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The anti-wasp immune response across the genus Drosophila.

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Abstract

The anti-wasp immune response across the genus Drosophila.
By Balint Kacsoh

One of the most common parasites of Drosophila in nature are parasitic wasps, which lay their eggs in Drosophila larvae and pupae. *Drosophila melanogaster* mounts an immune response against wasp eggs and larvae termed melanotic encapsulation, whereby hemocytes form a multi-cellular, multi-layered capsule around the intruder before turning it black with melanin. We were interested in whether this melanotic encapsulation response is conserved across the genus Drosophila. Thus, we assayed fly immune mechanisms and immune success in a panel of 26 Drosophila host species using a diversity of parasitic wasp species. We found that different Drosophila species have unique hemocyte types not found in *D. melanogaster*, and that certain unique hemocyte lineages are involved in wasp egg encapsulation. Additionally, we took an in depth look at *D. melanogaster*, due to its poor performance against the wasp panel. Given that larvae of the fruitfly Drosophila melanogaster consume yeasts growing on rotting fruit and have evolved resistance to yeast fermentation products such as ethanol, we decided to test whether ethanol protects fruitflies from parasitoids. Here, we show that exposure to ethanol reduces wasp oviposition into fruitfly larvae. Furthermore, if infected, ethanol consumption by fruitfly larvae causes increased death of wasp larvae growing in the hemocoel and increased fly survival without need of the stereotypical anti-wasp immune response. This multi-faceted protection afforded to fly larvae by ethanol is significantly more effective against a generalist wasp than a wasp that specializes on *D. melanogaster*. Finally, we found that fly larvae seek out ethanol-containing food when infected, indicating they use alcohol as an anti-wasp medicine. We also examined *D. suzukii* and found that *D. suzukii* constitutively produces up to five times more hemocytes than *D. melanogaster*. Using a panel of 24 parasitoid wasp strains representing fifteen species, four families, and multiple virulence strategies, we found that D. suzukii was significantly more resistant to wasp parasitism than D. melanogaster. Thus, our data suggest that the relationship between hemocyte production and wasp resistance is general. Finally, we examined *Z. indianus* and found a novel hemocyte type to be involved in encapsulation ability against parasitoid wasps.

The anti-wasp immune response across the genus Drosophila.

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Introduction

A natural parasite of Drosophila includes Hymenopteran parasitoids, which infect a wide range of insects. These parasitoid wasps are known to regulate their hosts' populations. In natural populations of Drosophila melanogaster, up to 50% of fly larvae are found to be infected, demonstrating a form of population regulation with respect to flies. Drosophila-specific parasitoids include larval parasitoids. The females of these obligate, solitary parasitoid wasps seek out fruit fly larvae, use a modified ovipositor to pierce the cuticle, and inject their eggs and venom directly into the larval hemocoel. Drosophila-specific parasitoids also include pupal parasites. These parasitoids can either be endo- or ectoparasites. Endoparasites will lay their egg into the pupal case of a fruitfly, while ctoparasites will lay their eggs on the pupal case itself.

The outcome of any infection depends on the immune system of the fly and the venom of the wasp: *D. melanogaster* successfully defend themselves using an immune response called melanotic encapsulation. Here, fly plasmatocytes are activated and bind to the egg, which signals the lymph gland, the hematopoietic organ, to produce specialized hemocytes called lamellocytes. Lamellocytes form a multi-cellular, multi-layered capsule around the wasp eggs (and attached plasmatocytes). Hemocytes inside the capsule release phenoloxidase (PO) enzyme around the egg. The release of PO inside the capsule causes melanization of the wasp egg, which is thought to asphyxiate and kill it. This response against parasitoid wasps has been recognized as the canonical immune response, and has been said to span across most of the genus Drosophila. If venom from female wasps successfully suppresses this fly immune response, the wasp eggs hatch, and the wasp larvae begin feeding from the larval fly hemolymph. When the host flies pupate, the wasp larvae commence rapid consumption of fly tissues, which in turn kills the flies. The wasp larvae

then pupate within the Drosophila pupal case and eventually eclose as fully formed wasps, beginning the cycle again.

Very little is known about what determines a host immune range, which is essential if we are to predict the fate of novel interactions. Even less is known about the defenses of fruit flies outside of the model species *D. melanogaster*.. Phenotypic analysis elucidate whether these species have the common immune cell types, such as plasmatocytes and/or lamellocytes. Additionally, little is known about the effects of toxins or secondary metabolites on a host's ability to fight off infect. As some species of fly live in toxic environments, such as one having high levels of ethanol, evolution may have selected for individuals able to utilize the toxins against parasites.

Cellular encapsulation is a conserved mechanism with respect to innate immunity. The Drosophila immune system has been widely adopted as a model for studying innate immunity, and is similar in many respects to human innate immunity (i.e., Toll receptors in flies and Toll-like receptors in humans). Thus, knowledge of interactions between flies and their parasites could be relevant to both invertebrate and vertebrate biology. These experiments might provide the basis of strategies to control vectors spreading human disease and to protect pollinating bee populations. To date, little is known of the host ranges of parasitoid wasps that attack Drosophila. These studies were aimed at building a foundation to study the host-parasite relationship that has evolved between parasitic wasps and Drosophila species across evolutionary time. Additionally, our aim was also to elucidate the possible use of toxins against parasitoids. Our analysis leads to conclusions about the historic selective pressures on wasp infection strategies and fruitfly immune systems, and provide clues to how these species groups have co-evolved in the evolutionary arms race.

Chapter 1

The evolution of immune responses against parasitic wasps in the genus Drosophila.

Abstract

One of the most common pathogens of Drosophila in nature are parasitic wasps, which lay their eggs in Drosophila larvae and pupae. *Drosophila melanogaster* mounts an immune response against wasp eggs termed melanotic encapsulation, whereby hemocytes migrate towards an egg to form a multi-cellular, multi-layered capsule, and generate melanin and free radicals inside the capsule to kill the developing wasp. We were interested in whether this melanotic encapsulation response is conserved across the genus Drosophila, and also whether the same hemocyte cell types used by *D. melanogaster* are used by other Drosophila species. Thus, we assayed fly immune mechanisms and immune success in a panel of 26 Drosophila host species infected by a diversity of parasitic wasp strains representing 14 wasp species. We found that different Drosophila species have unique hemocyte types not found in *D. melanogaster*, and that certain of these hemocyte lineages are involved in wasp egg encapsulation. Furthermore, there appear to be at least three distinct mechanisms Drosophila species use to kill wasp parasites: melanotic encapsulation, encapsulation without melanization, and cell-free killing. Our study uncovers specialized immune strategies in different Drosophila clades and suggests the canonical melanotic encapsulation of *D. melanogaster* is likely not the ancestral Drosophila anti-wasp immune response. This newfound complexity in Drosophila anti-wasp immune responses opens up the Drosophila-wasp system as a model for host immune system evolution.

Introduction

Innate immunity is common to all organisms. Our understanding of innate immune responses has been largely driven by studies focused on microbial pathogens, and the host responses are typically categorized into two groups, humoral or cellular. Humoral immunity takes place in the "humors", or body fluids, where molecules such as antimicrobial peptides and reactive oxygen species kill pathogens cell-autonomously. Cellular immunity, instead, is unique to animals and is reliant on pathogen killing by mobile immune cells, usually via phagocytosis. But what kind of immune responses are mounted against pathogens that are too large (or too numerous) to succumb to these standard antimicrobial responses? In invertebrates, the main response against such pathogens is encapsulation, whereby hemocytes (blood cells) wall off invading pathogen(s) by forming a multi-cellular, multi-layered capsule around them ([1], [2], [3]). Vertebrates mount an analogous response mediated by macrophages that leads to the formation of granulomas.

Over the last 20 years the lab fruit fly *Drosophila melanogaster* has emerged as a leading model system for understanding innate immunity. For example, the discovery of the role of the Toll pathway in Drosophila microbial immunity led to the identification of homologous Toll-like receptor pathways in vertebrates. Likewise, most of our understanding of immunity in important insects, such as those that vector human diseases, pollinate crops, or consume crops, is derived from studies using *D. melanogaster*. The fruit fly has also been used to understand the encapsulation response. In particular, *D. melanogaster* mounts a vigorous encapsulation response against parasitic wasps, which lay their eggs in the hemocoel of fly larvae and pupae.

D. melanogaster produces four classes of circulating hemocytes. Plasmatocytes comprise approximately 95% of constitutively produced hemocytes. They act as sentinels of infection, are responsible for phagocytosis, and form the primary layer of cells during an encapsulation response. Podocytes are an activated version of plasmatocytes that are larger and have cytoplasmic extensions termed filopodia to sense the environment. Lamellocytes are large flattened cells derived from podocytes or directly from pro-hemocytes in the lymph gland (the hematopoietic organ), and are responsible for building multiple layers of cells on top of the plasmatocyte layer in developing capsules. Finally, crystal cells make up the other 5% of constitutively produced hemocytes and are responsible for carrying precursor molecules for generating melanin.

The canonical *D. melanogaster* encapsulation response against parasitic wasps is thought to involve several steps ([4]): The response begins when circulating plasmatocytes recognize the wasp egg as foreign, bind to it, and signal to activate the production of lamellocytes. The lamellocytes migrate towards, and then attach, spread, and consolidate around the plasmatocyte-covered wasp egg. Inner cells in the capsule then lyse, releasing reactive oxygen species and an impermeable layer of melanin, resulting in death of the wasp egg. This response is termed "melanotic" encapsulation because of the obvious blackened wasp eggs that can be observed through the fly cuticle after a successful immune response. However, wasps attempt to actively suppress host encapsulation responses by injecting venom along with their eggs into the host hemocoel, which often has deleterious affects on host hemocyte function. Hatched wasp larvae begin consuming fly hemolymph, but after the fly has pupated the wasps consume solid tissues and eventually kill the fly, eclosing from its pupal case ([5]; [6]). Genetic variation within and between fly species for resistance

against wasp infection, together with genetic variation within and between wasp species for virulence against flies, determines the outcome of any particular fly-wasp interaction.

For fly species closely related to *D. melanogaster* (e.g. in the melanogaster subgroup), fly resistance to parasitic wasp infection resistance has been linked to a very simple mechanism: flies that produce a greater number of hemocytes, either constitutively or after induction post-infection, are more resistant. For example, there was a significant correlation (r^2 = 0.90) between constitutive plasmatocyte load in the melanogaster subgroup and ability to melanotically encapsulate the eggs of the parasitic wasp *Asobara tabida* ([7]). It was also found that *D. melanogaster* strains artificially selected for resistance against *A. tabida* showed a significant increase in plasmatocyte numbers ([8]). Furthermore, *D. simulans*, which makes significantly more plasmatocytes than its sister species *D. melanogaster*, was significantly more resistant against the more immune-suppressive wasp *A. citri* ([9]). Finally, *D. melanogaster* mutants producing a wide range of hemocyte counts showed a significant correlation (r^2 = 0.45) between constitutive plasmatocyte numbers and encapsulation ability against the wasp *Leptopilina boulardi* ([10]). Finally, we recently showed that *D. suzukii*, a member of the melanogaster group but not the melanogaster subgroup, had significantly greater numbers of plasmatocytes than *D. melanogaster* and is also significantly more resistant to a wide diversity of wasps, although specialist wasps can overcome the high hemmocyte load ([11]). Altogether, this work suggests that high constitutive production of hemocytes is an effective and relatively simple mechanism by which hosts can evolve general resistance to one of their most common groups of parasites. However, this idea has not been tested across a wide diversity of flies in the genus Drosophila, Is the relationship between hemocyte load and

anti-wasp resistance general, and if so, what kind of selection pressures might prevent particular fly species from having high hemocyte loads?

Results from a handful of studies using diverse Drosophila species have indicated that the collection of hemocyte classes found in *D. melanogaster* may not be representative for the entire genus, and that some Drosophila species may use alternative mechanisms of resisting wasp infection distinct from the canonical *D. melanogaster* melanotic encapsulation response. Hemocyte classification schemes for invertebrates in general are varied and can include more than 10 hemocyte classes that are largely identified on the basis of morphology under different staining and preparation techniques ([12]). Therefore, it is often difficult to identify homologous hemocyte classes across invertebrate lineages. In genus Drosophila, hemocytes from only a small number of species outside of *D. melanogaster* have been studied in detail. *D. willistoni* produces plasmatocytes, podocytes, oenocytoids (that look superficially similar to lamellocytes), and crystalloid cells (i.e. crystal cells), like *D. melanogaster* ([13]). However, two other cell types were described: spheroidocytes are large cells characterized by numerous cytoplasmic spherical inclusion bodies while nematocytes are extremely fusiform cells (long and thin, but widened in the middle for the nucleus) containing numerous cytoplasmic granules. We recently showed that nematocytes are common to a number of Drosophila subgenera and that they are involved in encapsulation of wasp eggs in *Zaprionus indianus*, which despite its name is a member of the Drosophila genus (Kacsoh 2012b). It also has been shown that members of the obscura group as well as *D. paramelanica* do not form capsules around wasp eggs or inert foreign bodies. In the obscura group, a lack of lamellocytes is thought to be responsible for their lack of encapsulation ability and susceptibility to infection by the wasps *A. tabida* and *Leptopilina heterotoma* ([14]). In *D.*

paramelanica, however, *L. heterotoma* larvae were successfully killed, but although lamellocytes were present, they did not form a capsule around wasp eggs or larvae. Instead, analysis of the reactive oxygen species nitric oxide (NO) demonstrated that NO was significantly increased following the early stages of infection, and that injection of a nitric oxide inhibitor into fly larvae prior to infection increased parasite survival. These data suggested D. paramelanica uses an alternative method of wasp killing based on reactive oxygen species production around wasp larvae ([15], [16]). Finally, we recently showed that *D. melanogaster*, which has relatively low numbers of hemocytes and is generally susceptible to parasitic wasp infection, uses a behavioral immune response to fight off parasitic wasp infection. *D. melanogaster* lives in rotting fruits and is highly resistant to alcohol, and actively seeks out and consumes alcohol food when infected by wasps to kill wasp larvae living within its hemolymph ([17]). Altogether, these studies demonstrate that flies in the genus Drosophila do not rely solely on melanotic encapsulation to fight wasp infection, but can instead invest in other kinds of immune responses or in other, non-immunological aspects of fitness.

Parasitic wasps are exceedingly diverse and often act as keystone species in natural ecosystems ([18]; [19]). In nature, the threat to juvenile Drosophila posed by parasitic wasps is not a simple one, as most Drosophila species are infected by multiple wasp species, each with their own venom cocktails, and infection strategies. At least four families of parasitoid wasps are known to infect members of the genus Drosophila ([20]). These wasps vary in their host ranges from specialists of particular Drosophila species to generalist of the genus. Members of the families Braconidae and Figitidae are larval parasites while members of the families Diapriidae and Pteromalidae are pupal parasites. Pupal parasites of the genus Trichopria (Family Diapriidae) lay their eggs in the Drosophila hemocoel, like larval

parasites, but those of the genus Pachycrepoideus (Family Pteromalidae) lay their eggs in the space between the Drosophila pupal case and the pupa, and act as ectoparasites in the early stages of their life by sucking fluids from the pupa externally ([20]). A lack of pupal immunity against wasps may explain in part why pupal parasitoid wasps are thought to have more generalist host ranges than larval parasitoid wasps ([21], [22]). Parasitic wasps exert extremely strong selection pressures on juvenile Drosophila, as greater than 50% of fly larvae have been found to be infected in natural populations ([23], [24]).

Given previous work suggesting diverse mechanisms of anti-wasp immune defense across the genus Drosophila, we decided to undertake a comprehensive phylogenetic-based study of fly immunity using 26 Drosophila species and 27 wasp species and strains, for a total of 702 pair-wise fly-wasp interactions, in replicate. By assaying fly immune ability against a diverse panel of wasps, we were able to document how the anti-wasp immune responses has evolved in the Drosophila lineage, describing new fly hemocyte repertoires and wasp killing mechanisms in the process. This study was aimed at building the Drosophila-wasp system as a model for the evolution of host immunity and its co-evolution with pathogen virulence strategies.

Materials and Methods

Insect Species

The indicated strains were acquired from the Drosophila Species Stock Center and were grown on standard cornmeal/yeast/molasses Drosophila medium. The *D. suzukii* and *Z.*

indianus strains originated from four wild-caught females collected in Atlanta, GA in the summer of 2010 by TAS (Figure 1). The *D. suzukii* strain was maintained on standard Drosophila medium supplemented with (thawed) frozen raspberries, which were found to enhance egg-laying but were otherwise unnecessary for fly development.

A total of 27 Drosophila parasitic wasp strains were used for infection trials (Figure 2). Strains LgG500 and LgG510 were provided by R. Allemand, strain LbG486 was provided by D. Hultmark , strains Lclav, Ajap, Apleu, and Acit were provided by J. van Alphen, strain GxUg was provided by J. Pool, and strain AtFr was provided by B. Wertheim. All other strains were collected by the Schlenke lab. These wasp strains represent: (1) at least 14 species, (2) all four Hymenopteran families known to infect Drosophila, (3) larval and pupal parasites, and (4) a worldwide range of collection localities. Morphology and *COI* sequences from the two *Trichopria sp.* strains suggested that they were representatives of the same species, perhaps *Trichopria drosophila*. Furthermore, morphology and *COI* sequences from the G1Fl, G1Haw, and G2Atl suggested that they were representatives of a single undescribed species. All wasp species were maintained in the lab on *D. melanogaster* strain Canton S, with the exception of *A. tabida* (Sw and Fr strains), *Aph1Atl.*, LcNet, LcAtl*, G2Atl*, and *Pac1Atl*, which were maintained on *D. virilis*. To grow wasps, adult flies were allowed to lay eggs in standard Drosophila medium for several days before they were replaced by adult wasps, which then attacked the developing fly larvae or pupae. Wasp vials were supplemented with approximately 500 μl of a 50% honey/water solution applied to the inside of the cotton vial plugs.

Hemocyte Counts

Third instar larvae were either untreated or were pierced with a sterile needle to simulate the wounding associated with wasp oviposition. Such wounding has been shown to induce the production of lamellocytes in *D. melanogaster* ([25]). For each of five replicates, 15 fly larvae were rinsed in 1X PBS, dried on kimwipes, and immobilized on double sided tape. Their posterior cuticles were then pierced with 0.1 mm diameter dissecting pins (Fine Science Tools), with care taken to avoid harming internal organs. Fly larvae were then removed from the tape with a wet paintbrush, and allowed to recover in a moist chamber for one hour before being moved to 35 mm diameter Petri dishes filled with 1 mL of Drosophila medium. Control larvae were treated identically except without piercing. Hemocytes and crystal cells were then counted 24 hours post-treatment.

To count hemocytes, 5 third instar larvae from each treatment replicate (including controls) were washed in Drosophila Ringer's solution and dried on a Kim wipe, and bled together into 20 μl of 1X PBS solution containing 0.01% phenylthiourea on a glass slide. Dissection into buffer limits evaporation, and phenylthiourea prevents the hemolymph from melanizing [26]. Mineral oil was also added to the opening of the hemocytometer to further limit evaporation. The buffer-hemolymph mixture was applied to a disposable hemocytometer (Incyto C-Chip DHC-N01) and allowed to sit for 30 minutes to allow hemocytes to settle. Hemocytes from each sample were counted from sixteen 0.25 x 0.25 x 0.1 mm squares, which make up a total volume of 0.1 μl. Thus, the number of hemocytes from the whole 20 μl sample is expected to be 200 times the number counted, or a per larva value of 40 times the number counted.

The addition of hemolymph to the 20 µl of buffer is expected to increase the total buffer-hemolymph volume to greater than 20 µl, leading to a downward bias in our absolute hemocyte counts. However, the amount of hemolymph from five third instar larvae is only approximately 2.5 µl, and in practice about this much liquid evaporates before 20 µl of the buffer-hemolymph mixture can be pipetted onto the hemocytometer. Hemocyte counts are presumably further underestimated because a large fraction of plasmatocytes are docked on host tissues (*i.e.*, sessile) [27], and may not leave the larval tissues upon dissection.

The crystal cells are rapidly lost upon dissection and the cells become difficult to recognize; however, crystal cells self-melanize when larvae are incubated at temperatures ranging from 55 to 65°C ([28]). Larvae were incubated at 63°C (in 63°C distilled water) for 45 minutes to ensure maximal self-melanization of the crystal cells. Therefore, crystal cells were quantified separately from hemocytometer counts, by counting black spots from incubated whole larvae (five larvae per replicate) at 24 hours post-pierce and control.

Hemocyte Imaging

Larvae underwent the piercing procedure, in addition to control, as described above. Three larvae were dissected into 15 µl of 1X PBS. Carcasses were removed and a glass slide was applied. Hemocytes were imaged approximately five minutes later with a phase contrast compound microscope.

Resistance Trials

All aspects of the resistance experiments took place in a 25°C incubator (unless indicated otherwise) on a 12:12 light cycle, and each fly-wasp infection combination was replicated three times. Adult female Drosophila from each species were allowed to lay eggs into molasses medium supplemented with yeast paste, cornmeal medium scored with honey, molasses medium supplemented honey glaze, or mollasas medium supplemented raspberries in 60 mm Petri dishes. The different treatments were found to enhance egg-laying but were otherwise unnecessary for fly development. All egg lays were all performed at 25°C with the exception of *D. subobscura*, which was performed at 18°C.

Method 1: Molasses medium + yeast paste

- A molasses medium plate was prepared. Yeast paste was smeared on to plate. Yeast paste was made with a 50:50 mix of yeast and honey water. 1% of the paste was cantaloupe juice, which was found to enhance egglaying.

Method 2: Cornmeal + honey score

- A cornmeal medium plate was prepared using the media on which the flies grow. The food plates where then scored with a probe covered in honey.

Method 3: Molasses medium + Honey Glaze

- A molasses medium plate was prepared. Yeast paste was smeared on to plate. Yeast paste was made with a 50:50 mix of yeast and honey water. 1% of the paste was cantaloupe juice, which was found to enhance egglaying. The yeast paste was then covered with honey. The plate was then placed into the -4°C incubator for 1 hr before the start of the egglay.

Method 4: Molasses medium + Raspberries

- A molasses medium plate was prepared. Raspberries were supplemented.

72-96 hours following the start of the egglay, adult flies were removed and second instar fly larvae were collected for infection trials.

For larval parasitoid infections, 50 fly larvae were moved into 35 mm diameter Petri dishes filled with 1 mL of Drosophila cornmeal medium. Three female wasps were placed into the Petri dish and immediately allowed to attack these fly larvae for approximately 72 hours. After attack, 10 of the 50 fly larvae were dissected to determine the number of wasp eggs laid per fly larva and the percent of larvae infected in each sample. Encapsulation percentage was also determined for applicable species by assaying the presence of encapsulation per larva. 30 of the 40 remaining larvae were then moved into Drosophila vials to complete development. For pupal parasitoid infections, 40 fly larvae were moved into vials containing Drosophila medium, and were allowed to develop another 72 hours to the wandering third instar stage, just before they began pupating on top of the medium or on the sides of the vials. Three female wasps were then allowed to attack the fly pupae for 72 hours, at which time the wasps were removed and the fly pupae were left to complete development. The infection conditions were chosen to be optimal for wasp success.

The total number of flies and wasps that eclosed from all wasp treatments were determined 15 days and 30 days post-infection, respectively, times by which all viable flies and wasps should have emerged. Fly-wasp interactions may yield one of three outcomes: (1) a successful immune response by the fly, (2) a successful parasitism by the wasp, or (3) death

of the fly and the wasp within it. Control uninfected flies from both species were reared under identical conditions and showed nearly 100% survival (data not shown).

Win Tactic Imaging

Fly-wasp pair-wise combinations were undertaken (using the previously described procedure) using wasps that failed to successfully infect selected fly species. Fly larvae were dissected into 20 μl of 1X PBS.

When a wasp egg was seen, a glass cover slip was applied directly onto the slide and imaging was performed immediately.

When a wasp larva was found, two procedures were undertaken. With one wasp larva, a glass cover slip was applied directly onto the slide. The second wasp larva was imaged by building a bridge over the larva (using the glass cover slides). These two samples were compared to assess the presence of cells on the outside of the larva. The two methods were performed to ensure that wasp larva death was not due to the glass cover slip.

Results and Discussion:

Blood Cell Classification

To characterize constitutive hemocyte numbers, we used uninfected fly larvae. To stimulate lamellocyte production in the absence of wasp attack, we pierced larvae from every species

with sterile needles. This was used as a proxy to avoid any possible inhibitory effects of wasp venom ([25]). We found changes in different classes of blood cells as a result of immune induction.

The terminology used in the classification of hemocytes across the genus Drosophila is borrowed from the Rizki study on *D. willistoni* and the Russo study on *D. melanogaster* ([13], [3]). To assay for blood cell type, we used immune-induced and immune-uninduced 3rd instar larvae. Immune-induced larvae were pierced with a sterile needle, while immune-uninduced larvae were not pierced. In addition to assaying for type of blood cell, we also used induced and uninduced larvae and performed hemocyte counts.

Class 1—Plasmatocytes. We defined plasmatocytes as cells that are clearly defined and round. These were the most common cell type and were present in every species we examined. The nucleus is visible through a phase contrast microscope. This cell type is morphologically similar to described *D. melanogaster* plasmatocytes. Plasmatocytes were approximately 5 μ in length. We found these cells in both induced and uninduced fly larvae of all species tested (Figure 4 A).

Class 2—Podocytes. The podocytes are larger in size than plasmatocytes. They are distinguished by their light shade and dark nucleus. These cells were found to be on approximately 18 μ in length. The podocytes we found were morphologically similar to *D. melanogaster* podocytes. These cells were found in both induced and uninduced fly larvae in all species tested (Figure 4 B).

Class 3—Pseudolamellocytes. We found a novel class of hemocytes present in some, but not all species of fly tested. These cells resemble the coloration of podocytes, but with a

darker nucleus, and resemble the circular shape of a lamellocyte, but fail to spread out on a glass slide in a similar way to a lamellocyte. This cell type is approximately 25 μ in length, and it is absent in *D. melanogaster*. These cell types were present in both induced and uninduced larvae (Figure 4 C).

Class 4—Lamellocytes. The lamellocytes were found to be present in some, but not all species. These cells flatten out on a dissection slide and resemble a fried egg. Lamellocytes were found to be approximately 45 μ in length. This cell type is morphologically similar to *D. melanogaster* lamellocytes, among all species tested. These cells were generally found in induced fly larvae (Figure 4 D).

Class 5—Nematocytes. The extremely fusiform cell type isolated from the fly larvae is termed as a nematocyte. Nematocytes were found constitutively and induced in a subset of Drosophila—specifically members of the Subgenus Drosophila and *Zaprionus indianus*. Once induced, all three fly strains contain nematocytes that have increased in size. This cell type is absent in *D. melanogaster* and its closely related species (Figure 4 E).

Class 6—Crystal Cells. These are small, circular cells that burst upon bathing the fly larva at 63°C, allowing cells to be scored. These cells congregate mostly at the posterior end of the fly larva. Crystal cells vary in color between black and brown, where some species have both black and brown and other have only black. These crystal cells are present across the genus Drosophila at varying levels. Larvae from all fly species, with the exception of *D. virilis* and *D. pseudoobscura*, had constitutive presence of crystal cells. Following pierce, larvae from all fly species, with the exception of *D. erecta*, decreased their total crystal count, suggesting that crystal cells are used in the wound healing response.

Evolution of the immune response and the Ancestral State

The outcome of the pairwise interactions we observed had three possible outcomes: 1) successful fly eclosion; 2) wasp eclosion; 3) death of both the fly and the wasp. We observed varying success patterns of fly eclosion across the genus Drosophila in response to the panel of wasps used. Some flies were very successful against the panel of parasitoids, showing high proportions of fly elcosion (*D. biarmipes, D. paramelanica*). This was in contrast to other fly species where a very small proportion of flies successfully eclosed following infection (*D. melanogaster, D. virilis*) (Figure 3, Figure 13). Successful fly eclosion has been correlated with a successful immune response. In our study, we observed three distinct immune responses, two of which are novel and different from the canonical melanotic encapsulation of *D. melanogaster*: A) melanotic encapsulation; B) cellular encapsulation without melanization; and C) non-cellular mediated killing of the parasitoid (Figure 4 F-H).

The evolution of the Drosophila immune response has undergone many changes. The canonical melanotic encapsulation we see in *D. melanogaster*, is a recent evolutionary phenomenon. We hypothesize that melanotic encapsulation had evolved from the common ancestor of *D. elegans*, as this is the first fly in the Melanogaster group which can do so. We postulate that *D. elegans* is the first fly species in the Melanogaster group with this ability due to the extent of its response—it can successfully encapsulate every wasp we tested, however, following immune stimulation, it begins to encapsulate its own tissues. This suggests an autoimmune response—the flies can mount the melanotic encapsulation response, but cannot turn it off. Thus, we hypothesize *D. elegans* is the first fly with this ability and lacks appropriate regulatory elements to control its response. *D. affinis* is a fly outside of the melanogaster group that can also melanotically encapsulate its parasites, though in a different

pattern than that of *D. melanogaster*. A final member of the genus that can melanotically encapsulate its parasites is *Z. indianus*. It is not known as to where this species falls on the tree. In addition, it is very unique with respect to its hemocyte content. All other melanotic encapsulators have plasmatocytes, podocytes, sometimes pseudolamellocytes, and lamelocytes. *Z. indianus* has plasmatocytes, podocytes, lamellocytes, and nematocytes. Yet, it still melanotically encapsulates, making in the only member of the genus to have this immune response and have nematocytes present. It is also important to note that every member of the genus that can melanotically encpasulate has lamellocytes present in their hemolymph, suggesting that lamellocytes are important to this melanotic capsule formation.

The ability to melanotically encapsulate varied between the attacking wasp strain, with respect to fly species that were found to melanotically encapsulate. Some fly species, such as *D. elegans*, could melanotically encapsulate a large portion of every wasp strain used in the study, whereas other species, such as *D. lutescens*, could only melanotically encapsulate a very small proportion of the wasps used (Figure 16).

Most of the fly species we examined killed their parasites through two alternative ways—cellular encapsulation with no melanization and through a non-cellular mediated mechanism. The species most closely related to *D. elegans* (the first member of the melanogaster group that can melanotically encapsulate), *D. ficusphila*, actually uses cellular encapsulation with no melanization as its immune response to wasps. Non-cellular mediated form of parasite combat is also present in the genus. In fact, this was the most common phenotype observed. Previous work has demonstrated that *D. paramelanica* does not use a cellular-mediated response, but instead uses reactive oxygen species (ROS) against parasites. We found that

response was present in *D. paramelanica* against every wasp used. We found similar phenotypes in many other fly species with respect to their immune response (*D. virilis, D. mojavensis, D. hydei, D. immigrans, D. funebris, D. willistoni, D. subobscura, D. pseudoobscura, D. annanassae, D. kikkwai, D. tsacasi)*, where no cells were present on the dead parasites. This suggests that these flies might use similar mechanisms to that of *D. paramelanica.*

Some flies employ multiple strategies in order to kill their parasites (*D. biarmipes, D. suzukii, D. affinis, D. subobscura, D. funebris*). These flies employ the use of some form of cellular encapsulation (some with melanization, some without) in addition to employing non-cellular mediated responses. Presumably, the ability to kill parasites multiple ways is indicative of what the ancestral state of the earliest common ancestor was.

We compared the success of each of the three immune responses utilized by each of the fly species against successful fly eclosion. We did not find a superior immune response, but instead we found variation between successful fly eclosion and each of the three possible immune mechanisms (Figure 5). Our analyses suggest that without a clear superior immune mechanism, other factors are also present that account for successful fly eclosion.

Based on our data, we hypothesize that the earliest common ancestor of the genus employed a non-cellular mediated form of killing its parasites. We can also hypothesize as to the original hemocyte composition of this ancestor—presumably it also had plasmatocytes and podocytes. Our data cannot confirm as to the presence or absence of pseudolamellocytes or lamellocytes. However, our data does suggest that the common ancestor did have nematocytes present in its hemolymph, as the most distant flies examined also have them

present. Nematocytes have also been seen in other insect species, such as the army ant ([29]). We hypothesize that the evolution of the immune response stemmed from non-cellular mediated to cellular mediated in order to be more effective in killing parasites as well as protecting the host from harmful chemicals used in the immune response. Further evolution employed the use of melanin in the encapsulation response, possibly to offer more protection from the immune response if not make it more effective.

Correlation Analyses

Previous studies have indicated that increased hemocyte load is correlated with increased resistance to parasitoid wasps ([11]). Thus, we performed hemocyte counts on the fly species to assay for both constitutive hemocyte counts and induced hemocyte counts (through the use of a sterile needle to simulate wasp attack). We also examined crystal cell number through the same means.

We performed statistical analyses to find correlations between total hemocyte count (THC) and overall fly success, with regard to each fly species. Our first analysis involved the entire genus Drosophila, measuring fly success against THC of constitutive and induced larvae(Figure 14, 15). We found a significant correlation between fly success and hemocyte load in both cases. We also ran this analysis against only those fly species that melanotically encapsulate. Here, we found a stronger, significant, correlation. Previous studies have indicated that the Melanogaster subgroup's success is based on hemocyte load (Prevost 2004). When we examined only the Melanogaster subgroup, the correlation was still present and significant, but was much weaker than previously described. We presume that this

difference is to a difference in species used from the subgroup from that of the previous study. Finally, we compared THC, both induced and constitutive, against melanotic capsule formation success of each fly that has the ability to melanotically encapsulate. We found no correlation with respect to constitutive hemocyte load, but did find a significant correlation with respect to the induced THC numbers compared to encapsulation ability (Figure 6 A-H).

In addition to examining THC, we also examined fly eclosion success (of all flies, melanotic encapsulators, Melanogaster subgroup, melanotic encapsulation ability) with respect to each class of hemocyte we identified. We found that every one of these interactions had a positive significant correlation in both induced and uninduced states with a few exceptions. Constitutive plasmatocyte load and induced pseudolamellocyte load was found not to correlate with encapsulation ability (Figures 7-11).

Our study further confirms the idea that successful fly eclosion is correlated with hemocyte count. Correlations were found to be even stronger in fly species that melanotically encapsulated, presumably because they use hemcoytes in their immune response, while not all fly species do (Figure 12).

The Obscura group

Previous work has described the Obscura group as the group that lacks an immune response because melanotic encapsulation was not observed ([14]). However, our work, which used a broader range of parasitoid wasps, demonstrates that the Obscura group is armed with two possible immune responses—melanotic encapsulation and non-cellular mediated killing of

the parasite. *D. affinis* utilizes both of these methods. *D. affinis* is a species outside of the melanogaster group that can also melanotically encapsulate its parasites, though in a different pattern than that of *D. melanogaster*. *D. affinis* encapsulation appears to lack a uniform melanization process, and instead has spots of melanization, while *D. melanogaster* encapsulation is a more uniform process. *D. affinis* is located in the Obscura group and its two closest relatives, *D. pseudoobscura* and *D. subobscura*, cannot melanotically encapsulate. These two species also lack a class of blood cells that *D. affinis* has—namely, the lamellocytes. This data indicates two evolutionary points. One is that the ability to melanotically encapsulate evolved independently in *D. affinis* from its two sister speices and *D. melanogaster*. Additionally, *D. subobscura* and *D. pseudoobscura* both are successful against some parasitoids and utilize non-cellular mediated form of wasp killing.

Flies that use toxins

D. melanogaster demonstrated very low levels of successful fly elcosion. This weakness could be attributed to the lack of natural conditions, where it would usually have access to ethanol in a fermenting fruit. Previous work has described seeking behavior in *D. melanogaster* towards food with high concentrations of alcohol. This feeding on ethanol food had been demonstrated to confer a fitness advantage, showing the larvae of *D. melanogaster* actually self-medicate ([17]). Presumably, the weakness we observe is due to the lack of ethanol for *D. melanogaster* larvae to utilize following infection.

The ability to melanotically encapsulate is conserved in the Melanogaster group from *D. elegans* through *D. melanogaster*, with one notable exception—*D. sechelia*. *D. sechelia* is an genetically isolated fly species (located on the Seychelles) and lives on a toxic plant known as the noni fruit ([30], [31], [32]). This fruit has high levels of octanoic acid. Presumably, *D. sechelia* has evolved the ability to utilize this toxin in a similar method to *D. melanogaster* using alcohol, with the key difference being that in this evolutionary process *D. sechelia* has lost its ability to melanotically encapsulate.

D. mojavensis larvae were generally found to be dead following wasp attack. In cases where they were not immediately dead, they failed to eclose as either fly or wasp, suggesting that the death of the host was incurred due to wasp attack. We hypothesize that this could be due to *D. mojavensis*'s cactus diet, where the bevy of toxin, ethanol, and antioxidant many incur a fitness advantage or necessity in the wild, but is lost under standardized lab conditions ([33]; [34]). *D. mojavensis* might also utilize its environment as a way of successfully fighting off parasitoids.

Conclusion

The outcome of any fly-wasp interaction is successful fly eclosion, successful parasite eclosion, or death of both fly and wasp. We found variation in each of these outcomes across fly species, suggesting little phylogenetic conservation. Interestingly, the canonical *D. melanogaster* melanotic encapsulation response is unique to the melanogaster group and two outliers. We have isolated three possible immune mechanisms as well as two novel classes of hemocytes. Our analysis leads to conclusions about the evolutionary selective pressures on

wasp infection strategies and fruitfly immune systems, and provide clues to how these species groups have co-evolved.

Supplement:

Figures 18-42 demonstrate each hemocyte class and mode of wasp killing across each member we tested.

Figure 1

Fly species used and collection sites of flies in this study.

Figure 2

Phylogenetic relationships and provenance of wasps used in this study. Tree topology is derived from previous phylogenetic studies of Hymenopteran families, the family Figitidae, and the family Braconidae. Branch lengths are approximated.

Figure 3

Evolutionary patterns between the genus Drosophila and their interaction with the parasitoid wasps used in this study.

Figure 4

Representative hemocytes and immune responses from the genus Drosophila; (a) plasmatocyte; (b) podocyte; (c) pseudolamellocyte; (d) lamellocyte; (e) nematocyte; (f) melanotic encapsulation; (g) encapsulation without melanization; (h) non-cellular mediated wasp death.

Figure 5

Immune strategy compared to fly success.

Figure 6

Associations involving total hemocyte counts: (a) total constitutive count compared to fly eclosion; (b) total induced hemocyte count compared to fly eclosion; (c) total constitutive count compared to flies that melanotically encapsulate; (d) total induced hemocyte count compared to flies that melanotically encapsulate; (e) total constitutive count compared to the Melanogaster subgroup; (f) total induced hemocyte count compared to the Melanogaster subgroup; (g) total constitutive count compared to average melanotic encapsulation ability; (h) total induced hemocyte count compared to average melanotic encapsulation ability.

Figure 7

Associations involving plasmatocyte counts: (a) constitutive plasmatocyte count compared to fly eclosion; (b) induced plasmatocyte count compared to fly eclosion; (c) constitutive plasmatocyte count compared to flies that melanotically encapsulate; (d) induced plasmatocyte count compared to flies that melanotically encapsulate; (e) constitutive plasmatocyte count compared to the Melanogaster subgroup; (f) induced plasmatocyte count compared to the Melanogaster subgroup; (g) constitutive plasmatocyte count compared to average melanotic encapsulation ability; (h) induced plasmatocyte count compared to average melanotic encapsulation ability.

Figure 8

Associations involving podocyte counts: (a) constitutive podocyte count compared to fly eclosion; (b) induced podocyte count compared to fly eclosion; (c) constitutive podocyte count compared to flies that melanotically encapsulate; (d) induced podocyte count compared to flies that melanotically encapsulate; (e) constitutive podocyte count compared to the Melanogaster subgroup; (f) induced podocyte count compared to the Melanogaster subgroup; (g) constitutive podocyte count compared to average melanotic encapsulation ability; (h) induced podocyte count compared to average melanotic encapsulation ability.

Figure 9

Associations involving pseduolamellocyte counts: (a) constitutive pseduolamellocyte count compared to fly eclosion; (b) induced pseduolamellocyte count compared to fly eclosion; (c) constitutive pseduolamellocyte count compared to flies that melanotically encapsulate; (d) induced pseduolamellocyte count compared to flies that melanotically encapsulate; (e) constitutive pseduolamellocyte count compared to average melanotic encapsulation ability; (f) induced pseduolamellocyte count compared to average melanotic encapsulation ability.

Figure 10

Associations involving lamellocyte counts: (a) constitutive lamellocyte count compared to fly eclosion; (b) induced lamellocyte count compared to fly eclosion; (c) constitutive lamellocyte count compared to flies that melanotically encapsulate; (d) induced lamellocyte count compared to flies that melanotically encapsulate; (e) constitutive lamellocyte count compared to the Melanogaster subgroup; (f) induced lamellocyte count compared to the Melanogaster subgroup; (g) constitutive lamellocyte count compared to average melanotic encapsulation ability; (h) induced lamellocyte count compared to average melanotic encapsulation ability.

Figure 11

Associations involving nematocyte counts: (a) constitutive nematocyte count compared to fly eclosion; (b) induced nematocyte count compared to fly eclosion.

Figure 12

Associations involving crystal cell counts: (a) constitutive crystal cell count compared to fly eclosion; (b) constitutive crystal cell count compared to flies that melanotically encapsulate; (c) constitutive crystal cell count compared to the Melanogaster subgroup; (d) constitutive crystal cell count compared to average melanotic encapsulation ability.

Figure 13

Infection outcomes for host larvae infected by each wasp strain: (a) *D. melanogaster*; (b) *D. sechelia;* (c) *D. simulans*; (d) *D. mauritiana*; (e) *D. yakuba*; (f) *D. erecta*; (g) *D. eugracilis*; (h) *D. suzukii*; (i) *D. biarmipes*; (j) *D. lutescens*; (k) *D. elegans*; (l) *D. ficusphila*; (m) *D. tsacasi*; (n) *D. kikkawai*; (o) *D. annanassae*; (p) *D. affinis*; (q) *D. pseudoobscura*; (r) *D. subobscura*; (s) *D. willistoni*; (s) *D. funebris*; (u) *D. immigrans*; (v) *D. hydei*; (w) *D. mojavensis*; (x) *D. paramelanica*; (y) *D. virilis*; (z) *Z. indianus*. Average (+) standard deviation shown.

Figure 14

Hemocyte count comparison species 24 hours after piercing with a sterile needle: (a) *D. melanogaster*; (b) *D. sechelia;* (c) *D. simulans*; (d) *D. mauritiana*; (e) *D. yakuba*; (f) *D. erecta*; (g) *D. eugracilis*; (h) *D. suzukii*; (i) *D. biarmipes*; (j) *D. lutescens*; (k) *D. elegans*; (l) *D. ficusphila*; (m) *D. tsacasi*; (n) *D. kikkawai*; (o) *D. annanassae*; (p) *D. affinis*; (q) *D. pseudoobscura*; (r) *D. subobscura*; (s) *D. willistoni*; (s) *D. funebris*; (u) *D. immigrans*; (v) *D. hydei*; (w) *D. mojavensis*; (x) *D. paramelanica*;

(y) *D. virilis*; (z) *Z. indianus*. Average (+) standard deviation shown. Numbers are approximately one fortieth of the number of cells per one fly larva (Methods).

Figure 15

Crystal cell count comparison 24 hours after piercing with a sterile needle: (a) *D. melanogaster*; (b) *D. sechelia;* (c) *D. simulans*; (d) *D. mauritiana*; (e) *D. yakuba*; (f) *D. erecta*; (g) *D. eugracilis*; (h) *D. suzukii*; (i) *D. biarmipes*; (j) *D. lutescens*; (k) *D. elegans*; (l) *D. ficusphila*; (m) *D. tsacasi*; (n) *D. kikkawai*; (o) *D. annanassae*; (p) *D. affinis*; (q) *D. pseudoobscura*; (r) *D. subobscura*; (s) *D. willistoni*; (s) *D. funebris*; (u) *D. immigrans*; (v) *D. hydei*; (w) *D. mojavensis*; (x) *D. paramelanica*; (y) *D. virilis*; (z) *Z. indianus*. Average (+) standard deviation shown.

Figure 16

Encapsulation success of wasp-infected fly larvae represented by the average proportion of fly larvae that encapsulated a wasp egg: (a) *D. melanogaster*; (b) *D. simulans*; (c) *D. mauritiana*; (d) *D. yakuba*; (e) *D. erecta*; (f) *D. eugracilis*; (g) *D. suzukii*; (h) *D. biarmipes*; (i) *D. lutescens*; (j) *D. elegans*; (k) *D. affinis*; (l) *Z. indianus*. Average (+) standard deviation shown.

Figure 17

Immune components and response of *D. melanogaster* to parasitoid wasps: (a) plasmatocyte; (b) podocyte; (c) lamellocyte; (d) melanotic capsule formation; (e) whole larva image; (f) crystal cells.

Figure 18

Immune components and response of *D. sechelia* to parasitoid wasps: (a) plasmatocyte; (b) podocyte; (c) pseudolamellocyte; (d) lamellocyte; (e) non-cellular mediated wasp death; (f) whole larva image; (g) crystal cells.

Figure 19

Immune components and response of *D. simulans* to parasitoid wasps: (a) plasmatocyte; (b) podocyte; (c) lamellocyte; (d) melanotic capsule formation; (e) whole larva image; (f) crystal cells.

Figure 20

Immune components and response of *D. mauritiana* to parasitoid wasps: (a) plasmatocyte; (b) podocyte; (c) pseudolamellocyte; (d) lamellocyte; (e) melanotic capsule formation; (f) whole larva image; (g) crystal cells.

Figure 21

Immune components and response of *D. yakuba* to parasitoid wasps: (a) plasmatocyte; (b) podocyte; (c) lamellocyte; (d) melanotic capsule formation; (e) whole larva image; (f) crystal cells.

Figure 22

Immune components and response of *D. erecta* to parasitoid wasps: (a) plasmatocyte; (b) podocyte; (c) pseudolamellocyte; (d) lamellocyte; (e) melanotic capsule formation; (f) whole larva image; (g) crystal cells.

Figure 23

Immune components and response of *D. eugracilis* to parasitoid wasps: (a) plasmatocyte; (b) podocyte; (c) lamellocyte; (d) melanotic capsule formation; (e) whole larva image; (f) crystal cells.

Figure 24

Immune components and response of *D. suzukii* to parasitoid wasps: (a) plasmatocyte; (b) podocyte; (c) lamellocyte; (d) melanotic capsule formation; (e) whole larva image; (f) crystal cells.

Figure 25

Immune components and response of *D. biarmipes* to parasitoid wasps: (a) plasmatocyte; (b) podocyte; (c) pseudolamellocyte; (d) lamellocyte; (e) melanotic capsule formation; (f) whole larva image; (g) crystal cells.

Figure 26

Immune components and response of *D. lutescens* to parasitoid wasps: (a) plasmatocyte; (b) podocyte; (c) pseudolamellocyte; (d) lamellocyte; (e) melanotic capsule formation; (f) whole larva image; (g) crystal cells.

Figure 27

Immune components and response of *D. elegans* to parasitoid wasps: (a) plasmatocyte; (b) podocyte; (c) lamellocyte; (d) melanotic capsule formation; (e) whole larva image; (f) crystal cells.

Figure 28

Immune components and response of *D. ficusphila* to parasitoid wasps: (a) plasmatocyte; (b) podocyte; (c) pseudolamellocyte; (d) lamellocyte; (e) encapsulation of wasp egg without melanization; (f) whole larva image; (g) crystal cells.

Figure 29

Immune components and response of *D. tsacasi* to parasitoid wasps: (a) plasmatocyte; (b) podocyte; (c) pseudolamellocyte; (d) lamellocyte; (e) non-cellular mediated wasp death; (f) whole larva image; (g) crystal cells.

Figure 30

Immune components and response of *D. kikkawai* to parasitoid wasps: (a) plasmatocyte; (b) podocyte; (c) pseudolamellocyte; (d) lamellocyte; (e) non-cellular mediated wasp death; (f) whole larva image; (g) crystal cells.

Figure 31

Immune components and response of *D. annanassae* to parasitoid wasps: (a) plasmatocyte; (b) podocyte; (c) lamellocyte; (d) non-cellular mediated wasp death; (e) whole larva image; (f) crystal cells.

Figure 32

Immune components and response of *D. affinis* to parasitoid wasps: (a) plasmatocyte; (b) podocyte; (c) pseudolamellocyte; (d) lamellocyte; (e) melanization spots on wasp egg; (f) melanotic capsule formation; (g) whole larva image; (h) crystal cells.

Figure 33

Immune components and response of *D. pseudoobscura* to parasitoid wasps: (a) plasmatocyte; (b) podocyte; (c) pseudolamellocyte; (d) non-cellular mediated wasp death; (e) whole larva image; (f) crystal cells.

Figure 34

Immune components and response of *D. subobscura* to parasitoid wasps: (a) plasmatocyte; (b) podocyte; (c) pseudolamellocyte; (d) non-cellular mediated wasp death; (e) whole larva image; (f) crystal cells.

Figure 35

Immune components and response of *D. willistoni* to parasitoid wasps: (a) plasmatocyte; (b) podocyte; (c) pseudolamellocyte; (d) lamellocyte; (e) non-cellular mediated wasp death; (f) whole larva image; (g) crystal cells

Figure 36

Immune components and response of *D. funebris* to parasitoid wasps: (a) plasmatocyte; (b) podocyte; (c) pseudolamellocyte; (d) lamellocyte; (e) nematocyte; (f) encapsulation without melanization; (g) whole larva image; (h) crystal cells.

Figure 37

Immune components and response of *D. immigrans* to parasitoid wasps: (a) plasmatocyte; (b) podocyte; (c) pseudolamellocyte; (d) nematocyte; (e) non-cellular mediated wasp death; (f) whole larva image; (g) crystal cells.

Figure 38

Immune components and response of *D. hydei* to parasitoid wasps: (a) plasmatocyte; (b) podocyte; (c) pseudolamellocyte; (d) nematocyte; (e) non-cellular mediated wasp death; (f) whole larva image; (g) crystal cells.

Figure 39

Immune components and response of *D. mojavensis* to parasitoid wasps: (a) plasmatocyte; (b) podocyte; (c) pseudolamellocyte; (d) nematocyte; (e) non-cellular mediated wasp death; (f) whole larva image; (g) crystal cells.

Figure 40

Immune components and response of *D. paramelanica* to parasitoid wasps: (a) plasmatocyte; (b) podocyte; (c) pseudolamellocyte; (d) lamellocyte; (e) nematocyte; (f) non-cellular mediated wasp death; (g) whole larva image; (h) crystal cells.

Figure 41

Immune components and response of *D. virilis* to parasitoid wasps: (a) plasmatocyte; (b) podocyte; (c) pseudolamellocyte; (d) nematocyte; (e) non-cellular mediated wasp death; (f) whole larva image; (g) crystal cells

Figure 42

Immune components and response of *Z. indianus* to parasitoid wasps: (a) plasmatocyte; (b) podocyte; (c) lamellocyte; (d) nematocyte; (e) melanotic capsule formation; (f) whole larva image; (g) crystal cells.

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Chapter 2

Alcohol Consumption As Self-Medication

Against Blood-Borne Parasites

In The Fruitfly

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Abstract

Plants and fungi often produce toxic secondary metabolites that limit their consumption [1-4], but herbivores and fungivores that evolve resistance gain access to these resources and can also gain protection against non-resistant predators and parasites [3, 5-8]. Given that larvae of the fruitfly *Drosophila melanogaster* consume yeasts growing on rotting fruit and have evolved resistance to yeast fermentation products such as ethanol [9, 10], we decided to test whether ethanol protects fruitflies from one of their most common natural parasites, endoparasitoid wasps [11-13]. Here, we show that exposure to ethanol reduces wasp oviposition into fruitfly larvae. Furthermore, if infected, ethanol consumption by fruitfly larvae causes increased death of wasp larvae growing in the hemocoel and increased fly survival without need of the stereotypical anti-wasp immune response. This multi-faceted protection afforded to fly larvae by ethanol is significantly more effective against a generalist wasp than a wasp that specializes on *D. melanogaster*. Finally, fly larvae seek out ethanol-containing food when infected, indicating they use alcohol as an anti-wasp medicine. Although the high resistance of *D. melanogaster* may make it uniquely suited to exploit curative properties of alcohol, it is possible that alcohol consumption may have similar protective effects in other organisms.

Results and Discussion

Ethanol levels found in natural *D. melanogaster* habitats range up to 6% ethanol by volume in rotting fruits, and 11% in wine seepages found at wineries [14, 15]. Fly consumption of food with moderate levels of ethanol (i.e. less than 4% by volume) results in increased fitness [16-18], but consumption of higher ethanol concentrations (i.e. greater than 4%) causes increasing fly mortality [18-20]. Given that secondary metabolites were shown to harm

endoparasitoid wasps in other systems [3, 7, 21, 22], and the suggestion that *D. melanogaster* living in fruits with high ethanol concentrations might experience less wasp parasitism [23], we decided to test whether natural levels of ethanol could act as a protective toxin in fly interactions with two wasp species: *Leptopilina boulardi* is a specialist parasite of *D. melanogaster* and its close relatives that was previously shown to have relatively high ethanol knockdown resistance, while *L. heterotoma* is a generalist parasite that infects a diversity of Drosophila species living in fermenting fruits, decaying plant materials, and sap fluxes [24-26]. Both wasp species are attracted to the odor of fermentation products such as ethanol, presumably as a means to locate hosts [25, 27], and they are each highly infectious in *D. melanogaster* lab strains [28]. We compared ethanol knockdown resistance of adult female flies and wasps over a 24 hr period using Drosophila food mixed with concentrations of ethanol ranging from 4% to 10% by volume (Figure 1A, Figure 2). At 6% ethanol, *D. melanogaster* adults and adults of the specialist wasp *L. boulardi* both showed significantly greater knockdown survival than adults of the generalist wasp *L. heterotoma* (Figure 1A). Considering all ethanol concentrations used, *D. melanogaster* is most ethanol resistant, followed by the specialist wasp *L. boulardi*, followed by the generalist wasp *L. heterotoma* (Figure 2).

Given wasps suffer knockdown by natural levels of environmental ethanol, we tested whether wasps also show a reduction in oviposition when presented with host fly larvae grown in 6% ethanol food (Figure 1B). There was a significant effect of ethanol in reducing oviposition in both wasp species. A significant ethanol-by-wasp interaction effect also indicated that ethanol had a stronger effect in reducing oviposition by the generalist *L. heterotoma* than the specialist *L. boulardi*. This difference is not explained by a difference in

wasp mortality, as there was no wasp death over the course of the two-hour trial. Wasps may lay fewer eggs because they are sickened by ethanol fumes and attack less, but it is also possible that they insert their ovipositors into fly larvae growing on ethanol food at a normal level and limit oviposition because they detect a hostile environment for their offspring. Given that wasp oviposition was not reduced in fly larvae briefly removed from ethanol (data not shown), we favor the former hypothesis. Thus, ethanol can provide protection to fly larvae from being attacked by endoparasitoid wasps.

We next considered whether ethanol can help flies kill wasp parasites in the hemocoel once flies are infected. First, we measured the hemolymph ethanol concentration of *D. melanogaster* larvae grown in 6% ethanol food and found that fly hemolymph ethanol concentration was significantly higher in flies grown on food containing ethanol, with concentrations reaching approximately 6 mM (0.02% hemolymph ethanol content by volume) (Figure 3A). This ethanol concentration is low relative to those found in adult flies and honeybees [29-32], suggesting *D. melanogaster* larvae may be particularly resistant to passage of ethanol across the gut wall or cuticle into the hemolymph, and/or may have very efficient ethanol detoxification mechanisms. Fly hemolymph ethanol returned to baseline level within 24 hrs of being removed from ethanol food, and wasp infection did not result in increased fly hemolymph ethanol concentration or prolong the presence of ethanol in the hemolymph (Figure 4A, 4B). Altogether, these data show that wasp eggs and larvae living in fly hemolymph are exposed to a moderate level of ethanol (and presumably to ethanol breakdown products such as acetaldehyde) when flies live in or consume ethanol. Any protective effect ethanol might have for infected flies is likely passive, as infected flies do not

appear to purposefully increase hemolymph ethanol levels, for example by down-regulating ethanol breakdown enzymes.

To determine whether host ethanol consumption affects wasp larval development, *D. melanogaster* larvae raised in food containing 6% ethanol were briefly removed from the food for attack by wasps before being returned to the food. There was a significant effect of host ethanol consumption on wasp larval mortality (Figure 3B). There was also a significant effect of wasp species and a significant interaction between ethanol treatment and wasp species, indicating that the increase in wasp larval mortality due to host consumption of ethanol was significantly greater for the generalist *L. heterotoma* than the specialist *L. boulardi.* To determine if wasp larval mortality was an effect of ethanol experienced by the host fly larvae before or after attack, a similar infection experiment was performed in which food treatments were switched after the fly larvae were attacked (Figure 4C). Although there was no overall effect of different ethanol treatments on wasp larval mortality, in a regression analysis stratified by wasp type there was a significant increase in death of *L. boulardi* larvae in hosts grown on ethanol food post-infection compared to pre-infection ($p = 0.003$), whereas *L. heterotoma* larvae suffered high mortality regardless of ethanol consumption timing ($p = 0.623$). Larval wasp death resulted in a decreased proportion of wasps surviving through eclosion and a significant increase in the proportion of flies that eclosed, despite an overall increase in ethanol-mediated mortality (Figure 3C). There were significant ethanol-by-wasp interaction effects on the proportion of flies and wasps eclosed, again indicating that ethanol has a stronger protective effect in flies infected by the generalist *L. heterotoma.* Altogether, these results indicate that ethanol consumption enhances fitness of wasp-infected flies, and that flies can receive maximal therapeutic benefit by consuming ethanol post-infection.

Wasp larvae dissected from singly infected control hosts invariably had defined internal organs and moved vigorously (Figure 4D). However, wasp larvae dissected from fly larvae grown on 6% ethanol food often did not move, showed amorphous internal organ structure, and had everted tissues, in many cases in close proximity to their anuses (Figure 4E), suggesting ethanol causes defects in wasp organ development or maintenance. Normally, flies attempt to kill wasps in a process termed encapsulation, and the increased mortality of wasps growing in ethanol-fed host flies might be the result of a heightened fly encapsulation response. Encapsulation involves constitutively produced plasmatocytes recognizing a wasp egg or larva as foreign and signaling to induce differentiation of lamellocytes, which spread over the wasp in a multi-layered capsule, leading to wasp death [33]. The wasp strains used here are highly virulent in *D. melanogaster* hosts and normally completely suppress the encapsulation response, but no wasp eggs or larvae dissected from ethanol-consuming fly larvae were found to be encapsulated by host hemocytes either. Although ethanol consumption was associated with a significant increase in fly plasmatocyte numbers, ethanol consumption was associated with a significant decrease in the number of lamellocytes, the hemocyte type specifically induced to mount the encapsulation response (Figure 4F, 4G). Lack of induction and/or death of host lamellocytes could be the result of ethanol toxicity, but it may be adaptive for hosts to purposefully suppress induction of an immune response that is un-needed in the presence of an anti-parasite toxin, given the presumed energetic cost of mounting an immune response [34].

Use of toxic secondary metabolites in defense against enemies is usually preventative, i.e. organisms consume a toxic food source as part of their normal diet and the presence of

toxin in their bodies results in internal host conditions that limit subsequent predation and infection. However, parasitized organisms can also therapeutically self-medicate, whereby they actively seek out compounds that help cure pre-existing infections [35, 36]. The fact that fly consumption of ethanol post-infection has strong protective effects (Figure 4C) led us to consider the possibility that *D. melanogaster* might self-medicate. To test this idea, infected and uninfected fly larvae were placed in bisected petri dishes containing half control food and half 6% ethanol food, and the proportions of fly larvae that moved to (Figure 5A) or remained on (Figure 5B) the ethanol food side of the dish were measured over time. Fly larvae initially placed on control food showed a significant effect of wasp treatment at 24 hrs, with fly larvae infected by each wasp species significantly more likely to have moved to the ethanol food side of the dishes (Figure 5A). Infected fly larvae initially placed on ethanol food moved off the ethanol food faster than uninfected fly larvae, but returned to the ethanol food in significantly greater numbers than uninfected fly larvae by 24 hrs (Figure 5B).

These results are not caused by an increased sensitivity to ethanol sedation in infected fly larvae, which might cause the ethanol half of the dishes to act as an "absorbing state" for these flies, because infected larvae were highly mobile and vigorously masticated the food once they were settled on the ethanol side of the dishes. Instead, these results show that infected flies self-medicate by actively sampling their environment for a food source containing levels of ethanol most suitable for fighting off wasp infection, despite the otherwise toxic effects of ethanol consumption on fly developmental rate and survival found by us (Figure 6) and others [18-20]. Interestingly, in both choice experiments, fly larvae infected by the generalist *L. heterotoma* showed a significantly stronger preference for ethanol

food than fly larvae infected by the specialist *L. boulardi* (Figure 5). These data suggest that fly larvae can distinguish between endoparasitoids with different levels of ethanol resistance, or that *L. boulardi* can better manipulate the ethanol seeking behavioral immune response of *D. melanogaster*.

Finally, we tested the eclosion success of infected flies allowed to self-medicate by giving them the option of 0% or 6% ethanol food in bisected petri dishes (Figure 7). Survival of self-medicating flies was significantly greater than that of flies given no ethanol, and equivalent to that of flies grown in dishes where both sides contained ethanol. Death of infected flies given a choice between control and ethanol food was significantly greater than that of flies given no ethanol, indicating the choice of ethanol food results in ethanol-mediated death, but death was significantly lower than for flies grown in dishes where both sides contained ethanol. Altogether, these data show that flies not only choose to consume ethanol as self-medication against wasp infection, but also balance their ethanol intake to limit toxic effects on themselves. Furthermore, there were significant effects of wasp species on infection outcomes, where flies infected by the generalist wasp *L. heterotoma* achieved a relatively greater increase in eclosion success due to self-medication.

It is not surprising that *D. melanogaster* are highly attuned to ethanol concentration [37-39] given the previously characterized fitness benefits and costs of different levels of ethanol [16-20], along with the variation in ethanol content across rotting fruits, within rotting fruits, and temporally during the fruit rotting process. We have shown here that ethanol provides novel benefits to flies by reducing wasp infection (Figure 1B), by increasing infection survival (Figure 3B, 3C), and by allowing for a behavioral immune response against wasps

based on consumption of it in toxic amounts (Figure 5, 7). To our knowledge, these data are the first to show that alcohol consumption can have a protective effect against infectious disease, and in particular against blood-borne parasites. Given that alcohols are relatively ubiquitous compounds consumed by a number of organisms, protective effects of alcohol consumption may extend beyond fruitflies. Although many studies in humans have documented decreased immune function in chronic consumers of alcohol [40-42], little attempt has been made to assay any beneficial effect of acute or moderate alcohol use on parasite mortality or overall host fitness following infection.

Experimental Procedures

Insect rearing

D. melanogaster strain Oregon R was used for all experiments. *L. boulardi* strain Lb17 and *L. heterotoma* strain Lh14 originated from single females collected in Winters, California in 2002 [28] and have been continuously maintained in the lab on *D. melanogaster* strain Canton S. Instant Drosophila medium (Formula 4-24, Carolina Biological Supply) in 0.25 g aliquots per 35 mm diameter Petri dish was used for most experiments, supplemented with approximately 20 granules of active baker's yeast and specific concentrations of ethanol. For standard experimental infections, Oregon R flies were allowed to lay eggs overnight; 48 hrs after egg lay, second-instar larvae were moved into Petri dishes containing the experimental medium in groups of forty per dish. 72 hrs after egg lay, early third-instar fly larvae were moved into new, non-ethanol food dishes to be attacked by groups of ten female wasps for two hrs, after which they were returned to the experimental food conditions. Insects were kept in a 25 degrees C incubator with 12 hr light-dark cycle for all experiments.

Adult Ethanol Resistance

We used ACS/USP grade 95% ethanol (#111000190, Pharmco-AAPER), which contains less than 0.001% methanol, for all experiments. Batches of ten female flies and wasps were collected 3-5 days post-eclosion and aspirated into food dishes containing 0, 4, 6, 8, and 10% ethanol in five dish replicates. Counts for dead flies and wasps were made 1.5,and 24 hrs later. Statistical comparison of survival curves between species was perusing the Kaplan-Meir survival analysis assuming constant hazard (Figure 1A, 2).

Wasp Oviposition

Batches of thirty 72 hrs old fly larvae grown on control food were placed in new dishes containing either control food or 6% ethanol food, in five dish replicates, and immediately exposed to 10 female wasps for 2 hrs. Fly larvae were then dissected to count the number of wasp eggs found inside. Multivariable Poisson regression was used to test the effects of ethanol, wasp species, and their interaction on wasp egg lay counts (Figure 1B). The 6% ethanol concentration was chosen for this and following experiments because this is the upper limit of ethanol concentrations found in naturally rotting fruits [14], the upper limit that adult *L. boulardi* and *L. heterotoma* can withstand for 2 hrs with minimal death (Figure 1A), and a concentration at which fly larvae experience moderate mortality (band 40%) during development [20]. Thus, it is an ecologically relevant ethanol concentration that should maximize patterns observed in our experiments.

Hemolymph Ethanol Content

D. melanogaster hemolymph (blood) was collected by bleeding groups of thirty larvae onto a cold glass slide and drawing up 2 uL of hemolymph with a micropipette. The level of ethanol found in fly hemolymph following various treatments was measured using a colorimetric

assay kit (#K620, BioVision) following the manufacturer's recommended protocol. Briefly, alcohol oxidase was used to oxidize ethanol and generate hydrogen peroxide, which reacted with a probe to generate colored product with an absorption maximum of 570 nm. Ethanol concentration was calculated by comparing experimental spectrophotometric readings to a standard curve. Statistical comparison of hemolymph ethanol content from flies grown on control versus ethanol food was made with five dish replicates using a one-tailed t-test with the Satterthwaite correction for unequal variances

(Figure 3A). Experiments in which flies were moved oin three dish replicates, and hemolymph ethanol contordinary least squares regression (Figure 4A, 4B).

Wasp Larval Mortality and Fly Eclosion

Fly larvae grown on control and 6% ethanol food were dissected 60 hrs post-attack, a time by which the majority of *L. boulardi* and *L. heterotoma* wasp eggs should have hatched. Fly larvae were also switched between control and ethanol food after wasp attack to compare the effects of hosts grown on ethanol pre- versus post-attack. Dead wasp larvae were scored as those that did not move and that did not have defined internal organ structure. All wasp larvae mortality experiments were run in five dish replicates, and multivariable logistic regression was used to model the effects of ethanol and wasp treatments on mortality (Figure 3B, 4C). To determine whether this wasp mortality results in increased fly eclosion, flies were grown on 0 or 6% ethanol in three dish replicates and exposed to wasps at 72 hours, before being moved into the food vials. The effects of ethanol and wasp treatments on fly eclosion success, wasp eclosion success, and death of both fly and wasp were modeled independently using multivariable logistic regression (Figure 3C).

Hemocyte Counts

Fly larvae were reared under different food and wasp exposure conditions in three replicates. Batches of five larvae from each replicate food dish were cleaned, dried, and bled onto a glass side into 20 uL of 1X PBS solution containing 0.01% phenylthiourea to prevent hemolymph melanization [S3]. This liquid was applied to a hemocytometer, and the hemocytes were allowed to settle for 30 minutes before plasmatocytes and lamellocytes were counted [S4]. Hemocyte numbers are approximately one fortieth of the number of cells per fly larva. Multivariable Poisson regression was used to monitor the effects of ethanol consumption and wasp infection on hemocyte numbers; because there were three wasp treatments, pairwise comparisons were statistically assessed in the model (Figure 4F, 4G).

Fly Development

Fly larvae were grown in food dishes containing varying concentrations of ethanol at 48 hours of age. At 72 hours, they were moved into Drosophila food vials containing the same ethanol concentration. These vials were then checked every day for one month, and the proportion of flies eclosed over time (a measure of fly developmental rate) was compared across ethanol concentrations using a Cox proportional hazards regression model (Figure 6A). The proportion of flies eclosed (a measure of fly mortality) was modeled across ethanol concentrations using logistic regression (Figure 6B).

Ethanol Food Choice

Divided 100 mm diameter petri dishes (#08-757-150, Fisher Scietwo distinct food compartments in each petri dish, across which ethanol cannot diffuse. For preference assays (Figure 5), each side of the dish was filled with 1 g of instant Drosophila medium, with one

side containing no ethanol and the other side containing 6% ethanol by volume. Batches of 100 uninfected or infected 72 hrs old fly larvae were placed in either the control food side or the ethanol food side of the dish, for three dish replicates of each treatment. Fly larvae were free to crawl over the divider and into either compartment, and counts for the number of larvae in each compartment were made 1.5, 3, 6, 12, and 24 hrs later. Logistic regression was used to model the proportion of flies on the ethanol side of the food plate at 24 hrs by treatment (Figure 5). For survival assays, (Figure 7), three ethanol treatments were used: both sides of the dish contained no ethanol, one side contained no ethanol and the other side contained 6% ethanol by volume, or both sides contained 6% ethanol by volume. Batches of 50 infected 72 hrs old fly larvae were placedon one side of each dish, always starting on the 0% ethanol side in mixed dishes, in three replicate food dishes. These flies were left for 72 hours before being moved into non- ethanol food vials to eclose. The effects of ethanol choice treatments on fly eclosion success, wasp eclosion success, and death of both fly and wasp were modeled independently using logistic regression; because there were three ethanol treatments, pairwise comparisons were statistically assessed in the model (Figure 4).

Statistical Analysis

All confidence intervals and standard deviations shown describe variation across true replicates, *e.g.* independent dishes, and all statistical analyses are based on these replicates.

For each statistical analysis, data were tested for goodness of fit in JMP version 9.0.0 to determine if distribution assumptions were met; Kolmogorov's D was used to test whether data from treatment groups followed Poisson or binomial distributions, while Shapiro-Wilk's W was used to test whether data from treatment groups followed normal distributions. No dataset was a significantly poor fit for the assumed distributions of the

statistical test conducted. Ethanol knockdown survival analyses were performed in R version 2.10.1. All other statistical analyses were performed in SAS version 9.2.

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Figure 1

The effect of ethanol on wasp knockdown and oviposition. Survival curves were generated for adult insects living in petri dishes with 6% ethanol food (A). Error bars indicate 95% confidence intervals. The numbers of wasp eggs laid per host (B) were counted by dissecting fly larvae grown on food containing 0 or 6% ethanol and exposed to wasps for two hours. Error bars indicate standard deviation. Dm = *D. melanogaster*, Lb = *L. boulardi*, Lh = *L. heterotoma*. There were five dish replicates for all treatments.

Figure 2

The effect of other ethanol concentrations on wasp knockdown. Survival curves (A,B,C) show a decrease in adult insect survival as ethanol levels increase, with the generalist wasp suffering the highest mortality, followed by the specialist wasp. Error bars indicate 95% confidence intervals across five dish replicates.

Figure 3

Increased hemolymph ethanol is associated with wasp death and fly survival. Hemolymph ethanol concentration was compared between 72 hrs old fly larvae grown on food with or without 6% ethanol (A). Error bars indicate standard deviation across five dish replicates. Infected fly larvae grown on control or ethanol food were dissected to determine the viability of wasp larvae growing within them (B). Error bars indicate 95% confidence intervals across five dish replicates. The proportion of infected fly larvae resulting in each of the three infection outcomes (fly eclosion, wasp eclosion, and death of both fly and wasp)

was compared across ethanol and wasp treatments (C). Error bars represent 95% confidence intervals across three dish replicates.

Figure 4

Effect of hemolymph ethanol on wasp death and fly hemocyte numbers. Hemolymph ethanol concentration was compared between specialist-infected fly larvae (A) and generalist-infected fly larvae (B) grown continuously on ethanol food (6 - 6%) and switched to non-ethanol food (6 – 0%) for 24 hours. Error bars indicate standard deviation across three dish replicates. The proportion of specialist and generalist wasp larvae that died (C) was compared in fly hosts fed 6% ethanol either before (6 - 0%) or after (0 – 6%) wasp infection. Error bars indicate 95% confidence intervals across five dish replicates. Wasp larvae dissected from control fly larvae (D) were visually compared to wasp larvae dissected from fly larvae grown on 6% ethanol (E). Images were taken at 200X. Plasmatocytes (F) and lamellocytes (G) were counted from fly larvae grown on control or 6% ethanol food, with and without wasp infection. Error bars indicate standard deviation across three dish replicates.

