Developing *Dalotia coriaria,* the greenhouse rove beetle, as a novel model organism

Thesis by

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In Partial Fulfillment of the Requirements for the

Degree of

Doctor of Philosophy

CALIFORNIA INSTITUTE OF TECHNOLOGY

Pasadena, California

2023

Defended August 9, 2022

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ACKNOWLEDGEMENTS

First and foremost I would like to thank Joe Parker, the fearless leader without whom this project would have never existed. I first heard about Joe and the lab (which at the time consisted of only Joe) in an email Liz Ayala (The Graduate Option Manager for Biology) sent out to all of BBE. In it Joe described a new system for studying symbiotic interactions between ants and beetles. The first bullet point of available projects read "Development of genetic tools for non-classical model organisms." As someone with an obsessive love of animals who spent his teenage years breeding different genetic lines of geckos, I had to find out more. I then attended his talk at the BBE retreat where he explained the rove beetle system he was building. I was totally captivated and set up a meeting with him to discuss a rotation. We met in a temporary office in the basement of BBB as our lab had not yet been built. He had Rubbermaid boxes full of dirt and beetles on the ground, and a small space heater blowing over them. My 10 week rotation project was to get CRISPR to work in *Dalotia*. Sure we only had a temporary lab space with minimal reagents (we didn't even have ethanol yet), neither one of us had ever worked with CRISPR, and we had absolutely no idea how to get it to work in our new model system, or if it could work at all; somehow none of that really mattered and we were both very, very excited. This first meeting was a perfect foreshadowing of what the rest of the PhD would entail. Every time I encountered a major problem or roadblocks (which happened A LOT) Joe and I would meet and figure out a path forward. No matter how impossible the problem seemed before our meeting, I would leave ready to approach it with sheer confidence, optimism, and determination. I want to thank Joe for teaching me not to fear the apparent impossibility of a problem. It doesn't matter if something has never been done before. As long as it is

interesting and exciting, just go for it. That is the story of my PhD and I will carry this mindset for the rest of my life. For that, I thank you Joe.

Of course, I also have to thank my lab sidekick Mina. Her official title is lab manager, but she was just as much a mentor, scientist, and friend without whom we would have never succeded in this project. I would also like to thank all of my fellow lab members for being awesome people. It was a joy to go to work and see you all every day. Thank you for the intellectual help, support, and friendship. I would also like to thank my biology cohort for all of our game nights, camping trips, scotch nights, and companionship.

Next, I would like to thank my family for all their support. I would not be here without them. I would like to thank my dad for teaching me calmness and steadiness. I would like to thank my mom for teaching me determination and grit. I would like to thank my brothers for keeping me grounded. I would like to thank Aitite, Abuela Merche, Grandpa, and Gramma for being my number one fans.

I, of course, would like to thank Eleni, my fiancée, for oh so much. This PhD was very hard a lot of the time. She provided more moral support than I could have imagined. Thank you for ALWAYS being there for me.

I would like to thank Irene, my undergrad mentor for guiding me into science and instilling a love of research in biology. I would like to thank Barb, Kyle, and Connor for keeping the musician in me alive during grad school. Thank you Barb for your infinite wisdom in the jazz improv group and jazz band. Thank you Kyle and Connor for all of our jams, concerts, and friendship. I would like to thank Mauricio, my kickboxing coach, for keeping mentally sane. Nothing clears the mind like your classes and I will also miss you as a friend.

I would also like to thank Liz Ayala, Raina Beaven, and Lauren Breeyar for all of their help navigating Caltech from day one. I don't know how the department would run without you.

Lastly, I would also like to thank my thesis committee: Marianne Bronner, David Anderson, and Paul Sternberg for your valuable time and feedback during our yearly meetings and on this thesis.

ABSTRACT

This thesis deals with the development of *Dalotia coriaria*, the greenhouse rove beetle, as a novel model organism. A fundamental characteristic of metazoan life is inter-species interactions. Chapter 1 explores why there is a need for *Dalotia* as a new model organism to study these interspecies interactions in ways that are intractable with current established models. It also explores the life history characteristics of *Dalotia* that make it amenable to development as a novel model organism as well as the need for genetic access in order to successfully make *Dalotia* an established laboratory model organism.

Chapter 2 explores how I have solved the husbandry techniques required for genetic manipulations in *Dalotia.* These include the ability to collect large amounts of early embryos, mount embryos on slides, micro inject embryos, raise single housed larvae to adulthood, and set up one-on-one adult crosses.

Chapter 3 explores how I have developed the Piggybac transposon system to successfully knock in trans-genes into the *Dalotia* genome. It also shows how I have developed the UAS Gal4Δ binary expression system to work in *Dalotia,* allowing for controlled high expression of inserted trans-genes.

Chapter 4 explores how I have developed the CRISPR Cas9 system to successfully perform targeted germline point mutations in the *Dalotia* genome. It also shows how I have developed fast and accurate genotyping techniques for producing and maintaining homozygous stocks of mutant *Dalotia* for long periods of time. The appendices of this

thesis include step-by-step protocols that allow for reproduction of all of the husbandry and genetic manipulation techniques covered in Chapters 2-4.

Lastly, Chapter 5 of this thesis explores olfactory receptor guided behaviors in *Dalotia.* I use RNA Smartseq techniques to produce a map of chemoreceptors across the *Dalotia* body. I also use the *Dalotia* CRISPR Cas9 protocol I developed to produce a homozygous line of olfactory receptor-deficient *Dalotia* by knocking out the olfactory receptor co-receptor. I show that the line of olfactory receptor-deficient *Dalotia* are incapable of olfaction, and then explore how it affects their defensive behavior when interacting with ants. These interactions are studied in a free moving arena and in a tethered beetle on the ball setup using machine learning to analyze pose.

PUBLISHED CONTENT AND CONTRIBUTIONS

Miller, D.R., Kanwall, J.K., Yousefelahiyeh, M., Omoto, J., and Parker, J. (2022). Olfactory guidance of chemical defense behavior in a rove beetle. Manuscript in preparation.

D.R.M. participated in the conception of the project, experimental design, data collection, data analysis, and writing.

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Chapter I

WHY DEVELOP *DALOTIA CORIARIA* AS A NOVEL MODEL ORGANISM?

Introduction

A fundamental characteristic of metazoan life is inter-species interactions. Animals navigate a living planet filled with other organisms. Ecosystems are communities of interacting species, and at each interaction an organism must decide how to respond or not respond (Jeffries and Lawton, 1984; Oliveira and Bshary, 2021). Is the other organism a predator, prey, parasite, a mate, sexual competitor, a symbiotic partner, neutral? All animals have a nervous system that has evolved to facilitate interactions with other organisms in an adaptive way, and have the capacity to navigate these interactions (Bernays and Wcislo, 1994). Very little is known about how an animal perceives another species and then chooses to behave during an interaction at a mechanistic level. These interspecies interactions likely work as potent selective forces on animal brains during evolution, as the stakes for executing the wrong behavior during an interaction can lead to death (Parker and Kronauer, 2021). This means sensorimotor pathways are likely under selection to generally identify other species correctly and then perform appropriate behaviors during ecological interactions. How these sensorimotor pathways are wired regarding an animals capacity to interact with a separate species however has been severely understudied. The most common laboratory model organisms (mice, fruit flies,

zebrafish, etc.) are typically raised and studied in the absence of any other species and are not necessarily the best candidates to study the question at hand. In this thesis, I will posit why the greenhouse rove beetle, *Dalotia coriaria*, is a suitable model organism to explore interspecies interactions at a molecular and mechanistic level.

Rove beetles, the family Staphylinidae, are an amazing corner of the tree of life. They are the most diverse animal family, containing over 64,000 species (Al Newton, pers. comm.). They are mostly dark-colored, predatory soil beetles with a modified body plan comprising short elytra and a highly flexible abdomen that enables them to move rapidly through the leaf litter. Soil and leaf litter are incredibly species-rich ecosystems, and interactions between soil living arthropods are commonplace (Kanwal and Parker, 2022). Rove beetles must constantly decide whether or not to interact with the species they run into, and how to interact when they do. They have also, like many other species, evolved structures to interact with other animals. In the case of free living rove beetles such as *Dalotia,* the structure is a tergal gland embedded in its flexible abdomen that can be used to direct defensive chemicals, typically benzoquinones, at other animals (Figure 1.1) (Brückner et al., 2021). Their frequent interspecies interactions in nature and specialized dedicated structures make rove beetles an excellent metazoan family to study these interactions at a mechanistic level (Naragon et al., 2022). For example, generalist insect species like *Dalotia* use circuits downstream from the sensorimotor periphery during interspecies interactions to decide which behavior to execute: hunt, avoid predation, evade a parasite, use physical defense, use chemical defense, or simply do nothing.

Dalotia will execute all of these behaviors in the lab depending on the species it interacts with. What sensory cues allow *Dalotia* to know which specific behavior to execute and how the decision is processed in the brain is unknown. By establishing *Dalotia* as a model species, we will be able to begin to explore what the important sensory cues are and how they are processed during distinct interspecies interactions.

1.1 *Dalotia coriaria* (right) encountering a *Liometopum occidentale* ant (left). *Dalotia* curls and flexes its abdomen when it encounters a dangerous organism in order to release noxious benzoquinones and defend itself during the interaction.

Another related important characteristic of metazoan life is symbiosis. From parasitic nematodes, mites, and wasps, to commensal remora-shark relationships and mutualistic aphid-farming ants, symbiotic interspecies relationships are extraordinarily diverse and widespread. A common attribute among these relationships is social interaction, where each species has evolved new behaviors specific to interacting with its symbiotic partner. Studying complex symbiotic interspecies interactions has previously proven intractable, given the lack of symbiotic relationships in traditional laboratory model organisms. This means basic questions about the molecular and neural architecture of symbiotic animals have been nearly impossible to study. Some of these questions include: How is the nervous system of a symbiont animal recalibrated for recognition of and interaction with its partner organism? How do specialized modes of interspecies communication evolve at the genetic and cellular levels? And what behavioral and life history traits enable symbiotic species to persist evolutionarily, despite the challenges of being dependent on other species?

Rove beetles have repeatedly and independently evolved to infiltrate and live inside colonies of social insects particularly ants and termites (Parker and Grimaldi, 2014). While most insects would be attacked and killed upon approaching or entering an ant colony, symbiotic rove beetles have evolved strategies to inhibit ant aggression and engage in a number of stereotyped, social behaviors, including being fed trophallactically (mouth to mouth) by worker ants, grooming worker ants, climbing onto worker ants and riding on their backs, and being carried to brood galleries inside nests where they can eat

ant eggs and larvae, or lay eggs for the ant workers to rear (brood parasitism) (Danoff-Burg, 1996; Kistner, 1990, 1993; Parker and Grimaldi, 2014). As a result, rove beetles present themselves as an obvious group from which to create a new model system for the study of interspecies symbioses.

In order to understand how these complex symbiotic species evolve, one must first understand the groundplan from which they emerged. Dozens of independent rove beetle lineages have evolved symbiotic life inside ant colonies, with dramatic behavioral, chemical and often anatomical adaptations (Maruyama and Parker, 2017). Given that *Dalotia* shares conserved anatomical, behavioral, and glandular phenotypic traits with other free-living aleocharines, it embodies the free-living groundplan capable of repeatedly evolving social symbiosis (Figure 1.2). Understanding how symbiotic social behaviors evolve from non-symbiotic ancestors requires an understanding of the ancestral state, represented by *Dalotia.*

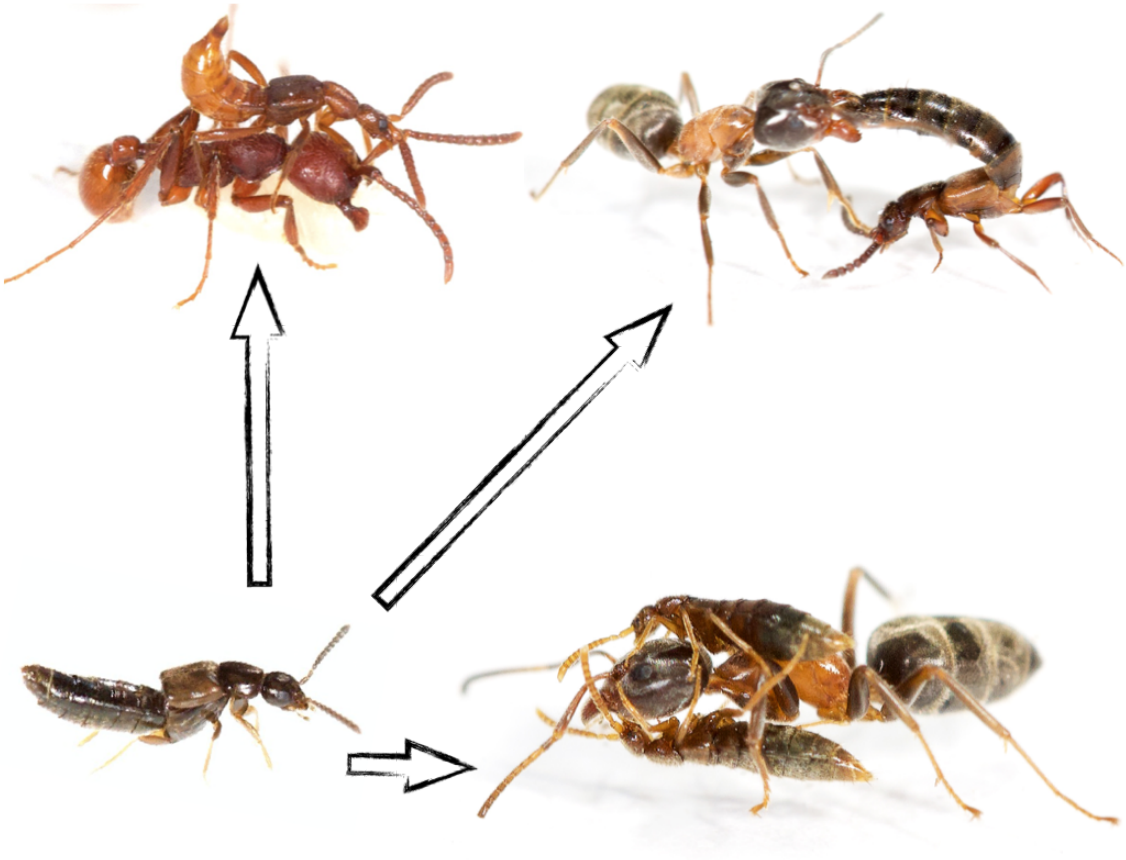

Figure 1.2 Examples of free-living and myrmecophilous rove beetles. *Dalotia coriaria* (bottom left) is a stereotypical free-living rove beetle. Myrmecophilous species have convergently evolved from a free-living common ancestor like *Dalotia.* Pictured are *Ecitonidia wheeleri* with its host ant *Neivamyrmex californicus* (top left), *Platyusa sonomae* with its host ant *Liometopum occidentale* (top right), and *Sceptobius lativentris* with its host ant *Liometopm occidentale* (bottom right). These are three of the four myrmecophilous rove beetles that can be found in the San Gabriel mountains 15 minutes north of Caltech.

Establishing *Dalotia* as a model organism would therefore allow us to investigate questions for which existing model organisms are not well suited ranging from general inter-species interactions to complex symbiotic interactions. In order to achieve the molecular granularity with which we would like to investigate these questions, genetic access to *Dalotia* will be required. In the most basic sense, this means developing the ability to knock genes out and knock genes in. This would allow us to tease apart everything from how *Dalotia's* gland to *Dalotia's* brain work. Studying knocked out genes has allowed us to investigate their function for over a century (Morgan, 1910). Knocking in tools such as G protein coupled Calcium sensors (GCaMP) will allow us to visualize brain circuits as *Dalotia* behaves (Nakai et al., 2001; Simpson and Looger, 2018). The ability to develop this genetic access is not guaranteed for any non-model species. Achieving access will require both a comprehensive understanding of the organism's husbandry and fundamental characteristics of the organism's life history which allow it to function as a laboratory model organism.

What makes a good laboratory model organism? Does *Dalotia* qualify?

While any organism brought into a laboratory can in theory be used as a model, there are certain characteristics that make some organisms more amenable than others. This question can be answered in two ways. The first is purely biological, and assumes that a

good laboratory model organism allows us to answer questions about the natural world in ways that could not be done with existing model organisms. This has already been introduced and will be expanded upon in the last chapter of this thesis. The second is technical, and involves the husbandry of said organism and whether or not it can be sustained in a lab. While genetic access is not necessarily required for a laboratory model organism, this section will assume that a good laboratory model organism is amenable to being maintained in cultures with homozygous mutations in desired genes of interest (Valentino and Omar, 2018).

The first necessary characteristic for a good model is the ability to mass-culture an organism at low cost. This allows for immediate availability of the organism year round. *Dalotia* passes this test as we can culture hundreds in a single 11 quart plastic tub (Figure 1.3). Our small temperature controlled room contains tens of boxes and thousands of *Dalotia*.

Figure 1.3 Coconut husk in an 11-quart plastic tub used to mass culture *Dalotia coriaria.*

Second, a fast generation time is very helpful. This allows for stocks to be replenished quickly when used, and it allows for efficient purification of mutant lines. *Dalotia* has a generation time of around 18 days, which is only days longer than Drosophila, and shorter than *Aedes aegypti's* 30-day generation time (Bassett et al., 2013; Sowilem et al., 2013) . Given the success of establishing genetic lines with the above-mentioned dipterans, *Dalotia's* generation time will not be a limiting factor in its establishment as a model organism.

Another important characteristic in model organisms is sexual dimorphism. Being able to determine the sex of an organism non-invasively is crucial to setting up one-on-one crosses required for establishing mutant genetic lines. As shown in Figure 1.4, male *Dalotia* exhibit a jagged concave 8th segment while female *Daltoita* exhibit a smooth convex 8th segment. This is easily seen under a dissection microscope and rapid screening works well.

Figure 1.4 Sexual dimorphism in *Dalotia.* The 8th segment of *Dalotia* pictured in green is jagged in males and smooth in females.

In line with one-on-one crosses, laboratory model organisms need to be tolerant to inbreeding. For one, multiple rounds of inbreeding allows for the creation of a more isogenic population. Furthermore, genetic lines are typically established from a single organism, which then requires multiple rounds of inbreeding. The lab's *Dalotia* population was generated through 7 rounds of backcrossing, and subsequent stable lines have been generated with no inbreeding issues as will be discussed in Chapter 3 of this thesis.

Next, an annotated chromosomal level genome is required to identify and manipulate an organism at the genetic level and the more compact the genome, the easier it is to work with. This is due to the reduced intron size, making sequencing and genotyping much more efficient. Our lab has produced an annotated chromosomal level genome for *Dalotia* and its 120 Mb size makes it very easy to work with (Figure 1.5). The size is comparable to Drosophila and is much smaller than other beetle species.

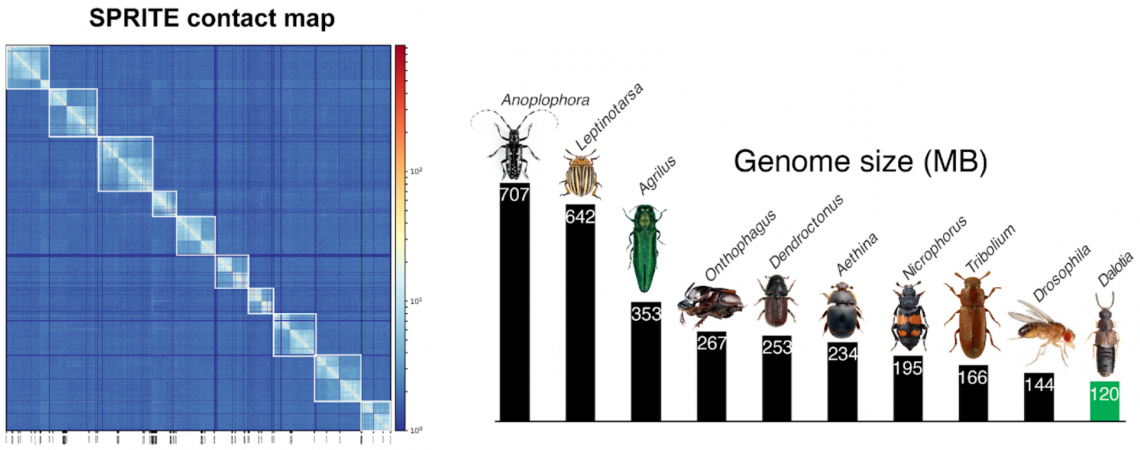

Figure 1.5 ***Dalotia genome.*** A sprite contact map is pictured left, showing chromosomal level structures in the *Dalotia* genome. The right panel shows the genomes size relative to other beetles and *Drosophila.*

The next characteristic of a laboratory model organism is less universal and is only necessary for organisms used in behavior. A good laboratory model organism will have a robust and reproducible set of behaviors that can be easily measured/quantified and replicated in a variety of conditions. For example, *Drosophila* will reproducibly fly and change its direction in response to visual stimuli (Frye and Dickinson, 2001). They will still do this while tethered and under a two photon microscope. This has allowed us to learn about how *Drosophila* navigates as well as what neural circuits encode the behavior. *Dalotia's* defensive behavior in response to a threatening organism, where it flexes its abdomen and secretes benzoquinones, is equally reproducible. It will still perform its defensive behavior head fixed on a ball with a two photon mount attached (Figure 1.6). The robustness of its behavior in synthetic settings will allow us to precisely

characterize the defensive behavior and investigate the underlying neural circuits that control it. *Dalotia's* robust and reproducible behavior when interacting with other species makes it a unique and effective laboratory model organism.

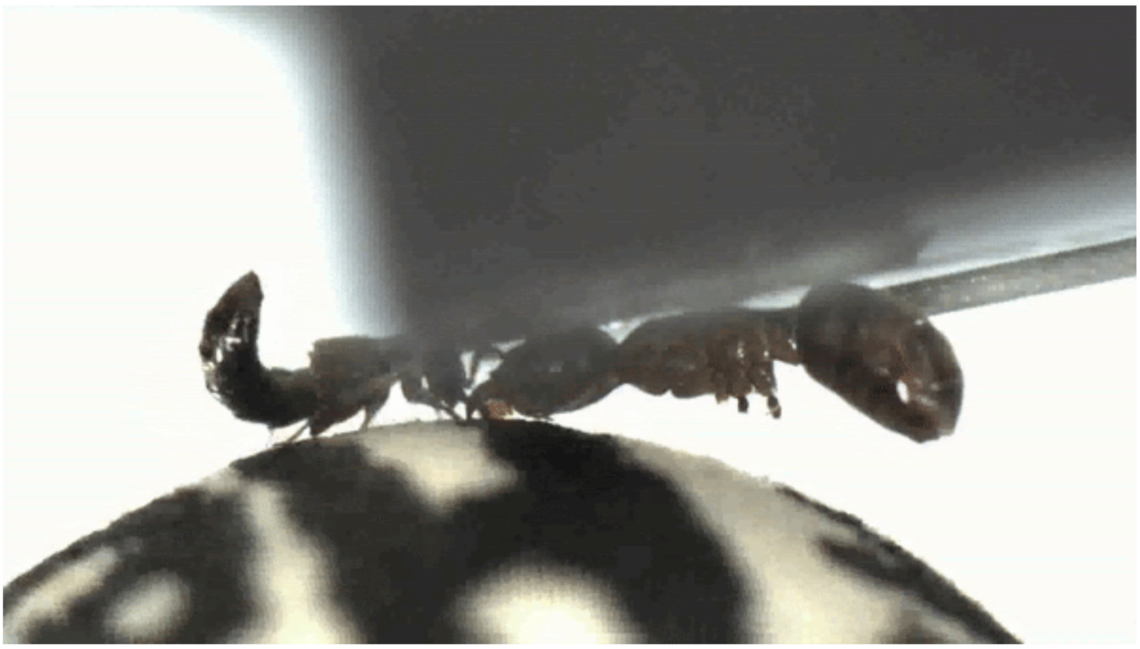

Figure 1.6 ***Dalotia* walking on a ball while head fixed interacting with an ant.** *Dalotia* will still perform its signature defensive abdomen flexing while head fixed on a two-photon mount.

