

The University of South Bohemia in České Budějovice

Faculty of Science

**Trade-offs in parasitoid resistance and
competitive ability in *Drosophila***

Master's thesis

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ANNOTATION:

Trade-offs in fitness traits in *Drosophila* were studied in a laboratory environment. Fly and wasp lines originated from an ecological community in a tropical rainforest in Queensland, Australia and were established in České Budějovice. Experimental evolution was used to impose selective pressures on populations of flies to select for parasitoid resistance and competitive ability. The selection regimes were followed by two separate phenotyping experiments to measure the resulting phenotypic changes and impose novel conditions to the evolved flies in order to identify trade-offs.

DECLARATION:

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

České Budějovice, 8 April, 2022



Jeni Sage Sidwell

Acknowledgements

First and foremost, I must express my deepest gratitude to my supervisor, Jan Hrček, for supporting me, challenging me, listening to me, trusting me, and drinking (a lot of) beer with me all throughout this one-of-a-kind experience. Working in his lab has given me incredible research experience and self-assurance, truly sacred gifts that I'll take with me into the next steps of my career and life.

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“Had I been present at the creation, I would have given some useful hints for the better ordering of the universe.”

-Alfonso X

Dedicated to Rho.

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Introduction

Ecological and evolutionary trade-offs

In nature, evolutionary adaptation is driven by ecological interactions, and those adaptations subsequently affect species' performance in novel ecological conditions (Ellers 2009, Hairston et al. 2005, Sigmund and Holt 2021). This overlap and positive feedback loop can be referred to as eco-evolutionary dynamics. Studying the dynamics of the two processes together is essential to forming an accurate picture of species adaptation and response to selective pressures, including environmental changes and community structure (De Meester et al. 2019).

Traditionally, ecology and evolution have been taught and studied separately. According to recent commentaries by Urban et al. (2020) and Hoffmann and Flatt (2022), it was previously believed that ecology and evolution operated on separate time scales: evolution was seen as a process operating on vast scales, unlike ecology, but increasing evidence shows that evolution can also operate on fine time scales simultaneously with ecological processes. Eco-evolutionary dynamics are difficult to investigate, but experimental approaches can be pivotal in investigating the relationship between ecology and evolution (Rudman et al. 2022). Trade-offs are a fundamental topic of study which can help us explore these eco-evolutionary dynamics and their broader implications. Organisms regularly experience multiple selective pressures, and theories related to fitness generally rely on the assumption that the development of one fitness trait occurs at the expense of another (McPeck 1996, Stearns 1989, Garcia-Robledo and Horvitz 2011). More directly, no genotype can have maximal fitness in its resource-limited, energetically costly environment, so energy is allocated to certain traits to optimize adaptation to selective pressures.

Selective pressures, driven by the environment and ecological interactions, influence the energy allocation for physiological or phenotypic changes in a population, but phenotypes can be unstable and are not always followed by changes in the genome (Bergelson 1994, Bohannan and Lenski 2000, Thompson 1991). In other words, the adaptations are often short-term. Studies on the mechanisms of adaptation commonly address phenotypic plasticity as being an example of a clever response to selective pressures in unstable or stochastic environments: certain advantageous traits are expressed, but these traits are already present in the genome and the

associated adaptations don't always result in genetic change (Stearns et al. 1991, Urban et al. 2013). When phenotypes are relatively unstable, and selective pressures persist, long-term genetic change may follow (Grether 2005, Torres-Garcia et al. 2019). Trade-offs can therefore be identified as physiological changes, reflecting different allocations of energy that we can identify at phenotypic level (sometimes referred to as “ecological” trade-offs), or evolutionary changes, which are reflected in the genome (referred to as “evolutionary” trade-offs; Fry 2003, Garcia-Robledo and Horvitz 2011, Schlenke et al. 2007). These short- and long-term adaptations are driven by eco-evolutionary dynamics, and are both heavily influenced by factors like genetic diversity and population size (see Torres-Garcia et al. 2019). The process of identifying whether trade-offs are ecological or evolutionary is complex, but with cutting-edge sequencing technology, we are now well-equipped to experimentally explore the means by which selective pressures can influence short- and long-term adaptation (Hoffman and Flatt 2022, Huang et al. 2018, Toquenaga et al. 2003), although most experimental approaches have investigated trade-offs by primarily focusing on single selective pressures at a time, rather than the multiple pressures that regularly occur simultaneously in nature.

Life history trade-offs

Trade-offs are central to life history theory. The well-known 1989 review by S.C. Stearns discusses life history trade-offs as being centered primarily around reproduction, or, more directly, on the energy allocation needed to survive long enough for successful reproduction. The review emphasizes that trade-offs can be measured intra-individually, or how an individual female in a population might change her behavioral patterns from one mating season to the next, and intra-generationally, which accounts for the probability of the offspring's survival. Trade-offs are often studied at the population level by means of genetic structure and mean phenotypic changes, and many researchers argue the need for ecological and evolutionary trade-offs to be studied in conjunction by linking phenotype, genotype, and the intermediate structure (see Fry 2003, Schwenke et al. 2015, Stearns 1989). Stearns goes on to list the commonly studied life history trade-offs: 1) Age vs size of maturity, where organisms must evolve a reaction norm for maturation in a heterogeneous environment. In many cases, faster growth indicates earlier

maturation and slower growth indicates later maturation. In all cases, these reaction norms for maturation and growth rate are dependent on environmental conditions. 2) Reproductive investment vs survival. Some life history strategies involve high reproductive investment which gives way to higher offspring survival. Other strategies include lower reproductive investment and lower survival, which is seen in conjunction with higher numbers of offspring for better intra-generational survival probability (Stearns 1989).

With these broad life history trade-offs come changes in traits such as body size, clutch size, lifespan, and other components of reproductive optimization (Lancaster et al. 2017, Stearns 1989). These traits can be measured with phenotyping and genetic methods, and with the development of increasingly sophisticated means of genotyping, more emphasis should be placed on linking phenotype and genotype to determine whether the trade-offs in a population are ecological or evolutionary (Fry 2003, Garcia-Robledo and Horvitz 2011, Schlenke et al. 2007). The research community has historically approached studies in trade-offs by examining a single pressure at a time with a focus on either ecological or evolutionary changes, but recent studies and commentaries, for example a review by Sigmund and Holt, argue that investigating the ecological drivers of evolutionary adaptation under multiple pressures is essential to understanding species response to environmental changes (Sigmund and Holt 2021). Therefore, it's important to also look at how trade-offs arise in a broader scope: apart from optimization in reproduction as it relates to life cycles, trade-offs can also be identified in optimization of other survival traits in fluctuating environments in communities, as multiple selective pressures regularly occur in communities.

Trade-offs in community ecology

Adaptive response in the real world is complicated, especially when examined through the lens of community ecology (Johnson and Stinchcombe 2007). Research by Pardikes et al. (2021) and Thierry et al. (2019) highlight the need for a community focus in eco-evolutionary theory: community structure is variable and dependent on compounding effects of multiple simultaneous interactions. For example, the performance and phenology of a host-parasitoid community under warming conditions is primarily dependent on community interactions and

intricate networks over pairwise interactions (Pardikes et al. 2021). In other words, rich biodiversity is essential under changing conditions, and the ecological drivers behind evolutionary adaptation gives us valuable insight into how populations in communities co-evolve and how they tend to respond to changes in environment and community composition. Because this allocation of energy is so variable in community ecology, trade-offs are a powerful method of investigating eco-evolutionary processes (Lancaster et al. 2017, McPeck 1996, Sterck et al. 2011, White et al. 2004).

Haloin and Strauss (2008) emphasize in their review that the feedbacks of ecology and evolution operate across simultaneous time scales, and species in communities are constantly experiencing multiple simultaneous selective pressures from their biotic interactions and the environment. Those community-wide selective pressures (e.g. resource availability, predator/parasite abundance, competition, environmental stressors, etc) can change gradually or abruptly and ultimately influence survival traits, so populations within the communities are forced to optimize their adaptive response to the present conditions in order to survive and reproduce (Egas et al. 2004, McPeck 1996). A recent study by Rudman et al. (2022) discusses the prevalence of abrupt changes in ecosystems and the subsequent species adaptive response. In the study, there is clear evidence that evolution can operate on a time scale compatible with that of rapid ecological change. Using a combination of field observations and a common garden experiment, rapid and continuous evolutionary adaptation was observed from 10 different fruit fly (*Drosophila melanogaster*) populations in response to strong environmental pressures. This quick development of adaptive fitness traits emphasizes the impact of evolutionary change on ecological outcomes. More research that builds on this idea of energy allocation in the context of eco-evolutionary dynamics can give us valuable insight into species' adaptive response to major environmental changes.

In addition to discussing the influence community structure and environmental changes have on fitness traits, it is important to note how those fitness traits vary, both between species and within species. Historically, interspecific (between species) trait variation has been commonly investigated in community ecology, but recent studies and commentaries increasingly advocate for a focus on the fact that intraspecific (within species) variation influences community structure as well. Reviews by Bolnick et al. (2011) and Violle et al. (2012) discuss the importance of giving intraspecific variation its deserved focus: investigating intraspecific

trait variation that arises from energy allocation in multiple pressure conditions provides a clearer picture of community dynamics than looking only at interspecific variation. According to these commentaries, high variability in functional traits can occur within species, and can subsequently alter community structure as directly as interspecific variation can. Therefore, it is important not to disregard intraspecific trait variation in studies regarding trade-offs in community ecology.

There are a large number of studies that examine trade-offs in communities. Some researchers approach this with observational studies in nature, and other research involves structured experiments in a laboratory setting. Given that trade-offs are such an essential component of ecology, evolutionary adaptation, and interacting mechanisms of both, research in trade-offs span across a wide variety of study systems, each offering a different focus on the same broad questions (see e.g. Sterck et al 2011, White et al. 2004, Schwenke et al. 2015), and further research in trade-offs would benefit from approaches that acknowledge intraspecific variation and consider the overlap in time scale in ecology and evolution. However, eco-evolutionary dynamics are difficult to investigate, so experimental approaches are powerful in identifying causal relationships (Kawecki et al. 2012, Kubrak et al. 2017, Rodriguez et al. 2017).

