote the rapid evolution of assortative mate choice, probably because other aspects of sexual selection act against the evolution of this reproductive barrier.

Key words: Experimental evolution, non-parallel evolution, mate choice, reproductive isolation, ecological speciation, *Poecilia reticulata*

4.2 Introduction:

Ecological speciation – the build-up of reproductive barriers owing to divergent natural selection between environments – is now thought to be a primary driver of speciation (1-3). It is also true, however, that many instances of divergent environments do not seem to generate much progress toward ecological speciation (4, 5). Current research efforts are thus increasingly focused on determining the factors that promote and constrain ecological speciation. One recently hypothesized promoter is increasing dimensionality of divergent selection (6), which is expected to increase the total strength of divergent selection and perhaps also the number of independent trait dimensions on which selection acts (i.e., multifarious selection). The consequence of both effects then should be increased progress toward ecological speciation (3, 5). To test these ideas, an ideal experiment would manipulate – in replicate natural populations – the strength and dimensionality of divergent selection, and thereby test which factors lead to the more rapid evolution of reproductive barriers. To gain some initial insight into this approach, we here used replicate experimental introductions in nature to test whether or not very strong multifarious divergent selection can lead to the rapid evolution of assortative mate choice. If such a result is obtained, the next step would be to experimentally manipulate the various dimensions of selection.

Our experiment (details below) simultaneously manipulates at least four major dimensions of selection in nature: diets, resource levels, predation, and parasitism. Diet variation is the classic dimension of selection considered in many studies of ecological speciation, including fish feeding on benthic versus limnetic diets (7, 8) and birds feeding on different types of food (9, 10). Resource levels (independent of specific diets) are less frequently considered but could be important owing to divergent selection for resource use efficiency leading to morphs

that are more competitive under high or low resource availability (11). Predation is increasingly considered in studies of ecological speciation, including studies of variation in predation regime (fish versus dragonfly) (12), and predator-present versus predator-free environments in amphipods (13) and fish (14). Finally, parasitism is increasingly suggested to be an important diver of ecological speciation. As this last aspect of selection is of intense recent interest (15-20), we now provide some more details on its expected role.

Parasites could drive ecological speciation in several ways (15). First, direct natural selection against migrants and hybrids can occur as a result of their maladaptation to local parasites (20, 21). Second, divergent natural selection on the immune system (e.g., MHC) can pleiotropically generate preferences for local mates (18). Third, parasite-mediated divergent sexual selection (i.e., mate choice based on divergent traits that better communicate resistance in each environment) can also generate assortative mating. Despite enthusiasm for these ideas, empirical support is generally weak as many studies have thus far been correlative. It is therefore important to add manipulations of parasitism to experimental investigations of the role of multi-dimensional divergent selection in contributing to ecological speciation.

An experimental manipulation of multiple dimensions of selection could track any number of reproductive barriers that might evolve, including natural selection against migrants, natural selection against hybrids, and assortative mating. We here focus on assortative mating as its evolution is thought to be necessary for substantial progress toward ecological speciation (3, 22). That is, selection against migrants and hybrids can exist without much restriction in gene flow between environments. Assortative mating could arise through several mechanisms, such as divergent habitat preferences, divergent reproductive timing, or divergent mate choice. However, in our empirical system (see below), the post-manipulated populations are not in contact

(negating a role for divergent habitat choice) and the organism reproduces continually (negating a role for divergent timing). We therefore focus on assortative mate choice, which is a common emphasis of studies testing for the role of divergent selection in driving ecological speciation (7, 14, 23-26).

Our study

We studied Trinidadian guppies (*Poecilia reticulata*) translocated between locations differing dramatically in each of the four aspects of selection listed above. Specifically, guppies were introduced from site characterized by high invertebrate -consumption diets (owing to high selectivity), high resources (owing to open canopies), high predation (from piscivorous fishes), and high parasitism (specifically from the monogenean ectoparasites *Gyrodactylus* spp) into two independent sites characterized by high diatom- and detritus-consumption diets (owing to lower selectivity), low resources (owing to closed canopies), low predation (no major piscivorous fishes), and low parasitism (no *Gyrodactylus*). Each these selective forces is known to drive dramatic adaptive divergence between populations: diet divergence shapes trophic morphology (27) and life-histories (28), resource level divergence shapes life histories (29), predation divergence shapes a great diversity of traits (30, 31) , and parasitism shapes patterns of resistance (32). Importantly, many of these adaptive differences have been shown – through experimental introductions – to evolve within very short periods of time, often just a few generations (33-36). Indeed, many of these precise effects have been observed in our specific study populations (35, 37, 38).

A number of these adaptive differences could form the basis for the evolution of assortative mating. For instance, divergence in predation regimes and resource levels strongly

influence divergence in male size and colouration (30, 31), traits that strongly influence female mate choice (39). In addition, divergence in parasite communities is known to cause divergence in MHC genotypes (40), which also have strong effects on female mate choice (41, 42). At the same time, however, several aspects of female preference might constrain the evolution of assortative mating (43, 44). For instance, females sometimes have conserved preferences (45), females sometimes prefer "novel" males (46), female preferences are frequency dependent (47), and males can use "sneaky" copulations to circumvent female choice (48). The influence of strong divergent selection along multiple dimensions on the evolution of assortative mate choice is therefore not certain *a priori* but rather a question ripe for empirical analysis.

Following the replicate experimental introductions (details below), the guppies were allowed to evolve in their new (introduced populations) and ancestral (source population) environments for 8-12 generations. We then collected guppies from the wild, reared them for two generations in a common-garden laboratory environment, and assessed divergence in male signaling traits and female preferences. We specifically address four questions. First, to what extent have male signaling traits that might influence mate choice diverged between the source and introduced populations? This question addresses the potential for male trait divergence to shape the evolution of assortative mating. Second, do females prefer sympatric (from the same population) over allopatric (from a different population) males? This questions tests whether assortative mating has evolved during the experiment. Third, is female preference for sympatric males contingent on the allopatric male population given as an alternative? This question addressed the possibility of context-dependence in the evolution of assortative mating. Fourth, is female preference for sympatric males influenced by male signaling traits? This question allows

us to consider whether observed patterns are exclusively shaped by the specific traits we measured or whether other, unmeasured, traits might also be important.

4.3 Methods:

Experimental introductions

We compared guppies from an ancestral population to guppies from two descendant populations established through a large scale translocation experiment (35, 37, 49). Briefly, juvenile guppies were collected in 2008 from the main channel of the Guanapo River in Trinidad ("Guanapo source" -10° 38′ 23″N, 61°14′54″W and 10° 39′ 14″N, 61°15′ 18″W). This site has four key features relevant to our study: [1] the monogenean ectoparasite *Gyrodactylus* spp. is present, [2] large predatory fishes (including *Crenicichla* sp.) are present, [3] resource availability is high and [4] invertebrate availability is high. The collected guppies were quarantined and treated for a wide spectrum of pathogens, and – once mature - were introduced (37 males and 37 females) into a tributary stream of the Guanapo River (Lower Lalaja). To increase genetic diversity, the introduced females had earlier been mated in groups of five with five non-introduced males from the source population. In 2009, this process was repeated, with the guppies being introduced into another tributary of the Guanapo River (Taylor – 64 guppies of each sex). The Lower Lalaja and Taylor environments, which are similar to each other, differed from the source environment in the above key features: [1] *Gyrodactylus* spp. are absent, [2] large piscivorous predators are absent, [3] resource availability is low and [4] invertebrate availability is low (35, 38, 50). Following introduction, the guppies were allowed to experience the new selective environments for three (Lower Lalaja) and two (Taylor) years, which are equivalent to approximately 12 and 8 guppy generations (51).

In 2011, guppies were collected from the source and introduction sites, transported to laboratory facilities, and raised for two generations in a common-garden environment (for details see 35). For this reason, any observed differences among the populations likely reflect genetic effects rather than plasticity or maternal effects (33). Field-collected guppies and F1 laboratory-reared guppies were bred and raised by C. Ghalambor at Colorado State University (Fort Collins, Colorado). The F1 guppies were then transferred to our laboratory at McGill University (Montreal, Quebec) to breed the F2 laboratory-reared generation and to perform the mate choice trials (see below). As the F2 guppies matured, males and females were separated so that the females remained virgin until the experiments described below. Families – individuals born from the same mother – were also kept separate from each other. All guppies were fed paste made from water and Tetramin Tropical Flakes (Tetra, Melle, Germany) and were housed in a flow-through system (Aquaneering Inc., San Diego, California) that standardized water conditions.

Mate choice trials

Mate choice trials were conducted in 5 gallon glass tanks (40.6 cm x 20.3 cm x 25.4 cm) filled with 8 l of municipal water that was carbon filtered and treated with Freshwater Biozyme (Mardel, Oklahoma city, USA) and Prime (Seachem Laboratories , Madison, USA). The tanks were divided into three compartments (Figure 4.1) by means of transparent acrylic sheets (0.080 inch Clear Acrylic Sheet - Plaskolite, Columbus, USA) bordered by 1.5 cm of 700 µm syrex net to allow for the flow of any chemical cues. At both ends of the central compartment, a 5 cm wide (approximately two female body lengths) preference zone was delineated on the exterior of the tank to quantify female association with males in the different compartments. Similar tank

designs are commonly used in studies of mate preferences in fish, including guppies (52-54), and association strongly correlates with probability of mating (55, 56).

Prior to a trial, a female guppy was placed into the central compartment to habituate overnight (20 hours). On the day of the trial, the experimental tank was illuminated from overhead by an 18" full spectrum fluorescent light (Aqueon Products, Franklin, U.S.A.). To prevent female-male interactions prior to the onset of a trial, the female was isolated in a 12 cm diameter opaque cylinder in the center of the tank, while males from two different populations were added into the side compartments and allowed to habituate for 10 minutes. After habituation, the opaque cylinder was gently removed to release the female and a thin layer of cheesecloth was placed on top of the experimental tank to simulate dusk/dawn conditions when courtship is highest in nature (57, 58). After starting the video recording equipment (Canon PowerShot SD1000 - Canon, Melville, USA), the experimenter left the room and allowed the trial to run for 20 minutes.