Lastly, a good laboratory model organism is amenable to genetic techniques, which allows for stable lines to be generated that have genes either knocked out or knocked in. The two main techniques discussed in this thesis will be Piggybac transposon insertions

and CRISPR Cas9 deletions. Chapters 2-4 will discuss how I have made Dalotia genetically tractable using the aforementioned techniques. Chapter 5 will discuss how I used the techniques to begin exploring the mechanisms that regulate interspecies interactions in *Dalotia.*

In conclusion, *Dalotia* is an excellent model for studying interspecies interactions with a robust set of replicable behaviors. It is mass culturable, has a fast generation time, is sexually dimorphic, is tolerant to inbreeding, and has a compact annotated genome. With the advent of genetic techniques I have developed, *Dalotia* has functionally become a laboratory model organism.

Chapter II

THE HUSBANDRY BARRIER TO GENETIC ACCESS

Introduction

When attempting to establish genetic access in a new model organism, one must first understand how it has been achieved in related model organisms. In our case there are three main references: *Tribolium castaneum, Drosophila melanogaster*, and *Aedes aegtypti* (Berghammer et al., 2009; Featherstone et al., 2009; Kistler et al., 2015). *Tribolium*, the red flour beetle, is the closest established model phylogenetically. *Drosophila* is by far the most established insect model, and *Aedes*, is the most recently established insect model.

There is a structurally similar pipeline to how genetic manipulation is performed in all three of these models. First, there is a method for collecting large amounts of syncytial embryos[1] (Gilbert, 2000). Next, there is a method for microinjecting relevant genetic constructs (CRISPR Cas9, transposon system, etc.) into the embryos and having a substantial percentage hatch. Subsequently larvae must be raised with high survival to adulthood. Lastly the mutant larvae must be screened/genotyped and selectively crossed one-on-one. This process is then repeated with F1 offspring. While this pipeline seems straightforward, not all insects (likely very few) are amenable to all of these steps. If a

[1] A syncytial embryo is one where nuclei divide in a common cytoplasm before cellularization. This is important because it means reagents injected into the embryo will have access to the nuclei without having to cross a cell membrane.

single one of these husbandry steps does not work efficiently, it will be impossible to generate any genetic lines. The next portion of this chapter will explain how each step was overcome. Step-by-step protocols are attached in Appendix A.

embryo collection

Given the different niches occupied by different insects, they all have a unique way in which they determine when and where to lay eggs. In our lab environment *Dalotia*, being a soil dwelling invertebrate, lays its eggs in the shredded coconut husk substrate in which it lives. While this rearing style works well for mass culturing *Dalotia,* it makes collecting eggs difficult. The eggs are about 100 uM in diameter (Figure 2.2) and are very soft and malleable for the first few hours after being laid (Figure 2.1). Other substrate dwelling organisms like *Tribolium* lay much bigger and more robust eggs (Gilbert, 2000; Gilles et al., 2015; Strobl and Stelzer, 2014) that can be filtered out by sieving substrate through different sized meshes leaving only eggs in one of them. I attempted this technique with *Dalotia* and tried modifying the substrate, box type, number of beetles per box, sieve size, temperature, food type, humidity, and day-night cycle. Unfortunately, none of the attempted permutations led to successful results. While a number of permutations led to eggs at the bottom of the sieve, they were always older embryos well past gastrulation like those seen in the rightmost panel of Figure 2.2. These embryos are too old for our reagents to reach nuclei during microinjection and therefore not usable for us. I hypothesize that the reason early pre-blastoderm embryos were never collected is that early *Dalotia* eggs are too soft and sensitive. Every time I tried to sieve them out,

they would rip during the process, and only older hardened older embryos would reach the bottom sieve.

Given that sieving eggs out of the soil was not working, I took a step back and tried to find a new way to culture *Dalotia* all together. I tried rearing Dalotia in multiple ways to see if they would reproducibly lay eggs, including on agar, in petri dishes with small amounts of dirt, in *Drosophila* culture bottles, and on hydrostone plates. None were particularly effective. Some eggs could be found in the dirt pile in the petri dish, but it was too tedious and time consuming to reliably remove old eggs and then collect large amounts of early eggs. I did however notice that in one of the petri dishes containing soil an egg had been stuck to the side of the petri dish. I used this as a starting point, and tried keeping beetles in these petri dishes with no soil. Again some eggs were laid against the side of the petri dishes (Figure 2.1). I then performed experiments modulating the food being fed, lighting conditions, temperature conditions, humidity conditions, and amount of beetles kept per dish. The optimal result occurs when 3–4 beetles are kept in a dish with ~3–4 frozen thawed drosophila as food and exchanged every 24 hours. The dishes are kept in the dark, at 50% humidity, at 26 °C. In these conditions *Dalotia* will reliably lay eggs in a way that they can be easily collected. Removing overnight eggs during a morning collection and doing subsequent 2-hour timed collections allows us to obtain eggs laid in the past 2 hours. As shown in the first panel of Figure 2.2, at this stage embryos are still pre-blastoderm syncytial and consist of only a few nuclei making them suitable for microinjection.

Figure 2.1 Female *Dalotia* with freshly laid egg against the side of a petri dish. When 3–4 *Dalotia* are kept in a petri dish, in the dark, at 26°C, at 50% humidity, and have their food changed out daily, they will reliably lay eggs against the side of a petri dish allowing for efficient timed collections.

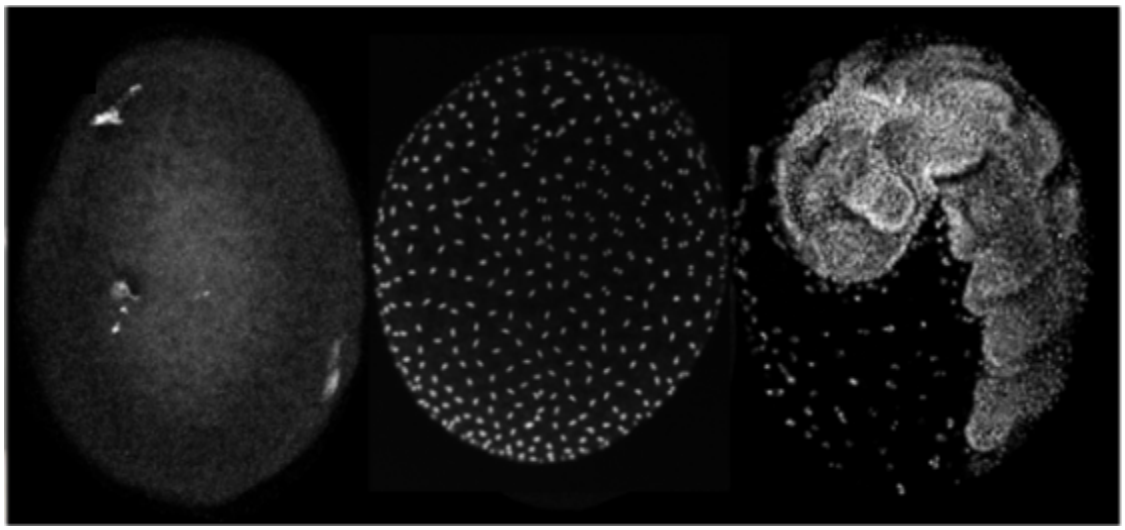

Figure 2.2 Time course of *Dalotia* embryos showing stained nuclei. The embryo on the left is an early syncytial blastoderm with only two clear nuclei showing. The embryo in the center is a later syncytial blastoderm. Nuclear division can be seen starting in the top right quadrant of the embryo and spreading outwards. Eggs collected during our timed collections are somewhere between these two embryos. The embryo on the right is an older embryo past gastrulation that is no longer suitable for microinjection.

Using this technique, 75–150 petri dishes are set up with 3–4 beetles per dish and left in a dark incubator overnight. The next morning, all of the old food is removed and replaced with fresh food. Additionally all overnight eggs are removed from the side of the petri dish. The dishes are then placed back in the incubator for 2–3 hours. During the egg collection, a wet brush is used to pick the eggs and mount them onto a slide for injections. Using this technique, two people working together can reliably collect close to 100 microinjectable embryos every day. This is on the lower end of what egg collections

are like in *Drosophila* (Featherstone et al., 2009). Given that *Drosophila* is an established genetic model organism, these numbers should also be sufficient for *Dalotia.*

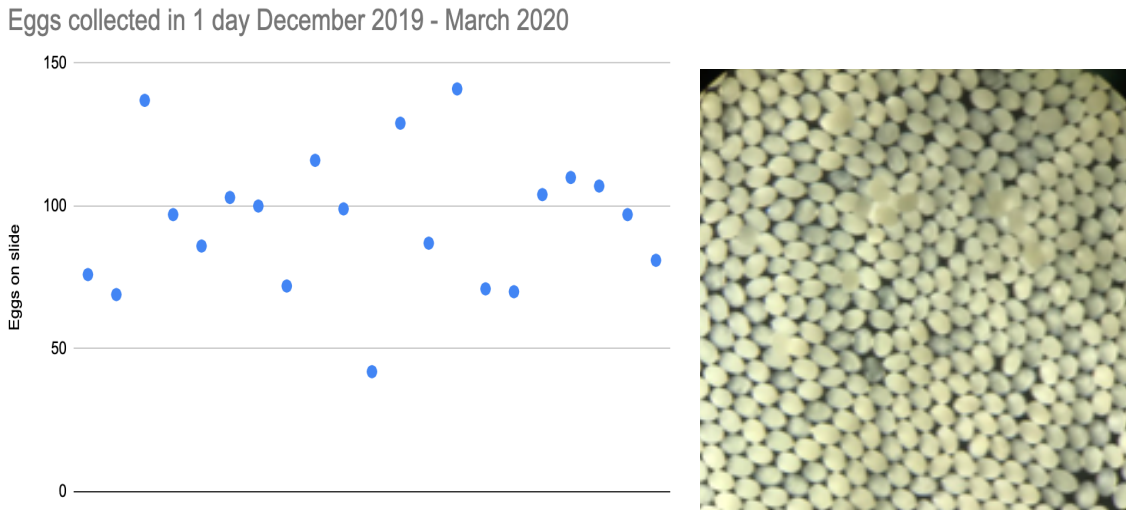

Figure 2.3 Number of *Dalotia* eggs collected each day. The plot on the left shows how many eggs were collected on a single day for a four month interval. The mean was 95 eggs in a day. The image on the right shows a batch of collected *Dalotia* eggs before they are mounted on a slide.

Slide mounting

Our reference organisms mount insect embryos onto double-sided tape to keep them in place during the microinjection process. I found that early *Dalotia* eggs are too fragile and rip easily when placed on double-sided tape. While attending a " New Genetic Tools for Non-Model Organisms" conference, multiple presenters said they used tegaderm on a slide as a more gentle but effective method for keeping insect embryos in place. This

worked much better for *Dalotia*, and allows eggs to be mounted without ripping while still keeping them in place during microinjection. Eggs are mounted in two rows on the slide oriented as shown in Figure 2.4.

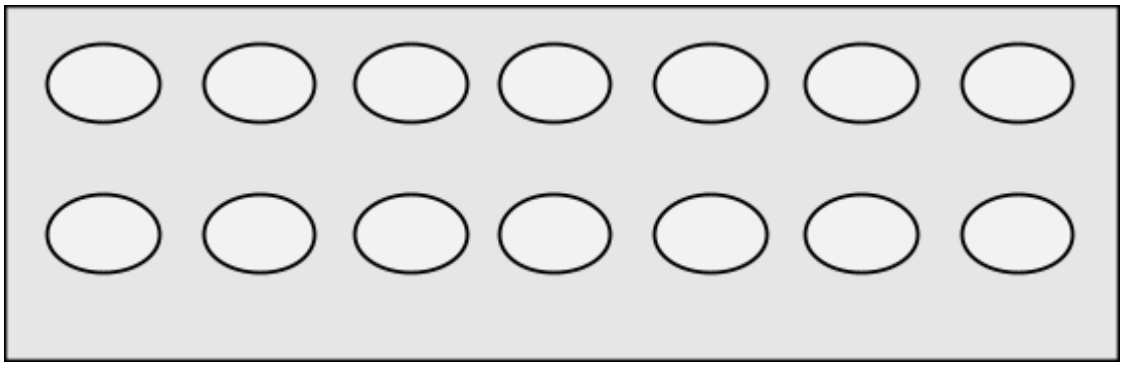

Figure 2.4 Orientation of collected *Dalotia* eggs on a slide. This schematic shows how to organize *Dalotia* eggs on a slide for subsequent microinjection.

Egg injection

During the early stages of this project, injections would kill 100% of collected eggs. Early *Dalotia* eggs are prone to ripping when coming in contact with a needle and bursting when injected. In order to optimize the process, we got a tour of Omar Akbhari's setup at UC San Diego and replicated the instrumentation. Additionally, Mina Yousefelahiyeh who performs the microinjections attended a microinjection workshop. The major advances that allowed us to achieve embryo survival were thinner/sharper

needles, desiccating the eggs for 10 minutes to create space for the injection solution, finding the right injection angle, and optimizing egg incubation.

In order to produce the correct needles, we pull borosilicate glass needles in a micropipette puller. After loading the injection solution into the needle, a micropipette beveler plate is used to bevel the tip until it opens, allowing for an open sharp needle. To desiccate eggs, mounted eggs are placed in a desiccation chamber for 10 minutes. The chamber is a 10cm plate filled with drierite and sealed with parafilm. The time in the desiccation chamber slightly shrinks the eggs and creates space for the injection solution to fill, preventing them from bursting when injected. For the injection, *Dalotia's* chorion is thin enough that dechorionation is not required unlike other insect species. By injecting into the apical end without penetrating the yolk, and injecting for no more than 0.2 seconds eggs will not burst or tear. After microinjection, the slide is placed in a moist hydrostone plate sealed with parafilm. This plate is then placed in a humid chamber inside of an incubator at 26 degrees. 72 hours later surviving embryos will hatch. Hatch rates with the current method average around 27% (Figure 2.5).

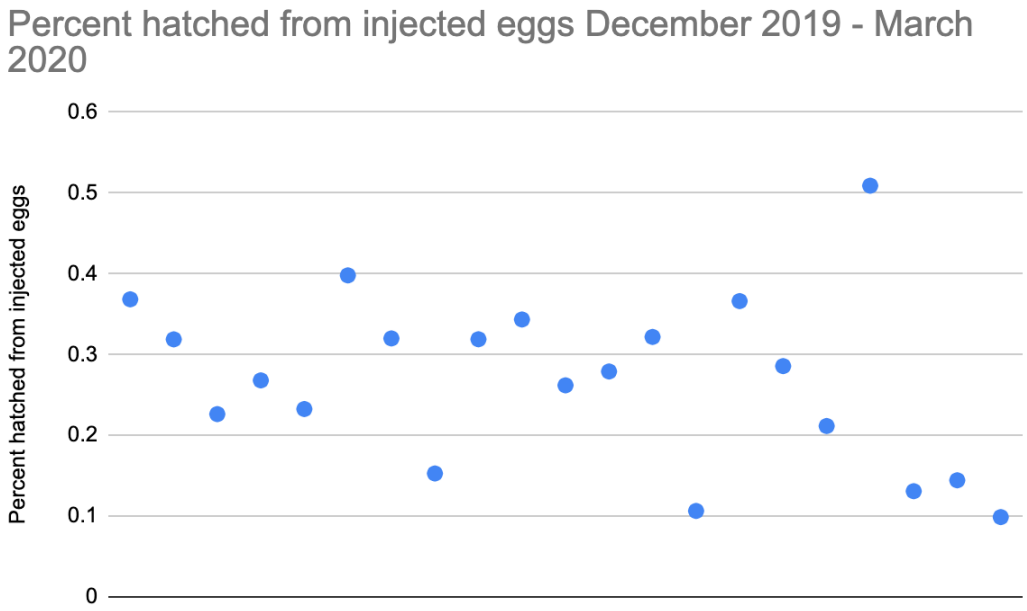

Figure 2.5 Hatch rate of *Dalotia* eggs per day. The plot shows the percentage of injected eggs that hatched on a single day. The average hatch rate is 27%.

Raising larvae to adulthood

Unlike the reference organisms mentioned above, *Dalotia* larvae are highly aggressive and cannibalistic while at the same time quite fragile. This is not an issue in the mass soil cultures, where a food abundant environment allows enough larvae to make it to adulthood to grow the population in spite of cannibalism and death. After egg injections, however, losing potentially transgenic larvae to cannibalism is not efficient given the amount of work required to produce each individual larva. It is therefore essential to raise them in single-housed conditions with high survival.

Dalotia larvae are sensitive to mold, humidity, and condensation. If food mold spreads, it easily traps and kills the larvae. If humidity is too low, they will dry out and die. If any condensation is present, it will trap and drown the larvae. Keeping a single larva in the usual 11 Quart box of soil is not practical because it takes up a large amount of space, and it is nearly impossible to find the larva once it is placed inside making adult retrieval inefficient. Keeping larvae in small containers makes regulation of food and mold much more difficult, as condensation and mold build up more rapidly in the small space. Additionally, the warm 26 degree environment also increases condensation.

For these reasons, I developed a method for rearing *Dalotia* larvae individually in petri dishes. I use the same petri dishes used for egg collections, but instead of a filter paper bottom I prepare a hydrostone bottom (Figure 2.6). This allows for humidity to be present while all condensation is absorbed into the hydrostone. Keeping these plates at room temperature instead of 26 degrees slows growth rate[2] but increases survival due to less mold growth and less condensation. By changing food every 24 hours to prevent any mold growth, over 75% of larvae survive to pupation.

[2] Larvae raised at room temperature take 3 more days on average to reach pupation, compared to their growth rate at the usual 26 ° C.

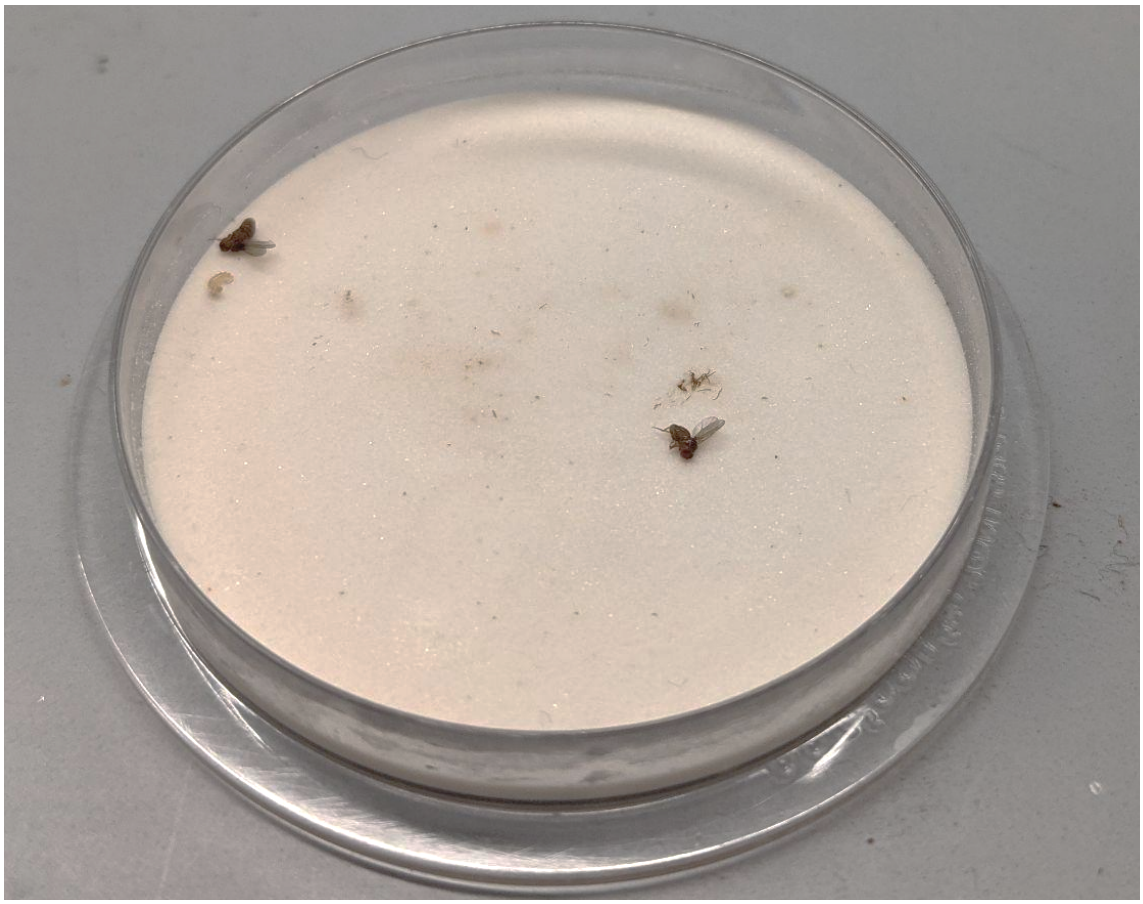

Figure 2.6 Petri dish with hydrostone bottom and *Dalotia* larva. The hydrostone bottom in the petri dish shown above is effective at maintaining high humidity in the dish while absorbing any condensation. This allows for optimal larval survival. The image also shows frozen thawed *Drosophila* used as food and a *Dalotia* larva in the top left portion of the dish.

Pupation takes around 7 days for *Dalotia*. While they can be allowed to pupate in the same hydrostone dish used to raise the larvae, only about 50% of larvae that reach pupation survive and eclose as adults in these conditions. A more efficient technique I discovered is to move them back into small boxes containing soil but no food (Figure

2.7). Given this is their natural habitat, pupation is more efficient. Additionally, since pupae no longer consume any food, the box can be kept sterile greatly reducing mold growth in the small space. In order to do this, larvae must reach the 3rd instar and be done consuming food. Given they are transparent and the gut is visible, this can be visually determined using a dissecting microscope because the gut will be empty. The larva will also become visibly fatter and move less, indicating it is about to enter pupation.

Figure 2.7 Pupation box for *Dalotia.* By adding a pre-pupal larva to a box as shown above, over 80% will reach adulthood. It is crucial to maintain the right humidity and not add food in order to prevent mold growth.

To prepare pupation soil, autoclaved coconut fiber substrate is added to a large container. Water is added and mixed until it feels wet to the touch, but moisture will not precipitate when squeezed in the hand. Then, a 175 mL box is filled with 25 mL of the prepared substrate. Using a brush, the pre-pupal larva is gently moved into the box. This box can be kept at 26 degrees. After around 6-9 days, the soil can be spread into a larger plastic container and gently shaken to spread the soil to a thin layer. An adult *Dalotia* should be easily visible and can be collected using an aspirator. Using this technique, around 80% of *Dalotia* survive pupation to adulthood. This means around 60% of hatched larvae make it to adulthood using the current technique. When I first started trying to single house larvae, 0% would survive to adulthood. After discovering plates and during the first rounds of microinjections, around 3 percent of hatched larvae made it to adulthood. The current 60% rate represents a 20-fold increase in survival and is critical for being able to efficiently microinject and produce genetically modified *Dalotia*.

Adult crosses

Of all the previous husbandry barriers, one-on-one crosses were the most straightforward to figure out. Once single-housed adults are found, they can be sexed as explained in Chapter 1. They can then be placed in the same petri dish used for egg collections in the dark, at 26 degrees with food exchanged every 24 hours. A single male can be placed with up to 3 females in a petri dish, and can be moved to a new dish every 24-48 hours to fertilize more females if required.

Eggs will be laid by the females against the side of the petri dish. The eggs can be allowed to hatch in situ, but previously hatched larvae will likely eat newly hatched larvae, reducing the amount of offspring obtained. In order to maximize survival, eggs can be moved daily to their own dish, where they will hatch three days after being laid. The larvae can then be single housed and raised as previously described.

Once a homozygous stable line is obtained, it can also be expanded. By adding at least 1 male and 1–2 females to a 250 mL box containing 50 mL of substrate, the population will grow exponentially. These lines are easy to maintain and only require feeding 3 times a week. They can be fed a 1:1 mixture of ground oats and chicken feed. While generating the line requires daily and time consuming work, maintaining it is easy. This is important since a goal is to produce a plethora of genetic lines that can be maintained over time for different experiments. A protocol detailing all of these steps is attached in Appendix A.