Experimental evolution

Experimentally testing eco-evolutionary processes that arise in intricate community structures is limited. However, we can achieve a glimpse into these mechanisms with experimental evolution. Experimental evolution is the study of processes of adaptation with conditions imposed by the experimenter (Burke and Rose 2009, Huang et al. 2018, Kawecki et al. 2012, Rodrigues et al. 2016). Essentially, populations can be evolved to develop traits of interest in a laboratory setting, making it a very powerful method for studying adaptation and species response to selective pressure, as seen in Richard Lenski's long-term experiment (Lenski et al. 1988) and its follow-ups, where different selective pressures have been imposed on strains of *Escherichia coli* over time, showing species adaptive response to chosen conditions. Experimental evolution has seen applications in various fields such as vaccine development, biotechnology, natural resources, and climate research. A review by Kawecki et al. (2012) notes

that experimental evolution is distinct from the widely-known approach of artificial selection, or selective breeding, where surviving individuals used to sire each generation are specifically chosen. This method strengthens the selective pressure and, as a result, often shows more dramatic results. The Kawecki et al. (2012) review emphasizes that experimental evolution differs in a few key ways: each generation is sired by randomly chosen individuals, and the natural environmental conditions (e.g. temperature, humidity, and light/dark cycle) are usually replicated in the lab. Although strong phenotypic and genotypic changes are not as well-guaranteed as with artificial selection, experimental evolution is arguably a more informative method for exploring natural processes, given that it more closely mimics nature (Kawecki et al. 2012).

Although experimental evolution and other selection experiments are used by researchers to study trade-offs (see an aforementioned follow-up from Lenski's lab, Bennett and Lenski 2007), most experiments do not explicitly focus on the multiple selective pressures that inevitably occur in community ecology, as has been noted to also occur in most observational studies on trade-offs in nature (see Brody 1997, Raffa and Barryman 1987). Building upon research with well-defined experimental evolution protocols to include this focus would be essential to better understanding evolutionary drivers of species adaptive response. Experimental evolution also allows for the possibility of studying eco-evolutionary feedbacks in a controlled setting.

Trade-offs in *Drosophila* systems

Overview

Fruit flies (*Drosophila*) are a model eukaryotic system for evolution experiments, including studies that examine trade-offs. Evolution experiments with *Drosophila* have been historically approached with artificial selection regimes, but experimental evolution is an increasingly utilized method. In a review by Burke and Rose, they discuss the effectiveness of the experimental evolution approach to explore the evolution of fitness traits in *Drosophila* systems. They introduce three forms these evolution experiments can take: direct selection for a trait, surveys of traits of interest in a population that was selected for other traits, and reverse

selection. Applications often include selecting for traits of interest in some populations and comparing these traits with a control population using a well-structured experimental design protocol. These experiments may be followed by phenotyping or genotyping experiments to analyze changes in phenotypes or allele frequencies in a population. Some of the commonly studied trade-offs in *Drosophila* evolution experiments include intraspecific competitive ability, stress tolerance (e.g. performance in different temperatures), and resistance to parasitoid wasps, other parasites, or pathogens. Typically, selective pressure is consistently imposed on populations of flies for several generations to encourage the development of these traits, encouraging adaptive response over time (Burke and Rose 2009).

Researchers commonly measure intraspecific competitive ability in *Drosophila* by rearing larvae in reduced food resources or higher population density, then counting the subsequent emergences (Kraaijeveld and Godfray 1997, Luong and Polak 2007). Resistance is commonly measured with emergence counts, larvae encapsulation and melanization (an immune response in some species which prevents successful parasitism by attacking a larval parasitoid and killing it, subsequently developing a darkened spot), and immune cell (hemocyte) counts (Boots and Haraguchi 1999, Fellowes et al. 1999, Kraaijeveld et al. 2001). As McGonigle et al. (2017) explain in a study which examines *Drosophila melanogaster* immune cell response when artificially selected for increased resistance, producing immune cells is very energetically costly, making it inevitable that the development of resistance occurs at the cost of other fitness traits. However, the study notes that immune responses differ quite widely between *Drosophila* species, and most of the data we have include only *D. melanogaster* (McGonigle et al. 2017).

D. melanogaster is the most widely used *Drosophila* species for experimental investigation, and most of what we know about trade-offs in *Drosophila* comes from data from this species. In a well-renowned study by Kraaijeveld and Godfray (1997), they identified a trade-off between parasitoid resistance and larval competitive ability in *D. melanogaster* using artificial selection. They exposed populations of flies to parasitoid infections to select for resistance, and reared other populations in varying levels of reduced food resources to select for increased competitive ability. These were followed by phenotyping assays, and their results show that increased resistance correlated with decreased competitive ability. Sanders et al (2005) did a similar study with *D. melanogaster*, investigating the reverse: whether there is a correlated response between increased competitive ability and decreased resistance. Interestingly, their

results indicated that flies with increased competitive ability didn't suffer from poorer performance in resistance conditions. This could be due to crowded conditions encouraging better wound response. Ultimately, studies such as these give us insight into trade-offs in crucial fitness traits in *Drosophila*, and this has implications for species response to extreme environmental stressors in this and other eukaryotic systems. The experimental design protocols in these studies are well-defined and have a wide-reaching impact for experimental approaches to understanding fitness costs, and further research could build on fundamental studies like this with a focus on better representing ecology and evolution as occurs in nature, such as more than a single selective pressure at a time and an experimental evolution approach rather than artificial selection.

Further examples of selection experiments in Drosophila

Luong and Polak (2007) explored evolutionary trade-offs between parasite resistance and larval competitive ability in *Drosophila nigrospiracula* and compared the performances in varying temperatures. This study compared evolved and control populations using an artificial selection experiment followed by phenotyping and genotyping assays. They tested for genetic differentiation at varying levels of selective pressure, and their results revealed a negative genetic correlation between resistance to an ectoparasitic mite (*Macrocheles subbadius*) and competitive ability in moderate to severe conditions. Their results show an environment-dependent trade-off between these two traits in populations undergoing a single-selective pressure regime approach.

In a follow-up study from the Kraaijeveld lab by Vijendravarma et al. (2009), they used an experimental evolution approach, unlike the artificial selection approach in the original study. Populations of *Drosophila melanogaster* were exposed to a microsporidian pathogen to increase resistance, and explored the resulting trade-offs with phenotyping assays. Their results, when compared with the control, suggested successful selection for resistance to the pathogen, which was reflected by increased densities of hemocytes, higher early-life fecundity, and longer-term survival under pathogen exposure. The evolved flies consequently showed a number of fitness costs, including lower fecundity and poorer larval competitive ability in harsh environmental conditions when compared with the control lines, as expected.

Intellectual merit

As is evident in these and other experimental studies on trade-offs in *Drosophila* systems, researchers often use artificial selection (but see studies like Stearns et al 2000), and focus on only one selective pressure at a time. Building on well-defined experimental protocols like these with closer attention to representing natural conditions (i.e. multiple selective pressures and experimental evolution) could give us a better indication of species response to stressors, especially with rapidly changing environments. Furthermore, this approach gives us a better eco-evolutionary vision of these adaptive mechanisms, which is also directly applicable to how communities could be evolving in nature. It would also benefit research in *Drosophila* experimental studies to use species other than *D. melanogaster* to compare adaptive responses between a wider variety of species in this model study system.

Aims

In this study, we first used an experimental evolution approach with two *Drosophila* species (*D. birchii* and *D. sulfurigaster*) that have been rarely used in selection experiments. Our selection regimes aimed to increase the development of the following traits: intraspecific larval competitive ability and parasitoid resistance. The evolved populations then underwent separate phenotyping experiments, during which we measured the resulting phenotypic changes in each population. The evolved flies were compared with a control. The aim of this approach was to 1) determine phenotypic changes associated with adaptation, then test those phenotypes in novel conditions to look for ecological trade-offs, and 2) to see how each population responds to conditions with multiple selection pressures. The following questions were addressed:

- 1) In our two new species, does increased performance in one condition cause decreased performance in the other condition?
- 2) Which phenotype (evolved or control) is more optimal for conditions with multiple selective pressures?

Our expectations included 1) identifying trade-offs between resistance and competitive ability, and 2) seeing better performance from the general phenotypes (control) than the more extreme phenotypes (evolved) in the multiple pressure environment.

Methodology

Study system

General system

Drosophila and their parasitoid wasps (Hymenoptera) are generally accepted as being a model study system for evolutionary experiments in eukaryotes (Nouhaud et al. 2018, Parsons 1991, Schlenke et al. 2007, Vijendravarma et al. 2009). In nature, *Drosophila* larvae feed on yeast growing on rotten fruits, but are rarely specialists. As a result, their natural ecology is relatively easy to mimic in a laboratory setting, giving us a reasonable representation of how larvae might develop and emerge in nature (Burke and Rose 2009). In a laboratory setting, many *Drosophila* species are extensively used and well understood, being easy to collect, rear, and manipulate. Their large population sizes and short life spans cultivate rich possibilities for evolutionary change, and there are extensive records of existing genetic and phenotypic data available for many *Drosophila* species (Burke and Rose 2009, Stearns et al. 2000, Vijendravarma et al. 2009).