Figure 5

Choice of ethanol food by wasp-infected fly larvae. Preference for food containing 6% ethanol was compared between infected and uninfected flies over time using bisected petri dishes, with fly larvae initially placed on the control food side (A) or ethanol food side (B) of the dish. Error bars indicate 95% confidence intervals across three dish replicates. EtOH = ethanol.

Figure 6

The effect of ethanol on fly oviposition and development. The proportion of flies eclosed over time (A) was used as a measure of the fly developmental rate. The total proportion of

flies that eclosed (B) was used as a measure of fly survival. Error bars represent 95% confidence intervals across three dish replicates. Significance groups are indicated by lower case letters.

Figure 7

The option of ethanol food enhances fitness of wasp-infected flies. Larvae were placed in bisected petri dishes with either 0% or 6% ethanol food on each side of the dish. The proportion of wasp-infected fly larvae resulting in each of the three infection outcomes (fly eclosion, wasp eclosion, and death of both fly and wasp) was compared across wasp and ethanol choice treatments. Error bars represent 95% confidence intervals across three dish replicates.

Chapter 3

High Hemocyte Load Is Associated With Increased Resistance Against Parasitoids in *Drosophila suzukii*, A Relative of *D. melanogaster*

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Abstract

Among the most common parasites of Drosophila in nature are parasitoid wasps, which lay their eggs in fly larvae and pupae. *D. melanogaster* larvae can mount a cellular immune response against wasp eggs, but female wasps inject venom along with their eggs to block this immune response. Genetic variation in flies for immune resistance against wasps and genetic variation in wasps for virulence against flies largely determines the outcome of any fly-wasp interaction. Interestingly, up to 90% of the variation in fly resistance against wasp parasitism has been linked to a very simple mechanism: flies with increased constitutive blood cell (hemocyte) production are more resistant. However, this relationship has not been tested for Drosophila hosts outside of the melanogaster subgroup, nor has it been tested across a diversity of parasitoid wasp species and strains. We compared hemocyte levels in two fly species from different subgroups, *D. melanogaster* and *D. suzukii*, and found that *D. suzukii* constitutively produces up to five times more hemocytes than *D. melanogaster*. Using a panel of 24 parasitoid wasp strains representing fifteen species, four families, and multiple virulence strategies, we found that *D. suzukii* was significantly more resistant to wasp parasitism than *D. melanogaster*. Thus, our data suggest that the relationship between hemocyte production and wasp resistance is general. However, at least one sympatric wasp species was a highly successful infector of *D. suzukii*, suggesting specialists can overcome the general resistance afforded to hosts by excessive hemocyte production. Given that *D. suzukii* is an emerging agricultural pest, identification of the few parasitoid wasps that successfully infect *D. suzukii* may have value for biocontrol.

Introduction

Fruitflies of the genus Drosophila are regularly attacked by parasitoid wasps. In natural *D. melanogaster* populations, upwards of 50% of fly larvae are found to be infected by wasps, suggesting they exert extremely strong selection pressures on Drosophila populations in nature [1,2,3]. Once infected, fruitfly larvae mount an immune response against wasp eggs, termed melanotic encapsulation, that is thought to involve several steps [4,5]: The response begins when circulating, constitutively produced plasmatocytes recognize the wasp egg as foreign and signal to induce the differentiation of larger lamellocytes from pro-hemocytes in the lymph gland (the fly hematopoietic organ) and from other circulating plasmatocytes (via the intermediate podocyte form) [6,7]. These newly derived lamellocytes migrate towards, and attach and spread around the wasp egg in a multi-layered capsule. In the final step, the inner cells of the capsule surrounding the wasp egg lyse and release reactive oxygen species and an impermeable layer of melanin, resulting in death of the wasp egg. However, parasitoid wasps can potentially evade host immune responses by using a non-reactive coating on their eggs, or suppress host immunity by injecting venom into hosts along with their eggs. There is both between and within species genetic variation in flies for resistance against wasps and among wasps for virulence against flies [8,9,10,11,12,13,14,15,16].

In previous work, Drosophila species from the melanogaster subgroup were found to have significantly different numbers of constitutively produced plasmatocytes, and there was a significant correlation ($r^2 = 0.90$) between plasmatocyte counts and ability to melanotically encapsulate the eggs of the immune-evasive parasitoid wasp *Asobara tabida* [12]. It was also found that *D. melanogaster* strains artificially selected for resistance against *A. tabida* showed a significant increase in plasmatocyte numbers [17]. Furthermore, *D. simulans*, which makes significantly more plasmatocytes than its sister species *D. melanogaster*, was significantly more

resistant against the more immune-suppressive wasp *A. citri* [18]. Finally, *D. melanogaster* mutants producing a wide range of hemocyte counts showed a significant correlation (r^2 = 0.45) between constitutive plasmatocyte numbers and encapsulation ability against the wasp *Leptopilina boulardi* [19]. Altogether, this work suggests that high constitutive production of hemocytes is an effective and relatively simple mechanism by which hosts can evolve resistance to one of their most common groups of parasites.

We were interested in whether the relationship between Drosophila standing immune defense (hemocyte production) and immune resistance against wasps is general across a large panel of diverse wasp lineages with unique infection strategies, and whether the relationship extends beyond the melanogaster subgroup of the genus Drosophila. Pilot data from a study aimed at characterizing hemocyte lineages across the genus Drosophila (unpublished) suggested *D. suzukii*, a member of the melanogaster group but not the melanogaster subgroup, constitutively produces an extremely large number of hemocytes compared to other Drosophila. Thus, the goal of this study was to confirm whether *D. suzukii* constitutively produces higher numbers of hemocytes than *D. melanogaster*, and if so, to determine whether *D. suzukii* was also more resistant against a large panel of parasitoid wasp species and strains.

D. suzukii is native to east Asia but has recently gained widespread attention due to its spread as an agricultural pest in Europe and North America (Figure 1) [20,21,22,23]. Although most of the ~1,500 described Drosophila species lay their eggs and feed on decaying plant and fungal tissues, including rotting fruits (like *D. melanogaster*), *D. suzukii* is one of a handful of species that live on ripe fruits, using its serrated ovipositor to lay eggs in

the flesh of soft-skinned fruits (Figure 1C). Its larvae subsequently burrow through the body of the fruit as they eat (Figure 1D), allowing bacteria and other microorganisms access to the inside of the fruit, which results in premature rotting. Because parasitoid wasps have been successfully used as biocontrol agents against a wide range of insect agricultural pests, including Coleopterans (*e.g.,* weevils, bean beetles), Hemipterans (*e.g.*, scale insects, whiteflies, aphids, leafhoppers, stinkbugs), Lepidopterans (*e.g.*, various moth and butterfly larvae), and Dipterans (*e.g.*, Tephritid fruitflies, blackflies) [24,25,26,27,28], study of *D. suzukii* resistance and susceptibility to parasitoid wasps may have added applied value.

At least four families of parasitoid wasps are known to attack Drosophila in nature [29]. These wasps use a variety of infection strategies to defeat the fly immune response, including immune suppressive and evasive tactics, and vary in their host ranges from specialists of particular Drosophila species to generalist of the genus. Members of the families Braconidae and Figitidae are larval parasites – they lay single eggs in Drosophila larvae and, if not killed, the hatched wasp larva begins to consume internal fly tissues before eventually killing the fly and eclosing from the fly pupal case. Members of the families Diapriidae and Pteromalidae are pupal parasites - they lay single eggs inside Drosophila pupae, and the hatched wasp larva consumes the fly pupal tissues, also eventually killing the fly and eclosing from the fly pupal case. It is unclear whether fly pupae can mount an immune response or otherwise defend themselves once infected by pupal parasites. Pupal parasites of the genus Trichopria (Family Diapriidae) lay their eggs in the Drosophila hemocoel, like larval parasites, but those of the genus Pachycrepoideus (Family Pteromalidae) lay their eggs in the space between the Drosophila pupal case and the pupa, and act as ectoparasites in the early stages of their life by sucking fluids from the pupa externally [29]. A lack of pupal immunity against wasps may

explain in part why pupal parasitoid wasps are thought to have more generalist host ranges than larval parasitoid wasps [30,31].

The Drosophila-wasp system is ripe for study as a model for the co-evolution of pathogen infection strategies and host immune responses across lineages and communities of pathogens and hosts [32]. We attempted to answer the following questions: Is the melanotic encapsulation response observed in *D. melanogaster* conserved in *D. suzukii*? Does *D. suzukii* have higher constitutive hemocyte production than *D. melanogaster*? Is increased hemocyte production by *D. suzukii* associated with stronger resistance against a panel of parasitoid wasps with diverse life histories and infection strategies? Do wasps make different oviposition choices depending on host species? Do wasp phylogeny and biogeography play any role in fly-wasp interactions? From an applied point of view, which parasitoid wasp species show the most potential for use in *D. suzukii* biocontrol in the field?

Materials and Methods

Insect Species

The *D. melanogaster* genome strain 14021-0231.36 was acquired from the Drosophila Species Stock Center and was grown on standard cornmeal/yeast/molasses Drosophila medium. The two additional *D. melanogaster* strains originated from single wild-caught females collected in Atlanta, GA in the summer of 2010. The primary *D. suzukii* strain tested originated from four wild-caught females collected in Atlanta, GA in the summer of 2010, while two additional isofemale strains were collected in Atlanta, GA in the summer of 2011. *D. suzukii* were maintained on standard Drosophila medium supplemented with (thawed)

frozen raspberries, which were found to enhance egg-laying but were otherwise unnecessary for fly development.

A total of 24 Drosophila parasitoid wasp strains collected from around the world were used for infection trials on *D. melanogaster* and *D. suzukii* (Figure 2). Strains LgG500 and LgG510 were provided by R. Allemand, strain LbG486 was provided by D. Hultmark, strains Lclav, Ajap, Apleu, and Acit were provided by J. van Alphen, strain GxUg was provided by J. Pool, and strain AtFr was provided by B. Wertheim. All other strains were collected by the Schlenke lab. These wasp strains represent: (1) at least 14 species, (2) representatives of all four Hymenopteran families known to infect Drosophila, (3) larval and pupal parasites, and (4) a worldwide range of collection localities (Figure 2). Morphology and *cytochrome oxidase I (COI)* sequences from the two *Trichopria sp.* strains suggested they were representatives of the same species, perhaps *Trichopria drosophila* (Ashmead). Furthermore, morphology and *COI* sequences from the two *Ganaspis sp.* strains suggest they are representatives of a single undescribed species. All wasp species were maintained in the lab on *D. melanogaster* strain Canton S, with the exception of *L. clavipes*, *A. tabida*, *Aphaereta sp.*, and *Pachycrepoideus sp.*, which were maintained on *D. virilis*. To grow wasps, adult flies were allowed to lay eggs in standard Drosophila medium for several days before they were replaced by adult wasps, which then attacked the developing fly larvae or pupae. Wasp vials were supplemented with approximately 500 uL of a 50% honey/water solution applied to the inside of the cotton vial plugs. *COI* sequences for *D. suzukii* and all wasp strains have been deposited in Genbank under accession numbers XXX-XXX. Wasp strains are available upon request.

Hemocyte Counts

Fly-wasp development for all experiments took place in a 25 ^{0} C incubator on a 12:12 light:dark cycle. For hemocyte count experiments, adult female *D. melanogaster* and *D. suzukii* were allowed to lay eggs into fly food supplemented with yeast paste (50:50 mix of baker's yeast and water) or raspberries, respectively, in 60 mm Petri dishes. After 72 hours, adult flies were removed and size-matched second instar fly larvae were collected for two independent experiments.

For hemocyte count experiments, *D. melanogaster* and *D. suzukii* larvae were either uninfected or were infected by the wasp strain LbG486, with three replicates per treatment. For parasitoid infections, 50 fly larvae were moved into 35 mm diameter Petri dishes filled with 1 mL of Drosophila medium. Ten female wasps were immediately allowed to attack these fly larvae for 3 hours, and five larvae per dish were later dissected to determine the number of wasp eggs laid per fly larva. Fourteen of fifteen *D. melanogaster* larvae across the three replicates were found to be infected by single wasp eggs, as well as fourteen of fifteen *D. suzukii* larvae, so we assumed the wasp infection rate was very similar across the two host fly species. Hemocytes were counted at two time-points, 12 and 24 hours post-infection, in which the induced cellular immune response was expected to be highly activated. Crystal cells, a distinct hemocyte type described below, were counted independently 33 hours post-infection.

In an experiment to test hemocyte induction absent wasp venom effects, *D. melanogaster* and *D. suzukii* larvae were either untreated or were pierced with a sterile needle to simulate the wounding associated with wasp oviposition. Such wounding has been shown to induce the production of lamellocytes [33]. For each of four replicates, 15 fly larvae were rinsed in 1X

PBS, dried on Kimwipes, and immobilized on double sided tape. Their posterior cuticles were then pierced with flame-sterilized 0.1 mm diameter stainless steel dissecting pins (Fine Science Tools 26002-10), with care taken to avoid harming internal organs. Fly larvae were then removed from the tape with a wet paintbrush, and allowed to recover in a moist chamber for one hour before being moved to 35 mm diameter Petri dishes filled with 1 mL of Drosophila medium. Control larvae were treated identically except without piercing. Hemocytes were then counted 24 hours post-infection, while crystal cells were counted independently 33 hours post-infection.

To count hemocytes, 5 third instar larvae from each treatment replicate (including controls) were washed in Drosophila Ringer's solution, dried on a Kimwipe, and bled together into 20μL of 1X PBS solution containing 0.01% phenylthiourea on a glass slide. Dissection into buffer limits evaporation, and phenylthiourea prevents the hemolymph from melanizing [34]. The buffer-hemolymph mixture was applied to a disposable hemocytometer (Incyto C-Chip DHC-N01) and allowed to sit for 30 minutes to allow hemocytes to settle. Hemocytes from each sample were counted from sixteen 0.25 x 0.25 x 0.1 mm squares (*e.g.,* Figure 3A, 3B), which make up a total volume of 0.1 μL. Thus, the number of hemocytes from the whole 20 μL sample is expected to be ~200 times the number counted, or a per larva value of 40 times the number counted.

The addition of hemolymph to the 20 μL of buffer is expected to increase the total buffer-hemolymph volume to greater than 20 μL, leading to a downward bias in our absolute hemocyte counts. However, the amount of hemolymph from five third instar larvae is only approximately 2.5 μL, and in practice about this much liquid evaporates before 20 μL of the

buffer-hemolymph mixture can be pipetted onto the hemocytometer. Our hemocyte counts may also underestimate true hemocyte loads because a large fraction of plasmatocytes are sessile (*i.e.*, docked on host tissues) [35], and may not detach from the larval tissues upon dissection. *D. melanogaster* and *D. suzukii* adults and larvae are similar in size, (Figure 1, 3), so we did not expect differences in species hemocyte counts to result from fly size differences, but we were careful to use larvae of the same size from both species for all experiments. Hemocytes were classified as plasmatocytes (small round cells with obvious nuclei), podocytes (activated plasmatocytes that are larger and refract more light than plasmatocytes), and lamellocytes (large, clear flattened cells) [7].

The fourth hemocyte cell type, crystal cells, are medium sized cells containing cytoplasmic crystals made up of the substrate that the phenoloxidase enzymatic cascade converts into melanin [36]. The crystals are rapidly lost upon dissection and the cells become difficult to recognize, so a separate method was used to count them. Crystal cells self-melanize when larvae are incubated at 60^0 C for 10 minutes [37]. Therefore, crystal cells were quantified separately by counting dark spots from the dorsal side of incubated whole larvae (*e.g.,* Figure 3C, 3D) at 33 hours post-infection. Crystal cells were counted and averaged from three larvae per replicate. It is not yet known whether crystal cells play a role in the melanotic encapsulation response [4].

Multivariable regression models assuming Poisson distributions were specified to model hemocyte counts by fly species and immune challenge (wasp infection, piercing). When hemocyte counts were overdispersed, negative binomial distributions were specified instead of Poisson distributions.

Resistance Trials

Each fly-wasp infection combination was replicated three times. Adult female *D. melanogaster* and *D. suzukii* were allowed to lay eggs into fly food supplemented with yeast paste (50:50 mix of baker's yeast and water) or raspberries, respectively, in 60 mm Petri dishes. After 72 hours, adult flies were removed and size-matched second instar fly larvae were collected for infections. For larval parasitoid infections, 50 fly larvae were moved into 35 mm diameter Petri dishes filled with 1 mL of Drosophila medium. Three female wasps were immediately allowed to attack these fly larvae for 72 hours. After attack, 10 of the 50 fly larvae were dissected to determine the percent of larvae infected, the number of wasp eggs laid per fly larva, and the proportion of fly larvae bearing encapsulated wasp eggs in each sample. 30 of the 40 remaining larvae were then moved into Drosophila vials to complete development. For pupal parasitoid infections, 40 fly larvae were moved into vials containing Drosophila medium, and were allowed to develop another 72 hours to the wandering third instar stage, just before they began pupating on top of the medium or on the sides of the vials. Three female wasps were then allowed to attack the fly pupae for 72 hours, at which time the wasps were removed and the fly pupae were left to complete development. The infection conditions were chosen to be optimal for wasp success. Control uninfected flies from both species were reared under identical conditions and showed nearly 100% survival (data not shown).

The total numbers of flies and wasps that eclosed from all wasp treatments were determined 15 days and 30 days post-infection, respectively, times by which all viable flies and wasps should have emerged. Fly-wasp interactions may yield one of three outcomes, which were

compared between *D. melanogaster* and *D. suzukii* infections: (1) a successful immune response by the fly, (2) a successful parasitism by the wasp, or (3) death of the fly and the wasp within it. Furthermore, for larval parasitoid infections, the numbers of wasp eggs counted from dissected fly larvae were assessed for evidence of under-dispersion, as wasps are known to preferentially choose un-infected hosts for oviposition [38,39,40,41]. If wasps layed eggs in fly larvae randomly, without regard to host infection status, the number of wasp eggs per larva would have been expected to follow a Poisson distribution, where the average number of wasp eggs per fly larva and the variance in the number of wasp eggs per fly larva should have been equal. Thus, for each fly-wasp pair, we compared the average number of wasp eggs laid per 10 dissected fly larvae to the variance in the number wasp eggs laid per 10 dissected fly larvae across the three replicates of each treatment, using one-tailed paired t-tests. Although some figures show data for each wasp strain separately, values for wasp strains of the same species were averaged into single species values for all statistical analyses unless otherwise noted.

Results

Hemocytes

D. suzukii hemocytes were morphologically similar to those of *D. melanogaster* (Figure 3). In normal *D. suzukii* larvae, there were an abundance of small round cells in the hemolymph that were presumably homologous to plasmatocytes. In *D. suzukii* infected by wasps, medium-sized round cells resembling podocytes became much more numerous, as well as large irregular shaped cells that resembled *D. melanogaster* lamellocytes. Heating *D. suzukii* larvae resulted in the formation of darkened cells throughout the hemocoel. In *D. melanogaster,* this phenomenon has been attributed to the self-melanization of crystal cells,

and suggested that *D. suzukii* also possesses hemocytes responsible for carrying melanization factors. Interestingly, while all self-melanized crystal cells in *D. melanogaster* were dark black (Figure 3C), *D. suzukii* showed both brown and black cells (Figure 3D, inset). Finally, *D. suzukii* larvae encapsulated and melanized wasp eggs with hundreds of hemocytes that flattened and spread over the wasp eggs to form a tight capsule (Figure 3E, 3F). Thus, the stereotypic melanotic encapsulation response used by *D. melanogaster* against parasitoid wasps appears to be conserved in its relative, *D. suzukii*.

Though hemocyte morphology was similar in the two fly species, we found significant differences in constitutive and induced hemocyte counts between *D. melanogaster* and *D. suzukii*. We used two methods for inducing immune responses in these flies. First, we infected flies with wasp strain LbG486, which is relatively avirulent in *D. melanogaster* and has been shown to induce production of hemocytes, and especially lamellocytes, in particular infected *D. melanogaster* strains [42,43]. Second, in order to stimulate lamellocyte production in the absence of any possible immune inhibitory effects of wasp venoms, we pierced *D. melanogaster* and *D. suzukii* larvae with sterile needles [33].

We tested the effects of fly species and immune challenge on fly hemocyte counts using standard regression methods (Figure 4). We found consistent, significant species effects on plasmatocyte, podocyte, and lamellocyte numbers. Across time-points and immune treatments, *D. suzukii* had significantly more plasmatocytes, producing up to five times more plasmatocytes than *D. melanogaster*. *D. suzukii* larvae also produced significantly more podocytes than *D. melanogaster*, including constitutively produced podocytes, which are not normally found in *D. melanogaster* larvae. Furthermore, *D. suzukii* larvae produced

significantly more lamellocytes than *D. melanogaster* larvae. We found no effect of immune challenge on plasmatocyte or podocyte numbers, although as expected there were significantly more lamellocytes in immune-challenged flies. Interestingly, the *D. melanogaster* genome strain used in the present study was not resistant to LbG486, unlike *D. suzukii*, (see below), and also showed no significant increase in lamellocyte numbers at two time-points post-infection when infected by LbG486 (Figure 4A, 4B). Finally, there were significant species-by-immune challenge interaction effects on podocyte and lamellocyte numbers in some experiments, usually due to significantly greater induction of these cell types after an immune challenge in *D. suzukii*. Thus, like *D. melanogaster*, *D. suzukii* induces hematopoiesis and/or hemocyte differentiation during a cellular immune response, although this induction is often stronger than that observed in *D. melanogaster*.

We next tested the effects of fly species and immune challenge on fly crystal cell counts using standard regression methods (Figure 5). There was a significant effect of species on crystal cell numbers in the piercing experiment, whereby *D. suzukii* had more than three times the number of constitutively produced crystal cells compared to *D. melanogaster* (Figure 5B). There was a similar, albeit non-significant trend in the wasp-attack experiment (Figure 5A). There were consistent, significant immune challenge effects of crystal cell counts, whereby both species showed significant reductions in crystal cell numbers following wasp infection or piercing, suggesting either crystal cells or their crystals (which are thought to contain the melanization precursors [36]) were spent during the wound healing or immune responses. Significant melanization was observed around the wound site in both species.

In order to confirm that hemocyte count differences between *D. melanogaster* and *D. suzukii* are general, we conducted further hemocyte counts experiments using two more strains of both fly species (Figure 6). Once again, we found a significant effect of species on constitutive numbers of plasmatocytes, podocoytes, and crystal cells, with the *D. suzukii* strains having greater numbers of these cell types in every case.

Fly Resistance

In the next experiment, both host species were infected with a panel of parasitoid wasps. Since we did not observe the flies and wasps for the duration of the infection period, it was important to know whether wasp infection rates were similar across the two fly species, so that any difference in fly eclosion could be attributed to a successful encapsulation response rather than a lack of infection. We compared the average number of eggs laid in the larvae of both fly species by the panel of parasitoid wasps. Although significant differences existed in the number of eggs laid by different wasp strains within a fly species (*D. melanogaster* ANOVA $p < 10^{-4}$, *D. suzukii* ANOVA $p < 10^{-4}$) (Figure 7), there was no overall difference between fly species in the number of eggs laid by the different wasp species (Figure 8), which averaged close to 1.25 eggs per fly larva in both fly hosts. Thus there was no evidence of an overall infection preference by wasps for one fly species over the other, and no evidence of differences in alternative mechanisms of host defense, such as behavioral or physical immunity (*e.g.*, a thickened cuticle) by the flies.

D. suzukii was able to melanotically encapsulate at least a small proportion of eggs from all 21 larval parasitoid wasp strains tested, whereas *D. melanogaster* was able to encapsulate some proportion of eggs from only 8 of 21 wasp strains (LbFr, LbG486, Lclav, GFl, GHaw, AtFr,

AtSw, and Aphae) and only 5 of 12 wasp species (Figure 9A). The difference in the proportion of wasp species that the flies could melanotically encapsulate was statistically significant (Fisher's exact test $p = 0.005$). Qualitative melanotic encapsulation differences between *D. melanogaster* and *D. suzukii* held across additional strains tested of both species (Figure 9B). As expected, the *D. suzukii* strains were able to encapsulate 3 of the 4 larval parasites tested (Lb17, GxUg, Apleu, but not Ajap), while *D. melanogaster* was not able to encapsulate any of the parasites.

D. suzukii was also consistently more resistant to our panel of parasitoid wasp species than *D. melanogaster* (Figure 10, 11). A greater proportion of *D. suzukii* eclosed after wasp infection compared to *D. melanogaster* for 20 of the 24 wasp strains tested, the exceptions being *D. suzukii* infected by wasp strains GFl and GHaw (for which no flies of either species eclosed), Ajap, and TriCal. This corresponded to a significantly higher fly eclosion rate for *D. suzukii* compared to *D. melanogaster* across wasp species (Figure 11A). Furthermore, a lesser proportion of wasps eclosed from infected *D. suzukii* larvae compared to *D. melanogaster* for 19 of the 24 wasp strains tested, the exceptions being *D. suzukii* infected by wasp strains GFl, GHaw, Ajap, TriCal, and Pachy. This corresponded to a significantly lower wasp eclosion rate in *D. suzukii* compared to *D. melanogaster* across wasp species (Figure 11B). The proportion of attacks that led to death of both the fly and the wasp growing within the fly was also lower in *D. suzukii*, with *D. suzukii* showing a lower proportion of death than *D. melanogaster* for 17 of the 24 wasp strains tested. However, this difference was not significant across wasp species (Figure 11C). When we tested additional strains of both *D. suzukii* and *D. melanogaster*, we found qualitatively similar eclosion results (Figure 12). As expected, a greater proportion of *D. suzukii* eclosed following infection

compared to *D. melanogaster* for 3 wasp strains (Lb17, GxUg, Apleu) *D. suzukii* was previously successful against, but not for two wasp strains *D. suzukii* previously did poorly against (Ajap, TriCal).

Given our understanding of the Drosophila immune response against wasp parasitism, we expect that flies that successfully encapsulate particular wasp species will also have greater eclosion success against those same wasp species. To test this expectation, we assayed for correlations between encapsulation success and fly eclosion for both flies species infected by the panel of wasp species. Although we found a trend in the expected direction for both fly species, there was no significant correlation in either fly species (Figure 13).

Wasp Choice

Previous work using *D. melanogaster* has shown that wasps can differentiate between infected and un-infected flies, and that they preferentially lay eggs in fly hosts that have not already been infected [38,39,40,41,44,45]. This preference is presumably adaptive because it limits competition between juvenile wasps that require the resources from an entire fly to complete development. Such preference should lead to an under-dispersion of wasp eggs in any group of infected fly larvae, *i.e.,* a more even distribution of eggs per larvae than expected by chance. We found significant under-dispersion of wasp eggs in *D. melanogaster* larvae for 15 of the 21 larval parasite wasp strains (Figure 7). The wasp strains that laid the most eggs in *D. melanogaster* tended to show the least under-dispersion, suggesting that the wasps could not differentiate between infected flies once they were infected with more than one wasp egg [38,39]. Only 4 of 21 wasp strains showed a significant under-dispersion of eggs across *D. suzukii* larvae. This suggests that whatever cue the wasps use to identify infected *D.*

melanogaster larvae, whether it is a tag left by the previous wasp or some aspect of the *D. melanogaster* response to infection [40], is generally missing in *D. suzukii* larvae. In no fly-wasp interaction was there a significant over-dispersion of wasp eggs.

Drosophila parasitoid wasps can also distinguish between fly host species, and preferentially lay eggs in host species in which their offspring have a higher chance of survival [29,46,47]. We tested whether larval parasitoid wasps tended to lay more eggs in the fly hosts that their offspring more successfully eclosed from in our trials (note that in our trials the wasps did not have a choice between host species, only whether or not to lay eggs in a single given host) (Figure 14). There was no relationship between wasp species success and the number of eggs laid per larva with *D. melanogaster* as host ($r^2 = 0.0327$, ANOVA $p = 0.5739$). For *D. suzukii*, however, there was a highly significant relationship ($r^2 = 0.5847$, ANOVA $p = 0.0038$) that was due in large part to the wasp species *A. japonica* (strain Ajap) and *Ganaspis sp.* (combined strains GFl and GHaw). Ajap in particular laid the highest number of eggs in *D. suzukii* in our infection trials, and also had the highest eclosion success.

Specificity In Fly-Wasp Interactions

As described above, we found significant differences in the number of eggs laid by different wasp strains within fly species but not between fly species. This could mean that wasps that lay higher numbers of eggs in *D. melanogaster* also lay higher numbers of eggs in *D. suzukii*, *i.e.,* some wasps could have generally higher egglay rates than others. However, there was no correlation between the number of eggs laid in *D. melanogaster* and the number of eggs laid in *D. suzukii* for the panel of wasp species ($r^2 = 0.0159$, ANOVA $p = 0.6964$), suggesting that egglay rate is a plastic wasp trait that wasps tailor to the host species they encounter.

There were significant differences across the panel of wasp strains in the infection outcomes within fly species (ANOVA $p < 10^{-4}$ for all six comparisons: fly survival, wasp survival, death in *D. melanogaster*, *D. suzukii*) (Figure 10). Although these differences in infection outcomes were due to significant variation both between wasp species and within wasp species (variation amongst strains), the largest differences in infection outcomes are seen between wasp species rather than wasp strains. For each fly host, some wasp species were very successful infectors, some were very susceptible to the fly immune responses, and some induced a large amount of death. As described above there were also significant differences in the infection outcomes between fly species. Despite the superior wasp resistance of *D. suzukii*, it is possible that wasps that were more successful in *D. melanogaster* were also more successful in *D. suzukii*, *i.e.,* some wasps are generally more virulent than others. However, there was no correlation in the proportions of any of the three infection outcomes between *D. melanogaster* and *D. suzukii* (fly success $r^2 = 0.1024$, ANOVA $p = 0.2648$; wasp success $r^2 = 0.0012$, ANOVA $p = 0.9077$; death $r^2 = 0.0408$, ANOVA $p = 0.4889$). This indicates there was specificity in the outcome of wasp infections depending on the particular host fly species, despite *D. melanogaster* and *D. suzukii* being part of the same Drosophila species group.

There is a strong influence of wasp phylogeny on *D. melanogaster* infection outcomes. Members of the Leptopilina clade that includes *L. boulardi* and *L. heterotoma* are very successful against *D. melanogaster*, showing an average of 69% wasp eclosion. Infections by *L. clavipes* and members of the genus Ganaspis, which are likewise members of the family Figitidae, did not result in high eclosion rates in *D. melanogaster*, but instead caused an average

of 79% death of *D. melanogaster* larvae (Figure 8). Thus, *D. melanogaster* appears to lack an immune mechanism to counter shared virulence strategies of Figitid parasitoids. There appeared to be little influence of wasp phylogeny on the ultimate outcome of *D. suzukii*–wasp interactions, as *D. suzukii* was resistant to the majority of wasps tested. However, the larval parasitoid that eclosed from *D. suzukii* at the greatest rate (79%), *A. japonica*, is endemic to Japan where it is sympatric with *D. suzukii*.

Discussion

Previous studies have shown that fly species and strains with a greater constitutive production of hemocytes are more resistant against and/or are better able to encapsulate parasitoid wasp eggs [12,18,19,48]. Although a correlation does not necessarily imply causation, these data suggest that evolution of higher constitutive production of hemocytes is a relatively simple way for hosts to defeat one of their most common classes of parasites. However, the previous studies were limited to flies in the melanogaster subgroup and to a few wasp species/strains that represent only a small fraction of the diverse virulence strategies used by Drosophila parasitoid wasps. To determine if increased hemocyte production by flies is a panacea against wasp infection, we first compared hemocyte numbers between *D. melanogaster* and *D. suzukii*, a relative of *D. melanogaster* outside the melanogaster subgroup.

We found that third instar *D. suzukii* larvae made constitutively greater numbers of plasmatocytes, podocytes, and crystal cells than *D. melanogaster* larvae, and also induce greater production of podocytes and lamellocytes (Figure 4, 5, 6). Compared to our recently wild-derived *D. suzukii* strains, the *D. melanogaster* genome strain we used may have had relatively

poor genetic immune ability for its species due to its homozyosity and its long-term selection in a lab environment. However, hemocyte counts from the two additional wild-caught *D. melanogaster* strains we assayed were very similar to those from the genome strain. The hemocyte numbers we observed in our *D. melanogaster* strains were also similar to those seen in a variety of other studies where the unit of measurement was cells per larva [33,35,42], and also appeared similar to numbers found in studies that counted cells per volume of hemolymph (using a rough conversion factor of approximately 0.5 uL hemolymph per third instar larva) [12,19,43,49,50]. Thus, we have no reason to believe that differences we observe between our *D. melanogaster* and *D. suzukii* strains were due to a biased sampling of strains rather than actual species differences. In comparison with hemocyte numbers from other studies, *D. suzukii* appears to have somewhat greater constitutive hemocyte counts than *D. simulans*, which has the highest counts of any member of the melanogaster subgroup [12].

Using a diverse panel of parasitoid wasp strains and species, we found that infection rates in *D. melanogaster* and *D. suzukii* were similar (Figure 7, 8), but that *D. suzukii* was significantly better at melanotically encapsulating, and surviving infection by, the wasps (Figure 9, 10, 11,12). The panel of wasps included relatively specialist and generalist wasp species, such as *L. boulardi* and *L. heterotoma*, respectively [15], as well as relatively immune evasive versus immune suppressive wasp species, such as *A. tabida* and *G. xanthopoda*, respectively [51,52]. Our data suggest that a general protection against parasitoid wasps is afforded to fly species that have higher constitutive hemocyte loads. The association between hemocyte load and encapsulation ability reported previously [12] also appears to extend beyond the melanogaster subgroup of fly hosts, as *D. suzukii* is part of the melanogaster group but not

the melanogaster subgroup. Future infection trials using the same panel of parasitoid wasps, but a much wider range of fly species, will be needed for determining the true extent of the relationship between hemocyte load and resistance against parasitoid wasps.

The current model for the melanotic encapsulation process is that plasmatocytes act as sentinels of wasp infection and signal to activate other circulating plasmatocytes as well as the lymph gland once infection is recognized [4,5]. The activated plasmatocytes develop cytoskeletal projections and become known as podocytes, which may be an intermediate form between the smaller plasmatocytes and larger lamellocytes [6,7]. Lamellocytes are also induced via differentiation of pro-hemocytes in the lymph gland. The lamellocytes then migrate towards and surround the wasp egg, forming a tight capsule. The capsule becomes melanized, but it is not yet known whether melanin precursors stored in crystal cells are used in this process. Thus, any or all of the hemocyte cell types that *D. suzukii* produced in excess may have been responsible for the relatively high resistance of *D. suzukii* against wasp eggs.

Flies with more hemocytes may suffer fewer effects of wasp venom for a variety of reasons, enabling them to mount a quicker and/or stronger encapsulation reaction against wasps. For example, venoms often alter hemocyte structure and function [50,53], and thus an increased number of hemocytes could potentially dilute the effects of a standard dose of venom. Alternatively, hemocytes may be responsible for destroying venom components found in the hemolymph, via endocytosis or some other mechanism, preventing the venom from exerting its effects on other tissues. It is unclear whether an excess of constitutively produced hemocytes (plasmatocytes, crystal cells) or the increased induced production of podocytes and lamellocytes drives the relationship between hemocyte counts and wasp

resistance, but the distinction may be unimportant given that constitutively produced cells can differentiate into induced cell types [6,7]. However, in support of the idea that constitutive production of hemocytes alone is not sufficient for wasp resistance, Drosophila species of the obscura group that make relatively high numbers of plasmatocytes, but apparently do not produce a lamellocyte class of cells, are unable to encapsulate foreign objects and are highly susceptible to wasp infection [54,55].

Unlike for *D. melanogaster*, larval parasitoid wasps rarely under-dispersed their eggs across *D. suzukii* larvae. Wasps are thought to discriminate naïve host larvae from previously infected larvae either by recognizing a mark left by the previous wasp, or by recognizing the host response to infection [40]. Given that *D. suzukii* has a significantly more robust immune response against wasp infection than *D. melanogaster*, it seems unlikely that these wasps use host immune cues to avoid superparasitism. If fly hemocytes are responsible for clearing wasp venom components from the hemolymph, wasp "possession marks" might also be lost in fly hosts that make abundant hemocytes, leading to more random dispersal of wasp eggs across host larvae.

We expected to find a correlation between encapsulation ability and fly success in both *D. melanogaster* and *D. suzukii*, but although there was a trend in this direction, the correlations were not significant (Figure 13). Three factors likely contribute to this lack of correlation. First, we counted fly larvae as having successful encapsulations if any encapsulation was seen, even if flies were super-parasitized and hadn't encapsulated all wasp eggs they were infected by. Thus, flies scored as showing encapsulation could still succumb to infection. Second, some fly-wasp combinations that yielded encapsulations culminated in neither fly

nor wasp eclosion, but high rates of death of by both fly and wasp. Third, wasp parasites sometimes die inside their fly hosts even if the fly has not encapsulated them by the time-point we assayed.

Interestingly, *D. suzukii* does not have a clear survival advantage over *D. melanogaster* when infected by the two pupal parasite species (three strains) in our panel of wasps. Very little is known about the determinants of infection outcomes with regards to pupal parasites of flies, or even whether venom plays an important role. Although Trichopria acts as a pupal endoparasitoid, the Drosophila pupal stage does not appear able to mount melanotic encapsulation responses against them. Furthermore, Pachycrepoideus lays its eggs in the space between the pupal case and the pupa, and acts as an ectoparasite for most of its development [29], which could negate any ability the flies have to mount an internal, physiological immune response. In other systems, pupal parasitoid wasps are known to have more generalist host ranges than larval parasites [30,31], but they do not have unlimited host ranges either, so some specificity in their utilization of host resources is inherent. Although our data suggests increased hemocyte load has little effect on fly resistance against pupal parasites, a definitive statement will require data from a greater range of pupal parasite species.

Still, if increased hemocyte load provides general protection against larval parasitoids, why do some fly species, such as *D. melanogaster*, produce such low numbers compared to their close relatives? Hosts face an evolutionary tradeoff between investing in immune responses against parasites versus investing in other aspects of fitness [56,57,58,59]. The constitutive production and maintenance of hemocytes must obviously impart an energetic cost on the

host, diverting resources from other aspects of host fitness. Thus, if hosts are rarely infected by wasps in nature, or are commonly infected by specialist wasps that can overcome hemocyte-based immunity, it may make evolutionary sense to invest in fecundity rather than immunity, or in other aspects of immunity, such as behavioral immunity. On the other hand, investment in high constitutive hemocyte levels might be selected in host species that are commonly infected by non-specialist parasites.

Although *D. suzukii* is generally more resistant against larval wasp parasites than *D. melanogaster*, there were a small number of obvious exceptions. *A. japonica* is sympatric with *D. suzukii* in its native east Asian range, and was significantly more successful at infecting *D. suzukii* than *D. melanogaster*. Previous studies showed *A. japonica* successfully parasitizes *D. suzukii* both in the field and in the lab [60,61]. *A japonica* also laid approximately three times more eggs in *D. suzukii* than in *D. melanogaster*, and laid the highest number of eggs in *D. suzukii* of any larval parasitoid wasp. Altogether, these data suggest *A. japonica* may have co-evolved a specialized virulence strategy able to overcome the high hemocyte load of *D. suzukii*, and may have evolved an infection preference for *D. suzukii* as well. The only other larval parasite able to eclose from *D. suzukii* hosts at any appreciable rate is *Ganaspis sp.*, an undescribed species collected in Florida and Hawaii. Although *G. xanthopoda* was found to emerge from *D. suzukii* pupae collected in the field in Japan [61], the two *G. xanthopoda* strains used in this study, from Hawaii and Uganda, were very poor infectors of *D. suzukii*, suggesting populations of this wasp species may have locally adapted to *D. suzukii* host use in Japan.

D. suzukii has recently spread into Europe and North America as a pest species [20,21,22,23]. It was first documented in the United States in California in 2008, from where it quickly spread to Oregon and Washington. In these west coast states, *D. suzukii* was responsible for up to 80% yield losses in berry and cherry crops depending on location, and is estimated to be causing yearly monetary losses in the range of 500 million dollars [62,63]. In 2009, *D. suzukii* became established in Florida, and in 2010 reports of collections were made from a handful of new states [23]. However, experimental studies testing the efficacy of various management strategies for *D. suzukii* are as yet lacking [64,65].

One common pest management strategy is the use of biocontrol agents such as natural enemies (parasites, predators) [66], and parasitoid wasps have successfully controlled numerous other arthropod pests in the past [24,25,26,27,28]. Furthermore, Drosophila parasitoid wasps often infect a large proportion of fly larvae in natural populations [1,2,3], and the potential for an endemic Figitid species (*L. boulardi*) to control native Drosophila populations in California was previously considered [67]. It appears the wasp species with the highest potential for use in biocontrol of *D. suzukii* are the larval parasites *A. japonica* and *Ganaspis sp.*, and the pupal parasite *Trichopria sp.*. *A. citri* might also be considered a potential biocontrol agent for *D. suzukii* because of the high death rates it caused in *D. suzukii*, but this wasp had much higher eclosion rates using *D. melanogaster* as host than *D. suzukii*. Because infection trial conditions in this study were designed to be ideal for success of the wasps, and such conditions (easy access to hosts, no competition with other parasites, controlled temperature, abundant resources, etc) are unlikely to be replicated in the field, extensive field experiments will be required to assess the efficacy of the use of parasitoid wasps in *D. suzukii* biocontrol in practice.

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Figure 1

Fly morphology and behavior. (A) Female *D. melanogaster*; (B) female *D. suzukii*; (C) serrated ovipositor from female *D. suzukii*; (D) tunnel excavated by *D. suzukii* larva through agar food plate.

Figure 2

Phylogenetic relationships and provenance of wasps used in this study. Tree topology is derived from previous phylogenetic studies of Hymenopteran families [68], the family Figitidae [69,70], and the family Braconidae [71]. Branch lengths are approximated.

Figure 3

D. suzukii hemocytes and encapsulation of wasp eggs. (A) A 0.25 x 0.25 x 0.1 mm hemocytometer field from normal *D. suzukii* larvae showing abundant plasmatocytes; (B) hemocytometer field from *D. suzukii* larvae 12 hours after infection by wasp strain LbG486

showing increased podocyte and lamellocyte numbers; (C) control *D. melanogaster* larva with melanized crystal cells; (D) control *D. suzukii* larva with melanized crystal cells, showing color variation in inset; (E) initiation of encapsulation of LbG486 egg by *D. suzukii* showing loose hemocyte aggregation and melanization at anterior and posterior tips of egg; (F) LbG486 egg melanotically encapsulated by *D. suzukii*, showing surrounding layer of tightly spread hemocytes.

Figure 4

Hemocyte count comparison between *D. melanogaster* and *D. suzukii*. (A) 12 hours after infection by wasp strain LbG486; (B) 24 hours after infection by wasp strain LbG486; (C) 24 hours after piercing with a sterile needle. Average (+) standard deviation shown. Numbers are approximately one fortieth of the number of cells per one fly larva (Methods).

Figure 5

Crystal cell count comparison between *D. melanogaster* and *D. suzukii*. (A) 33 hours after infection by wasp strain LbG486; (B) 33 hours after piercing with a sterile needle. Average (+) standard deviation shown.

Figure 6

Hemocyte counts in other *D. melanogaster* and *D. suzukii* strains. (A) Constitutive plasmatocyte, podocyte, lamellocyte counts; (B) constitutive crystal cell counts. Average (+) standard deviation shown.

Figure 7

Numbers of eggs laid by each wasp strain in *D. melanogaster* (A) and *D. suzukii* (B). Average number of eggs per larva (+) standard deviation shown. ANOVA results compare egglay numbers within fly species across wasp treatments. * = significant under-dispersion of wasp eggs in fly larvae at $p < 0.05$ using a one-tailed paired t-test (Methods).

Figure 8

Parallel plot comparing average egglay numbers for each wasp species between hosts. There was no overall difference between fly species in numbers of eggs laid by wasps, nor was there a correlation between the number of eggs laid in *D. melanogaster* and the number of eggs laid in *D. suzukii* across the panel of wasp species (as indicated by the non-parallel connecting lines).

Figure 9

Encapsulation success of wasp-infected fly larvae. (A) Average proportion of fly larvae that encapsulated a wasp egg; (B) average proportion of fly larvae from additional fly strains that encapsulated a wasp egg.

Figure 10

Infection outcomes for host larvae infected by each wasp strain. Average (+) standard deviation shown for *D. melanogaster* (A) and *D. suzukii* (B). ANOVA results compare fly eclosion, wasp eclosion, or death proportions within fly species across wasp treatments.

Figure 11

Parallel plot comparing outcomes between host larvae infected by each wasp species. (A) fly eclosion; (B) wasp eclosion; (C) death. There were significant overall differences between fly species in fly eclosion and wasp eclosion proportions, but not in proportion dead. There is no correlation between fly eclosion, wasp eclosion, or death proportions between *D. melanogaster* and *D. suzukii* across the panel of wasp species (as indicated by the non-parallel connecting lines).

Figure 12

Infection outcomes for host larvae of other strains. (A, B) *D. melanogaster* extra strain 1 and 2; (C, D) *D. suzukii* extra strain 1 and 2. Average (+) standard deviation shown.

Figure 13

Relationship between encapsulation rate and fly eclosion. Average proportion of fly larvae that encapsulated a wasp egg for *D. melanogaster* (A) and *D. suzukii* (B).

Figure 14

Relationship between wasp eclosion success and number of eggs wasps choose to lay in a host. There was no significant relationship for the panel of wasp species attacking *D. melanogaster* (A), but there was a significant relationship for the panel of wasp species attacking *D. suzukii* (B).

Chapter 4

A role for nematocytes in the cellular encapsulation response mounted against parasitic wasps by the Drosophilid *Zaprionus indianus*.

Abstract

Among the most common parasites of Drosophila in nature are parasitoid wasps, which lay their eggs in fly larvae and pupae. *D. melanogaster* larvae can mount a cellular immune response against wasp eggs, but female wasps inject venom along with their eggs to block this immune response. Genetic variation in flies for immune resistance against wasps and genetic variation in wasps for virulence against flies largely determines the outcome of any fly-wasp interaction. Interestingly, up to 90% of the variation in fly resistance against wasp parasitism has been linked to a very simple mechanism: flies with increased constitutive blood cell (hemocyte) production are more resistant, as they are better able to mount the canonical melanotic encapsulation response. However, this relationship has not been tested for Drosophila hosts that do not have the same hemocyte composition to that of *D. melanogaster*. We analyzed hemocyte levels in 3 strains of *Zaprionus indianus*, a host having a minimally describe class of hemocyte—the nematocyte. Using a panel of 24 parasitoid wasp strains representing fifteen species, four families, and multiple virulence strategies, we found that *Z. indianus* was significantly resistant to wasp parasitism, being able to mount the melanotic encapsulation ability as characterized in *D. melanogaster*, but can also use nemacytes. At least one sympatric wasp species was a highly successful infector of *Z. indianus*, suggesting specialists can overcome the general resistance afforded to hosts by excessive hemocyte production, and found that such wasp venom targets nematocytes. Given that *Z. indianus* an emerging agricultural pest, identification of the few parasitoid wasps that successfully infect *Z. indianus* may have value for biocontrol.

Introduction

Cellular encapsulation of pathogens by host hemocytes (blood cells) is a well-conserved aspect of invertebrate immunity ([1], [2]), is important for resistance against pathogens in insect vectors of human disease (Richman, 1996), and is functionally similar to granuloma formation in vertebrates ([3]). In this immune response, pathogens in the hemolymph (blood) are recognized as foreign and hemocytes then migrate, adhere to, and consolidate around the pathogen, forming a tight multi-cellular multi-layered capsule. Free radicals are generated inside the capsule to kill the pathogen ([4], [5]). Cellular encapsulation usually occurs in response to pathogens that are too large or too numerous to be phagocytosed by individual hemocytes.

The lab fruit fly *D. melanogaster* has been used as a model system for understanding the encapsulation process, as this species readily mounts encapsulation responses against foreign objects. In *D. melanogaster*, hemocytes have been classified into three subtypes: (1) plasmatocytes comprise ~95% of all hemocytes in un-induced flies, act as sentinels of infection, and are responsible for phagocytosis and initial cell layers of developing capsules; (2) crystal cells comprise the remaining 5% of standing hemocytes and carry precursors for generating melanin, which is important in wound healing and is deposited around encapsulated objects; (3) lamellocytes are large flattened cells induced after infection that are responsible for forming the outside cellular layers of developing capsules ([6], [7]).

Parasitic wasps lay their eggs in the hemocoel of Drosophila larvae and pupae and induce a cellular encapsulation response in their hosts. These wasps are a common threat to juvenile Drosophila as upwards of 50% of fly larvae are found to be infected In natural populations ([8], [9])Four wasp families are known to infect Drosophila in nature: members of the

Braconidae and Figitidae infect Drosophila pupae, while members of the Diapriidae and Pteromalidae infect Drosophila pupae. Wasps inject venom into flies along with their eggs to suppress the fly encapsulation response. If their eggs successfully hatch, the wasp larvae grow within the fly larvae and pupae for several days before ultimately consuming the fly pupae from the inside out, eclosing from the hosts' pupal cases. Interestingly, the success of the cellular encapsulation response against wasps across Drosophila species is strongly correlated with constitutive and induced hemocyte loads in these species ([10], [11], [12], [13], [14]).

The genus Zaprionus comprises more than 50 described species ([15], [16], [17]) and is known to be phylogenetically imbedded within the genus Drosophila, although it's affinity to particular Drosophila subgenera is still unclear (Markow and O'Grady 2006 book). Zaprionus is distinctive in that all species possess distinct (usually longitudinal) light-colored stripes (Figure 1A, Figure 1B). The species *Z. indianus*, in particular, is native to Africa, the Middle East, and southern Eurasia, but has recently gained attention because of its spread to North and South America ([18], [15]) where it causes millions of dollars of damage annually as a pest species of figs ([19], [20]). Parasitic wasps have been successfully used as biocontrol agents against a wide range of agricultural pests ([21]).

Given *Z. indianus* is part of a unique Drosophilid subgenus and is a growing agricultural threat, we decided to test its resistance against a diverse panel of Drosophila parasitic wasps (Figure 2). This panel includes representatives from all four wasp families that infect Drosophila and multiple strains from a total of 15 wasp species. We were interested in what types of hemocytes *Z. indianus* carries, whether it mounts the canonical encapsulation

response described from *D. melanogaster*, and whether the numbers of constitutive and induced hemocytes it produces are correlated with its general resistance ability. As of yet, very little is known about the interaction between *Z. indianus* and parasitic wasps in nature, other than it can be infected by the Figitid Dicerataspis grenadensis ([22]).

Results/Discussion

Strain Creation

Zaprionus inidanus flies samples were collected and three highly inbred lines were formed by picking 5 virgin females and placing them with 10 males. These fly lines were then inbred for ten generations and were maintained in the lab on standardized cornmeal media before experimentation began. Our goal was to ensure that any result we may find could be applied to the *Z. indianus* species as a whole and not just a single strain**.**

Fly Resistance

Each of the three fly strains of *Z. indianus* was infected with a panel of parasitoid wasps from the families *Braconidae* and *Figitdae* (Fig 2). Since we did not observe the wasps during the attack period, we assayed egg numbers and proportion of eggs encapsulated in infected fly larvae. This step was performed so that any difference in fly eclosion successful/unsuccessful infection. We compared the average number of eggs that were melanotically encapsulated by each of the three strains against each wasp (Figure 3, A-C). Although there were differences in encapsulation ability with respect to which wasp species egg were encapsulated, overall there was no significant difference among the three fly strains in their encapsulation ability. Overall, *Z. indianus* was able to encapsulate some proportion at least 16 of 27 types of wasps used.

A significant proportion of *Z. indianus* flies elcosed following infection. In all three strains of Z. indianus, the flies eclosed after being infected with 18 of the 27 types of wasps tested in all three strains. *Z. indianus* failed to eclose after being infected with wasps Lgcam, LgSA, G1Fl, G2Atl, AcIC, or Tric1Fr. These treatments corresponded to a significant wasp eclosion rate (Figure 4, A-C). As *Z. indianus* originates from Africa, it makes sense that the Lg strains (both from Africa) could successfully infect *Z. indianus*. Moreover, the Gx strain from Uganda (GxUg) was successfully encapsulated by each of the three fly strains. Interestingly, the Gx species (which did not originate from Africa) could either successfully infect or kill *Z. indianus*.

Encapsulation

When examining a whole *Z. indianus* fly larva that had been attacked by an avirulent wasp, wasp eggs appeared to be melanized in a similar manner to that of *D. melanogaster* (Figure 5 A-B). Thus, we decided to dissect encapsulated wasp larvae from *Z. indianus* larvae and assay the hemocyte type responsible for the melanotic encapsulation observed.

In order to determine a possible mode of action, dissected wasp eggs were viewed with phase contrast microscopy. We observed a very tight capsule encasing the melanized wasp egg (Figure 5 C-D). However, the capsule layer was too tight to discern what cell type was involved.

To discern the types of blood cells that surrounded the wasp egg, dissected wasp eggs were treated with a 1% solution of EDTA. EDTA is known to disrupt calcium mediated cell adhesion and has previously been shown to disrupt capsules ([23]). Eggs were observed 5

and 20 minutes post treatment (Figure 6 A-B). Hemocytes detached from the encapsulated wasp egg. We observed both lamellocytes and nematocytes among them, indicating a novel form of cellular encapsulation in *Z. indianus*. In addition, we also found wasp eggs encapsulated by a mass of cells that appeared to be melanized nematocytes. While we could not verify their identity, it suggests that nematocytes might be playing a role in melanization.

When wasp eggs were not encapsulated, wasp larvae successfully hatched and began to devour the fly tissue (Figure 5 E). No encapsulated wasp larvae were observed and no hemocytes were observed on the larvae. This suggests that nematocyte- and lamellocyte-mediated encapsulation in *Z. indianus* is specific for the egg and excludes the larva in every wasp we tested.

Classification of Blood Cells

Our morphological classification of hemocytes in *Z. indianus* is based on previous studies in *D. willistoni* and *D. melanogaster* ([24],[25]). To visualize *Z. indianus* hemocytes, we dissected immune induced (pierced with a sterile needle) and control third instar fly larvae and viewed hemolymph under phase-contrast optics. We found five hemocyte classes in *Z. indianus*:

Class 1—Plasmatocytes. The plasmatocytes (Figure 7A) are usually round and are the most common cells. The nucleus is visible through a phase contrast microscope. This cell type is morphologically similar to *D. melanogaster* plasmatocytes. We find these cells in both induced and uninduced fly larvae of all strains of *Z. indianus* tested.

Class 2—Podocytes. The podocytes (Figure 7B) are larger in size than plasmatocytes. They are distinguished by their light shade and dark nucleus. This cell type is

morphologically similar to *D. melanogaster* podocytes. These cells were found in both induced and uninduced fly larvae in all strains tested.

Class 3—Lamellocytes. The lamellocytes (Figure 7C) are even larger than podocytes. The cells flatten out on the slide and resemble a fried egg. This cell type is morphologically similar to *D. melanogaster* lamellocytes. These cells were only found in induced fly larvae in all strains tested.

Class 4—Nematocytes. The extremely fusiform cell type isolated from the fly larvae is termed the nematocytes (Figure 7D, Figure 7E). This cell type was found in two of the uninduced fly strains, but was essentially absent in one strain, suggesting that not all strains of *Z. indianus* has constitutive nematocyte production. Once induced, all three fly strains contain nematocytes that have increased in size. This occurred in the strains that contained constitutive nematocytes, suggesting that induction changes nematocyte physiology. Some nematocytes have extensions that are usually very fine. This cell type is absent in *D. melanogaster* and its closely related species. We have observed similar hemocytes in distant relatives of *D. melanogaster* (data not shown).

Class 5—Crystal Cells. Small, circular cells that burst upon boiling the fly larva, allowing cells to be scored (Figure 9B). These cells congregate mostly at the posterior end of the fly larva, and vary in color between black and brown.

Based on the identification of nematocytes by the Rizkis in *D. willistoni* and by our identification in *Z. indiaunus*, we were interested in identifying how wide spread the presence of nematocytes are in the genus *Drosophila*. We assayed hemocyte class across a panel of 25 species in the genus *Drosophila*, looking specifically for the nematocyte clade of hemocyte. Across the phylogeny, we found the nemocyte class to be localized to the subgenus

Drosophila on the phylogenetic tree (Figure 4). In particular, we found nematocytes in *D. immigrans* (Figure 5A), *D. mojavensis* (Figure 5B), *D. paramelanica* (Figure 5C), *D. virilis* (Figure 5D), *D. hydei* (Figure 5E), and *D. funebris* (Figure 5F). Our observations suggest that the nematocyte class is an ancient hemocyte clade that was lost in much of the genus *Drosophila.*

Nematocyte Size Change

Constitutive nematocyte size was found in the range between 4 and 25 μ in length. Once induced, all three fly strains contain nematocytes that have increased in size (Figure 9 A-C). This occurred in the strains that contained constitutive nematocytes, suggesting that induction changes nematocyte physiology. Some nematocytes have extensions that are usually very fine. Induced nematocyte size was found to range between 30 and 150 μ in length (Figure 10).

Hemocytes

To characterize constitutive hemocyte numbers, we used uninfected fly larvae (Figure 11 A,C,E). Plasmatocyte and podocyte levels were similar in the three strains of *Z. indianus* tested. No lamellocytes were observed in the absence of infection. Nematocytes were observed in high numbers in two of the three strains, while one strain had almost no constitutive nematocyte production (Figure 12 A-C), suggesting that constitutive production of these blood cells is regulated by an unknown induction mechanism. This also suggests that cellular encapsulation is highly variable in nature with respect to what hemocyte types are involved.

To stimulate lamellocyte production in the absence of wasp attack, we pierced *Z. indianus* larvae with sterile needles (Figure 11 B,D,F). This was used as a proxy to avoid any possible inhibitory effects of wasp venom ([26]). We found lamellocytes significantly increased in all three strains following wounding. In addition we found nematocyte levels of uninduced and induced larvae in two of the three strains did not change, suggesting that nematocytes are not part of the wound healing process. Finally, the last strain, which lacked constitutive levels of nematocytes, significantly increased its nematocyte count compared. This suggests that nematocytes are induced following wounding (Figure 12 A-C), leading us to hypothesize that they are involved in immunity, in a similar manner to lamellocytes.

To assess this hypothesis, we tested four different wasp species against the three strains of *Z. indianus*. We chose two avirulent wasps (LvHaw, Aph1Atl), and two virulent wasps (Lgcam, AcIC). Pairs of virulent and avirulent wasps came from the same family in order to see if any hemocyte changes were wasp-dependent, or a general response. When infected with avirulent waps, we found all three strains of *Z. indianus* larvae induced lamellocyte and nematocyte production significantly (Figure 12 A-C). Because nematocytes were produced upon infection by avirulent wasp also in a strain lacking constitutive nematocyte production, we concluded that nematocytes played a role in the cellular response to wasp attack (Figure 12 C). When infected with virulent wasps, we found all three strains of *Z. indianus* lacked both nematocytes and lamellocytes (Figure 12 A-C). The lack of lamellocytes in all three strains also suggests that lamellocyte differentiation is blocked by virulent wasp attack.

The lack of nematocytes allowed us to hypothesize two conclusions; one stems from the two strains that have constitutive nematocyte production. Since these strains lost their

nematocytes when attacked by virulent wasp strains, we conclude that wasp venom kills nematocytes. Our second conclusion regarding nematocytes stems from the strain that lacks constitutive nematocye levels—because nemactocytes were not present following virulent wasp attack, we postulate that the wasp venom not only acts to actively kill nematocytes, but might also target induction of nematocytes.

To assay the role of crystal cells, we assayed both uninduced and pierced *Z. indianus* larvae of all three strains. All uninduced fly larvae showed high levels of crystal cells (Figure 13, A-C; Figure 8, A-B). All pierced fly larvae showed significantly decreased crystal cell numbers, almost having no crystal cells left (Figure 13 A-C; Figure 8 C). The wound site contained melanin deposits, but the rest of each larva appeared empty (Figure 8 D). This suggested that crystal cells were involved in the wound healing response and were decreased following non-infectious injury (piercing). Previous studies have indicated that crystal cells are decreased following piercing and wasp attack ([26]).

Other Species

Based on the identification of nematocytes by the Rizkis in *D. willistoni* and by our identification in *Z. indianus*, we were interested in identifying how wide spread the presence of nematocytes are in the genus *Drosophila*. We assayed hemocyte class across a panel of 25 species in the genus *Drosophila*, looking specifically for the nematocyte clade of hemocyte. Across the phylogeny, we found the nemocyte class to be localized to the subgenus *Drosophila* on the phylogenetic tree (Figure 14). In particular, we found nematocytes in *D. immigrans* (Figure 15 A), *D. mojavensis* (Figure 15 B), *D. paramelanica* (Figure 15C), *D. virilis*

(Figure 15 D), *D. hydei* (Figure 15 E), and *D. funebris* (Figure 15 F). Our observations suggest that the nematocyte class is an ancient hemocyte clade that was lost in much of the genus *Drosophila*.

Previous work has also shown the presence of nematocyte like cells in the army ant ([27]). This suggests that nematocytes are not only in the genus Drosophila, but are spread far throughout the insect order.

Conclusions

Although *Z. indianus* is generally resistant against larval parasites, there are a few exceptions. *L.g* is sympatric with *Z. indianus* in its native Africa and is significantly more successful at infecting *Z. indianus* than other parasitoids tested. This data suggests that *L.g* has directly evolved virulence factors specifically for *Z. indianus* in order to overcome the high nematocyte load.

According to the canonical model of the melanotic encapsulation process in *D. melanogaster*, plasmatocytes act as the messengers following wasp infection by activating other plasmatocytes as well lamellocytes via the lymph gland. In addition, activated plasmatocytes develop projections and become known as podocytes (Honti 2010, Rizki 1957). The capsule becomes melanized by an unknown mechanism. Our study demonstrates that encapsulation ability is not limited to this rigid formula, but may involve different mechanisms. We made observations suggesting that nematocytes are also involved in cellular encapsulation following wasp infection. To our knowledge, this is a novel role for this poorly characterized

cell type.

Z. indianus spreads rapidly to newly colonized areas ([20]). There are large population sizes observed the second year of its presence in any colonized area. It has now invaded all suitable habitats in the southern USA ([19]). As a fig parasite, it has reduced fig production in many areas by 40-50%. The fly has decreased fig exports by up to 80% ([28]). Experimental studies testing the efficacy of various management strategies to contain the pest are as yet lacking.

One typical strategy is to use biocontrol agents that are natural enemies (e.g., parasites) of the pest (Dent 1995). In the past, parasitoid wasps have been used successfully to control other arthropod pests ([29],[30]; [31]; [32]; [33]). In addition, *Drosophila* larvae are infected up to 80% in nature, and the potential for endemic species to control populations should be considered. It appears that wasp species with the highest potential for use in biocontrol of *Z. indianus* are the larval parasites *A. citri*, *Ganaspis sp, L. guineaensis*, and the pupal parasite *Trichopria Fr.*

Methods

(a) Insects

Zaprionus inidanus flies collected from Atlanta, GA in the summer of 2011 by TAS. Three highly inbred lines were formed by picking 5 virgin females and placing them with 10 males. These fly lines were then inbred for ten generations and were maintained in the lab on

standardized cornmeal media. The insects were kept in a 25 °C incubator with 12-12 day-night cycles.

A total of 27 Drosophila parasitoid wasp strains were used for infection trials on *Z. indianus* (Figure 2). Strains LgG500 and LgG510 were provided by R. Allemand, strain LbG486 was provided by D. Hultmark, strains Lclav, Ajap, Apleu, and Acit were provided by J. van Alphen, strain GxUg was provided by J. Pool, and strain AtFr was provided by B. Wertheim. All other strains were collected by the Schlenke lab. These wasp strains represent: (1) at least 14 species, (2) representatives of all four *Hymenopteran* families known to infect *Drosophila*, (3) larval and pupal parasites, and (4) a worldwide range of collection localities (Figure 2). Morphology and cytochrome oxidase I (COI) sequences from the two *Trichopria sp.* strains suggested they were representatives of the same species, perhaps *Trichopria drosophila* (Ashmead). Furthermore, morphology and COI sequences from the two *Ganaspis sp.* strains suggest they are representatives of a single undescribed species. All wasp species were maintained in the lab on *D. melanogaster* strain Canton S, with the exception of *L. clavipes*, *A. tabida*, *Aphaereta sp.*, *Lg SA*, *bygan*, and *bylepto*, which were maintained on *D. virilis*. To grow wasps, adult flies were allowed to lay eggs in standard *Drosophila* medium for several days before they were replaced by adult wasps, which then attacked the developing fly larvae or pupae. Wasp vials were supplemented with approximately 500 μL of a 50% honey/water solution applied to the inside of the cotton vial plugs. Wasp strains are available upon request.

(b) Hemocyte Counts

In the first experiment, *Z. indianus* larvae were either untreated or were pierced with a sterile needle. This simulated the wound healing response normally associated with wasp attack.

Such wounding had been previously shown to induce lamellocyte production ([26]). Five replicates were undertaken, where 15 fly larvae were rinsed in 1X PBS and dried on Kimwipes. They were then immobilized on double-adhesive-sided tape and their posterior cuticles were pierced by a flame-sterilized 0.1mm diameter stainless steel dissecting pin. Care was taken to avoid piercing internal organs. The larvae were then removed with a wet paintbrush and placed into a moist chamber for one hour to allow for recovery before being moved to 35mm diameter Petri dishes filled with 1mL Drosophila medium. Control larvae were treated the same but without the pierce. Hemocytes and crystal cells were counted 24 hrs following treatment.

In the second experiment, 15 *Z. indianus* larvae were placed onto 35mm diameter Petri dishes filled with 1mL Drosophila medium, for a total of 3 replicates. We tested 2 virulent and 2 avirulent wasps in order to analyze hemocyte induction. We chose the wasps of which either the majority eclosed from *Z. indianus* or the majority were encapsulated by *Z. indianus*. This was based on our own resistance trials (Figure 5A-C). Three female wasps were then placed onto the plates for a 24 hr attack period. Hemocytes were counted 24 hrs following the end of the attack period.

In order to count hemocytes, 5 third instar larvae were removed from each replica plate. The larvae were rinsed in Drosophila Ringer's solution, dried on a kimwipe, and bled together into 20 ul of 1X PBS solution containing 0.01% phenylthiourea. This buffer-hemolymph mixture was then pippetted into a disposable hemocytometer (Incyto C-Chip DHC-N01) and allowed to sit for 30 minutes to allow the hemocytes to settle. Hemocytes from each sample were counted from sixteen 0.25 x 0.25 x 0.1 mm squares (Figure 4A-F). The number

of hemocytes counted from the whole sample is expected to be 200 times the number counted.

Hemocyte counts may be underestimated because a large fraction of plasmatocytes are sessile. Furthermore, lamellocytes counted from avirulent wasps are underestimated as they involved in the encapsulation of the wasp egg, meaning that at the point of counting hemocytes, lamellocytes could already be involved in the encapsulation process. Finally, we do not know the location of all nematocytes in the fly larva, making comprehensive numbers very difficult to acquire. Hemocytes were classified as plasmatocytes (small round cells with obvious nuclei), podocytes (activated plasmatocytes that are larger and refract more light than plasmatocytes), lamellocytes (large, clear flattened cells), and nematocytes (long, thin cells).

Crystal cells contain cytoplasmic crystals made up of the substrate that leads to the melanization of a wasp egg ([34]). The crystals are rapidly lost following dissection, making these cells virtually impossible to count in the hemocytometer. Crystal cells have been shown to self-melanize when larvae are incubated at 60 C for 10 minutes ([35]).

(c) Resistance Trials

Three replicates were performed for each fly-wasp interaction. Adult female *Z. indianus* were allowed to lay eggs into a molasses medium plate supplemented with yeast paste (50:50 mix of honey water and yeast) in 60 mm Petri dishes. After 96 hours, adult flies were removed and second instar fly larvae were collected to perform the wasp infections. For the larval parasite attacks, 50 fly larvae were moved onto a 35 mm Petri dish filled with 1 mL of

Drosophila medium. Three female wasps were then placed onto the dish and allowed to attack for 72 hours. After the attack period, 10 of the 50 larvae were dissected to assay the number of wasp eggs laid per larva. 30 of the remaining 40 larvae were then moved into vials containing Drosophila medium and allowed to complete development. For the pupal parasites, 40 third instar larvae were placed into a vial containing Drosophila medium. Three female wasps were then placed into the vial and allowed to attack for 72 hours, after which the fly development was allowed to proceed to completion. The infection conditions were designed to optimize wasp success. Control uninfected flies were reared under identical conditions and showed 100% survival (data not shown).

The total number of flies and wasps eclosed from the treatments were determined 17 and 32 days following infection, respectively. By these times, all viable flies and wasps emerged. The fly-wasp interaction yielded 3 possible outcomes: (1) fly eclosion following a successful immune response; (2) wasp eclosion following a successful immune suppression; (3) death of both the fly and the wasp.

(d) Egg Imaging

Encapsulated wasp eggs were dissected into 20 μl of 1X PBS solution containing 0.01% phenylthiourea. These eggs were allowed to settle for 5 minutes before imaging.

For the EDTA assay, we used a previously reported protocol from a study assaying the encapsulation ability in moths ([23]). The dissection media used was Drosophila Ringer's solution plus 1% ethylene diamine tetra-acetate disodium. Incubations were carried out for 5 minutes before imaging, and examined for a 30-minute period using phase contrast

microscopy. At the end of the incubation period, capsule dissociation was evaluated and hemocytes were classified. Eggs were also dissected into Ringer's solution lacking EDTA and were found to maintain their tight capsule structure.

(e) Statistical Analysis

Statiscal analyses comparing nematocyte size were performed in R version 2.10.1.

Figure 1

Fly morphology: (a) female *Z. indianus* (lateral view); (b) female *Z. indianus* (dorsal view).

Figure 2

Phylogenetic relationships and provenance of wasps used in this study.

Figure 3

Proportion of wasp eggs encapsulated in each strain of *Z. indianus* larva. Strain 1 (A); Strain 2 (B); Strain 3 (C). Average (+) standard deviation shown.

Figure 4

Infection outcomes for host larvae infected by wasps. Proportion of flies and wasps that eclosed from each strain of *Z. indianus* larvae infected by each wasp strain are shown. Strain 1 (A); Strain 2 (B); Strain 3 (C). Average (+) standard deviation shown.

Figure 5

Encapsulation of wasp egg by *Z. indianus*. (a) Whole body image with encapsulated wasp egg; (b) close up of encapsulated wasp egg; (c) dissected encapsulated wasp egg from *Z. indianus*;

(d) close up of encapsulated wasp egg showing tightly bound cell layer; (e) successfully hatched wasp larva.

Figure 6

Dissected encapsulated wasp eggs treated with EDTA. (a) 5 minutes after EDTA treatment; (b) 20 minutes after EDTA treatment; (c) close up of capsule surrounded by lamellocytes and nematocytes detached from the egg—egg in panel a-c is the same; (d) 15 minutes after EDTA treatment with visible, melanized, nematocytes.

Figure 7

Z. indianus hemocytes; (a) plasmatocyte; (b) podocyte; (c) lamellocyte; (d) nematocyte; (e) activated nematocyte

Figure 8

Larvae boiled to induce crystal cell lysis. (a) Control *Z. indianus* larva with melanized crystal cells; (b) posterior tip of *Z. indianus* larva showing color variation in crystal cells; (c) *Z. indianus* larva wounded by sterile needle; (d) melanized pierce wound.