Discussion

While not necessarily exciting scientifically, the 4 previously described husbandry steps of early egg collecting, embryo injecting, raising larvae to adults, and crossing adults, are required in order to begin testing genetic modification techniques. Everything learned here applies specifically to *Dalotia* and perhaps other closely related athetine rove beetles. Overcoming these four steps is typically the barrier that prevents scientists from gaining genetic access to their insect of choice, and there is no guarantee that any or all of these steps will be feasible in a laboratory setting. The data shown in this chapter represents multiple years of work with a lot of failure on the way to successfully overcoming the husbandry barrier.

Chapter III

PIGGYBAC TRANSPOSON MEDIATED INSERTIONS

Introduction

A future goal for the lab is to be able to image brain circuit activity in *Dalotia* while it interacts with other species, particularly ants while tethered and walking on a ball. Its abdominal movements, which can serve as a proxy for its internal state, can be measured while brain activity is recorded (Hoopfer et al., 2015; Seelig et al., 2010). This experiment requires transgenic *Dalotia* expressing genetically-encoded activity indicators such as GCaMP to be expressed in the brain (Tian et al., 2009). In order to achieve this, we need to be able to introduce exogenous DNA sequences into the genome of *Dalotia*. PiggyBac (pBac) transposons have been widely used in insects including beetles for this purpose (Kuwayama et al., 2006; Lorenzen et al., 2003; Matthews et al., 2020). Microinjecting plasmids containing inverted terminal piggyBac transposon repeats flanking genes of interest, as well as a source of piggyBac transposase causes a high frequency of transposon insertion events at random genomic sites (Figure 3.1) (Fraser, 2012).

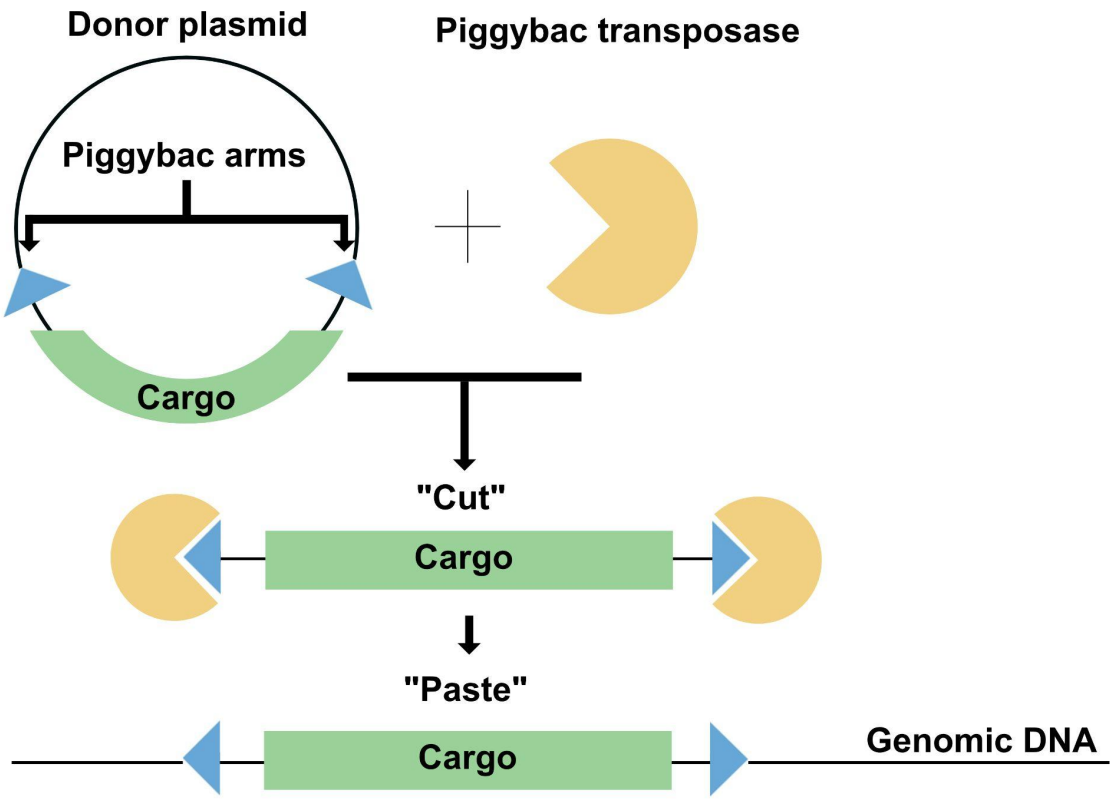

Figure 3.1 Piggybac transposon system. The schematic shows how cargo in a donor plasmid is excised by the piggybac transposase and inserted into genomic DNA.

In order to determine if the pBac system works in *Dalotia,* we need to find a functional promoter and a source of pBac transposase that works in our system. Furthermore, when expressing transgenes in a lab organism such as GCaMP, it is important to have the right endogenous promoters for the desired experiment. This promoter will allow one to control what cell types the transgene is expressed in. The transgene needs to be expressed at a high level in order to be functional. Endogenous promoters vary in strength, and

often are not strong enough to produce the expression levels of transgenes required in an experiment. The way this problem is overcome in other model organisms is through the use of a binary expression system, such as UAS Gal4 or the Q system (Kakidani and Ptashne, 1988; Potter et al., 2010). In these systems, the endogenous promoter is used to produce a transcription factor that then binds an activator sequence and leads to strong consistent expression of a transgene. Binary expression systems are not necessarily transferable from organism to organism (Matthews et al., 2020).

The experimental goals of this chapter are therefore to find a screenable promoter, successfully insert transgenic DNA into *Dalotia* using the pBac system, and develop a binary expression system that works in *Dalotia.*

Results

3.1 ie1 is an effective screening promoter in *Dalotia*

When building a genetic toolset from scratch in a new model organism, it is important to find positive controls for the toolset's effectiveness as early as possible. Without a screenable marker that can be used as a positive control it is very difficult to assess the effectiveness of a protocol, and therefore hard to modify and optimize it. Regarding promoters, initially 3xp3 was tried as it had proven effective in *Aedes* and *Tribolium* (Schinko et al., 2010). However, it seems to work better on lighter colored eyes, and given *Dalotia's* heavily pigmented eyes, it seemed like a sub-optimal candidate promoter.

Baculovrius immediate early gene (ie1) has proven to be an effective promoter expressing in the abdomen of *Bombyx mori* and *Drosophila melanogaster (Masumoto et al., 2012; Schinko et al., 2010)*. This was mentioned to me by Rory Coleman, a postdoc in Vanessa Ruta's lab, during a non-model organism conference at Janelia. A donor plasmid using ie1 to drive mcherry flanked by pBac arms was microinjected into *Dalotia* embryos (Figure 3.2).

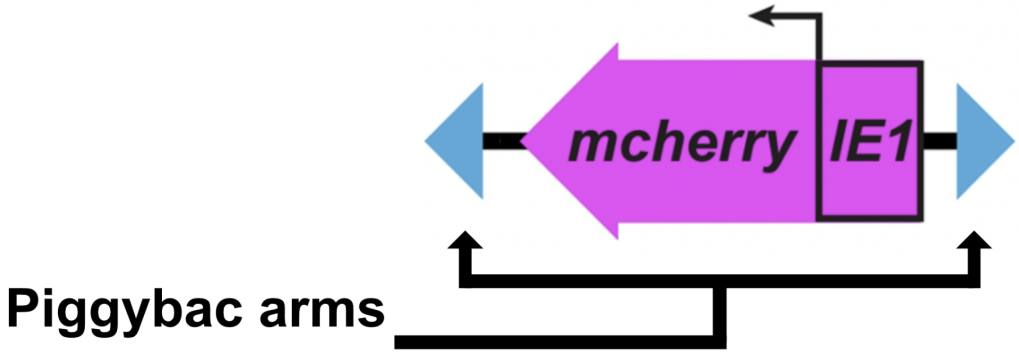

Figure 3.2 ie1-mcherry piggybac donor plasmid. The region between the piggybac arms is removed from the plasmid and inserted into the target genome. ie1 then drives the expression of mcherry.

As shown in Figures 3.3 - 3.5, ie1 successfully drives the expression of mcherry across most of *Dalotia's* body, not just the abdomen. It is easily visible in the larval and pupal stage. It is harder to see in the scleritized adult, but is still visible in between segments. ie1 is therefore a suitable promoter for screening whether a donor construct has been integrated into genomic DNA using the pBac system.

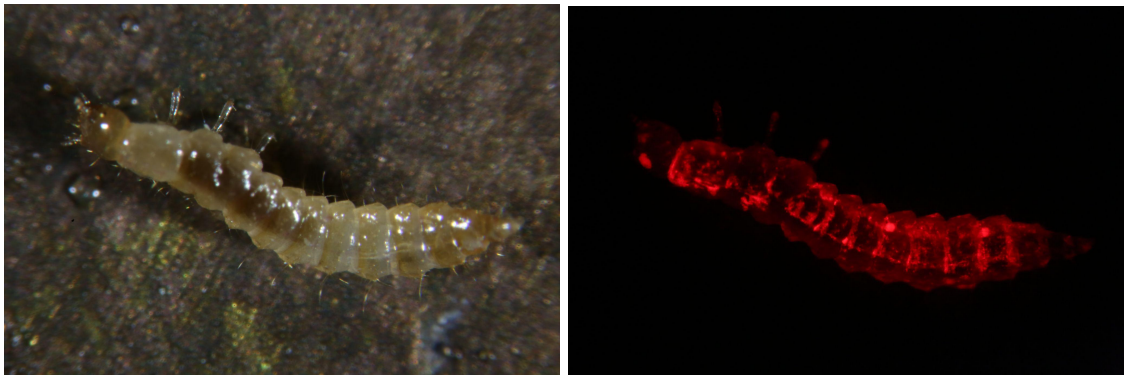

Figure 3.3 mCherry fluorescence driven by the ie1 promoter in a *Daloita* larva. Brightfield image of a larva (left) and mCherry epi-fluorescence of a larva (right) injected with the pBac-ie1-mcherry plasmid.

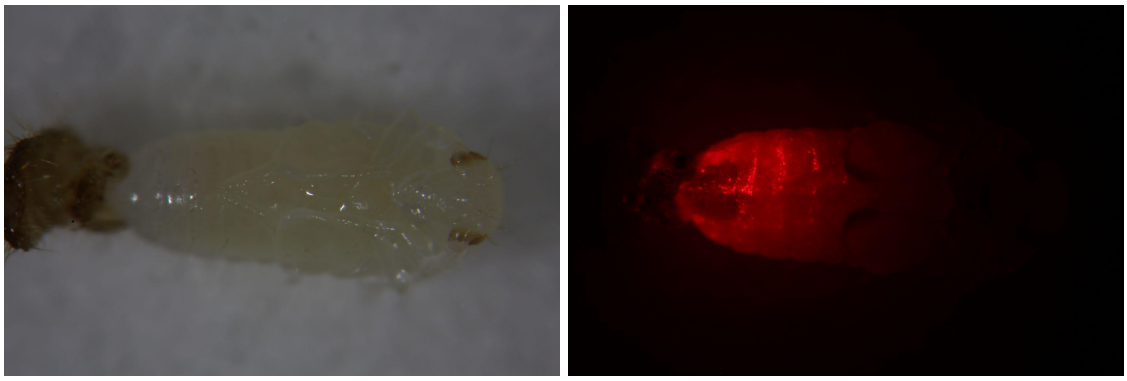

Figure 3.4 mCherry fluorescence driven by the ie1 promoter in a *Daloita* pupa. Brightfield image of a larva (left) and mCherry epi-fluorescence of a pupa (right) injected with the pBac-ie1-mcherry plasmid.

Figure 3.5 mCherry fluorescence driven by the ie1 promoter in a *Daloita* adult. Brightfield image of a larva (left) and mCherry epi-fluorescence of an adult (right) injected with the pBac-ie1-mcherry plasmid. Fluorescence is still visible through the scleritized cuticle.

3.2 Hyperactive transposase injected as mRNA is an effective transposase in *Dalotia*

When using the pBac system in insects, a transposase must be delivered alongside the donor plasmid. The transposase enzyme will cut the cargo out of the donor plasmid and insert it into genomic DNA (Figure 3.1) (Fraser, 2012). This transposase can be delivered in a plasmid with a universal promoter, as mRNA, or as a protein. Previous studies done in *Aedes* found that mRNA was the most efficient method (Matthews et al., 2020). Additionally, studies in a variety of insects have found that a hyperactive version of the transposase increased the amount of insertions leading to a higher proportion of hatched transgenic animals (Eckermann et al., 2018). This method of producing a mRNA of the hyperactive transposase was used. As shown in Figures 3.3-3.5, it is effective in

our *Dalotia* system. Within this system I found two parameters that greatly improved our efficiency. The first is preparing fresh transposase with minimal freeze thaw cycles. The second is the concentration of transposase. The higher the concentration, the higher the percentage of fluorescente larvae (Figure 3.6). When using high concentration (over 700 ng/uL**),** I achieved an average of 23% fluorescent larvae.

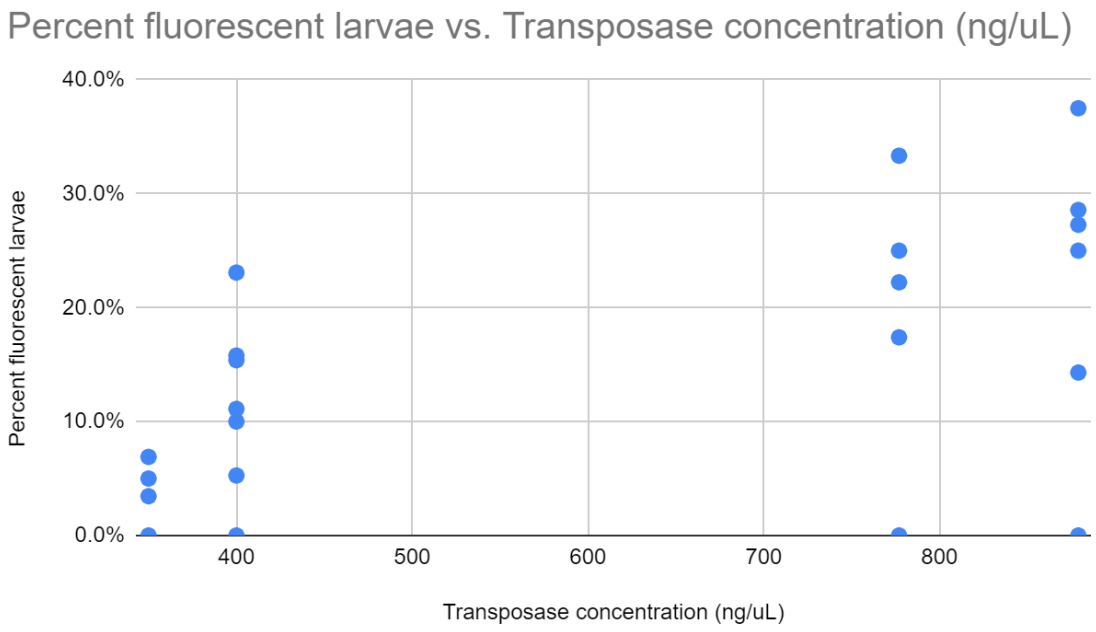

Figure 3.6 Number of fluorescent offspring for a given injection. As the final concentration of transposase in the injection solution increases, so does the percentage of fluorescent larvae that hatch from the given batch.

3.3 UAS Gal4Δ is a functional binary expression system in *Dalotia*

As referenced in the introduction, a binary expression system is crucial for driving high levels of a transgene in a given tissue type. Since binary expression systems are not necessarily transferable from organism to organism, I attempted to start with one that worked in *Dalotia*'s most closely related model organism, *Tribolium castaneum.* A modified version of UAS Gal4 was shown to be very effective in the *Tribolium*. The UAS sequence is the same, but the Gal4 has been cleaved to only express the C and N terminus portions of the protein and is called Gal4Δ (Schinko et al., 2010). Typically binary expression systems are used by having one genetic line express Gal4 in a given tissue, and another express UAS driving a given transgene. This allows for a lot of control and flexibility when doing experiments, but requires germ line insertions and stable lines, which I did not yet have in *Dalotia.* For this reason, I cloned the whole system into a single plasmid as shown in Figure 3.7. Given that ie1 was the only available validated promoter, I used it twice to drive both mcherry and Gal4Δ. The first ie1 driving mcherry will serve as a positive control, as any cell fluorescing in red should have received the plasmid. The second ie1 driving Gal4Δ should then produce enough Gal4Δ to drive UAS and express mCD8-GFP. If the system works, cells that fluoresce red should also have green fluorescent membranes.

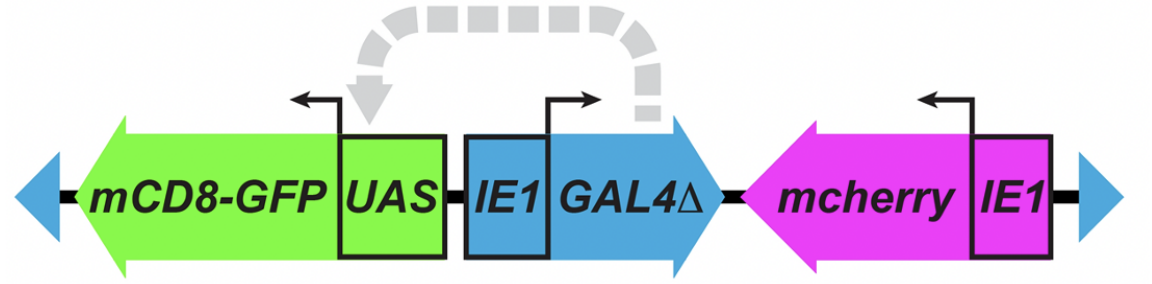

Figure 3.7 ie1-mcherry, ie-UAS-Gal4Δ-GFP piggybac donor plasmid. The region between the piggybac arms (blue triangles) is removed from the plasmid and inserted into the target genome. ie1 then drives the expression of mcherry and the expression of Gal4Δ. Gal4Δ in turn binds UAS and drives the expression of mCD8-GFP.

As shown in Figure 3.8, we are able to see red cells expressing green fluorescent membranes. This indicates that either the UAS Gal4Δ binary expression system is working as expected, or that the ie1 promoter is somehow driving the expression of the mCD8-GFP independent of the UAS sequence.

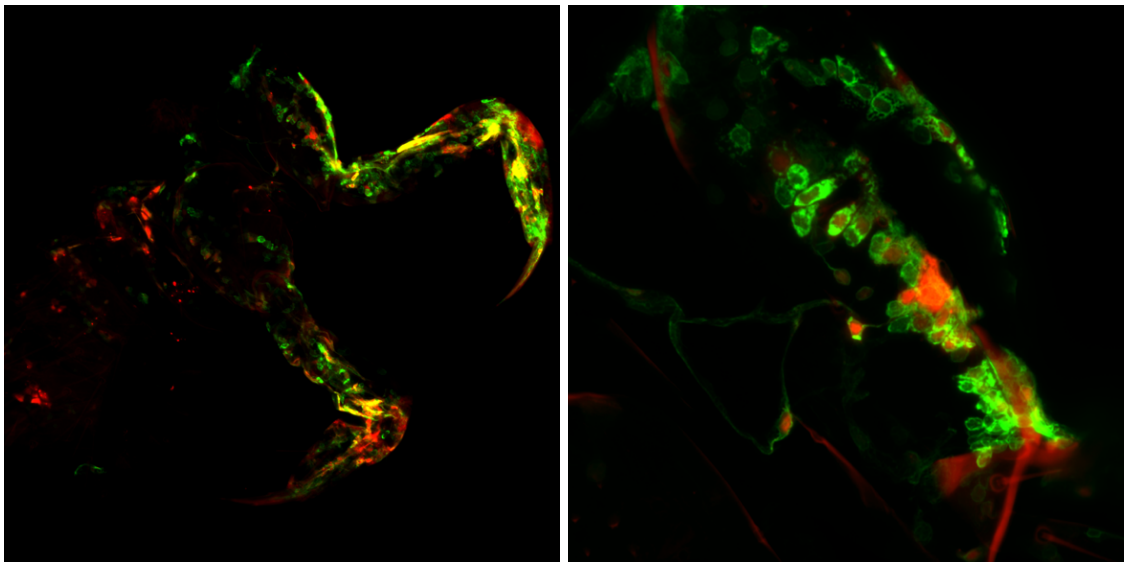

Figure 3.8 ie1-mcherry, ie-UAS-Gal4Δ-GFP expressed in adult *Dalotia.* Confocal images (10X Left, 40X Right) of *Dalotia* show the presence of cells expressing both cytosolic mCherry (red interior of cells) and membrane bound mCD8-GFP (green exterior of cells). Yellow areas show colocalization of both fluorophores.

In order to test if the expression of mCD8-GFP is actually being driven by UAS and not ie1, I produced a modified version of the construct. In this modified version, two stop codons are introduced early in the Gal4Δ sequence, meaning the protein will never be expressed (Figure 3.9). If red fluorescent cells still express green fluorescent membranes, then expression is being driven by ie1, as there is no Gal4Δ present to bind UAS. If however, green fluorescent membranes are removed, then the binary expression system is functional in *Dalotia.* As shown in Figure 3.10, red fluorescent cells no longer have green fluorescent membranes. The portions of visible green in the image are autofluorescent trachea. This indicates that indeed the UAS Gal4Δ binary expression system is functional in *Dalotia.* A full protocol for reproducing these results is attached in Appendix C.

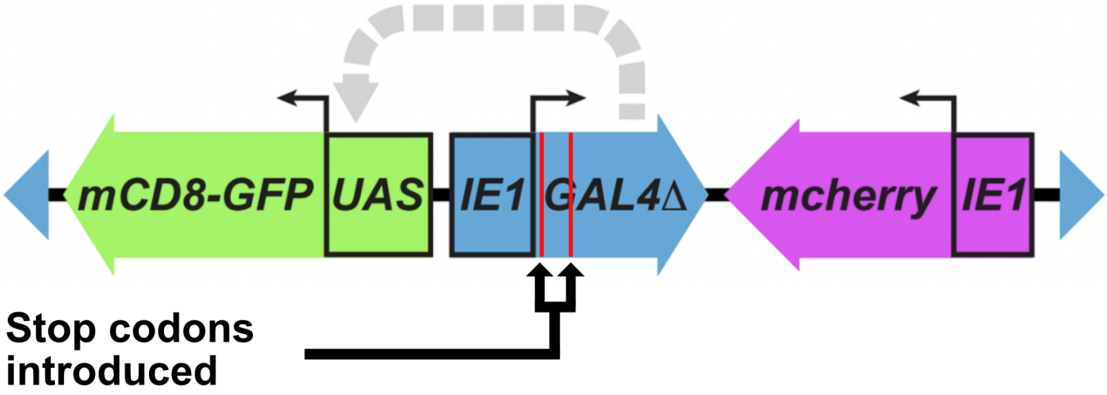

Figure 3.9 ie1-mcherry, ie-UAS-Gal4 STOP-GFP piggybac donor plasmid. The region between the piggybac arms (blue triangles) is removed from the plasmid and inserted into the target genome. ie1 then drives the expression of mcherry and the expression of Gal4Δ STOP. Since stop codons in Gal4Δ STOP prevent expression, it never binds UAS and does not drive the expression of mCD8-GFP.