Parasitoids are an exceptionally diverse group of taxa, representing roughly 10% of known insect species (Janssen 1989, Lue et al. 2018). These wasps parasitize a variety of hosts, including the larvae of *Drosophila* species. The wasp larvae develop and emerge from inside the *Drosophila* larvae, killing their host in the process (Green et al. 2000, Janssen 1989). These species have co-evolved in diverse ecological communities, making them a valuable study system for exploring eco-evolutionary dynamics (Lue et al. 2018, Rolf and Kraaijeveld 2008). Thus, this study system was selected in order to build upon existing research on these salient species and investigate the ecological drivers of evolutionary adaptation from an effective and accessible approach.

Australian system

For our evolution experiment, we selected 2 *Drosophila* species: *D. sulfurigaster* and *D. birchii*. These species coexist and are abundant in their natural communities, and are parasitized by a wide variety of shared wasp species. We have also selected 2 parasitoid wasp species: *Asobara* sp. (Braconidae: Alysiinae; strain 179C, reference voucher no. USNMENT01557099, 10

reference sequence BOLD process ID: N/A) and *Leptopilina* sp. (Figitidae: Eucolinae; strain 111F, reference voucher no. USNMENT01557117, reference sequence BOLD process ID: DROP053-21; Lue et al. 2021). Given that the parasitoid species are yet to be formally described, these details are provided so they can be precisely identified. Our species originate from a tropical rainforest community in Queensland, Australia, and we have established a large number of lines in the laboratory. After testing each combination at varying selective pressures, results suggested that the most interesting species combinations were *D. birchii* and *Asobara* and *D. sulfurigaster* and *Asobara*, both at medium selective pressure (2 wasps per vial). Ergo, final results from these combinations will be discussed in the most detail.

The natural areas in which our species are found have minimal impact from humans, are easily accessible, and have relatively mild seasonality and climate, with an average daily temperature of 23.5°C. Study sites range from rainforest areas along the northeast coast of Queensland between Cooktown and Townsville. These areas are species-rich and diverse, harboring intricate *Drosophila*-parasitoid networks in a well-preserved natural environment (Jeffs et al. 2021). The *Drosophila*-parasitoid system from this area has been established in our laboratory by a collection of isofemale lines in 2017-2018 and maintained in similar environmental conditions since. Given that this system is easy to replicate in a laboratory setting and representative of natural conditions, it is ideal for eco-evolutionary experiments.

Experimental evolution

Overview

During each generation of the evolution experiment, we used two selection regimes: 1) exposure to parasitoid wasps to increase resistance, 2) reduced food resources to increase competitive ability (figure 1). Surviving flies were used to sire the next generation. The *Drosophila* populations were founded with mass-bred isofemale lines. Mass bred populations of *Drosophila birchii* or *D. sulfurigaster* were founded with 10 (low diversity) or 40 (high diversity) recently established isofemale lines. Several combinations of lines were used to control for the sampling effect. 10 vials containing 100 eggs each were collected at the beginning of each generation. The intention was to keep population sizes fairly large in order to maintain genetic

diversity, and population density was kept as constant as possible across all lines. The flies were reared in vials with a standard diet to feed the larvae. The selection regimes lasted 16 discrete generations for resistance populations and 12 for the competition populations (fewer generations due to slower developmental time).

Experimental Evolution

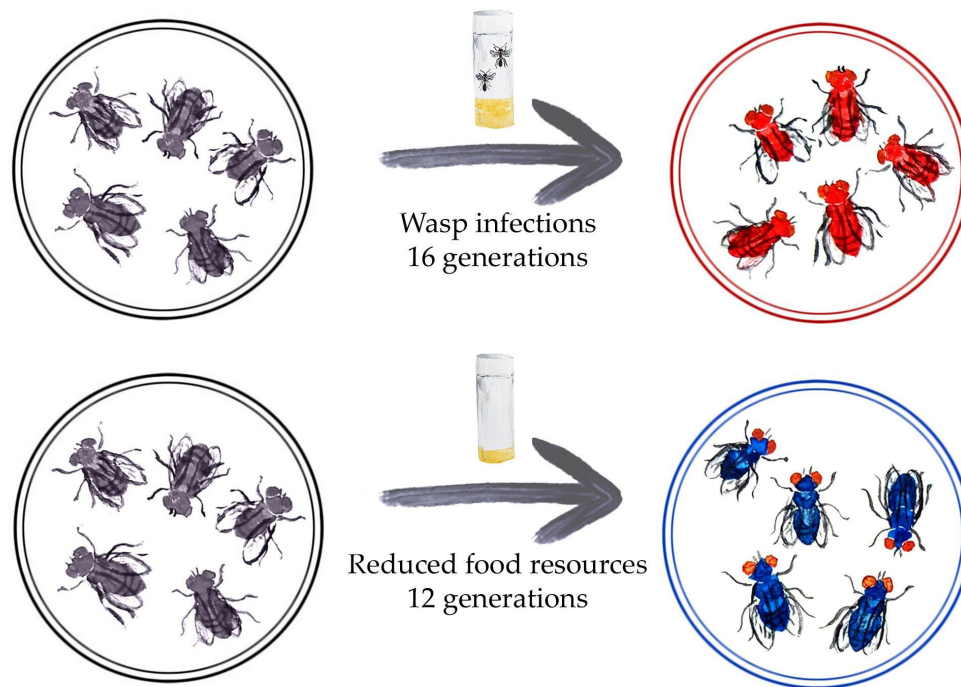


Figure 1: Representation of the selection regimes for the experimental evolution project. Flies selected for resistance were exposed to 2 parasitoids at their larval stage each generation, and flies selected for intraspecific competitive ability were reared in reduced food (4ml).

The wasp lines were kept separately to prevent co-evolution. Wasp lines were maintained on *D. melanogaster* and had no prior experience with *D. birchii* nor *D. sulfurigaster*. Twice per week, 15 female and ~1-2 male wasps from the line were introduced into six fresh vials with larvae from *D. melanogaster* to maintain our lines and keep the population sizes large. We used only female wasps in our experimental evolution infections.

The selection regimes were followed by a generation of relaxed selection before two separate phenotyping experiments to measure and compare the phenotypic changes from each population. Some of the data were inconclusive or insignificant (e.g. the combinations with *Leptopilina* wasps and the competition populations) and were omitted from the final analyses.

Experimental evolution conditions:

Drosophila birchii with *Asobara* infections (low and high pressure)

Drosophila birchii with *Leptopilina* infections (low and high pressure)

Drosophila birchii with reduced food resources

Drosophila sulfurigaster with *Asobara* infections (low and high pressure)

Drosophila sulfurigaster with *Leptopilina* infections (low and high pressure)

Drosophila sulfurigaster with reduced food resources

Environment parameter for all populations:

Temperature: 24°C

Humidity: 70%

Lighting: 12:12 light:dark regime

Populations selected for increased parasitoid resistance

The flies exposed to wasp infections included two replicates with different pressures: low (one female wasp per vial), and high (two female wasps per vial). *Drosophila* larvae were exposed to the wasps for 24 hours after reaching the second instar (about 50h after oviposition). This timing ensured susceptibility to parasitism, but also the likelihood of some larvae surviving. The wasps were generally chosen from the newest vials possible to ensure fertility and increased chance of parasitism. Flies were reared in 12ml of food per vial.

Populations selected for increased intraspecific competition

Drosophila larvae were exposed to reduced food resources each generation. We chose to reduce the amount of food rather than increase the number of individuals per vial so that we could keep our population numbers constant, thereby enhancing ease of analysis. We used 4ml of yeast mixture per vial for the 100 larvae, compared to the standard 12ml used for the resistance

and control treatments. Agar was used underneath the yeast mixture to keep it moist for longer, in order to accommodate the fact that the reduced food resources results in slower developmental time.

Control

A control population reared in benign conditions served as a reference for the final phenotyping assays. This population was founded with randomly obtained eggs from the box of mass-bred isofemale lines before the final phenotyping experiments, then was kept for 4 discrete generations with the same protocol as the rest of the populations (10 vials of 100 larvae, with surviving flies siring the next generation). The control flies were reared in 12ml of food per vial.

Phenotyping experiments

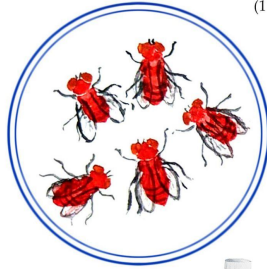
Overview

In order to ascertain whether phenotypic changes could be detected after the selection regimes, and how those changes influenced performance in different conditions, the experimental evolution was followed by separate phenotyping experiments. There were 2 phenotyping experiments: the first one aimed to test every population from the experimental evolution project, and the second one tested only the populations with the most significant results from the first phenotyping experiment. Although the experimental evolution portion of the project included populations evolved for increased competitive ability, they were ultimately removed from the final phenotyping analyses due to too few eggs and inconclusive data. Ergo, results from the analyses that reference populations labeled as “evolved flies” are all flies from only the resistance selection regime.

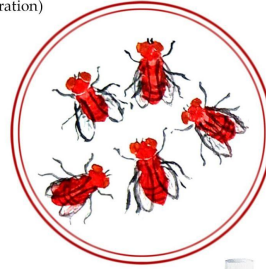
The phenotyping experiments involved testing flies from each population (evolved and control) in each of the different conditions (wasp infections, reduced food, and none/benign), as well as a combination of conditions (wasp infection and reduced food; Figures 2 & 3). Just as with the experimental evolution, 100 eggs were collected for each vial. Phenotypes were measured by emergence counts (flies and wasps that survived until adulthood). Emergences were counted by using CO₂ to anesthetize the individuals in each vial and logging the numbers, then transferring the adult flies to new vials with tweezers and aspirators.