Following each trial, the guppies were anesthetized in 0.02% Tricaine Methanesulfonate (MS-222 - Argent Chemical Laboratories, Redmond, USA) buffered to a neutral pH with NaCO3. Each guppy was then weighed (nearest 0.0001 g) and photographed on its left side with a Nikon D90 camera (Nikon, Mississauga, Canada) with an attached Speedlight Commander Kit R1C1 (Nikon, Mississauga, Canada). Additional illumination was provided by two full-spectrum fluorescent bulbs (Aqueon Products, Franklin, U.S.A.), and a scale and a X-Rite color checker card (X-Rite, Grand Rapids, U.S.A.) were visible in each image. To reduce and thoroughly mix any residual odours after each trial, two thirds of the water in the tank was replaced with fresh-prepared water and allowed to sit for a day before a new trial was performed.

Three types of "male population pairings" (MPPs: Guanapo vs. Lower Lalaja, Guanapo vs. Taylor, Lower Lalaja vs. Taylor) were generated and tested with females of each population, which led to nine possible "female population by male population pairings" (FMPPs). Each FMPP was replicated 10 times, generating a total of 90 trials conducted between December 2012 and February 2013. Each female was used in only one trial (n = 30 per population) and each male was used in a maximum of two trials (Guanapo source, n = 46; Taylor, n = 38; Lower Lalaja, n = 37). The specific guppies used in a given trial were selected at random with the following criteria: [1] sympatric (from the same population) males and females could not be from the same family, and [2] if more than one female from a family was used they had to be tested with males from different families. If a male was used twice, it could not be tested [1] for at least 10 days after the first trial, [2] against a female of the same population as in the first trial, or [3] against a male of the same family as the previous trial.

Phenotypic traits and female preferences

For each male guppy, we used ImageJ 1.46r (National Institutes of Health, Bethesda, USA) to measure (nearest 0.01 mm) standard length (SL – tip of lower jaw to end of caudal peduncle) and tail size (end of caudal peduncle to end of tail along the mid line). We also measured body mass (BM) to the nearest 0.0001 g. SL and BM were used to calculate individual condition (relative condition index, K_n) following Le Cren (59): K_n=(BM)/a(SL)^b, where a and b are the intercept and slope of a least-squares regression of BM on SL across all male guppies. We also measured the area of the body covered by three colours that influence mate choice in guppies: orange (including red), yellow, and black (53, 60, 61). Following standard procedures (e.g., 62), the colours were first visually identified and categorized. The area of each colour spot

was then measured in ImageJ and summed across all spots of that colour to obtain the total area of each colour on a guppy. Colour areas were then expressed as a relative proportion of guppy area: i.e. colour area divided by guppy area. All measurements were performed by the same person (LC) and were highly repeatable: orange area (R^2=0.86), yellow area (R^2=0.85), black area (R^2=0.83), total area (R^2=0.99), SL (R^2=0.94), and tail size (R^2=0.94).

We used JWatcher Version 1.0 (63) to analyze the video recordings for female preference (time spent in each preference zone). Each video was analyzed for 20 minutes, starting 30 seconds after the experimenter left the room. Two measures of female preference were quantified: [1] time with the focal male (details below) minus time with the non-focal male divided by time spent with both males (i.e., relative preference for focal male), and [2] time with focal male minus time with the non-focal male divided by the trial duration (i.e., absolute preference for focal male). These two metrics yielded qualitatively similar results (Appendices 4.A1, 4.A2 and 4.A3), and so we here present results based only on relative preferences. In sympatric-allopatric FMPP combinations (female from the same population as one of the males), the focal male was the sympatric male. In allopatric-allopatric FMPP combinations (female from a different population than both males), Taylor males were (arbitrarily) considered focal for Guanapo source and Lower Lalaja females, whereas Lower Lalaja males were (arbitrarily) considered focal for Taylor females.

Statistical analysis

To assess how males differed in signaling traits among populations, we used a multivariate analysis of variance (MANOVA), where the predictor variable was male population and the response variables were the male traits: orange area, yellow area, black area, SL, tail

size, and K_n. We also analyzed each trait individually in univariate analysis of variance (ANOVA) followed by Tukey's HSD tests. In addition, we used the ade4 (64) and MASS packages in R (65) to perform a linear discriminant analysis with male population as the categorical grouping variable. For males that were used in two trials, we here analyzed data from the first trial only - although similar results were obtained using data for both trials (Appendix 4.A4). Also, including SL as a covariate instead of as a response variable did not change qualitatively the results, with the exception of tail size (results not shown).

To assess the evolution of female preference, we first tested for whether or not preferences diverged from zero (i.e. no preference for either focal or non-focal male) within each FMPP, and then for how preferences differed between sympatric-allopatric FMPPs. For the first inference, we used one-sample t-tests. For the second inference, we performed an ANOVA with female preference as a response variable and FMPP as a fixed factor. Finally, to test if female preferences for (or against) sympatric males were context dependent (i.e., depended on the specific allopatric male population in the pairing), we performed planned-comparison t-tests that compared the two sympatric versus allopatric male pairs within each female population.

To assess how male signaling traits influenced female preferences for (or against) sympatric males, we modeled female preference (relative preference for or against sympatric males) using GLMs with a normal distribution and identity link function. The model included FMPP as a fixed factor and the difference between the sympatric and allopatric male canonical scores for the first and second canonical variates. All analyses were conducted in R (65).

4.4 Results:

Males differed in signaling traits among populations in MANOVA (Pillai's trace=0.651, $F_{2,118}$=9.17, p<0.001 – Figure 4.2) and in individual ANOVAs. In fact, with the exception of relative orange area ($F_{2,118}$=0.72, p=0.49), all measured traits differed among populations (relative black area: $F_{2,118}$=11.92, p<0.001; relative yellow area: $F_{2,118}$=25.68, p<0.001; SL: $F_{2,118}$=9.62, p<0.001; tail size: $F_{2,118}$=4.17, p=0.018; K_n: $F_{2,118}$=8.24, p<0.001). Specifically, [1] Guanapo source males had more relative black area and higher condition than did Lower Lalaja males, [2] Guanapo source males had more relative black area and higher condition but less relative yellow area and shorter SL than did Taylor males, and [3] Taylor males had more relative yellow area, larger SL and larger tails than did Lower Lalaja males. Although males from the three populations show some trait overlap, they could be discriminated from each other to some extent in discriminant analysis: Guanapo = 24% misclassified, Lalaja =43%, and Taylor = 39% (Figure 4.3). In general, Lower Lalaja males were generally intermediate between Guanapo source and Taylor introduced males. Indeed, classification improved considerably when Lower Lalaja males were excluded from the discriminant analysis (percent misclassified: Guanapo = 7% and Taylor = 24%).

The t-tests showed significant female preferences for a particular male population in some FMPPs but not others (Table 4.1, Figure 4.4). Females from the Lower Lalaja population did not show any significant preferences, whereas females from the other populations showed context-dependent preferences (Table 4.1A, Figure 4.4): they preferred allopatric males not from the Lower Lalaja. Specifically, Guanapo females preferred Taylor males over their own sympatric Guanapo males, and Taylor females preferred Guanapo males over their own

sympatric Taylor males (Table 4.1A, Figure 4.4). All females, when given the choice between two allopatric males, showed no preference (Table 4.1B).

Female preferences for sympatric males varied among FMPPs (ANOVA: $F_{5,54}$=3.16, p=0.014). In particular, Guanapo and Taylor females showed differences in the degree of preference for sympatric males between MPPs (Table 4.2). The first canonical variate (relative yellow and black areas -Figure 4.3) discriminating sympatric versus allopatric male traits explained some of the variation in female preference (Table 4.3), but did not change the above conclusions regarding variation in female preferences for sympatric males in relation to MPP.

4.5 Discussion:

After experimentally establishing populations whose environments differed along at least four major axes of selection (diets, resources, predators, and parasites), we assessed the degree to which male signaling traits, female preferences, and assortative mate choice evolved after 8-12 generations. We found that males from the introduced populations diverged in signaling traits relative to males from the Guanapo source population, and female preferences seem to be conserved. In particular, when females showed a clear difference in preference between males, they use the same cue in each case: they preferred novel (allopatric) males over their local (sympatric) males (Table 4.1, Figure 4.4). Thus, strong multifarious divergent selection has led to the contemporary evolution of multiple trait differences between these populations (35, 37; this paper) but not – at least not yet – assortative mate choice.

Non-parallel responses

The above assertions emerge from comparisons between the source population and one introduced population (Taylor). By comparison, the other introduced population (Lower Lalaja) showed less evolution of male signaling traits and was neither favored nor disfavored by females. This contrast between male populations indicates a substantial non-parallel component to evolution that is consistent with recent work on guppies (66-71) and other organisms (72). Such non-parallelism is frequently attributed to environmental variation (similar habitat types might not have similar selection), different evolutionary histories (different lineages might have different evolutionary potentials), or different time scales (longer times should allow more evolution). Such variation would seem minimal in the present study because the introduction sites were environmentally very similar (50), both introductions were from the same source population (albeit in different years), and the population (Lower Lalaja) that had longer in its new environment was the one showing less evolution. Our findings thus hint that even very subtle environmental or genetic differences can also be important in shaping non-parallel evolutionary responses, a conclusion increasingly emphasized in other recent studies (e.g., 72, 73, 74).

The limited divergence of Lower Lalaja males, and their resulting phenotypic intermediacy between the other two populations (Figure 4.2, Figure 4.3), is a likely explanation for why females did not distinguish between males from the Lower Lalaja versus males from the other populations (Figure 4.4). Similarly, Lower Lalaja females in particular did not discriminate between their sympatric males versus males from the other two populations (Figure 4.4, Tables 4.1, 4.2). That is, the magnitude of male trait divergence was predictive of the magnitude of female preference divergence, a result matching other guppy studies (45) as well as more general

meta-analyses (75). Importantly, however, trait divergence led to *negative* assortative mating (choosy females preferred novel allopatric males) whereas *positive* assortative mating would be predicted under ecological speciation. We suggest that female preferences for novel males, a common finding in guppies (47), were conserved in our experiment but were expressed as (negative) assortative mating only when male trait differences were large enough to form a basis for female discrimination. Non-parallel patterns of male signal divergence are thus likely to lead to non-parallel patterns of progress toward (or away from) ecological speciation.

Why no progress toward ecological speciation?

The general lack of progress toward speciation in guppies has been long ruminated upon (76, 77), including in relation to divergent selection and, hence, ecological speciation (43, 45, 78). The conundrum is that guppies rapidly and repeatedly evolve dramatic adaptive divergence between environments (30, 31), which should promote the evolution of reproductive barriers (1-3); yet guppies show only minimal, if any, progress toward ecological speciation (30). Our study reinforces this perspective in finding that dramatic multi-dimensional shifts in selection did not promote the contemporary evolution of positive assortative mating. Perhaps the simplest explanation for this result might be that not enough time has yet passed for a substantial response to divergent selection. However, this explanation can be dismissed – given that sexual traits had enough time to evolve (Figure 4.2), and that assortative mating (albeit negative) did actually evolve. Thus, the question that really needs answering is: Why did trait evolution following environmental change in multiple niche axes lead to *disassortative* mating between populations?