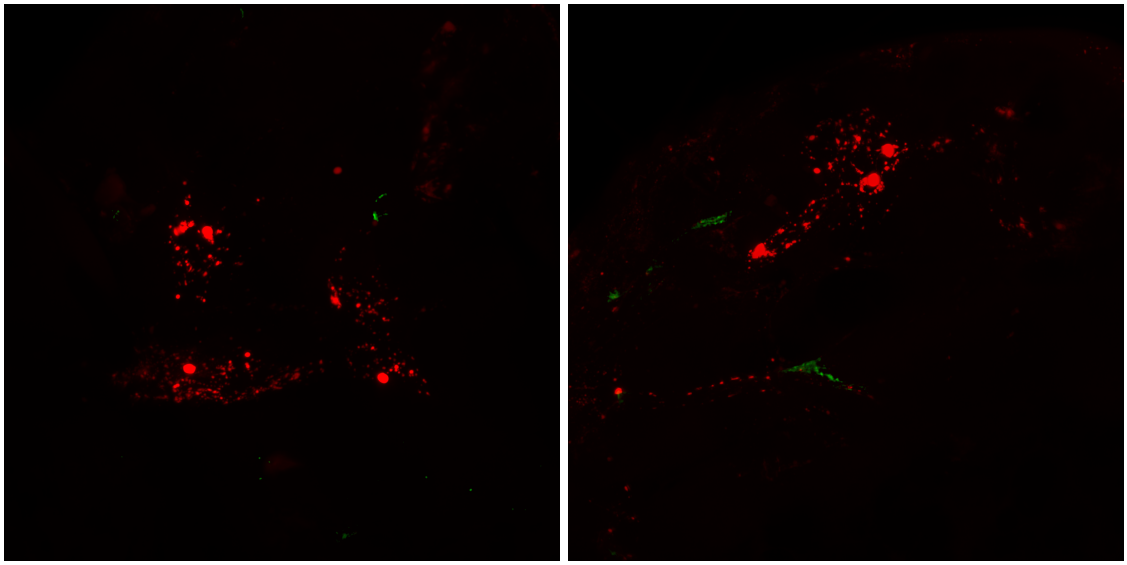

Figure 3.10 ie1-mcherry, ie-UAS-Gal4Δ STOP -GFP expressed in adult *Dalotia.* Confocal images (10X Left, 10X Right) of *Dalotia* show the presence of cells expressing cytosolic mCherry (red interior of cells) without expressing membrane bound mCD8-GFP (green exterior of cells) due to the presence of stop codons in the Gal4Δ portion of the construct.

Conclusion

The ability to introduce exogenous DNA sequences into the D. coriaria genome is crucial to the success of a variety of genetic manipulations. I have shown that ie1 is an effective screening promoter for determining when constructs have been inserted into *Dalotia's* genome. Next, I have shown that hyperactive transposase injections combined with donor plasmids are an effective way to introduce transgenes into the *Dalotia* genome, and have produced a reproducible protocol for the lab to use. Lastly, I have shown that UAS Gal4Δ is an effective binary expression system in *Dalotia.*

Discussion

When I started this project, nobody had ever successfully collected a batch of early *Dalotia* embryos. The husbandry troubleshooting alone took multiple years. Given that, having a reproducible protocol for transgene insertions and having a functional binary expression system are major steps forward. Nevertheless there are two major hurdles that remain, the first of which is germline transgenesis. None of the fluorescent adults produced to date have yielded transgenic offspring. This suggests that our reagents are not reaching the germline, which is required for the genomic insertion to be heritable. As will be shown in Chapter 4, CRISPR Cas9 germline mutations produced through microinjection are possible in *Dalotia,* suggesting it is not the delivery method itself that is not working. I hypothesize that the pBac transposase system may be slower than the

CRISPR Cas9 system, and therefore by the time the transposase is active, the germline has already been cellularized or sequestered. Potential solutions may include finding a tag that delivers pBac transposase specifically to the germline, finding a faster transposase, and modifying the way the embryo is injected.

The second remaining hurdle is to build a library of promoters that can be used to drive transgenes in different cell types. In order to facilitate this, the plasmid I built can be used (Figure 3.7). By swapping out the ie1 that drives Gal4Δ for DNA fragments upstream of genes of interest, genomic regions can be tested for promoter/enhancer activity. If a *Dalotia* cell expresses green fluorescence, it will have been driven by the DNA fragment. This fragment can now be used as a promoter, and its expression can be characterized by imaging the location of GFP.

Methods

A detailed protocol for reproducing all of the pBac system results in this chapter is listed in Appendix C. Appendices A and B also list protocols for general husbandry and egg collections required to reproduce results.

Chapter IV

CRISPR CAS9 MEDIATED GERMLINE DELETIONS

Introduction

When learning about the biological world, knocking genes out to determine their function is one of the most effective and widely used techniques across all aspects of Biology. When CRISPR came to the scene in the mid to late 2000s, it led to a frenzy of new research avenues, where targeted genomic deletions could be performed much more efficiently than ever before (Doudna and Charpentier, 2014). People developed CRISPR deletions in new organisms such as army ants (*Ooceraea biroi*), red flour beetles (*Tribolium castaneum*), and mosquitoes (*Aedes aegypti*) (Gilles et al., 2015; Kistler et al., 2015; Trible et al., 2017) . In *Dalotia* the questions that could be investigated by harnessing the power of single gene knockouts are endless. What sensory modalities are used to mediate inter and intra species interactions? What genes regulate the development and biosynthetic capabilities of the tergal gland? Developing this methodology in a new model organism from scratch requires four components: Identifying the right target genes, designing guide RNAs, screening for mutants, and maintaining stable knockout lines.

Identifying target genes can be broken down into two categories. The first and most common involves knocking out a gene that is involved in a biological process of interest. How that biological process is altered without the gene will give insight into the mechanism of said process. The second category which will be discussed here involves finding target genes that can serve as positive controls for the efficacy of the knockout tool itself. For *Dalotia,* this means finding a gene that is both visually screenable immediately after embryo hatching and not lethal. The reason it is important to screen immediately after embryo hatching, is that it allows us to know the extent to which the tested gRNA and injection procedure are effective. This is because experimental results from a round of injections are known within 3 days. If and when the protocol does not work, it can be quickly modified and optimized. If the gene knockout being screened for takes longer (only visible in the adult for example), it can take two to three weeks to have an experimental result, increasing the time it takes to troubleshoot a new protocol by an order of magnitude. Another reason the gene needs to be visually screenable is it will serve as a positive control during genotype screenings. A way of genotyping beetles while living so they can continue to breed is crucial to developing stable lines. Without a visual marker, it is hard to determine if a new genotyping protocol is functional. Once genotypes are known, producing stable homozygous lines is a matter of producing the right crosses. In summary, finding the correct gene to knock out will allow for optimization of the injection protocol, gRNA design, and genotyping protocol, which combined allow for the production of stable knockout lines.

Results

4.1 CRISPR Cas9 Distal-less knockouts

Given the prerequisites described above, I chose to attempt to knockout the Distal-less (DII) gene. Distal-less is a gene that is conserved across Arthropoda and regulates growth of distal tarsal segments (Cohen and Jürgens, 1989; Inoue et al., 2002; Panganiban et al., 1997; Williams et al., 2002). Previous studies in the crustacean *Parhyale hawaiensis* have shown that DII mutants are screenable from the day embryos hatch (Kao et al., 2016). The immediate screenability of the gene along with its conserved nature makes it a great candidate for troubleshooting CRISPR Cas9 deletions in new model arthropods. 4 guides were designed: the first three target exon 1, the last guide targets exon 2 (Figure 4.1).

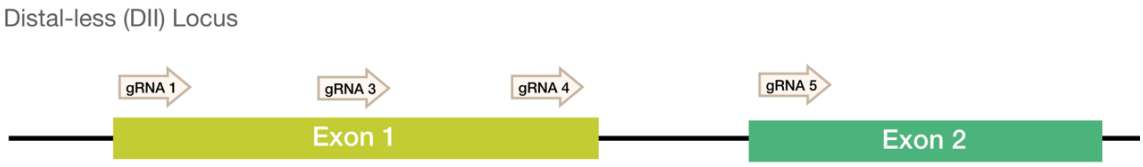

Figure 4.1 Distal-less (DII) Locus with gRNA binding sites. Four guides were designed to bind *Dalotia's* Distal-less locus. The arrow points to the location of the PAM site.

After protocol optimization, injection of all 4 guides led to 51% of hatched larvae missing some or all of their limbs (Figures 4.2 and 4.3). When each guide was injected

independently, only those larvae injected with gRNA 5 displayed the mutant phenotype, with 61% missing some or all of their limbs (Figure 4.3). This shows that the gRNA design for the last guide was effective. Additionally, it shows that the protocol is effective at producing G0 mutant *Dalotia.* At the time of performing this experiment, the *Dalotia* genome was still not complete enough to effectively troubleshoot a genotyping protocol. Additionally these larvae did not survive past the first instar due to their inability to walk and feed making them unsuitable for testing germline mutations.

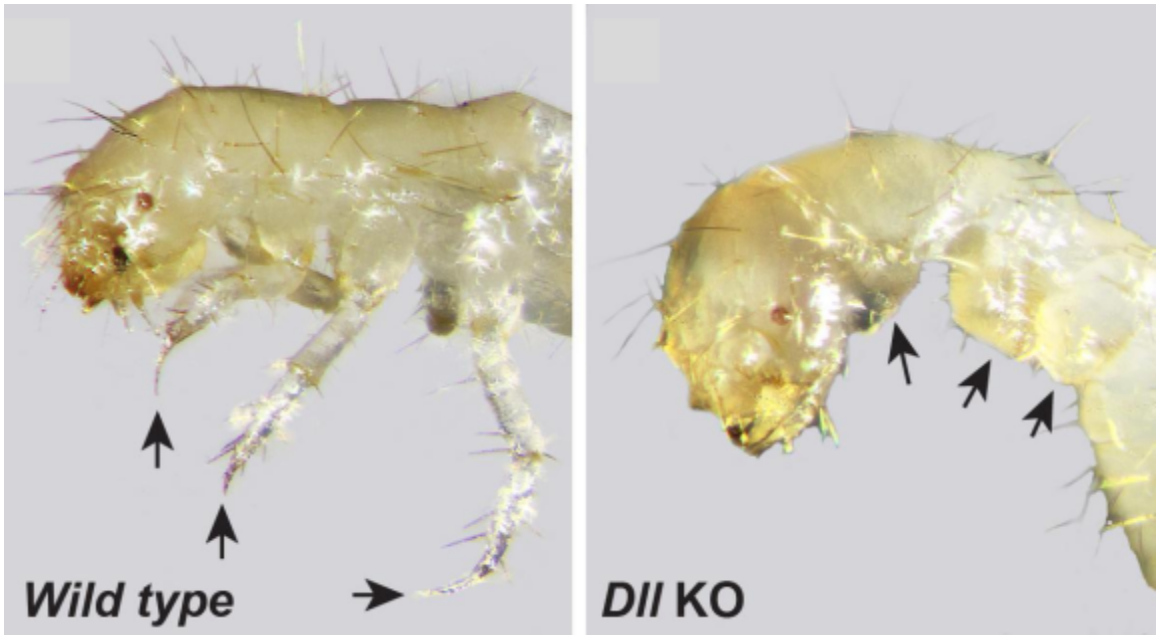

Figure 4.2 Distal-less mutant phenotype. The left panel shows a wild type larva, born with full leg segments. The right panel shows a Distal-less (DII) mutant larva that is missing limbs.

Injection solution	Live larvae hatched	Mutant larva	Percent mutant
Mix of all 4 gRNAs	37	19	51%
gRNA 1	27	0	0%
gRNA3	20	0	0%
gRNA4	20	0	0%
gRNA5	15	9	60%

Figure 4.3 Table of Distal-less gRNA effectiveness. Only the mix of 4 gRNAs and gRNA5 successfully create mutants, meaning only 1 guide worked in this batch.

4.2 CRISPR Cas9 White gene knockouts

Now that the CRISPR protocol has been optimized to produce a large amount of mutant offspring, the next step is to determine if the mutations are occurring in the germline, and are therefore heritable. This is crucial for the production of stable genetic knockout lines. The DII experiments were not informative in this regard because DII mutant larvae are unable to survive, as they cannot walk around to find food and grow. While in theory any non-lethal gene can be used to investigate whether or not a mutation is heritable, this requires an effective genotyping protocol. Since this model is brand new a genotyping protocol had not yet been established. It was therefore important to find a gene that was

both non-lethal and visually screenable. Visual screening would allow for proof of heritability independent of sequencing, and it would provide a positive control from which to optimize and validate a genotyping protocol.

The gene selected for this process is called white. In *Drosophila* it is commonly used as a marker gene because mutations in the gene lead to a loss of eye pigment which is easy to screen for (Mackenzie et al., 1999; Morgan, 1910). In *Dalotia* RNAi experiments had already shown that mutations in the gene lead to a lack of pigmentation during the pupal stage (Parker et al., 2017). Given its ease of screening, known function in *Dalotia*, and likely non-lethality, it is a great candidate gene.

Using guide design principles outlined in appendix D, 4 guides were designed to target the white gene in *Dalotia.* Larvae hatched out healthy, and 50% of pupae showed a mosaic phenotype in which only parts of the eye were pigmented (Figure 4.4). This suggested that our guides had successfully mutagenized DNA in parts of the developing embryo.

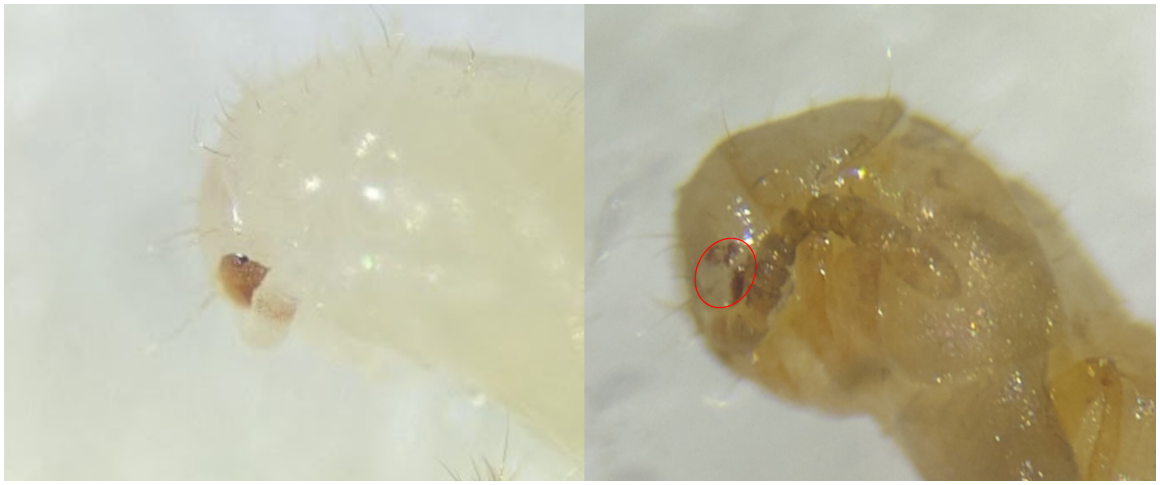

Figure 4.4 Mosaic white gene mutants. The panel on the left shows a wild type *Dalotia* pupal eye. Pigment is present in the entire eye. The panel on the right shows a mosaic mutant, where large parts of the eye are missing pigment (eye is circled in red). This phenotype persists into the adult stage.

Mosaic G0s were then outcrossed to wild type *Dalotia*. Sibling F1s were then crossed together as shown in Figure 4.5. If any germline mutations are present and both F1s are heterozygous, mendelian genetics would suggest that 25% of F2s would be mutant homozygotes from said cross. If no germline mutations are present, then all F2s should hatch out as wild type beetles.

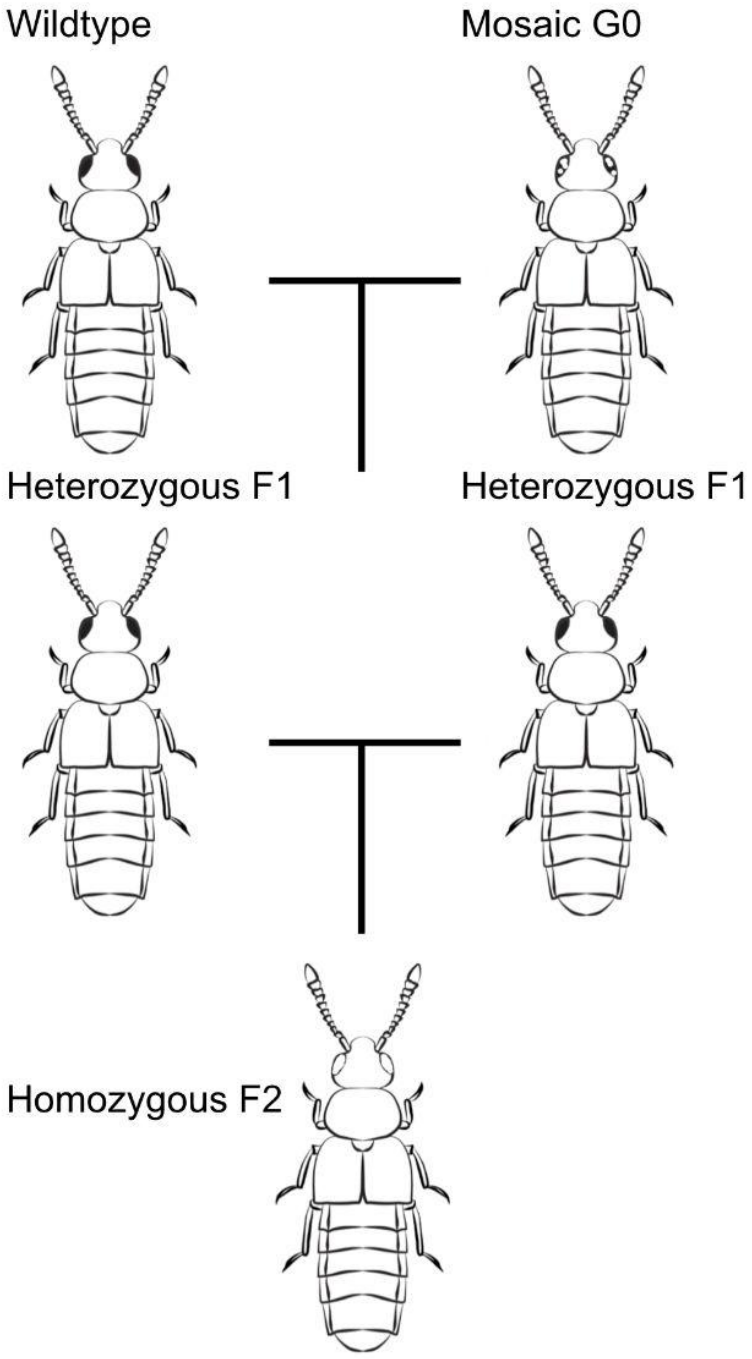

Figure 4.5 Crossing schematic for white gene mutants. Mosaic G0s are outcrossed to wild type beetles. If they carry germline mutations, heterozygous F1s can be crossed together to produce homozygous F2s.

For just under 43% of F1 crosses, 25% of larvae hatched missing larval eye pigment (Figure 4.6). These eye pigmentless larvae were raised to adults, where the eye pigment absence persisted (Figure 4.7). When crossed together, 100% of the offspring are also missing eye pigment. This result suggests that the protocol is effective at producing CRISPR Cas9 germline mutations in *Dalotia.* This population of white eye beetles has been maintained in culture for over a year, and can likely be maintained for much longer, even in the absence of balancer chromosomes.

Figure 4.6 Comparison of wild type larva and homozygous white mutant larva. The larva on the left is a wild type larva with a pigmented eye present. The larva on the right is a homozygous mutant in the white gene, and eye pigment is absent.

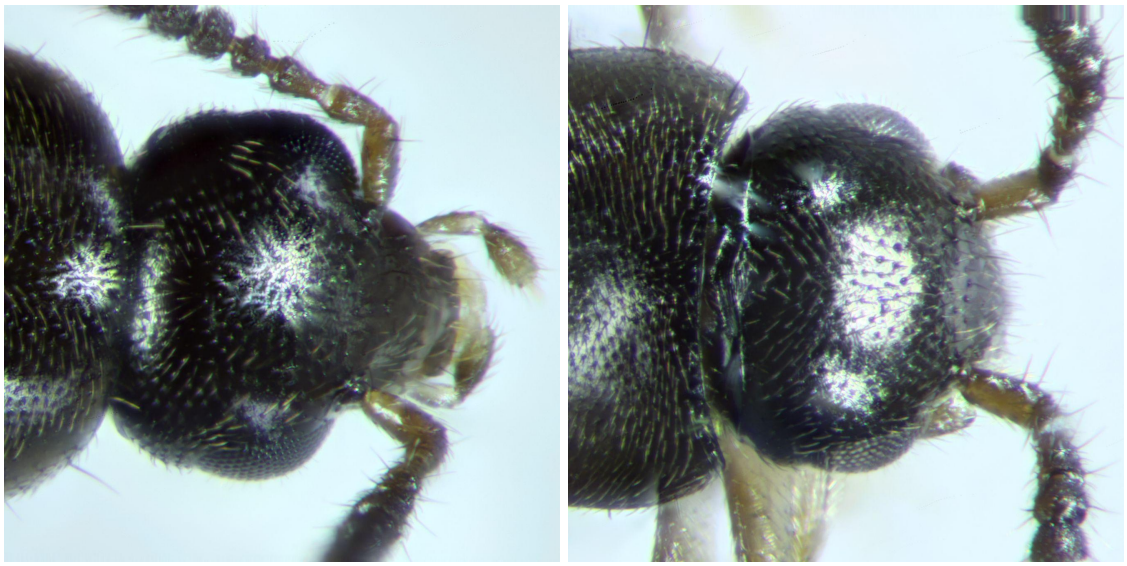

Figure 4.7 Comparison of wild type adult and homozygous white mutant adult. The adult on the left is a wild type *Dalotia* with a pigmented eye present. The adult on the right is a homozygous mutant in the white gene, and eye pigment is absent.

In order to validate that the pigmentless phenotype was in fact caused by a mutation in the white gene from one of the intended guides, a genotyping protocol was established. The tissue used in the genotyping protocol I developed is the metatarsus[3]. After being dissected, DNA is extracted and PCR primers flanking the gene of interest are used to amplify the sequence. The amplicon is sent out for sequencing, and a genotype can be obtained within 1 day of dissection. A detailed protocol is listed in Appendix D.

[3] The last segment of the middle limb.

Sequencing results from homozygous white mutant *Dalotia* show a 141 base pair deletion at the terminal end of exon 3 that completely removes exon 4 (Figure 4.8). gRNA 4 targeted that exact region of the gene, which shows that the mutation is in fact caused by the CRISPR Cas9 injection. Furthermore, the sequencing is replicable and consistent, validating the genotyping protocol.

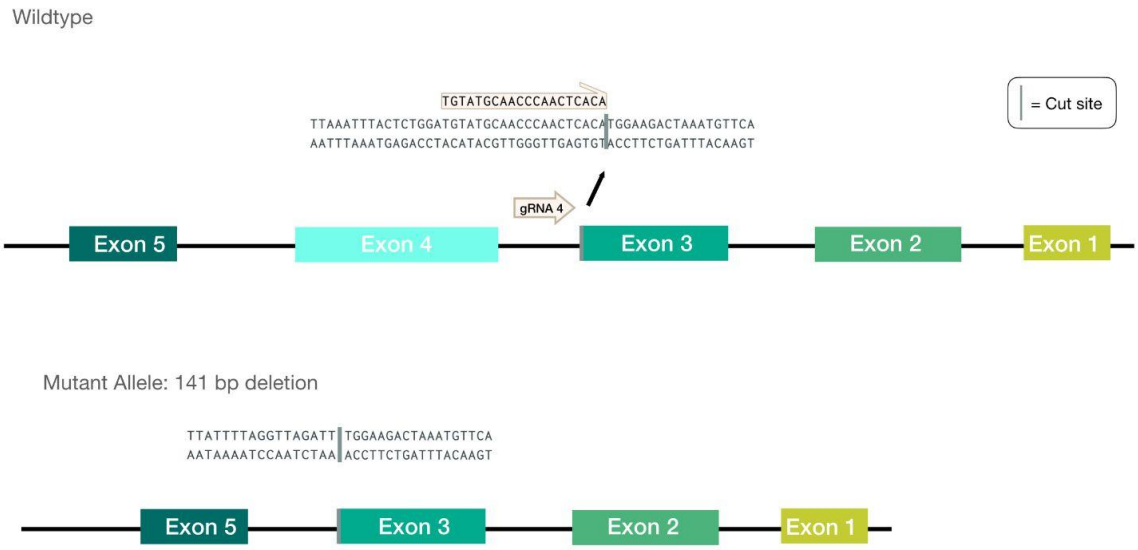

Figure 4.8 White gene locus in *Dalotia.* The top genome segment of the figure shows the wild type region of the white gene in *Dalotia* spanning exons 1 through 5. The guide RNA that produced the mutation is shown above exon 3. The bottom genome segment shows the mutant allele, missing 141 base pairs and completely missing exon 4.

Additionally, in order to confirm that the 141 base pair mutations found in the homozygous mutant was causing a reduction in the presence of white mRNA, qPCR was performed. The qPCR showed a two-fold reduction in levels of white mRNA in mutant *Dalotia* relative to wild type (Figure 4.9).