Phenotyping experiments: evolved flies

(1 generation)



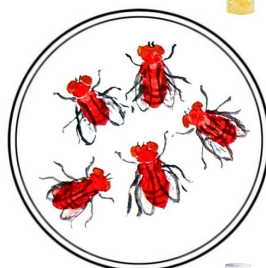
Reduced food



Wasp infections



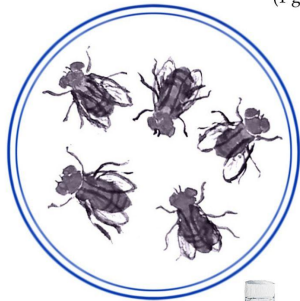
Reduced food and
wasp infections



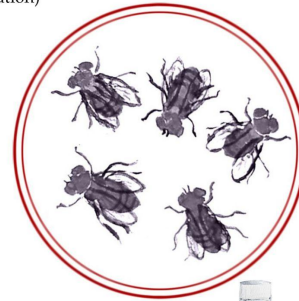
Benign

Phenotyping experiments: control flies

(1 generation)



Reduced food



Wasp infections



Reduced food and
wasp infections



Benign

Figure 2: Conditions imposed on the evolved (resistance, panel A) and control (panel B) populations for the phenotyping experiment to assess the optimal phenotypes for the selective pressures. This was repeated for all fly/wasp combinations.

Phenotyping experiment #1

The aim of the first phenotyping experiment was to test the phenotypic changes in all of the evolved and control populations from the evolution experiment and determine which combinations and conditions yielded the most significant results.

Species combinations used:

Drosophila birchii with *Asobara*

Drosophila birchii with *Leptopilina*

Drosophila sulfurigaster with *Asobara*

Drosophila sulfurigaster with *Leptopilina*

Conditions:

- 1) Parasitoid infections: low and medium pressure; 1 or 2 wasps (respectively) per vial introduced at the second instar (~50h after egg collection), removed after 24 hours.
- 2) Reduced food resources: 4ml of food per vial
- 3) Combined conditions: 4ml of food per vial with 1 wasp introduced at the second instar (72 hours after egg collection rather than 50, to account for slower developmental time in the reduced resources), wasp removed after 24 hours.
- 4) Benign conditions (no infections, 12ml of food)

Replicates: 4 per condition type.

Phenotyping experiment #2

In order to test whether our results were consistent, we repeated the phenotyping experiment with the most interesting populations. Two notable changes in this protocol included

1) increasing the number of replicates per condition type, and 2) easing the pressure for the reduced food conditions to encourage better survival in the multiple stressors conditions.

Species combinations used:

Drosophila birchii with *Asobara*

Drosophila sulfurigaster with *Asobara*

Conditions:

- 1) Parasitoid infections: medium pressure; 2 wasps per vial introduced at the second instar, removed after 24 hours.
- 2) Reduced food resources: 8ml of food per vial
- 3) Combined conditions: 8ml of food per vial with 1 wasp introduced at the second instar, removed after 24 hours.
- 4) Benign/none (no infections, 12ml of food)

Replicates: 6 per condition type.

Analysis

Statistical analyses were conducted in the software package R. Models were tested and compared to optimize the fit of each model to our data. Final selected model types included generalized linear models (GLMs) with a quasibinomial distribution to account for overdispersion.

The models were used to analyze the performance of each species from each of the phenotyping experiments. The generalized linear models were used to identify the best predictors (population, conditions, or interacting effects of population and conditions) for fly and wasp emergence. After the models were fitted and selected, more detailed examinations of the results of our tests could be done.

The first aim of these analyses was to ascertain whether our evolution experiment worked by determining whether the evolved flies performed significantly better in their accustomed

conditions (e.g. wasp infections) than the control. Performance of flies was measured both by emergence counts of flies (more surviving flies indicates better performance) and emergence counts of wasps (fewer surviving wasps also indicates better performance, i.e. resistance). The next aim of these analyses was to infer the existence of trade-offs by determining whether the evolved flies performed significantly poorer than the control in the conditions to which they were not accustomed (e.g. reduced food resources). Finally, we aimed to identify how the performance of evolved and control populations compared in the combination of conditions (i.e. which phenotype is most optimal for these multiple selective pressures).

Results

Overview

The experimental evolution regimes and both phenotyping experiments ultimately became a relatively extensive experimental protocol, and data were evaluated after all of the experiments concluded. The experimental evolution project involved individually counting 1000 fly eggs per population (12 populations for the majority of the selection regimes) for each generation over the span of about 10 months. The phenotyping experiments subsequently involved counting upwards of 2400 eggs per population to impose each of the conditions, all of which took about a month for each experiment. Finally, all flies and wasps were precisely counted over the following weeks as they emerged. The results from these data follow.

Phenotyping experiment #1

D. birchii

In the first round of results for *D. birchii*, some data were inconclusive due to low emergence counts, but others were more or less as we expected: according to the GLM results (McFadden's R-Squared value: 0.9108), the significant predictors for survival in this case were conditions ($p = 1.331e-11$, F value = 64.0932) and interacting effects of conditions and population ($p = 7.848e-06$, F value = 15.5587), and our evolved flies seemed to perform better in the infection conditions than the control (Figure 3). The control also performed better in the benign conditions, and seemed to perform better in the reduced food and combination conditions, but lack of data makes it hard to draw definitive conclusions. *Asobara* emergence counts were very low (mean = 0 for both resistance and combination conditions in evolved flies), and therefore not useful to supplement with fly emergence counts to measure *D. birchii* performance in this case. For that reason, a figure for these wasp emergence data is not included.

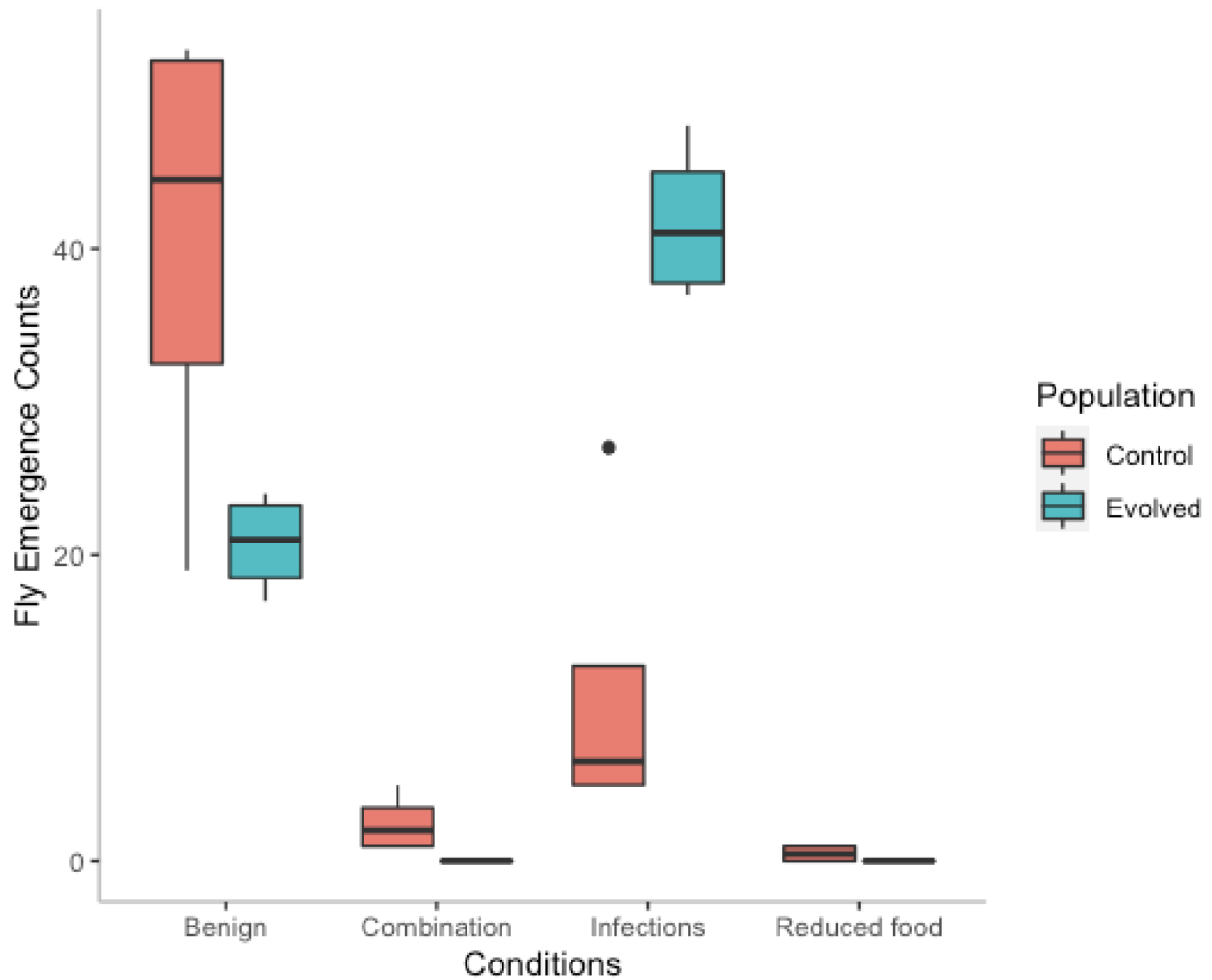


Figure 3: *D. birchii* fly emergences from phenotyping experiment #1 (N=4 in each condition). Box lines are the median and whiskers are showing variability outside the upper or lower quartiles.

D. sulfurigaster

In the first round of results for *D. sulfurigaster*, some fly emergences were again too low to be conclusive (i.e. in the reduced food and benign conditions). Other results show a similar pattern to the results from *D. birchii*, albeit smaller differences in the infection conditions (Figure 4). According to the GLM (McFadden's R-Squared: 0.9198), significant predictors for *D. sulfurigaster* survival are conditions ($p = 3.333e-13$, F value = 90.1455) and interacting effects

of conditions and population ($p = 0.02088$, F value = 3.9116), which again meets our general expectations.