Figure 9

Z. indianus nematocytes from each of the three strains at 100X magnification. (a) Control *Z. indianus* (strain 1) nematocyte; (b) *Z. indianus* (strain 1) larvae 24 hours after pierce with sterile needle showing nematocytes; (c) Control *Z. indianus* (strain 2) larvae nematocytes; (d) *Z. indianus* (strain 2) larvae 24 hours after pierce with sterile needle showing nematocytes; (e)

Control *Z. indianus* (strain 3) larvae nematocytes; (f) *Z. indianus* (strain 3) larvae 24 hours after pierce with sterile needle showing nematocytes.

Figure 10

Quantitative comparison of nematocyte size comparing constitutive and induced nematocytes from (a) strain 1, (b) strain 2, and (c) strain 3 of *Z. indianus*.

Figure 11

Z. indianus hemocytes from each of the three strains as seen in a 0.25 x 0.25 x 0.1 mm hemocytometer field. (a) Control *Z. indianus* (strain 1) larvae showing abundant plasmatocytes and nematocytes; (b) *Z. indianus* (strain 1) larvae 24 hours after pierce with sterile needle showing plasmatocytes, podocytes, lamellocytes, and nematocytes; (c) Control *Z. indianus* (strain 2) larvae showing abundant plasmatocytes and nematocytes; (d) *Z. indianus* (strain 2) larvae 24 hours after pierce with sterile needle showing plasmatocytes, podocytes, lamellocytes, and nematocytes; (e) Control *Z. indianus* (strain 3) larvae showing abundant plasmatocytes and no nematocytes; (f) *Z. indianus* (strain 3) larvae 24 hours after pierce with sterile needle showing plasmatocytes, podocytes, lamellocytes, and nematocytes.

Figure 12

Hemocyte count comparison between each of the three strains of *Z. indianus*. Strain 1 (A); Strain 2 (B); Strain 3 (C). Average (+) standard deviation shown.

Figure 13

Crystal cell count comparison between each of the three strains of *Z. indianus*. Strain 1 (A); Strain 2 (B); Strain 3 (C). Average (+) standard deviation shown.

Figure 14

Phylogenetic relationships of flies used in this study. Color indicates presence of nematocytes.

Figure 15

Nematocytes dissected out of (a) *D. immigrans*; (b) *D. mojavensis*; (c) *D. paramelanica*; (d) *D. virilis*; (e) *D. hydei*; (f) *D. funebris*.

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Conclusion

The outcome of any fly-wasp interaction is successful fly eclosion, successful parasite eclosion, or death of both fly and wasp. We found variation in each of these outcomes across fly species, suggesting little phylogenetic conservation. Interestingly, the canonical D. melanogaster melanotic encapsulation response is unique to the melanogaster group and two outliers. We have isolated three possible immune mechanisms as well as two novel classes of hemocytes. Our analysis leads to conclusions about the evolutionary selective pressures on wasp infection strategies and fruitfly immune systems, and provide clues to how these species groups have co-evolved. Additional studies involving transcriptome analyses will further elucidate as to the mechanism of successful wasp suppression.

Our work involving the toxin, ethanol, has also demonstrated novel use with respect to immunity. It is not surprising that *D. melanogaster* are highly attuned to ethanol concentration given the previously characterized fitness benefits and costs of different levels of ethanol, along with the variation in ethanol content across rotting fruits, within rotting fruits, and temporally during the fruit rotting process. We have shown here that ethanol provides novel benefits to flies by reducing wasp infection, by increasing infection survival, and by allowing for a behavioral immune response against wasps based on consumption of it in toxic amounts. To our knowledge, these data are the first to show that alcohol consumption can have a protective effect against infectious disease, and in particular against blood-borne parasites. Given that alcohols are relatively ubiquitous compounds consumed by a number of organisms, protective effects of alcohol consumption may extend beyond fruitflies. Although many studies in humans have documented decreased immune function in chronic consumers of alcohol, little attempt has been made to assay any beneficial effect of acute or moderate alcohol use on parasite mortality or overall host fitness following

infection. Much future work has been made possible with this discovery. For example, other flies also live in toxic environments. Perhaps they as well utilize their environment in a similar manner to that of *D. melanogaster*. Furthermore, we wonder whether there is a possibility of parental investment in offspring safety by choosing to lay in ethanol food in the presence of parasitoids. Further investigation will illuminate this question.

Our work with *D. suzukii* has demonstrated the use of increased hemocytes confers a fitness advantage against parasitoids. Flies with more hemocytes may suffer fewer effects of wasp venom for a variety of reasons, enabling them to mount a quicker and/or stronger encapsulation reaction against wasps. For example, venoms often alter hemocyte structure and function, and thus an increased number of hemocytes could potentially dilute the effects of a standard dose of venom. Alternatively, hemocytes may be responsible for destroying venom components found in the hemolymph, via endocytosis or some other mechanism, preventing the venom from exerting its effects on other tissues. However, this leads us to wonder: if increased hemocyte load provides general protection against larval parasitoids, why do some fly species, such as *D. melanogaster*, produce such low numbers compared to their close relatives? Hosts face an evolutionary tradeoff between investing in immune responses against parasites versus investing in other aspects of fitness, for example, utilization of ethanol. While our work on *D. suzukii* cannot fully answer this question, we can demonstrate the utilization of parasitoid wasps in the use of biocontrol against the emerging agricultural parasite. However, infection trial conditions in this study were designed to be ideal for success of the wasps, and such conditions (easy access to hosts, no competition with other parasites, controlled temperature, abundant resources, etc) are unlikely to be replicated in the field, extensive field experiments will be required to assess the efficacy of the use of parasitoid wasps in *D. suzukii* biocontrol in practice.

Our work with *Z. indianus* not only presents us with the use of parasitoids against this parasite (as with *D. suzukii*) but has also demonstrated a novel form of cellular encapsulation. As cellular encapsulation is a conserved mode of innate immunity, it is important to understand how the system has evolved. *Z. indianus* is very successful at fighting off most wasp infections. Perhaps the secrete lies in the nematocytes that seem to be involved in a successful immune response.

The genus Drosophila has been neglected as a whole—the model system *D. melanogaster* is very well studied while other species with novel immune responses and toxin use have been poorly characterized. We hope that our work will lay the foundation into increased interest and study into the origin and evolution of the immune system in the genus Drosophila.

Chapter 1

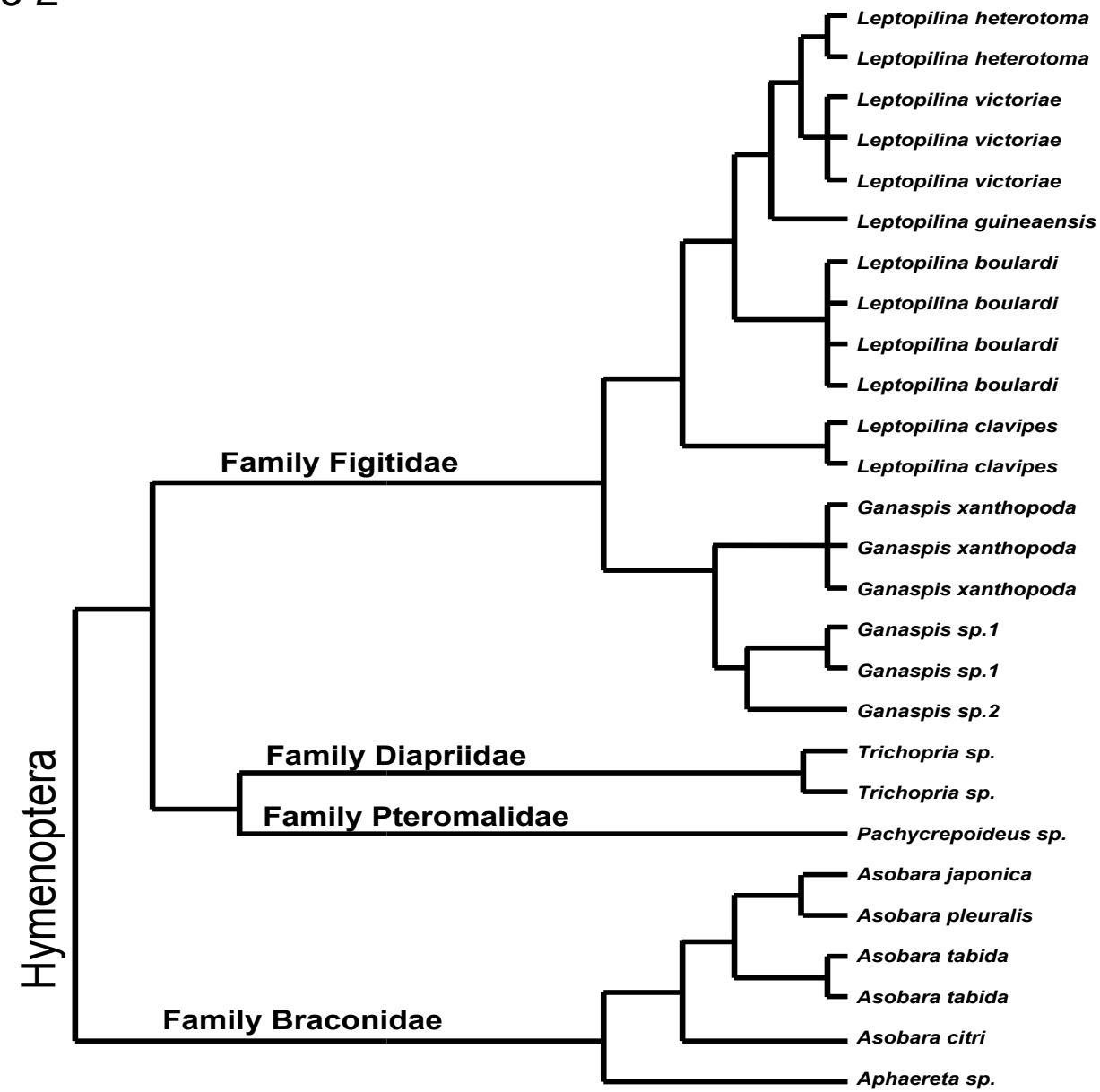
The anti-wasp immune response across the genus Drosophila.

Figures

Figure 1

Genus	Species	Drosophila Species Stock Center #	Collection City	Collection Date
Drosophila	affinis	14012-0141.08	Rocky Point, NY	July, 2004
Drosophila	ananassae	14024-0371.13	Hawaii	1945
Drosophila	biarmipes	14023-0361.09	Mysore, India	July, 1971
Drosophila	elegans	14027-0461.03	Hong Kong	unknown
Drosophila	erecta	14021-0224.01	unknown	unknown
Drosophila	eugracilis	14026-0451.10	Kuala Belalong, Brunei	2002
Drosophila	ficusphila	14025-0441.05	Taiwan	1961
Drosophila	funebris	15120-1911.07	Tehran, Iran	September, 1967
Drosophila	hydei	15085-1641.69	Berkeley, CA	November, 2007
Drosophila	immigrans	15111-1731.08	Tucson, AZ	June, 2003
Drosophila	kikkawai	14028-0561.14	Leticia, Colombia	unknown
Drosophila	lutescens	14022-0271.01	Mito, Japan	October, 1976
Drosophila	melanogaster	14021-0231.36	unknown	unknown
Drosophila	mojavensis	15081-1352.22	Catalina Island, CA	March, 2002
Drosophila	paramelanica	15030-1161.03	Muscatine, IA	June, 2003
Drosophila	pseudoobscura	14011-0121.94	Anderson, CO	1996
Drosophila	sechellia	14021-0248.25	Cousin Island, Seychelles	1980
Drosophila	simulans	14021-0251.195	unknown	unknown
Drosophila	subobscura	14011-0131.08	Cambridge, UK	April, 2005
Drosophila	suzukii		Atlanta, GA	August, 2010
Drosophila	tsacasi	14028-0701.00	Ivory Coast	October, 1955
Drosophila	virilis	15010-1051.87	unknown	unknown
Drosophila	willistoni	14030-0811.24	Guadaloupe Island, France	unknown
Drosophila	yakuba	14021-0261.01	Liberia	1983
Zaprionus	indianus		Florida City, FL	September, 2010

Figure 2



Strain	Place Collected	Date Collected
Lh14	Winters, California	2002
LhSw	Uppsala, Sweden	2007
LvHaw	Kaimuki, Oahu, Hawaii	2009
LvPhil	Consolacion, Cebu, Philippines	2007
LvUnk	unknown	unknown
LgG500	Yaounde, Cameroon	1998
LbFr	Marseilles, France	2007
LbG486	Brazzaville, Congo	1978
LbKen	Nairobi, Kenya	2009
Lb17	Winters, California	2002
LcNet	Heerenbergh, Netherlands	2000
LcAtl	Atlanta, Georgia	2011
GxHaw	Kaimuki, Oahu, Hawaii	2009
GxUg	Masindi, Uganda	2010
GxUnk	unknown	unknown
G1Fl	Homestead, Florida	2008
G1Haw	Kaimuki, Oahu, Hawaii	2009
G2Atl	Atlanta, GA	2011
Tri1Cal	Winters, California	2002
Tri1Fr	Marseilles, France	2007
Pac1Atl	Atlanta, Georgia	2008
AjJap	Tokyo, Japan	1995
ApIndo	Manado, Sulawesi, Indonesia	2005
AtFr	Sospel, France	>20 years ago
AtSw	Uppsala, Sweden	2007
AcIC	Lamto, Ivory Coast	1995
Aph1Atl	Atlanta, Georgia	2009

Figure 3

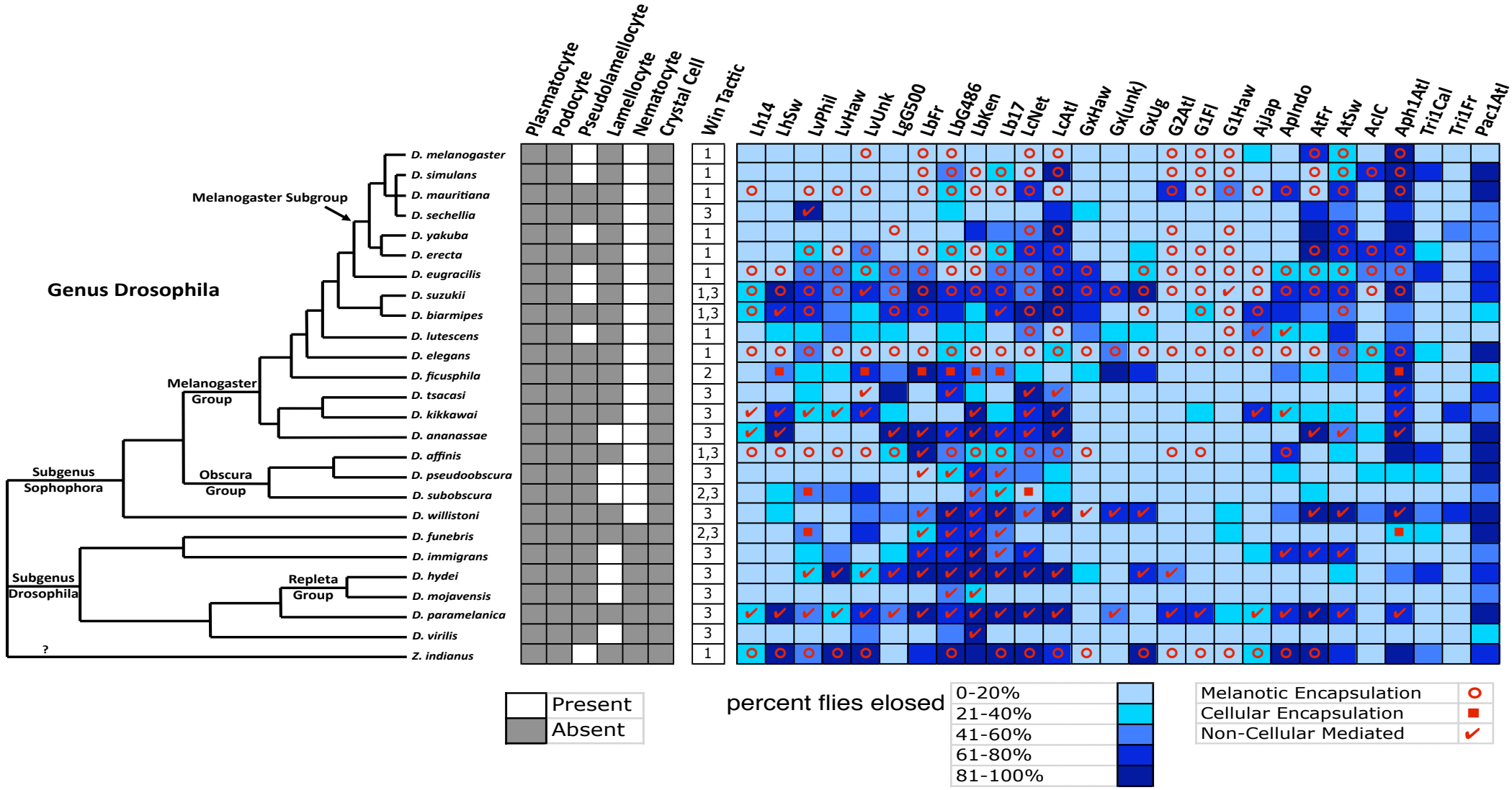

Plasmatocyte
Podocyte
Pseudolamellocyte
Lamellocyte
Nematocyte
Crystal Cell
Win Tactic
Lh14
LhSw
LvPhil
LvHaw
LvUnk
LgG500
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
Gx(unk)
GxUg
G2Atl
G1Fl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Genus Drosophila
Melanogaster Subgroup
Melanogaster Group
Obscura Group
Subgenus Sophophora
Repleta Group
Subgenus Drosophila
D. melanogaster
D. simulans
D. mauritiana
D. sechellia
D. yakuba
D. erecta
D. eugracilis
D. suzukii
D. biarmipes
D. lutescens
D. elegans
D. ficusphila
D. tsacasi
D. kikkawai
D. ananassae
D. affinis
D. pseudoobscura
D. subobscura
D. willistoni
D. funebris
D. immigrans
D. hydei
D. mojavensis
D. paramelanica
D. virilis
Z. indianus
?
1
1
1
3
1
1
1
1,3
1,3
1
1
2
3
3
3
1,3
3
2,3
3
2,3
3
3
3
3
3
1
Present
Absent
percent flies elosed
0-20%
21-40%
41-60%
61-80%
81-100%
Melanotic Encapsulation
Cellular Encapsulation
Non-Cellular Mediated

Figure 4

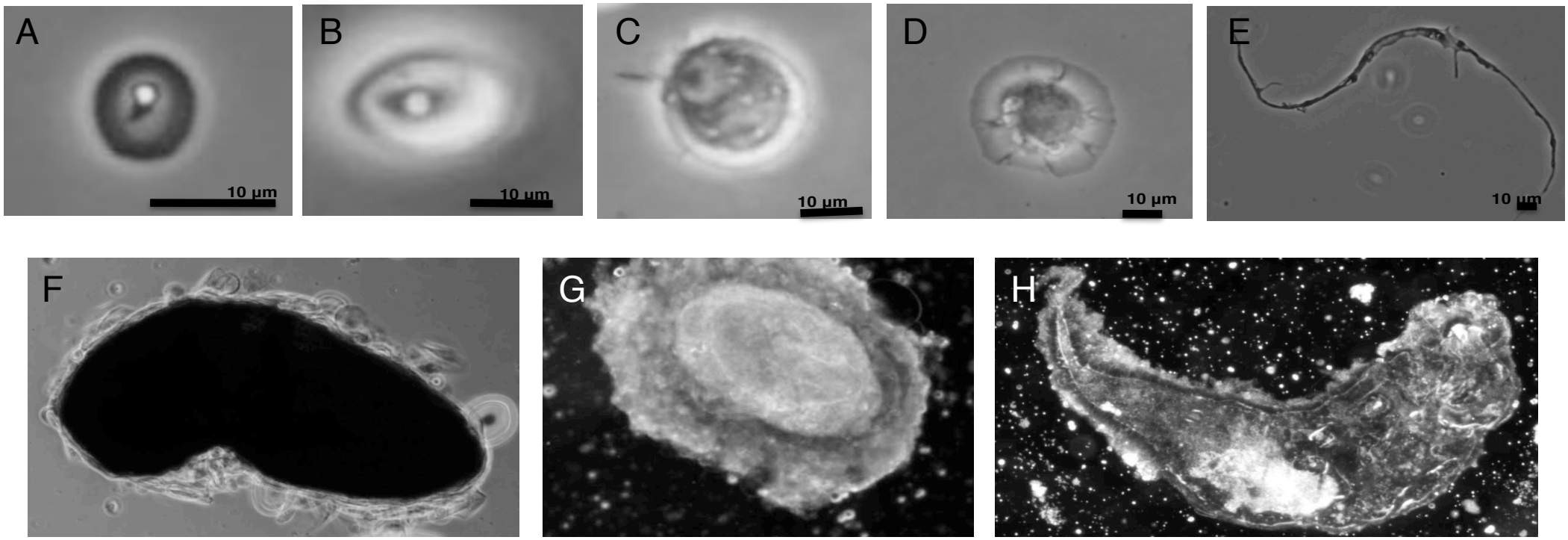


Figure 5

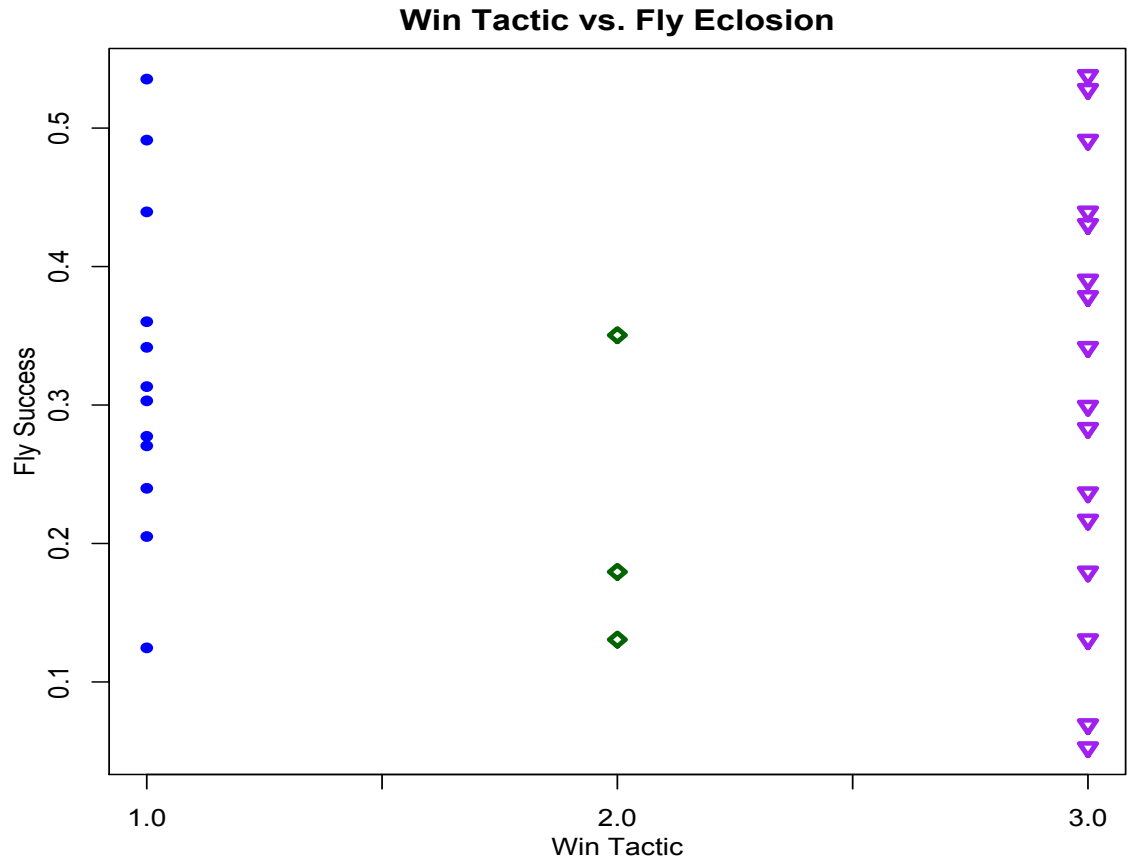
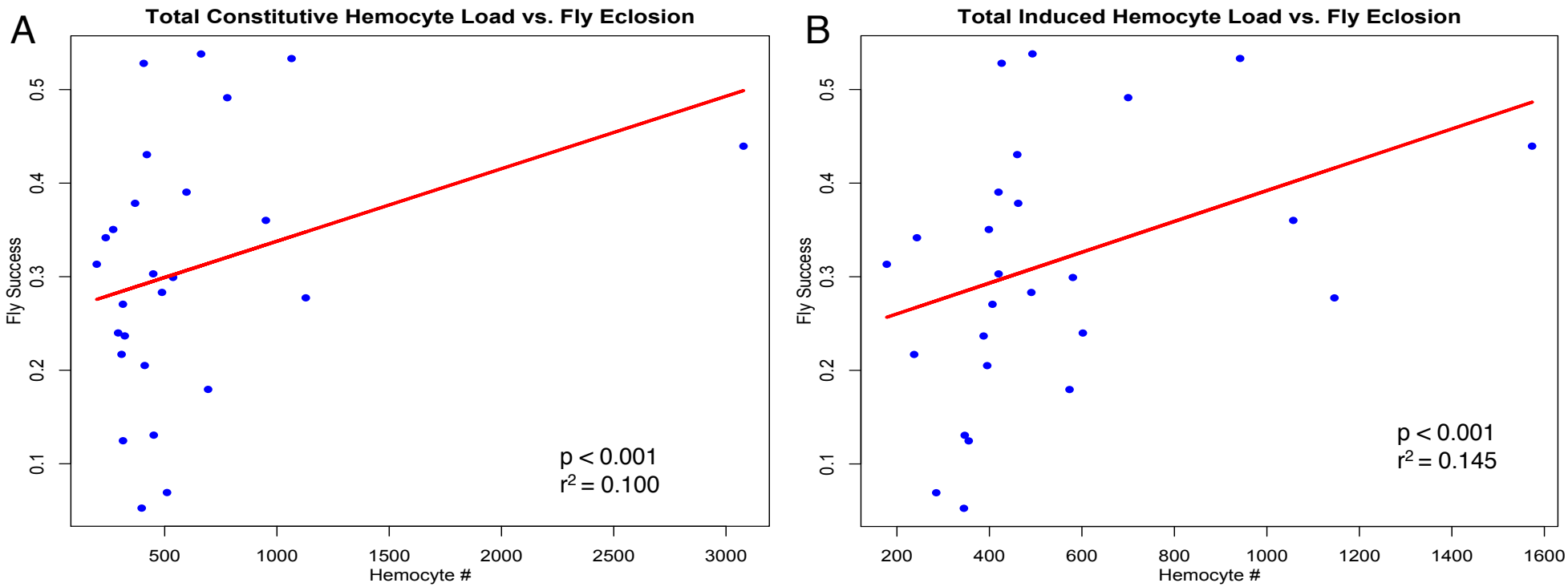
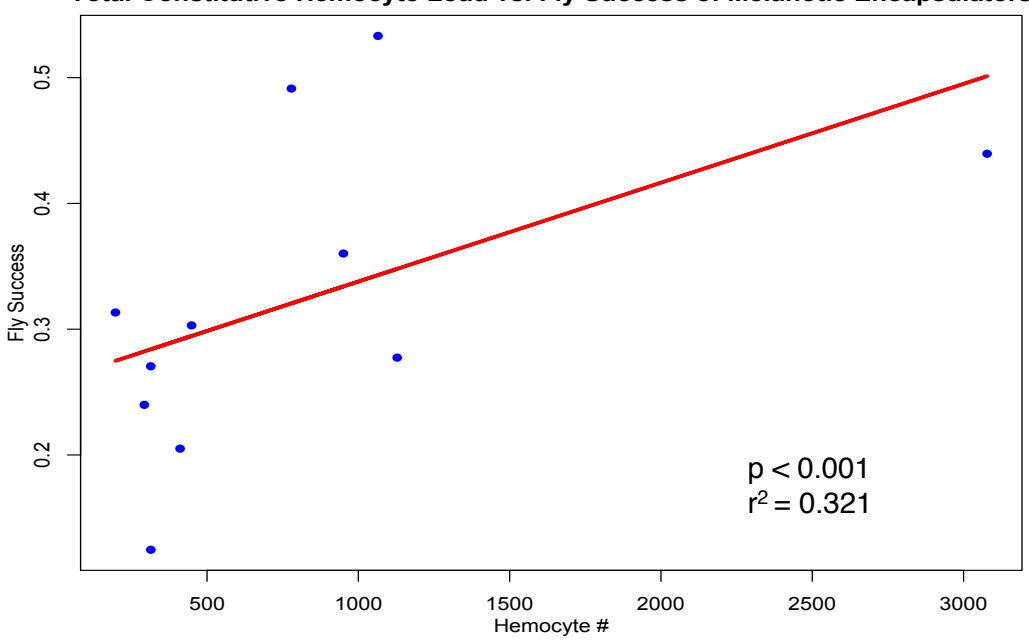

Win Tactic vs. Fly Eclosion
Fly Success
0.1
0.2
0.3
0.4
0.5
1.0
2.0
3.0
Win Tactic

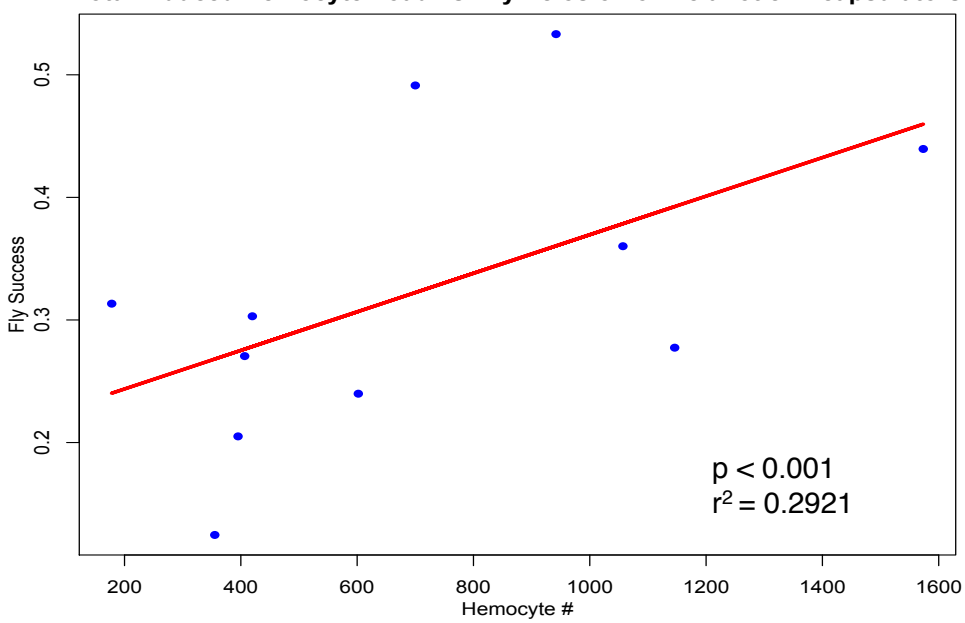
Figure 6


A
Total Constitutive Hemocyte Load vs. Fly Eclosion
Fly Success
Hemocyte #
p < 0.001
r2 = 0.100
B
Total Induced Hemocyte Load vs. Fly Eclosion
Fly Success
Hemocyte #
p < 0.001
r2 = 0.145

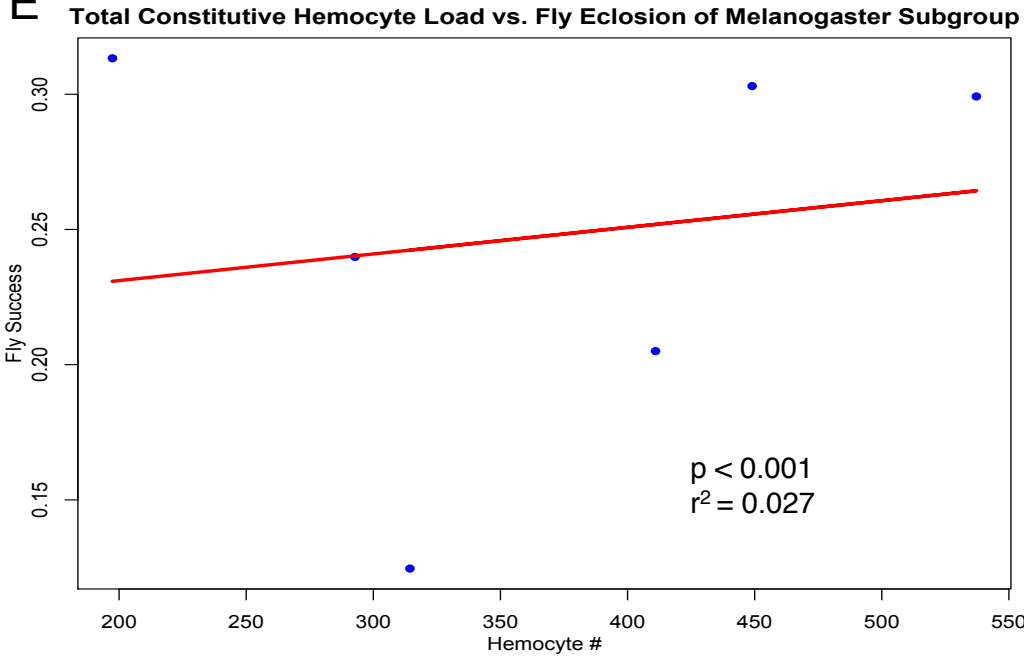
C


Total Constitutive Hemocyte Load vs. Fly Success of Melanotic Encapsulators
Fly Success
0.2
0.3
0.4
0.5
500
1000
1500
2000
2500
3000
Hemocyte #
p < 0.001
r2 = 0.321

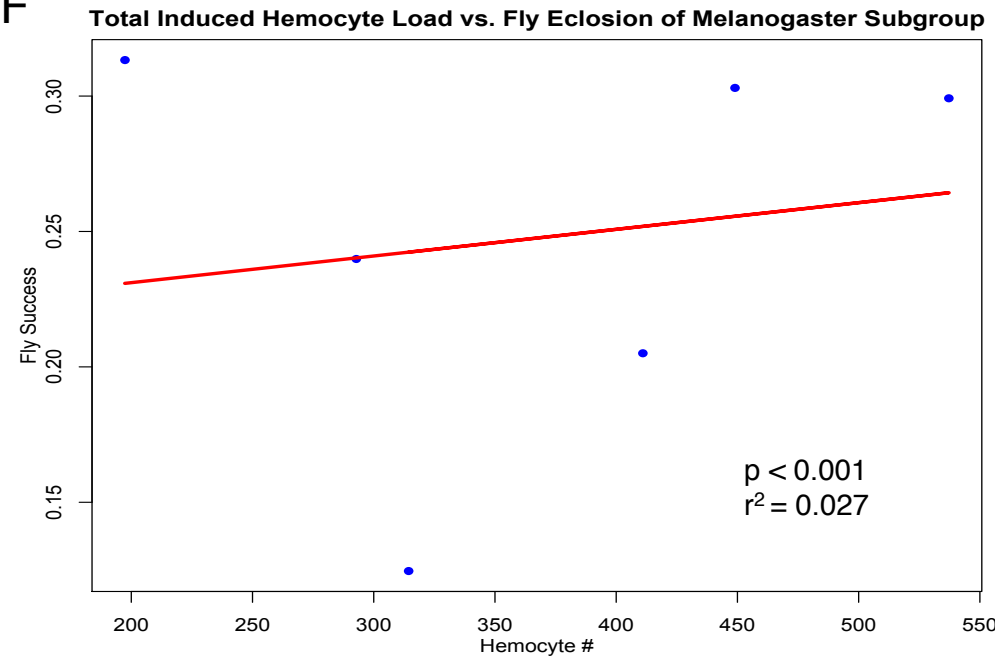
D


Total Induced Hemocyte Load vs. Fly Eclosion of Melanotic Encapsulators
Fly Success
0.2
0.3
0.4
0.5
200
400
600
800
1000
1200
1400
1600
Hemocyte #
p < 0.001
r2 = 0.2921

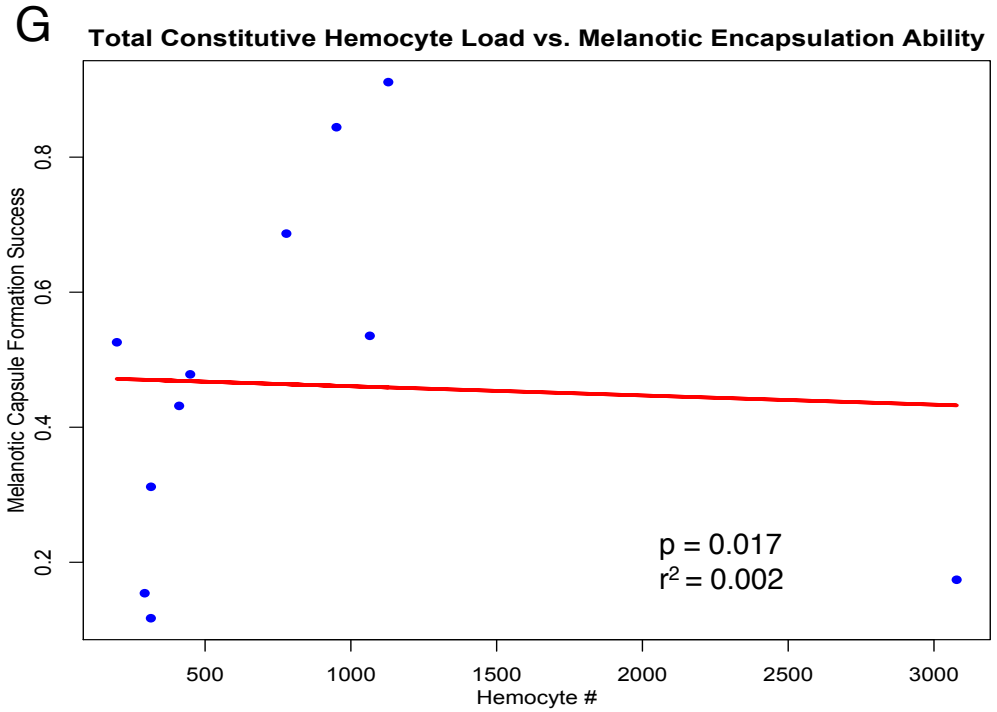
E


Total Constitutive Hemocyte Load vs. Fly Eclosion of Melanogaster Subgroup
Fly Success
0.15
0.20
0.25
0.30
200
250
300
350
400
450
500
550
Hemocyte #
p < 0.001
r2 = 0.027

F


Total Induced Hemocyte Load vs. Fly Eclosion of Melanogaster Subgroup
Fly Success
0.15
0.20
0.25
0.30
200
250
300
350
400
450
500
550
Hemocyte #
p < 0.001
r2 = 0.027

G


Total Constitutive Hemocyte Load vs. Melanotic Encapsulation Ability
Melanotic Capsule Formation Success
0.2
0.4
0.6
0.8
500
1000
1500
2000
2500
3000
Hemocyte #
p = 0.017
r2 = 0.002

H

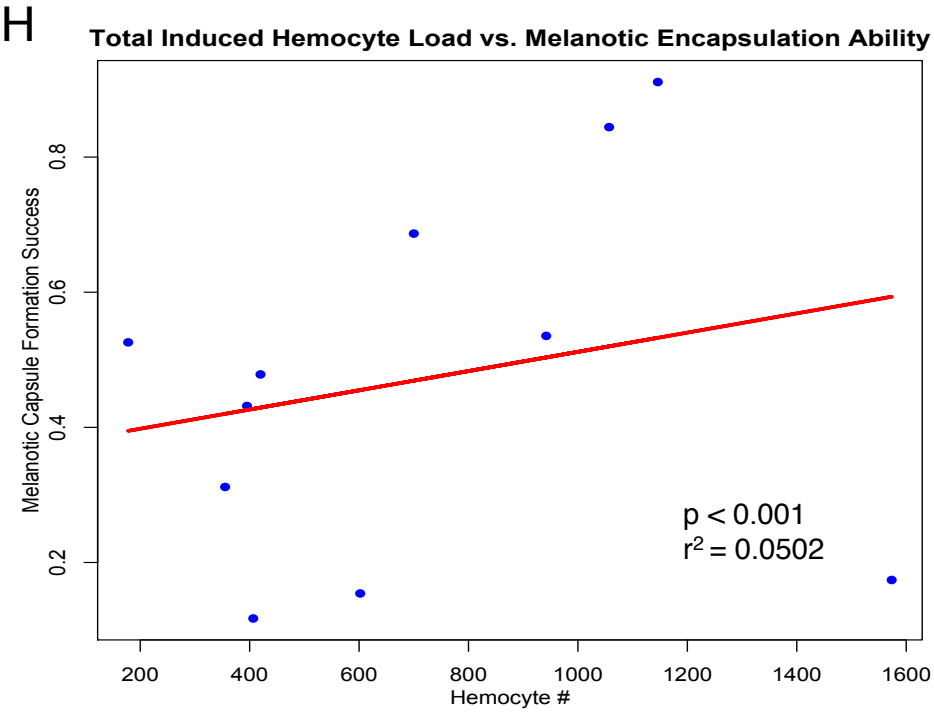
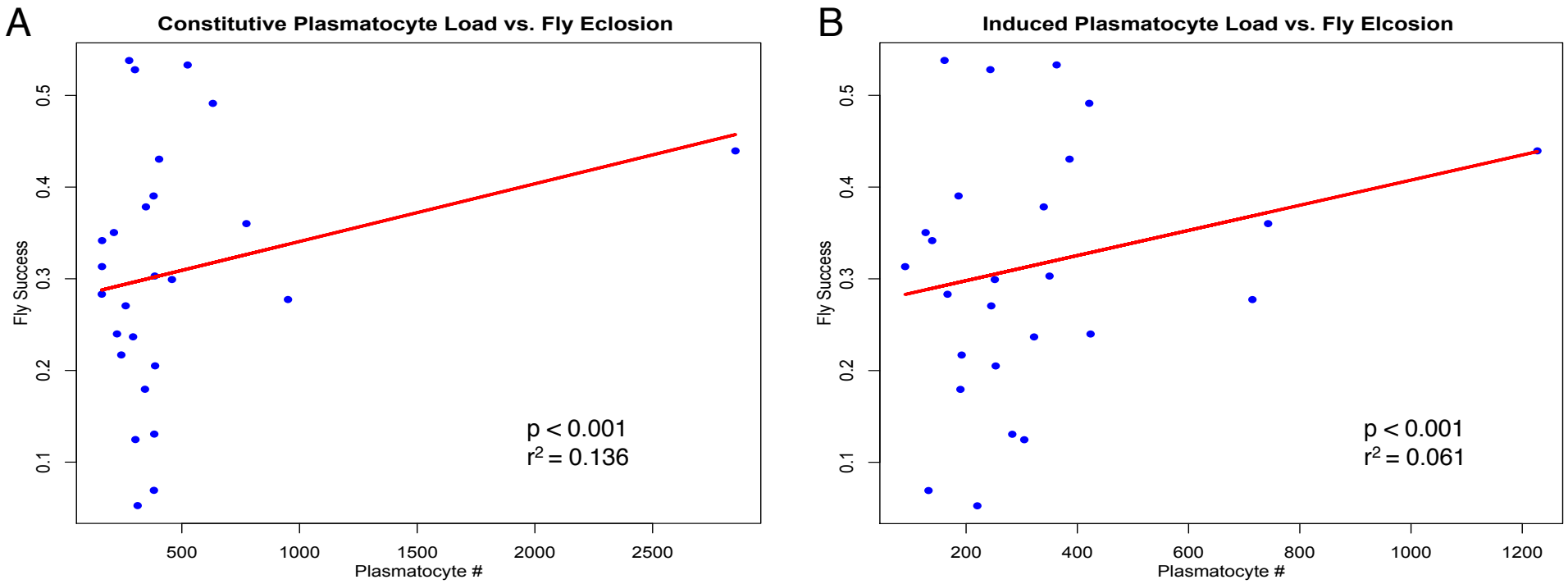
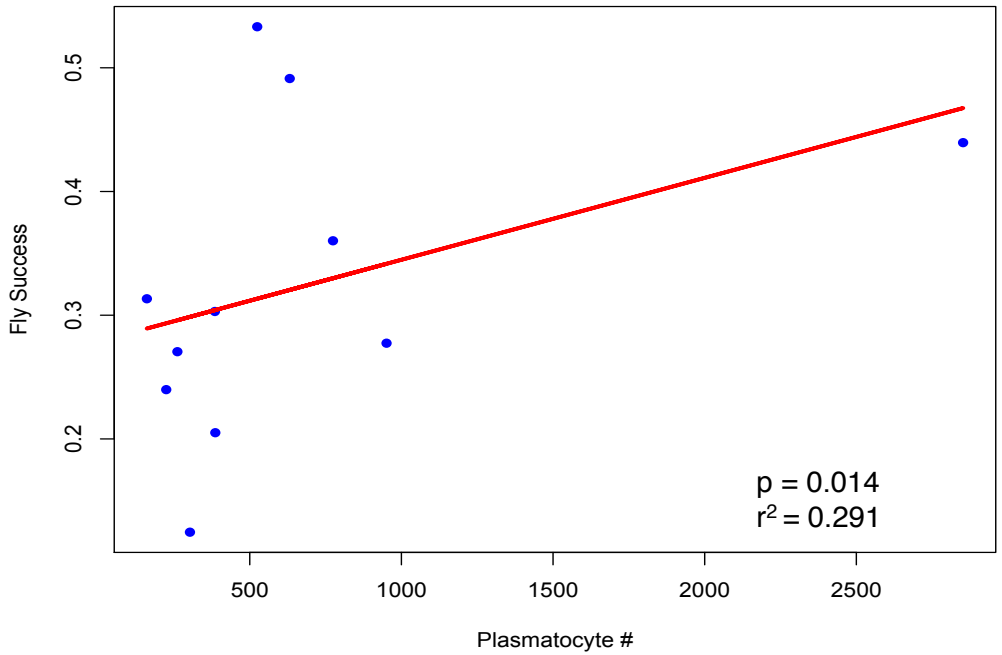

Total Induced Hemocyte Load vs. Melanotic Encapsulation Ability
Melanotic Capsule Formation Success
0.2
0.4
0.6
0.8
200
400
600
800
1000
1200
1400
1600
Hemocyte #
p < 0.001
r2 = 0.0502

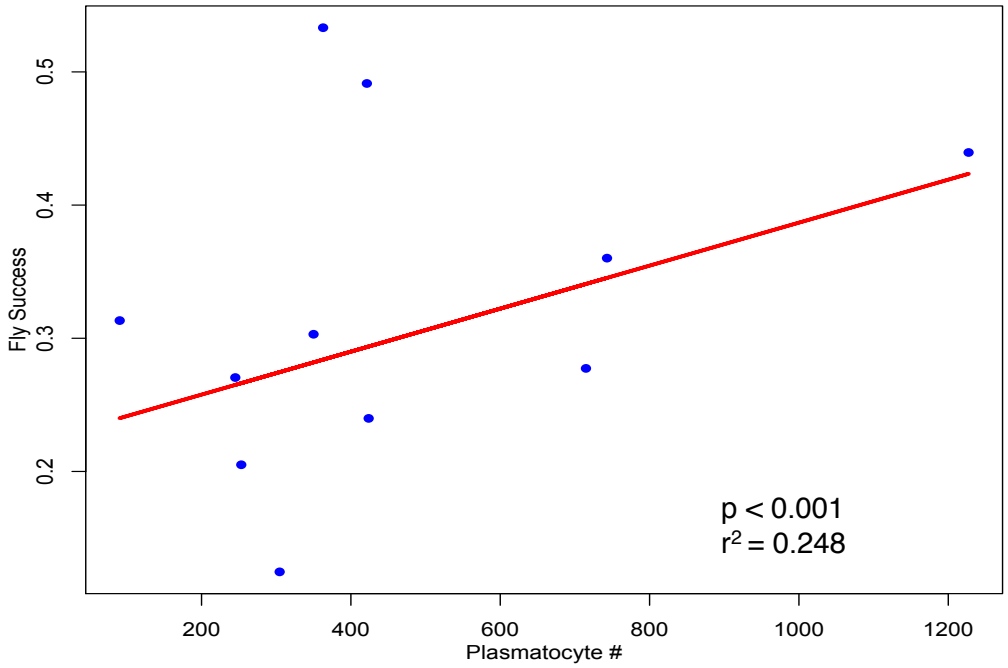
Figure 7


A
Constitutive Plasmatocyte Load vs. Fly Eclosion
Fly Success
Plasmatocyte #
p < 0.001
r2 = 0.136
B
Induced Plasmatocyte Load vs. Fly Elcosion
Fly Success
Plasmatocyte #
p < 0.001
r2 = 0.061

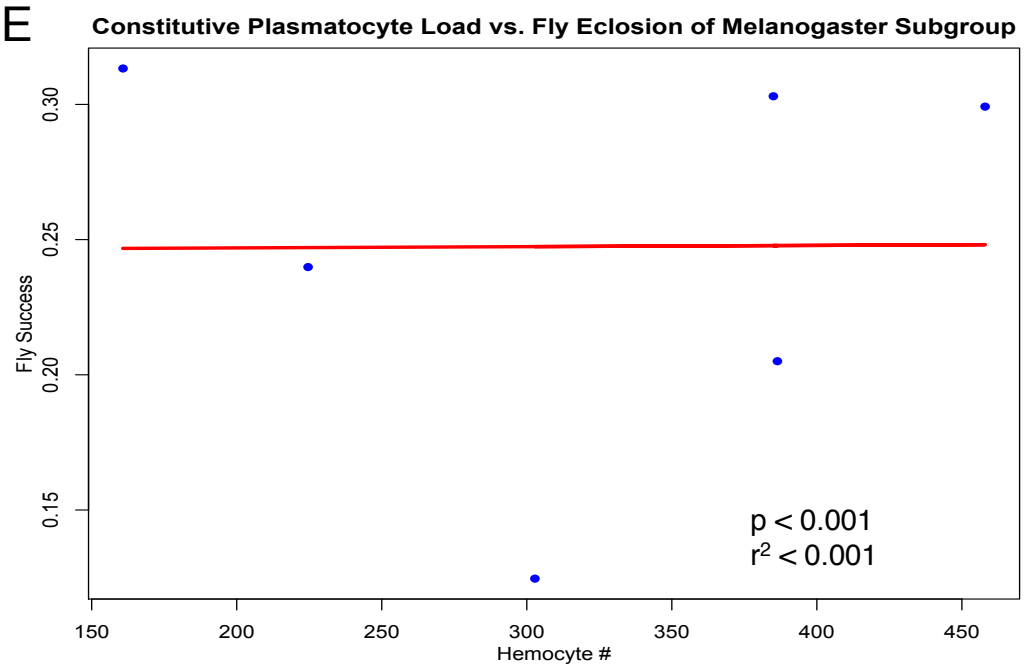
C **Constitutive Plasmatocyte Load vs. Fly Eclosion of Melanotic Encapsulators**



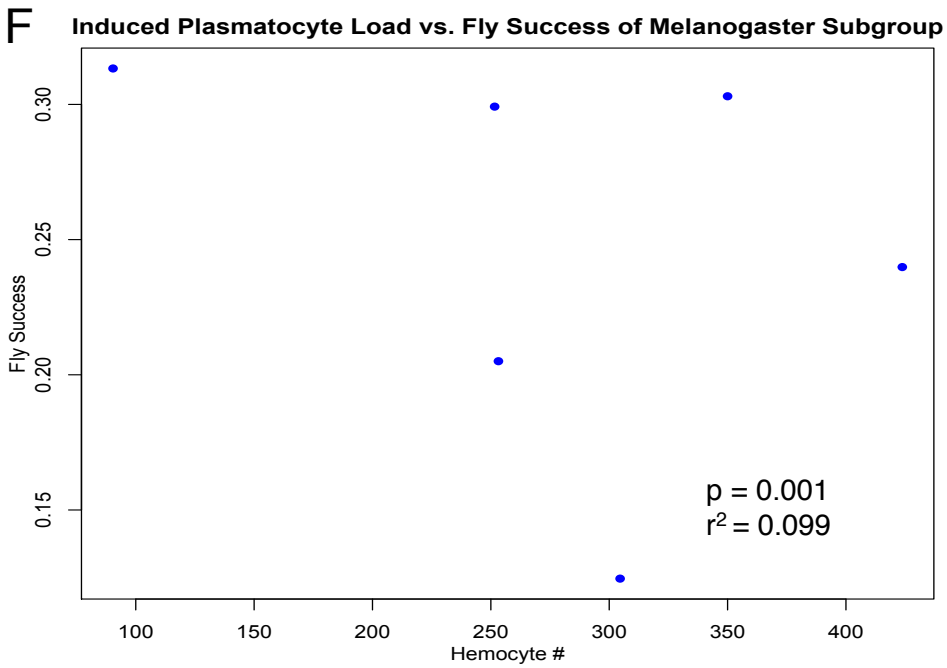
D **Induced Plasmatocyte Load vs. Fly Eclosion of Melanotic Encapsulators**



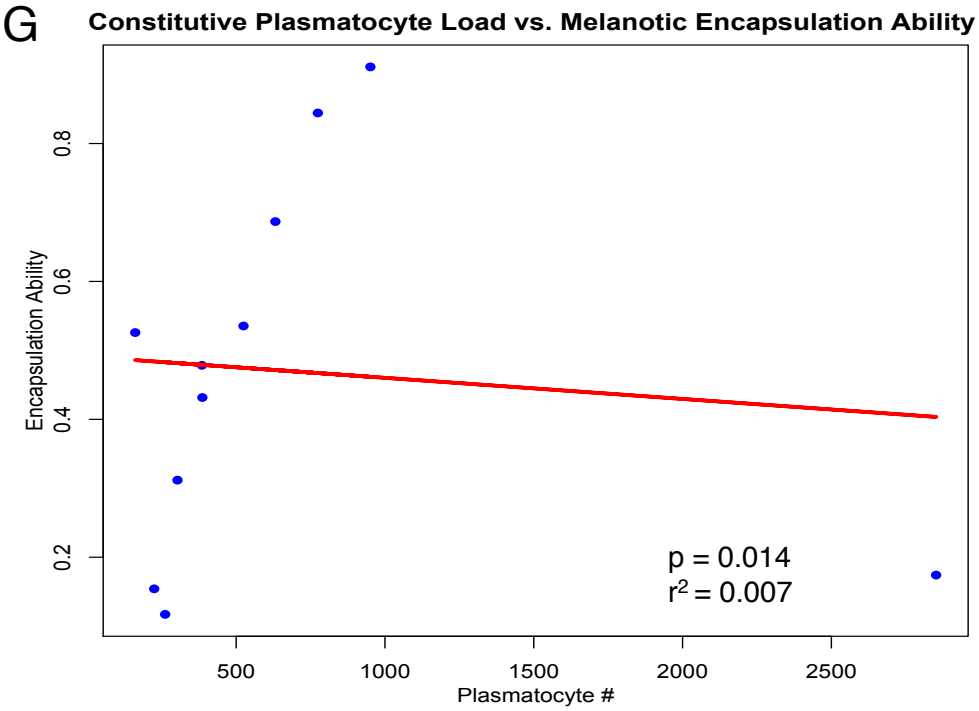
E


Constitutive Plasmatocyte Load vs. Fly Eclosion of Melanogaster Subgroup
Fly Success
0.15
0.20
0.25
0.30
150
200
250
300
350
400
450
Hemocyte #
p < 0.001
r2 < 0.001

F


Induced Plasmatocyte Load vs. Fly Success of Melanogaster Subgroup
Fly Success
0.15
0.20
0.25
0.30
100
150
200
250
300
350
400
Hemocyte #
p = 0.001
r2 = 0.099

G


Constitutive Plasmatocyte Load vs. Melanotic Encapsulation Ability
Encapsulation Ability
0.2
0.4
0.6
0.8
500
1000
1500
2000
2500
Plasmatocyte #
p = 0.014
r2 = 0.007

H

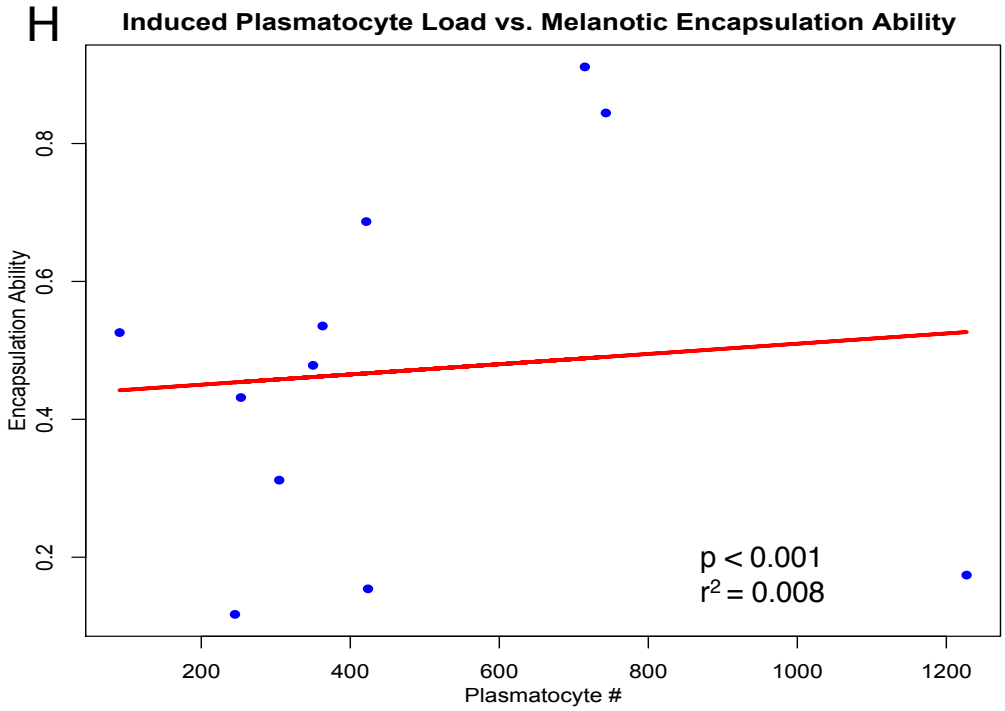
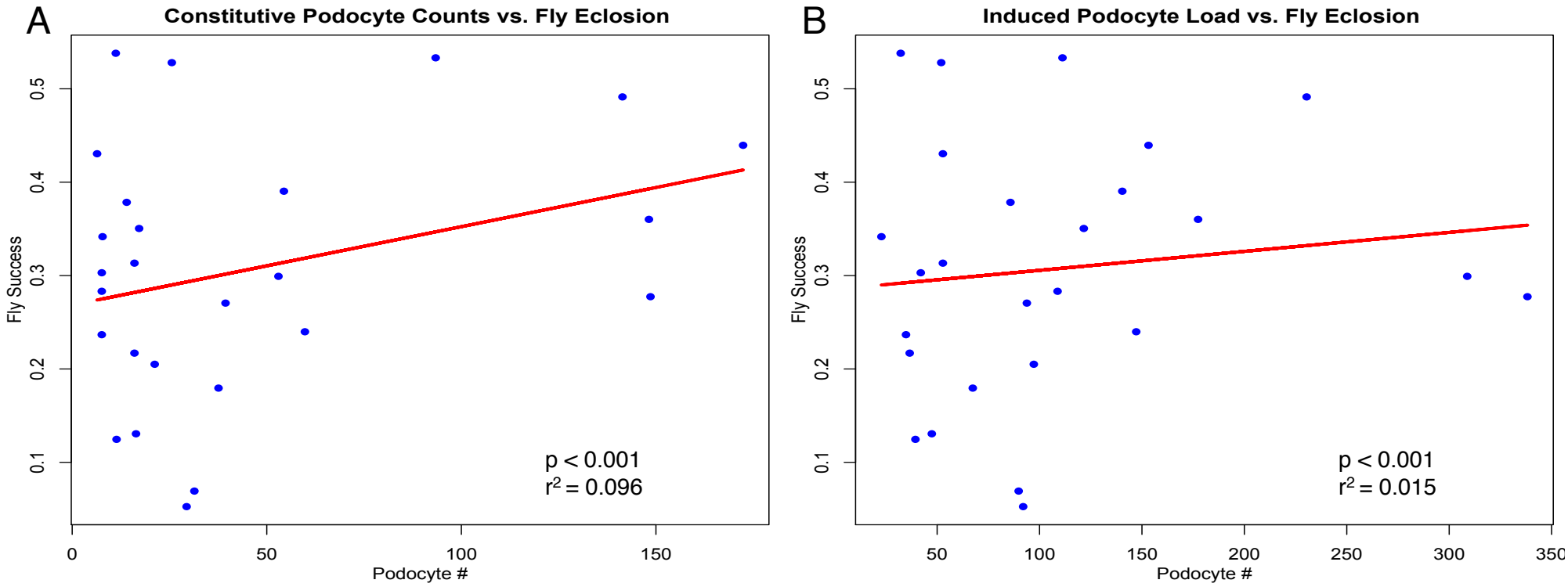
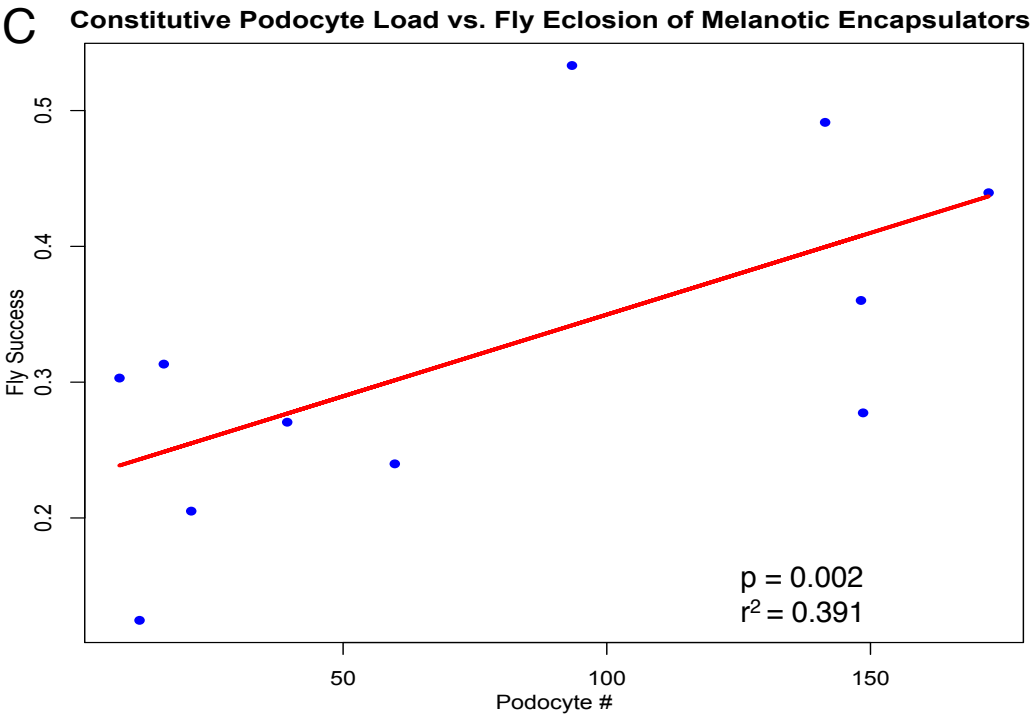

Induced Plasmatocyte Load vs. Melanotic Encapsulation Ability
Encapsulation Ability
0.2
0.4
0.6
0.8
200
400
600
800
1000
1200
Plasmatocyte #
p < 0.001
r2 = 0.008

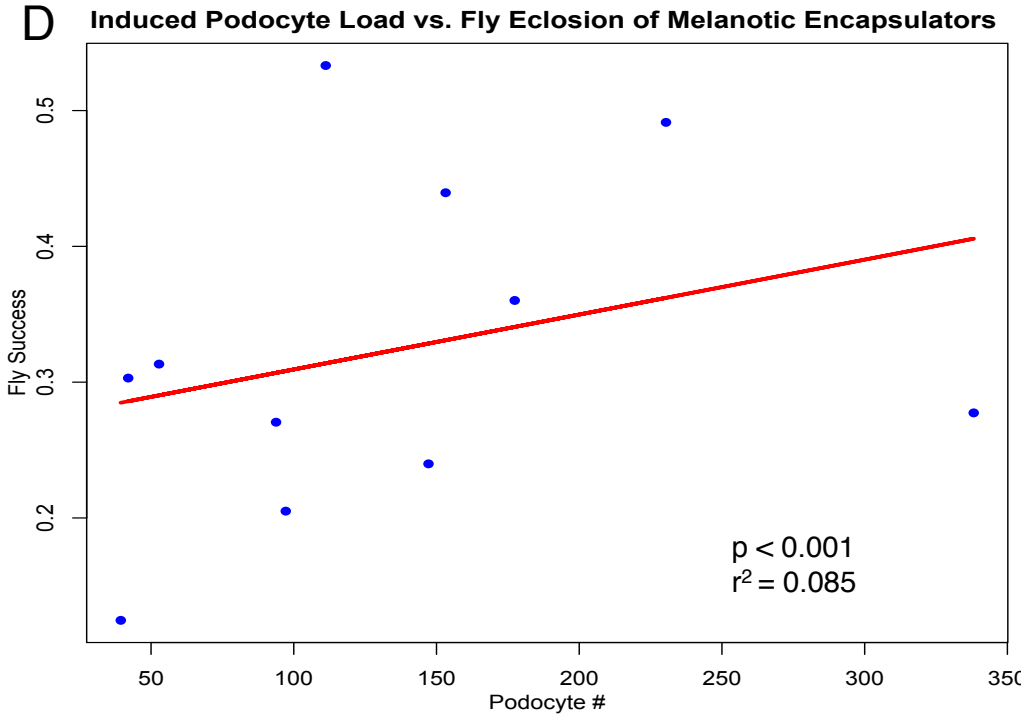
Figure 8


A
Constitutive Podocyte Counts vs. Fly Eclosion
Fly Success
0.1
0.2
0.3
0.4
0.5
0
50
100
150
Podocyte #
p < 0.001
r2 = 0.096
B
Induced Podocyte Load vs. Fly Eclosion
Fly Success
0.1
0.2
0.3
0.4
0.5
50
100
150
200
250
300
350
Podocyte #
p < 0.001
r2 = 0.015

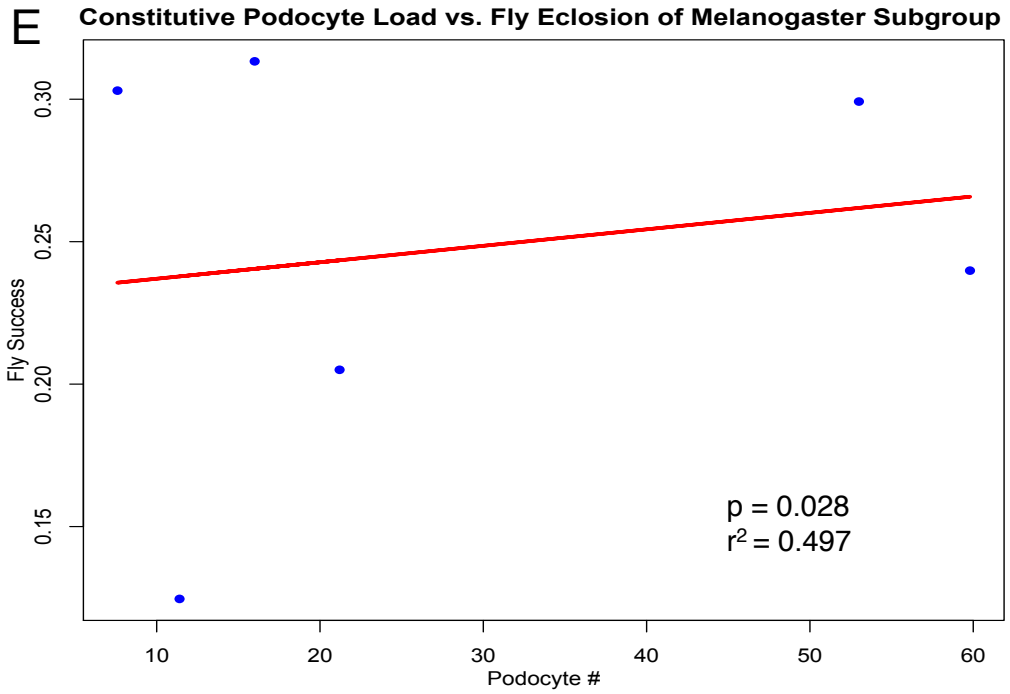
C


Constitutive Podocyte Load vs. Fly Eclosion of Melanotic Encapsulators
Fly Success
0.2
0.3
0.4
0.5
50
100
150
Podocyte #
p = 0.002
r2 = 0.391

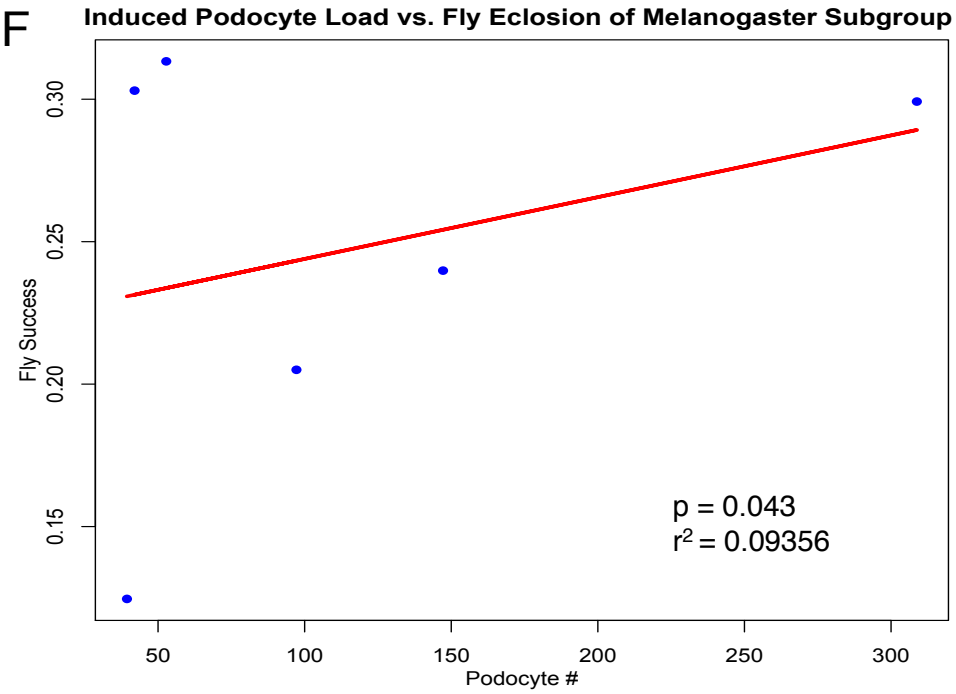
D


Induced Podocyte Load vs. Fly Eclosion of Melanotic Encapsulators
Fly Success
0.2
0.3
0.4
0.5
50
100
150
200
250
300
350
Podocyte #
p < 0.001
r2 = 0.085