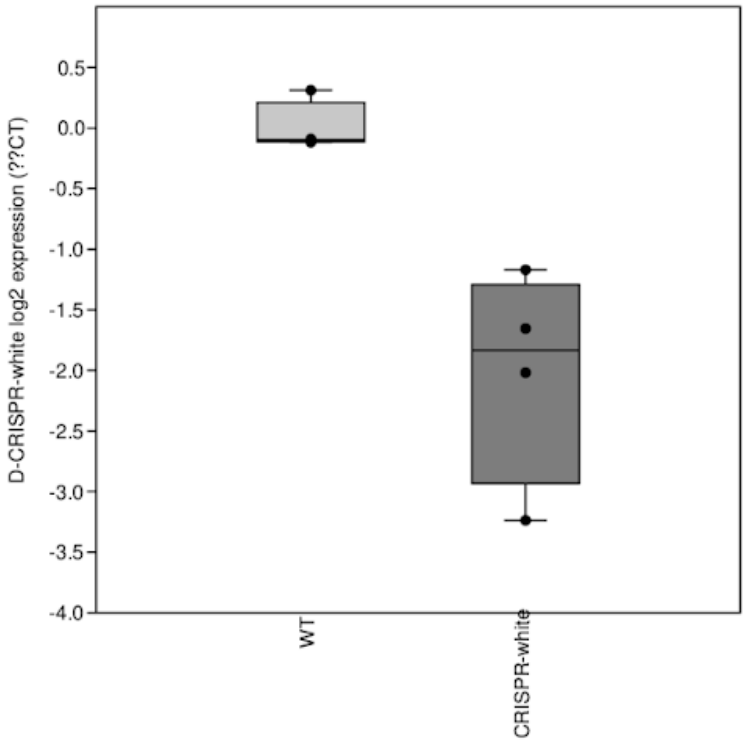

Figure 4.9 qPCR analysis of white gene in mutant *Dalotia*. qPCR primers targeting the white gene in *Dalotia* show a 2-fold reduction in expression of the white gene in homozygous mutants relative to wild type beetles.

Conclusion

The ability to introduce germline point mutations in any gene of interest is an important step towards turning *Dalotia* into an established model organism. In this chapter, I have shown that CRISPR Cas9 is an effective tool for generating such mutations, and my optimized protocol is efficient and reproducible. I have also shown that genotyping living beetles in *Dalotia* is both accurate and fast, allowing for homozygous mutants to be generated in 3-4 generations. I have also shown that homozygous mutant populations are stable and can be maintained in culture for extended periods of time. Lastly, I have shown that even small point mutations in *Dalotia* can knock out a gene both at a molecular level (qPCR) and phenotypically (lack of eye pigment).

Discussion

As discussed in Chapter 3, when I began this project there was no way of collecting early *Dalotia* embryos, raising larvae, performing one-on-one crosses, or any of the husbandry techniques required to start troubleshooting molecular techniques such as CRISPR Cas9. As such, having solved the husbandry and optimized a reproducible protocol for generating stable line mutants in *Dalotia* is a considerable advancement towards establishing *Dalotia* as a laboratory model organism. Additionally, the fact that lines can be maintained in culture for long periods of time means that a library of mutations can be created and maintained. This will allow for mutant *Dalotia* to be used for experiments

across different projects, and will allow for multiple mutations to be combined within the same beetle if required.

Since the generation of this method, other lab members have successfully reproduced it and made new lines of mutant beetles such as a decommissioned gene germline knockout, and are in the process of making a variety of other knockout lines (Brückner et al., 2021). What for years was a seemingly impossible outcome is now a common reproducible technique in *Dalotia.*

Methods

A detailed protocol for reproducing all of the CRISPR Cas9 mutations in this chapter is listed in Appendix D. Appendices A and B also list protocols for general husbandry and egg collections required to reproduce results.

Chapter V

EXPLORING OLFACTORY RECEPTOR GUIDED BEHAVIORS IN *DALOTIA CORIARIA*

Introduction

Most terrestrial animal life consists of soil dwelling invertebrates. Soil environments are complex multispecies assemblages, which are often dominated by social insects that exert top-down control over other arthropod populations by predation. To coexist in this environment, many species have invested in chemical defense mechanisms that rely on reliable detection of heterospecific threats, and accurate deployment (Arnam et al., 2018). Such interactions provide a neuroethological paradigm for studying how animals perceive and process sensory information emanating from other species, enabling them to efficiently navigate a world populated by other species (Kanwal and Parker, 2022).

One major radiation of such organisms are the rove beetles. Many have chemical defenses, and the success of these beetles has led to 64,000 species, explained at least in part by the effectiveness of their chemical defenses enabling them to proliferate in social-insect dominated ecosystems (Parker et al., 2017). How these beetles have evolved to correctly distinguish threats that require them to deploy chemical defense from neutral

stimuli that do not is unknown. This connects to the broader question of how animals have evolved to identify different species and interact appropriately.

Dalotia coriaria, the greenhouse rove beetle, recognizes ants as a threat and executes adaptive behaviors ranging from chemical defense to escape. It does not perform these behaviors when interacting with conspecifics or with other non-threatening insects. Previous studies of insect chemosensation have shown that olfactory receptors (ORs) play a major role in transducing long and medium range chemical cues (Vosshall et al., 2000). To what extent chemical cues, both long and short range, emanating from ants, are of relevance to components of ant recognition and downstream behavioral response is mysterious.

The goal of this chapter is to explore how olfactory receptors guide interspecies behaviors in *Dalotia.* To do so, I will build a map showing what olfactory receptors are expressed in different body parts. I will also use CRISPR Cas9 to knock out Orco, the olfactory receptor co-receptor. Orco is required for proper functioning of olfactory receptors, and without it all OR-mediated olfaction should be scrambled (Jones et al., 2005; Larsson et al., 2004). I will show that this Orco mutant has lost the ability to respond to odorant cues, and then explore how it handles interacting with ants.

Results

5.1 *Dalotia* expresses olfactory receptors across its body

Given the importance of olfactory receptors (ORs) in other species, we want to know what specific receptors are expressed in *Dalotia,* how they differ from other insect species, and where on the body they are expressed. I first built a phylogenetic tree of *Dalotia's* 106 ORs and populated the tree with annotated ORs from *Drosophila, Apis,* and *Tribolium* (Figure 5.1). The data shows that each species has its own nested expansions of ORs, which is expected given the evolutionary divergence of all four species.

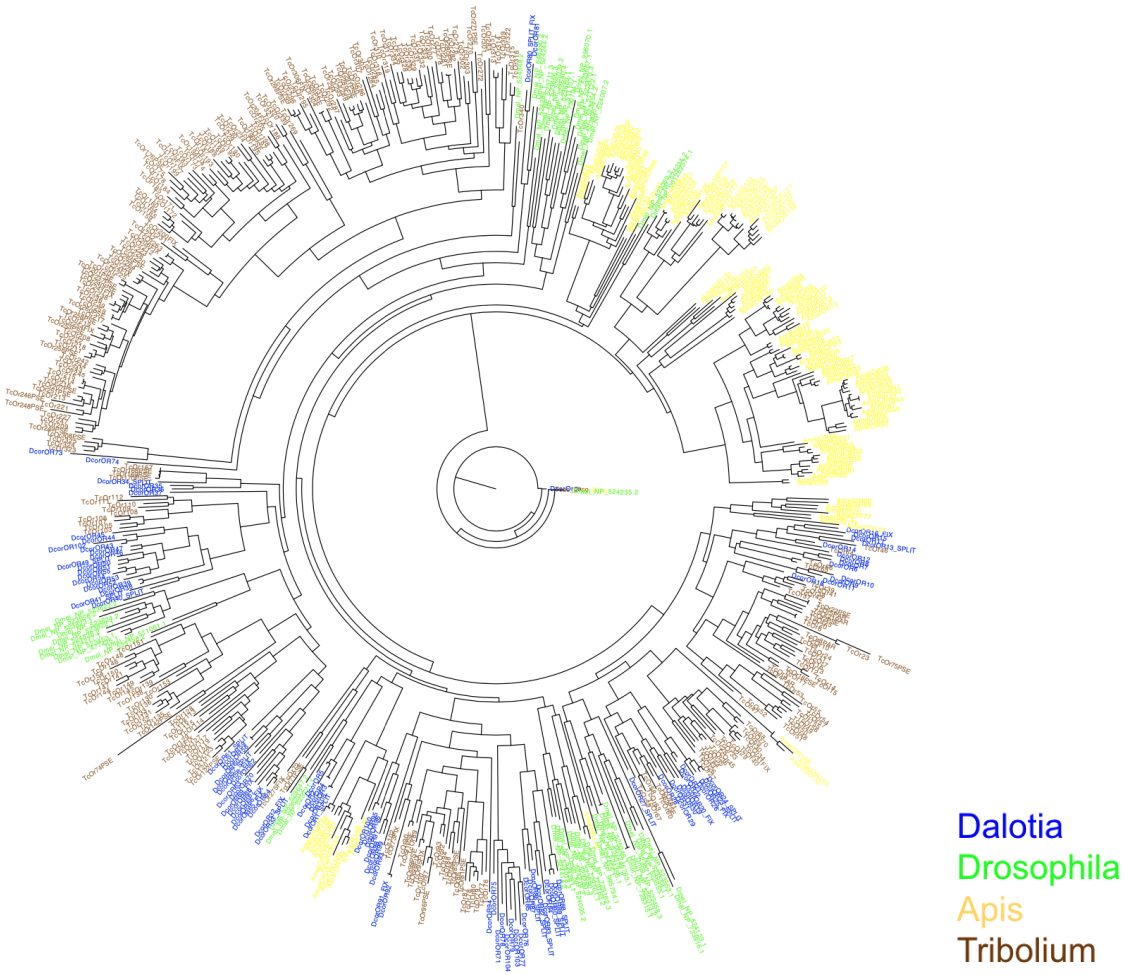

Figure 5.1 Phylogenetic tree of olfactory receptors (ORs) in *Dalotia, Drosophila, Apis,* and *Tribolium.* The tree is rooted at Orco (Olfactory receptor co-receptor), and shows how each species has expanded its own families of olfactory receptors.

Next, I used Smartseq RNA Seq to sequence different tissue types across *Dalotia's* body. Joe Parker performed dissections of *Dalotia* antennae, palps, protarsi, mesotarsi, metatarsi, segment 8, and segment 9. The first four tissue types were chosen because they have proven to be chemoreceptive centers in other more well studied insects (Vosshall et al., 2000; Wicher and Miazzi, 2021). Segments 8 and 9 were chosen to investigate if chemoreceptors are potentially involved in the targeting of the abdomen when flexing during defensive behaviors. 5 biological replicates were sequenced for each tissue type. I used kallisto to quantify the gene expression level in each tissue type and then separated counts for olfactory receptors (ORs), gustatory receptors (GRs), and ionotropic receptors (IRs) (Bray et al., 2016; Vosshall et al., 2000). By taking the median of all 5 biological replicates for each tissue type, I built the map displayed in Figure 5.2.

The highest expressed OR in *Dalotia* antennae relative to other ORs is Orco. The next two highly expressed ORs are OR83 and OR 66. OR101 is lowly expressed in the antenna relative to other ORs, but is very highly expressed in the six other sequenced tissues. Furthermore, OR14 is highly expressed only in the three sets of tarsi. Overall ORs are more highly expressed in segment 9 than in segment 8, with OR94 and OR78 being the most prominent ORs in segment 9.

On the gustatory receptor map palps showed the most diversity, with most GRs expressed. The highest expressed GR in the palps is GR1695541.218. The dominant GR in the tarsi is GR3119689.312. A large amount of GRs are also expressed on segment 9,

with the two dominant ones being GR1695541.218 which is also dominant in the palps, and GR6069552.784 which is the highest expressed GR in segment 8.

The ionotropic receptor (IR) map shows that IR3669713.437 is the dominant IR in the antennae, tarsi, and segment 8. As was the case for GRs, a variety of IRs are expressed in the palps, with IR 3669713.439 being dominant. IR6873940.324 is highly expressed in all tarsi, segment 8, and segment 9. The highest expressed IR in segment 9 however is IR1808848.48.

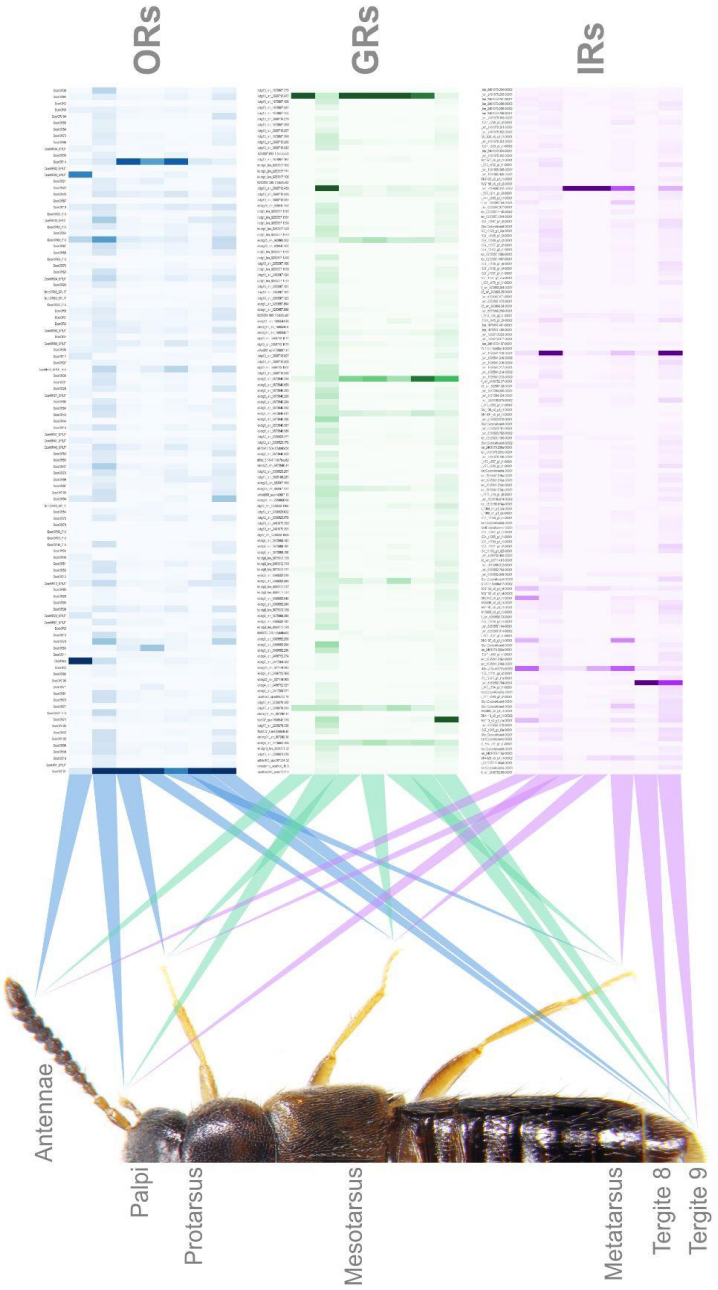

Figure 5.2 Map of olfactory (OR), gustatory (GR), and ionotropic (IR) receptors expressed in *Dalotia*. Smartseq libraries were prepared for *Dalotia* antennae, palps, protarsi, mesotarsi, metatarsi, segment 8, and segment 9. The map above shows what chemoreceptors are highly expressed in each tissue type. The value shown is the median of the 5 sequenced biological replicates for each tissue type.

5.2 Generating an Orco -/- *Dalotia*

Although we now have a map of the expression level and location of all of *Dalotia's* ORs, GRs, and IRs across its body, its is still unknown whether *Dalotia's* defensive behavior is governed by chemoreception at all, much less which class of receptors or which individual receptors. In order to begin to assess the extent to which chemoreception is a relevant sensory modality during this behavior I knocked out Orco, the olfactory receptor co-receptor. When Orco has been knocked out in *Drosophila, Aedes,* and *Oocerea,* it has removed the ability for ORs to function properly (DeGennaro et al., 2013; Trible et al., 2017; Yan et al., 2017). Given the conserved nature of the gene, it will likely do the same in *Dalotia* (Figure 5.1).

Using the CRISPR Cas9 knockout protocol discussed in Chapter 4, I designed guides and produced two independent stable homozygous Orco -/- lines (Figure 5.3). The first line (AE3 line) contains a 520 base pair deletion that removes most of exons 1 and 2. The second line (AA3 line) contains a 26 base pair deletion in exon 1. An analysis of the open reading frame after the mutation in the AA3 line reveals that it introduces 3 stop codons into exon 1, likely eliminating production of the entire Orco protein (Figure 5.4). Given that the AA3 line was generated first, it will be the main Orco -/- line used in subsequent experiments, except for control experiments in Figure 5.9.

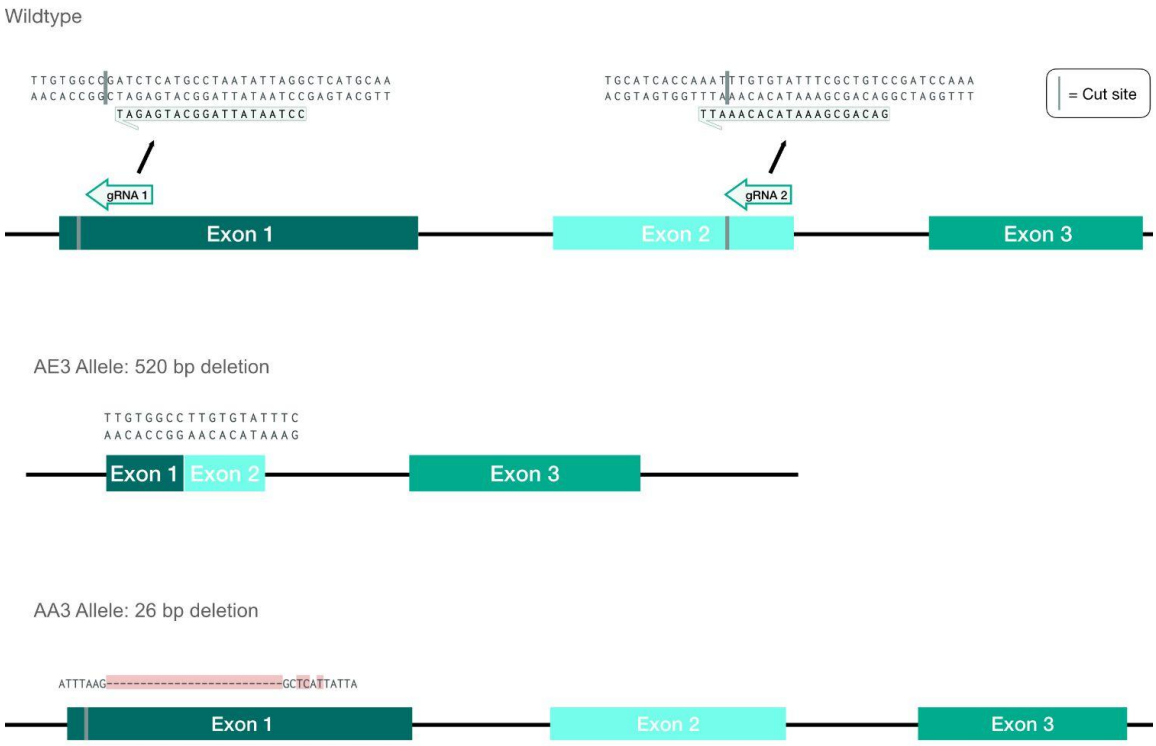

Figure 5.3 Orco gene locus in *Dalotia.* The top genome segment of the figure shows the wild type region of the Orco gene in *Dalotia* spanning exons 1 through 3. Both of the guide RNAs that produced cuts are shown above it, one on exon 1 and one on exon 2. The middle genomic segment shows one of the Orco mutant alleles with a 520 bp deletion eliminating most of exons 1 and 2. The bottom genomic segment shows the other Orco mutant allele with a 26 bp deletion in exon 1.

Exon 1 WT:

MMEFKVQGLVADLMPNIRLMQAAGHFMFNYHADNSGALH
TLRIGYSCLHLLLVLAQF

Exon 1 AA3 allele:

MMEFKAHY*AHASCRPFYVQLSCG**WCATYSQDWLFLLAF
VVGFSSVW

Figure 5.4 Amino acid translation of exon 1 comparing wild type to the AA3 allele. The 26 base pair mutations present in the AA3 allele causes a frameshift that introduces 3 stop codons in exon 1.

Now that an Orco -/- line has been generated, I used qPCR to test whether the 26 base pair mutation of the AA3 line actually led to reduced expression. As shown in Figure 5.5, Orco -/- *Dalotia* expresses almost 2 times lower levels of Orco relative to wild type. Given that a similar qPCR drop was shown in white -/- beetles, and the eye pigment was in fact eliminated, this suggests that the Orco protein is likely also no longer present in the AA3 line.

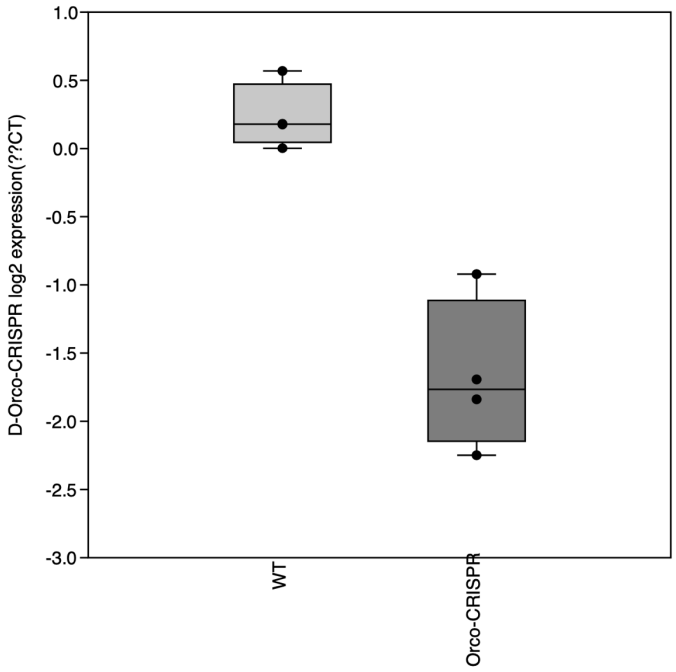

Figure 5.5 qPCR analysis of Orco gene in mutant *Dalotia*. qPCR primers targeting the Orco gene in *Dalotia* show a near 2 fold reduction in expression of the Orco gene in homozygous mutants relative to wild type beetles.

Next, I wanted to test whether Orco -/- *Dalotia* indeed had reduced olfaction. I built an odor preference arena, shown in Figure 5.6. This arena allows me to flow odors over two separate arena arms. If *Dalotia* are attracted to an odor they will spend more time in the arm flowing said odor. If *Dalotia* are repulsed by an odor they will spend more time in the control arm with no odor. Recordings are performed in the dark to eliminate any visual stimuli.

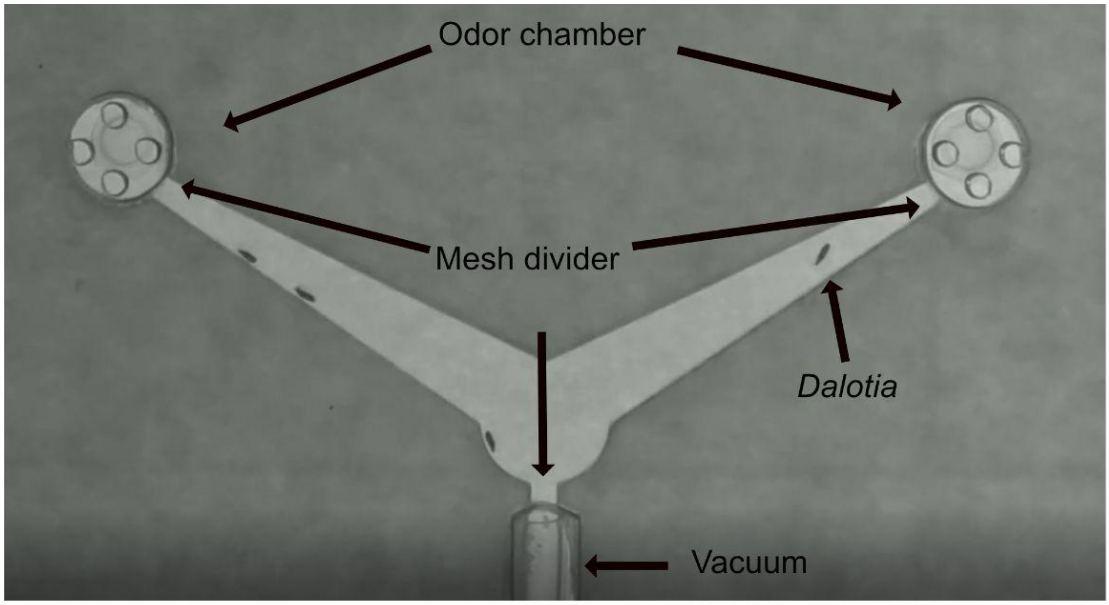

Figure 5.6 *Dalotia* odor preference arena. The two circles at the top of the arena are odor chambers. They contain wells holding paraffin oil in which odors can be dissolved. A vacuum at the bottom of the arena pulls air through the odor chamber through a mesh divider into the arena. Air flows from each odor well into the vacuum. *Dalotia* is free to explore both arms of the arena and can preferentially stay in or avoid the arm containing the tested odor. Images are captured in infra-red, as the behavior is recorded in complete darkness to eliminate any visual stimuli.