It is first important to remember that a number of studies have shown that females do often prefer rare (novel) phenotypes (46, 47, 79, 80). Suggested reasons for this tendency include

[1] inbreeding avoidance (81), [2] reduced probability of re-mating with the same male (80), and [3] a survival advantage of rare individuals (82). To these possibilities, we suggest adding another: mating with rare males likely increases MHC diversity (as is the case in many taxa – (83)), which could enhance resistance to parasites. Finally, it is reasonable to imagine that selection favours mating with rare males *within* populations, but that the resulting correlated effect of being more likely to mate males from other populations is maladaptive. However, this maladaptive effect would only impose selection in the presence of high dispersal (84), whereas our experimental populations were isolated by barriers to immigration and the source population was far from upstream low-predation sites. Thus, we might only expect the evolution of positive assortative mating if the populations undergoing adaptive divergence were also exchanging migrants (25).

Overall, our findings are congruent with recent suggestions that ecological speciation is not so easily accomplished (4, 5). That is, divergent selection might well be present but progress toward ecological speciation might be strongly constrained. First, high gene flow might prevent local adaptation despite divergent selection (85, 86). Second, adaptive divergence might occur but make only modest contributions to reproductive isolation. In guppies, for instance, migrants between predation environments can suffer low fitness, which can impose a reproductive barrier (87). However, conserved patterns of sexual selection can lead to dissassortative mating between those same populations (45) (46, 47; present study), effectively negating any serious progress toward ecological speciation. In short, guppies provide a particularly clear example of how divergent natural selection can simultaneously generate reproductive barriers (selection against migrants) and reproductive enhancers (disassortative mating), potentially leading to no net effect on gene flow (43, 88) and, hence, little or no progress toward speciation.

4.6 Implications:

We detected the contemporary evolution of guppy signaling traits after experimental introductions into a novel environment, but this male trait evolution was not coupled to female preference evolution. Our study highlights the importance of conserved sexual selection – here for novel males – in potentially counteracting the influence of strong and multifarious divergent natural selection. Theoretical and empirical studies of ecological speciation should pay additional attention to the various influences of mating system variation in enhancing or constraining progress to the evolution of reproductive barriers. Just as ecological speciation requires both divergent selection and assortative mating, positive assortment by mate choice requires a mating system that appropriately couples divergent trait evolution to female preferences.

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Ethical note:

All field collections were approved by the Trinidad and Tobago Ministry of Fisheries. All field and laboratory procedures were approved by McGill University's Animal Care Committee in accordance with the Canadian Council on Animal Care in Sciences guidelines (AUP #5759).

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Table 4.1: Female preference

		Female population	Male population-pair	t-value	df	p-value
A	Sympatric vs. Allopatric	Guanapo source	S-L	1.34	8	0.217
			S-T	-3.19	10	0.01
		Lower Lalaja	L-S	-0.12	9	0.91
			L-T	-0.36	9	0.729
		Taylor	T-S	-2.68	9	0.025
			T-L	1.54	9	0.157
B	Allopatric vs. Allopatric	Guanapo source	T-L	-0.78	9	0.455
		Lower Lalaja	T-S	1.82	9	0.102
		Taylor	L-S	-0.31	9	0.764

T-test for female preference (relative preference for focal male) between alternative males derived from different populations by female population and male pair combination. A significant difference represents female preference for males of a given population more than would be expected by chance. Positive t-values indicate preference for the population of the first male in the male population-pair column. Abbreviations for population names: Guanapo source (S) population, and Lower Lalaja (L) and Taylor (T) introduced populations.

Table 4.2: Planned comparisons of male pair differences within female population

Female population	Male paired populations 1	Male paired populations 2	t-value	df	p-value
Guanapo source	S-L	S-T	2.9	18	0.01
Lower Lalaja	L-T	L-S	0.11	18	0.91
Taylor	T-S	T-L	-2.76	18	0.013

T-test for differences in the strength of female preference for sympatric males between alternative sympatric-allopatric male pairings. A significant difference indicates that female preference for sympatric males is varies depending on the allopatric male population. Abbreviations for population names: Guanapo source (S) population, and Lower Lalaja (L) and Taylor (T) introduced populations.

Table 4.3: Female preference explained by male difference in canonical variates

	df	ss	F	p
CV1	1	0.51	4.39	0.041
CV2	1	0.15	1.27	0.266
Male pair by female population	5	0.29	2.46	0.045
Residuals	52	7.5		

Generalized linear model with a normal distribution of the errors and identity link of female preference for sympatric males (relative preference for focal male) as response variable, male pair combination by female population (MFPP) as factor and the difference in canonical scores between the sympatric and allopatric males for the first (CV1) and second (CV2) discriminant axes as covariates.

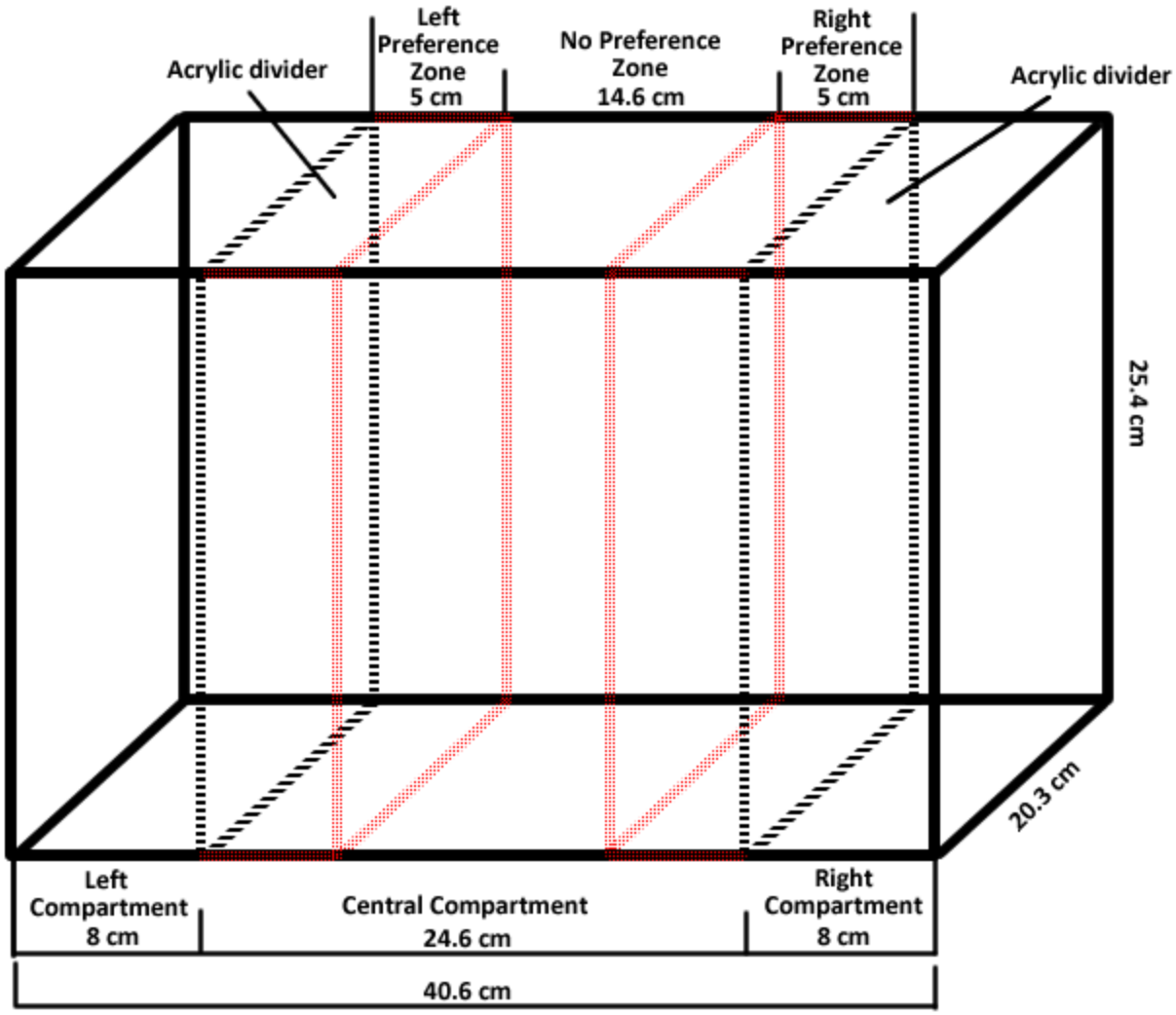

Figure 4.1: Mate choice apparatus

Females from each of the three populations (Guanapo source, Lower Lalaja and Taylor) were placed individually in the central compartment and allowed to acclimate for 20 hours. After acclimation one male was placed in each side compartment and female behaviour was recorded for 20 minutes. Preference was quantified as the time a female spent in the left or right preference zone. See manuscript for details.