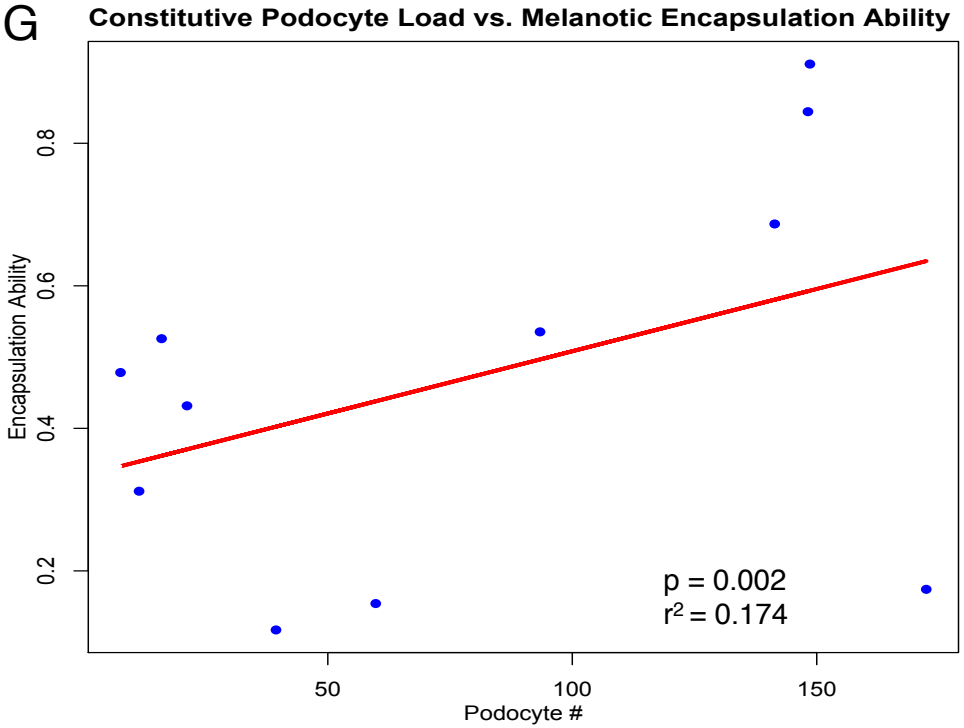
E


Constitutive Podocyte Load vs. Fly Eclosion of Melanogaster Subgroup
Fly Success
0.15
0.20
0.25
0.30
10
20
30
40
50
60
Podocyte #
p = 0.028
r2 = 0.497

F


Induced Podocyte Load vs. Fly Eclosion of Melanogaster Subgroup
Fly Success
0.15
0.20
0.25
0.30
50
100
150
200
250
300
Podocyte #
p = 0.043
r2 = 0.09356

G


Constitutive Podocyte Load vs. Melanotic Encapsulation Ability
Encapsulation Ability
0.2
0.4
0.6
0.8
50
100
150
Podocyte #
p = 0.002
r2 = 0.174

H

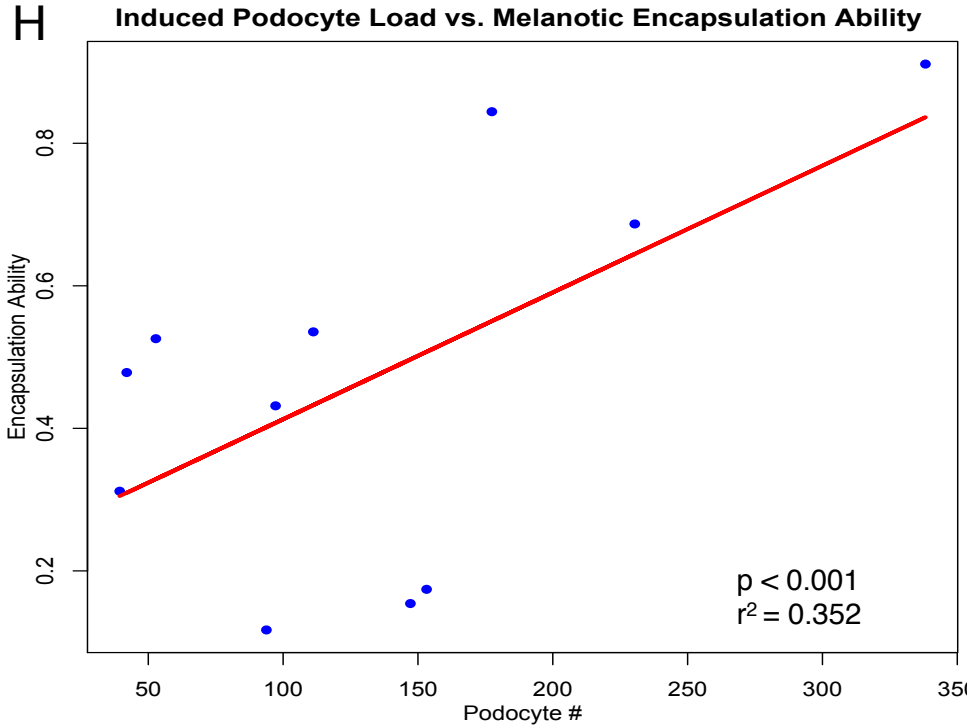
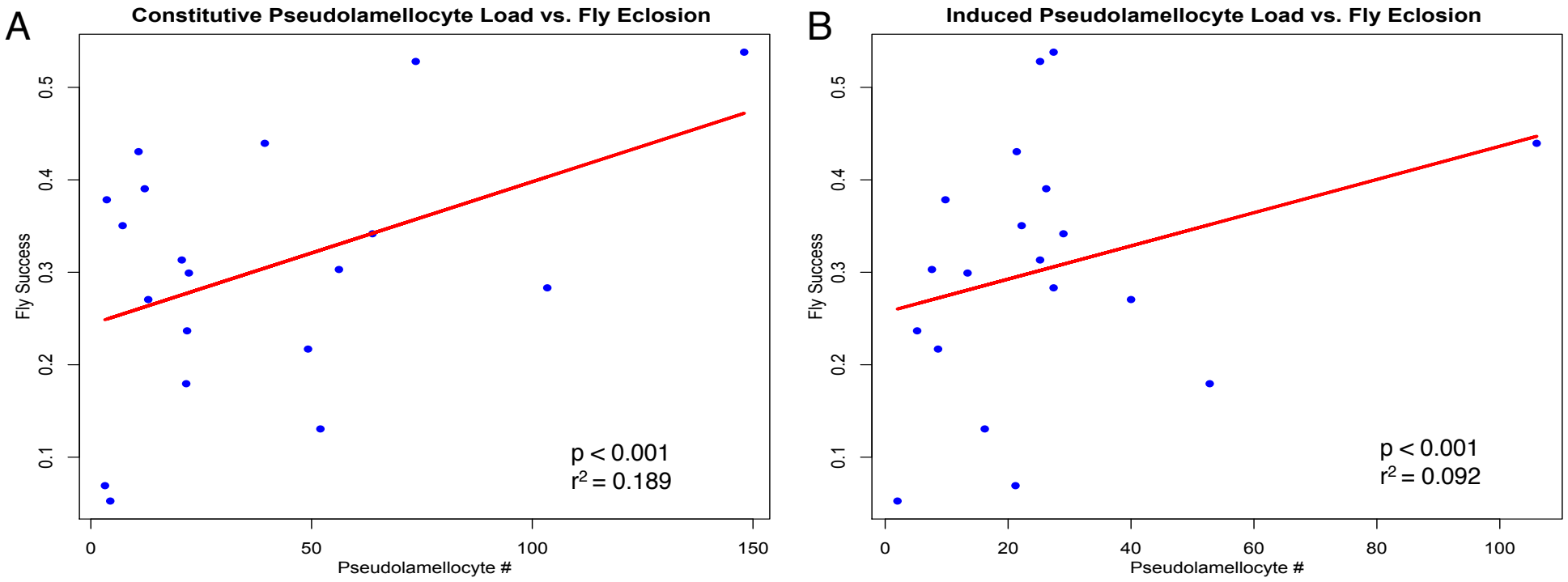
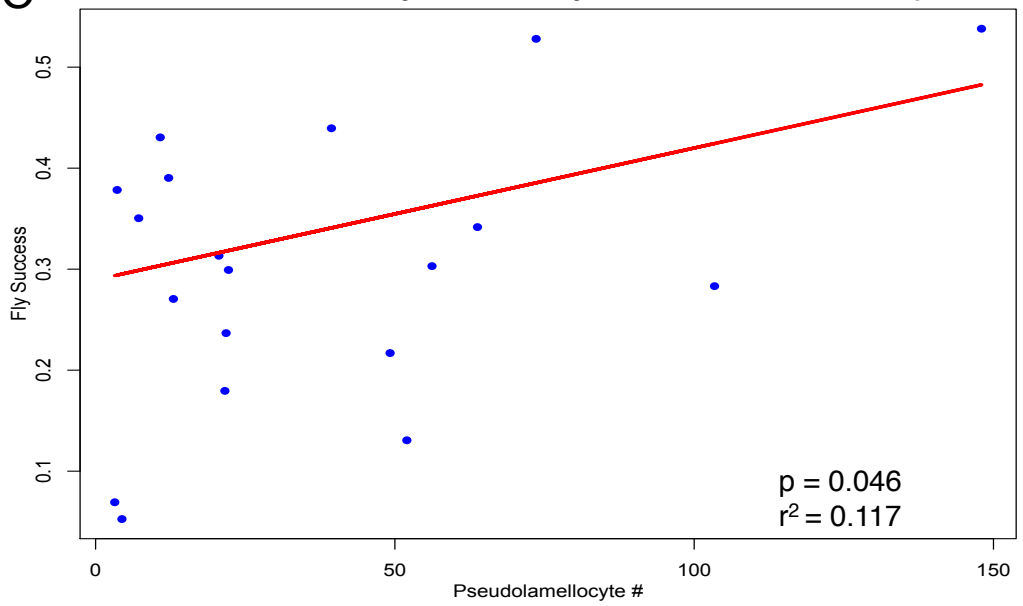

Induced Podocyte Load vs. Melanotic Encapsulation Ability
Encapsulation Ability
0.2
0.4
0.6
0.8
50
100
150
200
250
300
350
Podocyte #
p < 0.001
r2 = 0.352

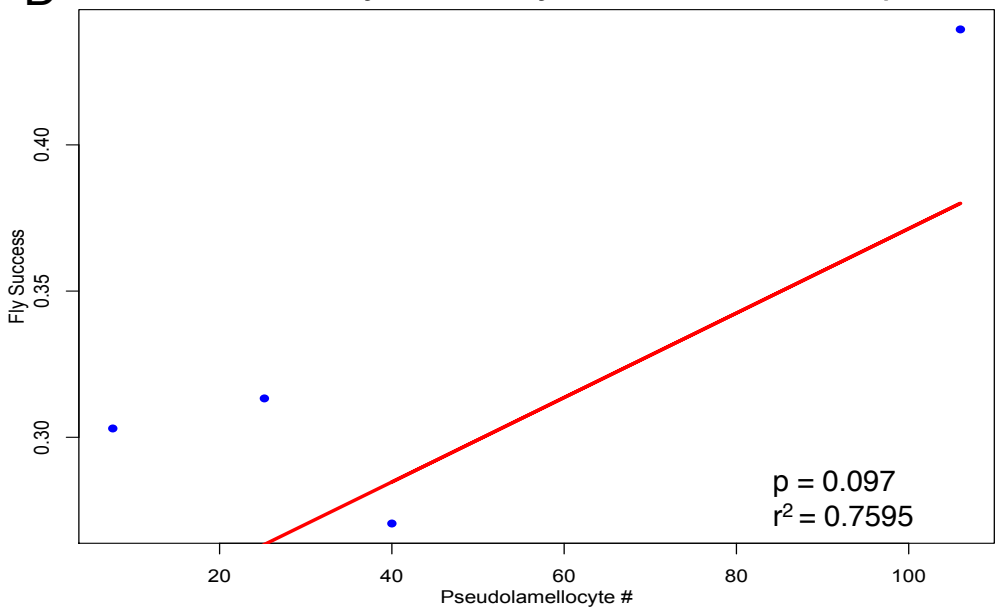
Figure 9


A
Constitutive Pseudolamellocyte Load vs. Fly Eclosion
Fly Success
Pseudolamellocyte #
p < 0.001
r2 = 0.189
B
Induced Pseudolamellocyte Load vs. Fly Eclosion
Fly Success
Pseudolamellocyte #
p < 0.001
r2 = 0.092

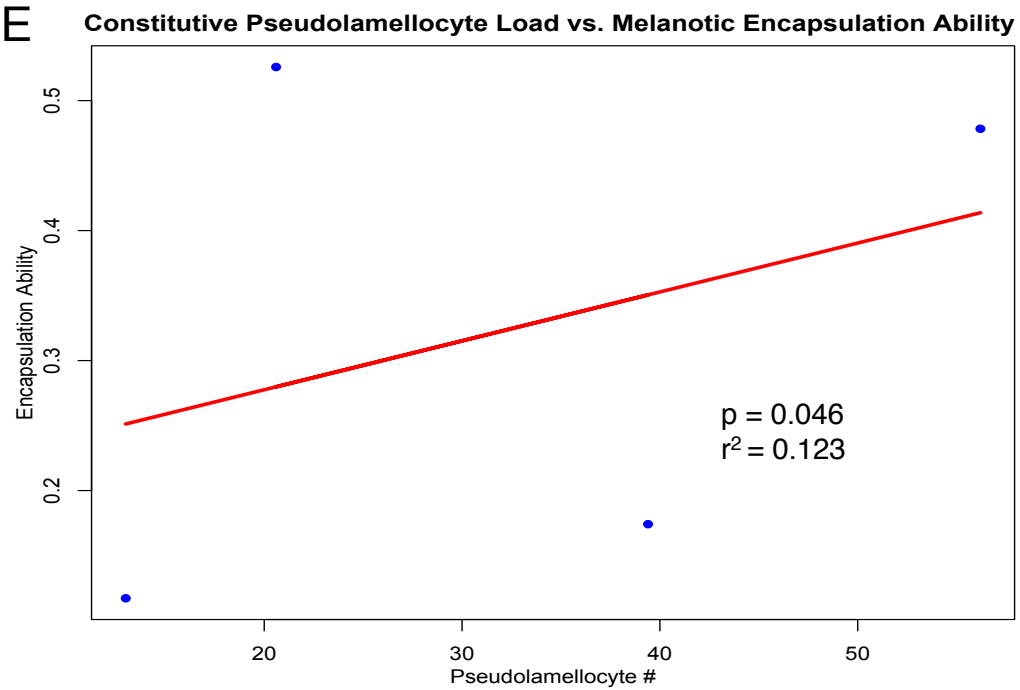
C


Constitutive Pseudolamellocyte Load vs. Fly Eclosion of Melanotic Encapsulators
Fly Success
0.1
0.2
0.3
0.4
0.5
0
50
100
150
Pseudolamellocyte #
p = 0.046
r2 = 0.117

D


Induced Pseudolamellocyte Load vs. Fly Eclosion of Melanotic Encapsulators
Fly Success
0.30
0.35
0.40
20
40
60
80
100
Pseudolamellocyte #
p = 0.097
r2 = 0.7595

E


Constitutive Pseudolamellocyte Load vs. Melanotic Encapsulation Ability
Encapsulation Ability
0.2
0.3
0.4
0.5
20
30
40
50
Pseudolamellocyte #
p = 0.046
r2 = 0.123

F

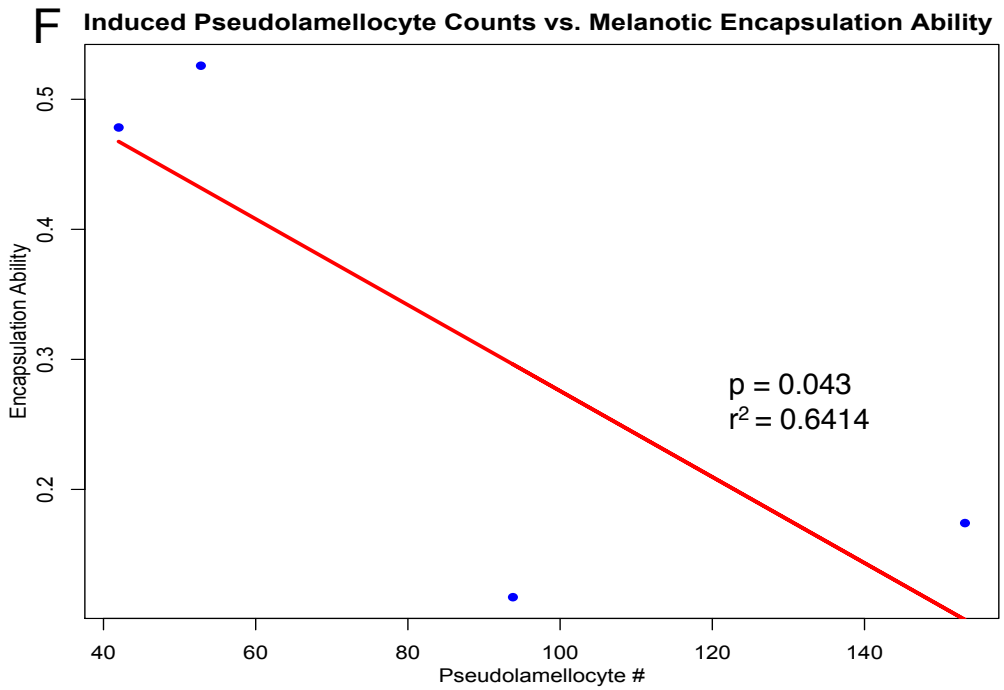
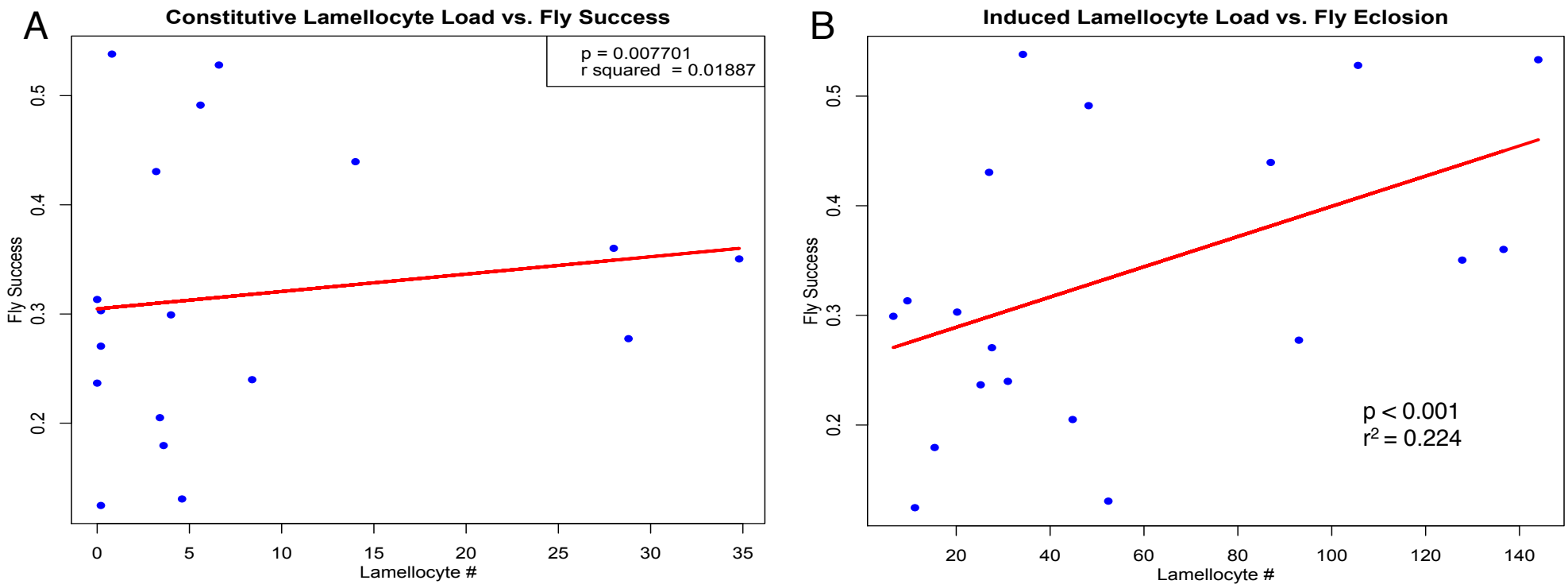
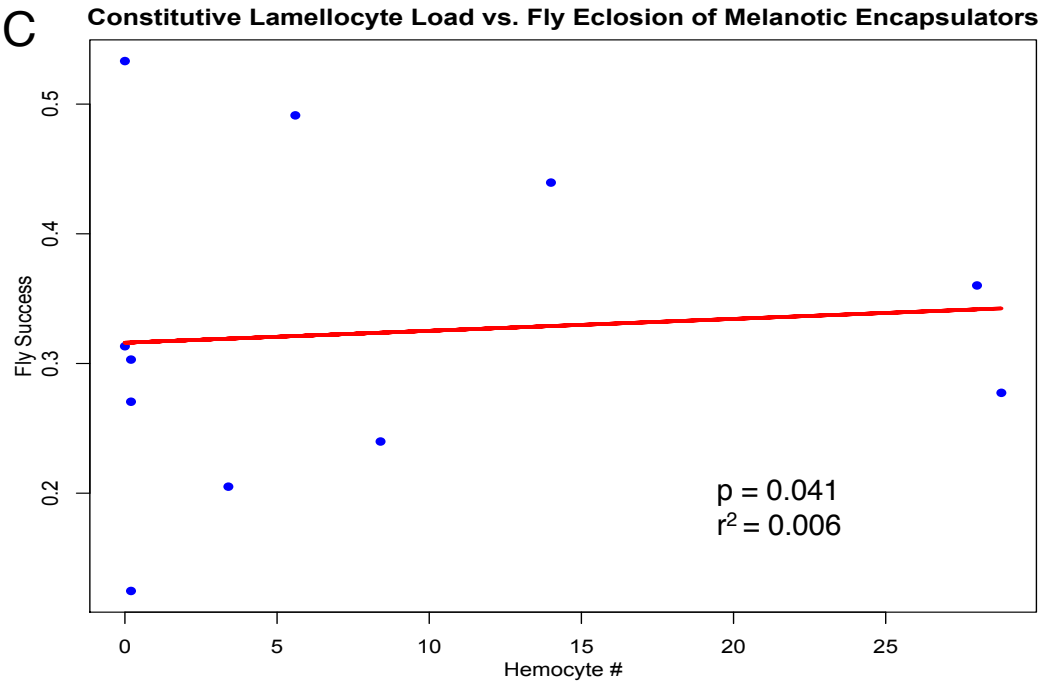

Induced Pseudolamellocyte Counts vs. Melanotic Encapsulation Ability
Encapsulation Ability
0.2
0.3
0.4
0.5
40
60
80
100
120
140
Pseudolamellocyte #
p = 0.043
r2 = 0.6414

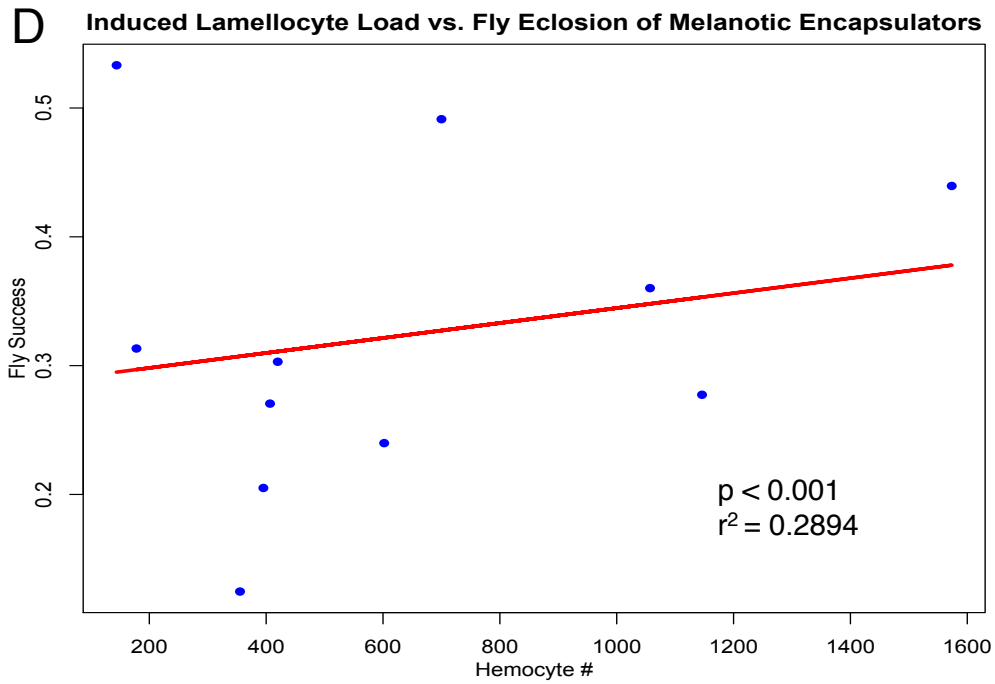
Figure 10


A
Constitutive Lamellocyte Load vs. Fly Success
p = 0.007701
r squared = 0.01887
Fly Success
0.2
0.3
0.4
0.5
0
5
10
15
20
25
30
35
Lamellocyte #
B
Induced Lamellocyte Load vs. Fly Eclosion
Fly Success
0.2
0.3
0.4
0.5
20
40
60
80
100
120
140
Lamellocyte #
p < 0.001
r2 = 0.224

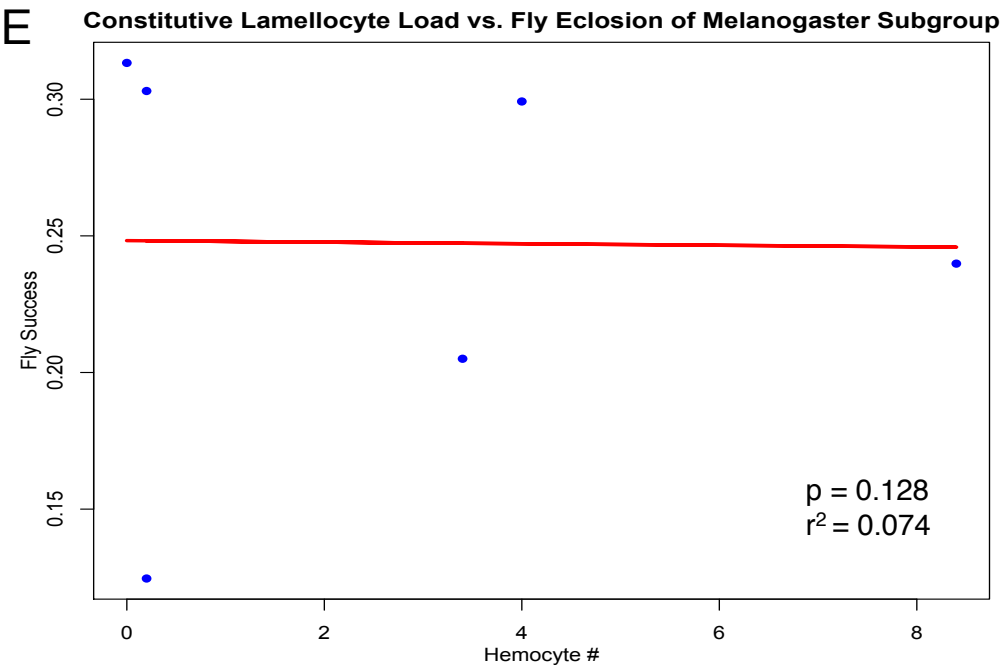
C


Constitutive Lamellocyte Load vs. Fly Eclosion of Melanotic Encapsulators
Fly Success
0.2
0.3
0.4
0.5
0
5
10
15
20
25
Hemocyte #
p = 0.041
r2 = 0.006

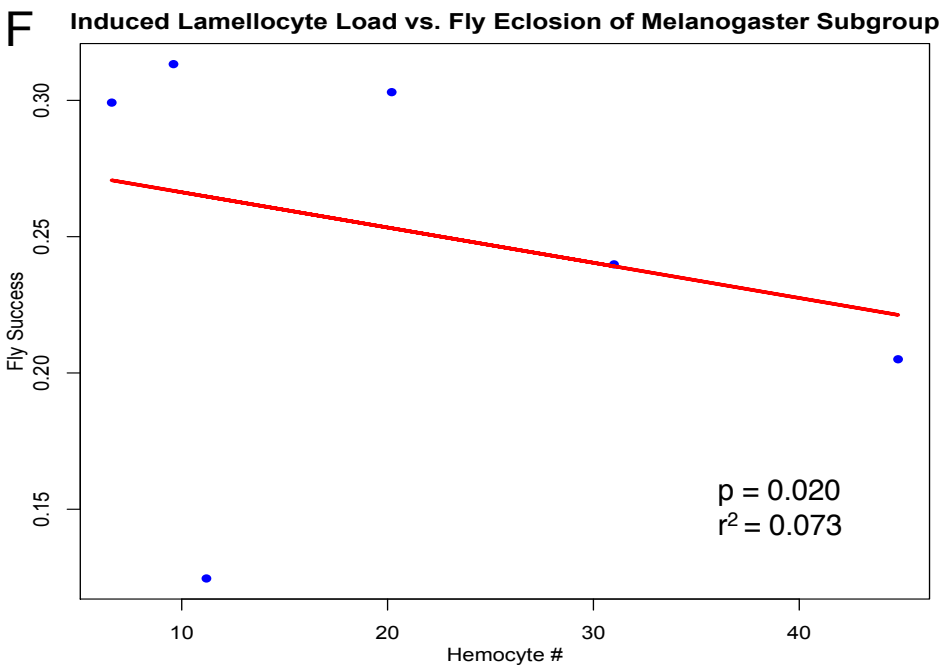
D

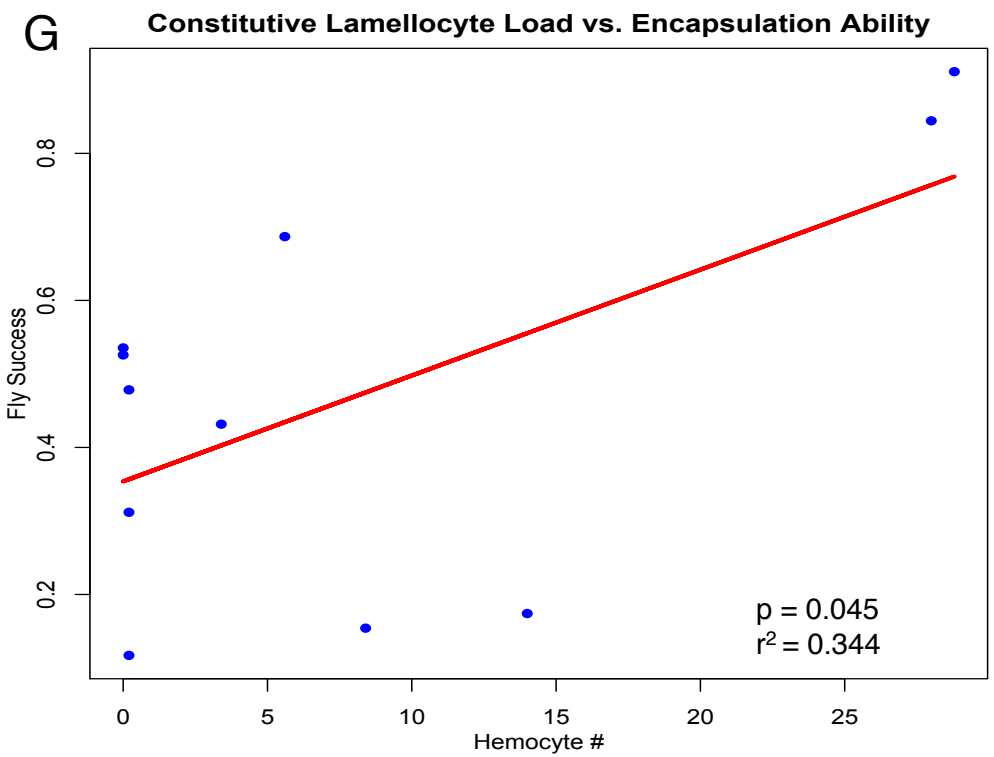

Induced Lamellocyte Load vs. Fly Eclosion of Melanotic Encapsulators
Fly Success
0.2
0.3
0.4
0.5
200
400
600
800
1000
1200
1400
1600
Hemocyte #
p < 0.001
r2 = 0.2894

E


Constitutive Lamellocyte Load vs. Fly Eclosion of Melanogaster Subgroup
Fly Success
0.15
0.20
0.25
0.30
0
2
4
6
8
Hemocyte #
p = 0.128
r2 = 0.074

F


Induced Lamellocyte Load vs. Fly Eclosion of Melanogaster Subgroup
Fly Success
0.15
0.20
0.25
0.30
10
20
30
40
Hemocyte #
p = 0.020
r2 = 0.073


G
Constitutive Lamellocyte Load vs. Encapsulation Ability
Fly Success
0.2
0.4
0.6
0.8
0
5
10
15
20
25
Hemocyte #
p = 0.045
r2 = 0.344

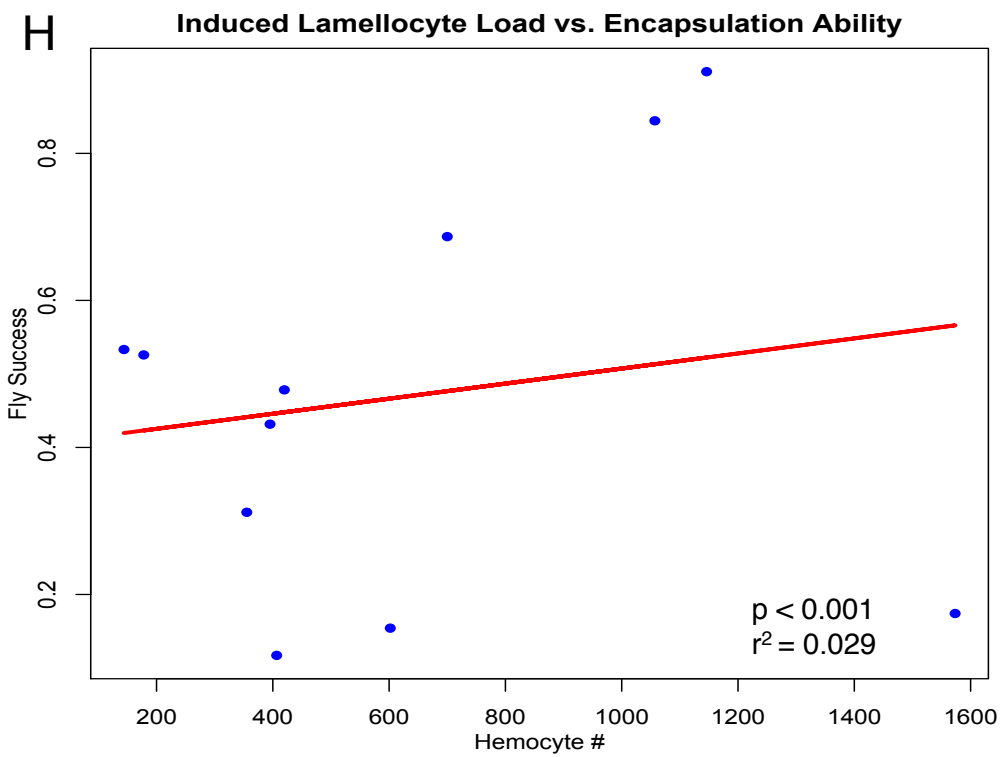
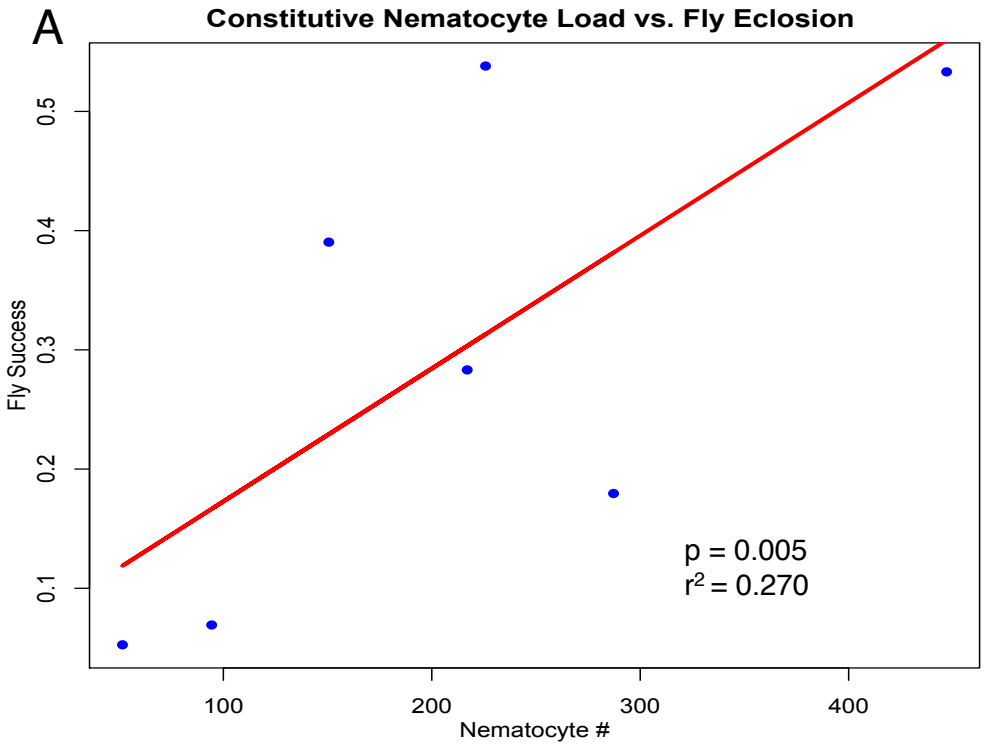

H
Induced Lamellocyte Load vs. Encapsulation Ability
Fly Success
0.2
0.4
0.6
0.8
200
400
600
800
1000
1200
1400
1600
Hemocyte #
p < 0.001
r2 = 0.029

Figure 11


A
Constitutive Nematocyte Load vs. Fly Eclosion
Fly Success
0.1
0.2
0.3
0.4
0.5
100
200
300
400
Nematocyte #
p = 0.005
r2 = 0.270

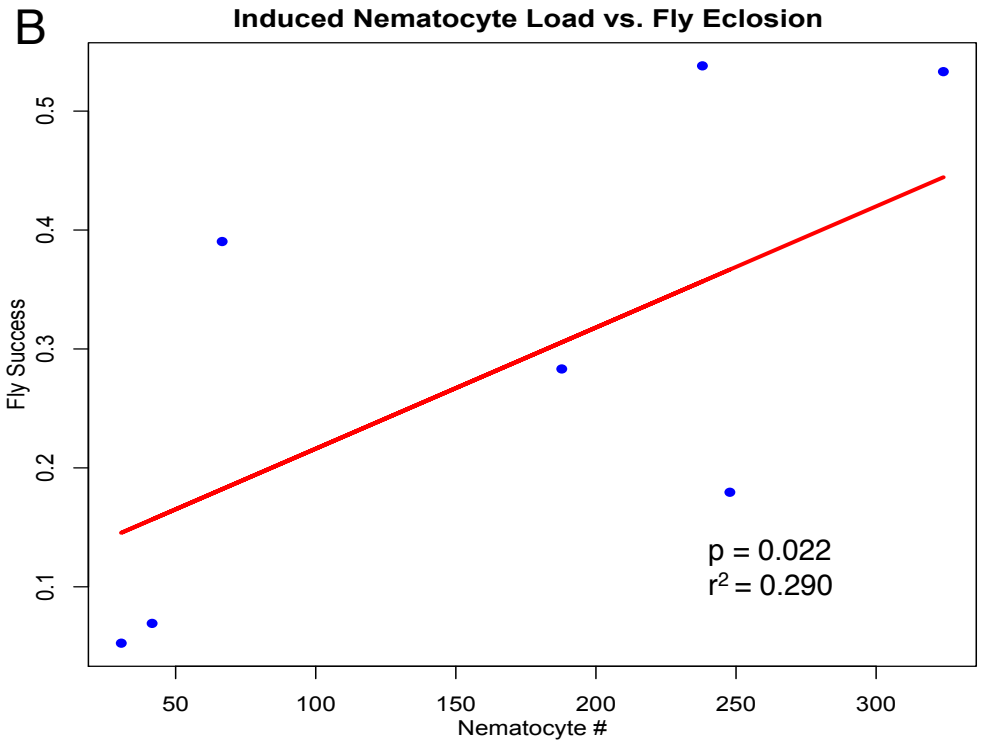
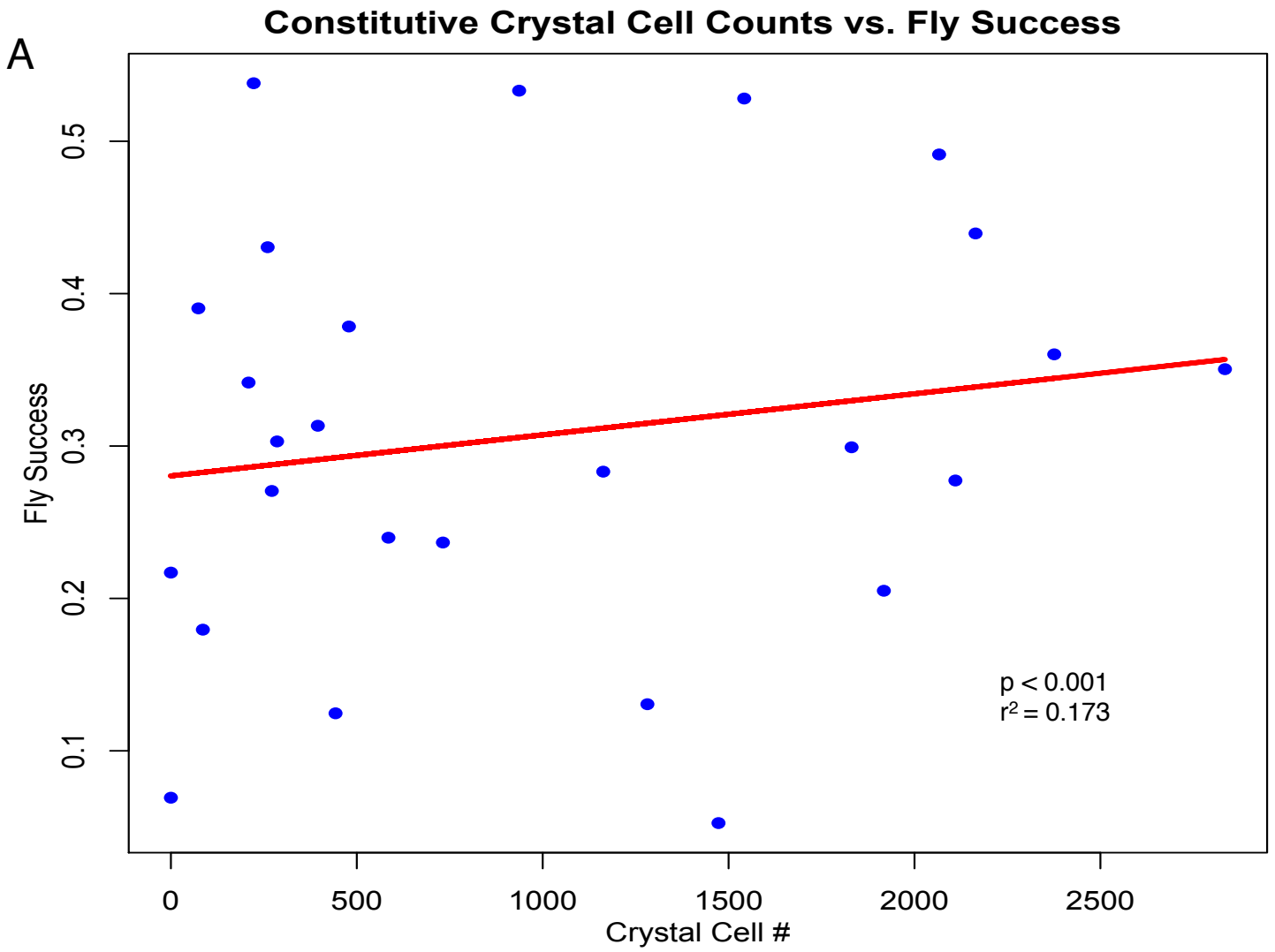
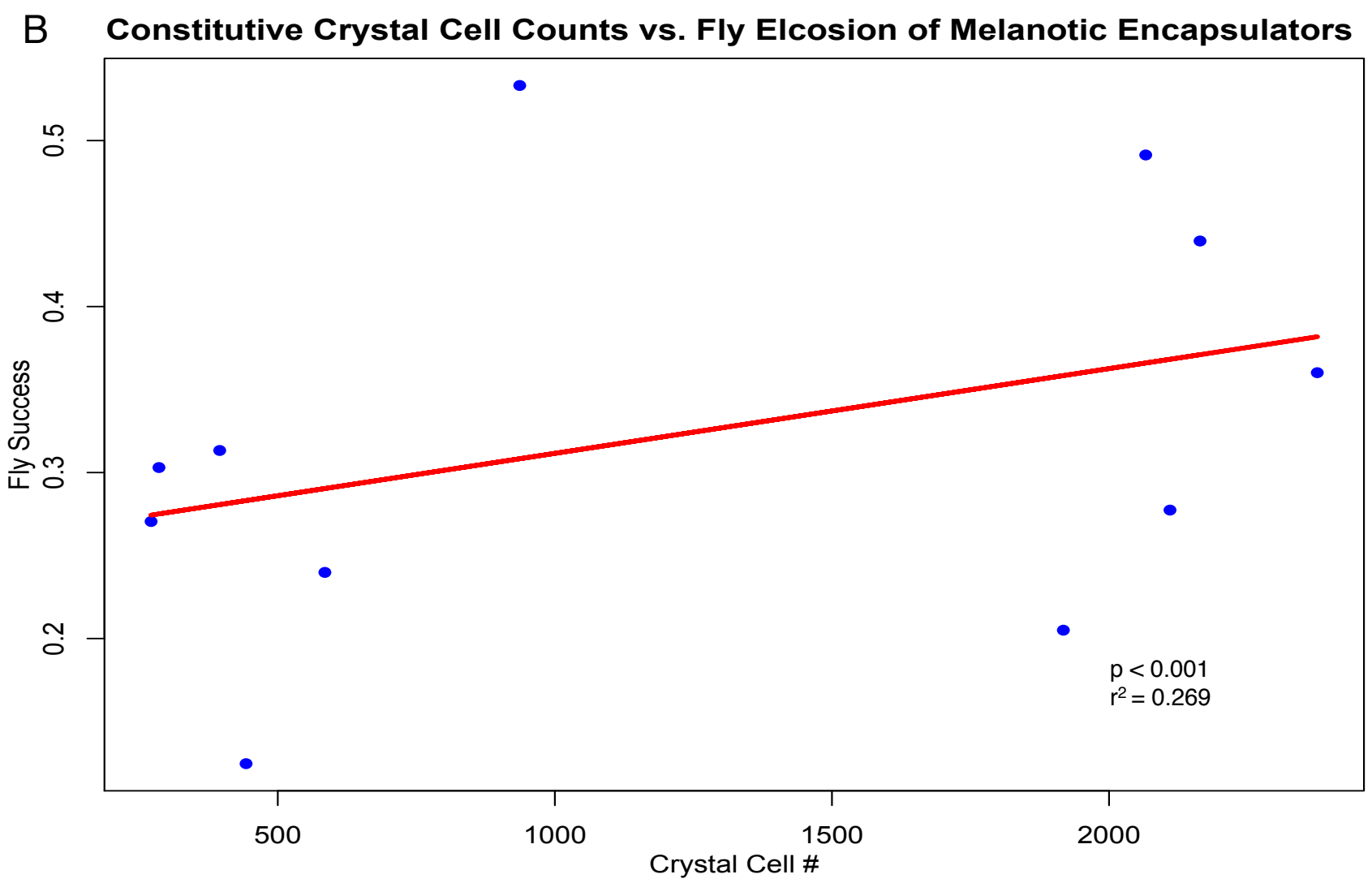
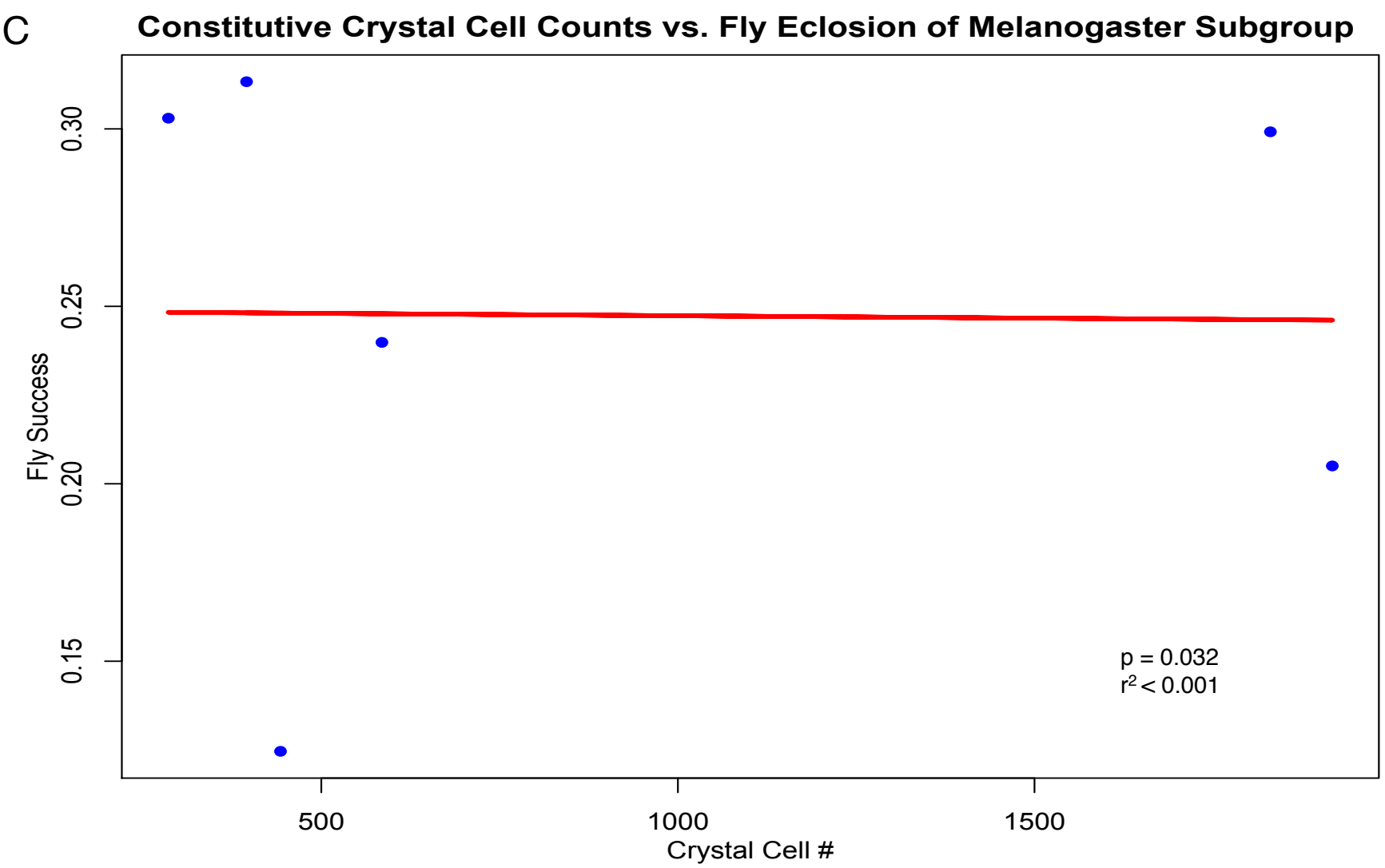

B
Induced Nematocyte Load vs. Fly Eclosion
Fly Success
0.1
0.2
0.3
0.4
0.5
50
100
150
200
250
300
Nematocyte #
p = 0.022
r2 = 0.290

Figure 12


A
Constitutive Crystal Cell Counts vs. Fly Success
Fly Success
0.1
0.2
0.3
0.4
0.5
0
500
1000
1500
2000
2500
Crystal Cell #
p < 0.001
r2 = 0.173


B
Constitutive Crystal Cell Counts vs. Fly Elcosion of Melanotic Encapsulators
Fly Success
0.2
0.3
0.4
0.5
500
1000
1500
2000
Crystal Cell #
p < 0.001
r2 = 0.269

C


Constitutive Crystal Cell Counts vs. Fly Eclosion of Melanogaster Subgroup
Fly Success
0.15
0.20
0.25
0.30
500
1000
1500
Crystal Cell #
p = 0.032
r2 < 0.001

D

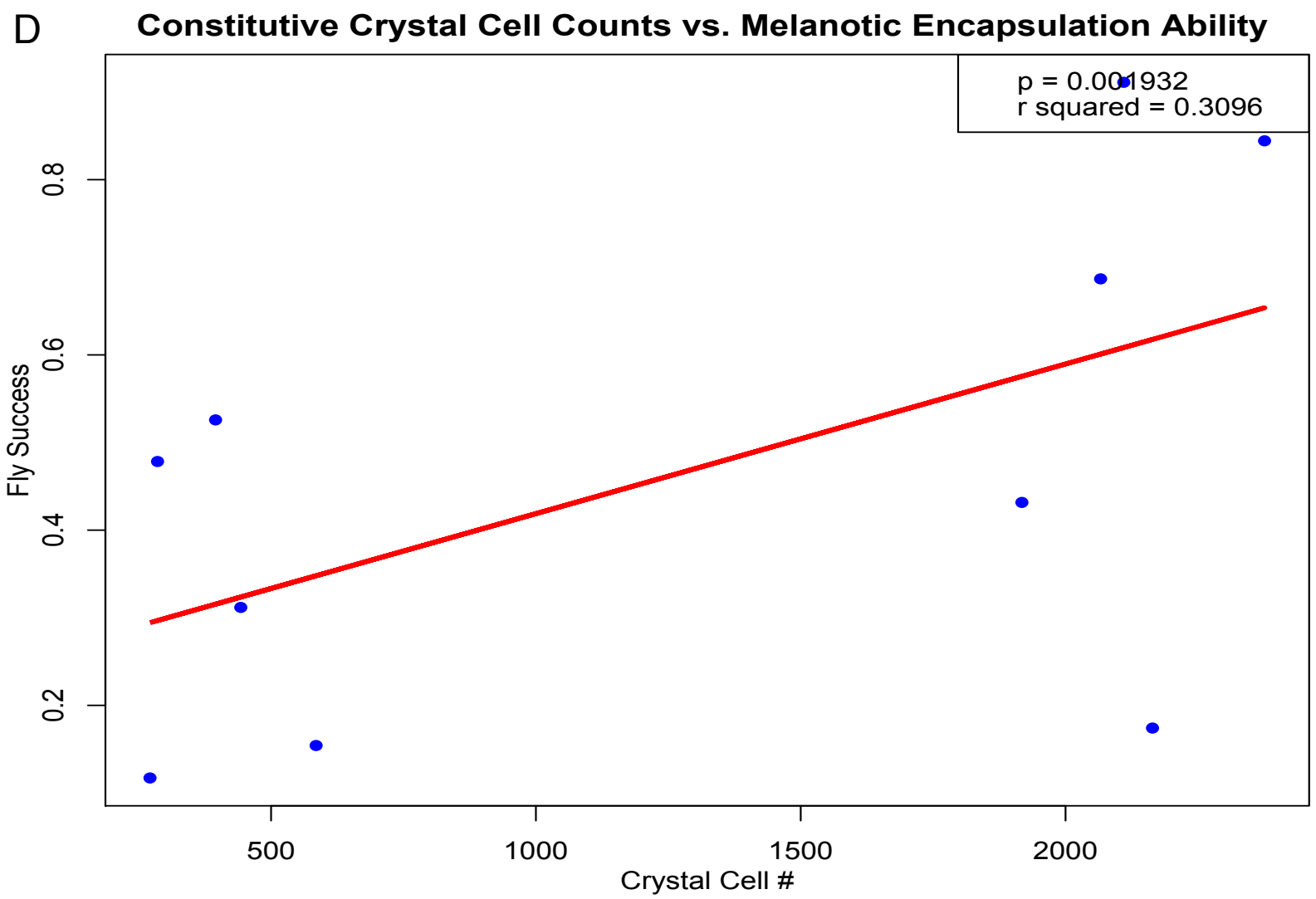
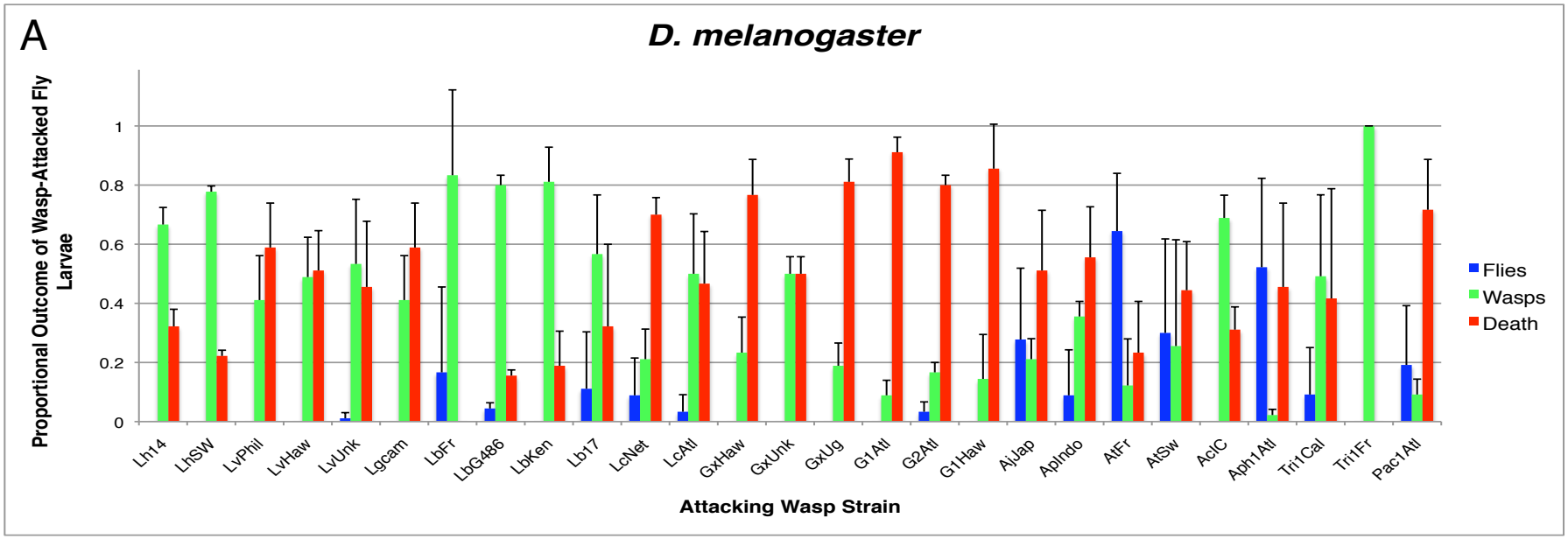
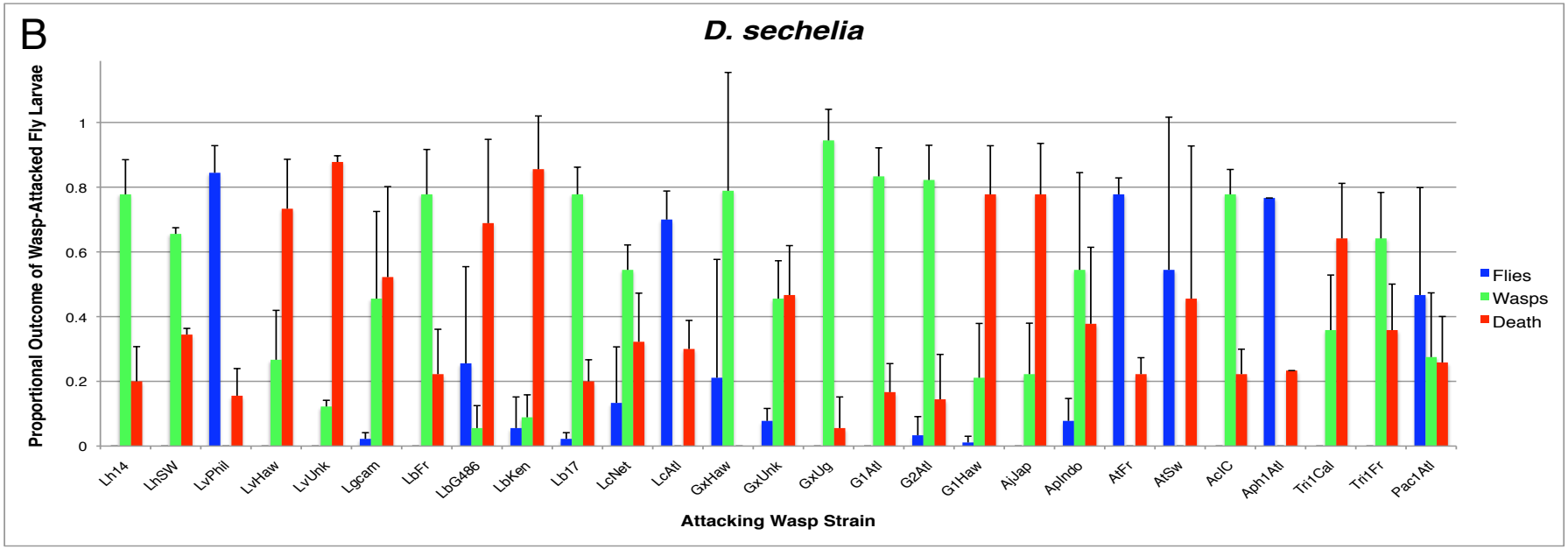
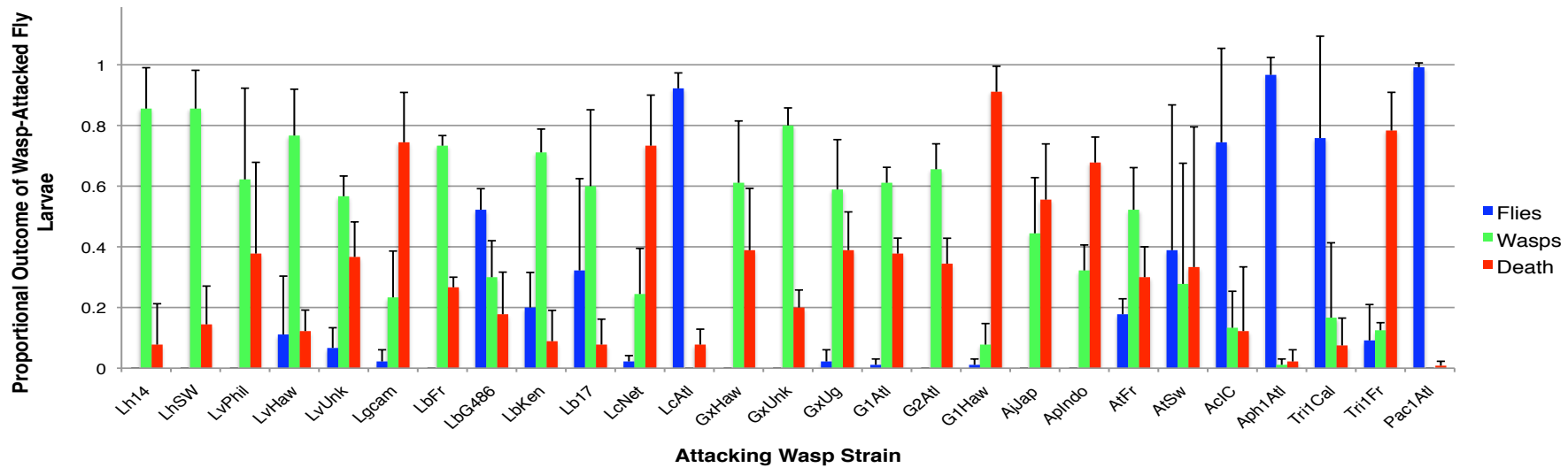

Constitutive Crystal Cell Counts vs. Melanotic Encapsulation Ability
p = 0.001932
r squared = 0.3096
Fly Success
0.2
0.4
0.6
0.8
500
1000
1500
2000
Crystal Cell #

Figure 13

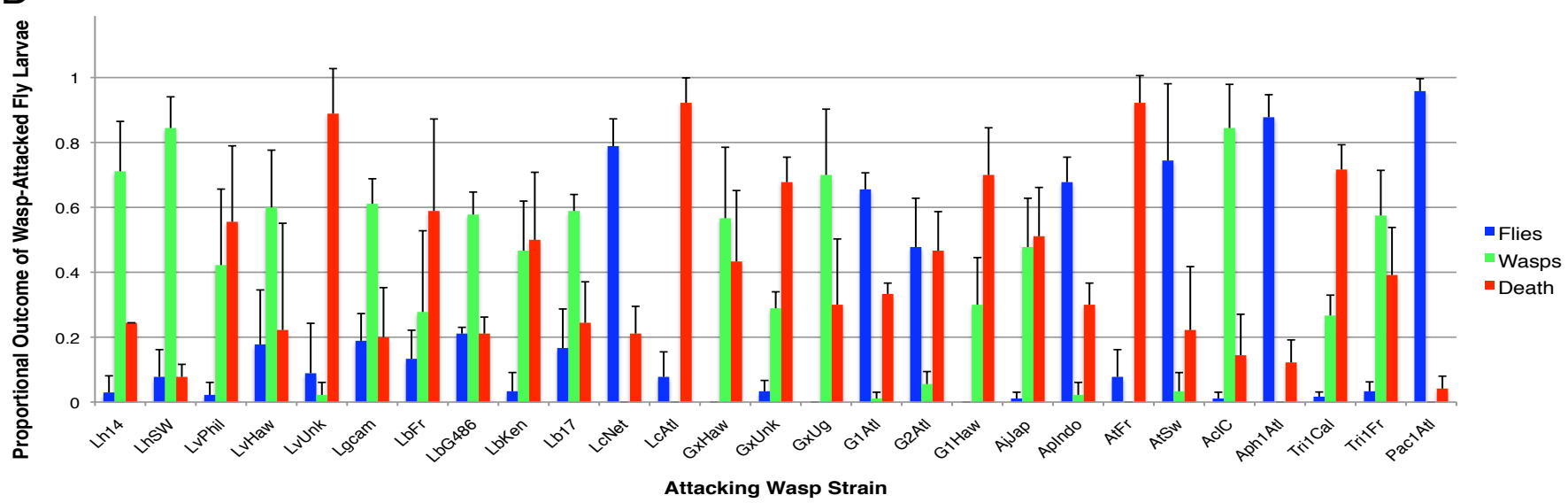

A
D. melanogaster
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Attacking Wasp Strain
Flies
Wasps
Death


B
D. sechelia
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Attacking Wasp Strain
Flies
Wasps
Death

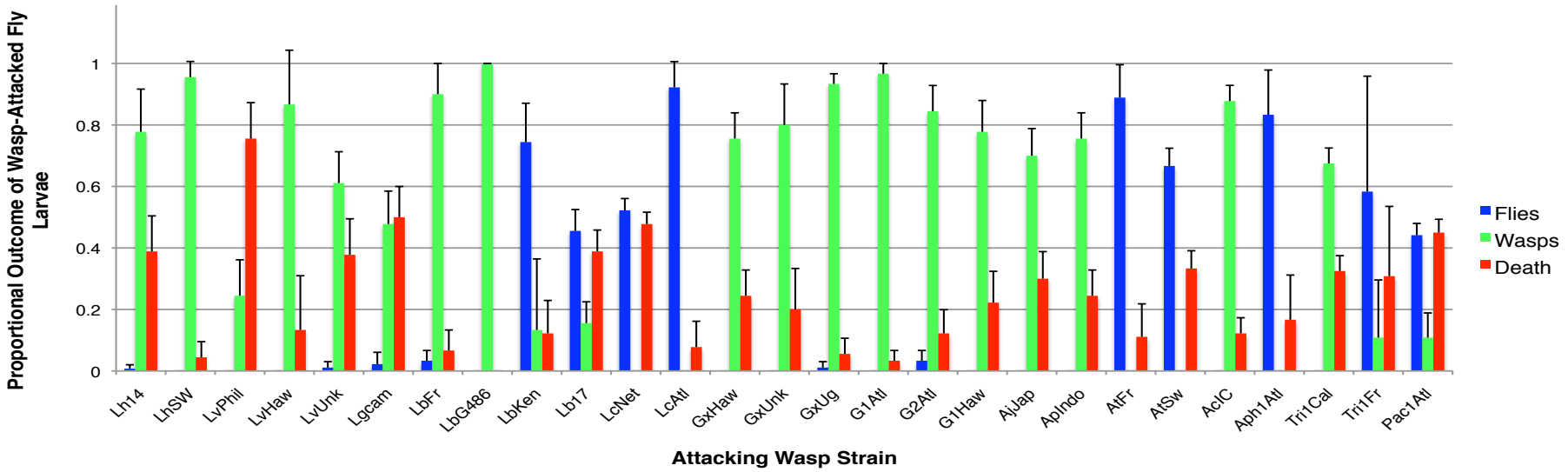
C


D. simulans
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Attacking Wasp Strain
Flies
Wasps
Death

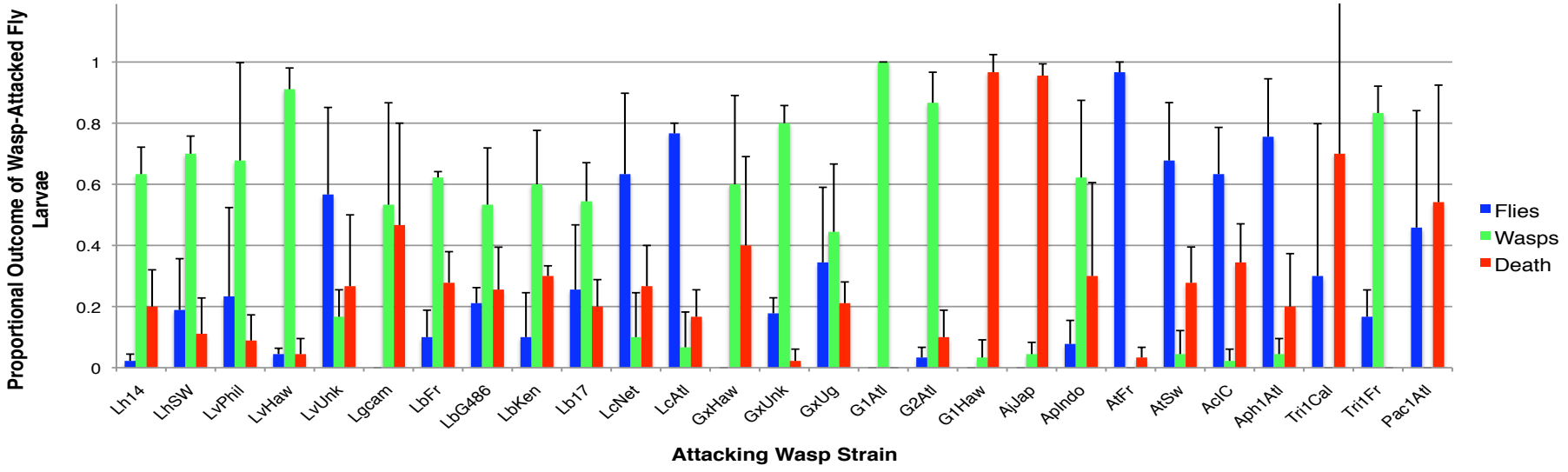
D


D. mauritiana
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Attacking Wasp Strain
Flies
Wasps
Death

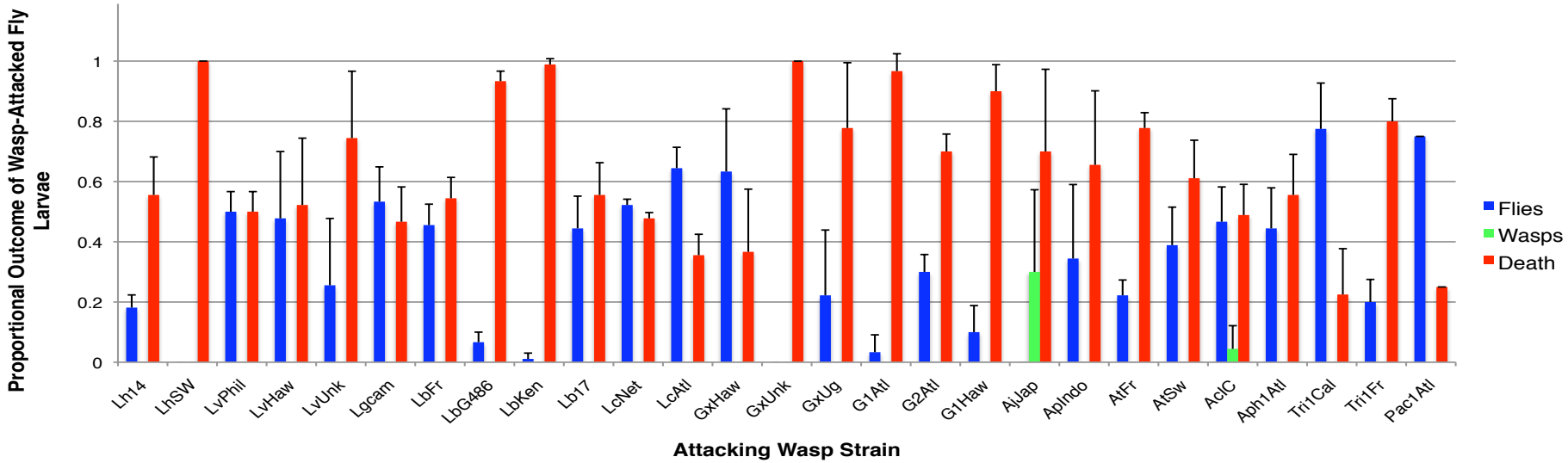
E


D. yakuba
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AcIC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Attacking Wasp Strain
Flies
Wasps
Death