I initially attempted to find an attractive odor, but everything I tried was either neutral or aversive. When using 1-octen-3-ol (mushroom alcohol), I found that wild type *Dalotia* was strongly repulsed (Figure 5.7). This compound has been shown to be OR-mediated in other species (Xu et al., 2015). I therefore hypothesized that if olfaction was in fact reduced in Orco -/- beetles, the repulsive phenotype should disappear.

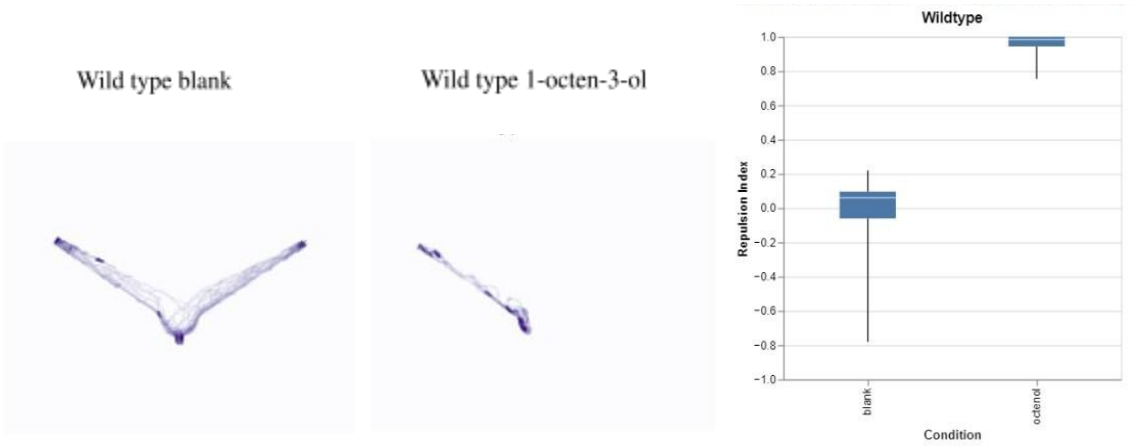

Figure 5.7 Wild type *Dalotia* response to 1-octen-3-ol (octenol) in odor preference arena. 10 trials were performed, each with 4 *Dalotia* for each condition. In the blank condition, both odor chambers contain 5 µL of paraffin oil. In the octenol condition, 1-octen-3-ol was diluted 1:500 in paraffin oil, and 5 µL was placed only in the right odor chamber. The left odor chamber contains 5 µL of paraffin oil. The repulsion index measures the difference in pixel intensity between the right side and the left side, and divides it by the total pixel intensity in the image. Values of 1 indicate that all time is spent in the left arm and indicate repulsion from the right odorante chamber. Values of -1 indicate all time is spent in the right odorant arm and indicate attraction to the right odorant chamber. The data shows that wild type *Dalotia* is strongly repulsed by the octenol in the right odor chamber.

As hypothesized, AA3 line Orco -/- beetles show no aversion to 1-octen-3-ol (Figure 5.8). Given the strong aversion exhibited by wild type beetles, this result suggests that AA3 line Orco -/- beetles are either incapable of perceiving the odor or are not processing the odor in the same way, and therefore interpret it as a neutral odor. Regardless, the result suggests that olfaction does not work properly in AA3 line Orco -/- *Dalotia.*

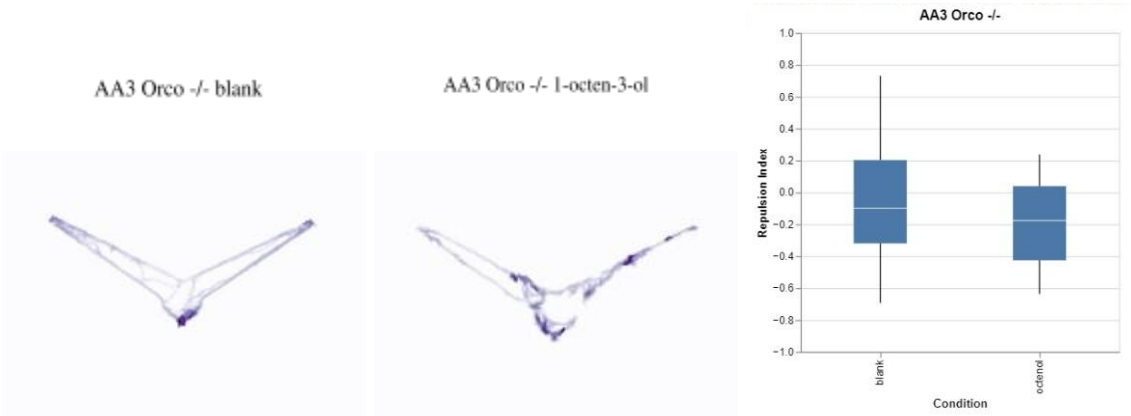

Figure 5.8 AA3 line Orco -/- *Dalotia* response to 1-octen-3-ol (octenol) in odor preference arena. 10 trials were performed, each with 4 *Dalotia* for each condition. In the blank condition, both odor chambers contain 5 µL of paraffin oil. In the octenol condition, 1-octen-3-ol was diluted 1:500 in paraffin oil, and 5 µL was placed only in the right odor chamber. The left odor chamber contains 5 µL of paraffin oil. The repulsion index is explained in Figure 5.7. The data shows AA3 line Orco -/- *Dalotia* show no aversion to the octenol in the right odor chamber.

In order to validate that the phenotype exhibited in Figure 5.8 is in fact due to the 26 base pair deletion in Orco exon 1 and not an off target effect, I repeated the experiment with Orco AA3 +/- beetles and Orco AE3 -/- beetles. As shown in Figure 5.9, AA3 line Orco +/- beetles show normal wild type aversion to 1-octen-3-ol. This suggests that there are no off target dominant mutations affecting the phenotype. Next, I tested AE3 Orco -/- beetles, and found the same lack of aversion found in AA3 Orco -/- beetles. The odds of an off target mutation leading to a lack of olfaction phenotype occurring in two independent mutant lines is extremely low. This suggests that AA3 Orco -/- beetles are in fact olfaction deficient as a result of the CRISPR Cas9 induced 26 base pair deletion in exon 1.

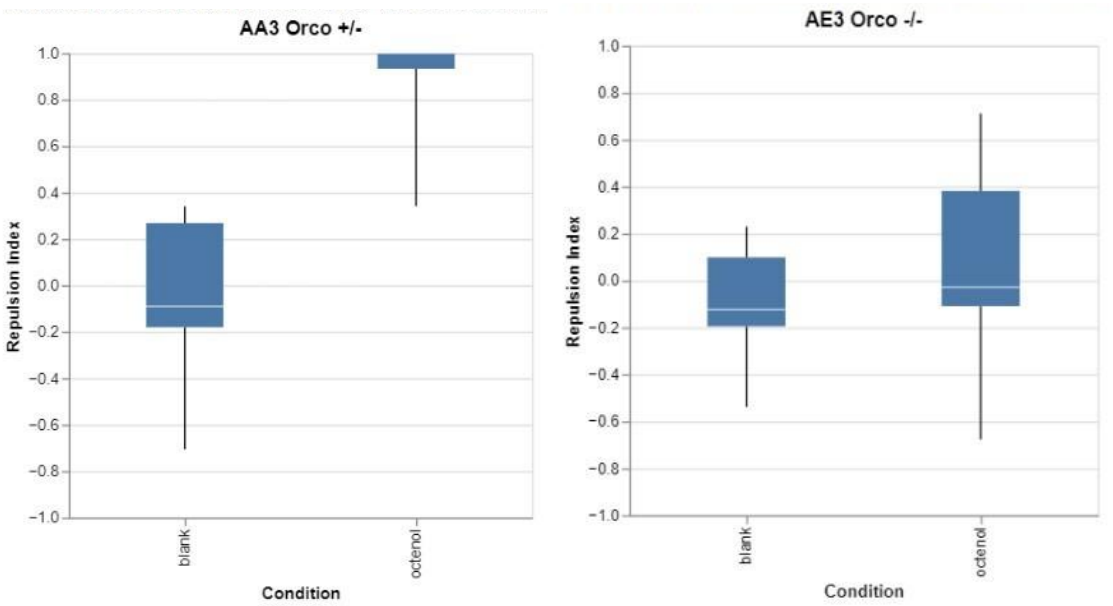

Figure 5.9 AA3 line Orco +/- *Dalotia* and AE3 line Orco -/- *Dalotia* response to 1-octen-3-ol (octenol) in odor preference arena. 10 trials were performed, each with 4 *Dalotia* for each condition. In the blank condition, both odor chambers contain 5 µL of paraffin oil. In the octenol condition, 1-octen-3-ol was diluted 1:500 in paraffin oil, and 5 µL was placed only in the right odor chamber. The left odor chamber contains 5 µL of paraffin oil. The repulsion index is explained in Figure 5.7. The data shows that AA3 line Orco +/- *Dalotia* are strongly repulsed by the octenol, just like wild type beetles. The data also shows that AE3 line Orco -/- *Dalotia* show no aversion to the octenol.

5.3 Orco -/- effects on the development of the antennal lobe:

Dipteran studies of Orco knockouts have shown no effect on the development of the antennal lobe in mutant organisms, while Hymenopteran studies of Orco knockouts have shown significant reductions in the volume of the antennal lobe in mutant organisms (Chen et al., 2021; Trible et al., 2017). Dipterans and Hymenopterans are two very divergent orders within insects (Figure 5.10) (Ishiwata et al., 2011). Orco had only ever been knocked out in these two orders, and the basal function of Orco in insect development was unknown.

Both wild type and AA3 line Orco -/- *Dalotia* brains were dissected and imaged by Jaison Omoto. I then used Imaris to quantify antennal lobe volume for both populations (Figure 5.11). There was no qualitatively perceivable difference in the antennal lobes in the mutant beetle relative to wild type, and antennal lobe volumes also showed no significant difference. This result suggests that the use of Orco in development was either a hymenopteran development, or was lost in a common ancestor of dipterans and coleopterans.

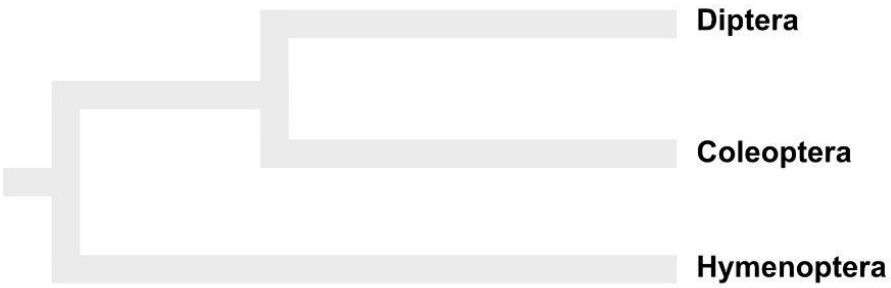

Figure 5.10 Phylogenetic relationship of insect orders in which Orco has been knocked out. Hymenopterans are in the earliest branching tree of insects, with Dipterans and Coleopterans being more recent. Orco has only ever been knocked out in these three orders, with developmental defects seen in Hymenoptera and not seen in Diptera.

Wild type antennal lobe **Orco -/- antennal lobe**

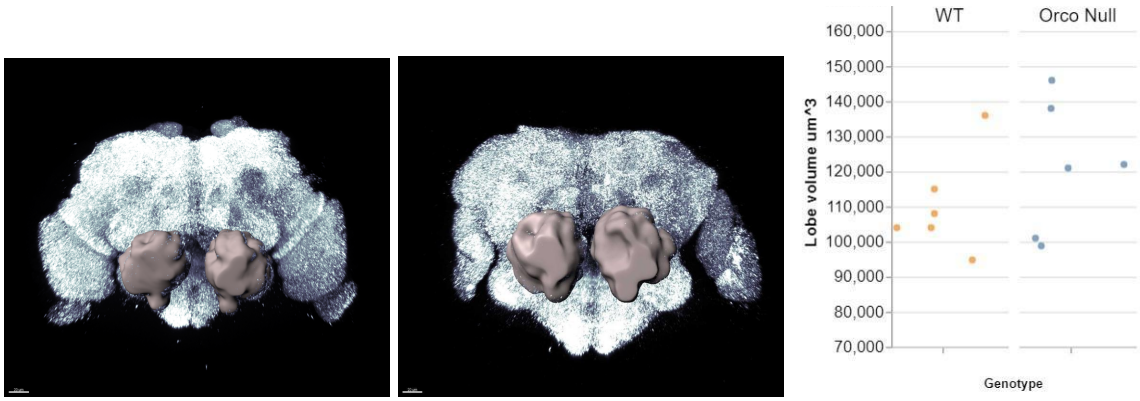

Figure 5.11 3D Reconstruction of antennal lobe in wild type *Dalotia* and Orco -/- *Dalotia*. The left panel shows the 3D reconstruction of an antennal lobe in a wild type male *Dalotia.* The center panel shows a 3D reconstruction of an antennal lobe in a AA3 line Orco -/- *Dalotia.* The right panel plots lobe volume comparing wild type and Orco -/- *Dalotia* and shows no difference between the genotypes.

5.4 Effect of Orco -/- mutation on interactions with ants in an open arena

Cuvette experiments were performed with Jessleen Kanwall, a postdoc in the lab, who designed the setup. When placed in a cuvette arena with an ant, wild type and Orco -/- are both capable of defending themselves from ants and escaping (Figure 5.11). Previous studies have shown that disarmed *Dalotia*[4] show reduced survival in the presence of ants (Brückner et al., 2021). Orco -/- *Dalotia* however show no reduction in their ability to survive encounters with ants in the cuvette arena. The duration of interactions with ants as well as the time between interactions (which could be an indication of ant avoidance) are also not significantly different between wild type and Orco -/- *Dalotia* (Figure 5.12).

There is however a significant difference in the body angle of *Dalotia* when comparing both phenotypes. Both wild type and Orco -/- flex at the onset of an interaction, but wild type beetles start flexing at a distance before the interaction while Orco -/- beetles do not (Figure 5.13). At a far enough distance from the ant, both phenotypes maintain the same level of non-flexion, but between 0 and 400 pixels apart before interaction, wild type beetles flex around 8 degrees more.

[4] Disarmed *Dalotia* have had all gland contents removed, and therefore are incapable of using chemical defense to defend themselves from threats.

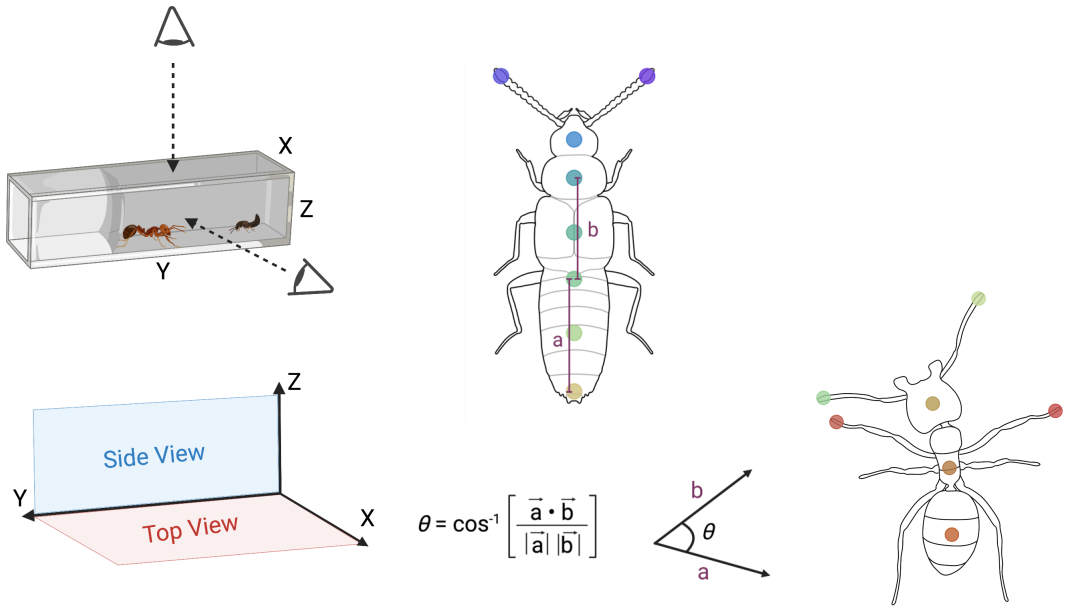

Figure 5.11 Schematic of cuvette arena. The arena pictured in the top left is coated on all sides with 2% agarose. A *Dalotia* and an ant are placed inside, and top and side view angles are recorded to capture a 3D image. These videos are analyzed using deeplabcut, which gives the body positions of the beetle as shown in the top center as well as the pose and position of the ant. The pose tracking from deeplabcut can be used to measure the body angle of *Dalotia* through the video.

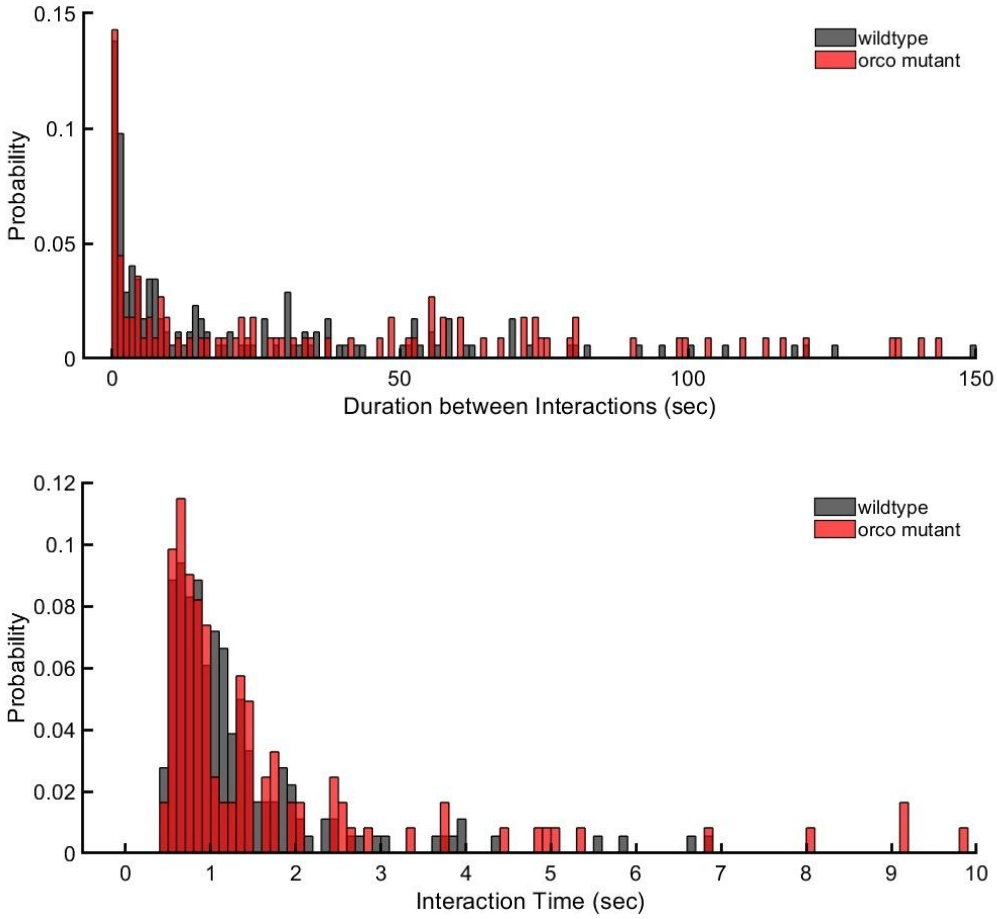

Figure 5.12 Distribution of interactions in cuvette arena. The top histogram shows the duration of time between interactions for both wild type and Orco-/- *Dalotia.* The bottom histogram shows the duration of interactions for both wildtype and Orco-/- *Dalotia.*

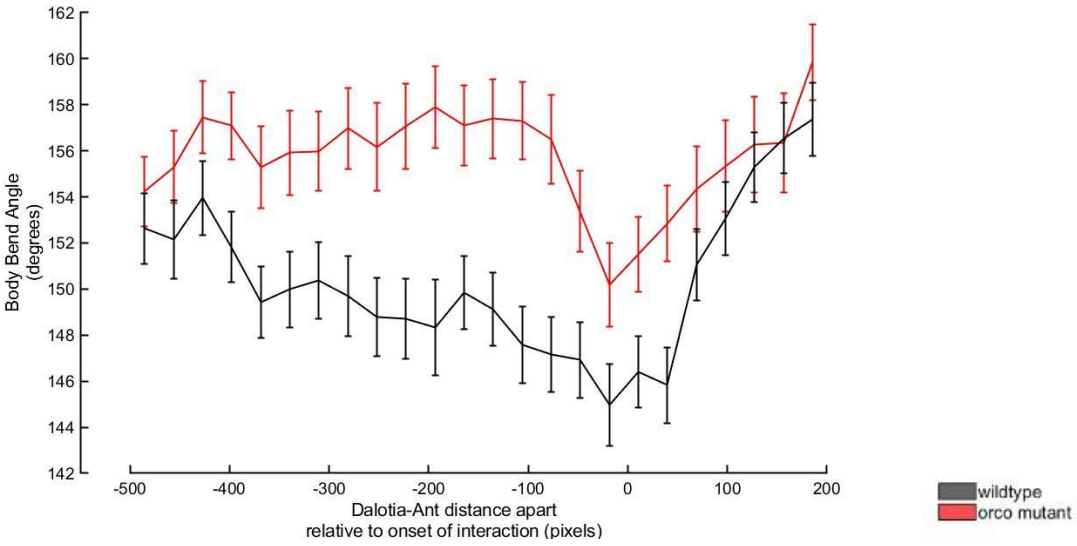

Figure 5.13 Body angle of *Dalotia* relative to distance apart before interaction with an ant. Wild type *Dalotia* (BLACK) and Orco -/- *Daloita* (RED) both start and end with similar body angles. At time 0, which is the time of interaction, both beetles flex. At the point of interaction and the time preceding, wild type *Dalotia* has a smaller angle indicating more flexion.

5.5 Effect of Orco -/- mutation on interactions with ants in a tethered beetle on the ball setup

Beetle on the ball experiments were also performed with Jessleen Kanwall. When placed on a ball setup, *Dalotia* will continuously walk with an unflexed abdomen (Figure 5.14). When presented with an aversive stimulus such as an ant, *Dalotia* will flex its abdomen in order to defend itself using its tergal gland (Figure 5.15). When presented with a neutral stimulus such as a glob of cured UV glue, *Dalotia* will also flex its abdomen, but typically not as intensely as it does when presented with an ant (Figures 5.16). Larger sample sizes are still needed for this experiment, but preliminary data shows no difference in the level of flexion toward either the neutral stimulus (UV glue) or the aversive stimulus (ant) between wild type and Orco -/- *Dalotia* (Figure 5.17 and Figure 5.18).