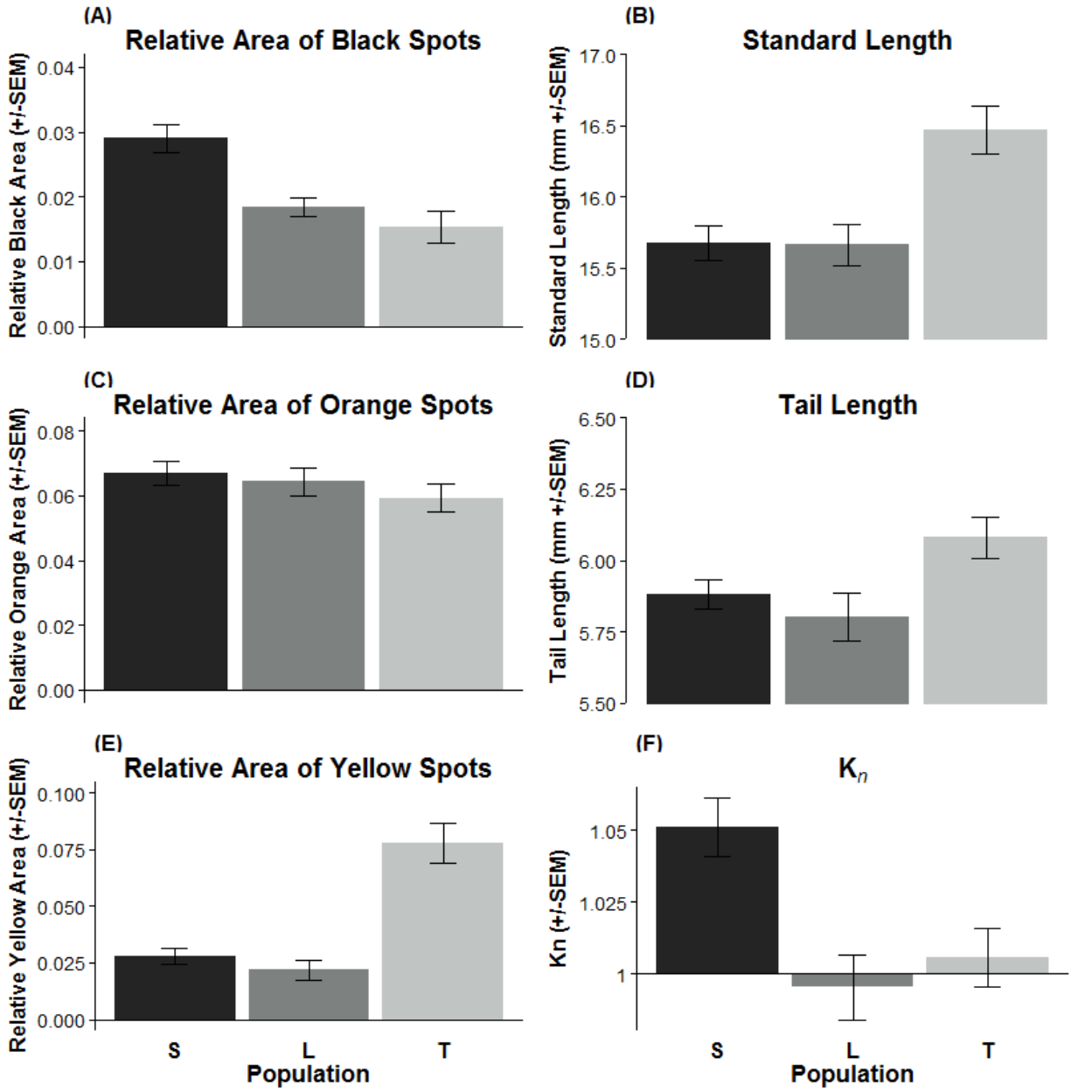

Figure 4.2: Male signaling trait divergence

Mean signaling trait values for males from the Guanapo source population (S) and the Lower Lalaja (L) and Taylor (T) introduced populations. Traits previously shown to influence guppy mate choice: Relative area of (A) black, (C) orange and (E) yellow spots, and (B) standard length (in mm), (D) tail length (in mm), and (F) condition (relative condition index – K_n).

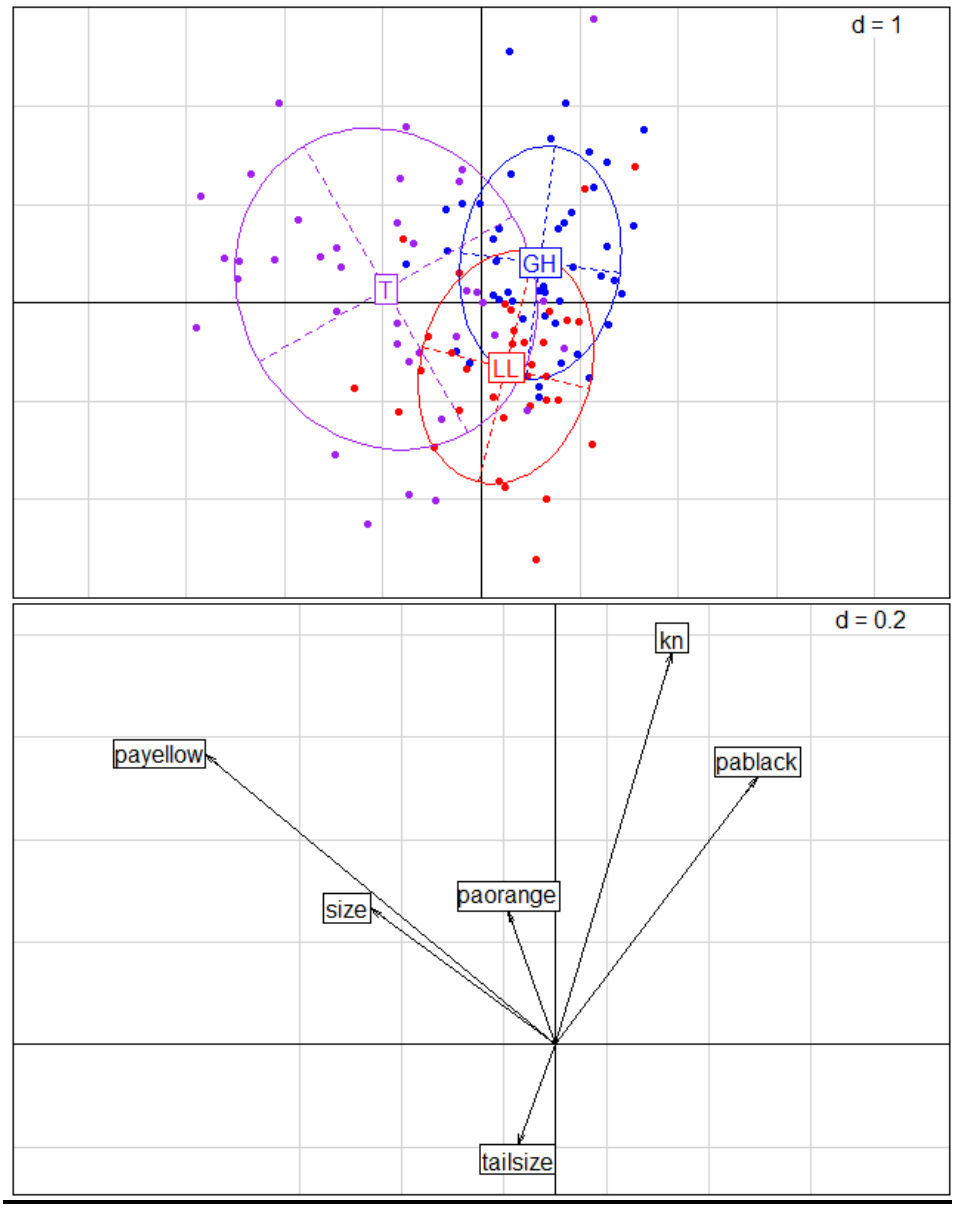

Figure 4.3: Discriminant analysis plot of male guppies differentiated by their signaling traits

(A) Divergence between male guppies from the Guanapo source (S), and the Lower Lalaja (L) and Taylor (T) introductions in the first (X) and second (Y) discriminant axes, differentiated by their signaling traits (relative black area, relative orange area, relative yellow area, standard length, relative condition index and tail size). (B) Loading plot of signaling trait weights in the first (X) and second (Y) discriminant axes.

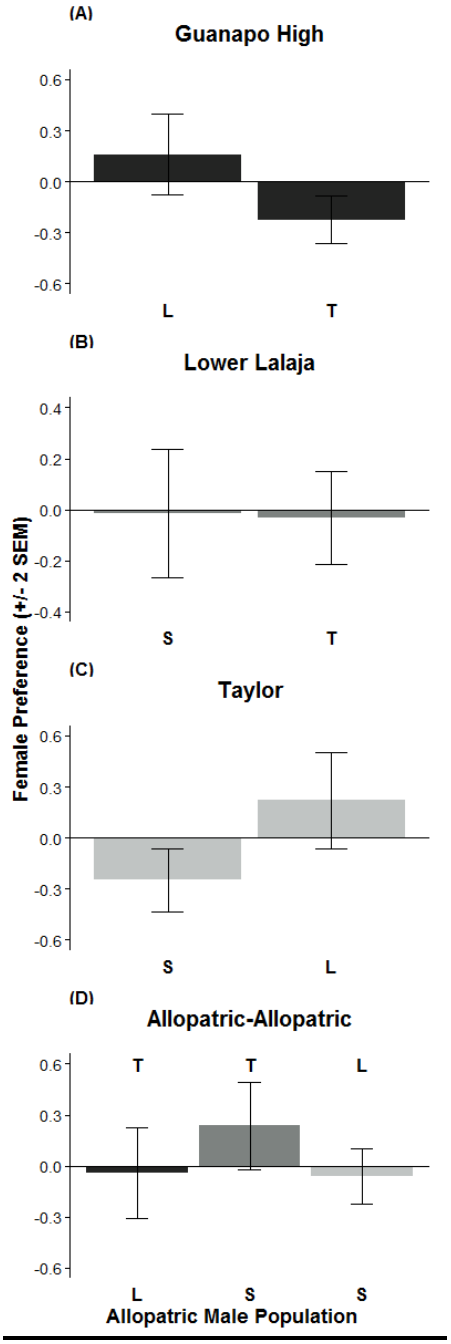

Figure 4.4: Female preference between alternative male choices.

Female preference measured as time spent with a sympatric male minus time spent with an allopatric male divided by the total time spent with both males (relative preference for focal

male). Positive values represent preference for the sympatric male and negative values preference for allopatric males. Abbreviations for population names: Guanapo source (S) population, and Lower Lalaja (L) and Taylor (T) introduced populations.

APPENDIX 4.A

An experimental manipulation in nature testing whether multifarious divergent selection promotes the contemporary evolution of assortative mating

Felipe Dargent*, Lisa Chen*, Gregor Fussmann and Andrew Hendry. (in prep). An experimental manipulation in nature testing whether multifarious divergent selection promotes the contemporary evolution of assortative mating.

*Equally contributing authors.

Table 4.A1: Female preference (absolute preference for focal male)

		Female population	Male population-pair	t-value	df	p-value
A	Sympatric vs. Allopatric	Guanapo source	S-L	1.49	8	0.174
			S-T	-3.15	10	0.01
		Lower Lalaja	L-S	-0.09	9	0.93
			L-T	-0.28	9	0.783
		Taylor	T-S	-2.59	9	0.029
			T-L	1.68	9	0.127
B	Allopatric vs. Allopatric	Guanapo source	T-L	-0.71	9	0.497
		Lower Lalaja	T-S	1.65	9	0.133
		Taylor	L-S	-0.52	9	0.619

T-test for female preference (absolute preference for focal male) between alternative males derived from different populations by female population and male pair combination. A significant difference represents female preference for males of a given population more than would be expected by chance. Positive t-values indicate preference for the population of the first male in the male population-pair column. Abbreviations for population names: Guanapo source (S) population, and Lower Lalaja (L) and Taylor (T) introduced populations.