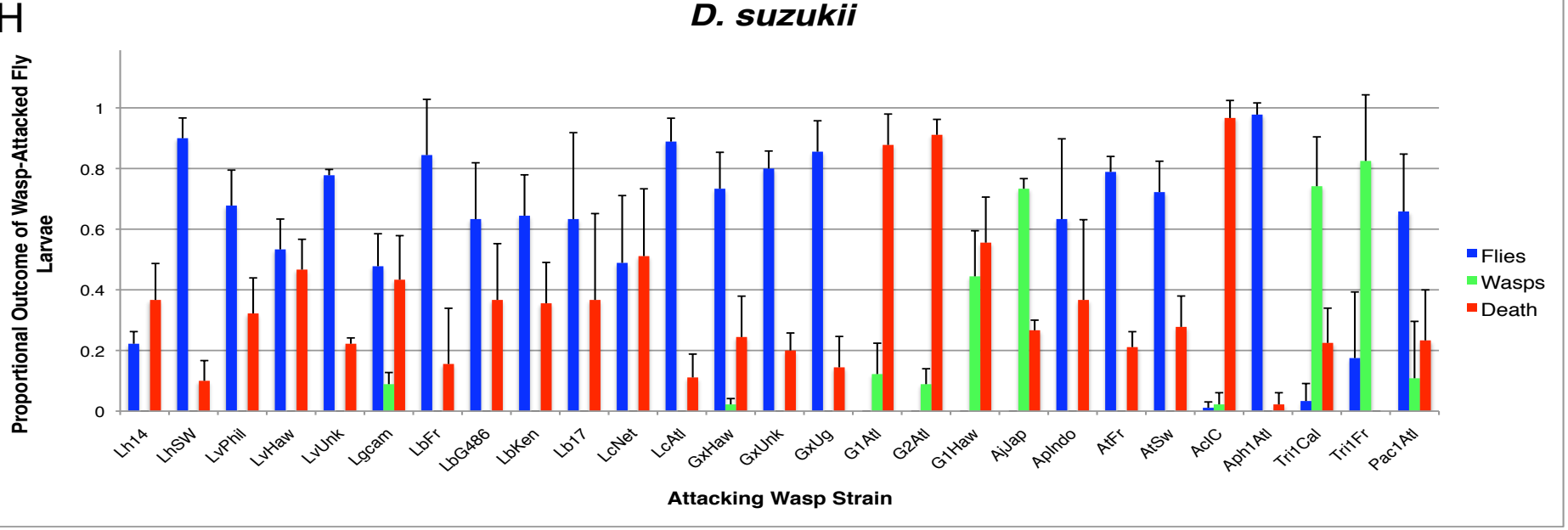
F


D. erecta
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AcIC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Attacking Wasp Strain
Flies
Wasps
Death

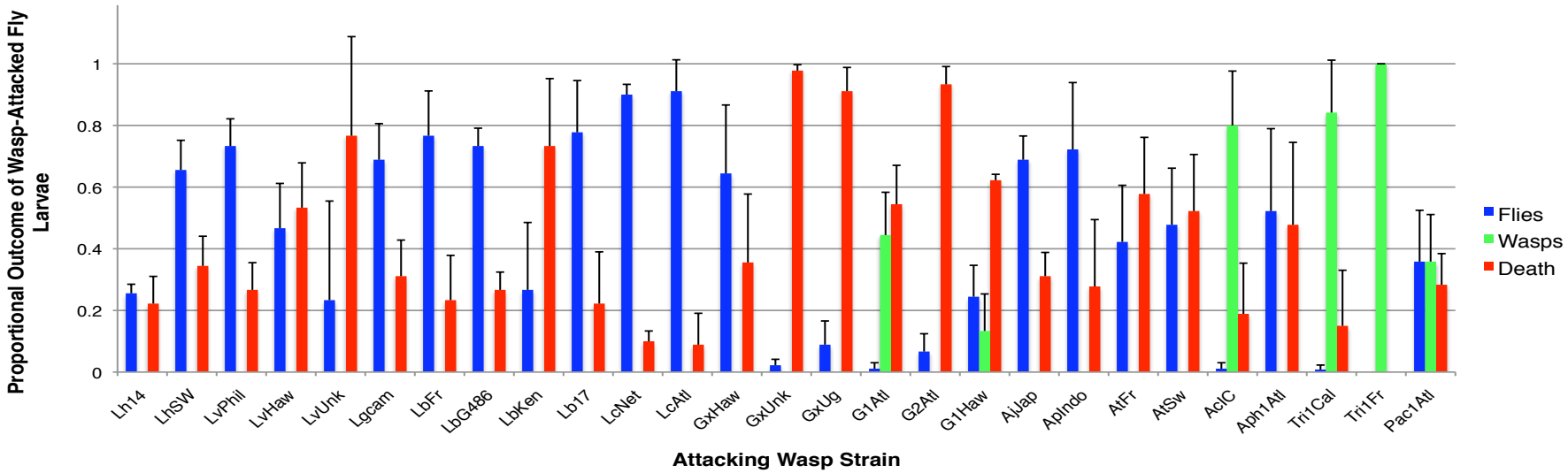
G


D. eugracilis
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Attacking Wasp Strain
Flies
Wasps
Death

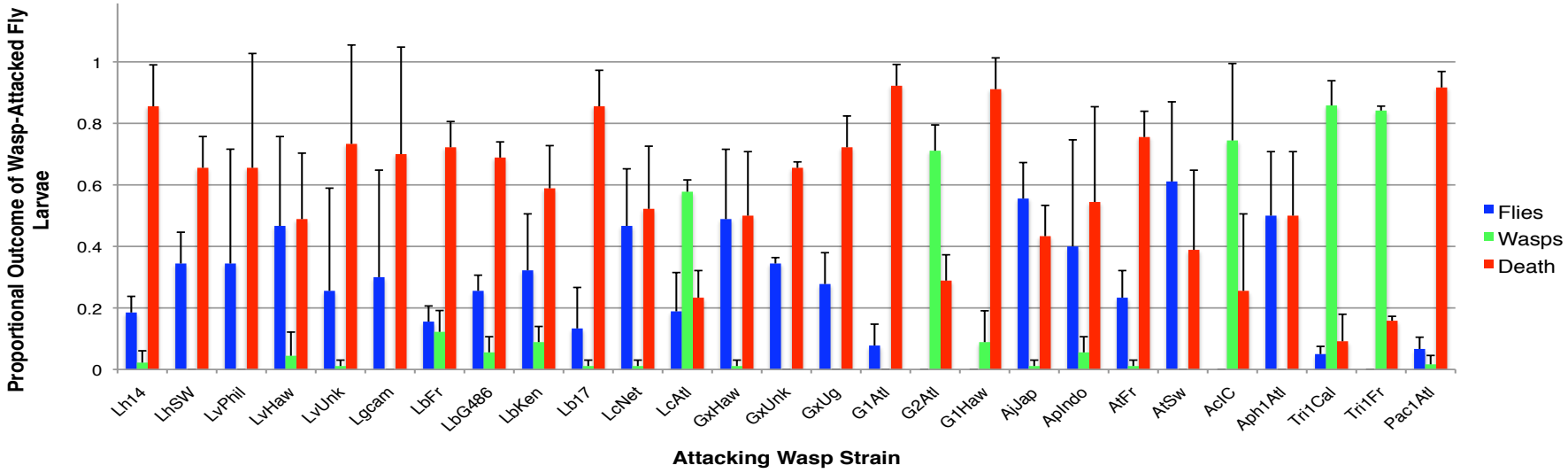
H


D. suzukii
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Attacking Wasp Strain
Flies
Wasps
Death

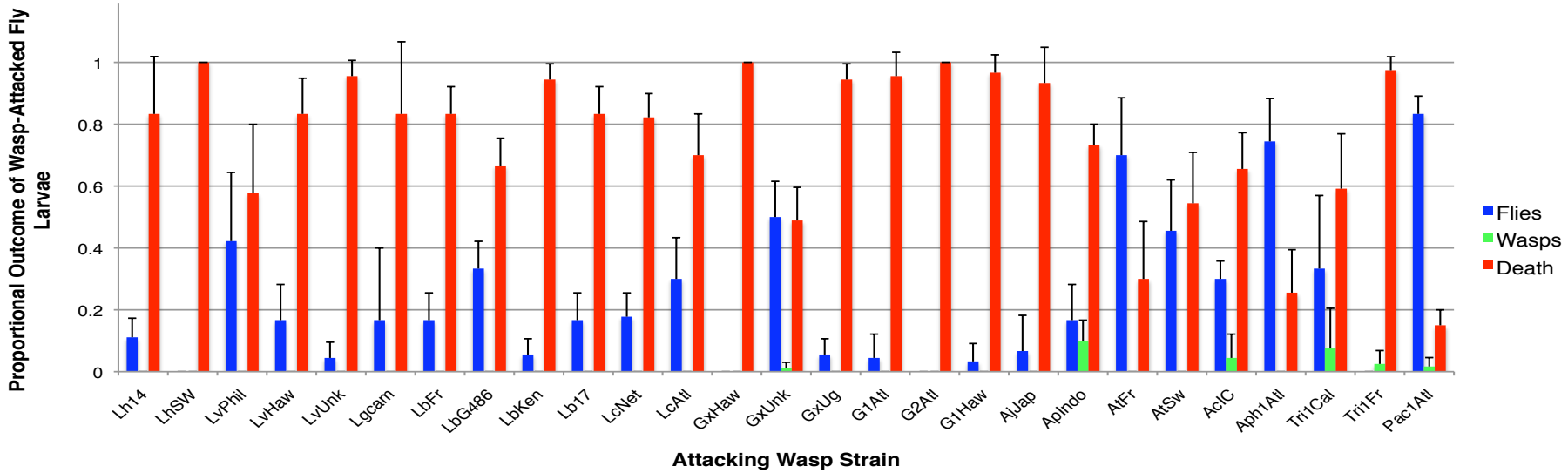
I


D. biarmipes
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Attacking Wasp Strain
Flies
Wasps
Death

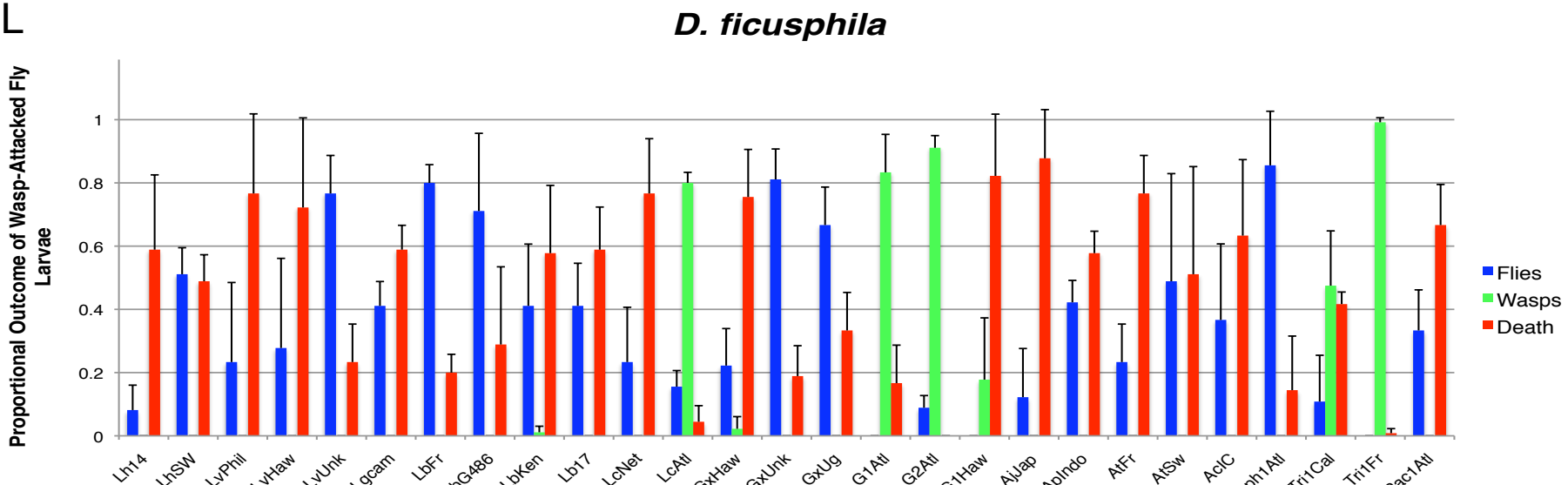
J


D. lutescens
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Attacking Wasp Strain
Flies
Wasps
Death

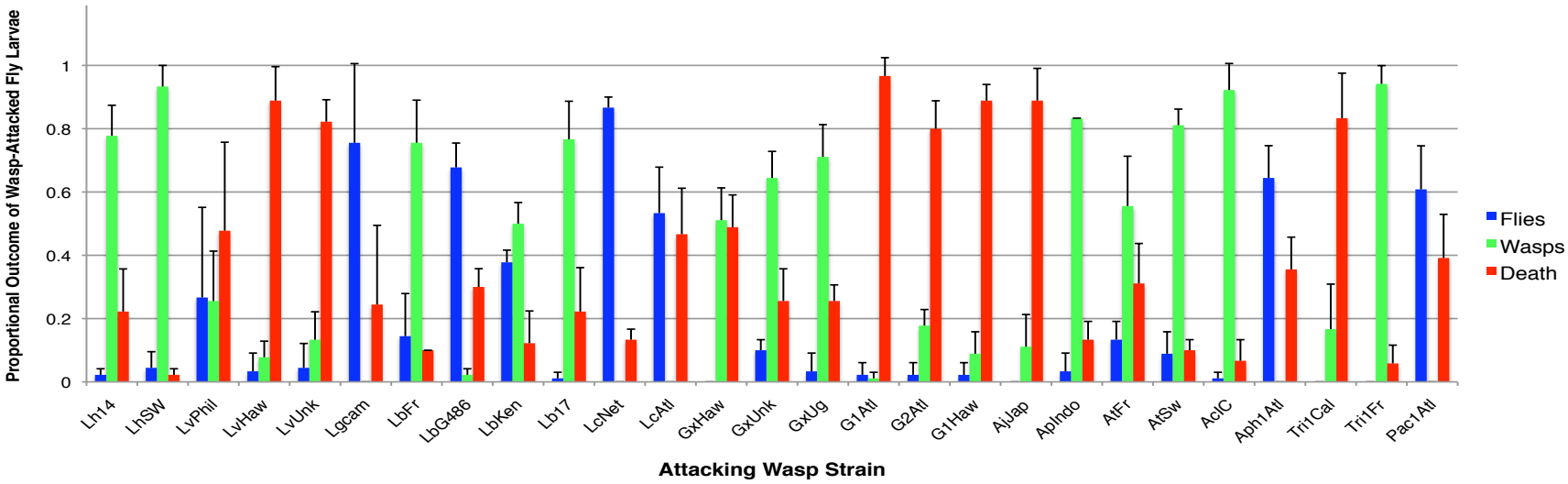
K


D. elegans
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Attacking Wasp Strain
Flies
Wasps
Death

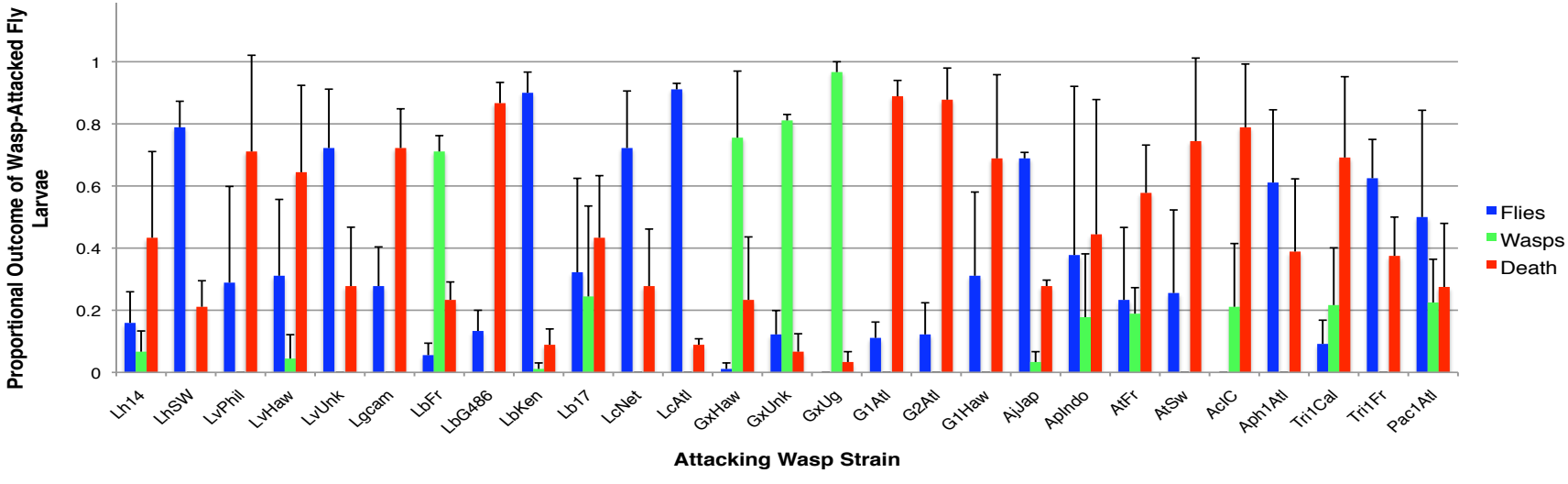
L


D. ficusphila
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Attacking Wasp Strain
Flies
Wasps
Death

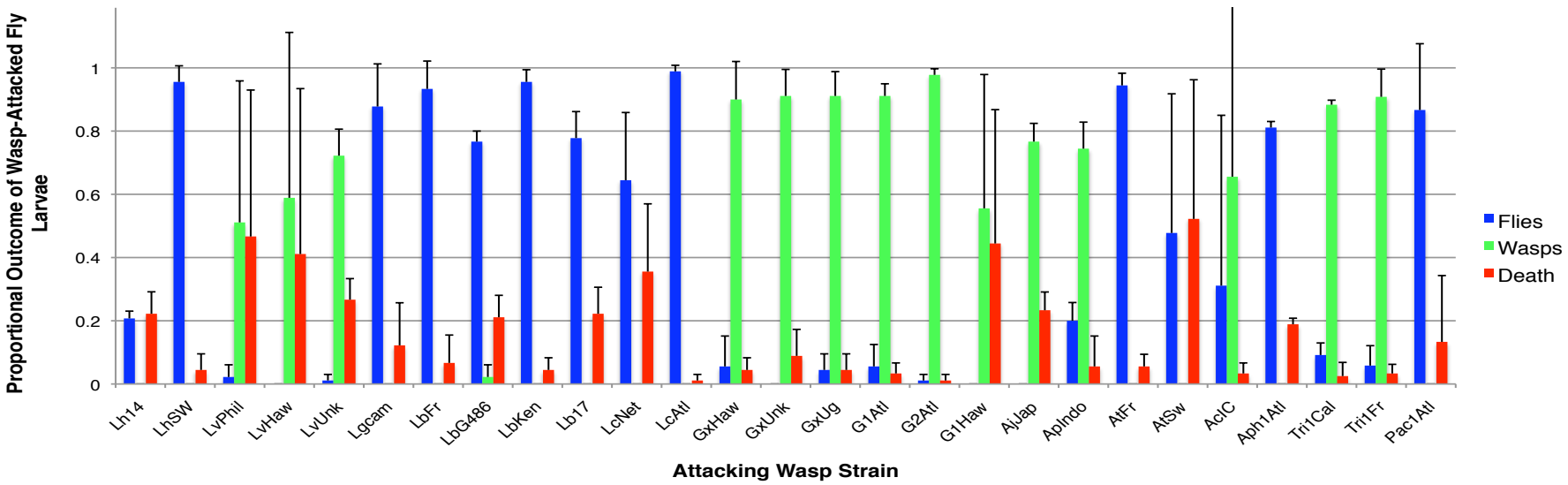
M


D. tsacasi
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Attacking Wasp Strain
Flies
Wasps
Death

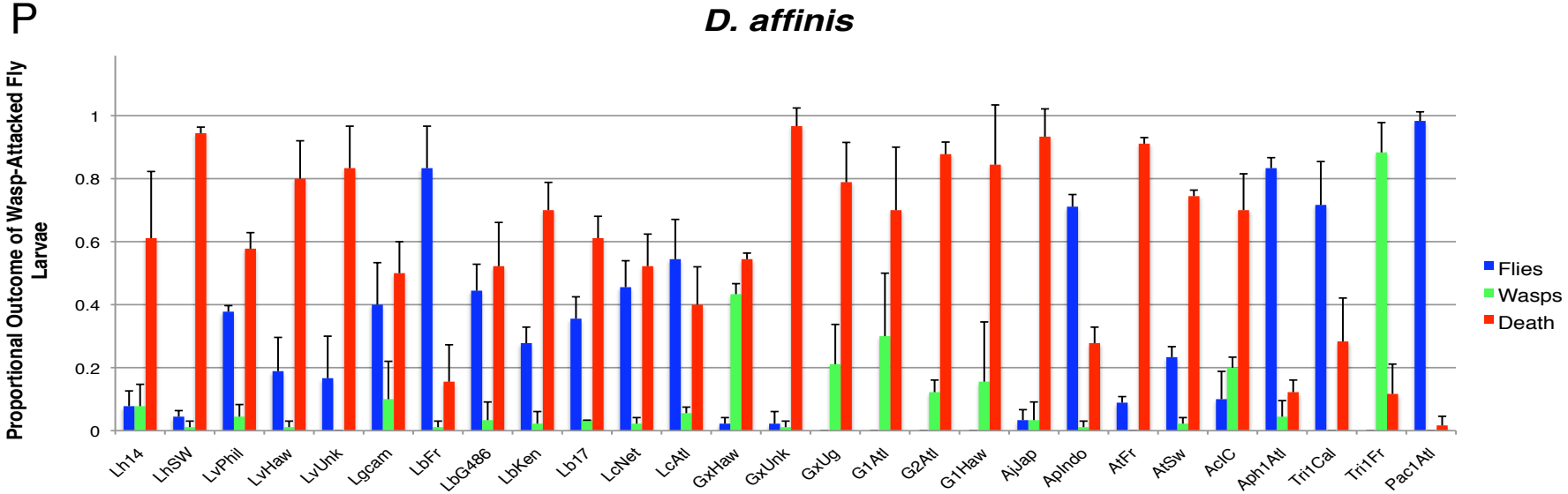
N


D. kikkawai
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Attacking Wasp Strain
Flies
Wasps
Death

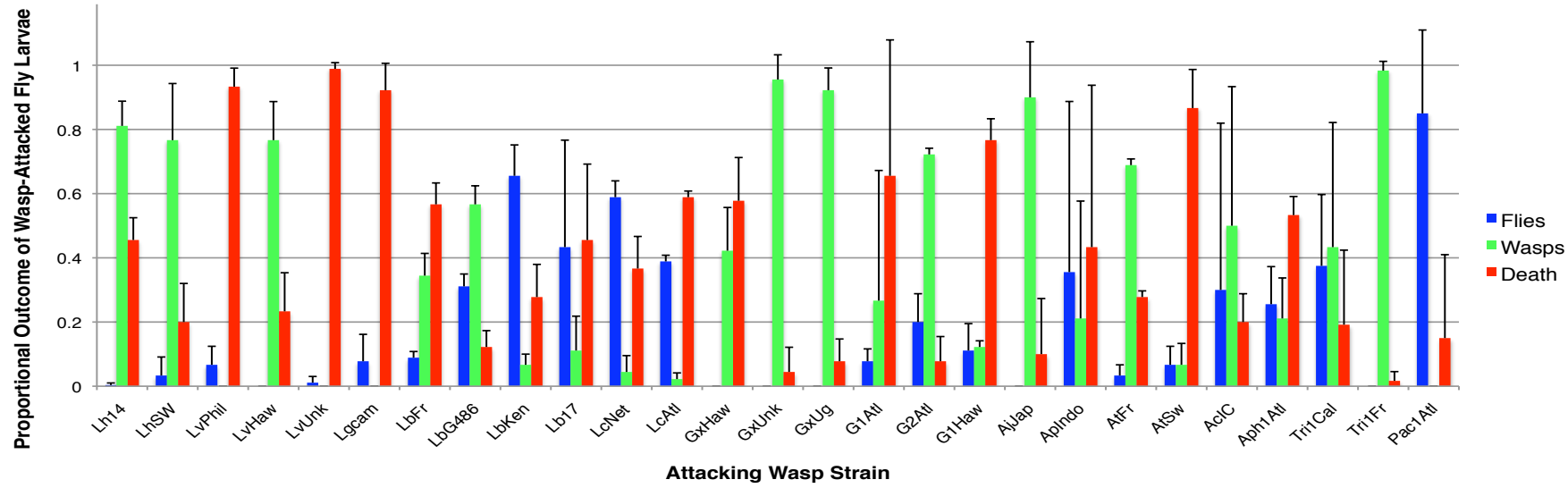
O


D. ananassae
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Attacking Wasp Strain
Flies
Wasps
Death

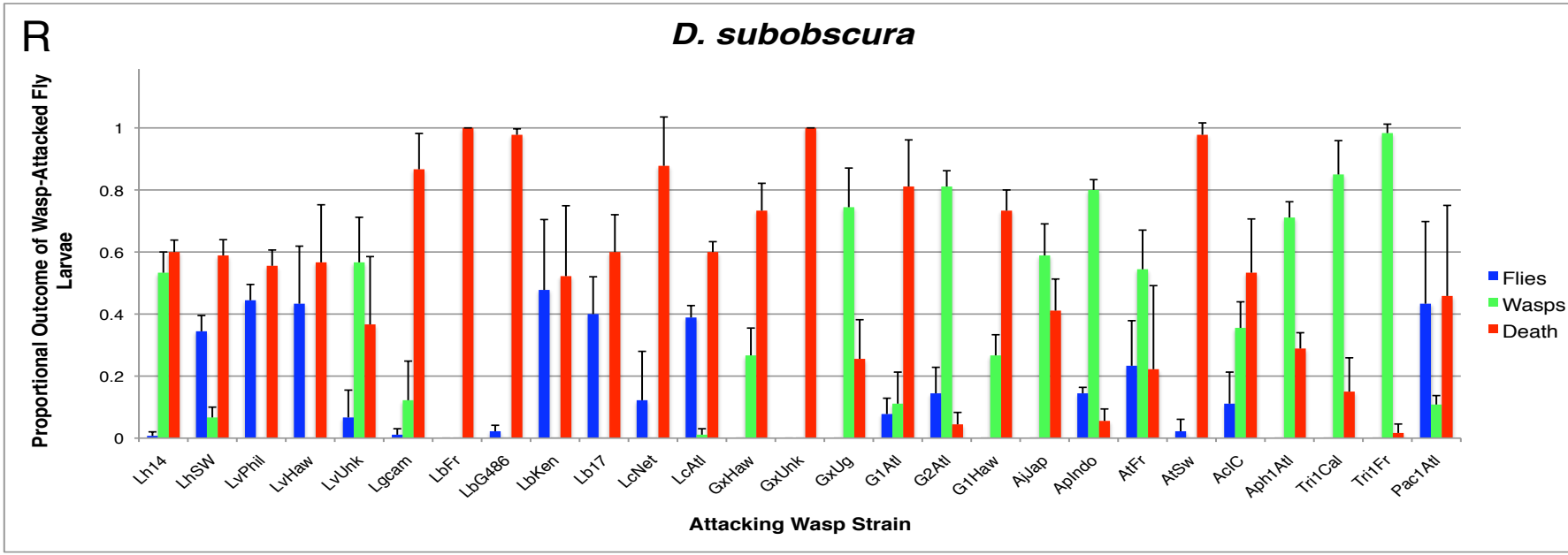
P


D. affinis
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Attacking Wasp Strain
Flies
Wasps
Death

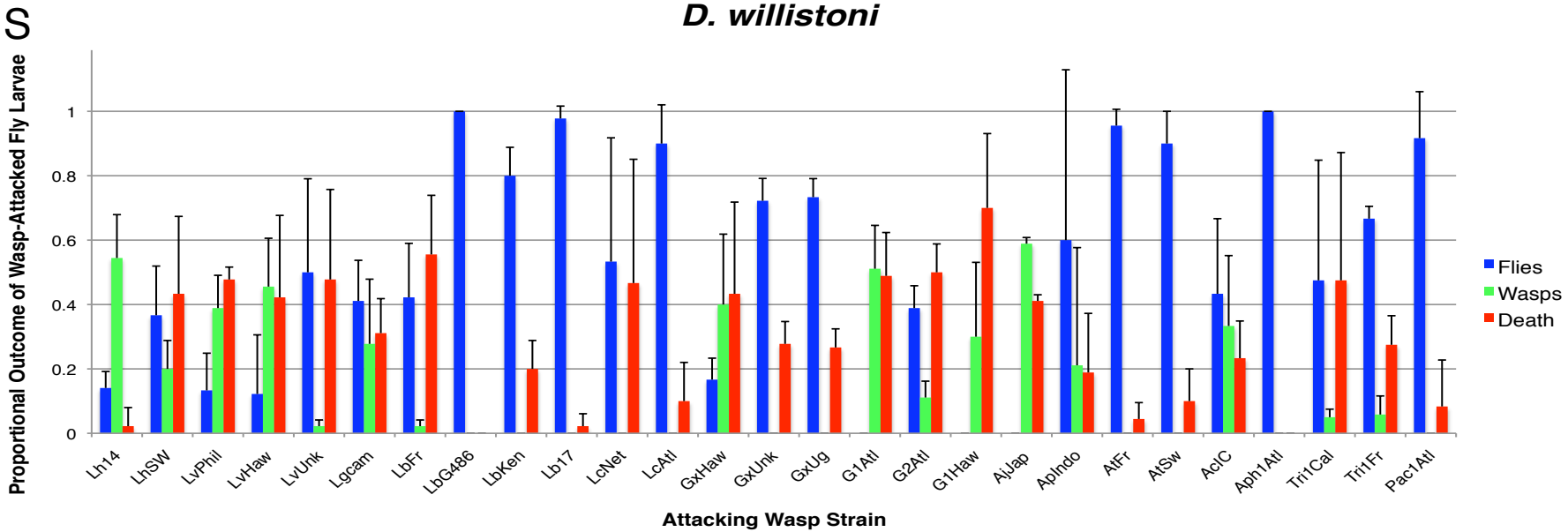
Q


D. pseudoobscura
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Attacking Wasp Strain
Flies
Wasps
Death

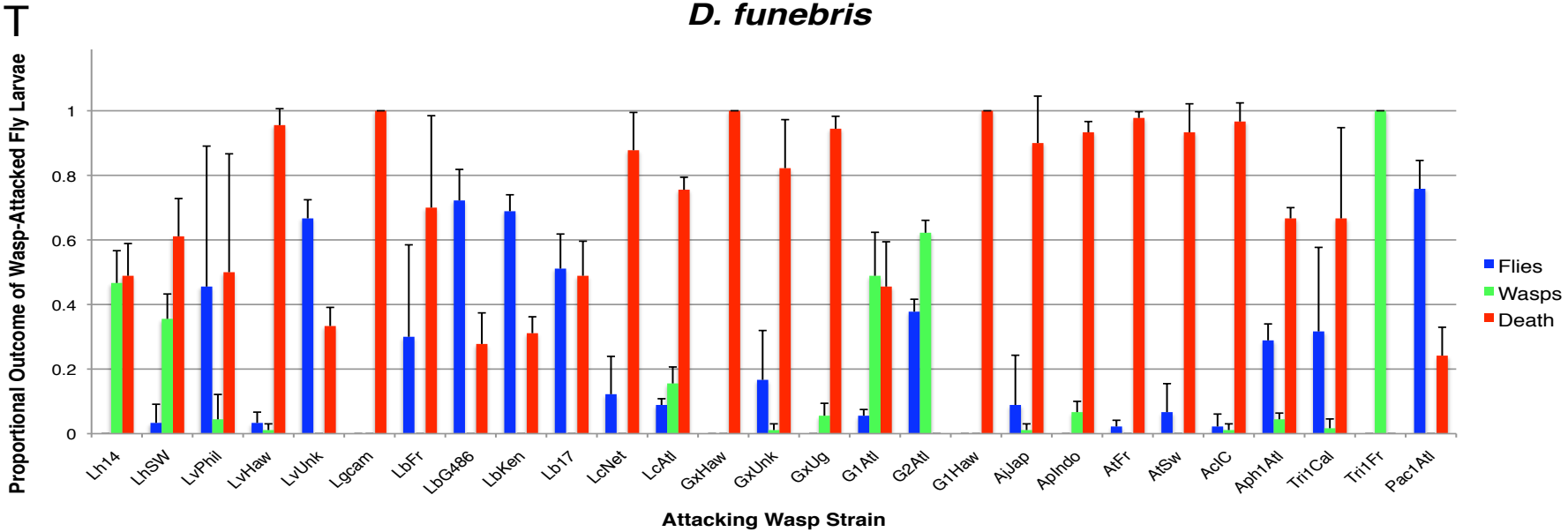
R


D. subobscura
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Attacking Wasp Strain
Flies
Wasps
Death

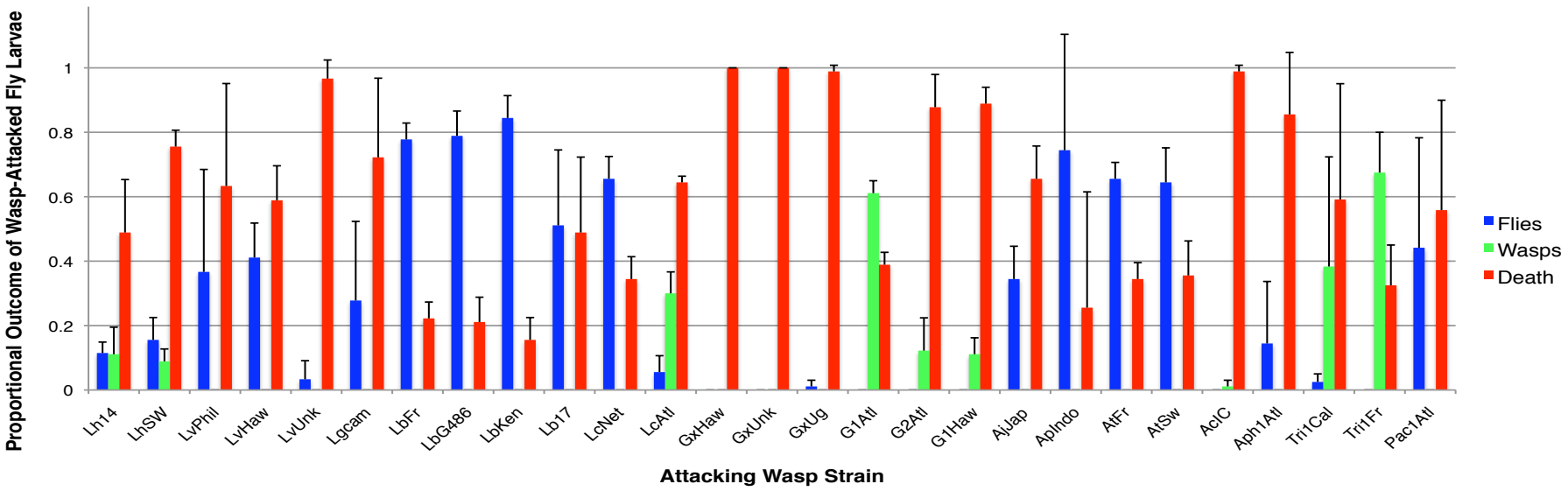
S


D. willistoni
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Attacking Wasp Strain
Flies
Wasps
Death

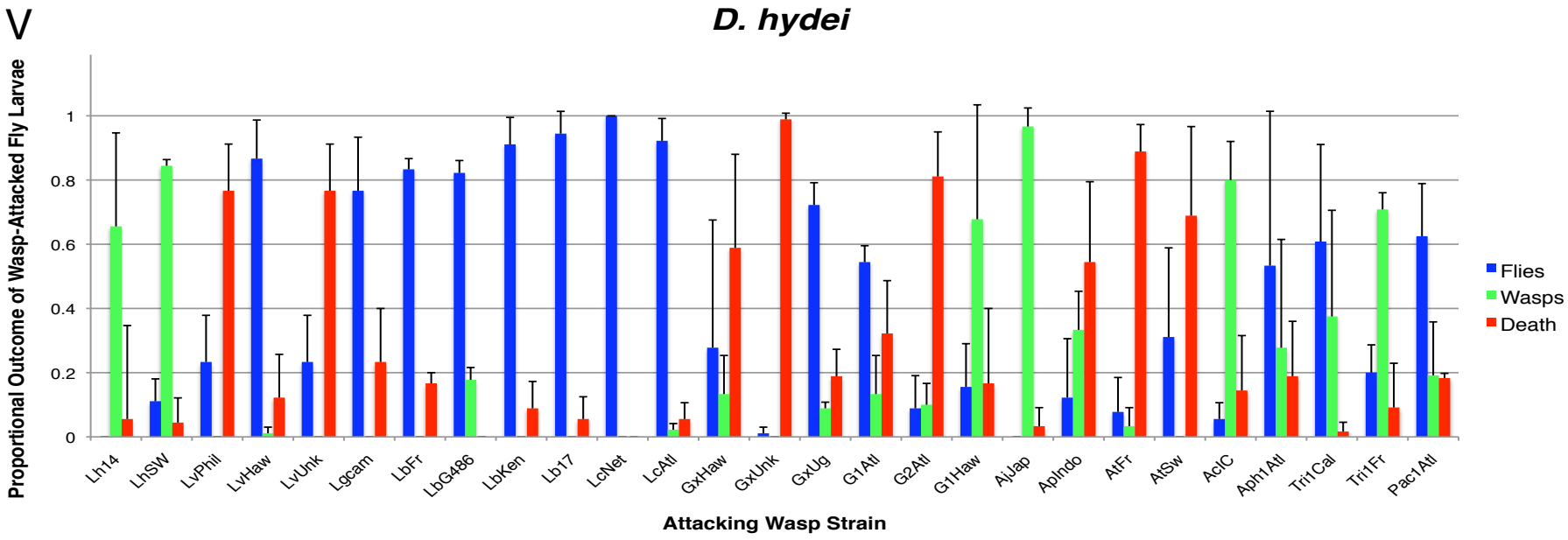
T


D. funebris
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Attacking Wasp Strain
Flies
Wasps
Death

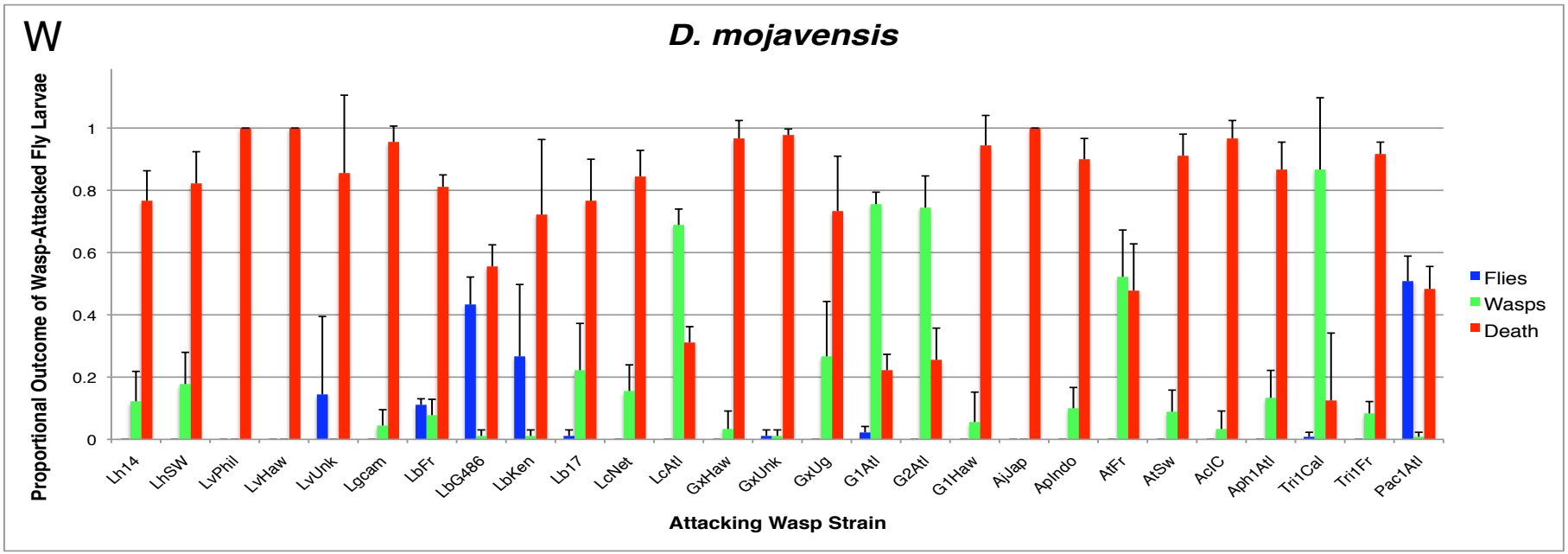
U


D. immigrans
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Attacking Wasp Strain
Flies
Wasps
Death

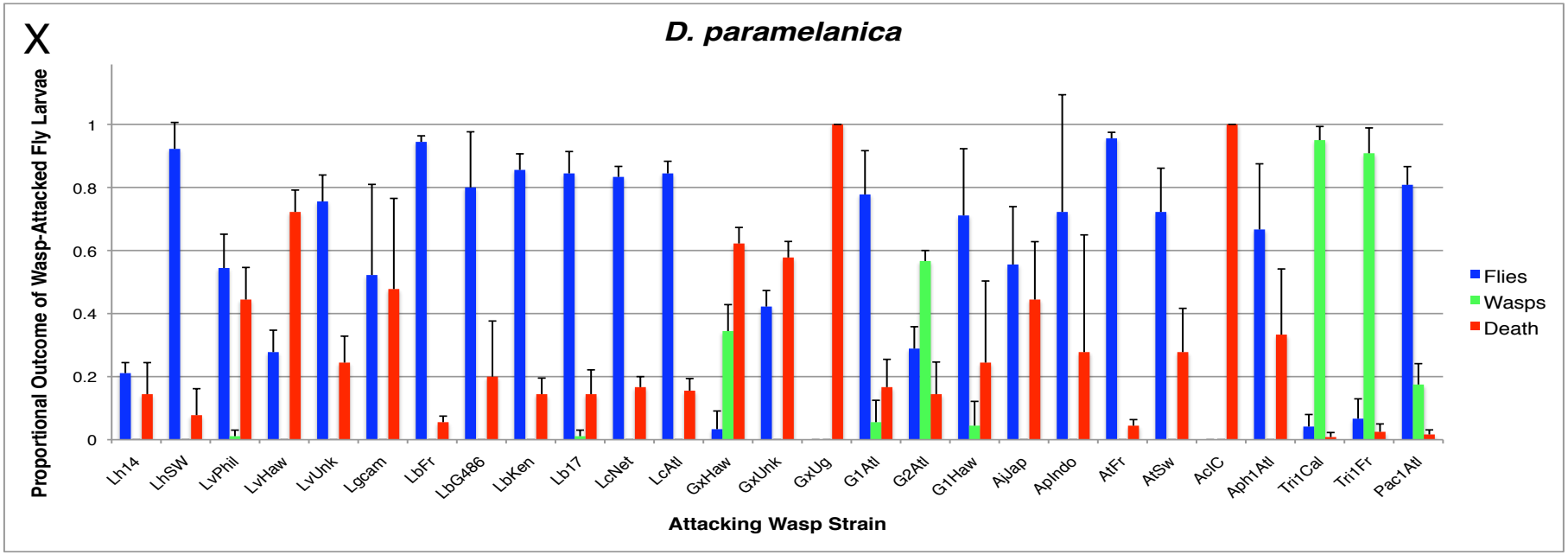
V


D. hydei
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Attacking Wasp Strain
Flies
Wasps
Death

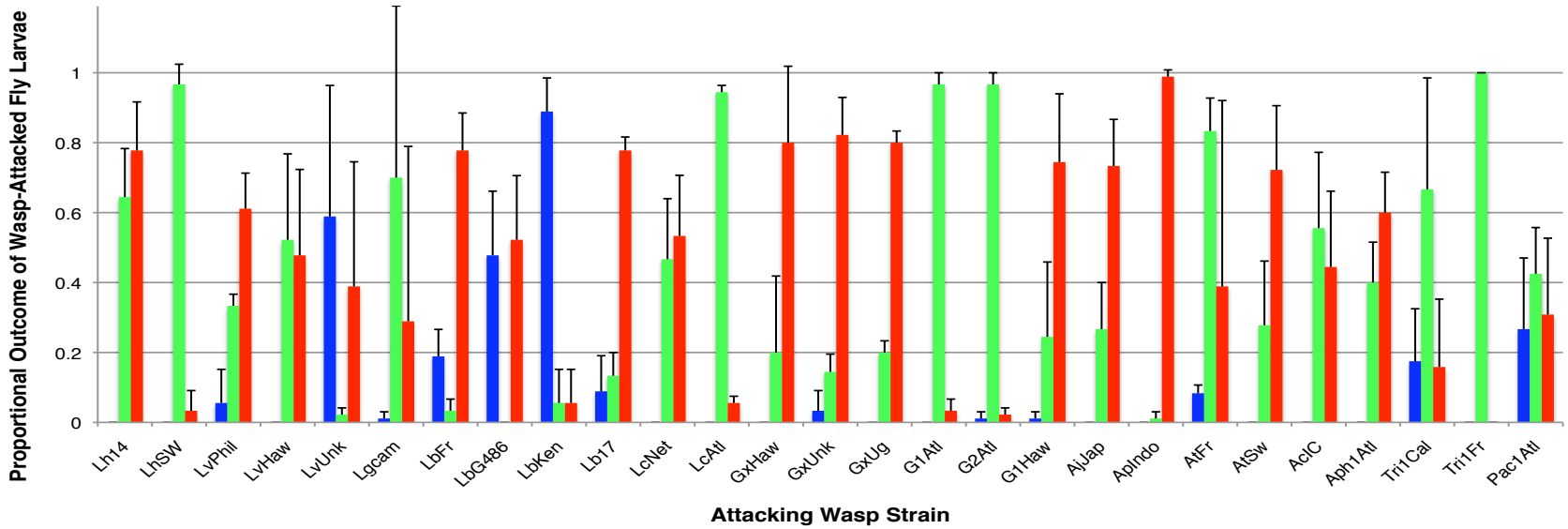
W


D. mojavensis
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Ln14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Attacking Wasp Strain
Flies
Wasps
Death

X


D. paramelanica
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Ln14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Attacking Wasp Strain
Flies
Wasps
Death

Y


D. virilis
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
AplndoAtFr
AtSw
AclC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Attacking Wasp Strain
Flies
Wasps
Death

Z

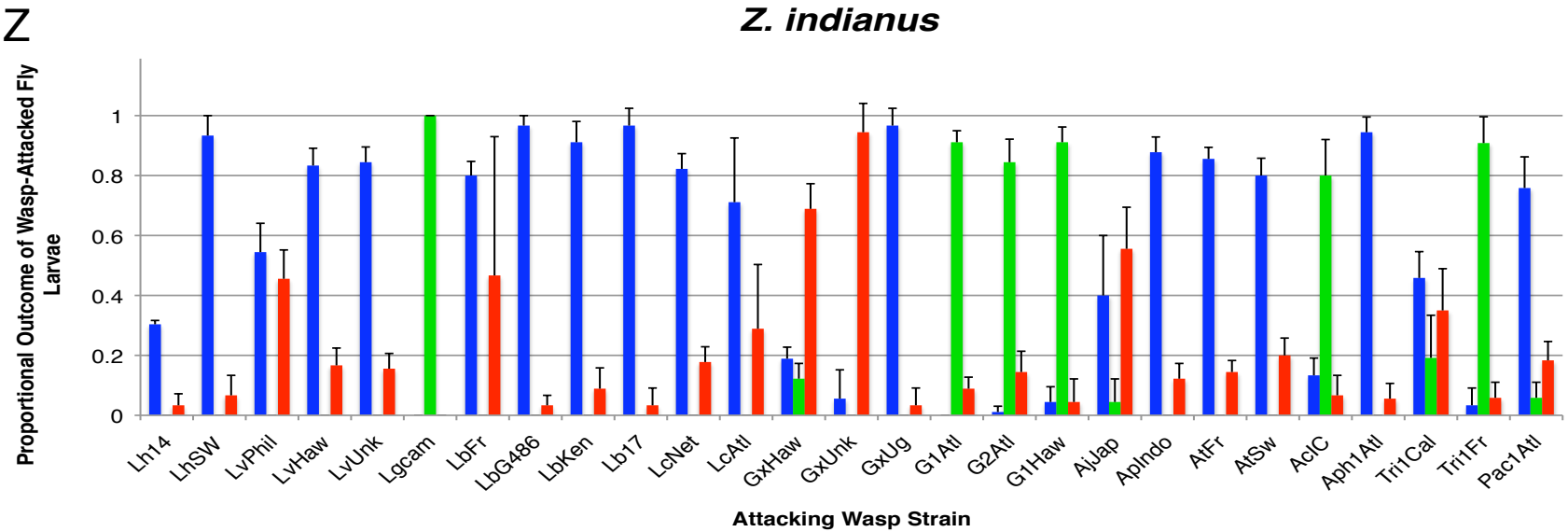
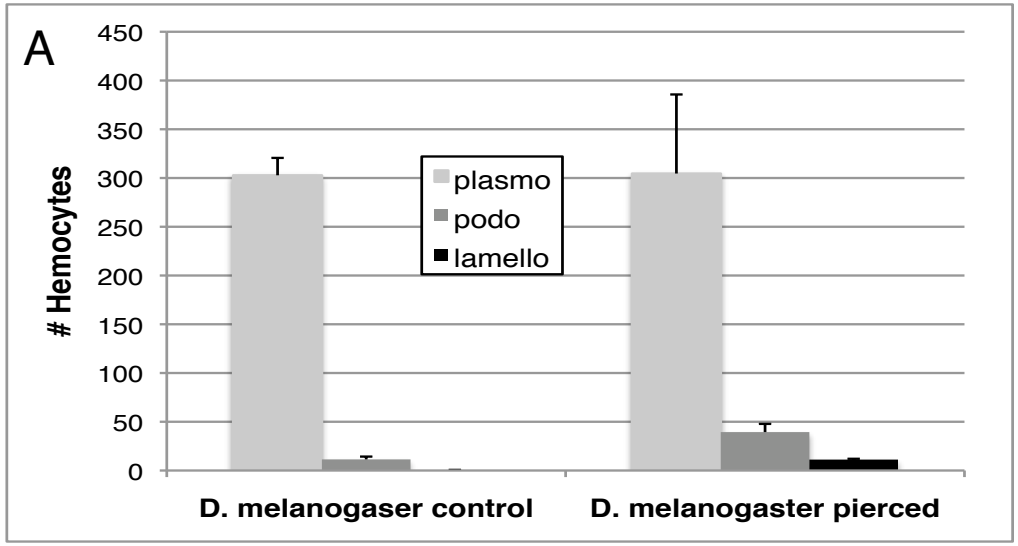

Z. indianus
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
AplndoAtFr
AtSw
AclC
Aph1Atl
Tri1Cal
Tri1Fr
Pac1Atl
Attacking Wasp Strain
Flies
Wasps
Death

Figure 14


A
450
400
350
300
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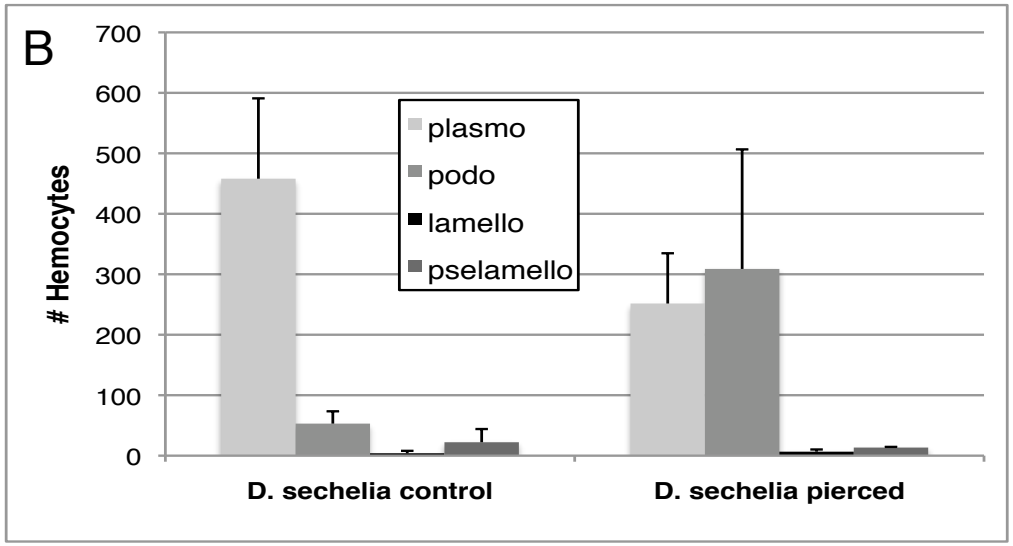
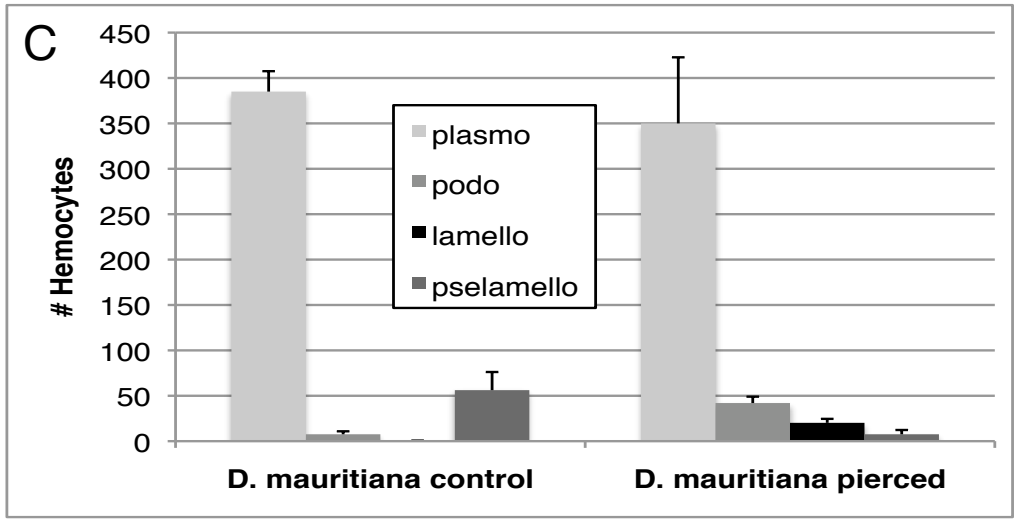
200
150
100
50
0
Hemocytes
plasmo
podo
lamello
D. melanogaser control
D. melanogaster pierced


B
700
600
500
400
300
200
100
0
Hemocytes
plasmo
podo
lamello
pselamello
D. sechelia control
D. sechelia pierced


C
450
400
350
300
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200
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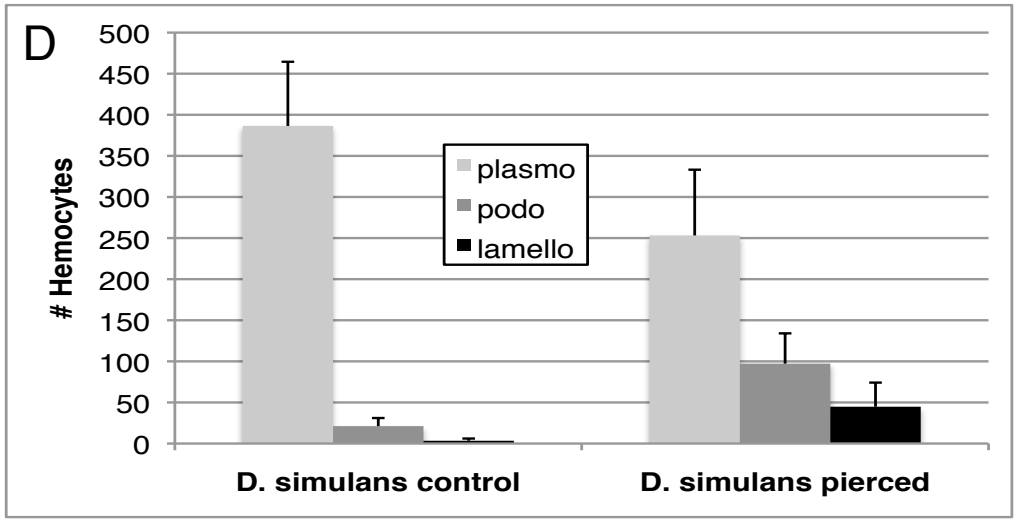
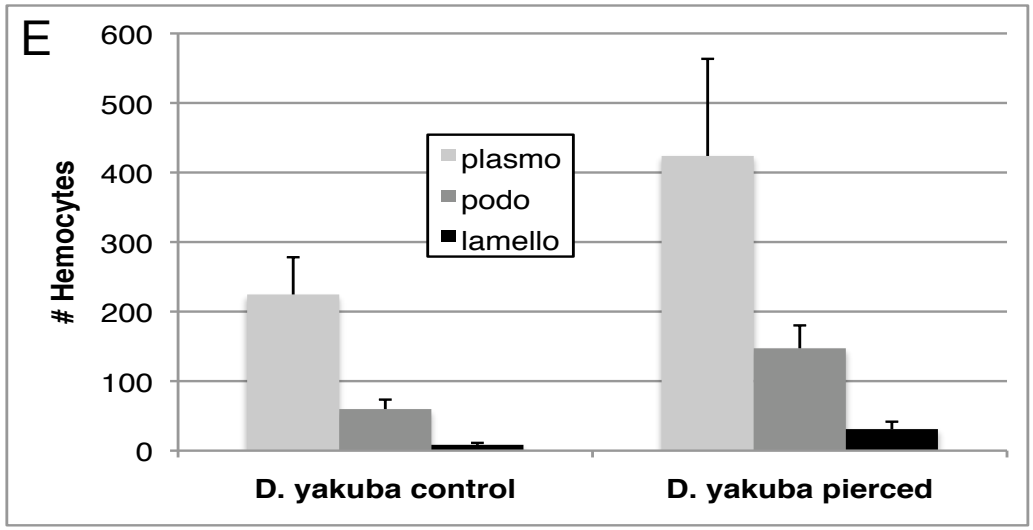
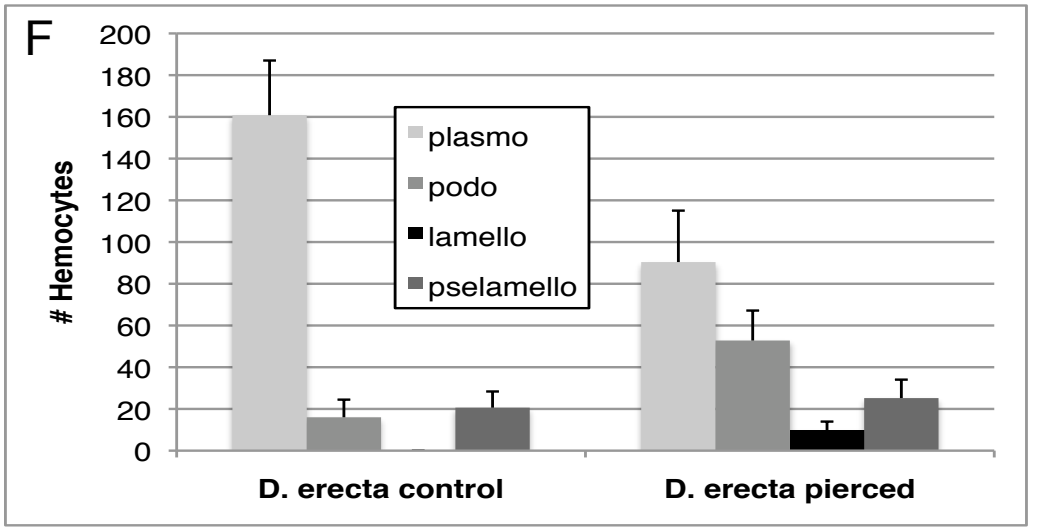
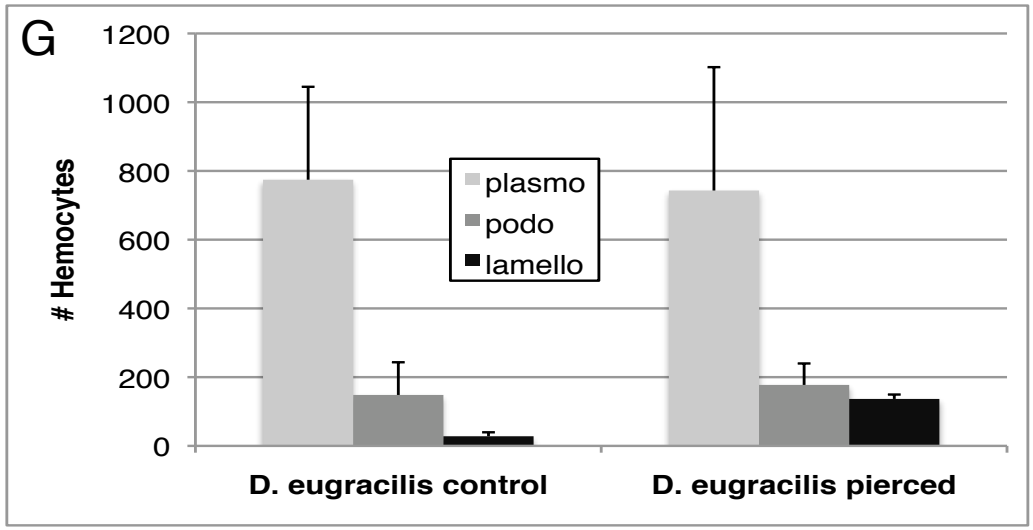
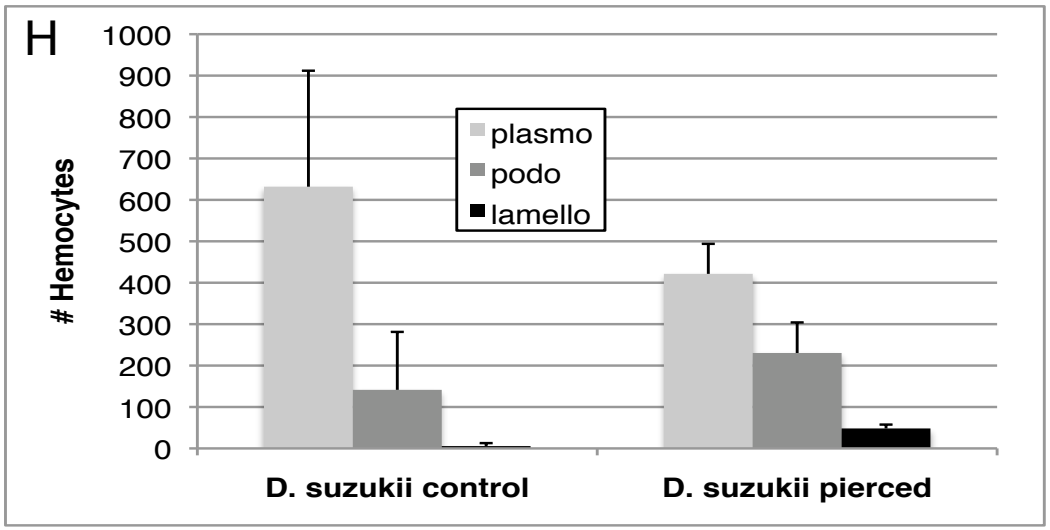
50
0
Hemocytes
plasmo
podo
lamello
pselamello
D. mauritiana control
D. mauritiana pierced

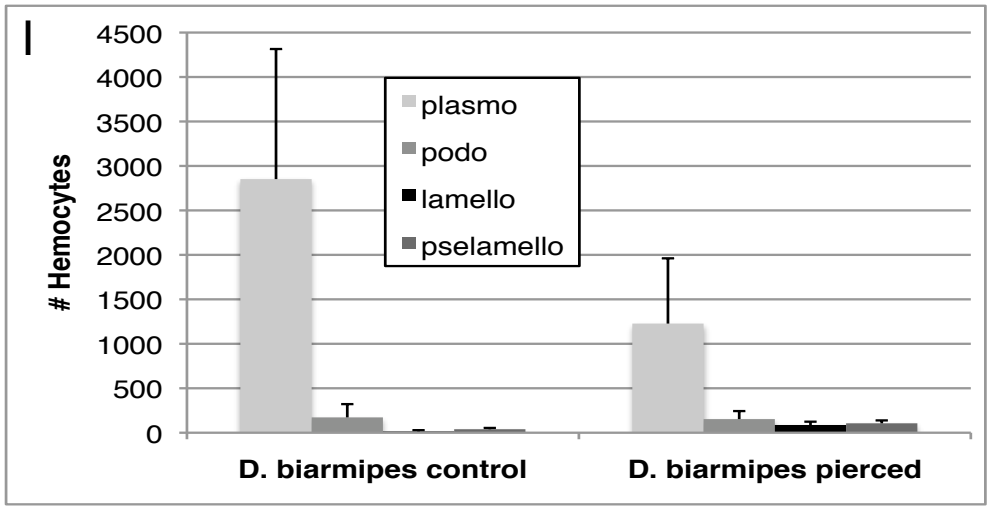

D
500
450
400
350
300
250
200
150
100
50
0
Hemocytes
plasmo
podo
lamello
D. simulans control
D. simulans pierced

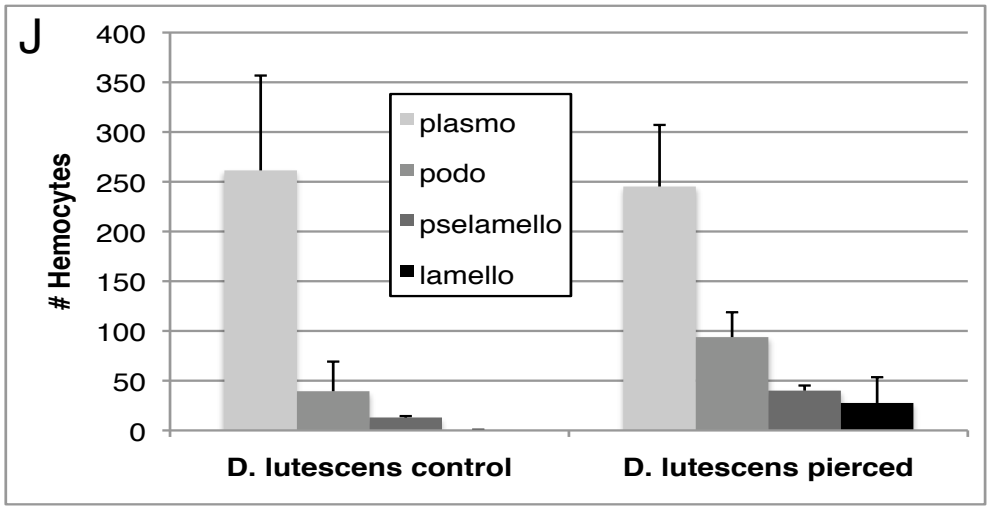

E
600
500
400
300
200
100
0
Hemocytes
plasmo
podo
lamello
D. yakuba control
D. yakuba pierced

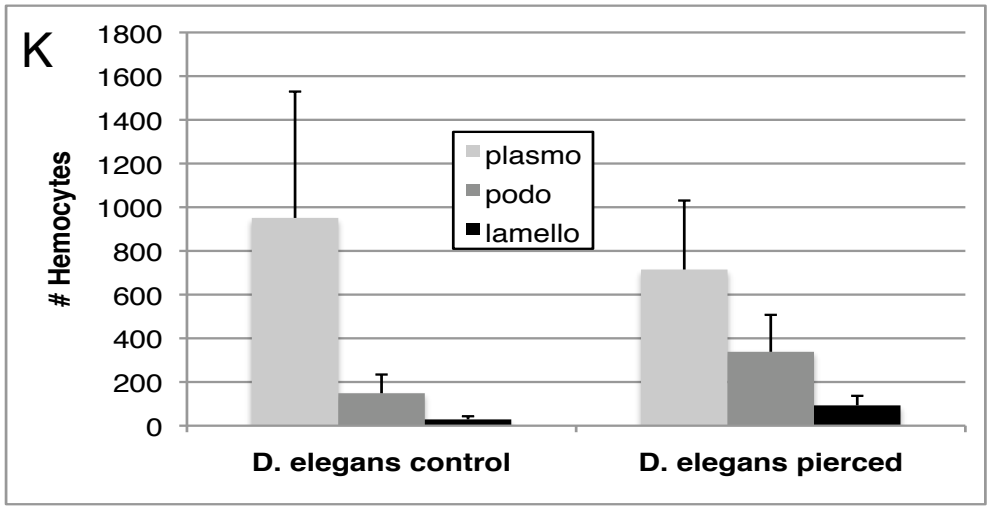

F
200
180
160
140
120
100
80
60
40
20
0
Hemocytes
plasmo
podo
lamello
pselamello
D. erecta control
D. erecta pierced

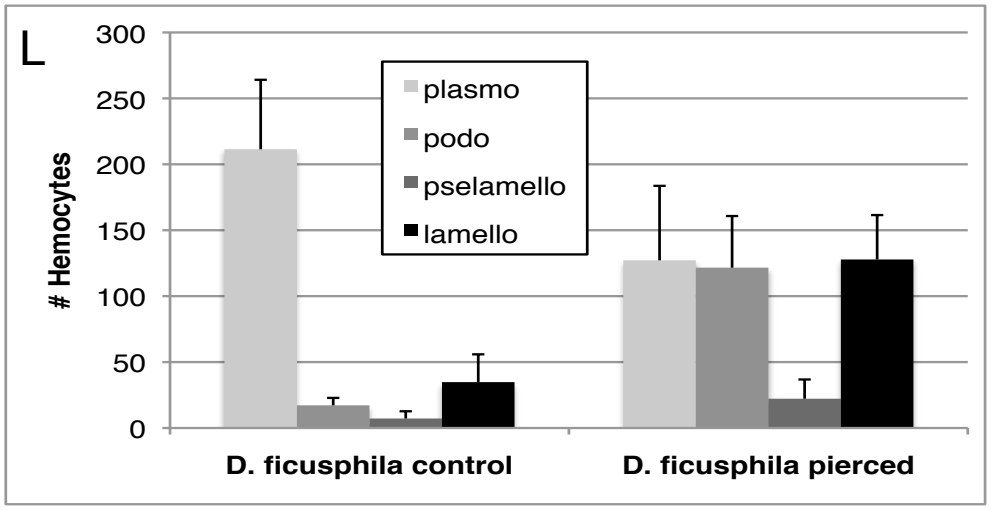

G
1200
1000
800
600
400
200
0
Hemocytes
plasmo
podo
lamello
D. eugracilis control
D. eugracilis pierced