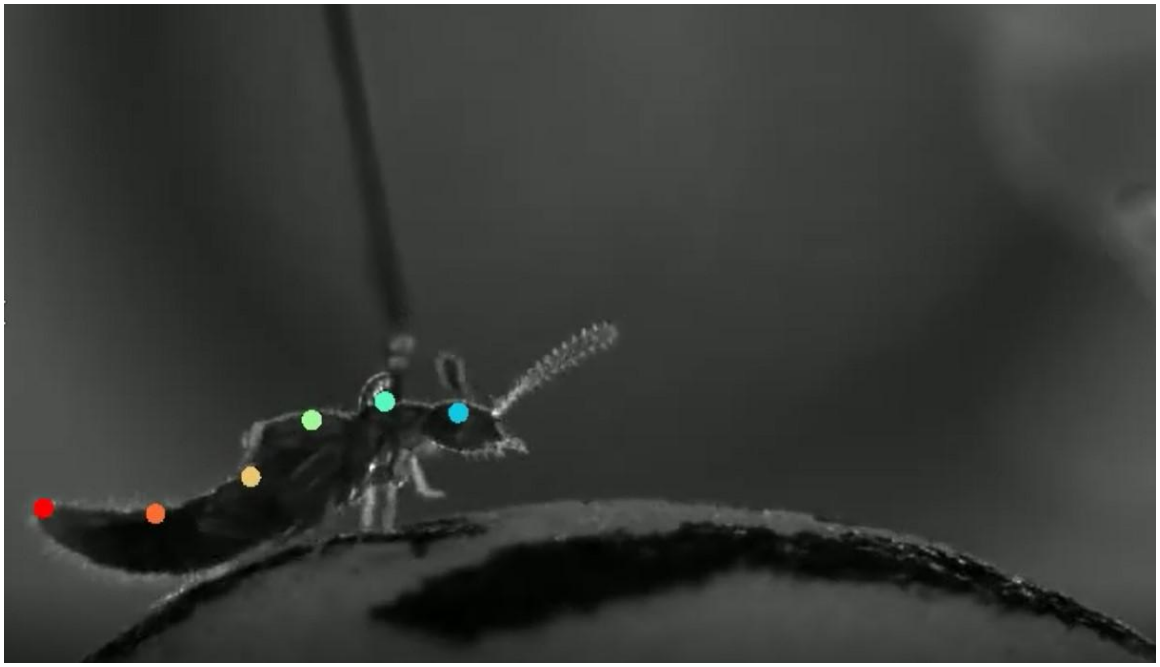

Figure 5.14 *Dalotia* tethered walking on a ball in the absence of stimulus. After being acclimated, *Dalotia* will walk continuously on a ball. The angle between its body and its abdomen will stay large, just under 180 degrees. Deeplabcut tracking allows us to quantify the angle of flexion during the video, and is visualized by the colored dots along the body.

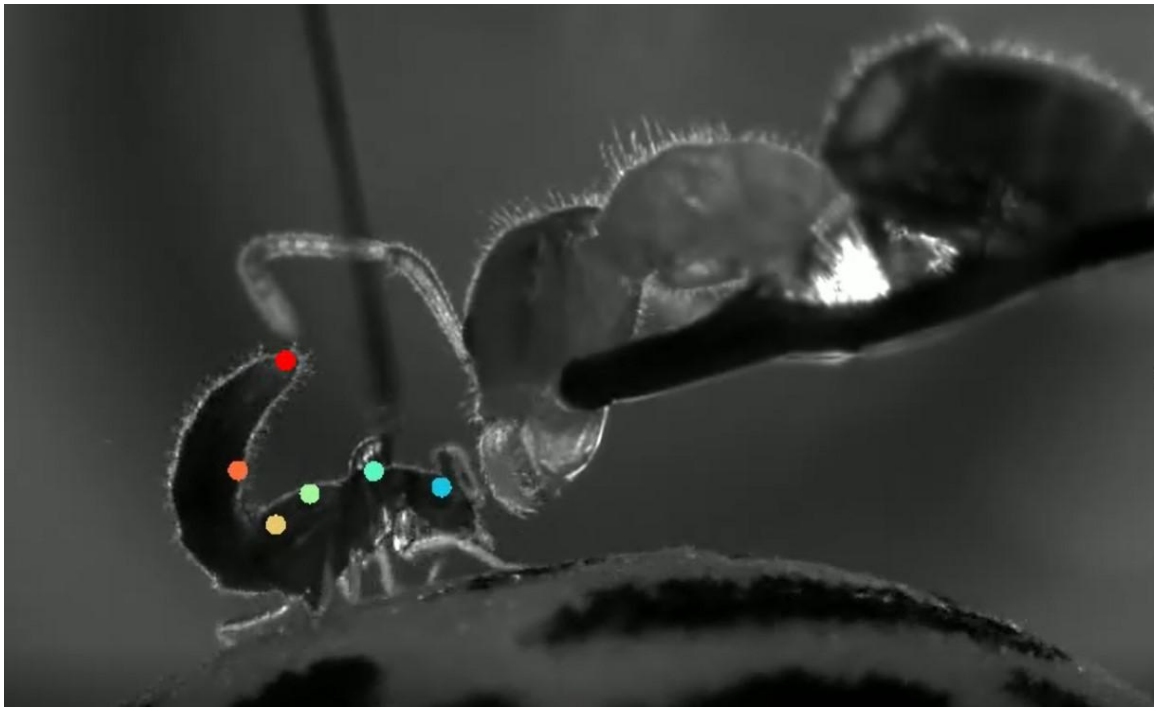

Figure 5.15 *Dalotia* tethered walking on a ball in the presence of an ant. An acclimated *Dalotia* will flex its abdomen to defend itself from a threat, in this case an ant, while walking on a ball. Deeplabcut tracking allows us to quantify the angle of flexion during the video, and is visualized by the colored dots along the body.

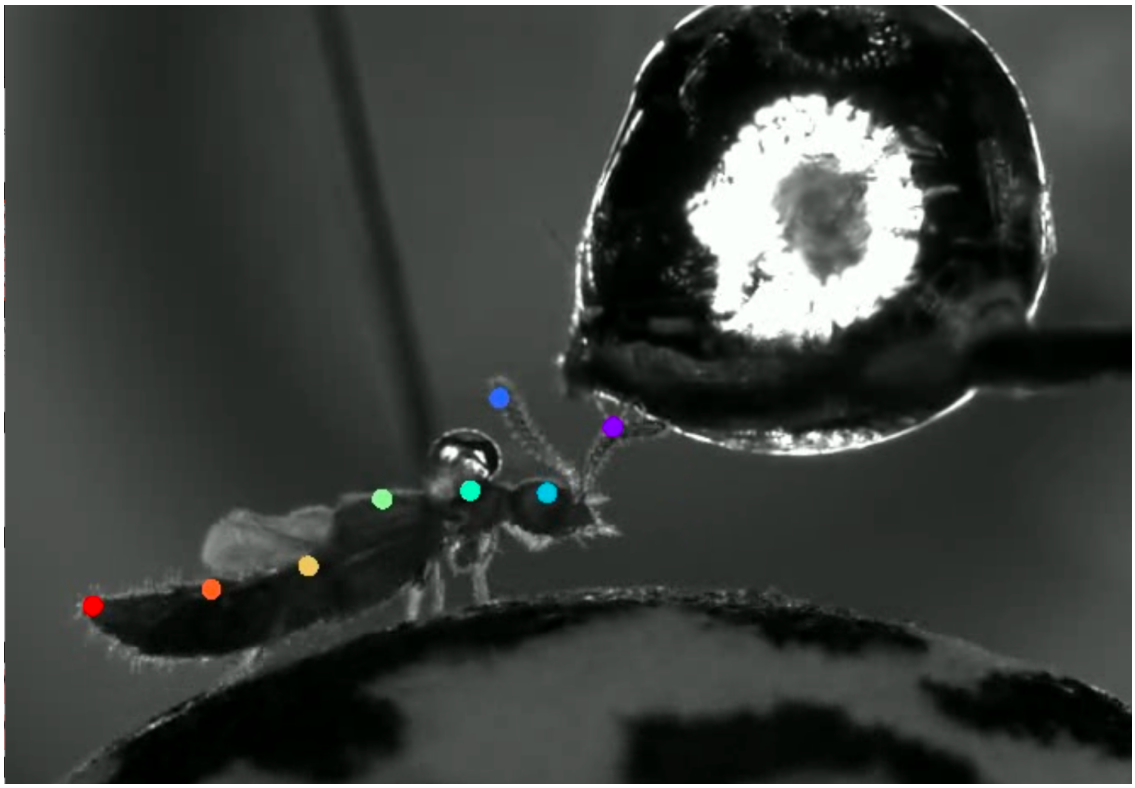

Figure 5.16 *Dalotia* tethered walking on a ball in the presence of a neutral stimulus. After being acclimated, *Dalotia* will walk continuously on a ball. The angle between its body and its abdomen will stay large, just under 180 degrees. When presented with a neutral stimulus, a glob of cured UV glue in this case, *Dalotia* will occasionally flex but typically continue walking in a non-flexed position. Deeplabcut tracking allows us to quantify the angle of flexion during the video, and is visualized by the colored dots along the body.

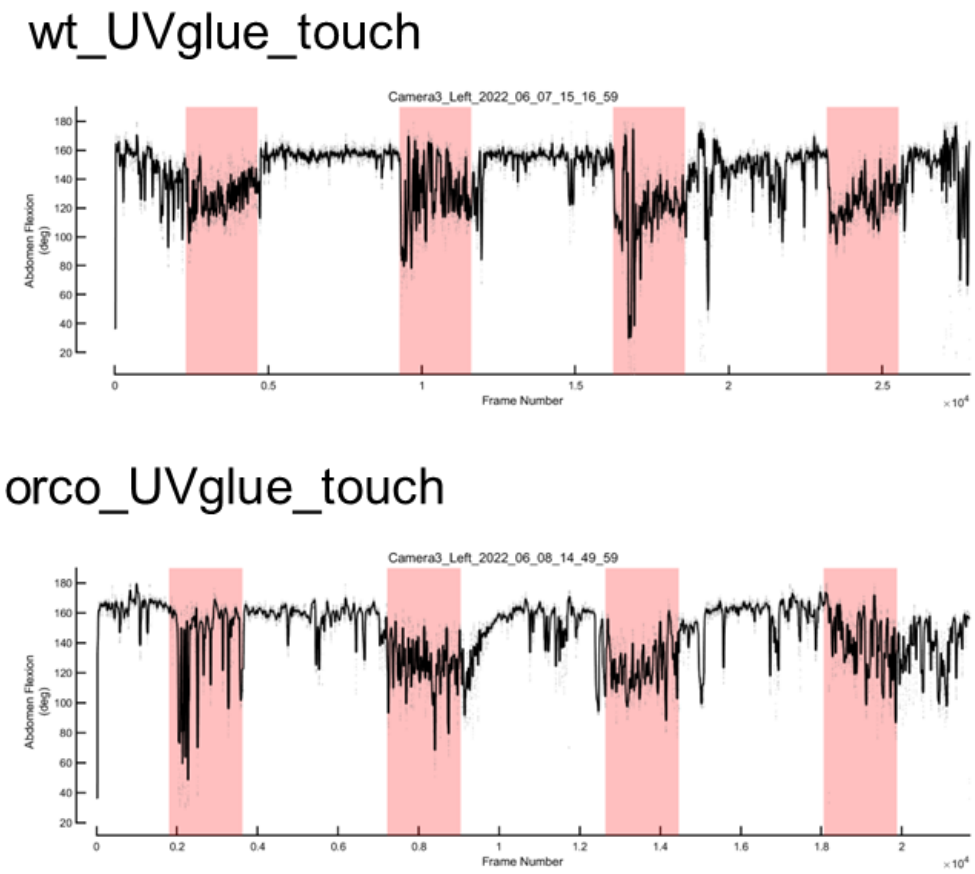

Figure 5.17 Comparison of abdominal flexion in response to cured UV glue of wild type and orco -/- *Dalotia* while tethered walking on a ball. Stimulus presentation time is marked in red. Lower abdomen flexion degrees indicate more flexing.

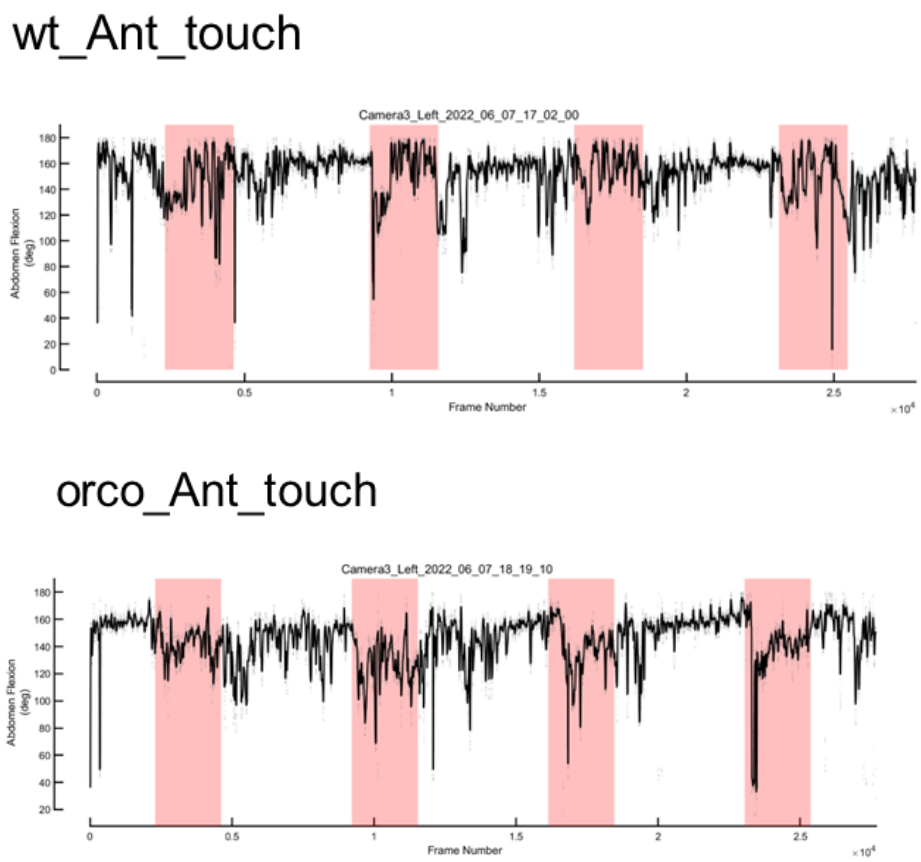

Figure 5.18 Comparison of abdominal flexion in response to an ant of wild type and orco -/- *Dalotia* while tethered walking on a ball. Stimulus presentation time is marked in red. Lower abdomen flexion degrees indicate more flexing.

Conclusion

As shown by the RNA seq expression map, *Dalotia's* antennae, palps, tarsi, and abdomen are all appendages that likely transduce chemosensory information through the use of olfactory, gustatory, and ionotropic receptors. Additionally, each tissue type is likely specialized for chemoreception of specific compounds by using the upregulated chemoreceptors shown in the map.

Next, Orco -/- *Dalotia's* behavior suggests that Orco, the olfactory receptor co-receptor, is required for proper functioning of olfactory receptors. The lack of olfaction ability is caused by the intended mutation and not an off target effect. Additionally, 3D reconstructions of the antennal lobe in mutant Orco -/- *Dalotia* show that antennal lobe development is not affected by the gene knockout. This suggests the advent or loss of Orco as a developmental transcription factor for the antennal lobe happened before Coleoptera and Diptera diverged.

Lastly, ORs may play a smaller role in *Dalotia's* defensive behavior than previously hypothesized. While flexing is reduced at a distance from ants, Orco -/- beetles are still capable of deploying the gland and fleeing from ants upon contact. This behavior is reproducible while tethered on a ball, where preliminary data shows there is no significant difference in the level of flexion between wild type and Orco -/- beetles. This result suggests *Dalotia* likely uses other sensory cues in order to perceive threats and execute defensive behaviors. These are likely mechanosensory, but could also involve

GRs and IRs. Visual cues are not necessary as the experiments were all performed in the dark.

Discussion

The RNA Seq map of chemoreceptors across the body is a useful starting point for a variety of downstream experiments. By using the CRISPR Cas9 technique detailed in Chapter 4, highly expressed chemoreceptors in specific tissues can be knocked out to determine their function. Additionally both of the Orco -/- lines are stable and can still be used for a plethora of experiments to tease apart the importance of ORs in other behaviors. These include interspecies interactions with prey, neutral insects, parasitic insects, conspecifics, as well as interspecies interactions with conspecifics.

Given the importance of ORs in mediating medium and long range olfactory cues in other insects as well as their role in mediating intraspecies behaviors in ants, we hypothesized that they would play a role in *Dalotia's* defensive interspecies interaction with ants. Data analyzed to this point however, suggests *Dalotia* is fully capable of recognizing an ant as a threat and executing a defensive flexing and fleeing response in the absence of functional ORs. Data is preliminary at this point, and further permutations of ant delivery may reveal a difference in defensive behavior in the absence of OR function. Additionally, data from the cuvette arena indicates flexing is reduced at a distance from the ant. This was not replicated on the ball, but with the correct setup flowing odor towards *Dalotia* may be reproducible. If in fact wild type *Dalotia* flexes more than Orco

-/- *Dalotia* at a distance from a threat, this would suggest *Dalotia* uses olfactory cues to detect the presence of a predator and pre-emptively raises its abdomen in preparation of a defensive behavior.

Another possible explanation is that after *Dalotia* is attacked the first time by an ant, it learns the olfactory profile associated with the attacker, and will preemptively flex any time it encounters the odor. This would suggest that while ORs are not necessary for the initial response, they are important for the learning of threats in order to execute quicker defensive responses in subsequent interactions.

Methods

Tissue-type specific RNA Seq (Smartseq)

Male *Dalotia* were dissected using a light dissecting microscope. The NEBNext Single Cell/Low INput RNA Library Prep Kit for Ilumina was used, and following dissection, tissue was immediately placed in the provided cell lysis buffer and flash frozen. Tissue was kept at -80°C until processing. The NEBNext kit was used for converting dissected tissues into cDNA libraries. cDNA amplification was performed for 12 cycles and final library amplification was performed for 8 cycles. The quality and concentration of the resulting libraries were assessed using the Qubit High Sensitivity dsDNA kit (Thermo Scientific) and Agilent Bioanalyzer High Sensitivity DNA assay. Libraries were sequenced on an Illumina HiSeq2500 platform (single-end with read lengths of 50 nt) with 20-25 million reads per library. Illumina sequencing reads were pseudo aligned to the bulk transcriptome and quantified (100 bootstrap samples) with kallisto (Bray et al., 2016).

Antennal lobe imaging

Male *Dalotia* brains were dissected in cold PBS and fixed in 4% PFA. Samples were left overnight with primary antibody 3C11 (anti SYNORF1) obtained from the Developmental Studies Hybridoma Bank. Imaging was performed on a Zeiss LSM 800. 3D reconstructions of the antennal lobe were performed manually using imaris software.

Odor preference arena

4 *Daloita* were cooled down at 4°C for 5 minutes. During the cooling, paraffin oils (with or without diluted odor) were placed in the appropriate odor chamber. After cooling, beetles were placed in the center of the arena. They were allowed to acclimate for 5 minutes, and then recorded for 3 minutes. Videos were recorded in the dark using an infrared camera. Videos were analyzed using a background subtraction python script written by Julian Wagner in the lab. The pixel intensity difference between the left and right sides was divided over the total pixel intensity of the image to generate a repulsion index.

Cuvette arena

Dalotia is single-housed on a hydrostone dish with no food for 24 hours. It is then placed at 4°C for 5 minutes, then moved into the cuvette. It is given 10 minutes to acclimate during which a *Liometopum occidentale* ant is removed from its colony and cooled at 4°C for 5 minutes. When cooling and acclimating is done, the ant is added to the cuvette and the recording is started. Videos are recorded in the dark using an infrared camera. Deeplabcut is used to analyze the body pose and position of both the ant and the beetle during the interaction (Mathis et al., 2018).

Beetle on the ball arena

Dalotia is cooled at 4°C for 5 minutes, then placed on a 4°C peltier with a custom mount. This is used to attach a tether to its pronotum using UV glue. The beetle is placed on the ball and allowed to acclimate for 30 minutes. A *Liometopum occidentale* ant is removed from its colony and anesthetized using CO_2. Under a dissection microscope, its limbs and antennae are removed, and its mandibles are glued together using UV glue. The ant is then attached to a tether using UV glue. During the behavior, an actuator is used to move the ant close enough for *Dalotia* to antennate it but not so close it pushes against its head. Videos are recorded in the dark using an infrared camera. Pose of *Dalotia* is analyzed using deeplabcut.

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Appendix A

DALOTIA CORIARIA HUSBANDRY PROTOCOL: EGG INCUBATION, RAISING LARVAE TO ADULTHOOD, CROSSES, AND EXPANDING/MAINTAINING MUTANT LINES

Materials:

- Watercolor paint brush
- Wash bottle filled with water
- Aspirator
- Petri dish (with absorbent pad): VWR CAT# 25388-640
    - All petri dishes referred to in this protocol include the absorbent pad.
- Container store tellfresh boxes (Plastic containers):
    - 175 mL (Tellfresh Baby): SKU:10062524
    - 250 mL: SKU #10070349
    - 1.125 L: SKU #10014831
    - 1.75 L: SKU #10014832
- Hydro-stone dish:
    - Remove filter paper from petri dish or batch of petri dishes.
    - Prepare a mixture of hydro-stone.
        - 1 part water, 3 parts hydro-stone by weight.

    - Use a 3 mL plastic transfer pipette to pour a thin layer (1-3 mm) of hydro-stone on the bottom of each dish.
    - Leave dishes uncovered for a minimum of 4 days to fully cure and set the hydro-stone.
        - Uncured hydro-stone can be toxic to insects.

- Pupation substrate:
    - Autoclave a box of coconut fiber substrate to remove mites, mold, and other contaminants.
    - Fill a 1.75 Liter tellfresh box half full by volume with autoclaved substrate.
    - Spray water and shake/mix until substrate is a darker color from the moisture, but no condensation is present.
        - Substrate should feel moist to the touch but when squeezed in the hand should release little to know condensation.
- Frozen drosophila:
    - Aggregate drosophila into a culture bottle with no agar base (just a plastic culture bottle).
    - Place bottle in -20°C freezer.
- *Dalotia* powdered food:
    - Add oats and chicken feed in a 1:1 mixture to coffee grinder.
    - Grind until a fine powder.
    - Store in -20°C freezer.

Egg incubation:

- Use a wash bottle to add water to the absorbent pad on a new petri dish.
    - The pad should darken in color uniformly from the water.
    - Make sure the pad does not curl or fold, and is flush with the bottom of the dish.
    - Applying pressure with a finger on the pad should not lead to a release of condensation.
- Using a wet brush, place egg on absorbent pad.
- If eggs were laid within less than a day of each other, multiple eggs can be placed in the same dish.
- Place dish in 26°C degree incubator.
    - Make sure water tray in the incubator is full.
- Larvae will hatch 3 days after eggs were laid.
- Larvae must be removed immediately after hatching or they will eat other eggs and other larvae.
    - Larvae can be moved gently using a wet brush.

Raising larvae to adults:

- Use a wash bottle to add water to a hydro-stone dish.
    - The hydro-stone should uniformly darken in color due to the moisture.
    - The hydro-stone should feel moist to the touch, but there should be no condensation anywhere inside the dish.

- Add 2-3 frozen drosophila to the dish.
- Using a wet brush, gently place the larvae inside the dish.
- Leave the dish at room temperature.
    - Larvae will grow faster at 26°C, but condensation and mold will reduce survival.
- Remove drosophila and replace with fresh frozen drosophila every day.
- When changing food, check whether there is still moisture in the hydro-stone. If it is dry, add more water as described in the first step of this section.
- After 5-8 days larvae will reach 3rd instar.
- Check larvae daily to see when they turn whiter in color, have rounder segments, and have a transparent gut. At this point they no longer need food and are ready for pupation.
- Prepare a pupation box by adding ≈ 25mL of pupation substrate to a 175 mL tellfresh container.

- Using a brush, gently move pre-pupal larva into pupation box.