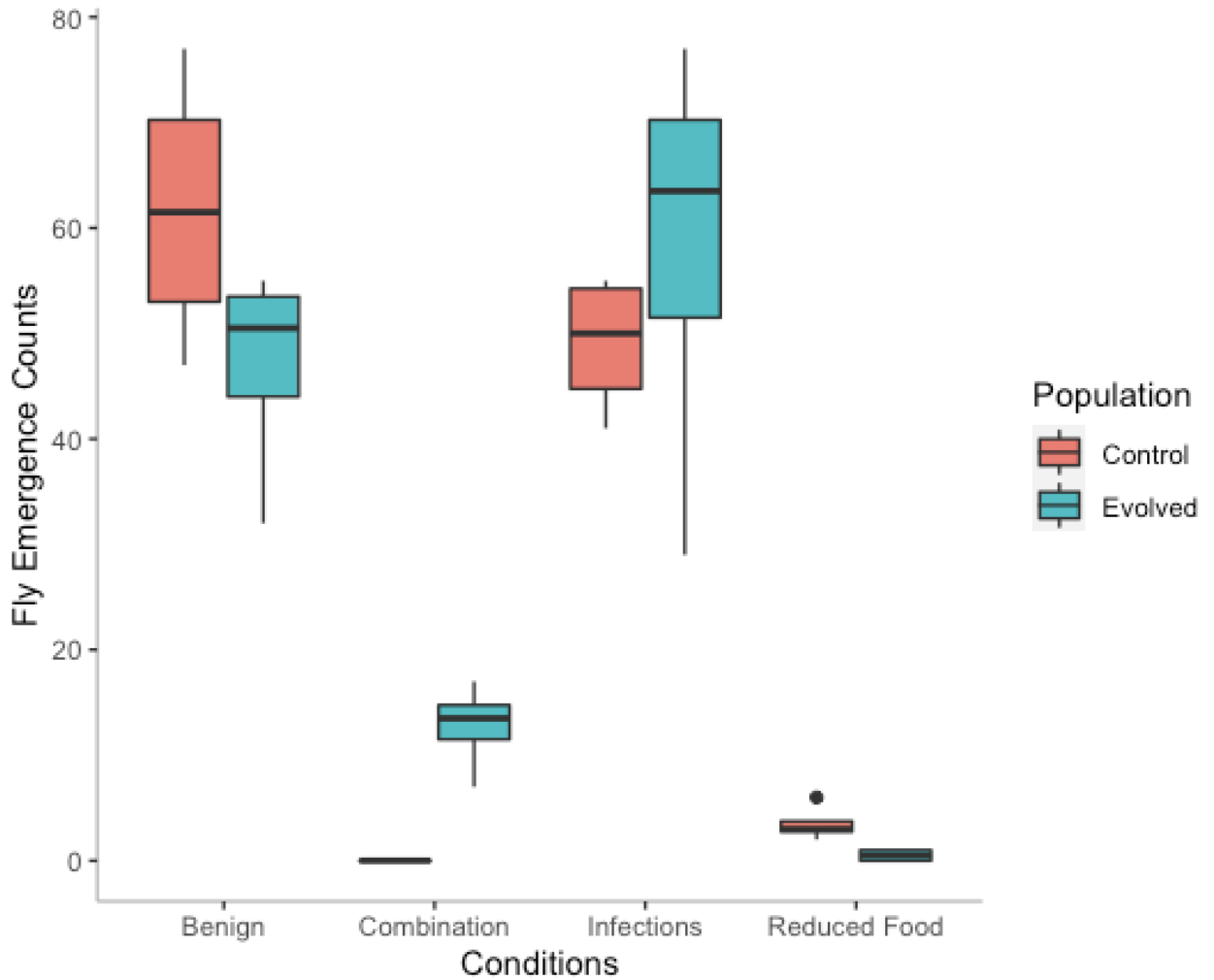


Figure 4: *D. sulfurigaster* fly emergences from phenotyping experiment #1 ($N=4$ in each condition).

Resistance in a population can increase even if fly survival does not: low wasp survival is also indicative of better fly resistance. It is common for infected hosts to die, but they can still prevent successful wasp emergence with effective immune responses. To supplement the fly emergence data on *D. sulfurigaster*, we also have results from *Asobara* wasp emergences (McFadden's R-Squared: 0.7278). In this case, the significant predictor was conditions ($p = 9.583e-05$, F value = 32.7353). See Figure 5 for details; trends look promising (i.e. fewer wasp

emergences in the evolved lines than control) but more data are needed to draw conclusions on significance for the combination conditions, as the means were within a margin of error.

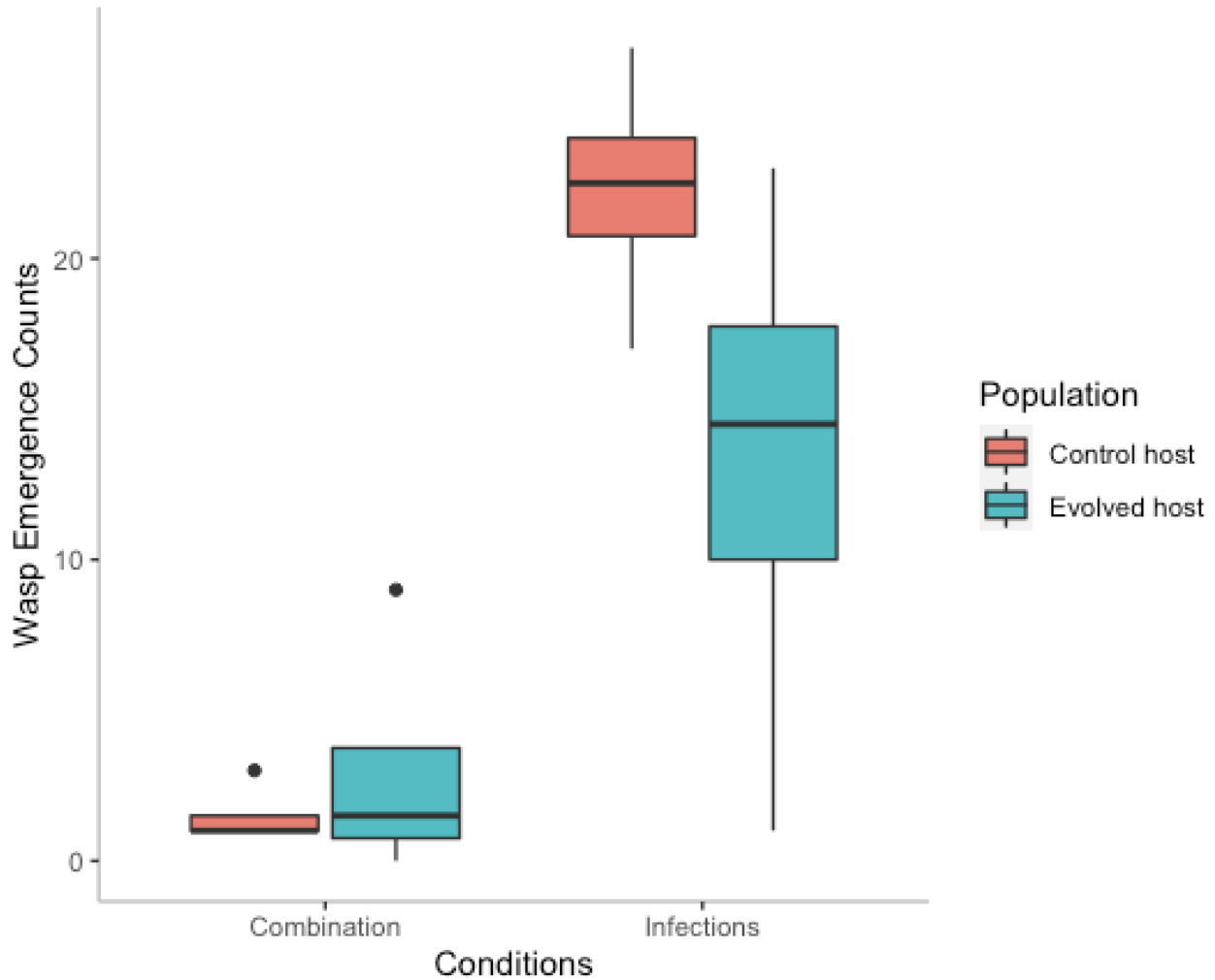


Figure 5: Wasp emergences from *D. sulfurigaster* hosts in phenotyping experiment #1 ($N=4$ in each condition).

Phenotyping experiment #2

For the second phenotyping experiment, we chose the populations that showed the most promising results. With the second experiment, the intention was to identify whether our results were consistent and to increase the number of replicates for more powerful statistical analyses.

D. birchii

In our second phenotyping experiment, results from *D. birchii* conflict with that of the first phenotyping experiment (Figure 6). Control flies emerged more in the infection treatment and evolved flies emerged more in the benign experiment. The significant predictors for *D. birchii* survival in this case were conditions ($p = 7.576e-06$, F value = 12.3170) and interacting effects of conditions and population ($p = 0.001556$, F value = 6.1383), but our model fit was not as tight as the first round of *D. birchii* analyses (McFadden's R-Squared: 0.5599).