Table 4.A2: Planned contrasts of male pair differences within female population (absolute preference for focal male)

Female population	Male paired populations 1	Male paired populations 2	t-value	df	p-value
Guanapo source	S-L	S-T	2.81	18	0.011
Lower Lalaja	L-T	L-S	-0.08	18	0.94
Taylor	T-S	T-L	-2.83	18	0.011

T-test for differences in the strength of female preference (absolute preference for focal male) for sympatric males between alternative sympatric-allopatric male pairings. A significant difference indicates that female preference for sympatric males is varies depending on the allopatric male population. Abbreviations for population names: Guanapo source (S) population, and Lower Lalaja (L) and Taylor (T) introduced populations.

Table 4.A3: Female preference explained by male difference in canonical variates (absolute preference for focal male)

	df	ss	F	p
CV1	1	0.51	4.39	0.074
CV2	1	0.15	1.27	0.14
Male pair by female population	5	0.29	2.46	0.056
Residuals	52	7.5		

Generalized linear model with a normal distribution of the errors and identity link of female preference for sympatric males (absolute preference for focal male) as response variable, male pair combination by female population as factor and the difference in canonical scores between the sympatric and allopatric males for the first (CV1) and second (CV2) discriminant axes as covariates.

Figure 4.A4: Male signaling trait divergence (including males that were tested more than once)

Results for signaling trait divergence analysis for all males used in the mate choice trials (including the values of the first and second measurements of males that were used more than once) reports qualitatively similar results to those tests that only included the values of the first time the males were used. Males differed in signaling traits among populations in MANOVA (Pillai's trace=0.652, $F_{2,177}$=13.94, p<0.001) and ANOVAs. In fact, with the exception of relative

orange area ($F_{2,177}$=2.67, p=0.07), all measured traits differed among populations (relative black area: $F_{2,177}$=21.32, p<0.001; relative yellow area: $F_{2,177}$=47.87, p<0.001; SL: $F_{2,177}$=13.99, p<0.001; tail size: $F_{2,177}$=6.65,p=0.002; and K_n: $F_{2,177}$=10.28, p<0.001). Post hoc Tukey's HSD tests for each individual ANOVA showed that Guanapo source males differed from Lower Lalaja males in two traits (relative black area: S>L, K_n: S>L), and from Taylor males in four traits (relative black area: S>T, relative yellow area: S<T, SL: S<T, and K_n: S>T), whereas Taylor males differed from Lower Lalaja males in three traits (relative yellow area: T>L, SL: T>L, and tail size: T>L).

CONNECTING STATEMENT

The experiments in previous chapters were designed to assess the evolutionary implications of enemy release at one end of the spectrum: full removal of the previous source of selection. Yet, often victims have to coexist with their enemies. In the following chapter I assess the effects of an untested mechanism for enemy release in group-forming species: forming groups of more than one species (i.e. mixed-species groups) as an antiparasite strategy.

Although mixed-species group formation is a common behaviour that has been reported in various taxa, it is usually regarded as an anti-predator strategy (especially when the number of conspecifics is limited) or as a foraging advantage strategy. Yet, the possible role of parasitism in mixed-species group formation remains relatively unexplored. In the following chapter I move away from the introduction experiments but continue to work with Trinidadian poeciliids, to specifically test whether victims that engage in group forming behaviour (guppies and their sister species *Poecilia picta*) can reduce their contagious parasite loads by forming mixed-species groups.

CHAPTER 5

Can mixed-species groups reduce individual parasite load? A field test with two closely related poeciliid fishes (*Poecilia reticulata* and *Poecilia picta*)

Felipe Dargent, Julián Torres-Dowdall; Marilyn E. Scott; Indar Ramnarine and Gregor F. Fussmann. (2013). Can mixed-species groups reduce individual parasite load? A field test of two closely related poeciliid fishes (*Poecilia reticulata* and *Poecilia picta*). *PLOS ONE*. **8**(2): e56789. DOI: 10.1371/journal.pone.0056789

5.1 Abstract

Predation and parasitism are two of the most important sources of mortality in nature. By forming groups, individuals can gain protection against predators but may increase their risk of being infected with contagious parasites. Animals might resolve this conflict by forming mixed-species groups thereby reducing the costs associated with parasites through a relative decrease in available hosts. We tested this hypothesis in a system with two closely related poeciliid fishes (*Poecilia reticulata* and *Poecilia picta*) and their host-specific monogenean ectoparasites (*Gyrodactylus spp.*) in Trinidad. Fish from three different rivers were sampled from single and mixed-species groups, measured and scanned for *Gyrodactylus*. The presence and abundance of *Gyrodactylus* were lower when fish of both species were part of mixed-species groups relative to single-species groups. This is consistent with the hypothesis that mixed-species groups provide a level of protection against contagious parasites. We discuss the importance of potentially confounding factors such as salinity and individual fish size.

Keywords

Poecilia reticulata, *Poecilia picta*, *Gyrodactylus*, infection.

5.2 Introduction

Forming groups is an adaptive strategy seen in many animal species (1). Individuals that join a group of conspecifics often gain benefits that increase their fitness compared to those that do not join a group; however group formation also involves interactions that are costly to the individual. Two major benefits of group forming behaviour are a decrease in an individual's probability of being preyed upon and an increase in the probability of acquiring food (2). A major cost that individuals experience when joining a group is increased contagious parasite transmission (3). Contagious parasites lack mobile dispersal stages (3), therefore their transmission depends on the number and frequency of contacts between hosts. As group size increases, increased contact between suitable hosts (4) results in higher mean number of contagious parasites per individual and higher percentage of infected individuals (3, 5, 6). Conversely, mobile parasites need to search or ambush their hosts; as group size increases this behaviour will likely decrease a host individual's probability of receiving a mobile parasite attack (7, 8).

Individuals also form groups with heterospecifics (1, 4, 9-11) when members of more than one species overlap spatially and temporally (1, 9). Active behavioural choice of at least one participating species is inferred for the formation of these groups as their occurrence is far more frequent than would be expected by chance (9, 12). Attempts to explain the advantages of forming mixed-species groups focus on anti-predation mechanisms and, less prominently, on foraging advantages (12, 13). When anti-predator mechanisms are evoked individual benefits derive from the numerical increase in group members; these are essentially the same mechanisms that are at work in single-species groups and cannot explain the particular advantage of a heterospecific association. For example, this is the case for anti-predation mechanisms where the

benefit resides in diluting the risk of attack (14) or in reducing exposure to predators (15). Foraging advantages, in contrast, result from the heterospecific nature of the association. For example, individuals in mixed-species groups can acquire more resources than those in equally sized single-species groups by reducing competition (i.e. interspecific competition is weaker than intraspecific competition (13, 16)).

Contagious host-specific parasitism provides an alternative explanation of why individuals in mixed-species groups would experience higher fitness compared with equally sized single-species groups (4). When individuals face the combined threat of predation and parasitism, joining a mixed-species group may be an adaptive strategy that, on aggregate, outperforms either of the pure protective strategies. In mixed groups, under certain circumstances (i.e. when not strongly phenotypically dissimilar (17, 18)), individuals can enjoy similar anti-predator protection as in single-species groups ("all individuals count") but can also decrease their risk of being parasitised due to dilution of the group with individuals of the second species that are not susceptible to infection ("only individuals of the host species count"(4)). This effect has been largely overlooked as an explanation for mixed-species group formation, although increasing evidence of the negative correlation between species diversity and parasite transmission has been accumulating during the last decade ("dilution effect" e.g. (19-21) but see (22)).

In this study we compared contagious parasite infections in single- versus mixed-species groups of two closely related poeciliid fishes in the wild. We assumed that parasite transmission and/or parasite intrinsic rate of increase was higher on the co-evolved species-specific host-parasite pairs (23). Therefore we hypothesised that individuals of each host species would experience lower levels of contagious parasites (presence and abundance) by engaging in mixed-species groups.

The study system

To test our hypothesis, we used as model organisms two poeciliid fishes that in their native habitat in Trinidad (24) form mixed-species groups, that are infected by different species of contagious parasites and that face the risk of predation by multiple piscivorous fishes. *Poecilia reticulata* ("Trinidadian guppy") is found in freshwater whereas *Poecilia picta* ("swamp guppy") is found across a salinity gradient from freshwater to brackish water (24). In the zones where their distributions overlap, they form mixed-species groups (25). These two fish species are parasitised by *Gyrodactylus spp*., a group of viviparous monogenean ectoparasites of the skin and fins that are transmitted mainly through host to host contact (26, 27). Wild *P. reticulata* are infected with *Gyrodactylus turnbulli* and *G. bullatarudis* (28), whereas *G. pictae* have been found only on wild *P. picta* (29). Under controlled lab experiments *G. turnbulli* and *G. bullatarudis* can survive on *P. picta* but only for short periods of time and with lower reproductive rates than on *P. reticulata* (30, 31). There is no field evidence of cross-infection.

5.3 Materials and methods

We sampled approximately 20 female and 20 male individuals of each *Poecilia* species present at three types of sites on each of three rivers using butterfly nets (Table 5.1). Two of the three rivers included a *P. reticulata*-only site, a *P. picta*-only site and two sites where mixed-species groups are formed. In the third river (Nariva), only one mixed-species site was sampled. Each collection site was less than a 10-metre longitudinal section of the river. At mixed-species sites, we observed no signs of spatial segregation among fish, so we consider these sites to be representative of individuals of the two species grouping together. Since salinity is a major environmental variable that drastically changes across the *P. picta* distribution, we measured

specific conductivity at each site, as a proxy of salinity (YSI probe model 85-50 FT), and report the direct measurement (specific conductivity). After capture, fish were first placed individually in 18 oz Whirlpak bags to avoid parasite transmission during transport to the lab. The total capture period at single species sites was no longer than 2 hours, and at mixed species sites was no longer than 4 hours. This included the time it took to find fish shoals, to capture a random sample of its members, to place them individually in Whirlpak bags, and to measure specific conductivity.

On the day of capture, each fish was anaesthetized in 0.02% Tricaine Methanesulfonate (MS-222) buffered to a neutral pH before counting all *Gyrodactylus* under a dissecting scope and taking photographs with a Nikon D80 camera for later standard length (SL) measurements to nearest 0.1 mm using ImageJ v.1.44. The fish was then euthanized in MS-222. We confirmed that the parasites were from the genus *Gyrodactylus* but were unable to identify them to the species level given the limited resolution of our field equipment.