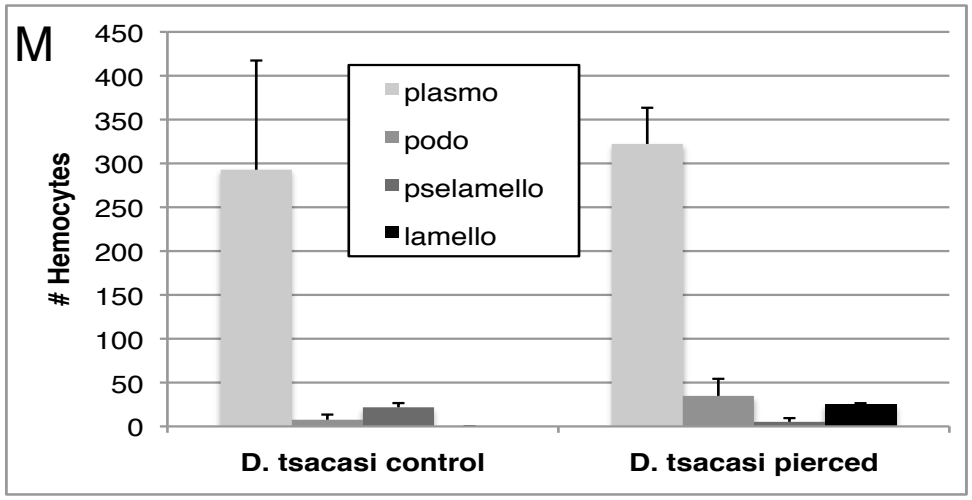

H
1000
900
800
700
600
500
400
300
200
100
0
Hemocytes
plasmo
podo
lamello
D. suzukii control
D. suzukii pierced

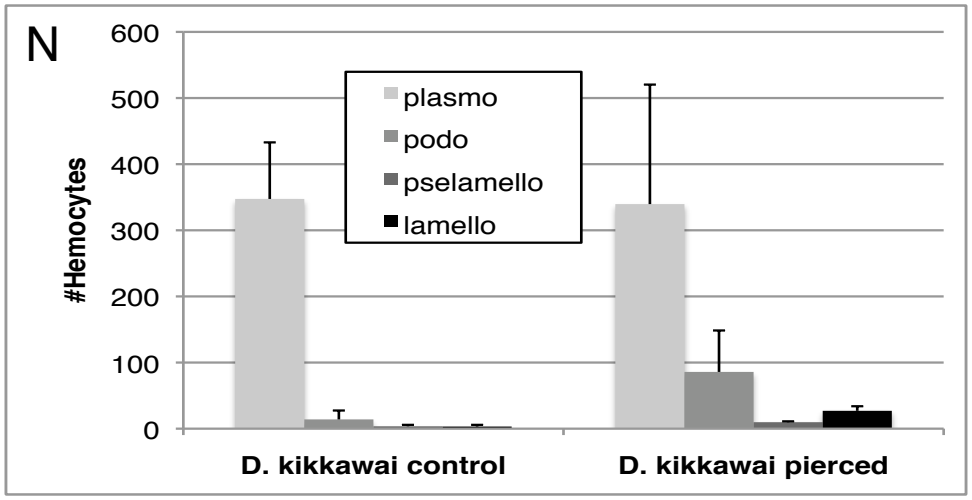

I
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Hemocytes
plasmo
podo
lamello
pselamello
D. biarmipes control
D. biarmipes pierced

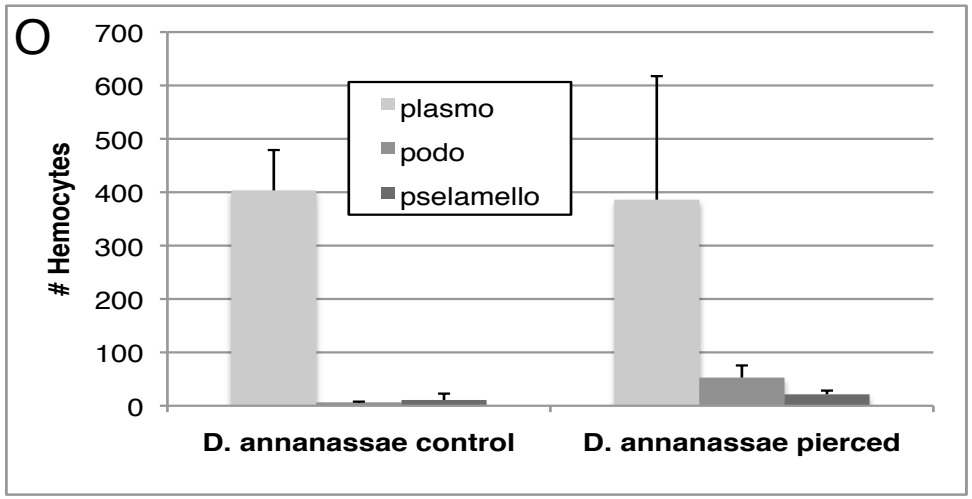

J
400
350
300
250
200
150
100
50
0
Hemocytes
plasmo
podo
pselamello
lamello
D. lutescens control
D. lutescens pierced

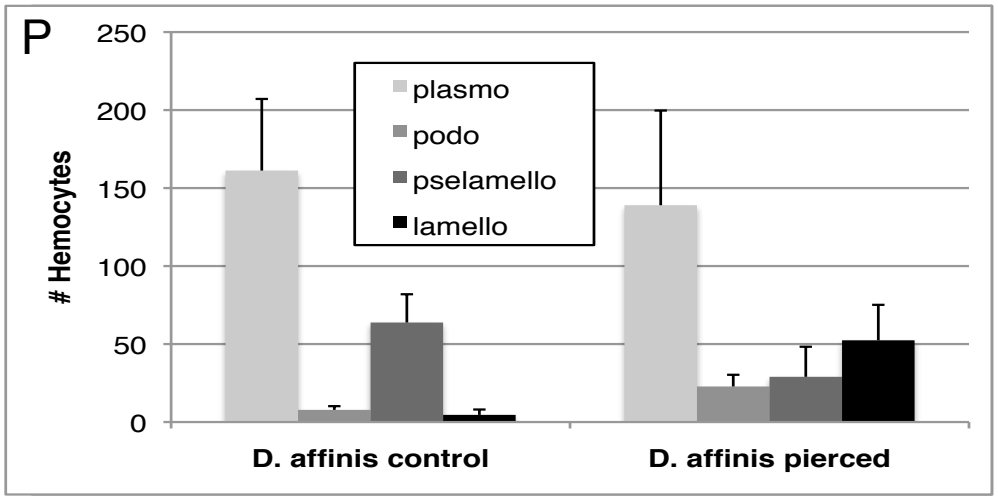

K
1800
1600
1400
1200
1000
800
600
400
200
0
Hemocytes
plasmo
podo
lamello
D. elegans control
D. elegans pierced

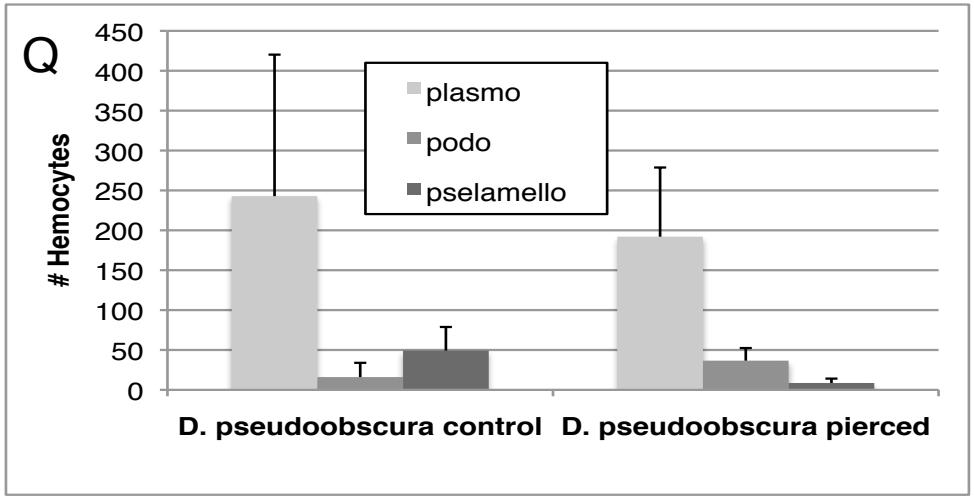

L
300
250
200
150
100
50
0
Hemocytes
plasmo
podo
pselamello
lamello
D. ficusphila control
D. ficusphila pierced

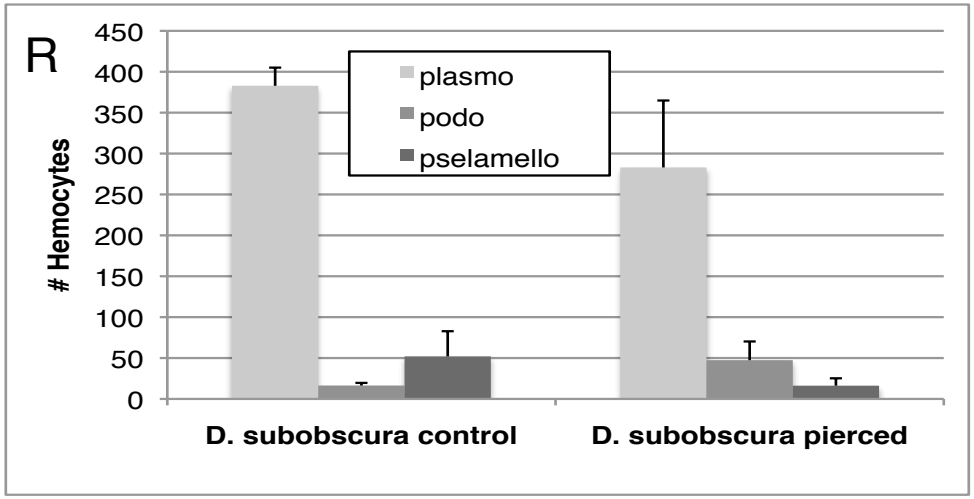

M
450
400
350
300
250
200
150
100
50
0
Hemocytes
plasmo
podo
pselamello
lamello
D. tsacasi control
D. tsacasi pierced

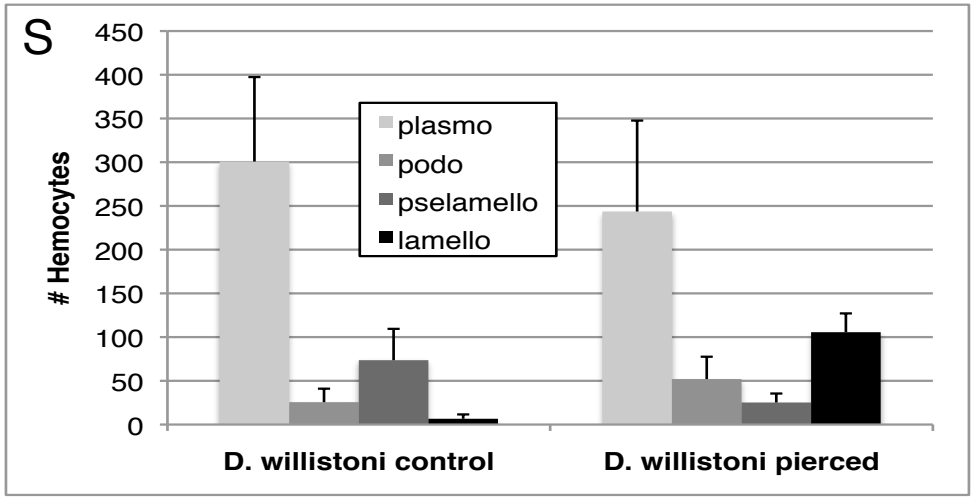

N
600
500
400
300
200
100
0
#Hemocytes
plasmo
podo
pselamello
lamello
D. kikkawai control
D. kikkawai pierced

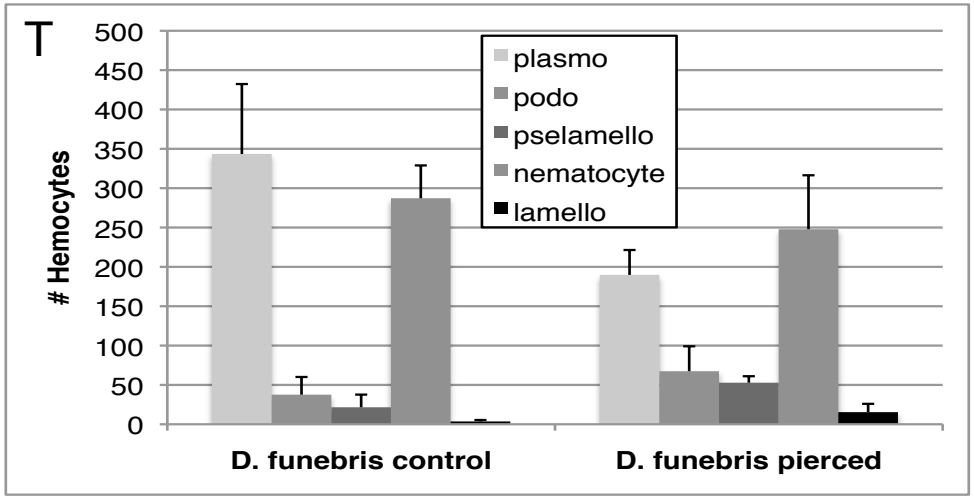

O
700
600
500
400
300
200
100
0
Hemocytes
plasmo
podo
pselamello
D. annanassae control
D. annanassae pierced

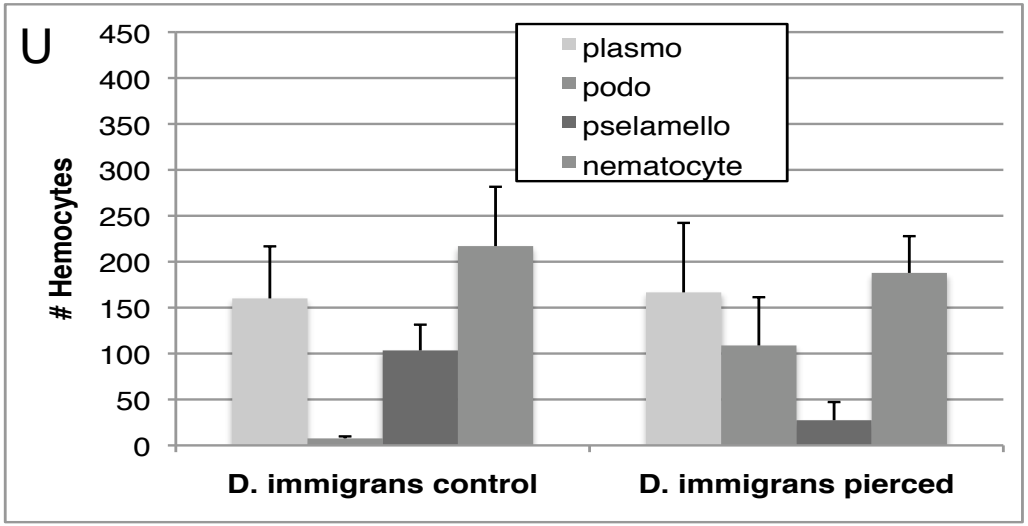

P
250
200
150
100
50
0
Hemocytes
plasmo
podo
pselamello
lamello
D. affinis control
D. affinis pierced

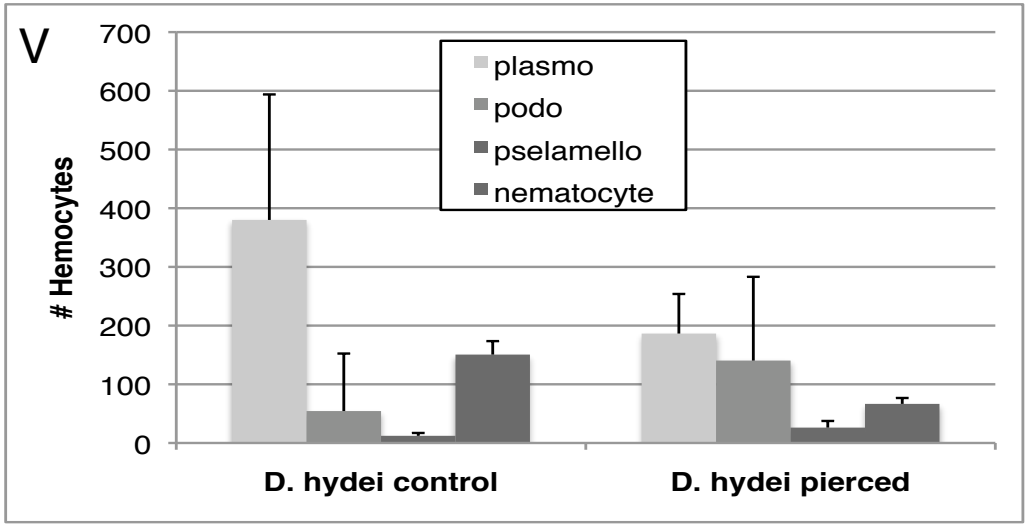

Q
Hemocytes
450
400
350
300
250
200
150
100
50
0
plasmo
podo
pselamello
D. pseudoobscura control
D. pseudoobscura pierced

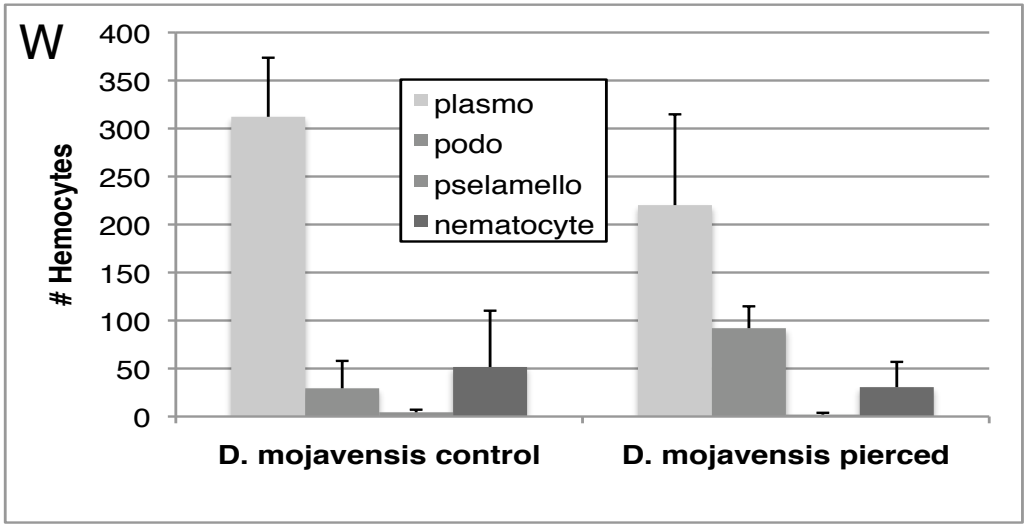

R
Hemocytes
450
400
350
300
250
200
150
100
50
0
plasmo
podo
pselamello
D. subobscura control
D. subobscura pierced

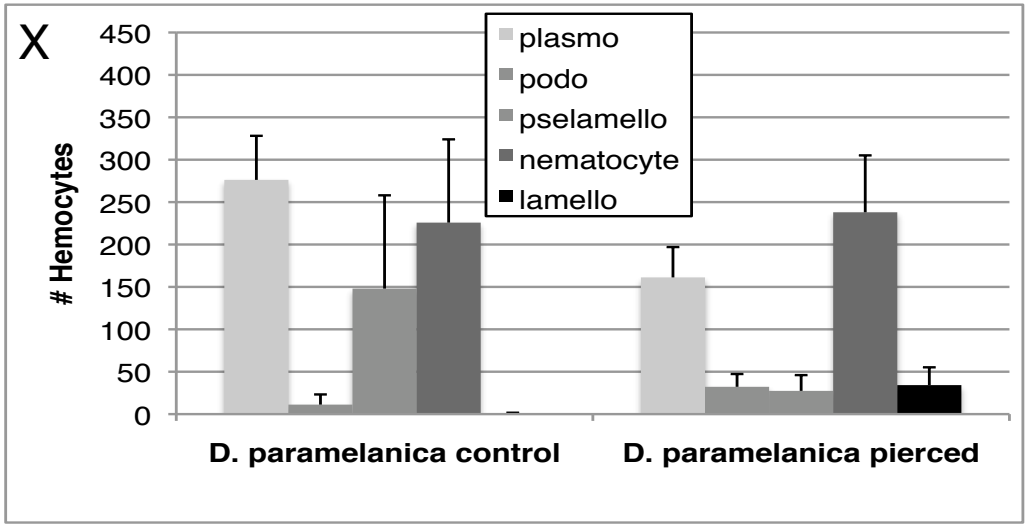

S
Hemocytes
450
400
350
300
250
200
150
100
50
0
plasmo
podo
pselamello
lamello
D. willistoni control
D. willistoni pierced

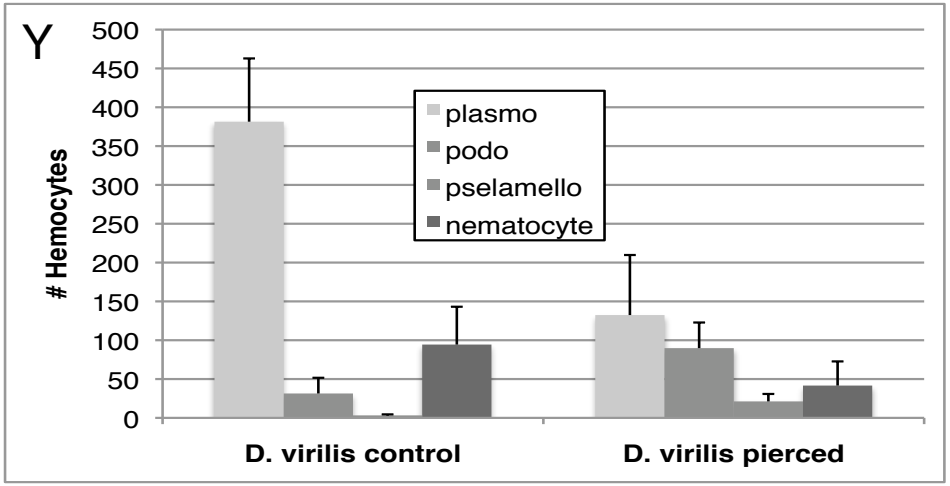

T
Hemocytes
500
450
400
350
300
250
200
150
100
50
0
plasmo
podo
pselamello
nematocyte
lamello
D. funebris control
D. funebris pierced


U
Hemocytes
450
400
350
300
250
200
150
100
50
0
plasmo
podo
pselamello
nematocyte
D. immigrans control
D. immigrans pierced


V
Hemocytes
700
600
500
400
300
200
100
0
plasmo
podo
pselamello
nematocyte
D. hydei control
D. hydei pierced


W
Hemocytes
400
350
300
250
200
150
100
50
0
plasmo
podo
pselamello
nematocyte
D. mojavensis control
D. mojavensis pierced


X
Hemocytes
450
400
350
300
250
200
150
100
50
0
plasmo
podo
pselamello
nematocyte
lamello
D. paramelanica control
D. paramelanica pierced


Y
Hemocytes
500
450
400
350
300
250
200
150
100
50
0
plasmo
podo
pselamello
nematocyte
D. virilis control
D. virilis pierced

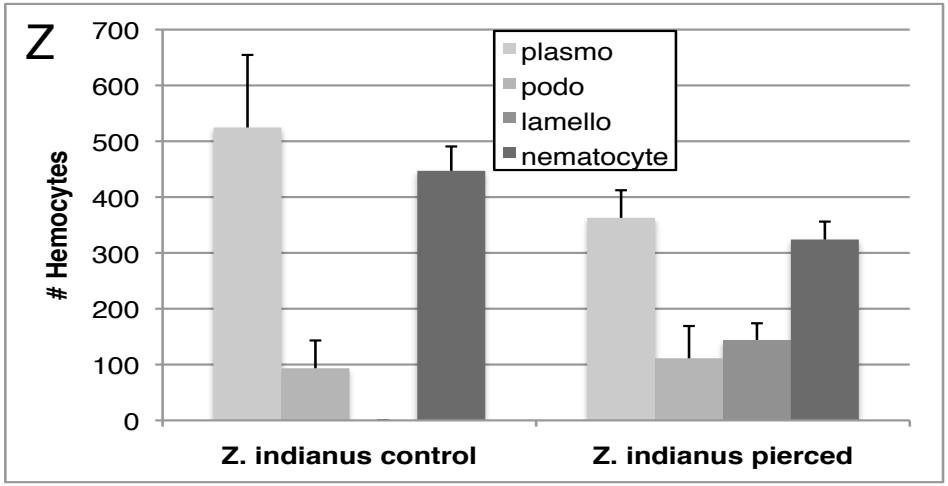
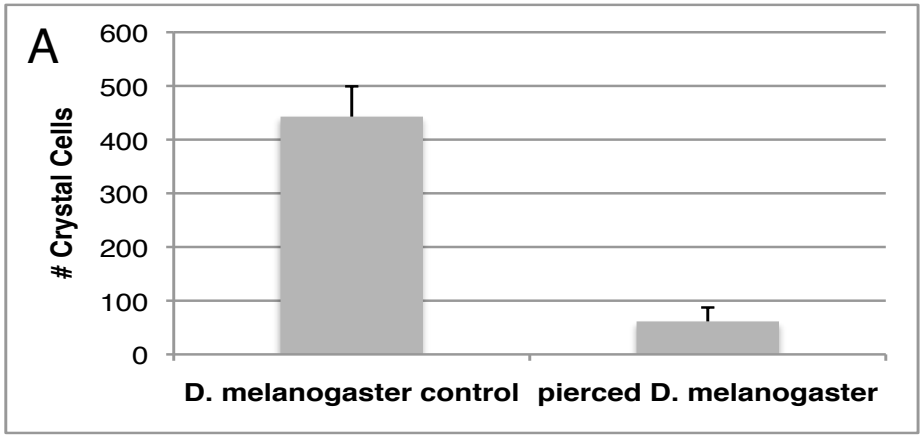
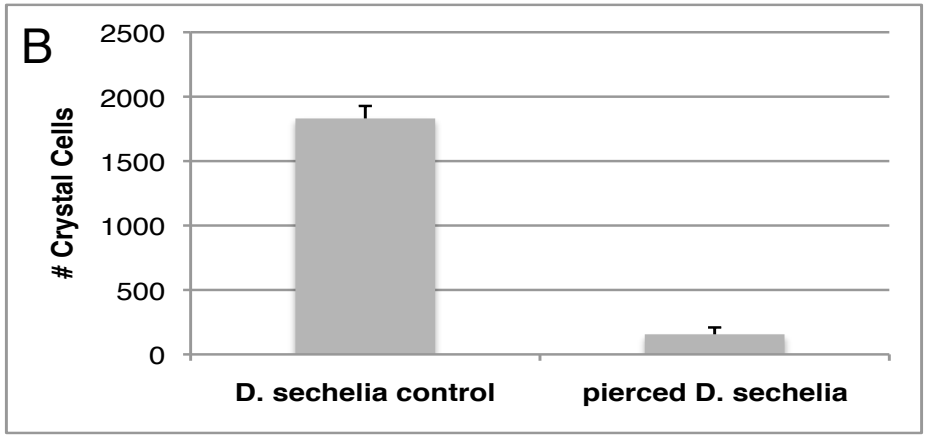
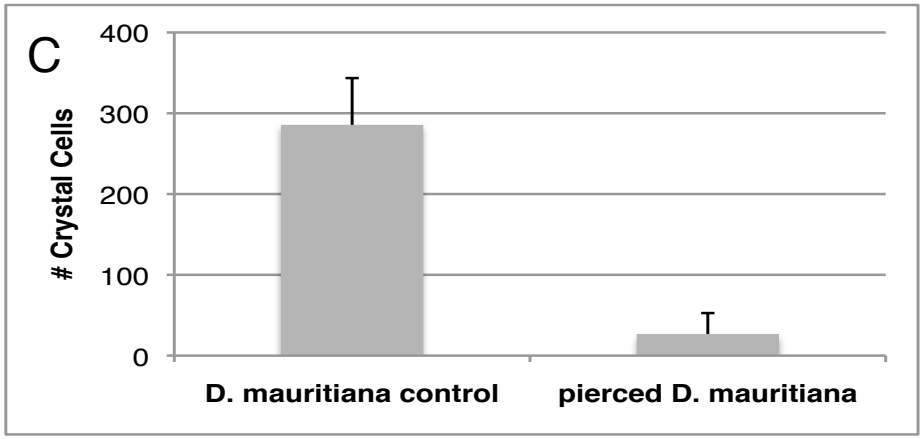
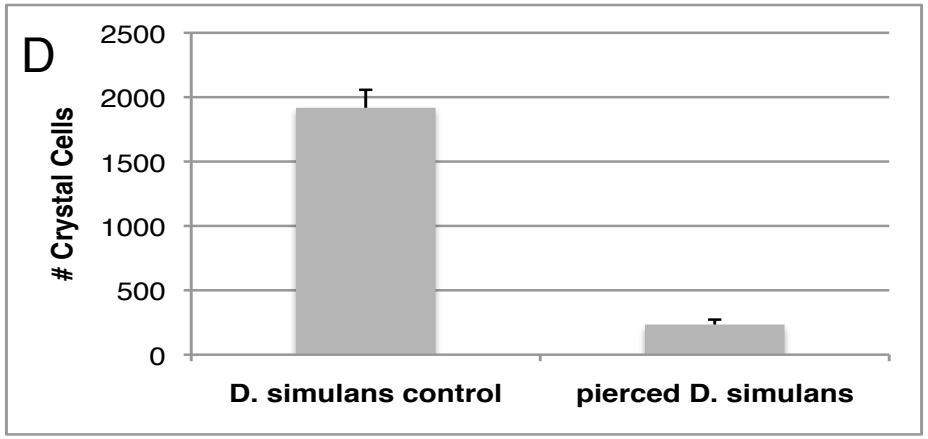

Z
Hemocytes
700
600
500
400
300
200
100
0
plasmo
podo
lamello
nematocyte
Z. indianus control
Z. indianus pierced

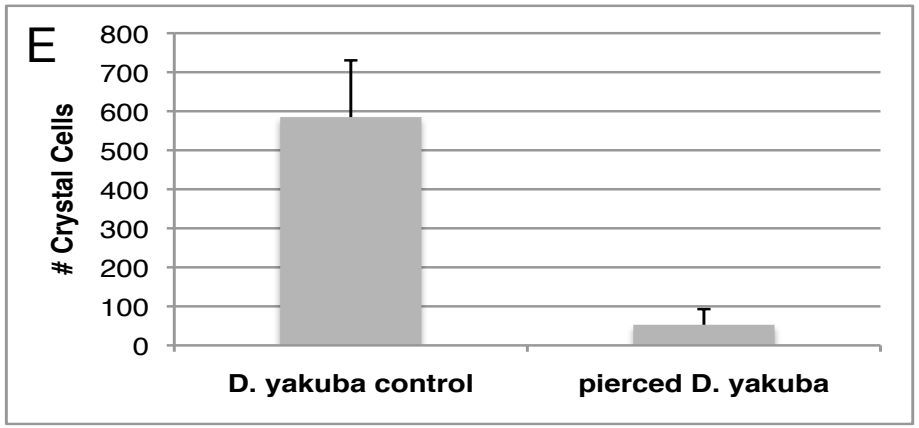
Figure 15

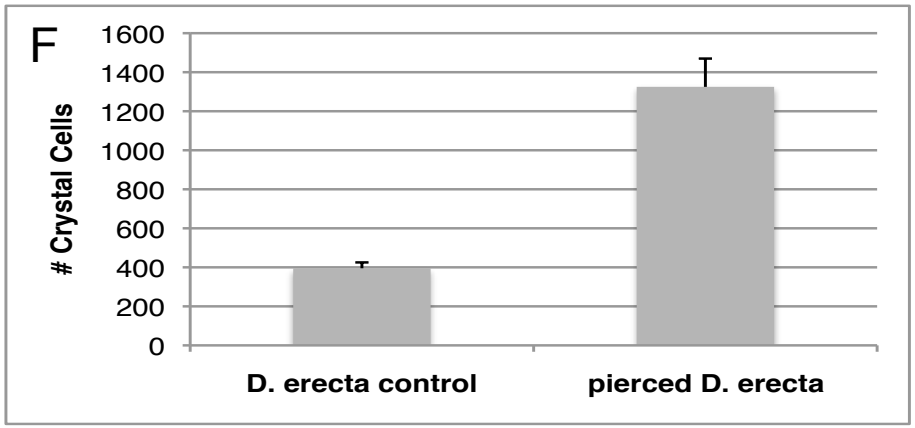

A
600
500
400
300
200
100
0
Crystal Cells
D. melanogaster control
pierced D. melanogaster

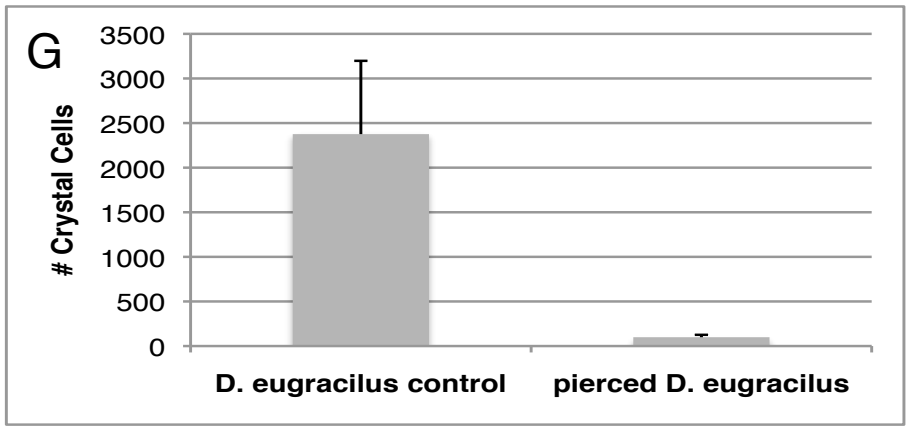

B
2500
2000
1500
1000
500
0
Crystal Cells
D. sechelia control
pierced D. sechelia

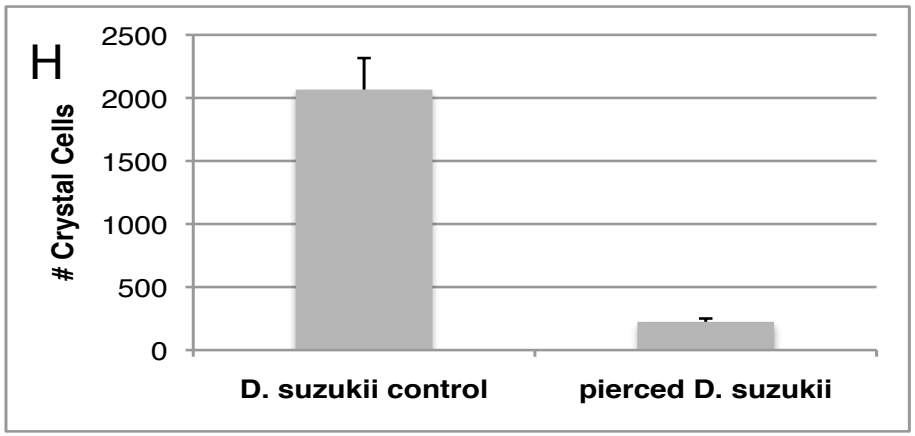

C
400
300
200
100
0
Crystal Cells
D. mauritiana control
pierced D. mauritiana

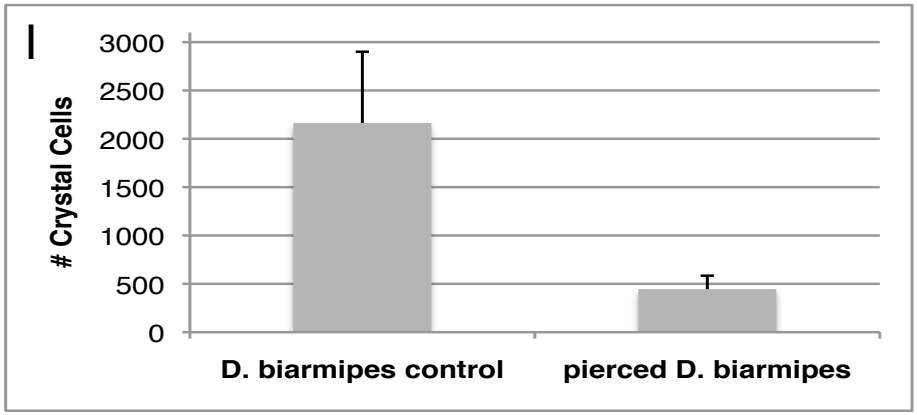

D
2500
2000
1500
1000
500
0
Crystal Cells
D. simulans control
pierced D. simulans

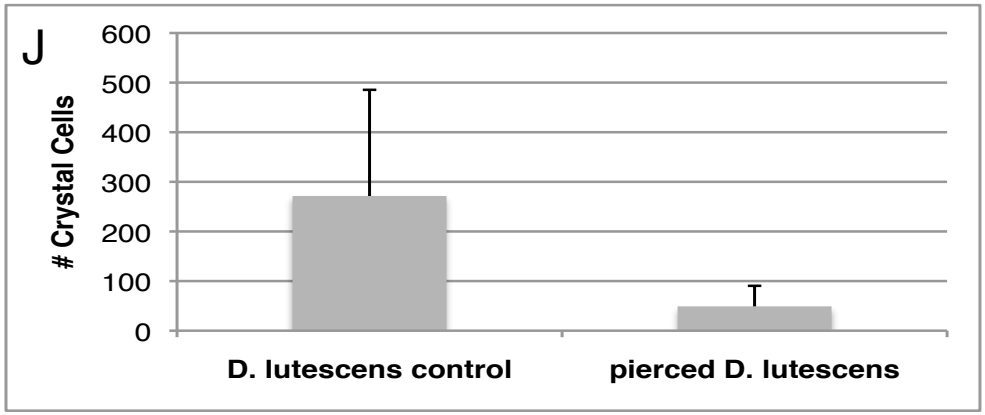

E
800
700
600
500
400
300
200
100
0
Crystal Cells
D. yakuba control
pierced D. yakuba

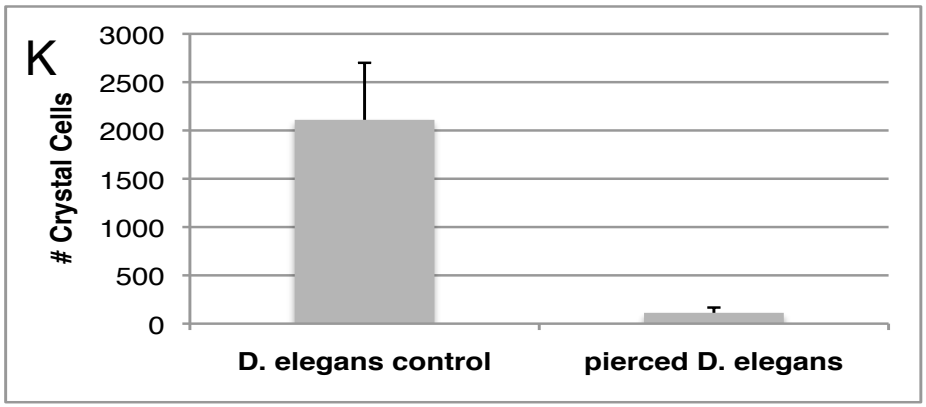

F
1600
1400
1200
1000
800
600
400
200
0
Crystal Cells
D. erecta control
pierced D. erecta

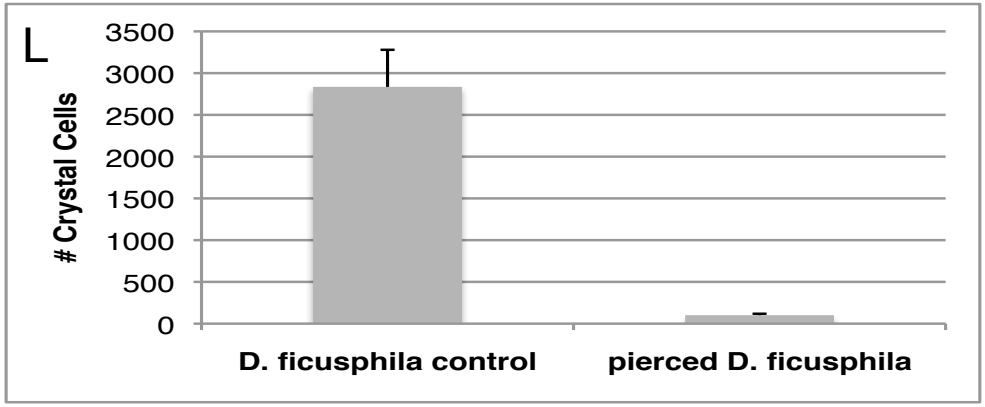

G
3500
3000
2500
2000
1500
1000
500
0
Crystal Cells
D. eugracilus control
pierced D. eugracilus

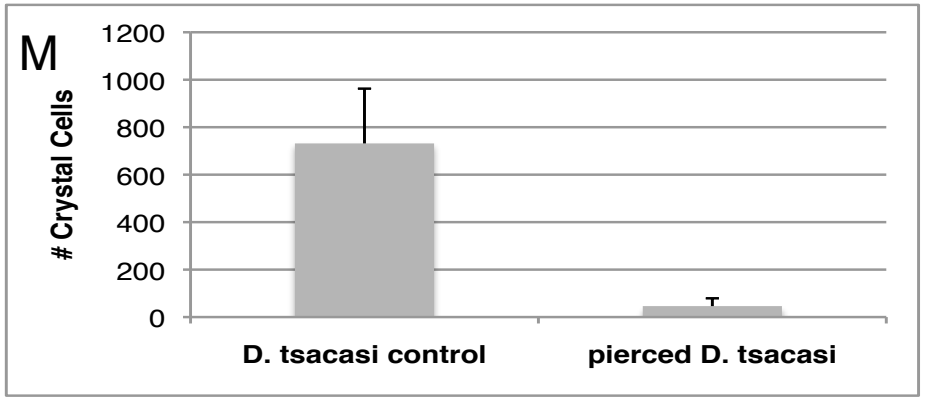

H
2500
2000
1500
1000
500
0
Crystal Cells
D. suzukii control
pierced D. suzukii

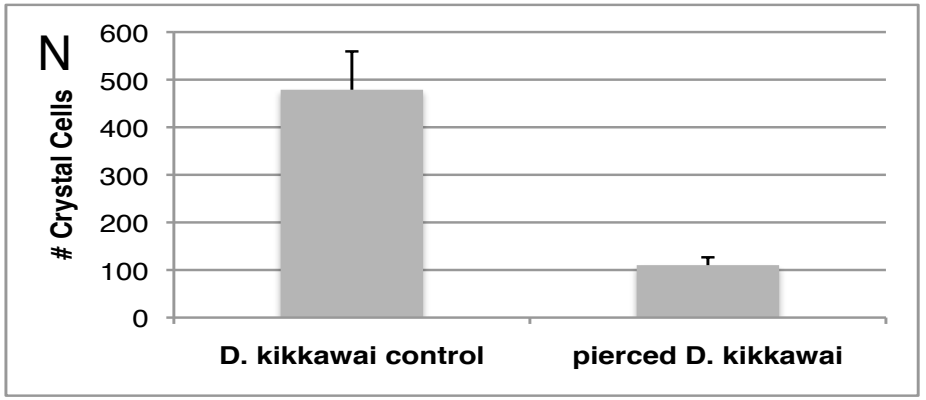

I
Crystal Cells
3000
2500
2000
1500
1000
500
0
D. biarmipes control
pierced D. biarmipes

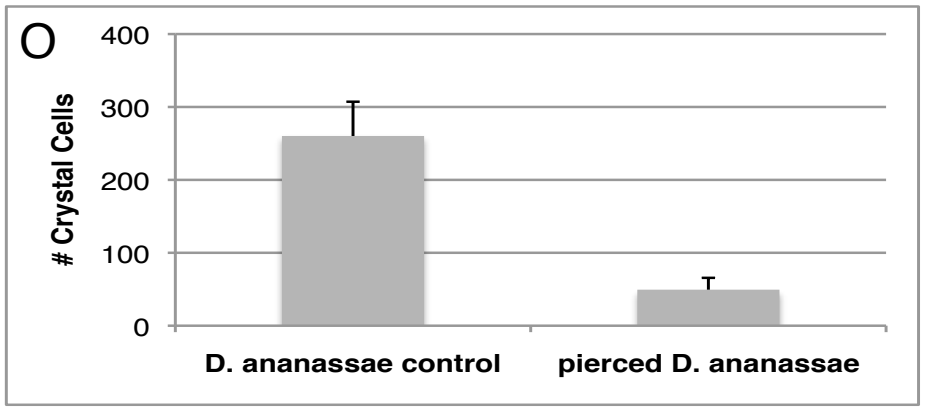

J
Crystal Cells
600
500
400
300
200
100
0
D. lutescens control
pierced D. lutescens

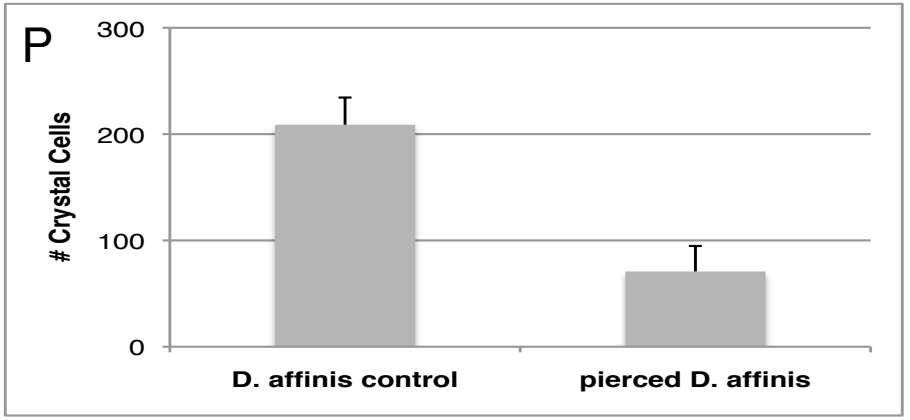

K
Crystal Cells
3000
2500
2000
1500
1000
500
0
D. elegans control
pierced D. elegans

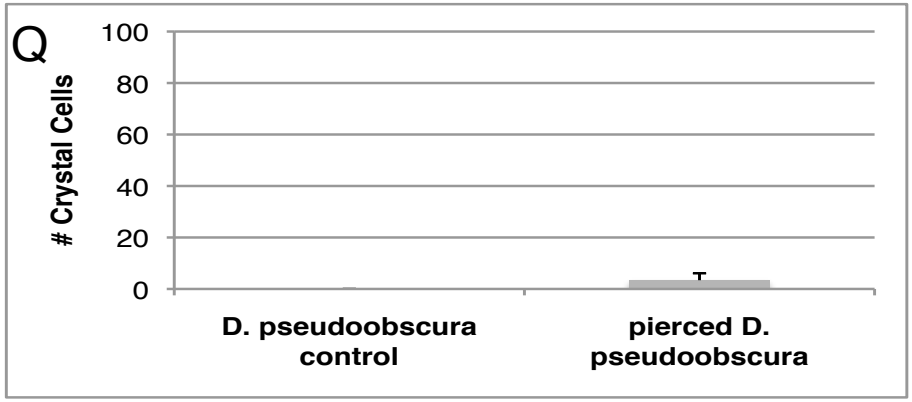

L
Crystal Cells
3500
3000
2500
2000
1500
1000
500
0
D. ficusphila control
pierced D. ficusphila

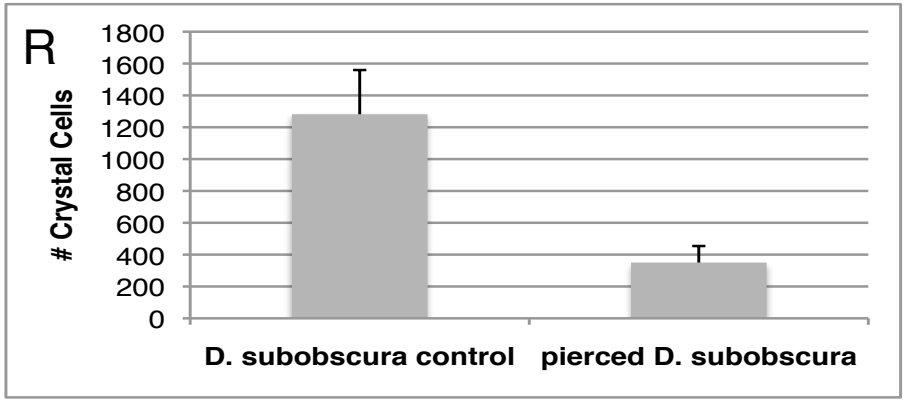

M
Crystal Cells
1200
1000
800
600
400
200
0
D. tsacasi control
pierced D. tsacasi


N
Crystal Cells
600
500
400
300
200
100
0
D. kikkawai control
pierced D. kikkawai

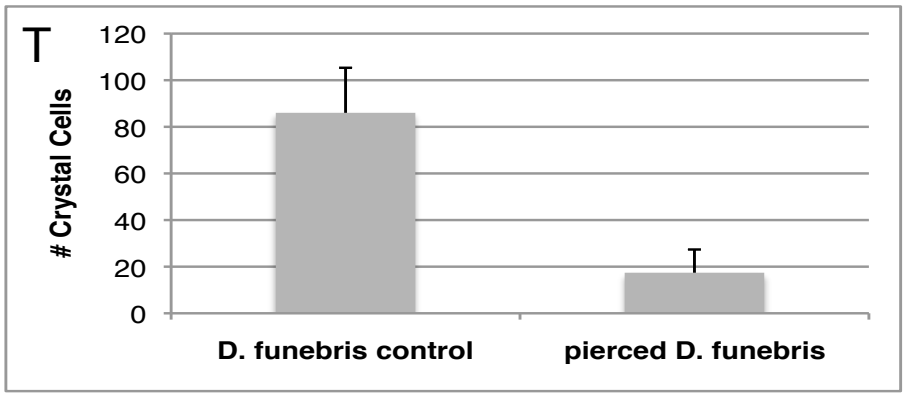

O
Crystal Cells
400
300
200
100
0
D. ananassae control
pierced D. ananassae

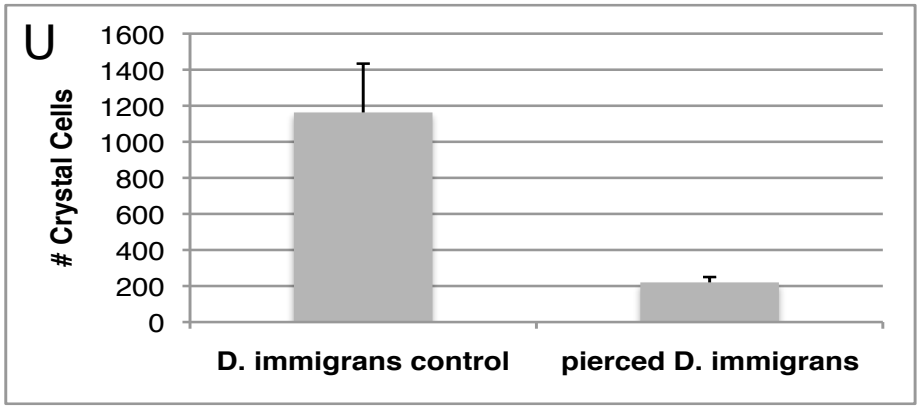

P
Crystal Cells
300
200
100
0
D. affinis control
pierced D. affinis

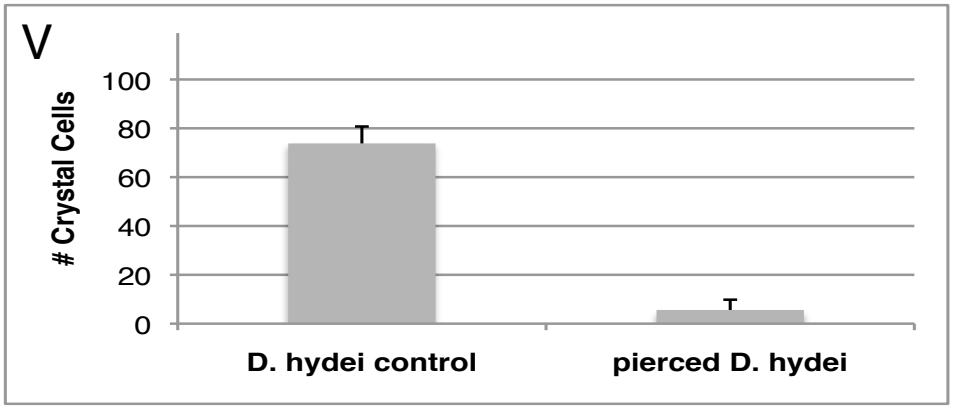

Q
Crystal Cells
100
80
60
40
20
0
D. pseudoobscura control
pierced D. pseudoobscura

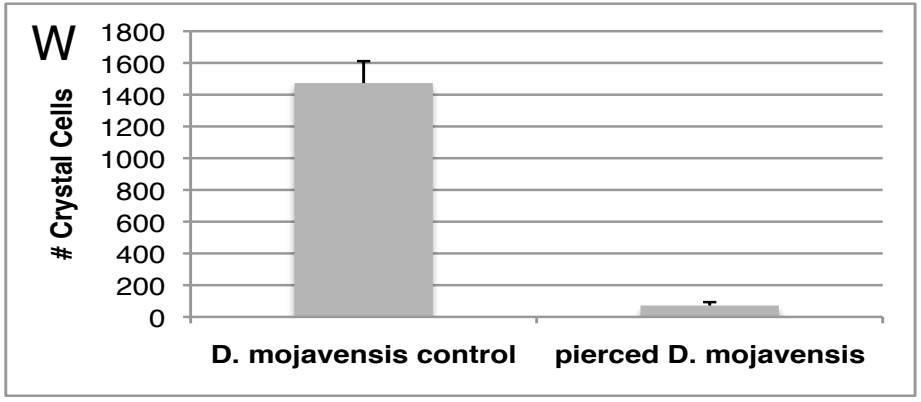

R
Crystal Cells
1800
1600
1400
1200
1000
800
600
400
200
0
D. subobscura control
pierced D. subobscura

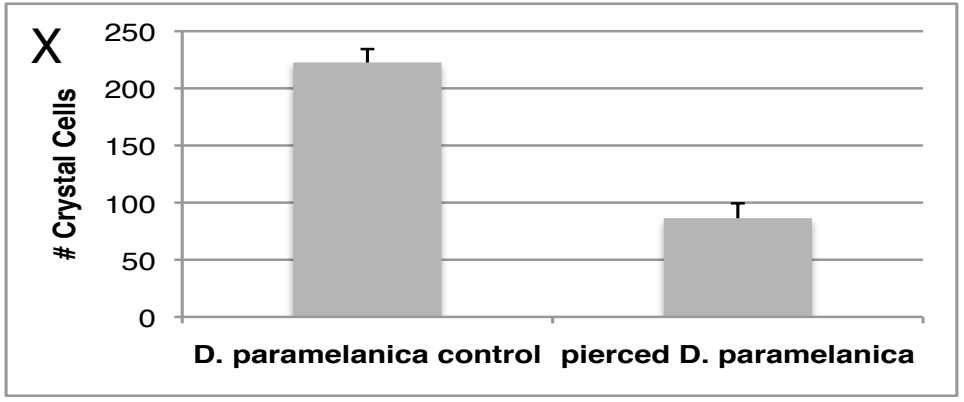

S
Crystal Cells
2000
1500
1000
500
0
D. willistoni control
pierced D. willistoni

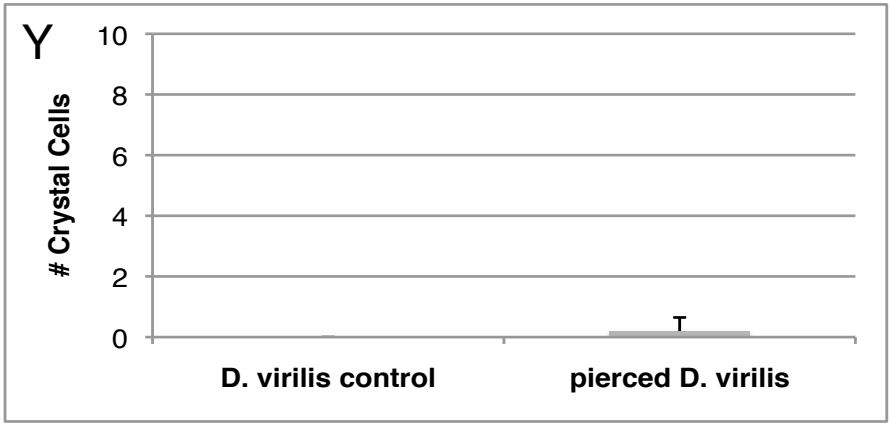

T
Crystal Cells
120
100
80
60
40
20
0
D. funebris control
pierced D. funebris


U
Crystal Cells
1600
1400
1200
1000
800
600
400
200
0
D. immigrans control
pierced D. immigrans


V
Crystal Cells
100
80
60
40
20
0
D. hydei control
pierced D. hydei


W
Crystal Cells
1800
1600
1400
1200
1000
800
600
400
200
0
D. mojavensis control
pierced D. mojavensis


X
Crystal Cells
250
200
150
100
50
0
D. paramelanica control
pierced D. paramelanica


Y
Crystal Cells
10
8
6
4
2
0
D. virilis control
pierced D. virilis

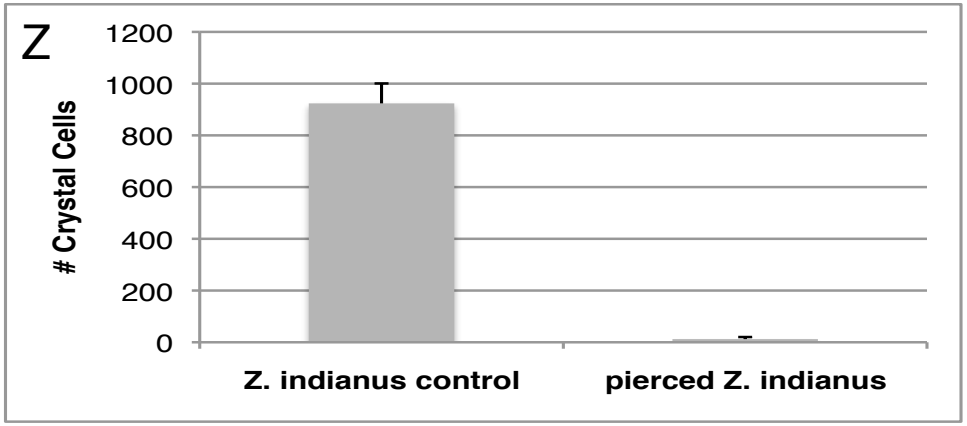
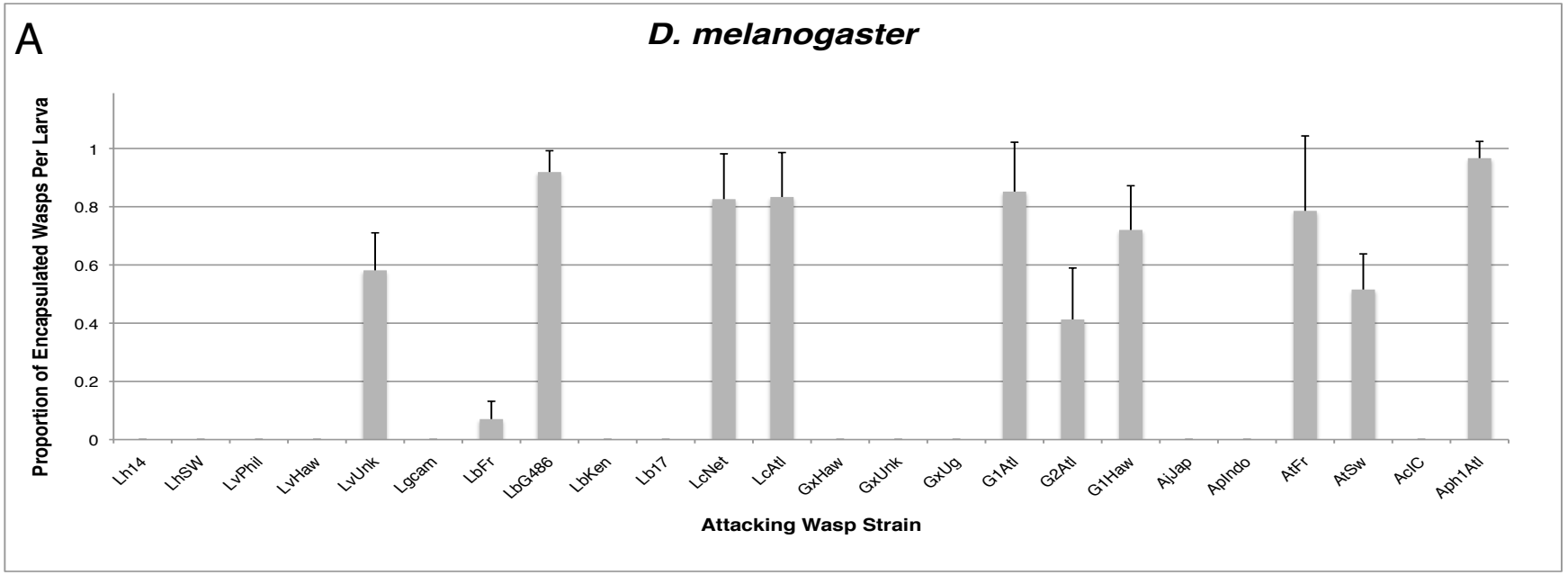
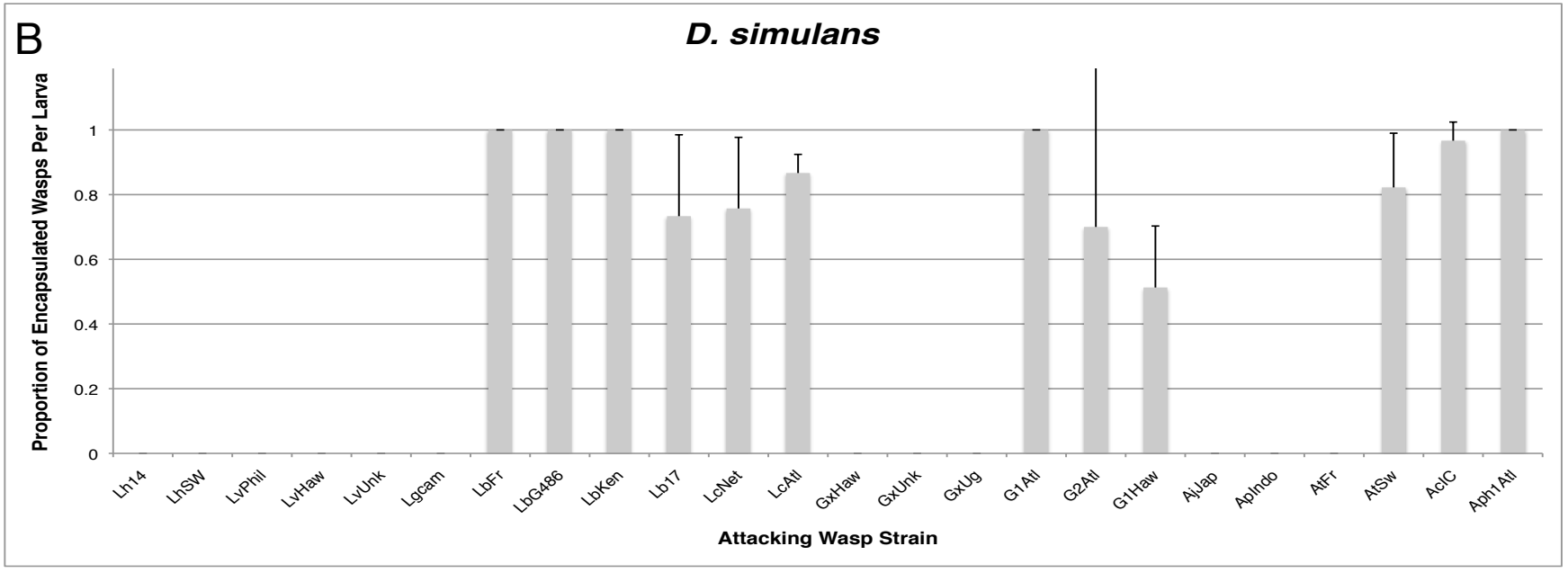
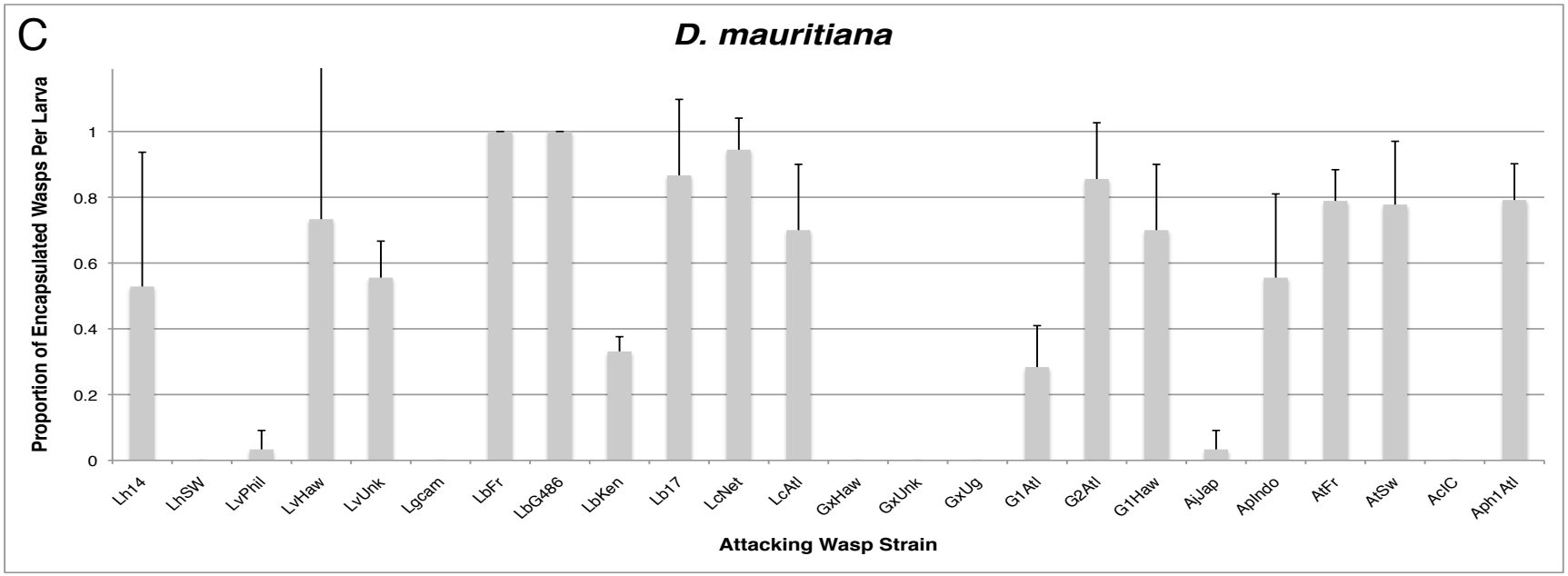
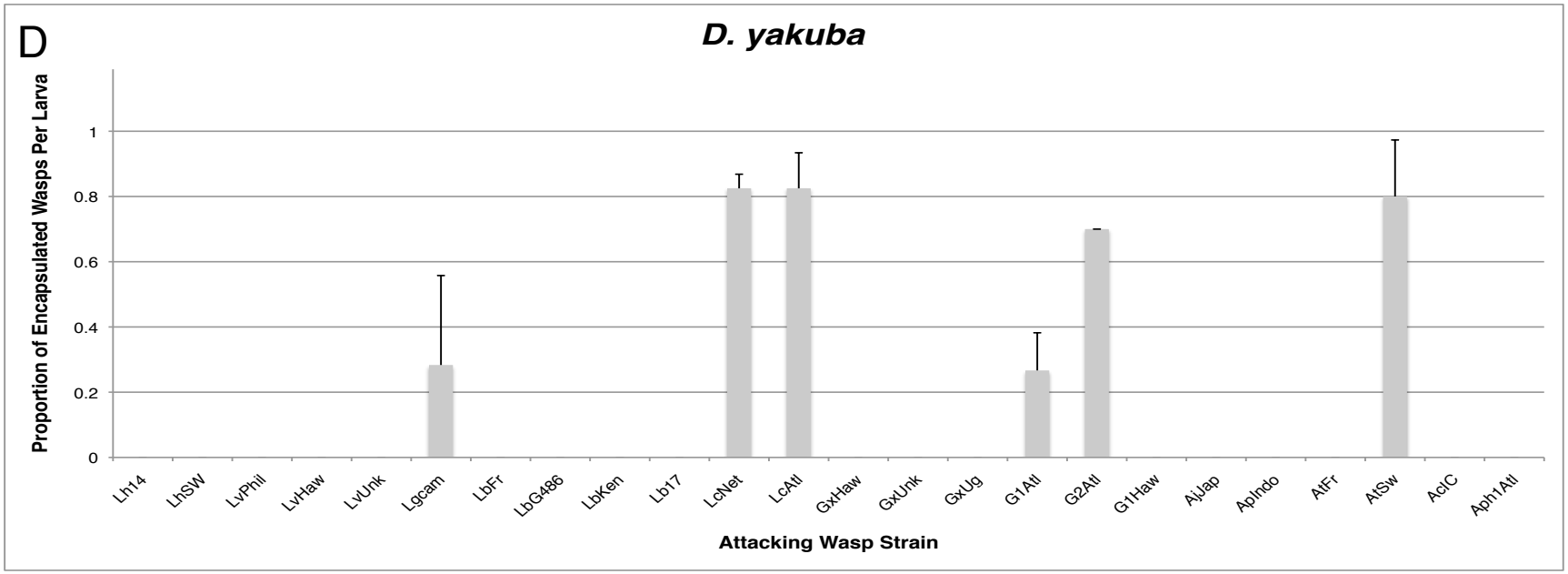

Z
Crystal Cells
1200
1000
800
600
400
200
0
Z. indianus control
pierced Z. indianus