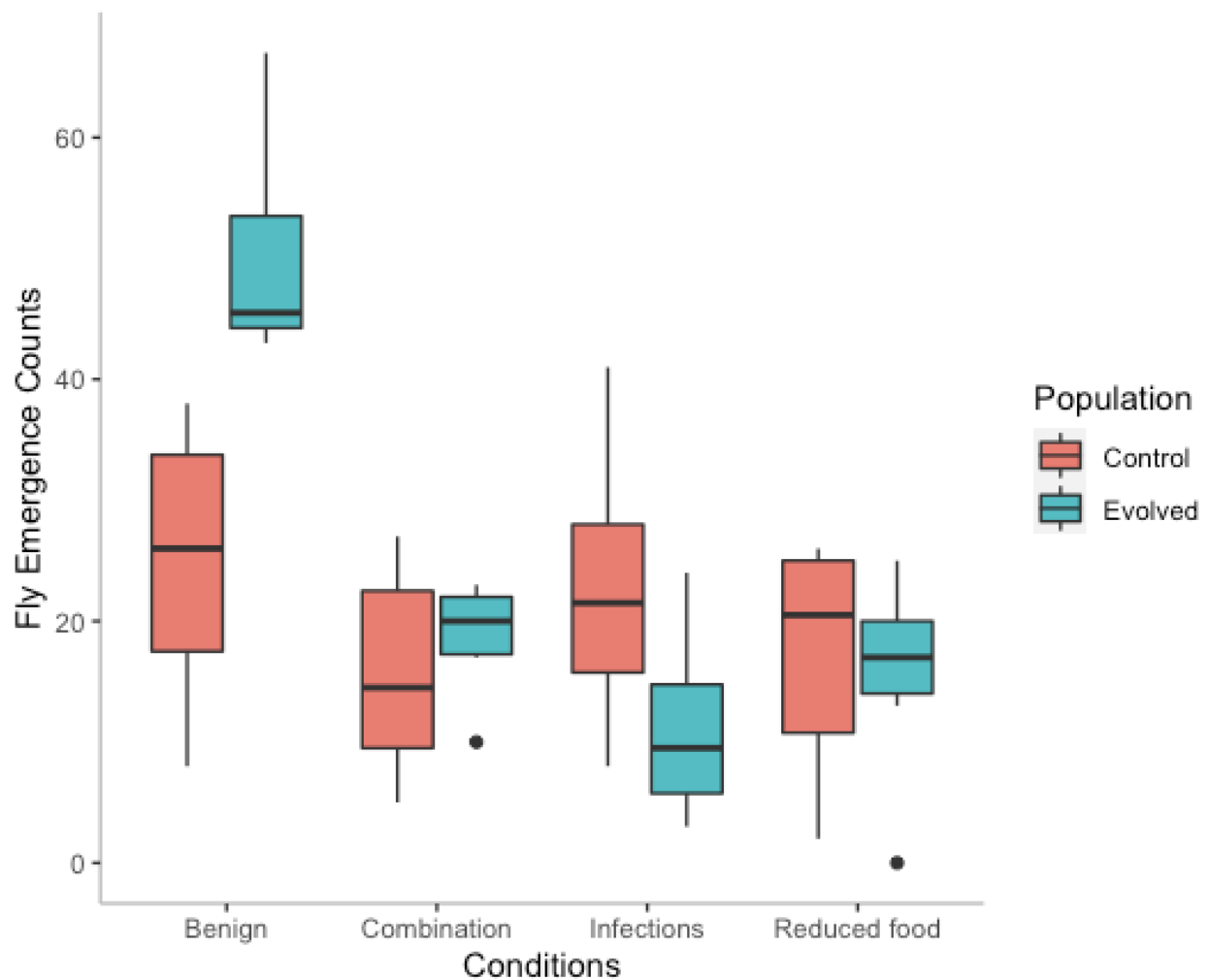


Figure 6: *D. birchii* fly emergences from phenotyping experiment #2 ($N=6$ in each condition).

Although we saw opposite results in the second phenotyping experiment for *D. birchii* fly emergences, *Asobara* emergence patterns seemed closer to what we had expected (Figure 7). The significant predictors for wasp emergence with *D. birchii* hosts in this case were conditions ($p = 3.779e-06$, F value = 39.6776). McFadden's R-Squared: 0.6660.

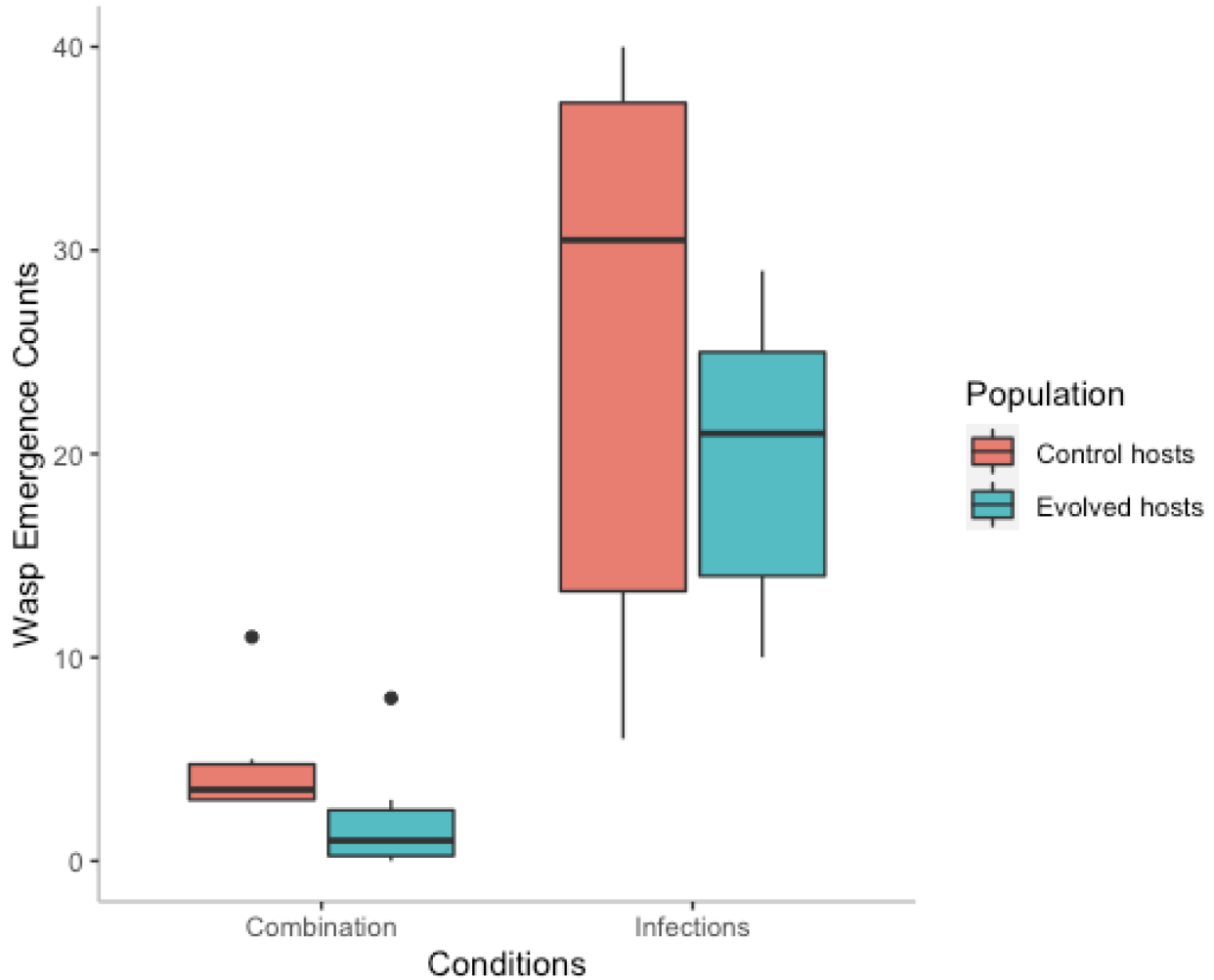


Figure 7: Wasp emergences from *D. birchii* hosts in phenotyping experiment #2 ($N=6$ in each condition).

D. sulfurigaster

Results from the second phenotyping experiment in *D. sulfurigaster* flies were very close to the expected patterns and the model fitted well (McFadden R-Squared value of 0.8612).

There was marginally better survival from evolved flies in infected conditions than the control, but control flies emerged better in the reduced food conditions (Figure 8). The significant predictors for *D. sulfurigaster* survival in this case were population ($p = 6.969e-09$, F value = 53.435), conditions ($p = 4.066e-14$, F value = 53.927), and interacting effects of conditions and population ($p = 7.198e-06$, F value = 12.384). Given both the model quality and consistent patterns, the results from *D. sulfurigaster* analyses will be discussed in the most detail.