Generalised linear mixed-effect models (GLMM) were used to test whether group type (mixed- or single-species) had an effect on whether or not an individual was infected (presence) and on the number of parasites per individual of a given species (abundance). All models included fish SL and group type as fixed effect variables, and river as a random effect variable to control for unaccounted differences among rivers. Due to variation in the difference between mixed- and single-species group types among rivers, the *P. reticulata* abundance model also included a group type within river random effect instead of a river random effect alone (i.e. the best fit model in this case included "group type|river" rather than "1|river"; χ^2=20.1, df=2, p<0.001). For *P. picta* models, specific conductivity was included as a fixed effect variable in addition to group type and SL. We used a binomial distribution of the errors with a logit link

function to analyse *Gyrodactylus* presence. Abundance data was square root transformed and we used a Poisson distribution of errors with a log link function for the analysis. All analyses were performed using R Language and Environment for Statistical Computing and the lme4 package (32).

5.4 Results

Both *P. reticulata* and *P. picta* individuals were more likely to be infected in single-species groups than in mixed-species groups, and for both fish species, fish with higher SL were more likely to be infected (Table 5.2 - presence). For *P. picta,* the likelihood of infection decreased with increasing specific conductivity (Table 5.2). *Gyrodactylus* mean abundance ranged among sites from 0.05 to 2.43 parasites per fish, with average site mean abundance of 0.83 for *P. reticulata* and 0.81 for *P. picta* (Figure 5.1). Across single-species sites, mean *Gyrodactylus* abundance was 1.66 per fish for *P. reticulata* versus 0.89 for *P. picta*. Across mixed species sites, mean abundance was 0.34 *Gyrodactylus* per fish for *P. reticulata* versus 0.76 for *P. picta*. After accounting for the influence of SL, river and specific conductivity, the abundance of *Gyrodactylus* on individuals of both fish species was significantly higher in single-species groups than in mixed-species groups (Figure 5.1, Table 5.2). Larger *P. reticulata*, but not *P. picta*, had more parasites than smaller ones, and *P. picta* individuals at sites with higher specific conductivity had lower numbers of *Gyrodactylus* than at sites with lower specific conductivity (Table 5.2).

The time required to catch 40 fish of both species from mixed-species sites was twice (or less) that required to catch 40 fish at single-species sites. Therefore we have no reason to presume that fish density differed between single- and mixed-species sites. We performed

additional analyses to exclude the possibility that differences in presence and abundance of *Gyrodactylus* between single and mixed-species groups merely reflect size differences of fish among sites. A one-way ANOVA on the SL data with site type nested within river revealed significant size differences among rivers ($F_{2,267}$=12.956, p<0.001 for *P. reticulata*; $F_{2,289}$=5.097, p<0.01 for *P. picta*; Figure 5.2) and among site types ($F_{5,267}$=3.513, p<0.005 for *P. reticulata*; $F_{5,289}$=5.293, p<0.001 for *P. picta*; Figure 5.2). However, Tukey's HSD post-hoc test showed that differences occurred chiefly among sites of different rivers. No significant size differences were found between single- and mixed-species sites within the same river for *P. reticulata*. *P. picta* on the other hand were significantly larger in the single-species site of the Caroni river (19.83 mm mean SL) than in one of the mixed-species sites (17.02 mm –Holstein site mean SL; Figure 5.2).

5.5 Discussion

We found that *P. reticulata* and *P. picta* individuals in mixed-species groups had a lower abundance of *Gyrodactylus* and were less likely to be infected than conspecifics in single-species groups. Independent of the proximate mechanisms that determine mixed-species associations, these findings are consistent with the hypothesis that mixed-species groups provide protection for each host species from host-specific contagious parasites. However, the two fish species we studied differed in the degree of support that they provided to our hypothesis. *P. reticulata* showed clear and consistent differences between single- and mixed-species groups for both response variables (presence, abundance). For *P. picta* these differences became apparent only after controlling for specific conductivity.

In our field system, both fish species experience predation risk at all sites (33) and both species are known to be infected by host-specific contagious parasites (29, 34). Forming groups is a common behavioural strategy to reduce the risk of predation (1) but as anti-predator benefits increase with group size, so does the risk of contagious parasite transmission. The selection imposed by these two sources of mortality will therefore affect the grouping decisions made by individuals. Forming mixed-species groups could provide an improved strategy that reduces the associated costs of contagious parasitism through a dilution effect. Contact and transmission between conspecifics is less frequent than in similarly sized single-species groups, which reduces the per-capita likelihood to contract the parasite. In addition, effective spread of the parasite through the susceptible part of the mixed-species group is probably hampered by the frequent encounter with non-susceptible hosts, a mechanism akin to the herd immunity provided to a population through vaccination (35, 36). The dilution effect seems to be pervasive at different scales of biodiversity, from the genetic level where within population diversity increases resistance to parasites (37, 38), to the community level where higher species diversity dilutes the availability of suitable hosts (19). The dilution effect is typically suggested for higher levels of biodiversity (e.g. (21, 39)) than our two-host-species system but the general principle of protection through relative rarity applies to our case. We suggest that the combined pressures of predation and contagious parasitism have selected for poeciliid individuals that express a behavioural strategy, forming mixed-species groups, by which they reduce the costs (increased parasite burden) while maintaining the benefits (protection from predation) of joining a group. Empirical evidence relevant to our system provides support for this idea. For example, teleost fish show the ability to actively modify their grouping preferences in response to parasites (40-42). More specifically, *P. reticulata* are able to discriminate individuals based on their history of

infection with *Gyrodactylus* (43-45) and their shoaling behaviour has been shown to be heritable (46). It is therefore likely that they can choose to preferentially associate with heterospecifics to reduce the costs of contagious parasitism.

We found that larger fish were more likely infected than smaller individuals. In addition, larger *P. reticulata* had more *Gyrodactylus*, in agreement with a previous study (47). Larger fish provide more living space for ectoparasites (48) and size may also be associated with increased probability of contacts among hosts or the availability of energetic resources for the parasites (49). *P. reticulata* did not significantly differ in SL within rivers although we detected a trend of slightly larger sizes at mixed-species groups (Figure 5.2). Therefore it is unlikely that size differences among individuals in different populations could explain the lower infections observed in mixed-species vs. single-species groups.

Our statistical models suggest that specific conductivity also played a role in the observed infection patterns. All *P. reticulata* mixed- and single-species groups occurred in the freshwater zone, thus salinity could not have driven the differences in infection between group types. However, *P. picta* single-species groups were all in sites with higher specific conductivity than mixed-species groups. Higher specific conductivity in the *P. picta* only sites could have reduced gyrodactylid survival and intrinsic rate of increase, and therefore decreased presence and abundance of the parasite. If specific conductivity is not taken into account (Figure 5.1), lower abundance of *Gyrodactylus* in mixed-species sites is only evident in one river (Nariva – Figure 5.1). Although the direct effects of salinity are unknown for *G. pictae*, Schelkle et al. (50) found that modest increases in salinity (from 0 to 12.1 µS/cm approximately) had a negative impact in *G. turnbulli* and *G. bullatarudis* growth rate and establishment on *P. reticulata*. Such effects may also occur with *G. pictae.*

A potential point of criticism is that we were not able to distinguish between the three Gyrodactylus species present in our system. This would be a point of concern regarding our results and conclusions if the parasites were generalists that can do equally well in all hosts. However, previous experiments in this system (30, 31) revealed that *G. turnbulli* and *G. bullatarudis* have faster rates of increase and their populations survive longer in *P. reticulata*.

Our study, in the light of previous experimental results and field evidence from the same host-parasite system, provides strong evidence in support of the hypothesis that individuals in mixed-species groups have lower levels of parasitism than those in single-species groups. Further research should focus on experimental studies within this system that test the effect of *Gyrodactylus* infection on host decisions to join single- or mixed-species groups and quantify the impacts of the relative proportions of the two host species on each of their *Gyrodactylus* parasite population dynamics. In addition, field studies in other systems are needed to confirm the generality of our findings. We hypothesize that as infection levels and parasite virulence increase individuals will shift preference from single- to mixed-species shoals due to the increased fitness gained through the dilution effect.

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Ethics Statement

This study was carried out in accordance with McGill University's Animal Care Committee and the Canadian Council on Animal Care in Sciences guidelines. Our protocol was approved by the Macdonald Campus Facility Animal Care Committee of McGill University (AUP #5759). Field sampling also received approval of the Ministry of Agriculture, Land and Marine Resources - Fisheries Division of the Republic of Trinidad and Tobago.

5.7 References

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Table 5.1: Location characteristics and standard lengths (SL) for *Poecilia reticulata* and *Poecilia picta* at sites where they formed single- or mixed-species groups.

		River Characteristics			*P. reticulata*			*P. picta*		
River	**Group type**	**Site**	**Coordinates**	**Specific conductivity (µS/cm)**	**n**	**Mean SL (mm) (SE)**	**Mean *Gyrodactylus* abundance (SE)**	**n**	**Mean SL (mm) (SE)**	**Mean *Gyrodactylus* abundance (SE)**
Caroni	Single	Bend	N10 34.940 W61 16.250	0.33	41	15.4 (0.4)	2.3 (0.4)	-	-	-
		Swamp	N10 36.307 W61 25.366	62.4	-	-	-	45	19.8 (0.5)	0.5 (0.1)
	Mixed	Bridge	N10 37.037 W61 24.324	0.31	39	16.7 (0.4)	1.2 (0.3)	38	18.6 (0.4)	1.7 (0.3)
		Holstein	N10 36.527 W61 23.261	0.27	10	16.8 (1.1)	0.3 (0.2)	14	17 (0.5)	0.6 (0.3)
Guayamare	Single	Coconut	N10 34.055 W61 20.468	0.35	40	14.7 (0.4)	2.4 (1)	-	-	-
		Barrier	N10 35.548 W61 24.984	60.8	-	-	-	40	18 (0.2)	0.2 (0.1)
	Mixed	Dusty	N10 35.548 W61 24.972	0.41	29	15.9 (0.6)	0.1 (0.1)	41	18.5 (0.4)	0.5 (0.2)
		Rusty	N10 35.336 W61 24.225	0.37	40	14.2 (0.3)	0.1 (<0.1)	40	18.1 (0.2)	0.8 (0.2)
Nariva	Single	Navet	N10 20.238 W61 11.462	0.37	41	14.6 (0.4)	0.2 (0.1)	-	-	-
		Outlet	N10 24.484 W61 01.547	46.00	-	-	-	41	18.6 (0.4)	2 (0.3)
	Mixed	Poole	N10 27.822 W61 04.340	0.33	36	13.8 (0.3)	0.1 (0.1)	38	16.9 (0.4)	0.3 (0.1)

Table 5.2: Presence and abundance of *Gyrodactylus* spp. on *Poecilia reticulata* and *Poecilia picta*.