Figure 16

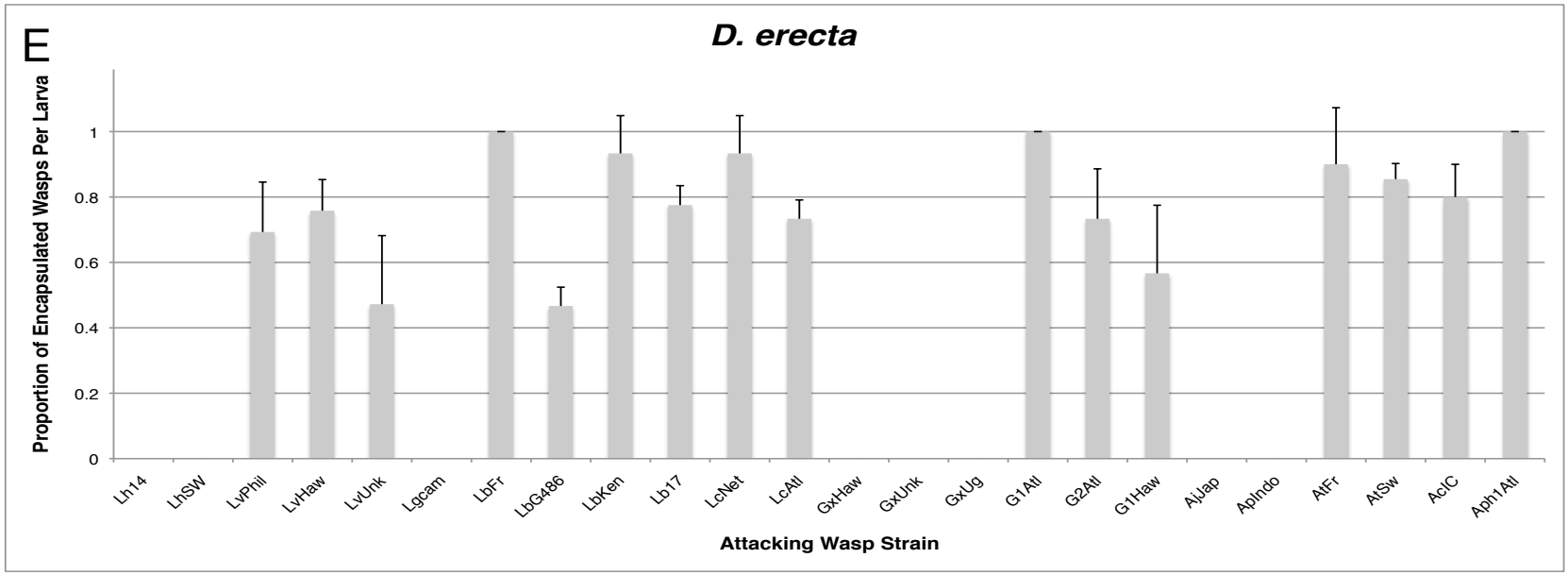

A
D. melanogaster
Proportion of Encapsulated Wasps Per Larva
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Attacking Wasp Strain


B
D. simulans
Proportion of Encapsulated Wasps Per Larva
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Attacking Wasp Strain

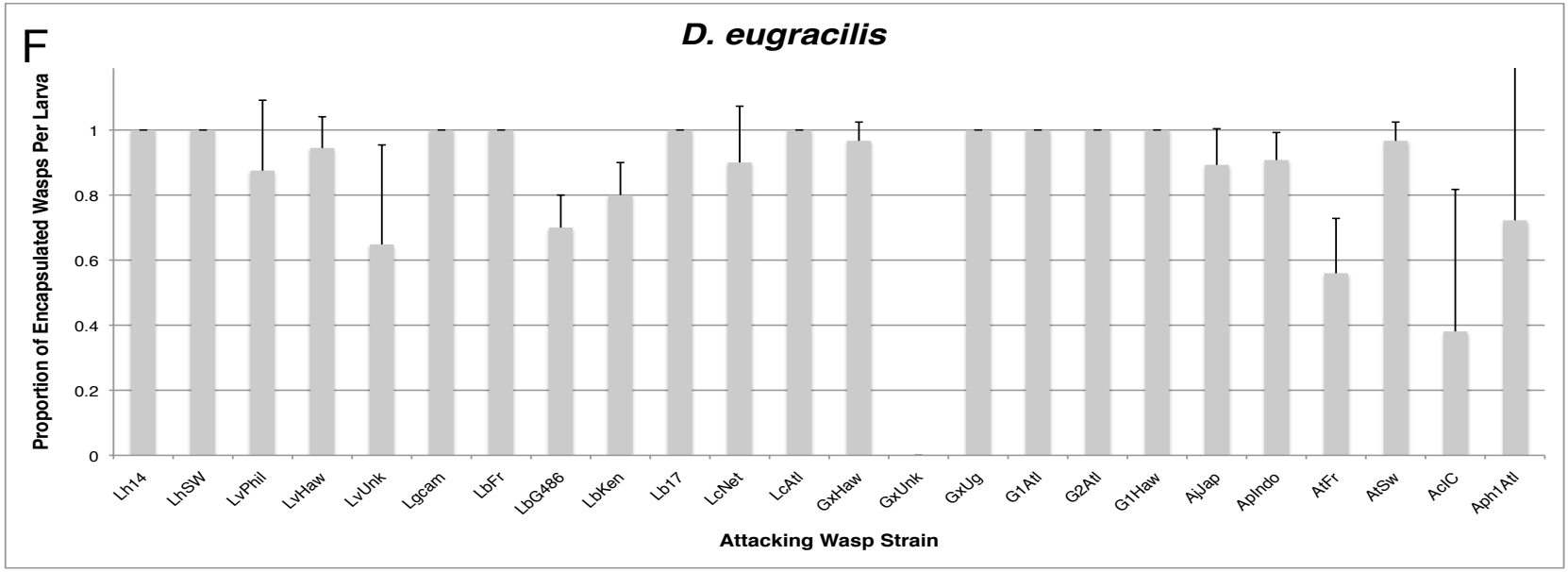

C
D. mauritiana
Proportion of Encapsulated Wasps Per Larva
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AcIC
Aph1Atl
Attacking Wasp Strain


D
D. yakuba
Proportion of Encapsulated Wasps Per Larva
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AcIC
Aph1Atl
Attacking Wasp Strain

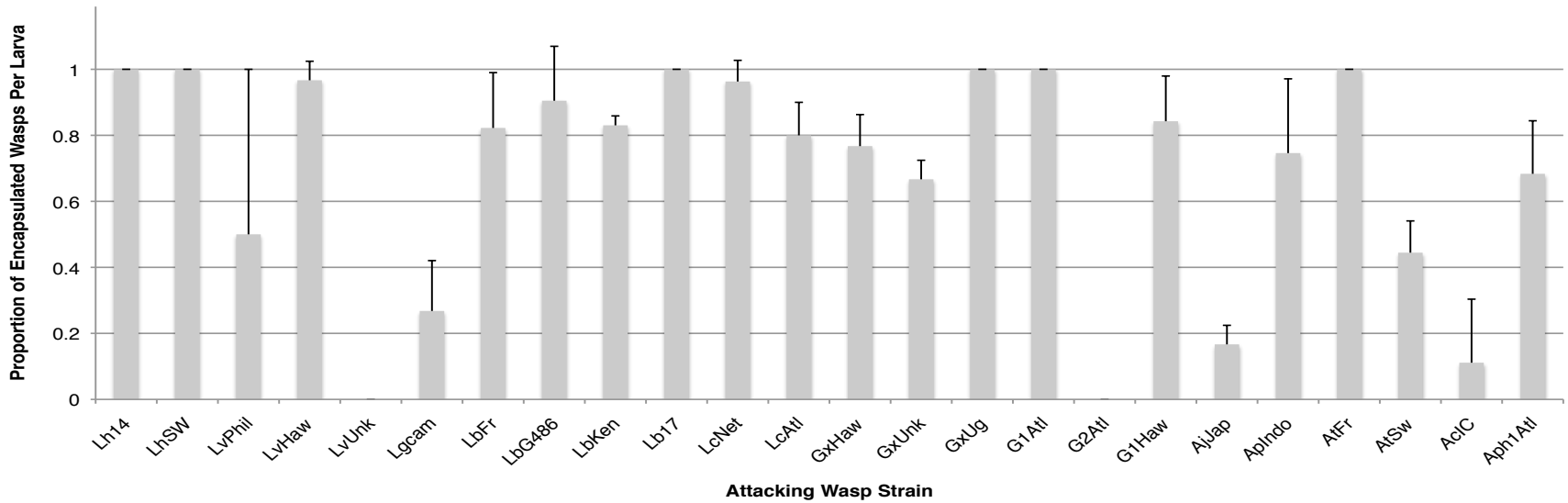
E


D. erecta
Proportion of Encapsulated Wasps Per Larva
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
AplndoAtFr
AtSw
AclC
Aph1Atl
Attacking Wasp Strain

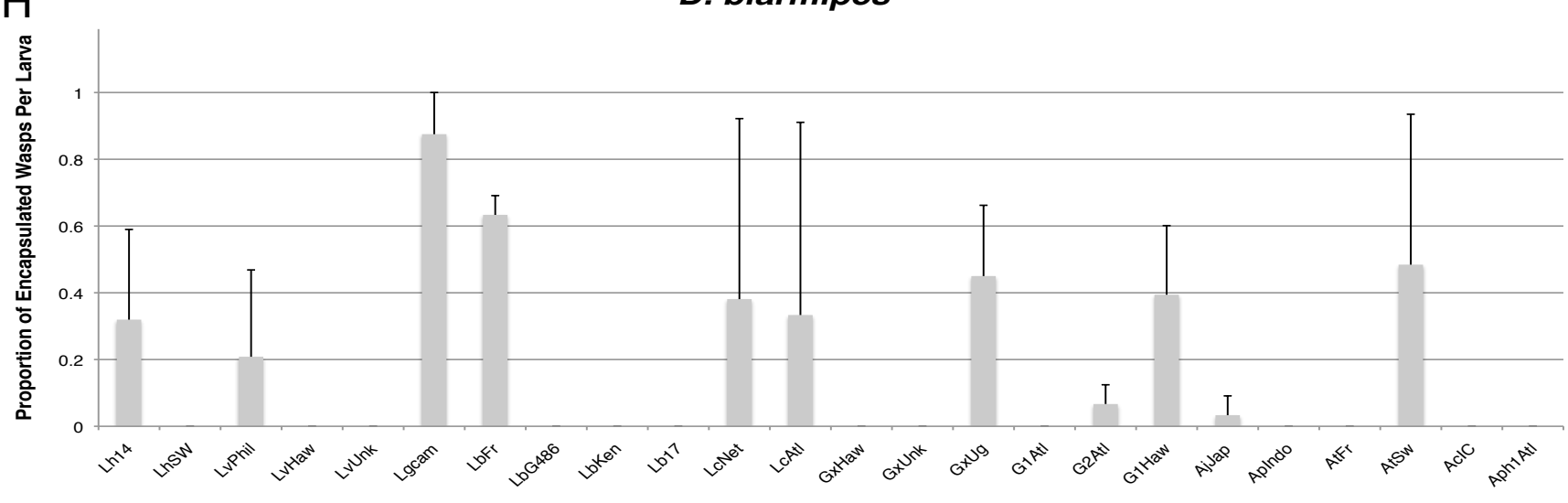
F

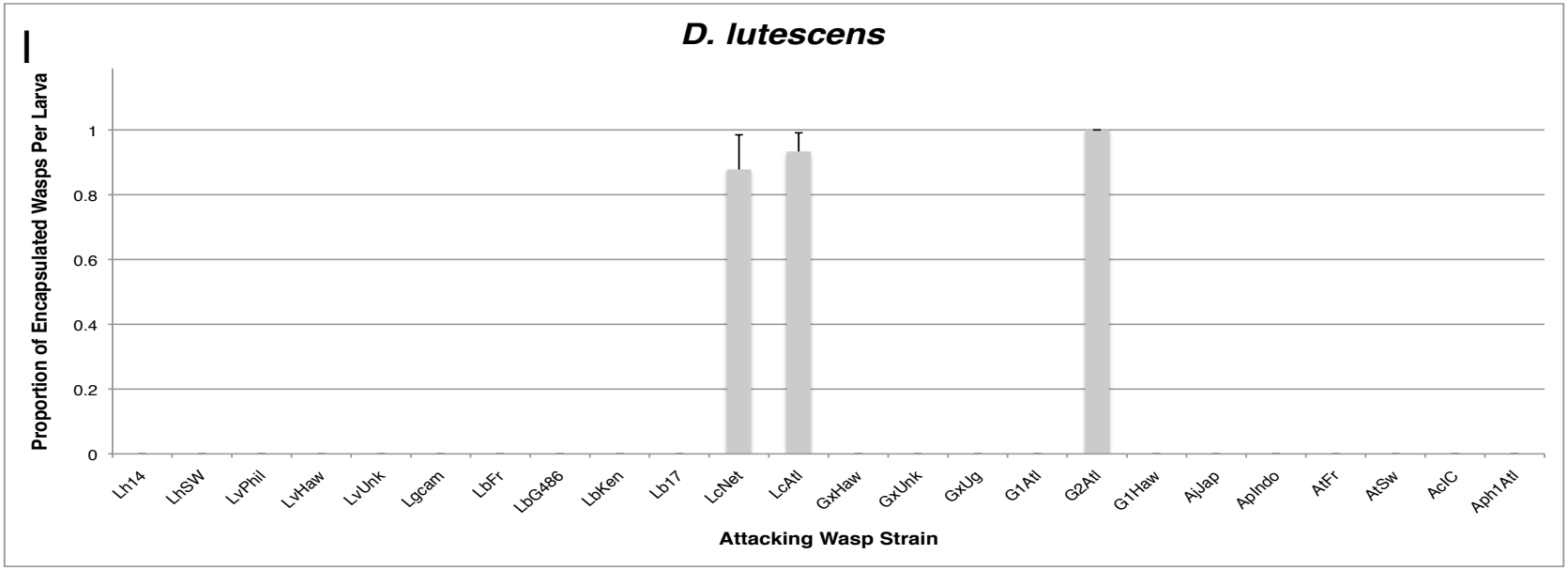

D. eugracilis
Proportion of Encapsulated Wasps Per Larva
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
AplndoAtFr
AtSw
AclC
Aph1Atl
Attacking Wasp Strain

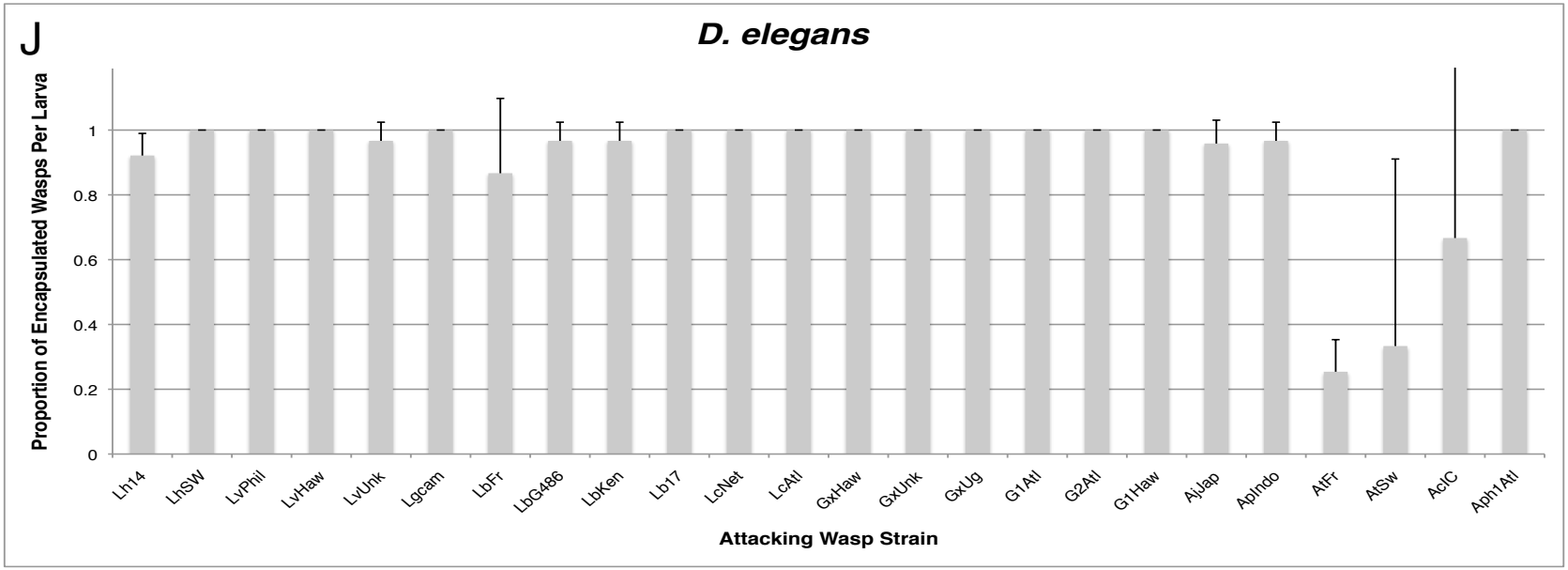
G


D. suzukii
Proportion of Encapsulated Wasps Per Larva
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Attacking Wasp Strain

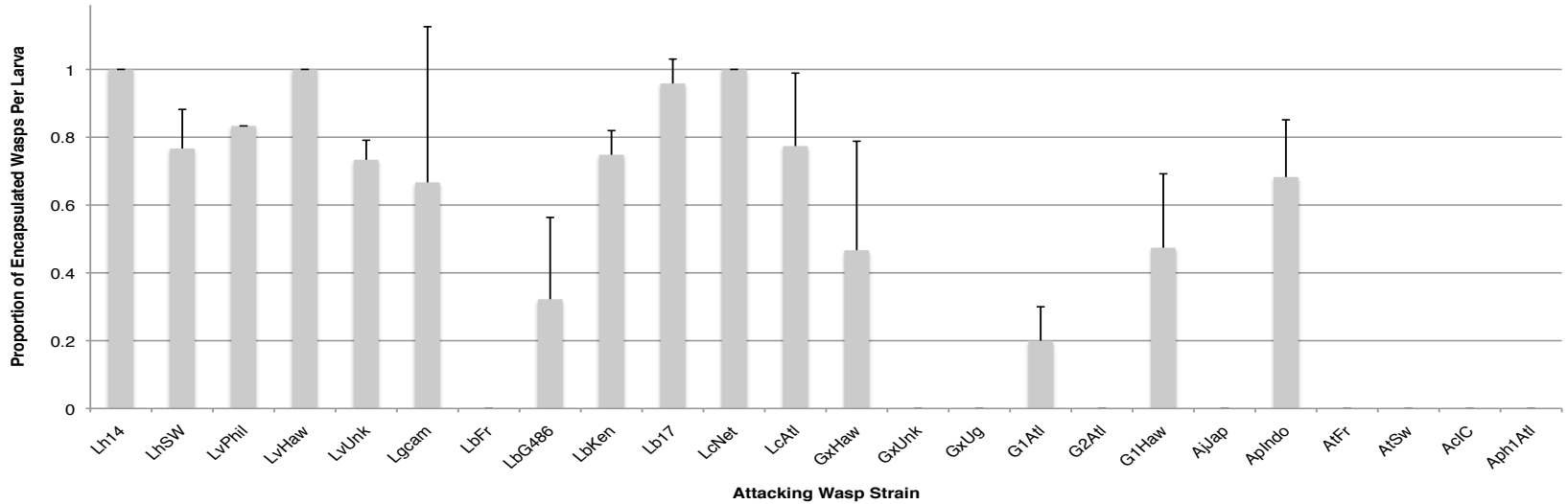
H


D. biarmipes
Proportion of Encapsulated Wasps Per Larva
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Attacking Wasp Strain


I
D. lutescens
Proportion of Encapsulated Wasps Per Larva
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AcIC
Aph1Atl
Attacking Wasp Strain


J
D. elegans
Proportion of Encapsulated Wasps Per Larva
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AcIC
Aph1Atl
Attacking Wasp Strain

K


D. affinis
Proportion of Encapsulated Wasps Per Larva
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Attacking Wasp Strain

L

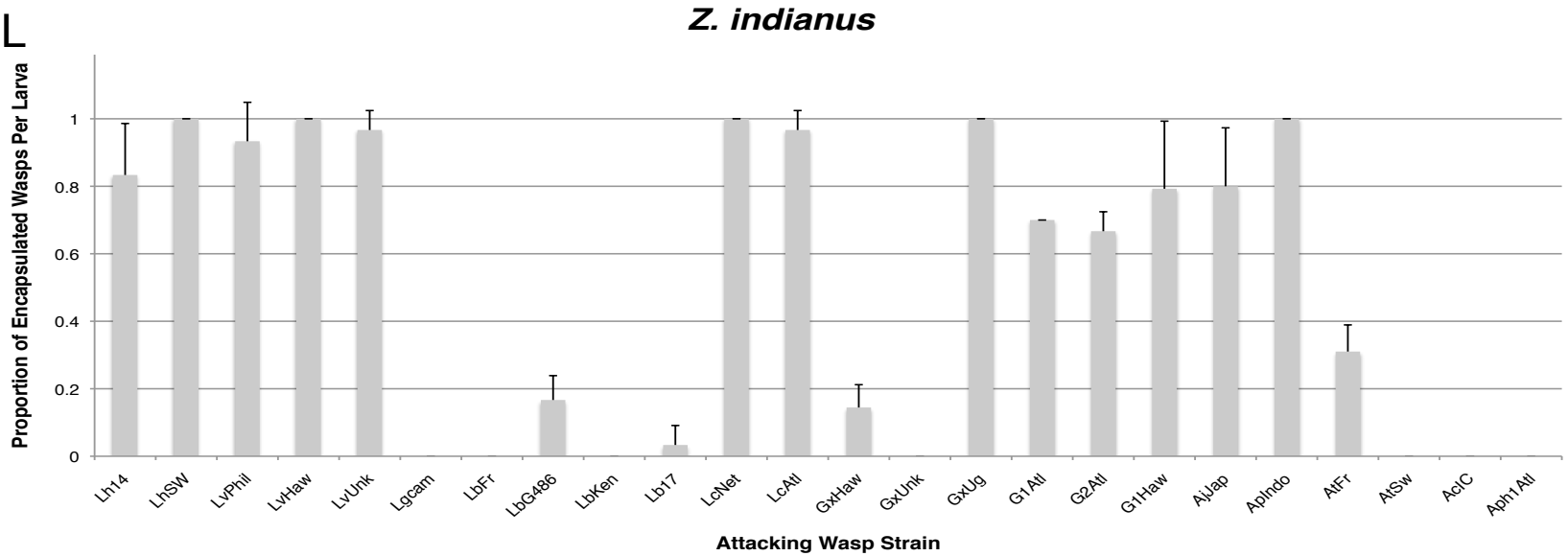
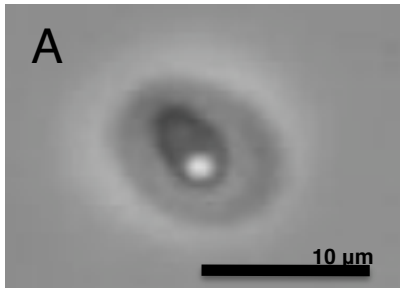
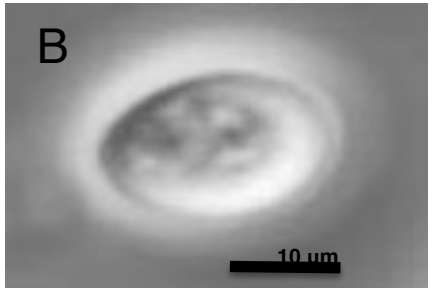

Z. indianus
Proportion of Encapsulated Wasps Per Larva
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AclC
Aph1Atl
Attacking Wasp Strain

Figure 17

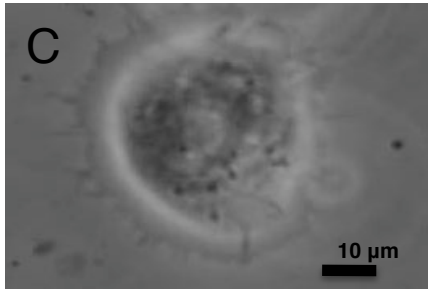
D. melanogaster



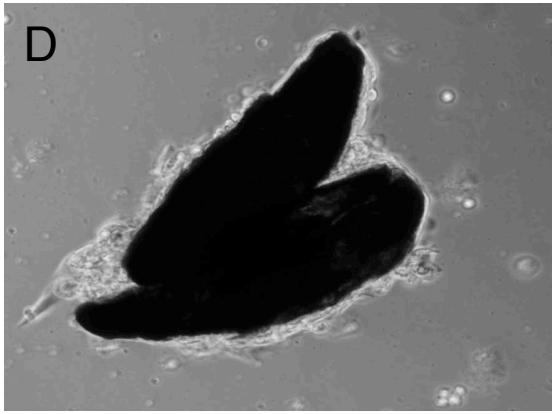
plasmatocyte



podocyte

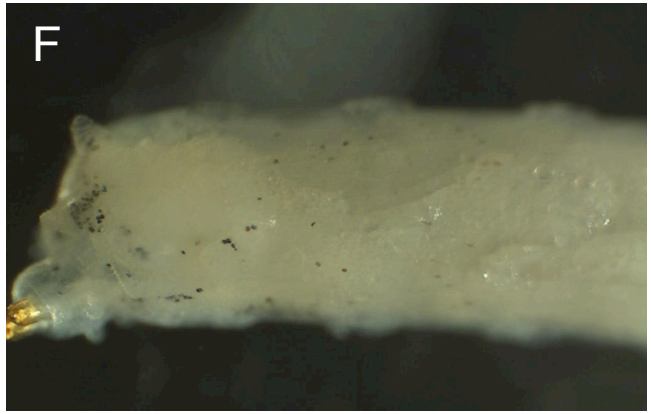


lamellocyte



melanotic encapsulation

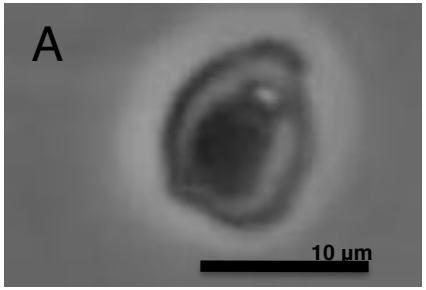
larva



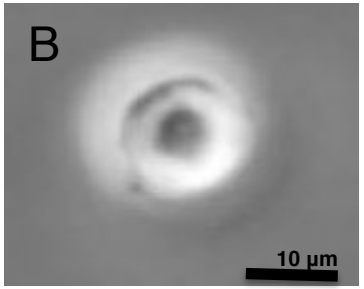
crystals

Figure 18

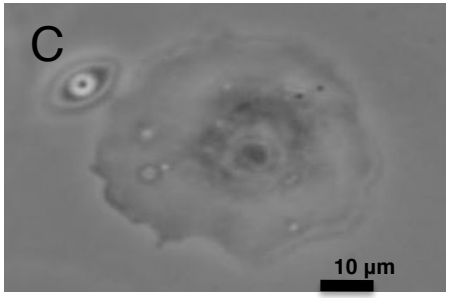
D. simulans



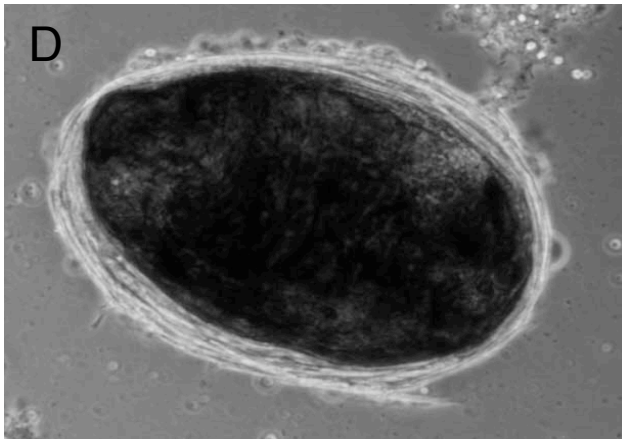
plasmatocyte



podocyte

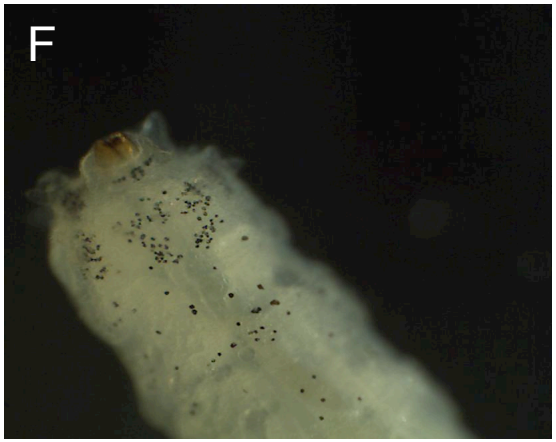


lamellocyte



melanotic encapsulation

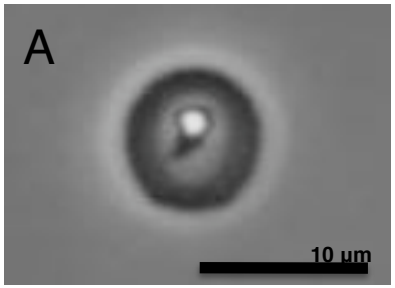
larva



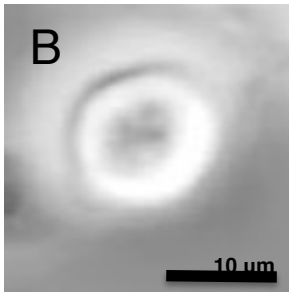
crystals

Figure 19

D. mauritiana



plasmatocyte



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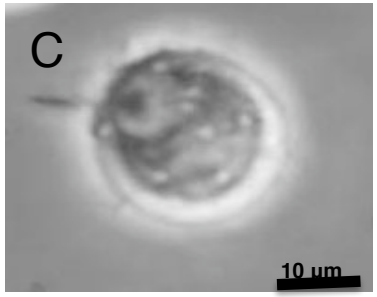
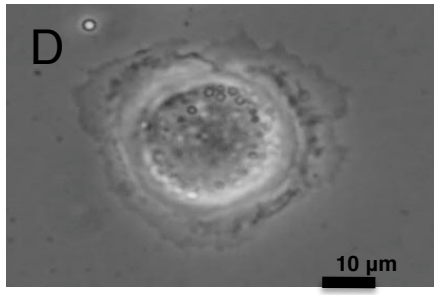
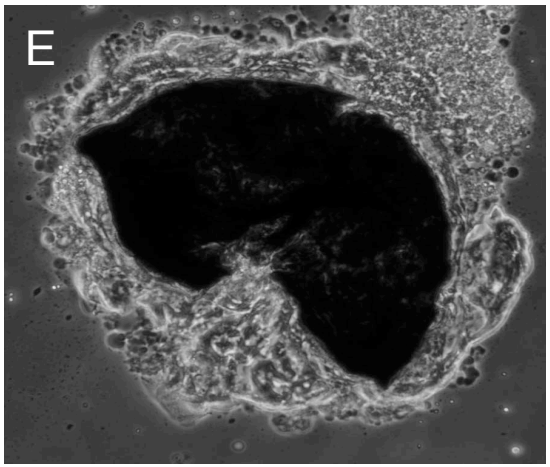


pseudolamellocyte

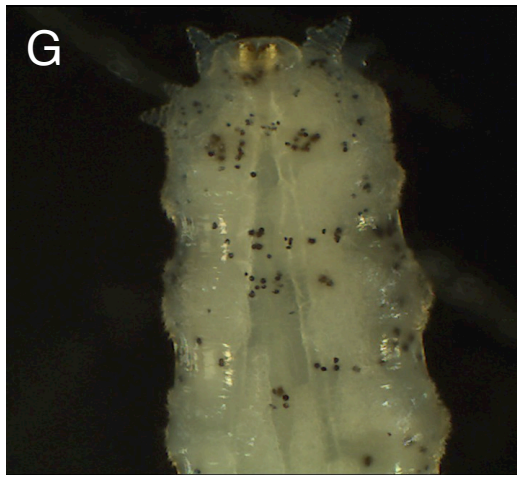


lamellocyte



melanotic encapsulation

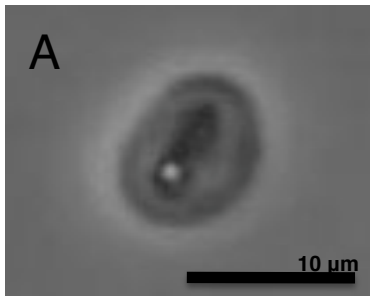
larva



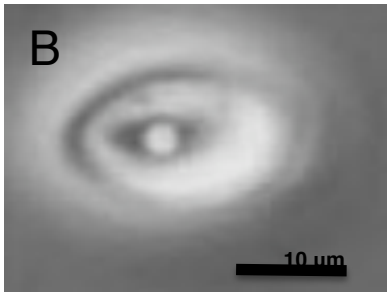
crystals

Figure 20

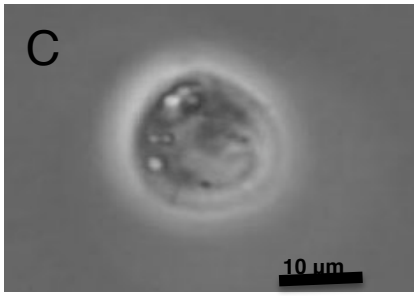
D. sechelia



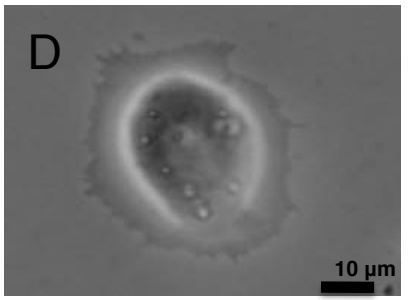
plasmatocyte



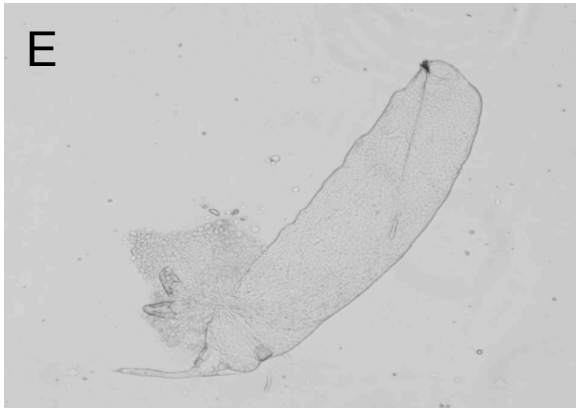
podocyte



pseudolamellocyte

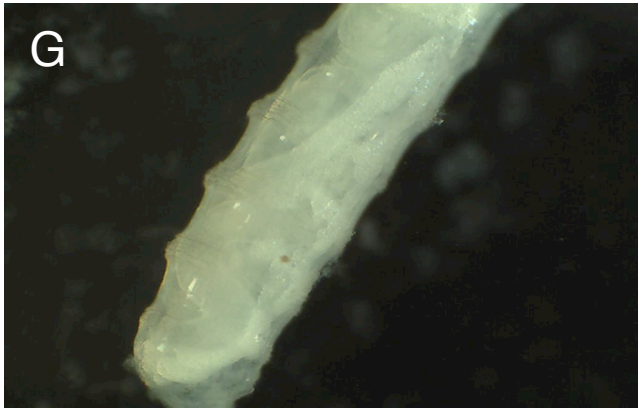


lamellocyte



non-cellular mediated

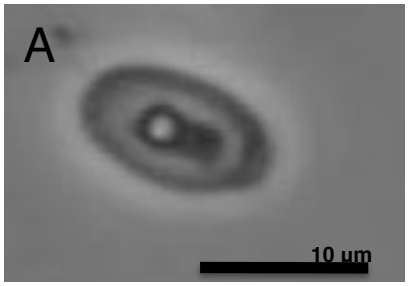
larva



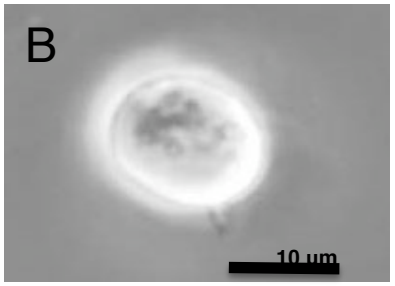
crystals

Figure 21

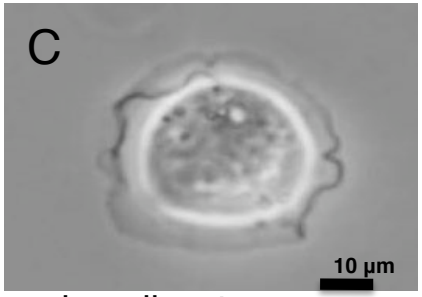
D. yakuba



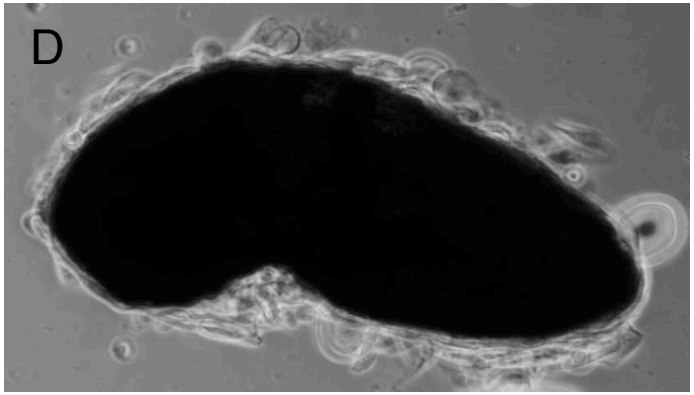
plasmatocyte



podocyte

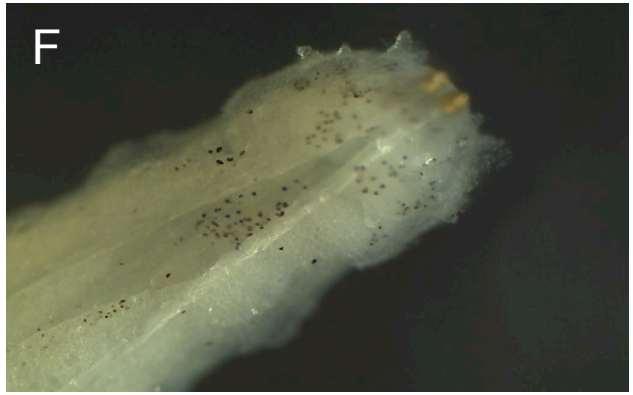


lamellocyte



melanotic encapsulation

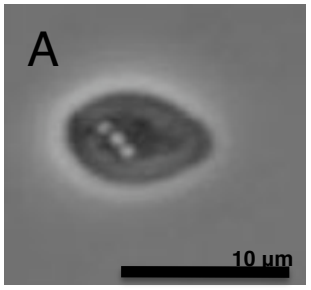
larva



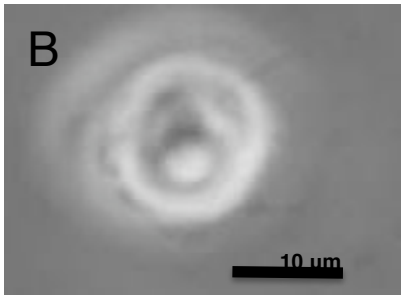
crystals

Figure 22

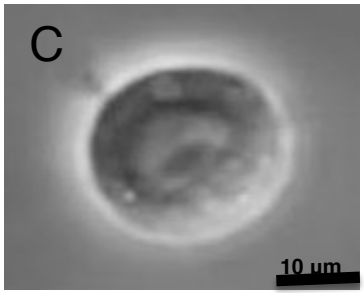
D. erecta



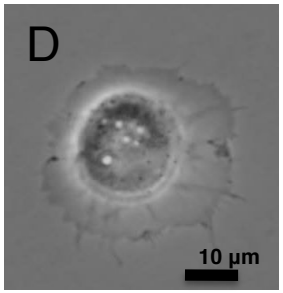
plasmatocyte



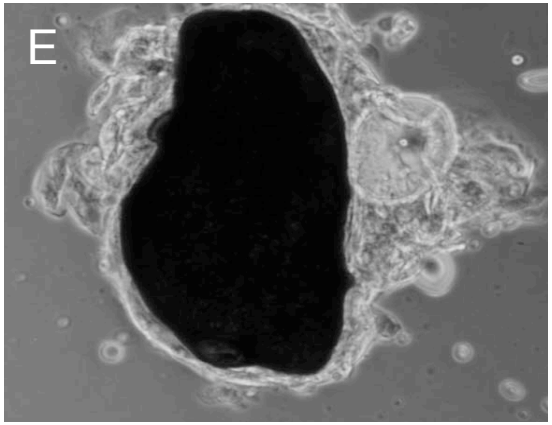
podocyte



pseudolamellocyte

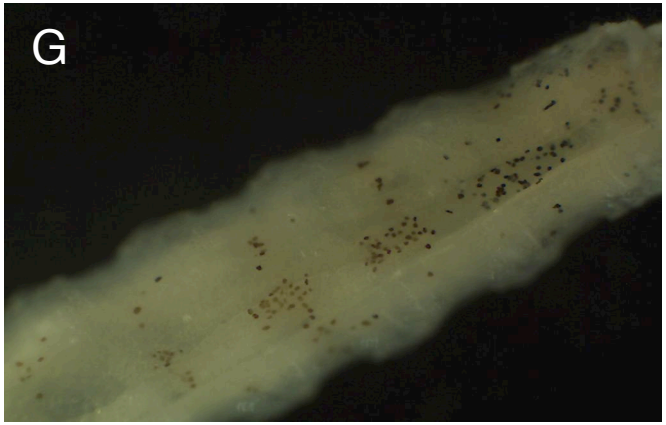


lamellocyte



melanotic encapsulation

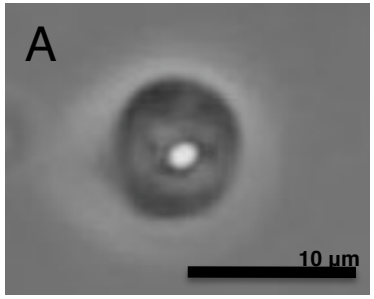
larva



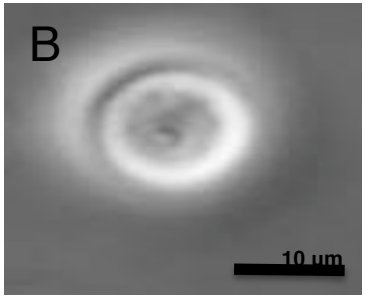
crystals

Figure 23

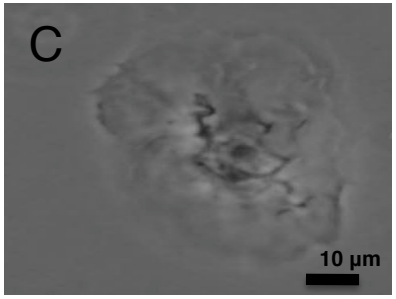
D. eugracilis



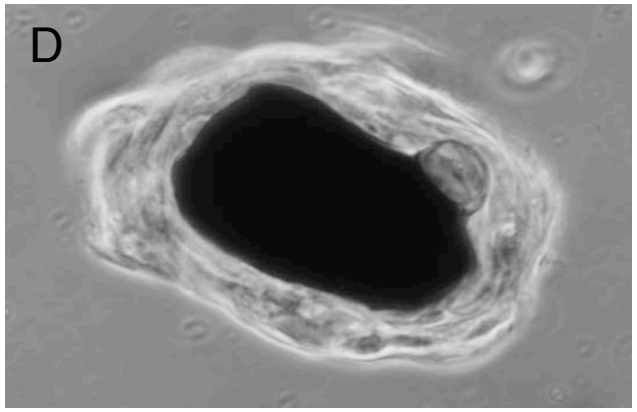
plasmatocyte



podocyte

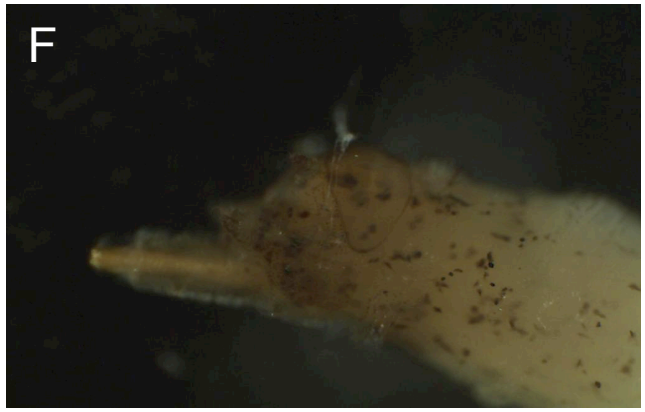


lamellocyte



melanotic encapsulation

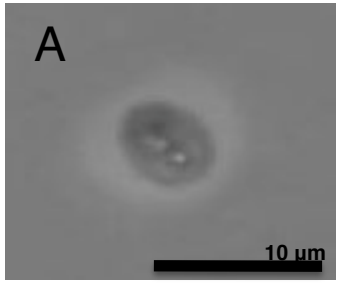
larva



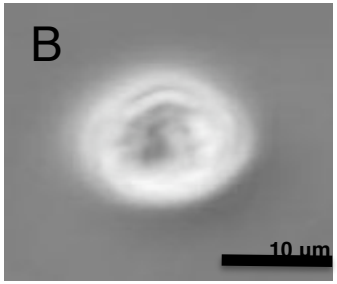
crystals

Figure 24

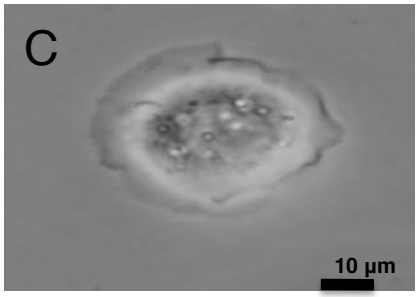
D. suzukii



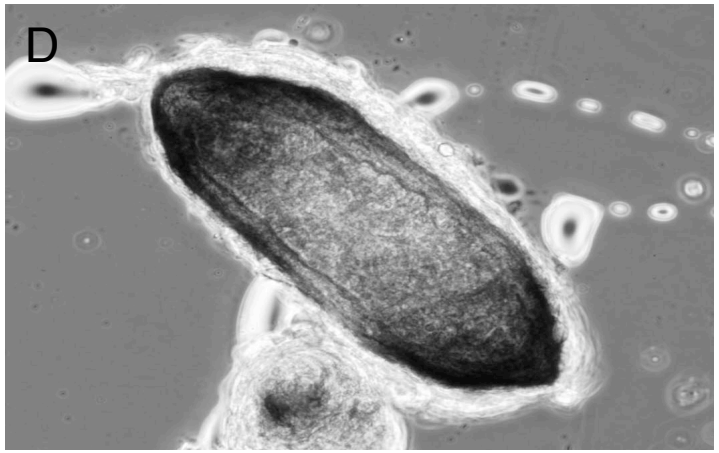
plasmatocyte



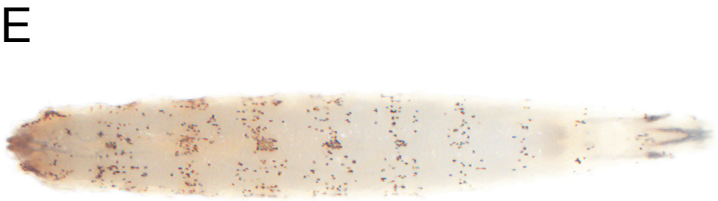
podocyte



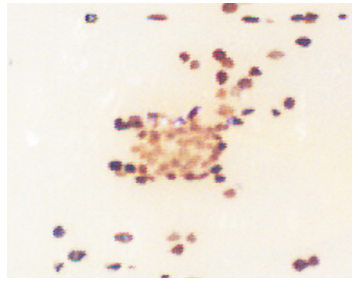
lamellocyte



melanotic encapsulation



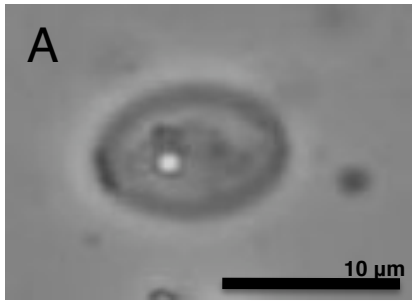
larva



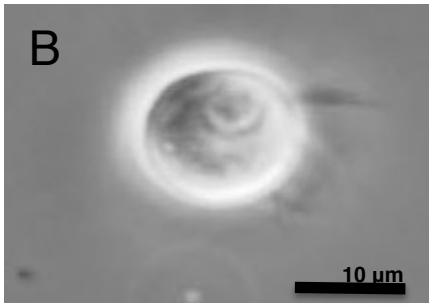
crystals

Figure 25

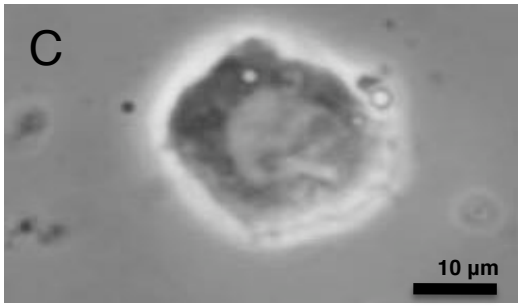
D. biarmipes



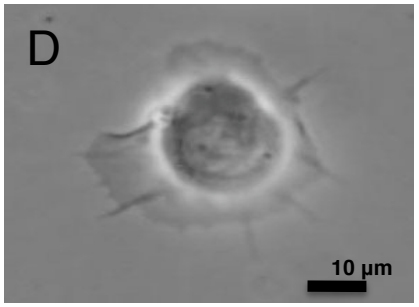
plasmatocyte



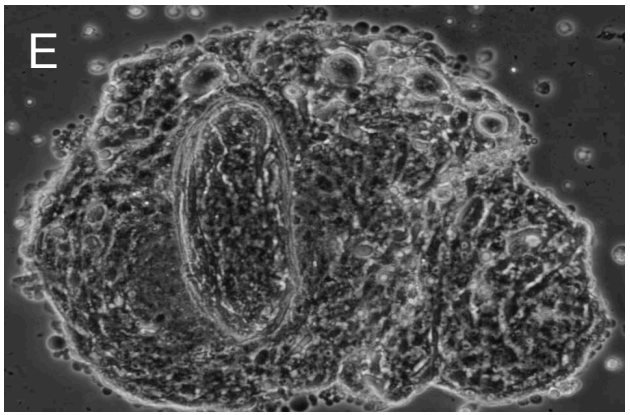
podocyte



pseudolamellocyte

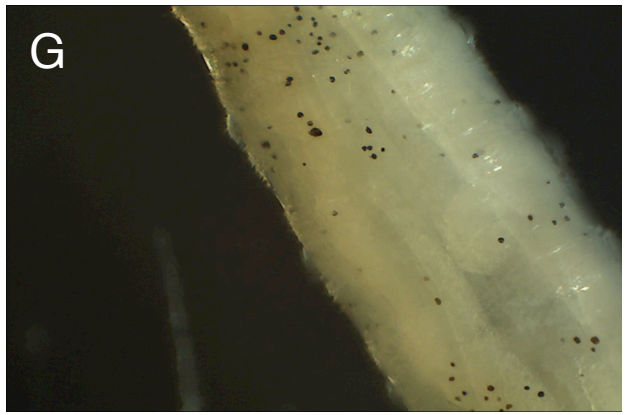


lamellocyte



melanotic encapsulation

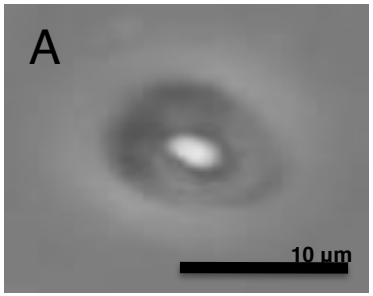
larva



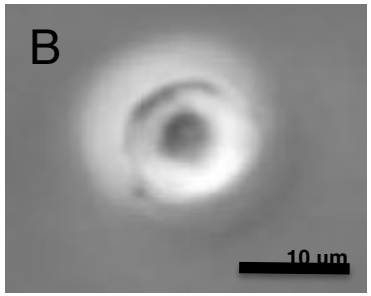
crystals

Figure 26

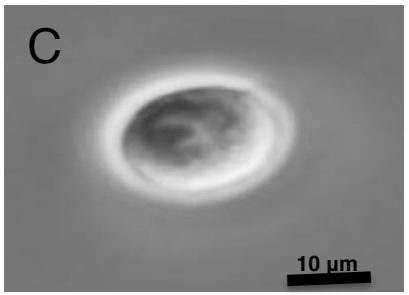
D. lutescens



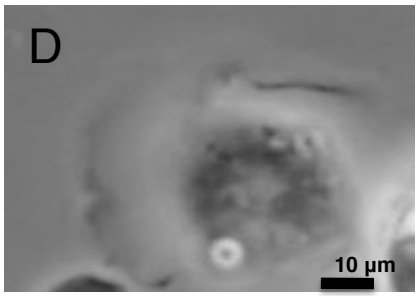
plasmatocyte



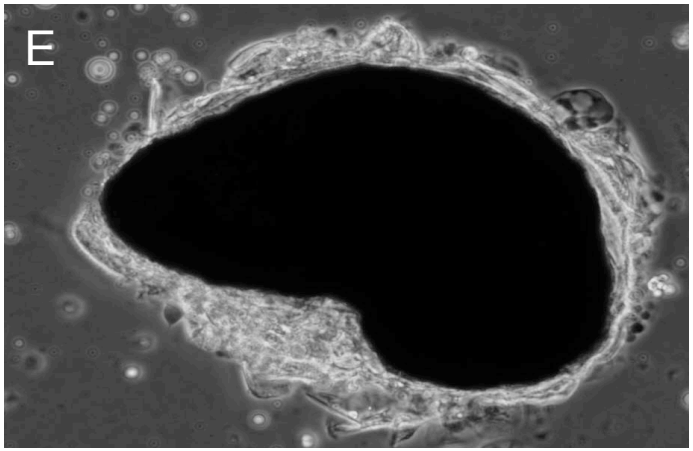
podocyte



pseudolamellocyte

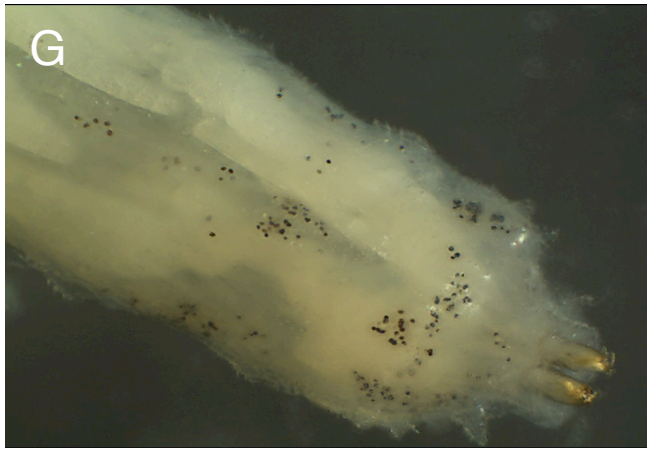


lamellocyte



non-cellular mediated

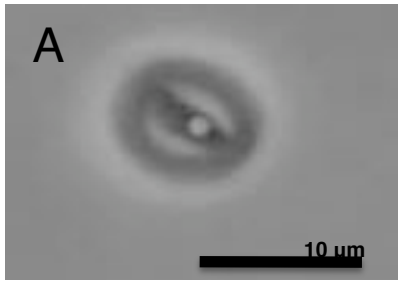
larva



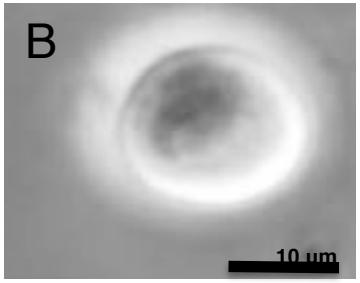
crystals

Figure 27

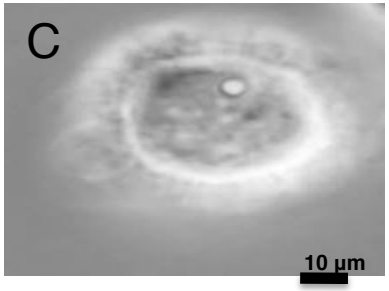
D. elegans



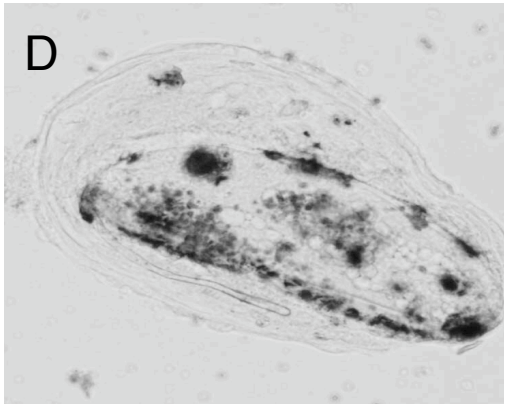
plasmatocyte



podocyte

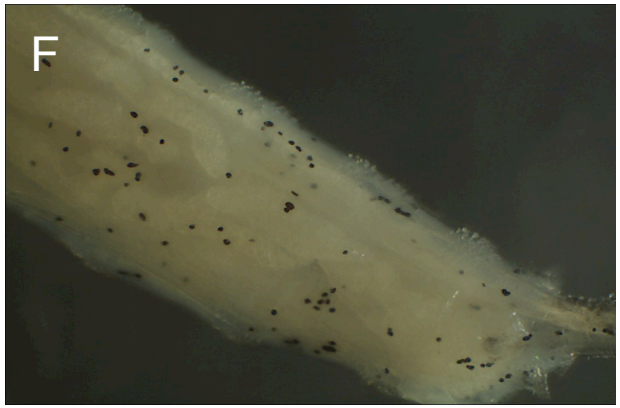


lamellocyte



non-cellular mediated

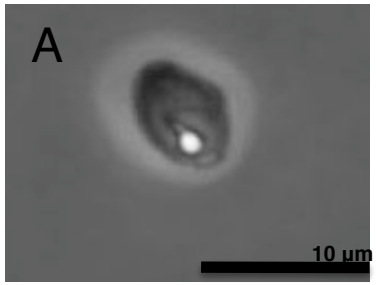
larva



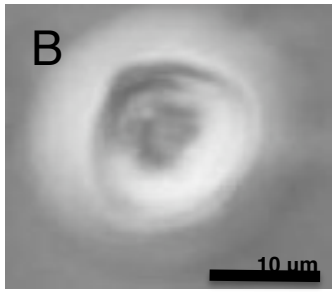
crystals

Figure 28

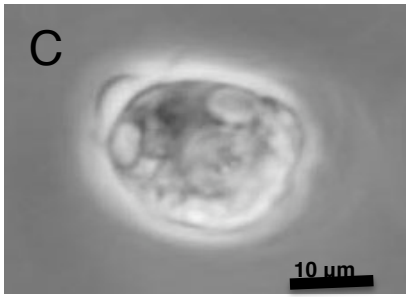
D. ficusphila



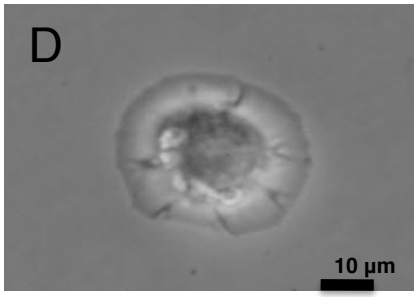
plasmatocyte



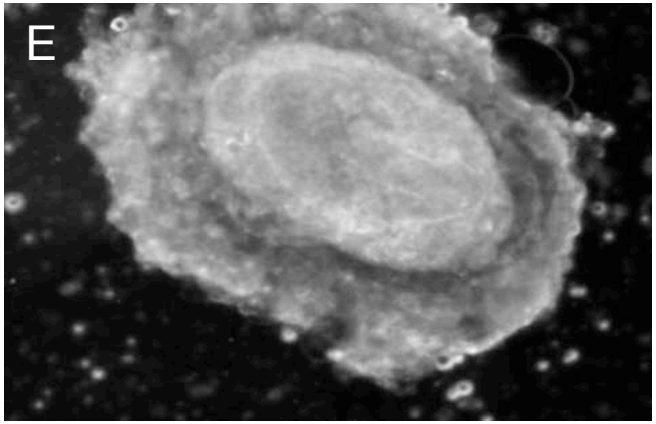
podocyte



pseudolamellocyte

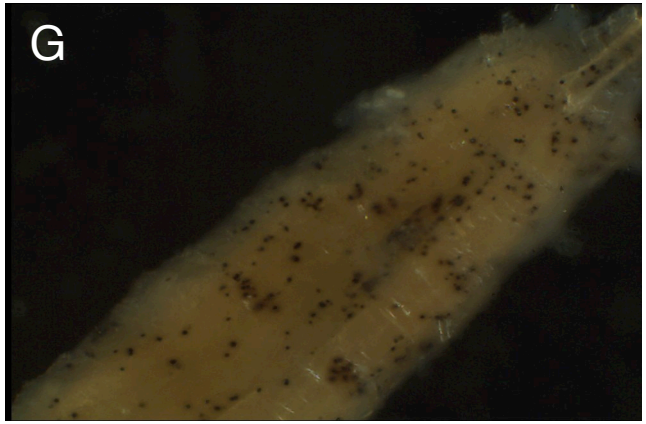


lamellocyte



cellular encapsulation

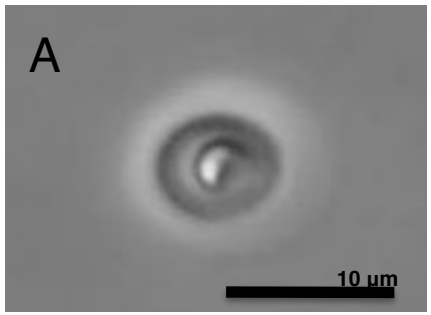
larva



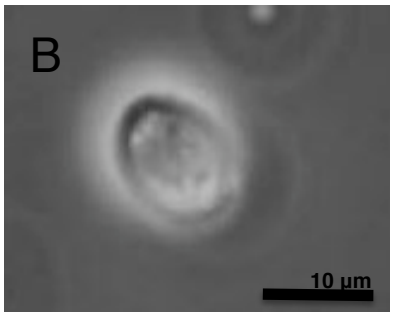
crystals

Figure 29

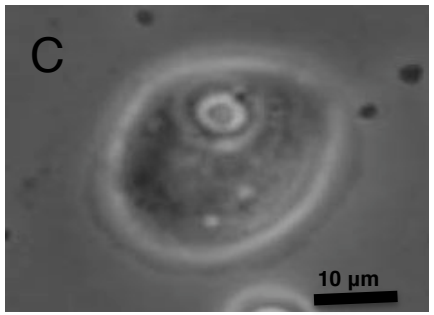
D. tsacasi



plasmatocyte



podocyte



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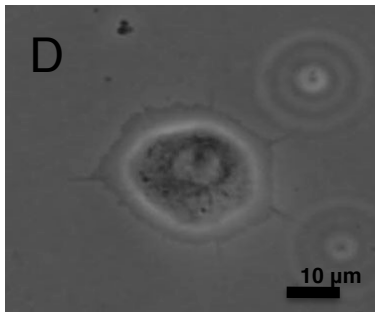
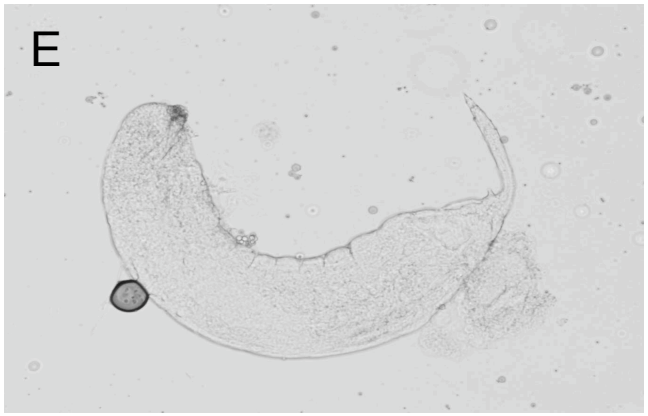
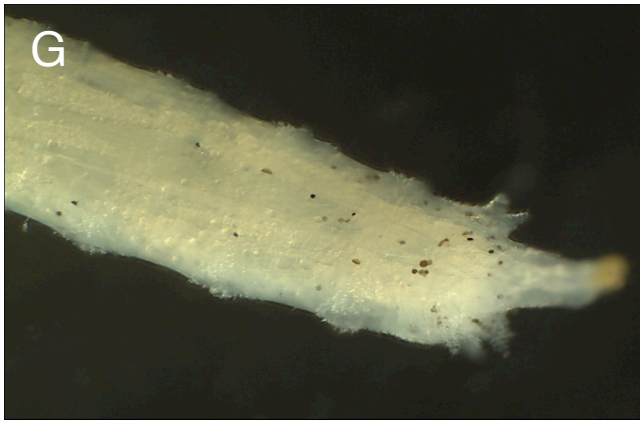


lamellocyte



non-cellular mediated

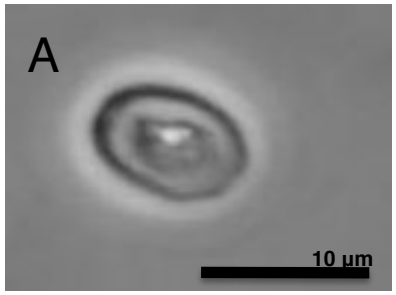
larva



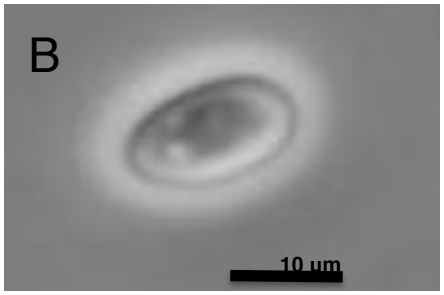
crystals

Figure 30

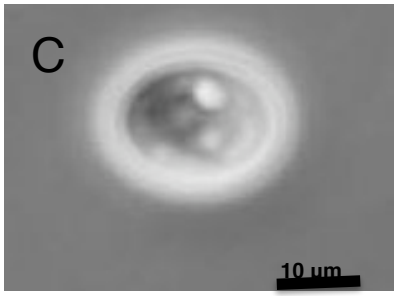
D. kikkawai



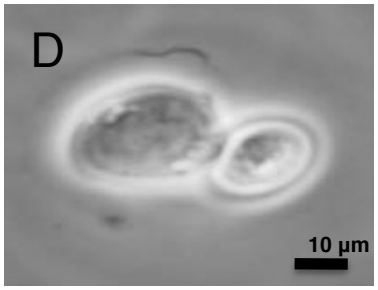
plasmatocyte



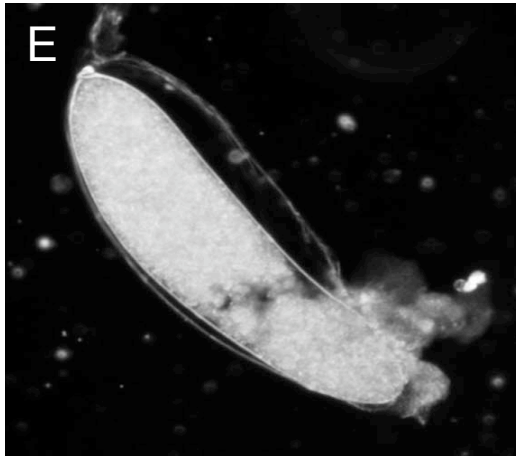
podocyte



pseudolamellocyte

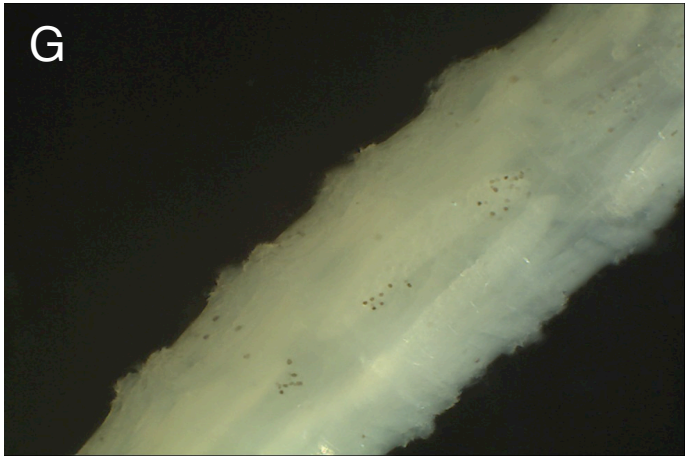


lamellocyte



non-cellular mediated

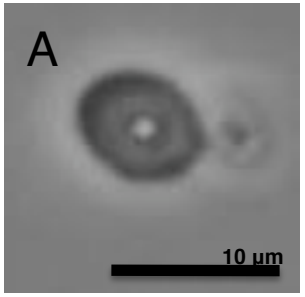
larva



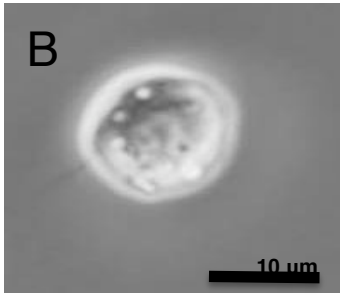
crystals

Figure 31

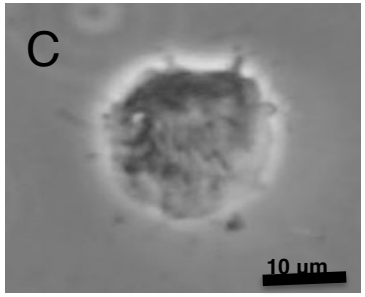
D. annanassae



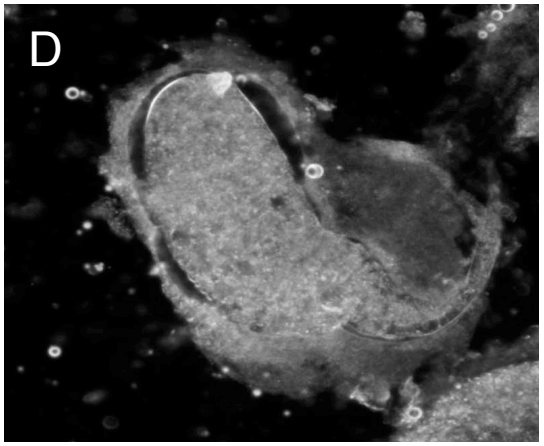
plasmatocyte



podocyte

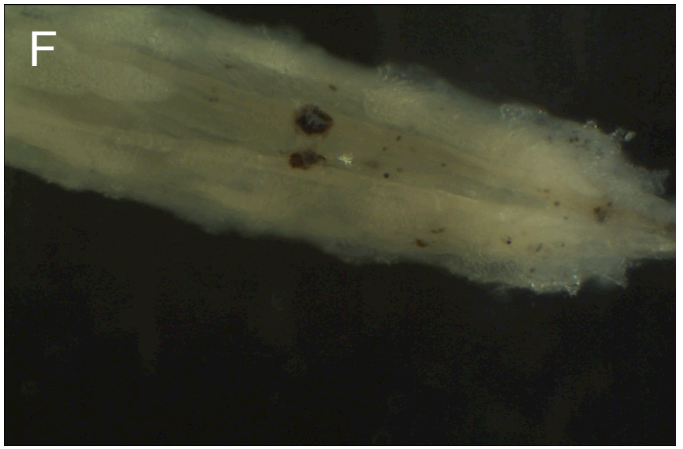


lamellocyte



non-cellular mediated

larva



crystals

Figure 32

D. affinis

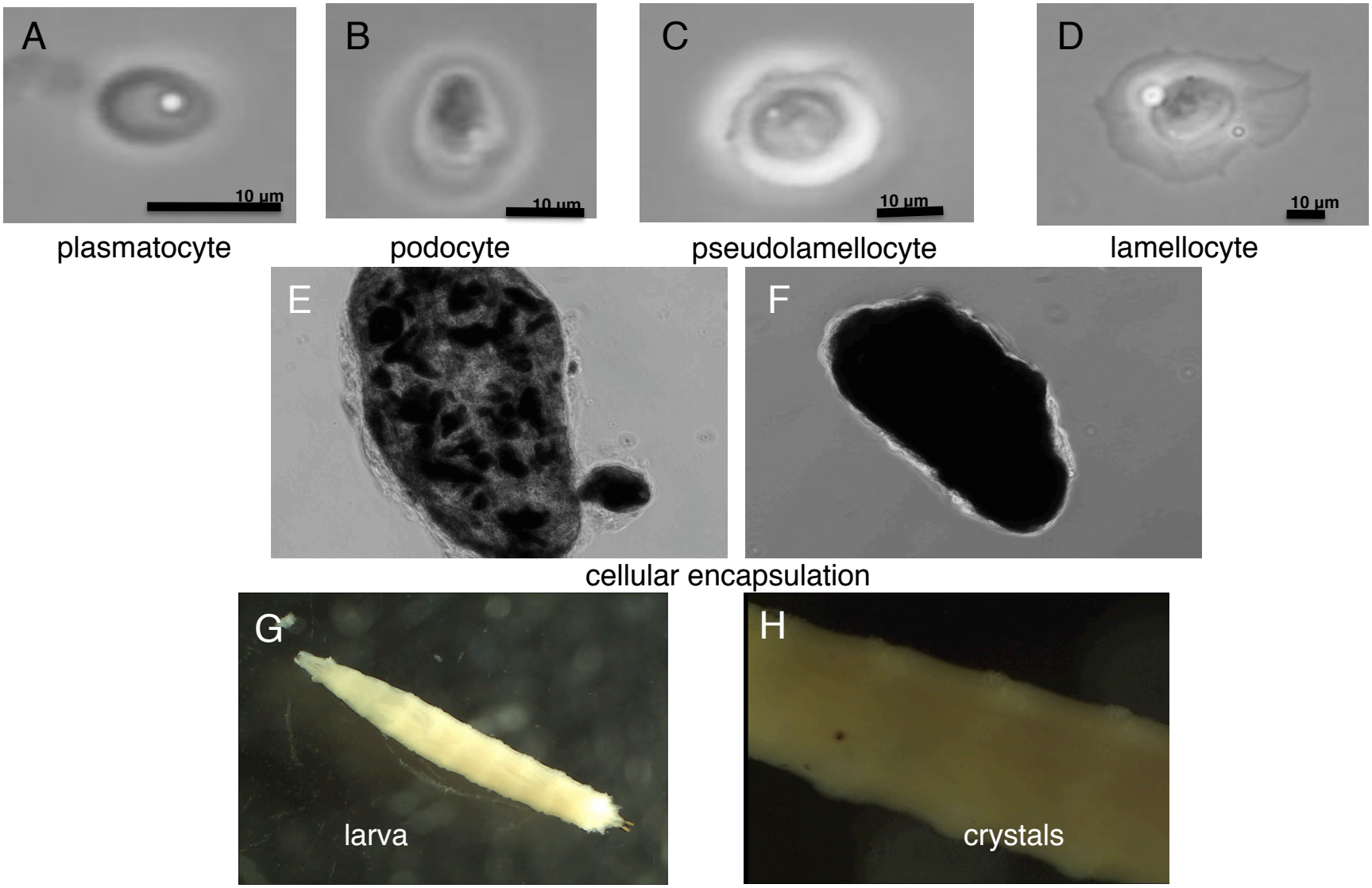
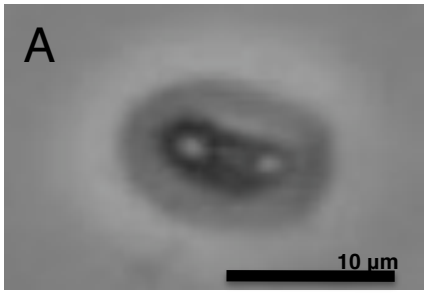
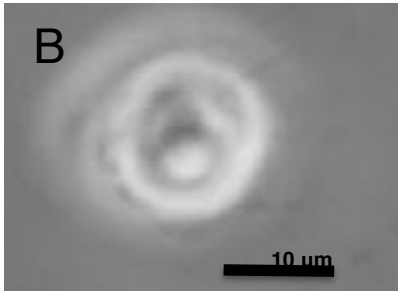