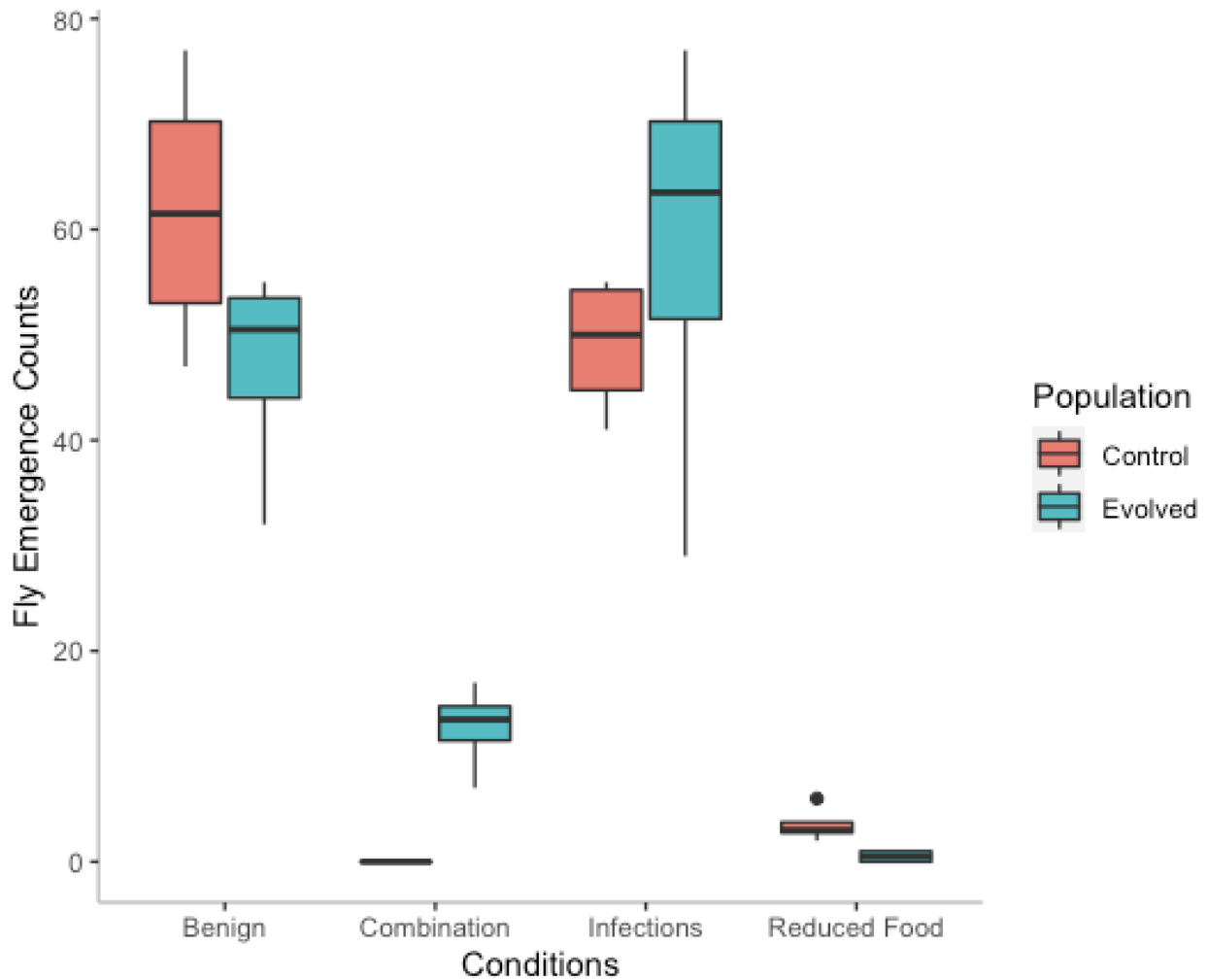


Figure 8: *D. sulfurigaster* fly emergences in phenotyping experiment #2 ($N=6$ in each condition).

Furthermore, the parasitoid emergences from *D. sulfurigaster* also fit our expectations, given that they were significantly dependent on the population ($p = 0.0001$, F value = 22.8380), and conditions ($p = 5.387e-07$, F value = 52.2448), with fewer wasps consistently emerging from

evolved hosts than control hosts in each of the conditions (Figure 9). McFadden's R-Squared for this analysis: 0.7851.

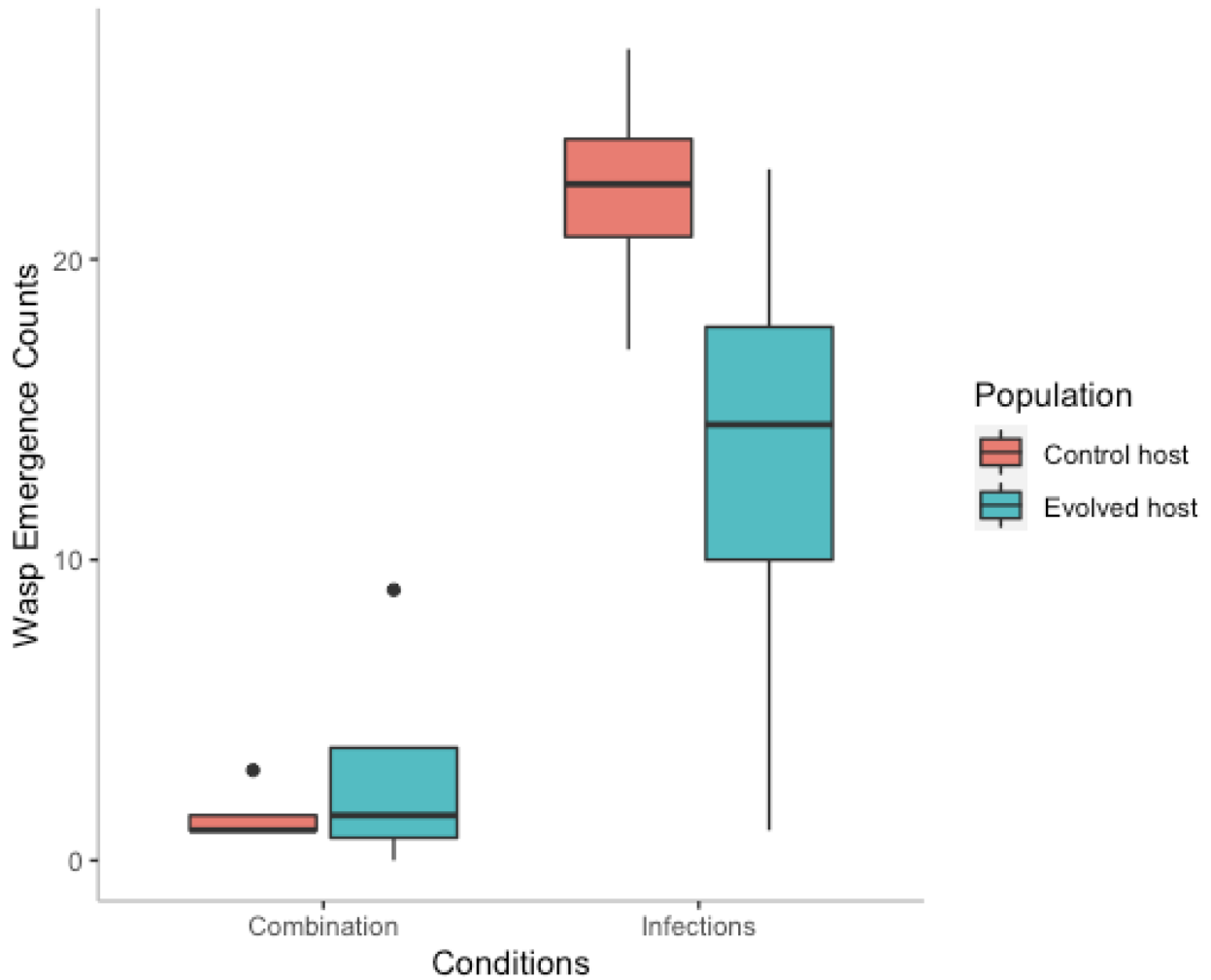


Figure 9: Wasp emergences from *D. birchii* hosts in phenotyping experiment #2 ($N=6$ in each condition).

Discussion

Summary and implications

Broad implications

Evolving and testing multiple phenotypes in the same experiment gave us a distinct advantage in identifying trade-offs and inferring which phenotypes are better-suited to each of the conditions we used, especially the combined conditions with multiple pressures. In ecological communities in nature, environmental stressors influence community structure (Jefferies et al 2020, Pardikes et al 2021, Thierry et al 2019). These environmental and network changes in turn drive species response by means of energy allocation (Johnson and Stinchcombe 2007, Lancaster et al 2017). In biodiverse communities with intricate network structures, such as the ones our study species originate from, more generalist phenotypes in *Drosophila* often have an advantage, as studies including Condon et al. (2013) and Parsons (1991) have found, and our results from the multiple selective pressure conditions seems to support this idea. Further, according to commentaries like Egas (2004), Lancaster et al (2017), and Thompson (1991), these trends can be applied to the wider field of community ecology as well (but see studies like Garcia et al 2011 and Seebacher et al 2015 that address the importance of the type of landscape, study system, and background environmental conditions for the generalist-specialist trade-off). Ultimately, we can infer that rich biodiversity in community networks encourages the development of optimal phenotypes through effective energy allocation, contributing to species adaptive response to environmental changes in this and other study systems (Chapin et al 2000, Johnson and Stinchcombe 2007, Norberg et al 2012).

Aim 1: identifying trade-offs

Based on our results, we can see evidence of ecological trade-offs in both *Drosophila* species. The general patterns reveal that the control populations were better competitors than the evolved populations when exposed to the reduced food conditions, indicating that the evolved flies' energy allocation was directed towards their resistance abilities, and as a result, their competitive ability suffered in that single pressure environment. We can also see that the evolved

flies generally performed better than the control flies when infected with parasitoids, which tells us that the evolved phenotype is better suited to that single selective pressure environment. The overall performance of the flies was measured not only by the fly emergence counts, but also the wasp emergence counts; although sometimes the number of fly emergence counts were comparable or even contradictory in our infection and combination conditions, patterns from our results indicate that the control flies were more susceptible to parasitism than evolved flies, given the consistently higher number of wasp emergence counts from control hosts. This could indicate better encapsulation abilities in the evolved flies: parasitism appeared to be more frequently prevented here, as previous studies on parasitoid immune response support (see e.g. Rolff and Kraaijeveld 2008). However, as we can infer from the aforementioned fly emergence data, prevention of successful parasitism may not necessarily guarantee better survival.

Aim 2: performance in multiple selective pressures

The better-performing phenotypes for the single selective pressures are relatively easy to understand given the general patterns in our results, but we were also interested in identifying the most optimal phenotype for the combination of pressures. Given our most significant populations' results, fewer evolved flies emerged in the combination pressure conditions than control flies. This could indicate that competitive ability was more imperative to survival than parasitoid resistance in these species when faced with this combination of conditions (see Sanders et al 2005), and the less extreme phenotype was favored (see Kubrak et al 2017, Toquenaga et al 2003). However, given the limited number of populations that showed conclusive results from the combination condition, this idea would benefit from further exploration in future studies.

Results from D. birchii

The results from *D. birchii* were the most contradictory, especially in the evolved population: they performed significantly better than the control when subjected to parasitoid infections in the first phenotyping experiment, then significantly worse in the second experiment. Also, the evolved flies emerged quite a lot more than the control in the benign conditions in the second experiment. It is possible that some illness in the flies or infertile wasps (noted in more detail later) were to blame for these unexpected outcomes, or perhaps there were errors made

when implementing the protocol (e.g. mislabeling or collecting the wrong number of eggs). The *D. birchii* performance in the first experiment and the *Asobara* data from the second experiment both appeared to match our expectations, but it's difficult to draw definitive conclusions given the contrast in fly emergence results. Another follow-up experiment would be necessary for uncovering a clearer picture of these results.