Presence

		Poecilia reticulata				*Poecilia picta*			
Fixed effects		**df**	**Estimate**	**SE**	**z-value**	**df**	**Estimate**	**SE**	**z-value**
	Intercept		-4.27	1.27	-3.35***		-2.72	1.01	-2.7**
	Single	1	2.13	0.35	6.08***	1	9.44	1.01	4.59**
	SL	1	0.14	0.06	2.32*	1	0.13	0.05	2.44*
	Conductivity	-	-	-	-	1	-0.17	0.04	-4.68***
Random effects			**Std Dev**	**Corr**			**Std Dev**	**Corr**	
	River		1.3	-			0.62	-	
	Single		-	-			-	-	

Abundance

		Poecilia reticulata				*Poecilia picta*			
Fixed effects		**df**	**Estimate**	**SE**	**z-value**	**df**	**Estimate**	**SE**	**z-value**
	Intercept		-3.21	0.82	-3.9***		-1.6	0.6	-2.65**
	Single	1	1.46	0.61	2.4*	1	6.83	1.3	5.24***
	SL	1	0.08	0.03	2.54*	1	0.05	0.03	1.67
	Conductivity	-	-	-	-	1	-0.12	0.02	-5.35***
Random effects			**Std Dev**	**Corr**			**Std Dev**	**Corr**	
	River		1.06	-			0.45	-	
	Single		0.93	-0.66			-	-	

*=p<0.05; **=p<0.01; ***=p<0.001

Generalized linear mixed model results for presence (binary variable with binomial distribution) and abundance (square root transformed discrete variable with Poisson distribution) of *Gyrodactylus spp.* on *Poecilia reticulata* and *Poecilia picta*. Values given for individuals in single- relative to mixed-species groups.

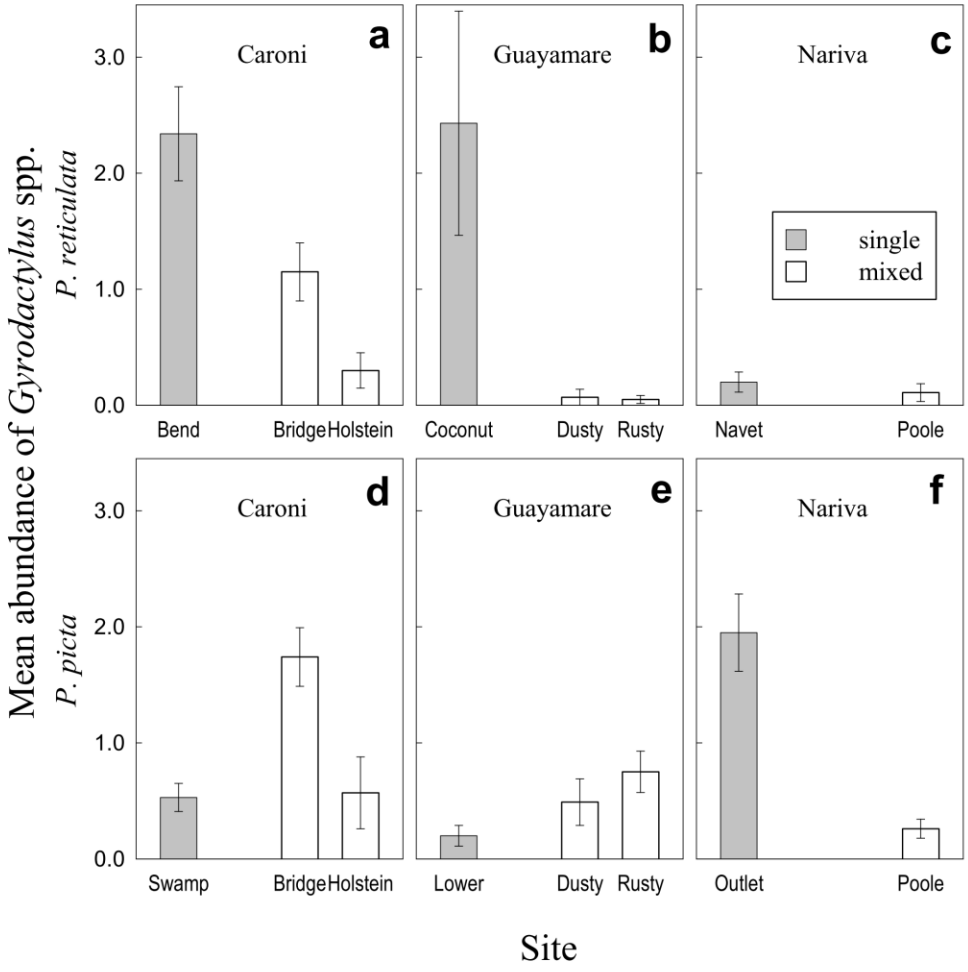

Figure 5.1: *Gyrodactylus* mean abundance.

Mean abundance (+/- 1 SE) of *Gyrodactylus* spp. on *P. reticulata* (**a, b, c**) and *P. picta* (**d, e, f**) in single- and mixed-species sites from three rivers in Trinidad.

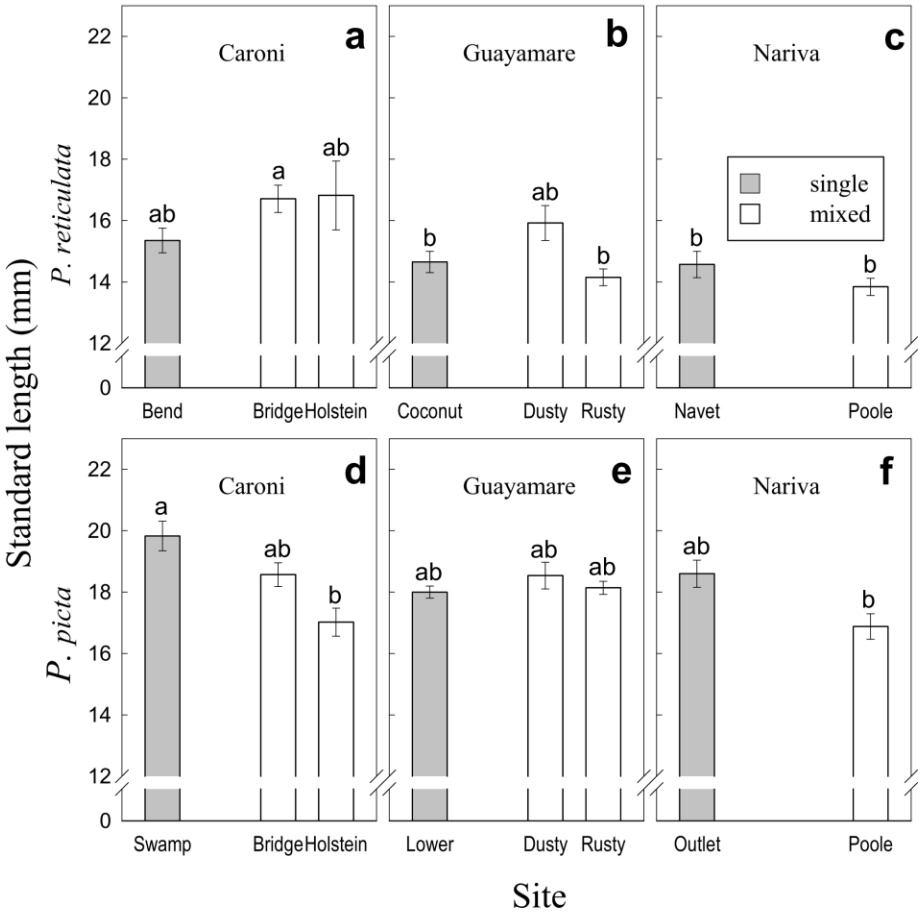

Figure 5.2: *Poecilia reticulata* and *Poecilia picta* standard length by site.

Standard length (+/- 1 SE) in mm of *P. reticulata* (**a, b, c**) and *P. picta* (**d, e, f**) in single and mixed-species sites from three rivers in Trinidad. Different letters denote significant pairwise differences (Tukey HSD) between sites for a given species.

CHAPTER 6

Conclusion

6.1 Overview:

In this thesis I have focused on the effects of ecology and evolution on enemy-victim interactions in nature. Specifically, I concentrated on the [1] implications of relaxed selection on the contemporary evolution of defence against parasites; [2] the degree to which contemporary evolution of defence is a sexually dimorphic (i.e. non-parallel) process; [3] the consequences of enemy release - under multidimensional-selection - on the build-up of reproductive barriers (i.e., speciation); and [4] the effects of inter-specific social interactions on contagious parasitism. I have used a variety of approaches for these purposes which combine experimental evolution in nature, multi-generation common-garden rearing, experimental infections, mate choice trials and field sampling.

In Chapter 2, I argue that the rapid and repeatable evolution of increased resistance in female guppies (*Poecilia reticulata*) after removal in the wild of a common and deleterious parasite (*Gyrodactylus* spp) was not a consequence of resistance-tolerance trade-offs or driven by differences in productivity. I contend that the results of Chapter 2 were not caused by artificial selection or guppy density (see Appendix A). In fact, I claim that the most likely explanation is that the rapid life-history evolution typical in such introductions (caused by release from predation) pleiotropically increases parasite resistance. In Chapter 3, I argue that past selection for higher resistance in males than females constrained the parallel evolution of the sexes towards increased resistance (i.e. males did not evolve resistance while females did) and led to a decrease in sexual dimorphism in defence. In Chapter 4, I suggest that conserved patterns of sexual selection (i.e. preference for novel males) can lead to disassortative mating between populations subjected to strong multidimensional divergent selection, even when adaptive divergence is detectable, and therefore constrain speciation. Finally, in Chapter 5, I claim that the

decision to form mixed-species groups is a mechanism of defence against contagious parasites that reduces their presence and abundance.