- Place pupation box in climate controlled beetle room at 26°C.
- Every 2-3 days open pupation box lid and gently shake box to aerate soil and prevent mold formation.
- After 6-9 days pupa should eclose as adult.
- To find adult, pour substrate into a larger plastic container and gently shake to spread it thinly.
- The beetle should be easily seen walking around and can be collected using an aspirator.

***Dalotia* crosses:**

- Use a wash bottle to add water to the absorbent pad on a new petri dish.
    - The pad should darken in color uniformly from the water.
    - Make sure the pad does not curl or fold, and is flush with the bottom of the dish.
    - Applying pressure with a finger on the pad should not lead to a release of condensation.
- Add 2-3 frozen drosophila to the dish.
- Add 1 male *Dalotia* with 1-3 virgin female *Dalotias* in the dish.
- Place dish in 26°C degree incubator.
    - Make sure water tray in the incubator is full.
- Remove drosophila and replace with fresh frozen drosophila every day.

- Once a day check the plate under a dissection microscope. Eggs will be laid against the side of the dish.
- Using a wet brush, gently remove the eggs and incubate as detailed in the egg incubation portion of this protocol.

Expanding and maintaining mutant line:

- This requires at least 1 homozygous mutant male and 1 homozygous mutant virgin female.
- For 1 male and 1-2 mutant females:
    - Add ≈ 50mL of pupation substrate to a 250 mL tellfresh box.

- For 1- 4 males and 3-8 mutant females:
    - Add ≈ 150mL of pupation substrate to a 1.125 Liter tellfresh box.

- Sprinkle a small pinch of powdered food.
    - Too much food will mold. The volume of the pinch should not be more than the volume of the number of beetles in the box.
        - For example, if there are 5 beetles in the box, a volume of powder approximate to the volume of 5 beetles is appropriate.
- Toss substrate by tapping box on a counter until clumps of powdered food are not visible.
- Repeat food sprinkling and mixing every 2 -3 days.
    - Monday, Wednesday, and Friday is appropriate.
- If substrate appears dry while feeding, use a wash bottle to add water and toss to mix the water as is done with the powdered food.
- After 3-4 weeks the number of adult beetles in the box should have at least doubled and there should be a plethora of larvae in the substrate as well.
    - Colonies in 250 mL boxes can be moved to 1.125 L boxes with ≈ 150mL of substrate.
    - Colonies in 1.125 Liter Boxes can be moved to 1.75 L boxes with ≈ 300mL of substrate.
- Colonies can be maintained in 1.75 L boxes for 2-3 months. After this amount of time, beetles must be removed using an aspirator and placed in a new 1.75 L box with freshly autoclaved pupation substrate to avoid mite proliferation.

- Colonies can also be expanded by removing 15-30 beetles from original colony and starting a new 1.75L box.

Appendix B

DALOTIA CORIARIA EGG COLLECTING AND MICROINJECTION PROTOCOL

Materials:

- Watercolor paint brush
- Wash bottle filled with water
- Aspirator
- 40 uM cell strainer: Fisherbrand catalog # 22-363-547
- Microscope slide
- Double sided tape
- Tegaderm
- Petri dish (with absorbent pad): VWR CAT# 25388-640
    - All petri dishes referred to in this protocol include the absorbent pad.
- Frozen drosophila:
    - Aggregate drosophila into a culture bottle with no agar base (just a plastic culture bottle).
    - Place bottle in -20°C freezer.

Collecting beetles:

- Find a stock beetle box or set of beetle boxes with large amounts of beetles and larvae.

    - **o** Make sure box was fed generously in the 48 hours previous to collecting beetles as well fed beetles lay more eggs.
- Add substrate from the box to the top layer of a sieve, shake, and aspirate beetles from the bottom layer.
    - **o** Try to aspirate as little substrate as possible.
    - **o** If too much substrate is collected, pour aspirator contents into a plastic bin and re-aspirate beetles avoiding the substrate.
- Repeat until enough beetles are collected.

Dish setup:

- Typically performed on Monday to allow for egg collections on Tuesday, Wednesday, Thursday, and Friday.
- Place 75-100 petri dishes on bench and remove their lids.
    - o This number can scale linearly with the amount of people collecting eggs. For example, if two people are collecting, 150-200 plates can be set up.
- Use a wash bottle to add water to the absorbent pad on each petri dish.
    - o The pad should darken in color uniformly from the water.
    - o Make sure the pad does not curl or fold, and is flush with the bottom of the dish.
    - o Applying pressure with a finger on the pad should not lead to a release of condensation.
- Add 2-4 frozen drosophila to each dish.

- Using a brush, move 3-4 beetles from the aspirator bottle to a dish, place the lid back on, and repeat for each dish.
    - If static is causing frozen drosophila to stick to the lid, rub a wet Kimwipe on the inside top of the lid to remove charge. Do this to lids as needed.
- Stack petri dishes and place in an appropriately sized plastic bin.
- Place plastic bin in 26°C degree incubator with the lights off.
    - Make sure the water tray in the incubator is full.
- Leave the beetles in the incubator overnight.

Dish with Dalotia and frozen Drosophila.

Egg collecting (pre-collection):

- Move plastic bin containing petri dishes to a dissection scope.
- For each dish perform the steps below:
    - Using a wet brush, remove all drosophila.
    - Add 2-3 frozen drosophila

- Using a wet brush, remove all beetle eggs stuck to the side of the petri dish.

- When overnight eggs have been removed and all food has been replaced, place bin back in the incubator and start a 2 hour timer.
  - Up to 3 hours is OK.

Dalotia next to egg laid against side of petri dish.

Egg slide preparation:

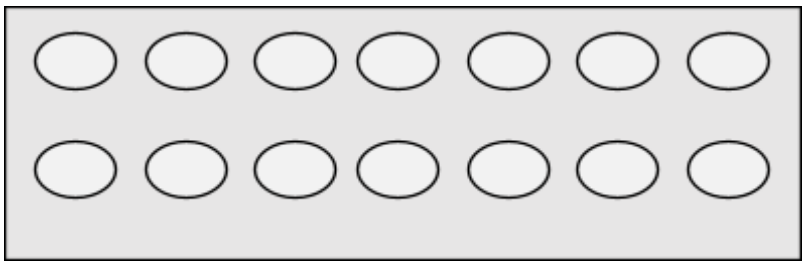
- If working alone, prepare before starting egg collection.
- Place a strip of double sided tape on a microscope slide.
- Place a strip of tegaderm on the double sided tape.

- Sticky tegaderm side should be facing up, with the non-sticky side facing down.

Egg collecting (collection 2 hours after pre-collection):

- Fold a Kimwipe into a small square, place on bench and saturate with water.
- Clean brush with 70% ethanol and rinse well.
- Clean cell strainer with 70% ethanol and rinse well. Place on top of wet Kimwipe square.
- Move plastic bin containing petri dishes to a dissection scope.
- For each dish perform the steps below:
  - Using the cleaned wet brush, gently move eggs from the side of the petri dish into the cell strainer.
  - Eggs should be malleable, wet, and soft with no clear embryo visible inside.
  - If an egg is abnormally firm and/or shows an embryo discard.
    - This is an older egg that was missed during the pre-collection.

- When all dishes have been checked, use a wet brush to align the collected eggs on the tegaderm slide.
    - Align the eggs following the pattern and orientation shown below:
    - Eggs are ready for micro-injection.



Appendix C

PROTOCOL FOR GENERATING PIGGYBAC-MEDIATED INSERTIONS IN *DALOTIA CORIARIA*

This protocol is a modified version of Ben Matthews' Aedes aegypti PiggyBac protocol. The protocol can be found here: http://mosquitolab.zoology.ubc.ca/assets/pdf/Aedes_pBac.pdf

Materials:

HiScribe™ T7 ARCA mRNA Kit (with tailing) - New England Biolabs (NEB) item # E2060S Protocol: https://www.neb.com/protocols/2015/03/25/standard-mrna-synthesis-e2060

ZymoPURE Plasmid Miniprep Kit Cat# D4211

Protocol:https://files.zymoresearch.com/protocols/_d4208t_d4209_d4210_d4211_d4212_zymopure_plasmid_miniprep.pdf

Monarch® PCR & DNA Cleanup Kit Cat # T1030L

Protocol:

https://www.neb.com/-/media/nebus/files/protocols/t1030_quick_protocol_card_monarch_pcrdna_cleanup.pdf?rev=a562d8f1f21741b0ac6d59ea9020cef3&hash=E4845F80E8B2BE925A09DAD6562A41EC

Donor plasmid:

- The donor (integration) plasmid contains the desired DNA insert flanked by PiggyBac sequence arms.
- This protocol requires stock donor plasmid to be clean and at a concentration of at least 1800 ng/uL. A nanodrop is sufficient to spec concentration, but Qbit can also be used.
- Donor plasmid can be made from either a midiprep or multiple minipreps pooled together and then concentrated using a Speedvac.
- To ensure donor plasmid is clean, use endo-toxin remover during the plasmid mini/midi prep. When midi/mini prep is complete, centrifuge sample at max speed for 10 minutes and take the top 90% of the sample as clean stock, discarding the rest.

Generating transposase mRNA by in vitro transcription:

- PCR: To generate DNA template for IVT, perform PCR from a plasmid containing transposase using the primers listed below:

- pBac forward primer (bold/italic represents T7 initiation sequence and necessary linkers):

  GAAACTAATACGACTCACTATAGGGAGAGCCGCCACATGGGT AGTTCTTTAGACGATG
- pBac reverse primer (hyperactive PBac, obtained from Martin Beye): TCAGAAACAACTTTGGCACATATCA

- Perform a PCR reaction (scaled to 100µL), using ~1 ng plasmid as template. I use Hotstart taq polymerase with an annealing temperature of 56°C and extension time of 40 seconds.

All following steps should be performed with nuclease free water/tips/tubes/pipettes!

- Cleanup PCR product using Monarch PCR and DNA cleanup kit, or equivalent.
- Check concentration with a Nanodrop or Qubit, ideally >250ng/µL.
- Perform standard mRNA Synthesis using the NEB Hiscribe T7 ARCA with tailing kit. Protocol is copied below.
- Thaw the necessary kit components, mix and pulse-spin in microfuge to collect solutions to the bottoms of tubes.
- Assemble the reaction at room temperature in the following order:

Nuclease-free water	To 20 µl	
2X ARCA/NTP Mix	10 µl	1 mM GTP, 4 mM ARCA, 1.25 mM CTP, 1.25 mM UTP, >1.25 mM ATP final
Template DNA	X µl	1 µg
T7 RNA Polymerase Mix	2 µl	
Total reaction volume	20 µl	

- Mix thoroughly and pulse-spin in a microfuge. Incubate at 37°C for 30 minutes.
- Do not heat the reaction. Do not purify the RNA. Proceed to DNase treatment and Poly(A) tailing steps. Or you can store the reaction at –20°C for a few days.
- Reaction time depends on template amount, quality and RNA transcript length. For reactions with transcripts longer than 0.5 kb, 30 min incubation should give you the maximum yield.
- For reactions with short RNA transcripts (< 0.5 kb), incubation time of 1 hour or longer is necessary to achieve good yield. It is safe to incubate the reaction for 16 hours (overnight).

- For reaction times of 60 minutes or less, a water bath or heating block may be used; for reaction times longer than 60 minutes, please use a dry air incubator or PCR machine.
- DNase treatment to remove template DNA. Add 2 µl of DNase I, mix well and incubate at 37°C for 15 minutes.
- DNase treatment is optional if the template does not interfere with downstream experiment. If left untreated, DNA template containing eukaryotic promoters may produce a background in mRNA transfection experiments.
- Save 1 µl for gel analysis if desired. Do not heat the reaction or purify the RNA. Proceed to tailing reaction.
- Poly(A) tailing. Set up the tailing reaction as below. The unpurified IVT reaction contains enough ATP, no extra ATP is necessary for the tailing reaction. Standard tailing reaction volume is 50 µl. Tail length is slightly longer in a 100 µl reaction volume for some transcripts.

H2O	to 20 µl	65 µl
IVT reaction	20 µl	20 µl
10X Poly(A) Polymerase Reaction Buffer	5 µl	10 µl

Poly(A) Polymerase	5 µl	5 µl
Total reaction volume	50 µl	100 µl

- A 30 min tailing reaction will give 150 nt or longer poly(A) tail for majority of mRNAs. Due to the nature of the tailing reaction, tail length will vary depending on RNA sequence, structure, yield, length, etc. For very short RNA (< 300 nt), tailing time can be extended to 1 hour to achieve sufficient tailing.
- The 3′ end of RNA must be exposed for efficient tailing. If the 3′ end is buried inside the RNA structure, it will not be available for tailing. Redesigning the mRNA 3′ end sequence may resolve the problem.
- Proceed with mRNA purification.

LiCl precipitation mRNA purification is modified slightly as listed below (modifications are underlined):

1. To the 50 µl tailing reaction, add 25 µl LiCl solution and mix well.
2. Incubate at –20°C for 30 minutes
3. Centrifuge at 4°C for 15 minutes at top speed to pellet the RNA.
4. Remove the supernatant carefully.
5. Rinse the pellet by adding 500 µl of cold 70% ethanol and centrifuge at 4°C for 10 minutes.

6. Remove the ethanol carefully. Spin the tube briefly to bring down any liquid on the wall.
7. Remove residual liquid carefully using a sharp tip (e.g., loading tip).
8. Air dry the pellet and resuspend the mRNA in 25 µl of 0.1 mM EDTA or nuclease free water.
9. Heat the RNA at 65°C for 5-10 minutes to completely dissolve the RNA. Mix well.
10. Use a the nanodrop to spec concentration (Should be >1000ng/µL)
11. Proceed immediately to final injection mix preparation. Any leftover mRNA should be aliquoted and stored at -80°C.

Injection solution preparation:

- Prepare on ice.
- Final concentrations should be as follows:
    - Donor plasmid 400 ng /µL
    - mRNA transposase > 600 ng/µL

Donor plasmid	X µL	1600 ng
mRNA transposase	X µL	>2400 ng

Nuclease free injection dye	to 4 µL (Minimum 0.25 µL)	
Total volume	4 µL	

- Aliquot injection mix into 4µL aliquots in small nuclease-free tubes.
- Freeze at -80° C.
- Keep frozen until immediately before injection.
- Injection solution can also be assembled on ice immediately before injection from aliquoted mRNA transposase.

Appendix D

PROTOCOL FOR GENERATING CRISPR DELETIONS IN *DALOTIA CORIARIA*

Finding gene of interest in *Dalotia* genome and preparing a benchling file:

- Find homologous gene in *Drosophila* on https://www.uniprot.org/ .
    - Find amino acid sequence.
- BlastP the amino acid sequence against the *Dalotia* protein database on http://scepto.caltech.edu:4567/
- Pick the top candidate:
    - Example of how protein white is named in the *Dalotia* transcriptome: Dcor_evm.model.scaffold143_size401101.33 Protein_white
- Open *Dalotia* genome browser: http://bioinformatics.caltech.edu/jbrowse/index.html?data=Dalotia_coriaria%2Fdata&loc=hic_scaffold_18%3A47093..47774&tracks=DNA%2CDcor_assembly_v2_200326_genes&highlight=
- Find *Dalotia* protein candidate by searching for the name found above without the annotation.
    - For example: Dcor_evm.model.scaffold143_size401101.33
- Click on the gene in the genome browser.

- Under Attributes, there should be a box labeled “Region Sequence” with the full gene sequence in FASTA format.
- Create a new DNA sequence in benchling and paste the full gene sequence from the genome browser into benchling.
    - Sequence information for regions before and after the gene can be found in the full genome fasta file on the scepto server, and copied into the benchling file.
- The genome browser also has all exons labeled. Under subfeatures there will be boxes with Type: exon.
- Copy the exon sequence from the genome browser, and use the find feature in benchling to annotate each exon.
    - I usually start at the first one and label them exon1, exon2, exon3 etc.
- The benchling file with the gene of interest and all exon information is complete.

Designing CRISPR guides:

- Open the file containing the gene of interest with all exon information annotated.
- Click the CRISPR button in the vertical panel on the right side of the page, and click design and analyze guides.
    - Design type: single guide
    - Guide length: 20
    - Genome: Tcas3

        - At the time of writing this protocol, the *Dalotia* genome is not on NCBI. When it is on NCBI, import it into benchling and select it instead.
    - PAM: NGG(SpCas9,3' side)
- In the sequence map select the region from exon1 to the final relevant exon (usually no more than exon4 is needed).
- Return to the DESIGN CRISPR panel and create the region of interest clicking the green plus sign.
- Select all found sequences by checking the top left checkbox.
- The sequence map panel will now show the location of all possible guides in the region of interest.
- The objective is to find 4 guides:
    - Guides targeting exon 1 and exon 2 are preferred.
    - PAM sites of CGG and TGG are preferred.
    - The 5 nucleotides next to the PAM site are preferably around 60% GC.
    - Cut site is typically 3-4 nucleotides upstream of PAM site.
    - Targeting ag splice acceptor site and gt splice donor site is effective.
- When a guide candidate is found click on the guide to select the region. Right click on the selected region and click create annotation. Make sure the annotation direction matches the direction of the guide. Repeat this for all 4 guides.
- Open the IDT website (https://www.idtdna.com) and go to the CRISPR-Cas9 section under CRISPR genome editing.

- Under CRISPR-Cas9 gRNA, click ORDER IN TUBES.
- For each guide, copy the 20 base pair DNA sequence in a 5’ 3’ direction.
    - The PAM site should be at the 3’ end.
    - Do not copy the PAM site.
    - Select Alt-R CRISPR-Cas9 crRNA, 10nmol.
- The cart should contain 4 different crRNAs.
- If not in stock in the lab, add Alt-R CRISPR-Cas9 tracrRNA, 20nmol and Nuclease Free Duplex buffer to the order.

Preparing microinjection solution

Alt-R RNA preparation modified from the following protocol: https://sfvideo.blob.core.windows.net/sitefinity/docs/default-source/user-submitted-method/crispr-cas9-rnp-delivery-b-tryoni-a-choo.pdf?sfvrsn=bb30b07_9

- Prepare stock solutions of crRNA and tracrRNA.
    - Spin down RNA pellet.
    - Resuspend in Nuclease Free Duplex Buffer to 200 uM as described in the table below.
    - Store at -20

Normalized amount delivered (nmol)	Volume of resuspension buffer (uL)
2	10
5	25
10	50
20	100
100	500

- Mix crRNA and tracrRNA in equimolar concentration in a sterile microcentrifuge tube to create a final duplex concentration of 100uM:

Component	Amount	Final concentration
200 uM crRNA	2 uL	~1180 ng/uL
200 uM tracrRNA	2 uL	~2217.5 ng/uL
Final volume	4 uL	100 uM

- Heat at 95°C for 5 min
- Remove from heat and allow to cool to room temperature.

- The bound crRNA and tracrRNA will be referred to as gRNA.
- Mix 4 gRNAs (4 uL each, 16 uL total)
    - 25 uM each
- Aliquot into 2 uL microcentrifuge tubes and store at -80°C

Injection solution:

Prepare on ice:

Component	Amount	Final concentration
gRNA cocktail	2 uL	40 uM (10 uM each)
Cas9 protein (2000 ng/uL)	2 uL	800 ng/uL
Dye	1 uL	

Proceed to "*Dalotia* embryo microinjection protocol" for injections and "*Dalotia* husbandry protocol" for larval rearing. Protocols can be found on the Parker Lab Wiki.

Genotyping *Dalotia*

Genotyping is performed using the Phire Tissue Direct PCR Master Mix kit. Below are links for the kit and its protocol. The *Dalotia* specific protocol is summarized below.

Kit:

https://www.thermofisher.com/order/catalog/product/F170S

Protocol:

https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2FF-170_QR_TS_5.pdf

1. Place tissue in a PCR tube and freeze at -20. Tissue can consist of a whole adult beetle, a larval beetle, or a dissected tarsus.
    1. To dissect a tarsus, anesthetize beetle on a CO2 pad and use forceps to remove a single meso tarsus. Tarsus can be placed directly into a PCR tube.
    2. Beetles will still be able to survive and breed after this dissection.
2. When preparing tissue samples, always add two empty PCR tubes to serve as negative controls.
3. Place water, tips, pipettes, tubes, sharpie, and tube racks under the PCR hood and sterilize with UV for 15 minutes to eliminate contaminant DNA. Also prepare a 15% bleach solution in a spray bottle, and spray/clean any item before it enters the hood during the protocol (gloves, primer tubes, dilution buffer, master mix tube, PCR tubes etc).
4. Prepare a dilution buffer master mix with 20 uL of dilution buffer and 0.5 uL of DNARelease additive per sample. Remember to make enough for both blanks as well as your samples.
5. Add 20.5 uL of the above master mix to each sample tube and both blank tubes.
6. For all samples, but especially dissected tarsi, check under the dissecting scope that the tarsus is in the liquid. If it is stuck against the side of the tube flick the liquid around until you see the sample submerged or floating on the buffer.

7. Incubate at room temperature for 2-5 minutes.
8. Place in a PCR machine and heat 98 °C for 2 minutes.
9. Sample can be frozen at -20 °C if not used immediately.
10. Prepare a PCR master mix containing 2 uL of 10uM primer (forward and reverse mixed), 22 uL of PCR grade water, and 25 uL of 2X Phire tissue direct PCR master mix for each sample. Remember to make enough for both blank samples.
11. For each sample, add 1 uL of extracted DNA/dilution buffer and 49 uL of PCR master mix to a new PCR tube.
12. Briefly vortex and spin down samples.
13. Place in thermocycler using times and temperatures listed below:

Table 2. Cycling protocol

Cycle step	2-step		3-step		Cycles
	Temp.	Time	Temp.	Time	
Initial denaturation	98 °C	5 min	98 °C	5 min	1
Denaturation	98 °C	5 s	98 °C	5 s	40
Annealing (see 6.3)	-	-	X°C	5 s	
Extension (see 6.4)	72 °C	20 s ≤1 kb 20 s/kb >1 kb	72 °C	20 s ≤1 kb 20 s/kb >1 kb	
Final Extension	72 °C +4 °C	1 min hold	72 °C +4 °C	1 min hold	1

- Run 5 uL of each sample on a gel. Blank samples should show no band. If blank samples contain a band contamination occurred and protocol must be repeated.
- Samples containing multiple bands suggest large CRISPR Cas9 deletions. Smaller deletions will not be visible on the gel and require sequencing.
- For sequencing, the 45 uL of remaining PCR product can be sent to laragen along with the appropriate primer or primers.
- In homozygous mutants, sequencing will show either deletions or insertions with high confidence for each base pair. Only one trace should be visible in the chromatogram. For heterozygous mutants, insertions and deletions may or may not be visible, but two traces will be visible on the chromatogram and base pair confidence will be low in the mutated area. This is due to the presence of different alleles, wild type and mutant.

Generating a stable knockout line:

1. Genotyping all G0s is not strictly necessary. While it provides information on the efficiency of the injection, it will not inform which G0s have germline mutations. It is useful to genotype 4-8 G0 adults at first to make sure the CRISPR Cas9 is cutting as expected.
2. Once it is established the injections are working, cross all adult G0s. Male G0s must be crossed to virgin female *Dalotia* that were single housed during pupation.

They can be either WT or other G0s from the same batch. Female G0s can be crossed to any G0 or WT male. Make sure to document the lineage of each F1.

3. Raise F1s from each cross to adulthood and genotype. Once a mutant is found in an F1, sequence its siblings to try to find another beetle with the same mutation for the opposite sex.
    1. If heterozygous mutant siblings with the same mutation are found, they can be crossed back to each other. 25% of their offspring will be homozygous mutants which can be crossed and expanded into a stable line as explained in the husbandry protocol.
    2. If heterozygous mutant siblings with the same mutation are not found, outcross the mutant F1 to a wild type beetle. 50% of F2s generated will be heterozygotes and can be crossed to each other. 25% of their offspring will be homozygous mutants which can be crossed and expanded into a stable line as explained in the husbandry protocol.
2. While sequencing chromatograms are useful for identifying heterozygous beetles, they are not enough to determine if a beetle is a homozygous mutant. In order to validate a homozygote, primers need to be designed that will only amplify the sequence of a wild type beetle, but not a homozygous mutant. These can be made by targeting either the sequence deleted or the region disrupted by an insertion. Homozygous mutants will only show bands with the general primers but not the WT specific primers. If both bands show up, the beetle is either WT or heterozygous. These primers must be designed for each specific mutant line.