Results from D. sulfurigaster

The results obtained from *D. sulfurigaster* were more consistent across both phenotyping experiments than *D. birchii*, and more closely aligned with expected outcomes. The evolved *D. sulfurigaster* generally performed better when infected with parasitoids than the control, and worse when reared in reduced food resources, as anticipated. Furthermore, *D. sulfurigaster* and their *Asobara* parasitoids yielded the most clear and significant results, particularly in the second experiment. These populations were able to give us the best data in our exploration of *Drosophila* performance in a multiple selective pressure environment, and the inferences and comparisons made of different phenotype performances in these conditions are best backed by these results.

Caveats

Discussion of the methods: addressing the inconclusive data

As previously mentioned, some of the data from the experimental evolution portion of the project did not make it into the results. Specifically, 1) the *Drosophila* populations selected for increased competitive ability, 2) the *Leptopilina* wasps, and 3) some of the wasp emergence counts from the first phenotyping experiment. These data were inconclusive or insignificant, and there are a number of possible explanations. About halfway through the evolution experiment, competition populations began consistently decreasing in the number of emerging flies. When the phenotyping experiments began, these populations did not yield enough eggs to be included. This could have been caused by inbreeding depression and lack of genetic diversity, spurring a population crash.

There was also little to no significance in the fly populations infected with *Leptopilina* wasps: all fly emergences and *Leptopilina* emergences were comparable during the first phenotyping experiment when all populations were assayed. It is possible that *Leptopilina* was simply less effective at parasitizing than *Asobara* and the pressure was not strong or consistent enough as a result. Further studies exploring the differences in parasitism rates between these parasitoid species could be interesting to explore in order to gain better insight on this phenomenon.

Finally, few wasps emerged from the *D. birchii* populations in the first experiment, which is one of the reasons why we sought to repeat it. Although we generally tried to choose young, healthy wasps from our parasitoid lines to infect our *Drosophila* larvae with, it's possible the wasps we used in this instance were older or simply less fertile for any number of reasons. It is also possible that *D. birchii* is more resistant in general than *D. sulfurigaster*, resulting in the selection regime being less effective. Based on previous experiments done with our *D. birchii* populations (see Pardikes et al 2019, Thierry et al 2022), this species has shown varying parasitoid resistance, but it could be context-dependent and possibly shows better resistance when the intricate community structure is simplified and environmental conditions are kept constant as was done here. Again, more research would expand this limited picture.

Discussion of the methods: broader caveats

Although we tried to mimic natural conditions to the best of our ability in our experimental evolution approach, selection experiments are restrictive and it is impossible to account for all of the processes that normally occur in nature. For example, we did not attempt to replicate unpredictable or stochastic changes in conditions within the laboratory setting. This study also uses only a few species, and communities in nature have much richer species diversity and more interactions. As emphasized in previously mentioned studies including Thierry et al. (2019), intricate community interactions are more important to predicting species response to stressors than pairwise interactions.

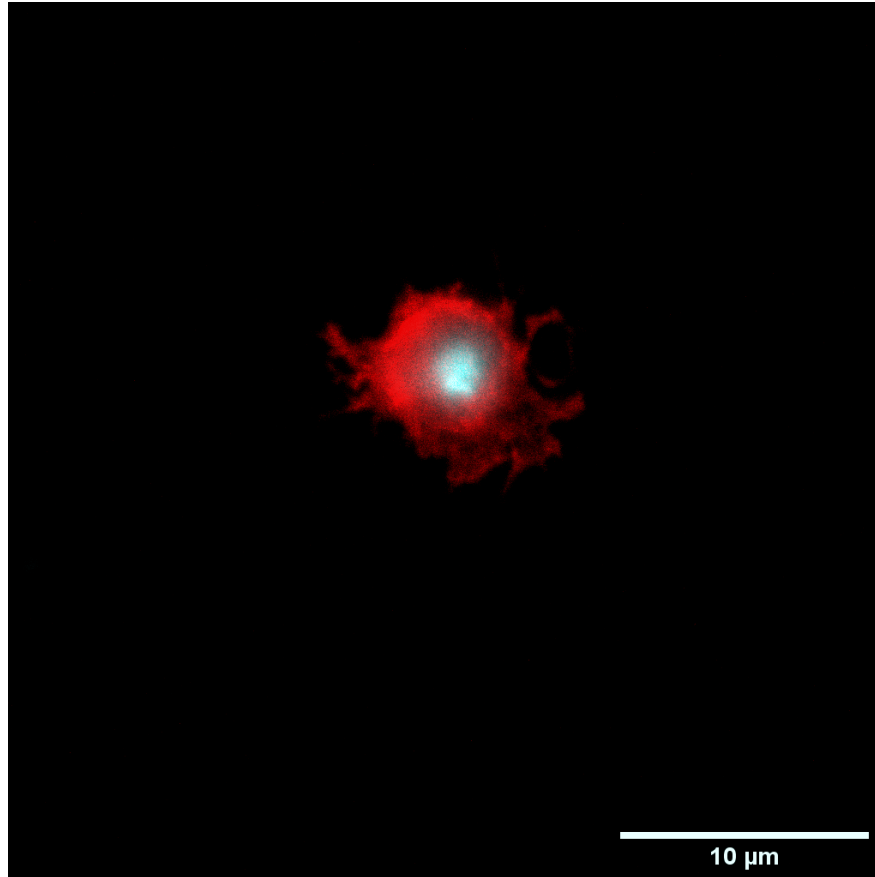
Finally, although we attempted to account for phenotypic plasticity and maternal effects by relaxing the selection regime for one generation before each phenotyping experiment, those possibilities may not have been eliminated: we could just be seeing phenotypic changes and perhaps not long-term changes in the genome. Although our experimental setup was not an

identical representation of natural conditions, we believe that it mimicked nature well enough to allow us to make reasonable inferences about the results we've seen.

Future directions

There are some avenues for supplemental data that could give us a bigger picture of some of the general processes we managed to glimpse during this experiment. Specifically, there are many alternative measurements of phenotype, such as body size, reproductive rate, encapsulation counts, and hemocyte (immune cell) counts (Burke and Rose 2009), which will be discussed in more detail shortly. There are also ways to measure genotypic changes, which would allow us to link the phenotypes and genotypes of our *Drosophila* populations, thereby giving us a more detailed picture of evolutionary response (Mackay and Huang 2017, Harrison et al. 2020).

Hemocyte counts are a fairly common phenotyping assay used in *Drosophila*-parasitoid experiments to measure fly immune response when exposed to parasitoid infections, as seen in several studies that use this system (McGonigle et al. 2017, Yang et al. 2020). Researchers often count plasmatocytes, which account for most circulating hemocytes, and lamellocytes, or encapsulation cells that are activated when the host is infected. There is also emerging research on multinucleated giant hemocytes (MGH), another encapsulation cell seen in some species of *Drosophila* that kill the parasite without melanization (Markus et al. 2015). We have already begun developing a protocol for taking these measurements together with the Laboratory of *Drosophila* Molecular Integrative Physiology and Adam Bajgar (see Figure 10). We would expect the evolved flies to have a higher and faster immune response than the control flies, which would supplement our parasitoid emergence data. This, coupled with encapsulation counts, would provide a thorough picture of phenotypic changes associated with our selection regimes.



*Figure 10: Image of a plasmatocyte from a larvae from one of the evolved *D. birchii* populations.*

Photo credit: Mgr. Pavla Nedbalová, PhD, Laboratory of Drosophila Molecular Integrative Physiology, 2021.

In addition to using more measurements of phenotype, genotyping would be an invaluable method of further exploring the relationship between fitness costs and evolutionary change in our *Drosophila* populations. We measured ecological trade-offs in this study, but based on previous studies and commentaries (Chippindale et al. 2004, Flatt 2011, Kawecki 2009, Stearns 1989), the research community has less understanding of the mechanisms behind evolutionary trade-offs and the link thereof, and it would serve future studies on fitness costs well to build on experiments like this one to include a genetic component. As was highlighted earlier, long-term adaptations are reflected in the genome, and to better address the plasticity caveat and explore how phenotypic changes lead to long-term evolutionary adaptation, we could use modern sequencing methods to determine which regions of the genome are associated with the changes in phenotype and analyze how the allele frequencies in the populations change over

time with selective pressure (Chippindale et al. 2004). This could be done with sequencing assays taking place during different parts of the experiment, including the beginning of the selection regimes, again partway through, and finally at the end. This approach would help us better understand the processes driving development of trade-offs in this and other eukaryotic communities (Guirao-Rico and González 2019).

Conclusion

Broadly speaking, experiments like this which explore trade-offs with eco-evolutionary underpinnings can help us gain a general understanding of species response to changes in the environment and community composition (Bergelson 1994, Johnson and Stinchcombe 2007, Kellermann 2009, Ketola and Saarinen 2015, Lancaster et al. 2017, Rudman et al 2022). In our case, the inevitable species interactions that occurred during our selection regimes drove the evolutionary adaptations we've seen in our fly populations, and those resulting adaptations gave rise to a change in performance when exposed to different ecological and environmental conditions. More specifically, we've seen *Drosophila* phenotypes optimize to their conditions over time as a result of environmental pressure and intra- and interspecific interactions, and the subsequent fitness costs. We may infer that extreme phenotypes may be generally less optimal in similar settings in natural communities, where there are multiple selective pressures and abrupt environmental changes, similar to what we saw in this study. By building on research such as this to explore both ecological and evolutionary trade-offs, we can achieve mechanistic insight into the drivers of adaptation in community ecology.

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