6.2 Specific implications:

Chapter 2 and Appendix A highlighted the importance of bridging the two common approaches that inform our current empirical understanding of the evolution of defence against parasites: [1] laboratory studies and [2] field surveys. Experimental evolution studies in the laboratory allow researchers to manipulate parasite levels while removing variation in all other environmental factors (1), yet evolution in nature occurs in the context of all these factors which could have potentially strong effects on the evolution of defence (2). While field surveys do incorporate natural complexity they rarely manage to disentangle cause and effect (3). By experimentally manipulating infection levels in nature, and thus bridging the two approaches, I documented evolutionary patterns that are inconsistent with theoretical models and laboratory experiments. This result strongly indicates that it is in the context of natural variation in other environmental factors (i.e., multidimensional selection) that we need to understand the evolution of defence against parasites.

Despite sharing their autosomes, males and females often show differences in genetic variation for the same traits (4) such as resistance (5). Chapter 3 merges the growing tradition of quantifying the degree to which evolution is (non)parallel (6, 7) with the idea of considering parallelism between the sexes (8-10) instead of only among populations and species. The sexually dimorphic evolution of resistance that I report suggests that studies of parallel evolution would benefit from increasing attention to sexual differences and their potential causes and consequences. Furthermore, humans have created many situations in which selection by parasites

has been relaxed through the use of antibiotics, antivirals, pesticides, and herbicides, as well as through improved hygiene. In the few instances that have considered the fitness effects of relaxed selection from parasites discussion follows the classical expectation that resistance should decrease, and do so in a similar fashion for both sexes (11, 12). My results challenge this simple interpretation and could therefore have diverse applications for disease control and conservation programs.

Evidence of repeatable adaptation to environments that strongly diverge in predation and productivity is a common feature of the guppy system (3, 13, 14), and yet assortative mating is not a common outcome of guppy evolution, even after increasing the dimensionality of selection by experimentally manipulating parasite levels. Chapter 4 underlines the importance of conserved mate preferences in thwarting the influence of strong and multifarious divergent natural selection. These results should encourage future studies of ecological speciation to pay increased attention to the mechanisms through which variation in mating systems and mate choice preferences constrain the development of reproductive barriers, thus increasing our understanding of "ecological speciation, or the lack thereof" (15).

Chapter 5 highlights the importance of interspecific social interactions and their role not only as an antipredatory strategy but also as a novel and previously untested resistance mechanism against contagious parasites. Although most species managed for conservation and productive purposes tend to be held in single-species groups, my findings suggest that mixed-species groups are a potentially cost-efficient strategy to control parasite burdens.

6.3 General implications:

Parasites, the type of enemy that is the focus of my dissertation, are a pervasive and influential driver of ecological (16-18) and evolutionary (19-22) processes, with the potential to initiate major ecosystem alterations (23, 24). Thus, by extending our understanding of the ecological and evolutionary interactions that shape host defence, this thesis contributes to our predictive capacity and can inform conservation (25, 26), food security (27), disease control (28), invasive species management (29) and poverty alleviation (30) strategies. Furthermore, the studies in this thesis are connected by more than the model system and the ecological and evolutionary implications of enemy release in nature: they present a nuanced reality that is counterintuitive to simple theoretical and empirical expectations under univariate selection. Thus, my results expand our understanding of selection and host-parasite interactions in the wild and highlight the need for increased consideration of multiple sources of selection and pleiotropy when studying evolution in natural contexts.

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APPENDIX A

Felipe Dargent, Marilyn E. Scott, Andrew P. Hendry and Gregor F. Fussmann (in review). A reply to Stephenson (2014): Experimental evolution of parasite resistance in wild guppies: natural and multifarious selection. Proceedings of the Royal Society B. (281) 1794. DOI 10.1098/rspb.2014.1820

A.1 Reply:

In Dargent et al. (1) we reported the evolution of increased resistance in populations of wild female guppies after the removal of an ectoparasite (*Gyrodactylus turnbulli*) and discussed possible mechanisms for this unexpected outcome. In her comment (2), Stephenson proposes two additional mechanisms that could have led to increased resistance: [1] artificial selection of more resistant individuals both prior to introduction and during laboratory rearing, and [2] differences in per capita resource availability due to different guppy densities between source and introduction sites. Additionally, the author suggests that our experimental design was not optimal for testing the effects of parasite removal on the evolution of resistance because we did not control for some other sources of variation.

Stephenson's suggested alternative mechanisms are certainly valid *a priori* hypotheses that might also occur to other readers. We therefore welcome the opportunity to further strengthen our conclusions, first by explaining why these alternatives do not apply to our study, and second by showing how several of the concerns raised by Stephenson support, in a broad sense, a central point of our paper. That is, multifarious selection (i.e. the presence of multiple other variables that could interact with parasitism) is the very context in which selection by parasites should be more often considered. In particular, studies in nature that include these other variables are informative of the conditions under which resistance actually evolves in nature, insight that thereby allows one to assess the importance of parasitism in the evolution of defence when multiple sources of selection, not only parasitism, might matter. We now discuss each of the points raised by Stephenson.

Stephenson suggests that artificial selection could have been imposed by selective mortality due to previous infection, handling, and antipathogen treatments. As an example of

artificial selection, the author cites a study by van Oosterhout et al. (3) that reported 14% handling-induced mortality for wild guppies and 43% handling-induced mortality for lab-reared guppies. We agree that 14% mortality is high and could impose unwanted selection. Mortality levels were much lower in our study. Between collection of fish from the source population and release into the introduction streams mortality was <2% (4). Furthermore, no deleterious effect of antipathogen treatments was detected, marking-induced mortality was below 1% (5), and fry mortality prior to infection in our laboratory flow-through system was below 3%. These low levels of mortality, even if selective, could not explain our finding of rapid and repeatable evolution of increased resistance to the now-absent parasite.

Stephenson suggested that a temporal pattern of increasing resistance would be one way to substantiate our conclusions by negating concerns regarding artificial selection. As the two collection years were separate experiments, it would have been invalid to directly compare them (1). However, changes between years/experiments in the magnitude of among-population differences, makes the point just as effectively. In particular, the difference between introduced and source populations increased from the first collection year/experiment to the second, and a population (Lower Lalaja) that was not significantly different from the source population after one year had significantly higher resistance on the second year. This temporal comparison, provides independent support (in addition to the above-noted low mortality) removing artificial selection as an explanation for our findings.

Stephenson's second point is that resource availability mediated by guppy density might have contributed to the differences in parasite resistance we observed. We agree that per capita resources availability matters, but we did not emphasize this metric as it (in contrast to Stephenson's suggestion) would not be lower in the introduction sites. First, population growth

within the introduction streams was rapid because half of the guppies introduced were gravid females, which would have led to at least a five-fold increase in density within the first month. Indeed, by the time our assays were conducted (and likely much earlier), guppy densities were already very similar to those suggested by Stephenson for low-predation sites (Table A.1 (5)), and did not correlate with infection-assay peak loads (R^2=0.15, $F_{1,6}$=0.69, p=0.45). Second, population densities in high-predation sites are generally lower, and productivity higher owing to more open canopies, than are densities and productivities in low-predation sites (6, 7). As a result, Stephenson suggestion that per capita resource availability would be higher in the low-predation introduction populations than in the high-predation source population is not supported by data for guppies, including our specific populations.

Stephenson's third point is that our study was not optimally designed to detect effects of altered parasitism because other factors differed between the introduction and source populations. This concern certainly would be valid if our goal was to isolate the effects of parasitism from other sources of natural variation, which can be achieved only through laboratory experimental evolution. However, our specific goal was to assess the evolution of parasite resistance in the presence of all that other variation, which can be achieved only through experimental evolution *in nature*. Resistance to a common and deleterious parasite is traditionally and theoretically expected to be more strongly influenced by the parasite itself than by correlated factors (8); yet, as we show, this effect is overridden by other factors (most likely predation) when placed in a natural context. Thus, our study was appropriately designed to explore the questions that we intended to test. In fact, the contrast between Stephenson's concern and our actual conclusions nicely highlights the significance of our study – it specifically tests something that controlled laboratory studies cannot. We agree that disentangling the effects of

each axis of variation independently would be an interesting next step having now shown that, under multifarious selection, a pattern expected under univariate selection does not hold.

Interestingly, and as noted in the original paper, our seemingly surprising findings actually mesh quite well with previous findings. In particular, low-predation guppies have lower *Gyrodactylus* loads than do high-predation guppies (9) and previous field introductions from *Gyrodactylus*-present sources (which did not report any anti-parasite treatments), all have no presence of *Gyrodactylus* (exception El Cedro - prevalence 1.4%) (9), which confirms a general higher resistance to parasitism in low-predation populations. Moreover, the high frequency of an MHC allele (10) that correlates negatively with *Gyrodactylus* load (11) is retained in these populations. Even more directly, the historic Turure introduction used an ancestral population from the same site as we did (Guanapo source) over 60 years ago, and these fish remain *Gyrodactylus* free (9, 10). Similarly, guppies introduced from the Yarra River high-predation site into the previously guppy-free Damier River remained infected in the high-predation reach (prevalence 16%) but not the low-predation reach (9). Finally, the low-predation population of the Guanapo River (upstream from the ancestral source population but downstream from the introduced populations) was *Gyrodactylus* free before and during the experiment (9, 10). In short, populations introduced from high-predation sites to low-predation sites, either by colonization or by translocation seem to repeatedly evolve increased resistance to *Gyrodactylus* parasites, a pattern inconsistent with traditional ideas but that we were able to experimentally confirm.

We appreciate the comments by Stephenson and the opportunity provided to enhance support for our results by showing that – in our specific study at least – the otherwise valid concerns do not apply. Moreover, they have allowed us to further highlight the importance of

considering the role of multifarious selection in natural contexts, such as can only be achieved through experiments in nature. We anticipate that our initially surprising results will continue to spawn alternative explanations, and we hope that these emerging ideas allow the opportunity for further discussion.

A.2 Acknowledgments:

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Table A.1: Guppy densities at the introduction streams (5):

Collection	Population	Density m^{-2} (95% C.I.)
2009, 1 year after introduction	Lower Lalaja	3.41 (2.79-4.02)
	Upper Lalaja	2.40 (1.80-3.01)
2010, 2 years after introduction	Lower Lalaja	8.32 (7.81-8.84)
	Upper Lalaja	9.97 (9.42-10.52)
2010, 1 year after introduction	Taylor	6.36 (5.17-7.56)
	Caigual	4.01 (3.81-4.21)

Guppy densities based on mark-recapture models at the introduction streams one and two year after introduction for individuals larger than 14mm standard length. Data from (5).