Figure 33

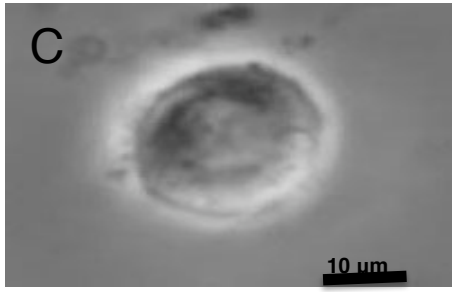
D. pseudoobscura



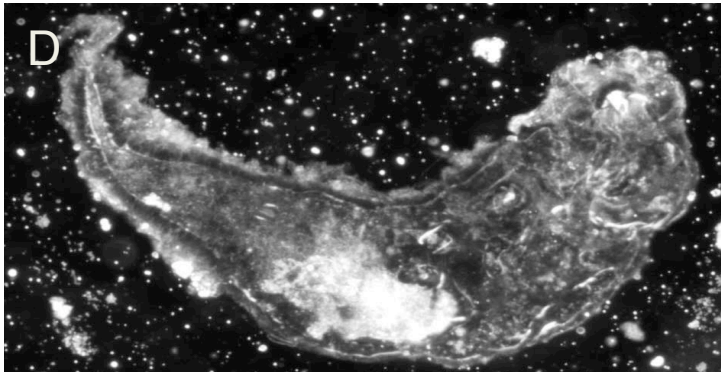
plasmatocyte



podocyte

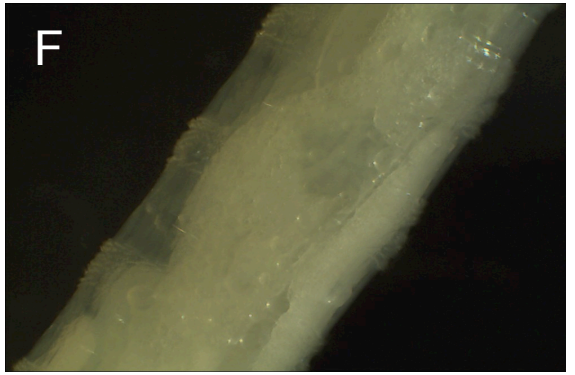


pseudolamellocyte



non-cellular mediated

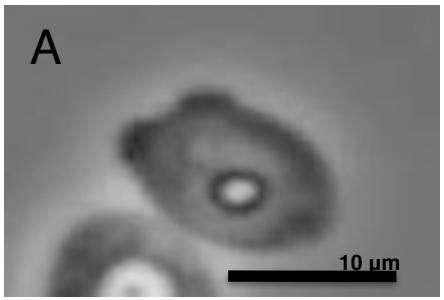
larva



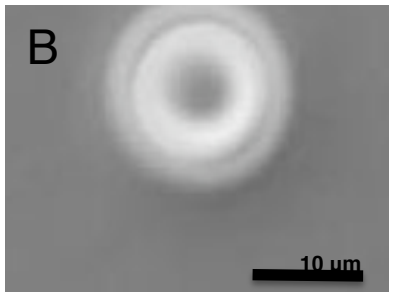
crystals

Figure 34

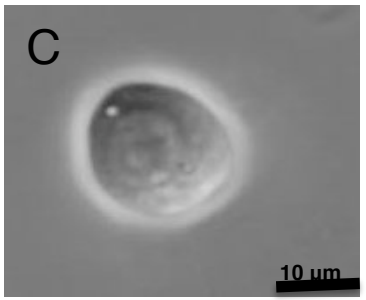
D. subobscura



plasmatocyte



podocyte



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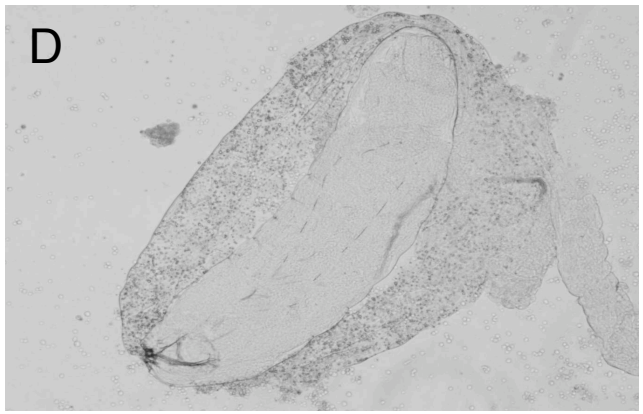
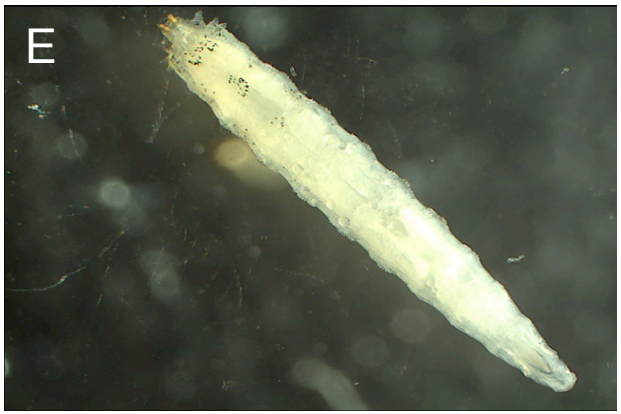
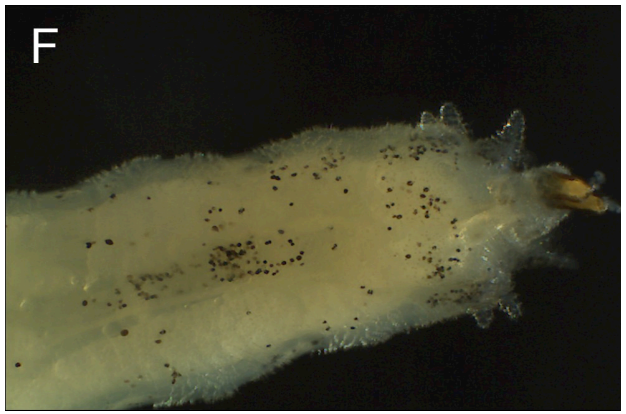


non-cellular mediated



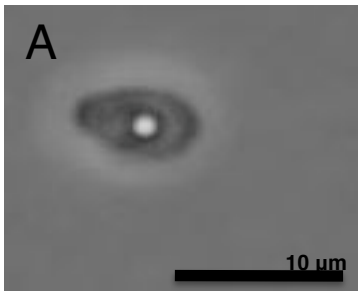
larva



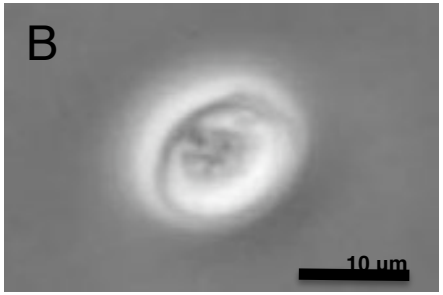
crystals

Figure 35

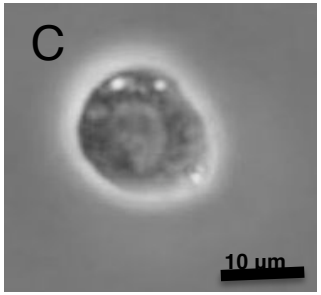
D. willistoni



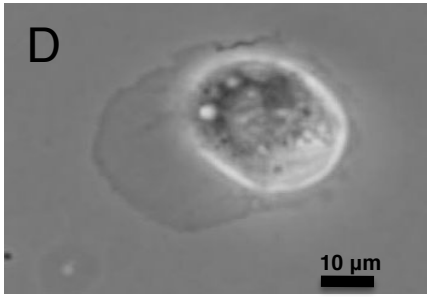
plasmatocyte



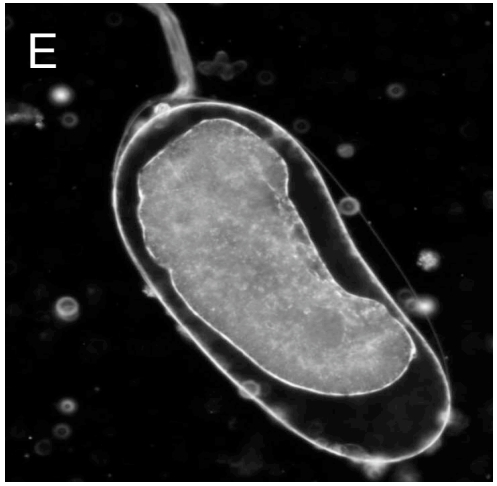
podocyte



pseudolamellocyte

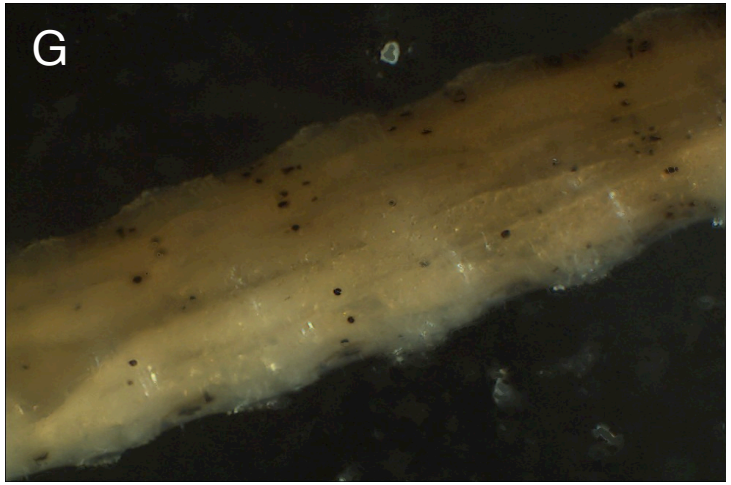


lamellocyte



non-cellular mediated

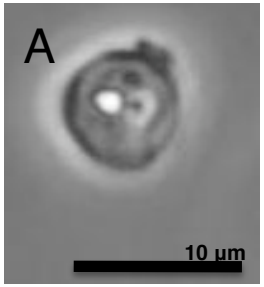
larva



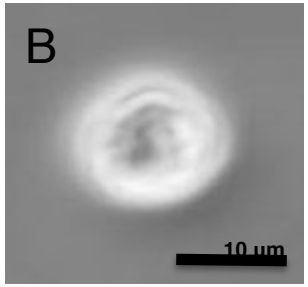
crystals

Figure 36

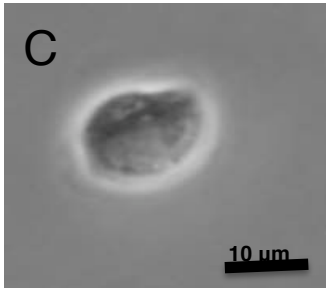
D. funebris



plasmatocyte



podocyte



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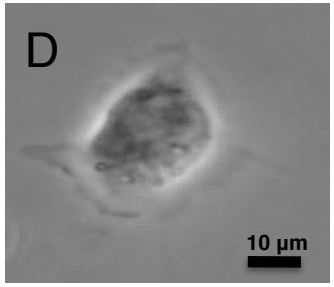
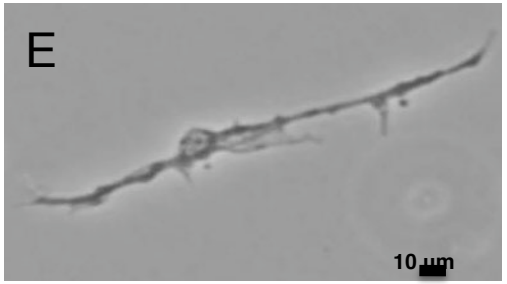
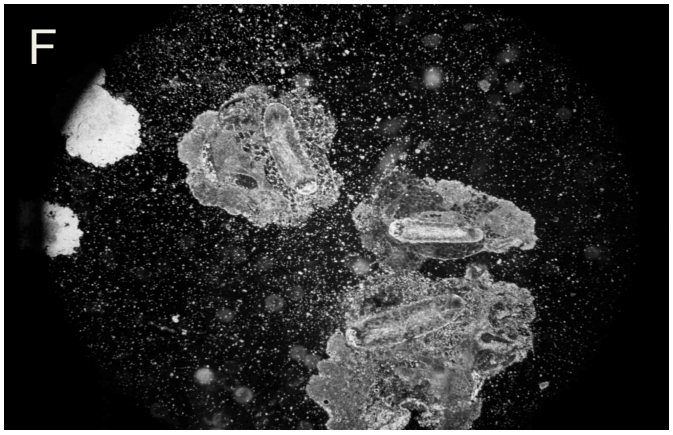


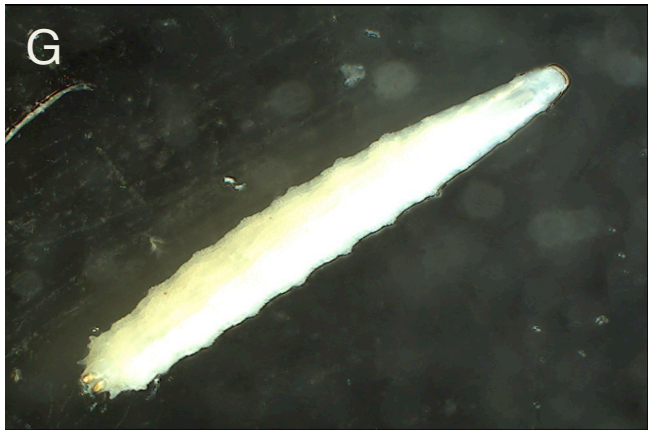
lamellocyte



nematocyte



cellular encapsulation

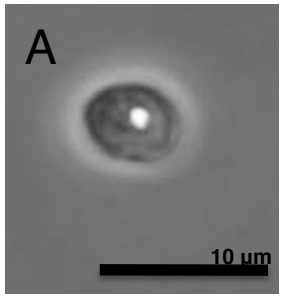


larva

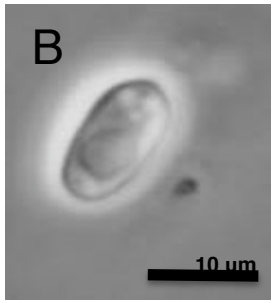
crystals

Figure 37

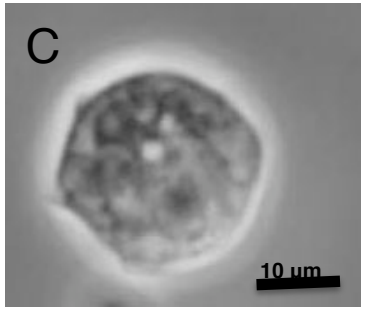
D. immigrans



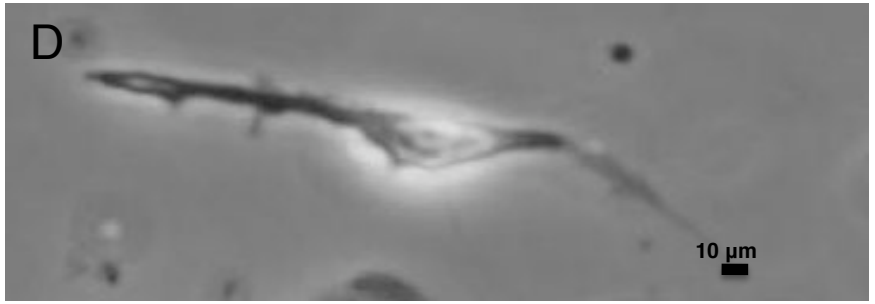
plasmatocyte



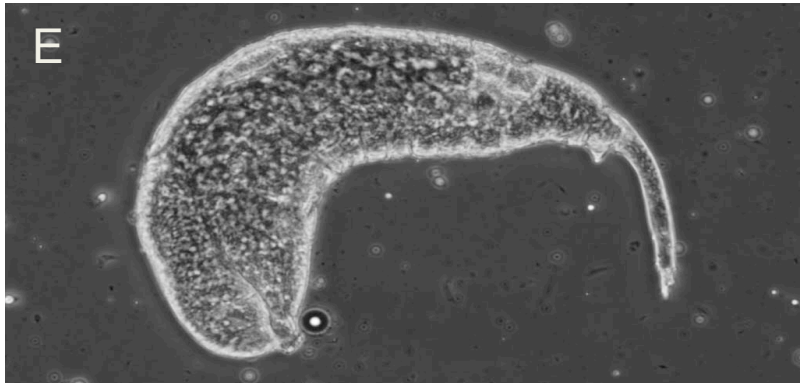
podocyte



pseudolamellocyte

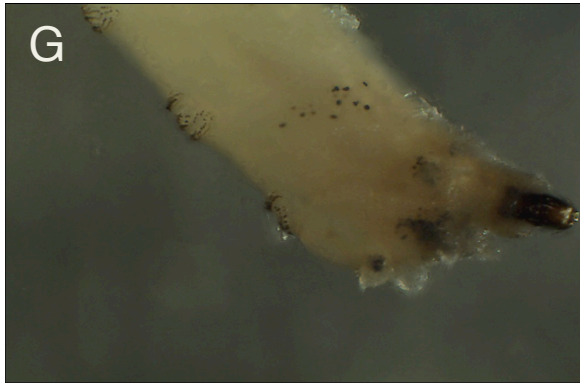


nematocyte



non-cellular mediated

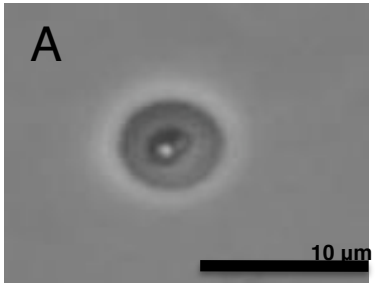
larva



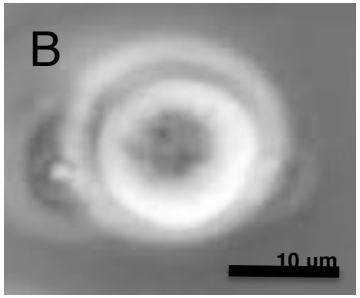
crystals

Figure 38

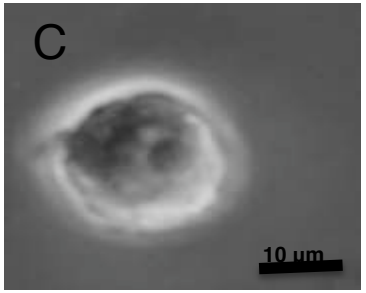
D. hydei



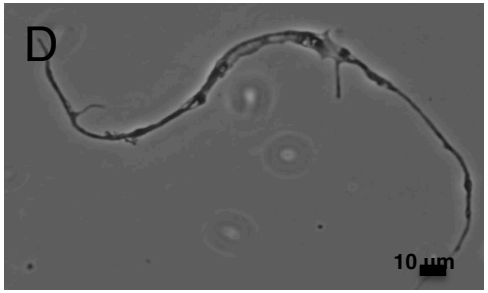
plasmatocyte



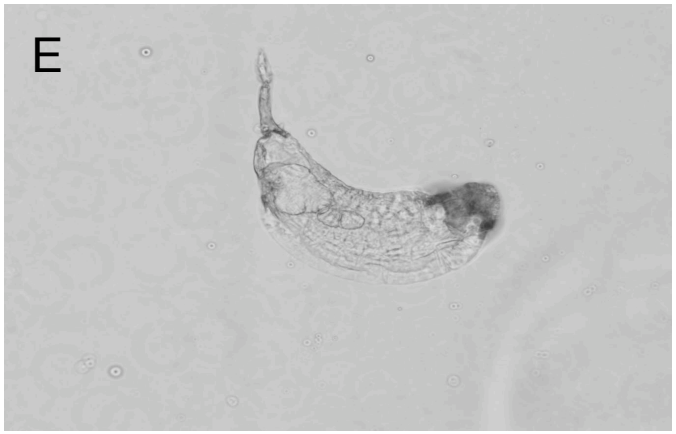
podocyte



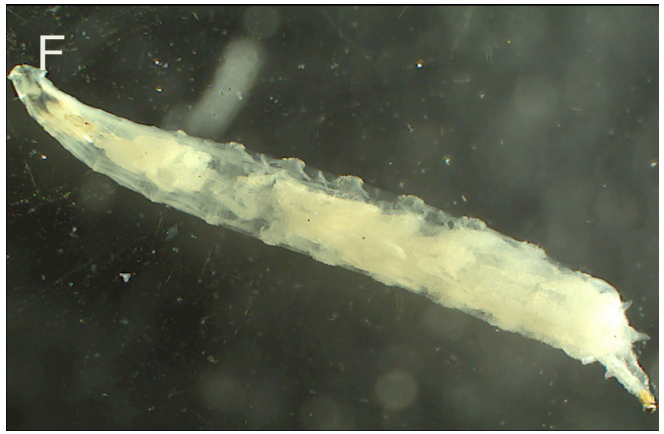
pseudolamellocyte



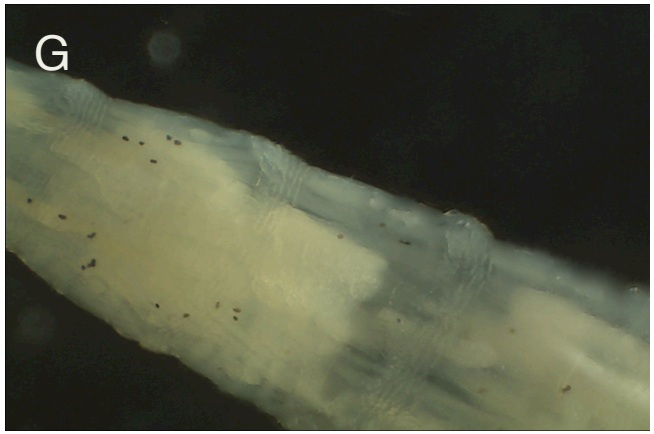
nematocyte



non-cellular mediated



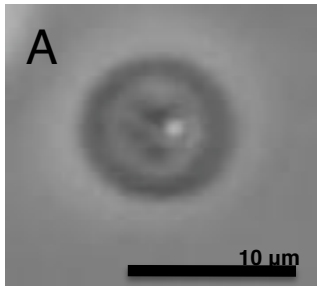
larva



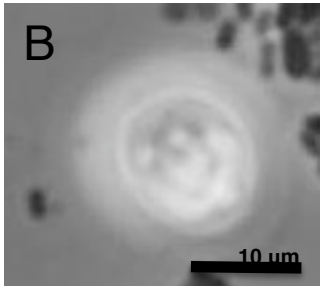
crystals

Figure 39

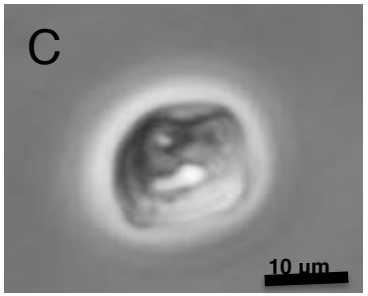
D. mojavensis



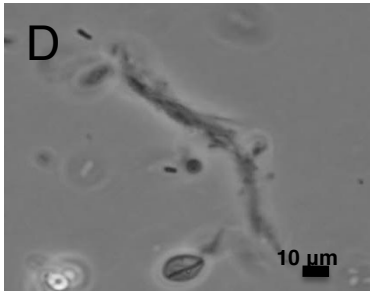
plasmatocyte



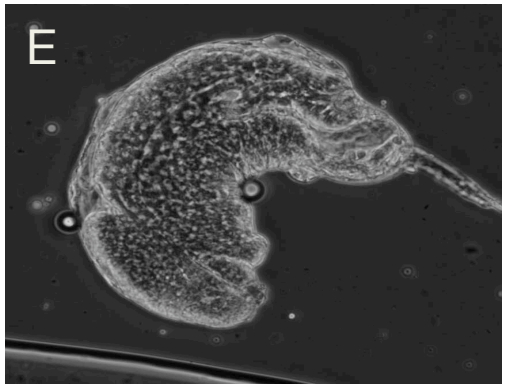
podocyte



pseudolamellocyte

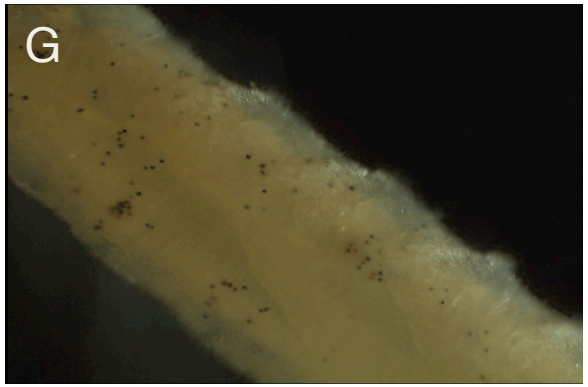


nematocyte



non-cellular mediated

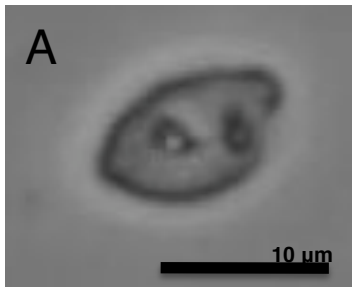
larva



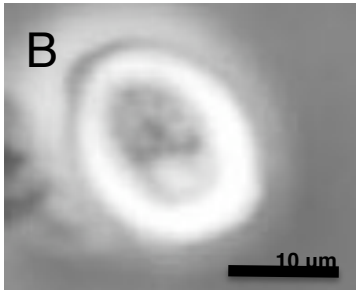
crystals

Figure 40

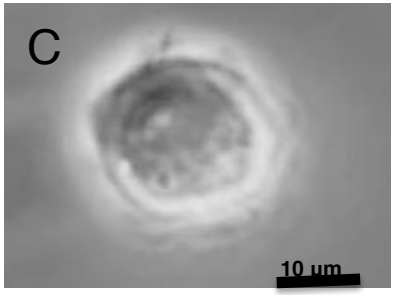
D. paramelanica



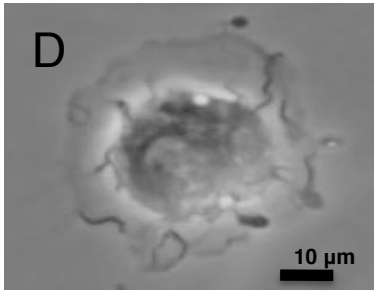
plasmatocyte



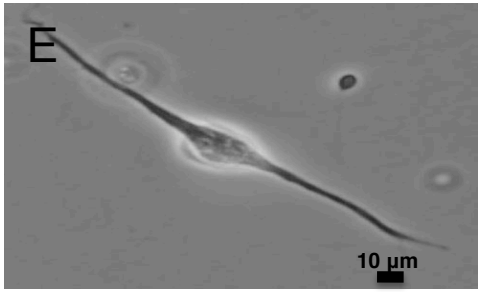
podocyte



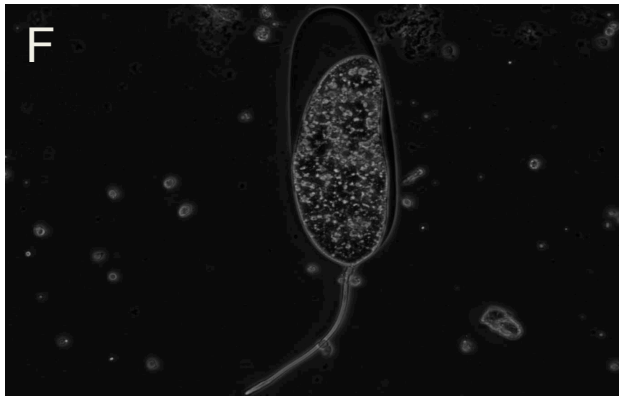
pseudolamellocyte



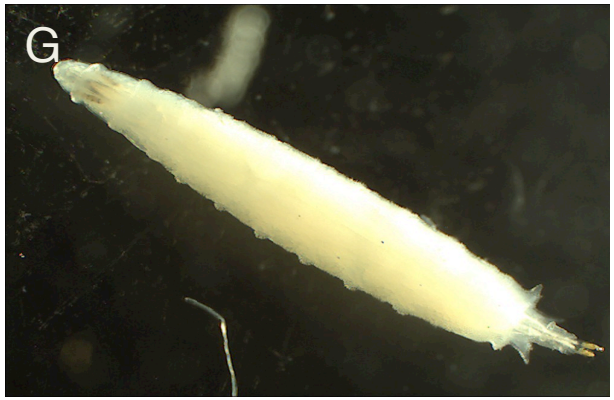
lamellocyte



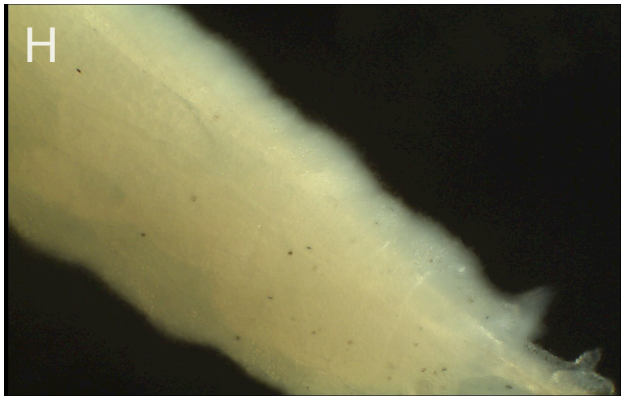
nematocyte



non-cellular mediated



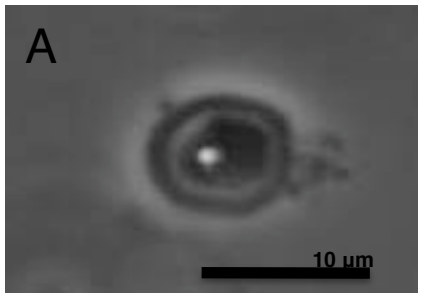
larva



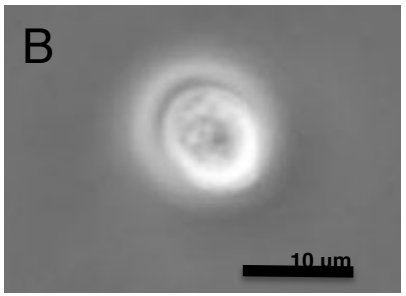
crystals

Figure 41

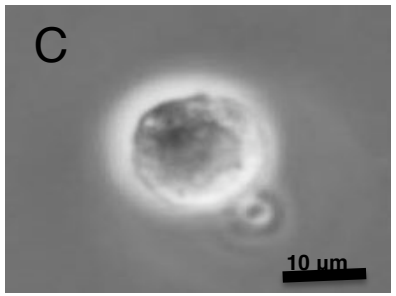
D. virilis



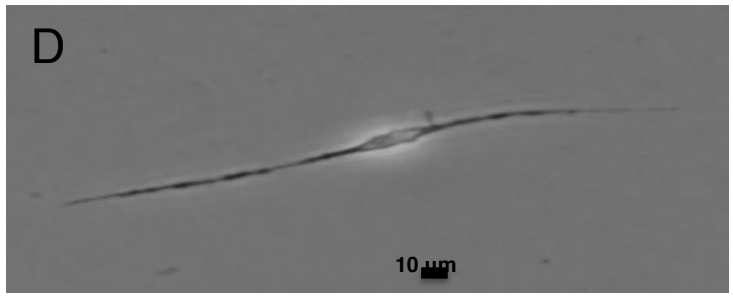
plasmatocyte



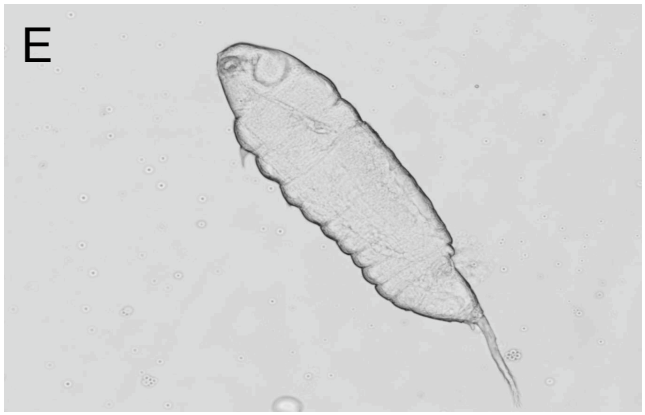
podocyte



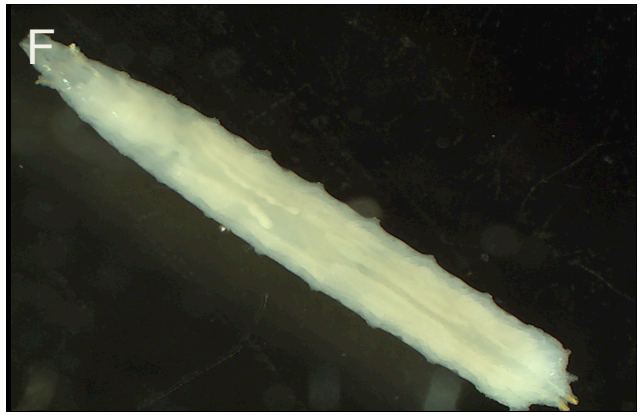
pseudolamellocyte



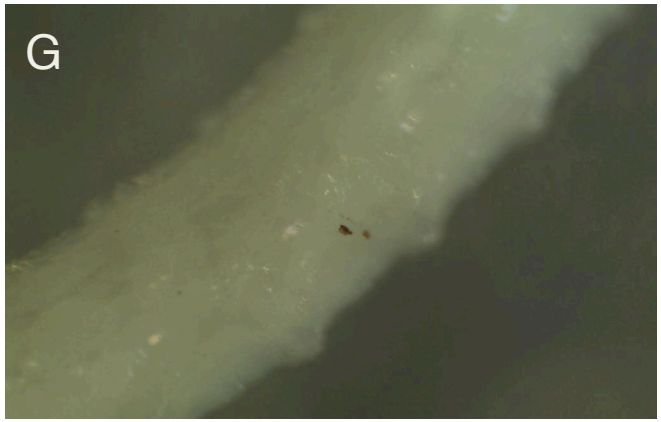
nematocyte



non-cellular mediated



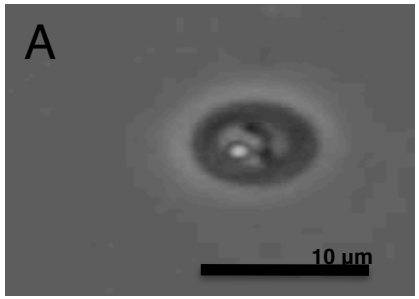
larva



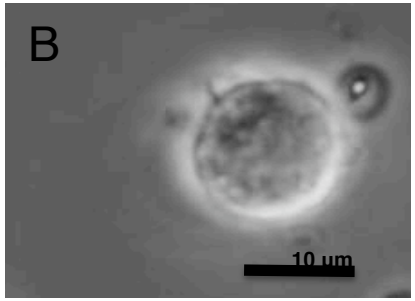
crystals

Figure 42

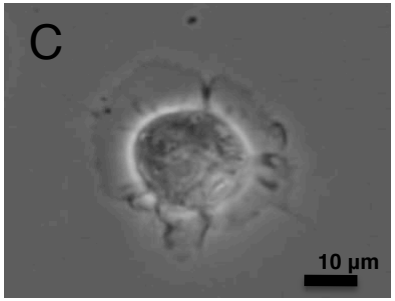
Z. indianus



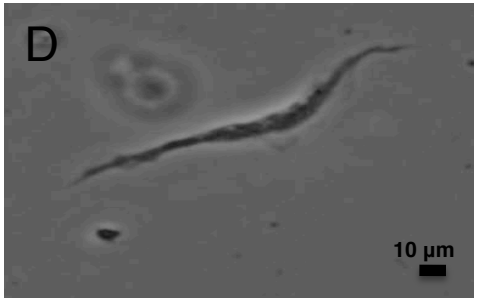
plasmatocyte



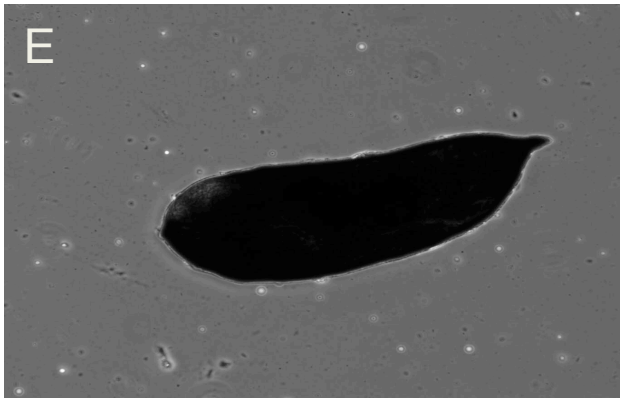
podocyte



lamellocyte

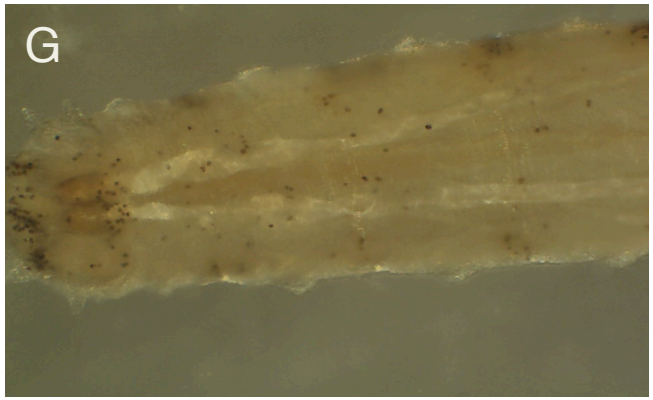


nematocyte



melanotic encapsulation

larva



crystals

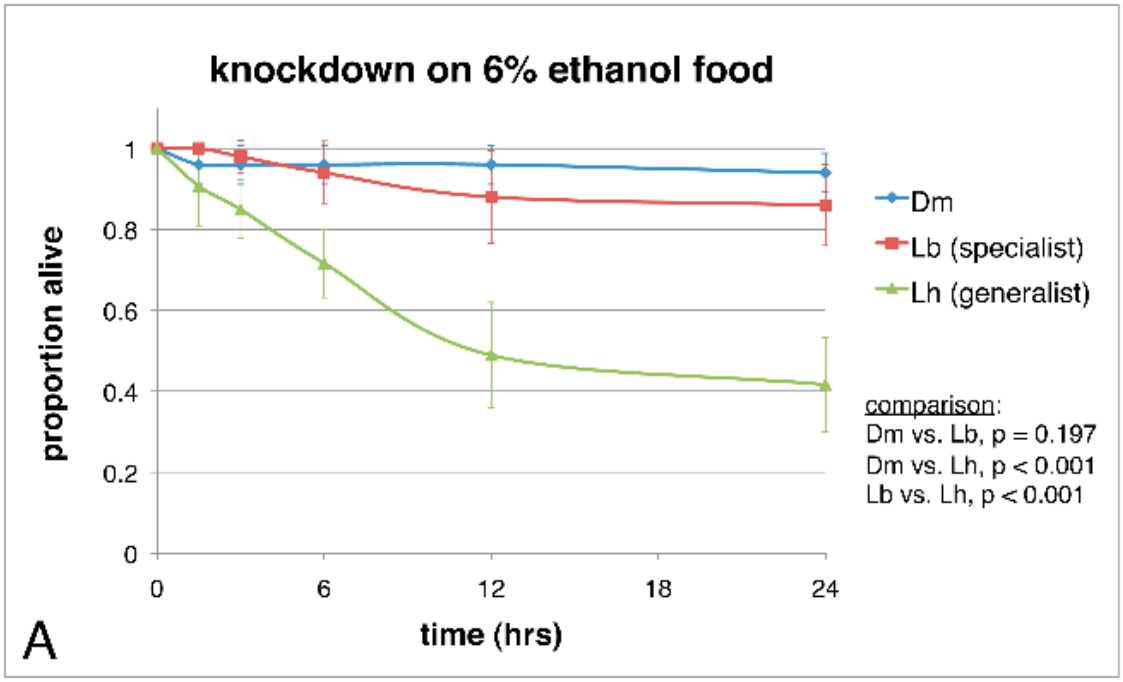
Chapter 2

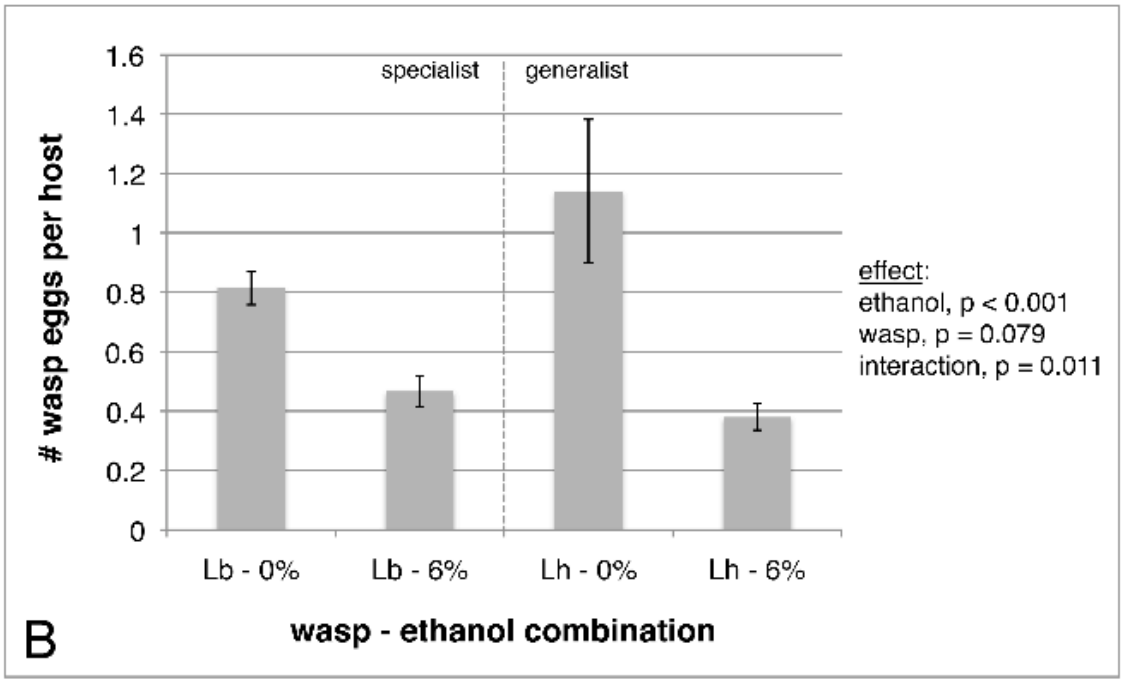
Alcohol Consumption As Self-Medication

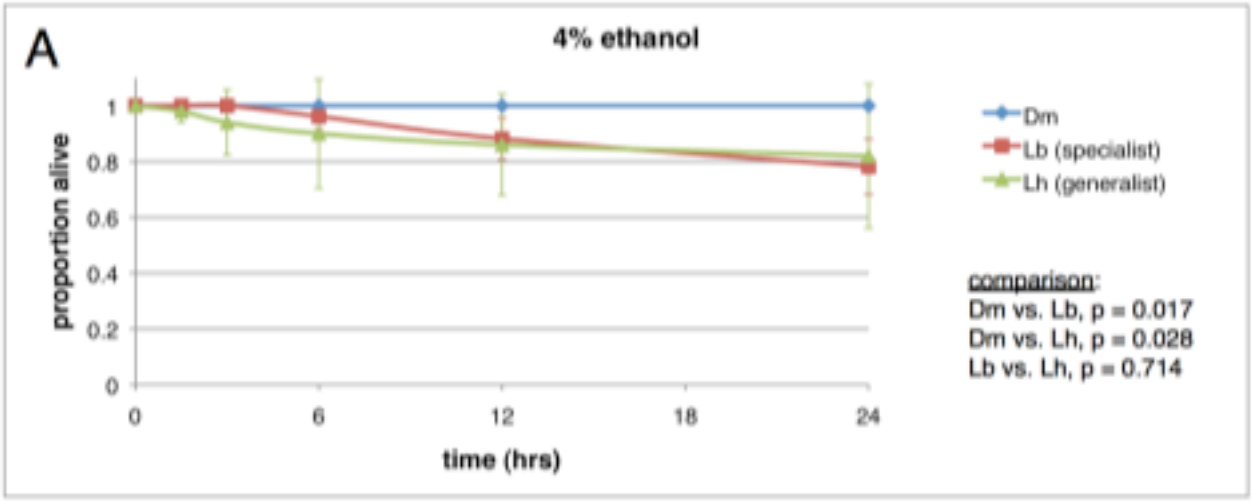
Against Blood-Borne Parasites

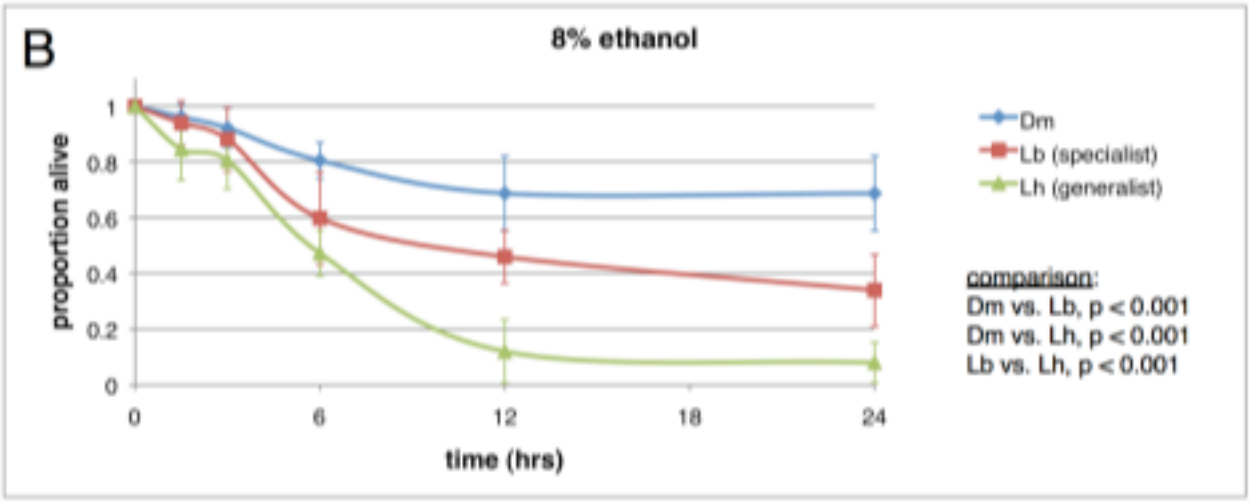
In The Fruitfly

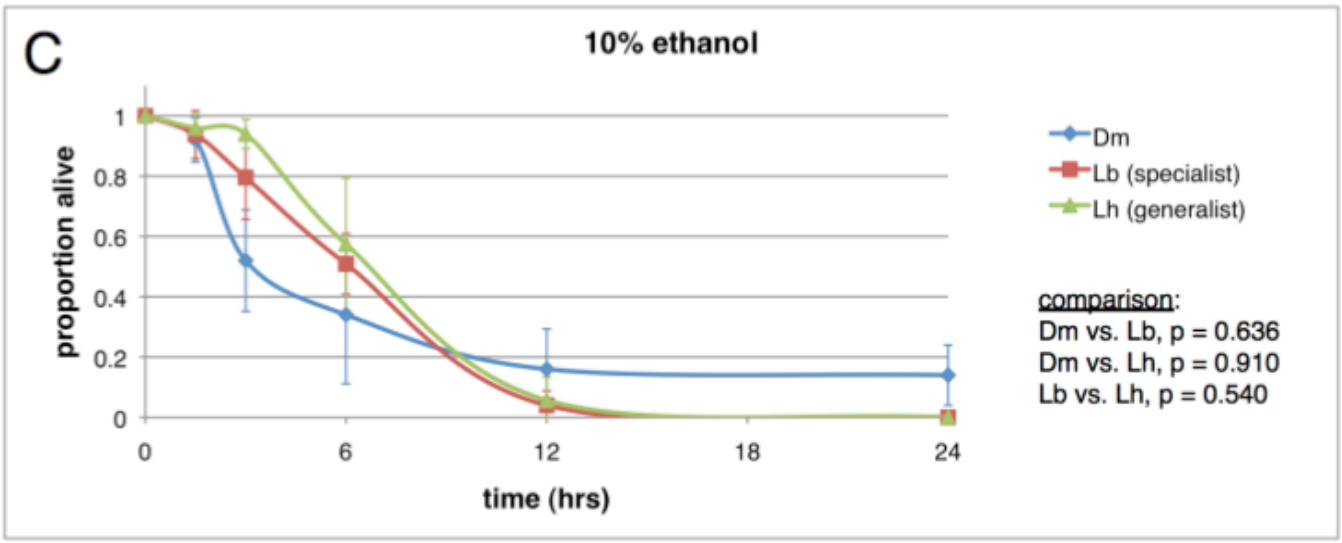
Figures

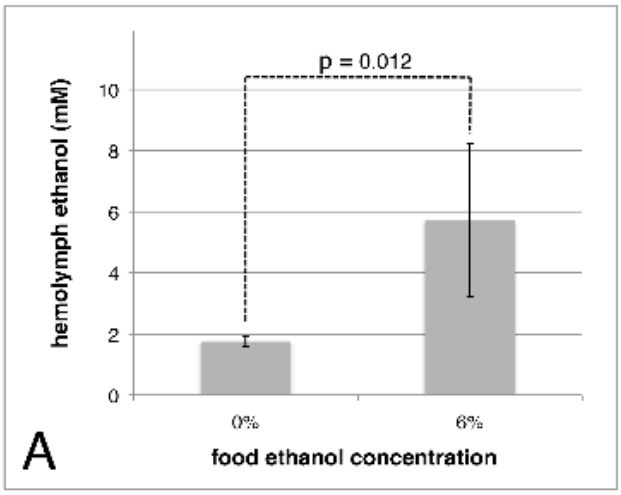

knockdown on 6% ethanol food
proportion alive
1
0.8
0.6
0.4
0.2
0
0
6
12
18
24
time (hrs)
Dm
Lb (specialist)
Lh (generalist)
comparison:
Dm vs. Lb, p = 0.197
Dm vs. Lh, p < 0.001
Lb vs. Lh, p < 0.001
A

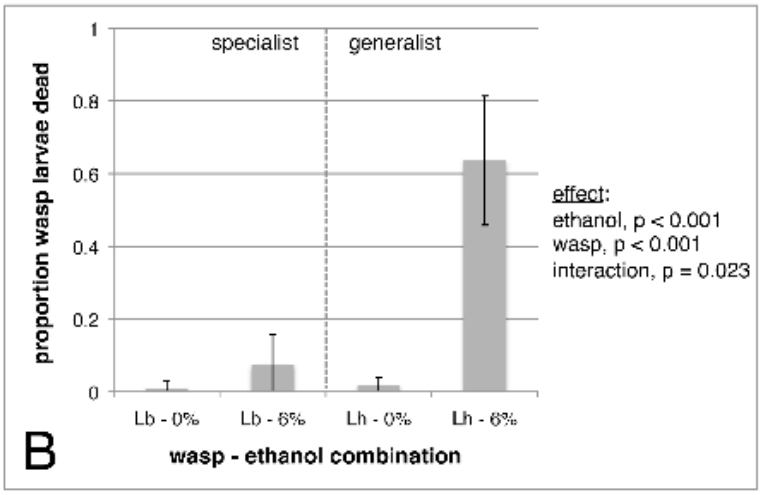

wasp eggs per host
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
specialist
generalist
Lb - 0%
Lb - 6%
Lh - 0%
Lh - 6%
wasp - ethanol combination
effect:
ethanol, p < 0.001
wasp, p = 0.079
interaction, p = 0.011
B

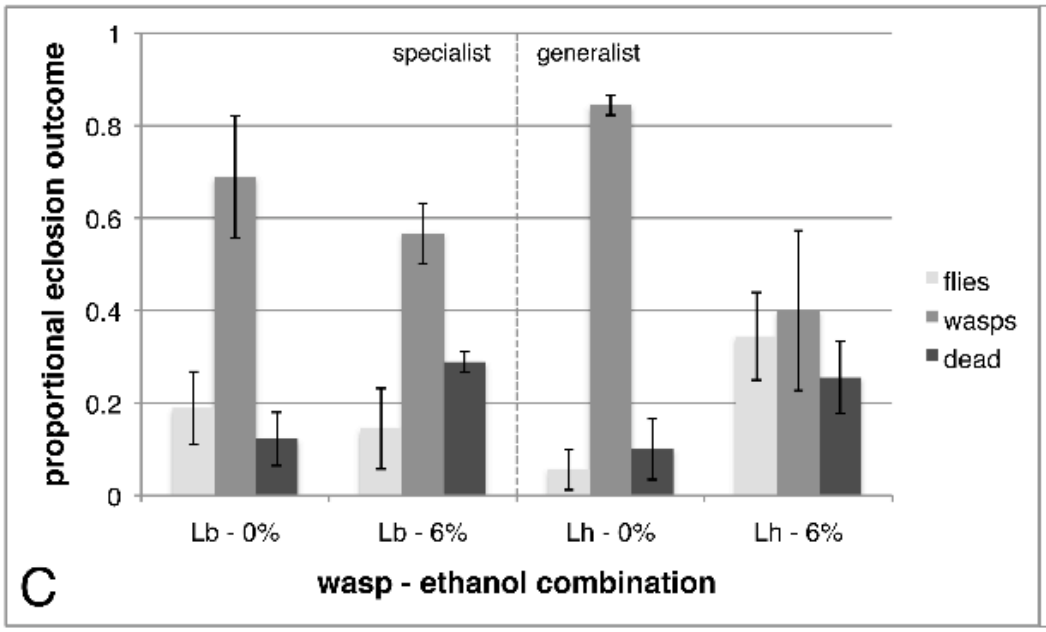

A
4% ethanol
proportion alive
1
0.8
0.6
0.4
0.2
0
0
6
12
18
24
time (hrs)
Dm
Lb (specialist)
Lh (generalist)
comparison:
Dm vs. Lb, p = 0.017
Dm vs. Lh, p = 0.028
Lb vs. Lh, p = 0.714


B
8% ethanol
proportion alive
1
0.8
0.6
0.4
0.2
0
0
6
12
18
24
time (hrs)
Dm
Lb (specialist)
Lh (generalist)
comparison:
Dm vs. Lb, p < 0.001
Dm vs. Lh, p < 0.001
Lb vs. Lh, p < 0.001


C
10% ethanol
proportion alive
1
0.8
0.6
0.4
0.2
0
0
6
12
18
24
time (hrs)
Dm
Lb (specialist)
Lh (generalist)
comparison:
Dm vs. Lb, p = 0.636
Dm vs. Lh, p = 0.910
Lb vs. Lh, p = 0.540



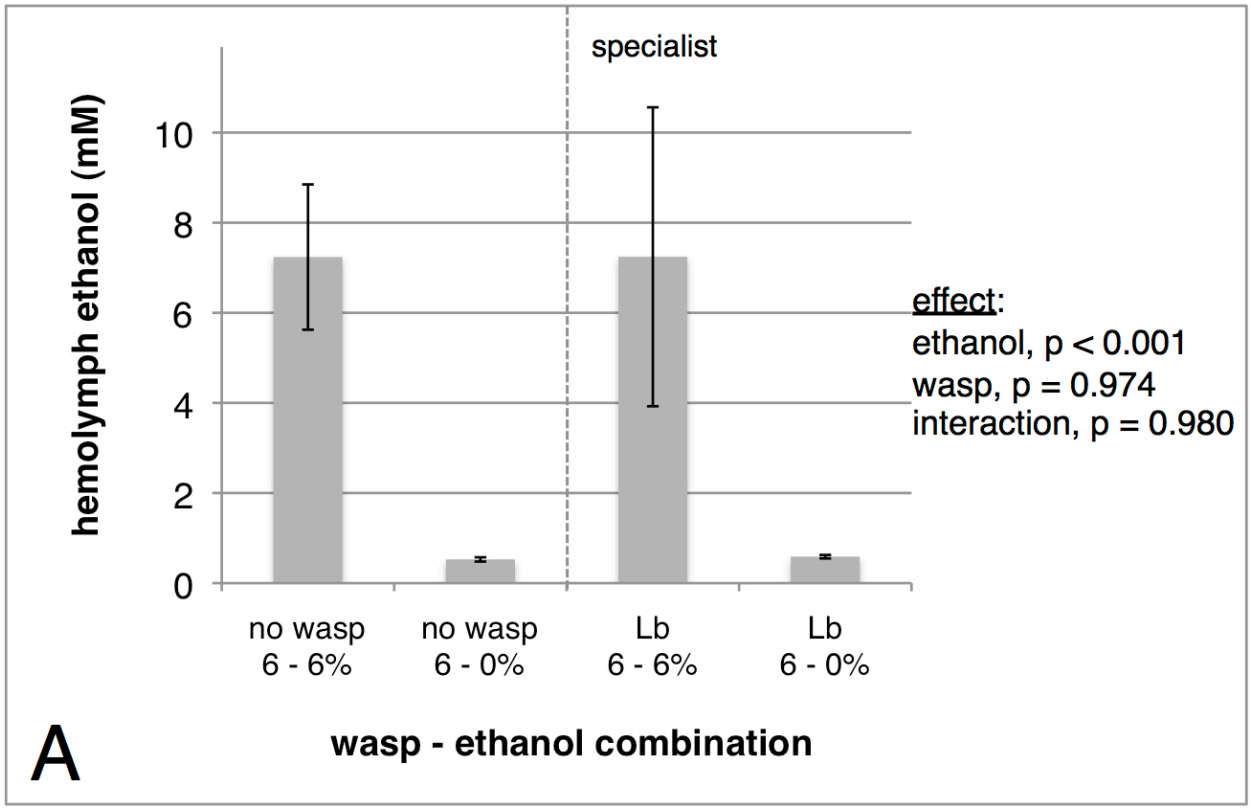


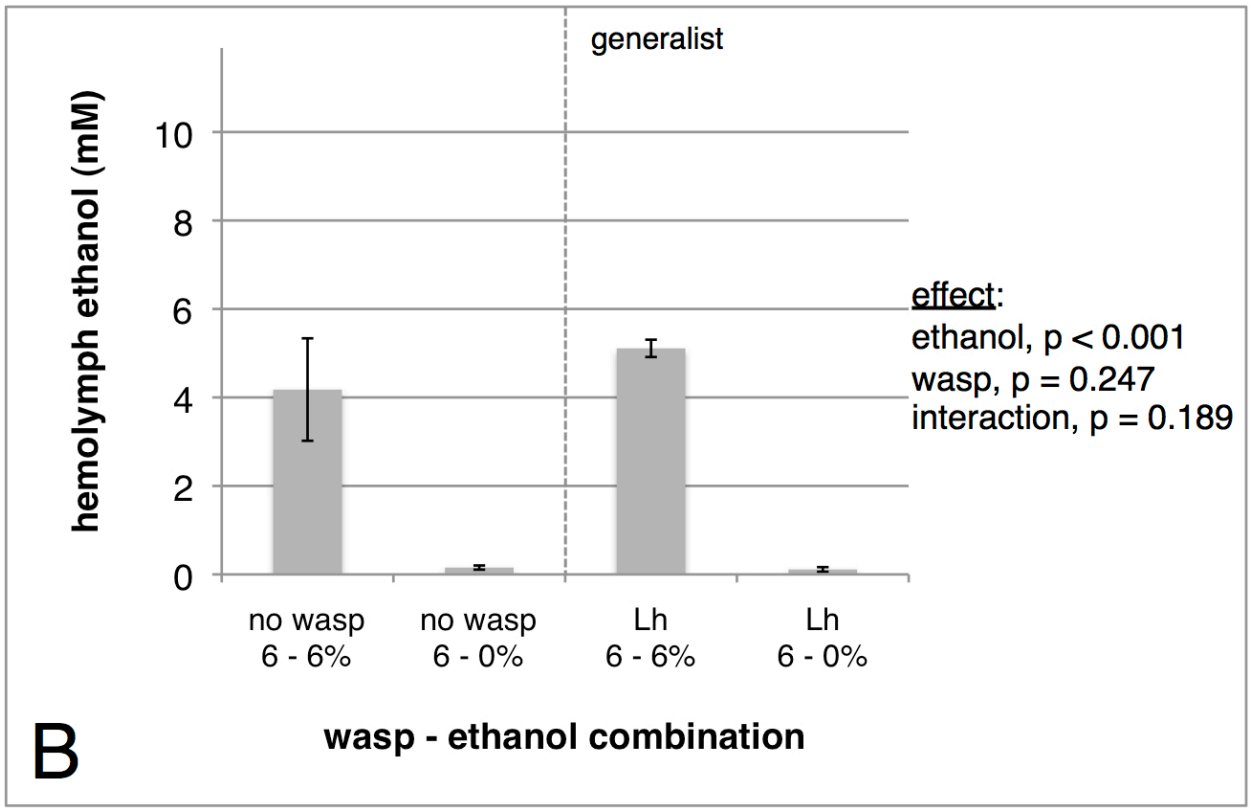


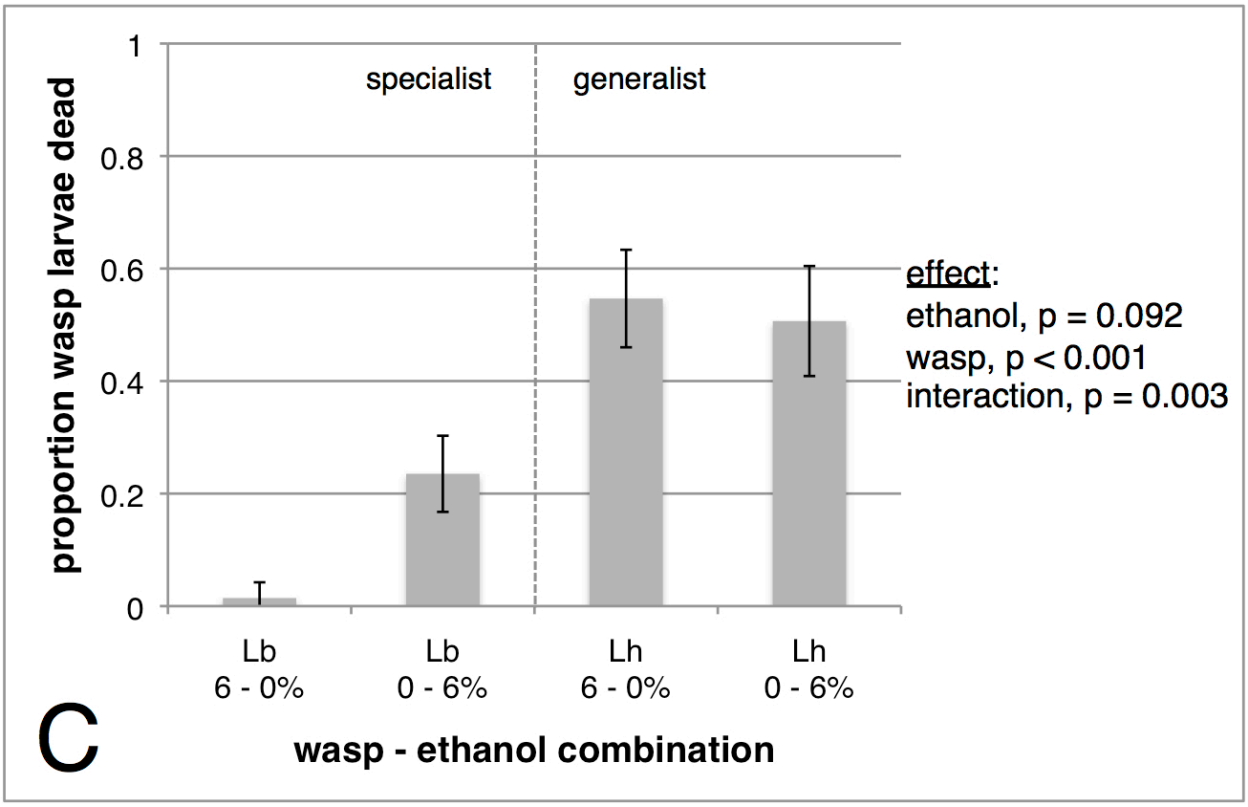
effect on fly eclosion:
ethanol, p = 0.008
wasp, p = 0.461
interaction, p < 0.001

effect on wasp eclosion:
ethanol, p < 0.001
wasp, p = 0.947
interaction, p = 0.046

effect on death:
ethanol, p < 0.001
wasp, p = 0.548
interaction, p = 0.884


specialist
hemolymph ethanol (mM)
10
8
6
4
2
0
no wasp 6 - 6%
no wasp 6 - 0%
Lb 6 - 6%
Lb 6 - 0%
effect:
ethanol, p < 0.001
wasp, p = 0.974
interaction, p = 0.980
A
wasp - ethanol combination


generalist
hemolymph ethanol (mM)
10
8
6
4
2
0
no wasp 6 - 6%
no wasp 6 - 0%
Lh 6 - 6%
Lh 6 - 0%
effect:
ethanol, p < 0.001
wasp, p = 0.247
interaction, p = 0.189
B
wasp - ethanol combination


proportion wasp larvae dead
1
0.8
0.6
0.4
0.2
0
specialist
generalist
Lb
6 - 0%
Lb
0 - 6%
Lh
6 - 0%
Lh
0 - 6%
wasp - ethanol combination
effect:
ethanol, p = 0.092
wasp, p < 0.001
interaction, p = 0.003
C


D
0.1 µm

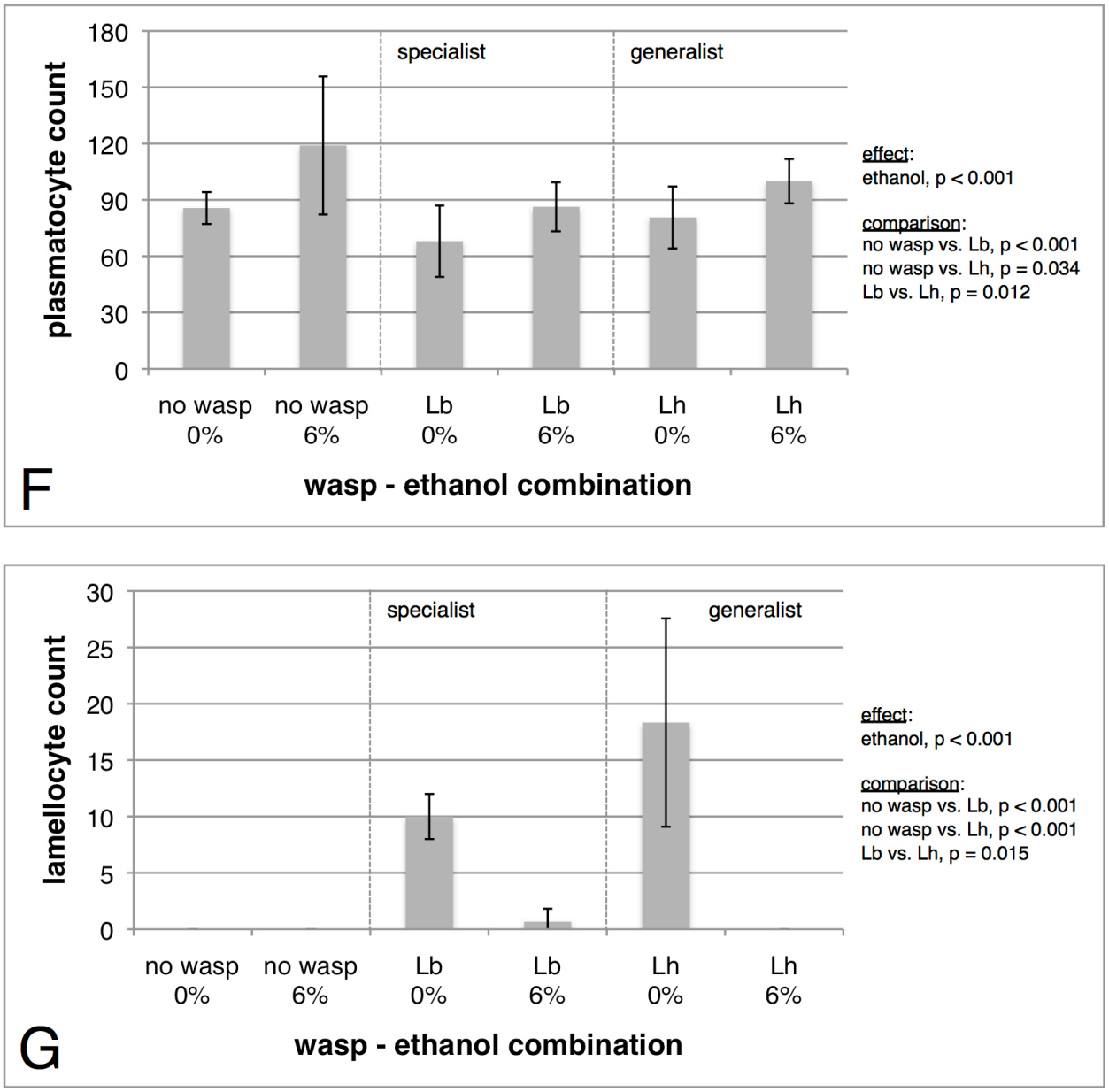
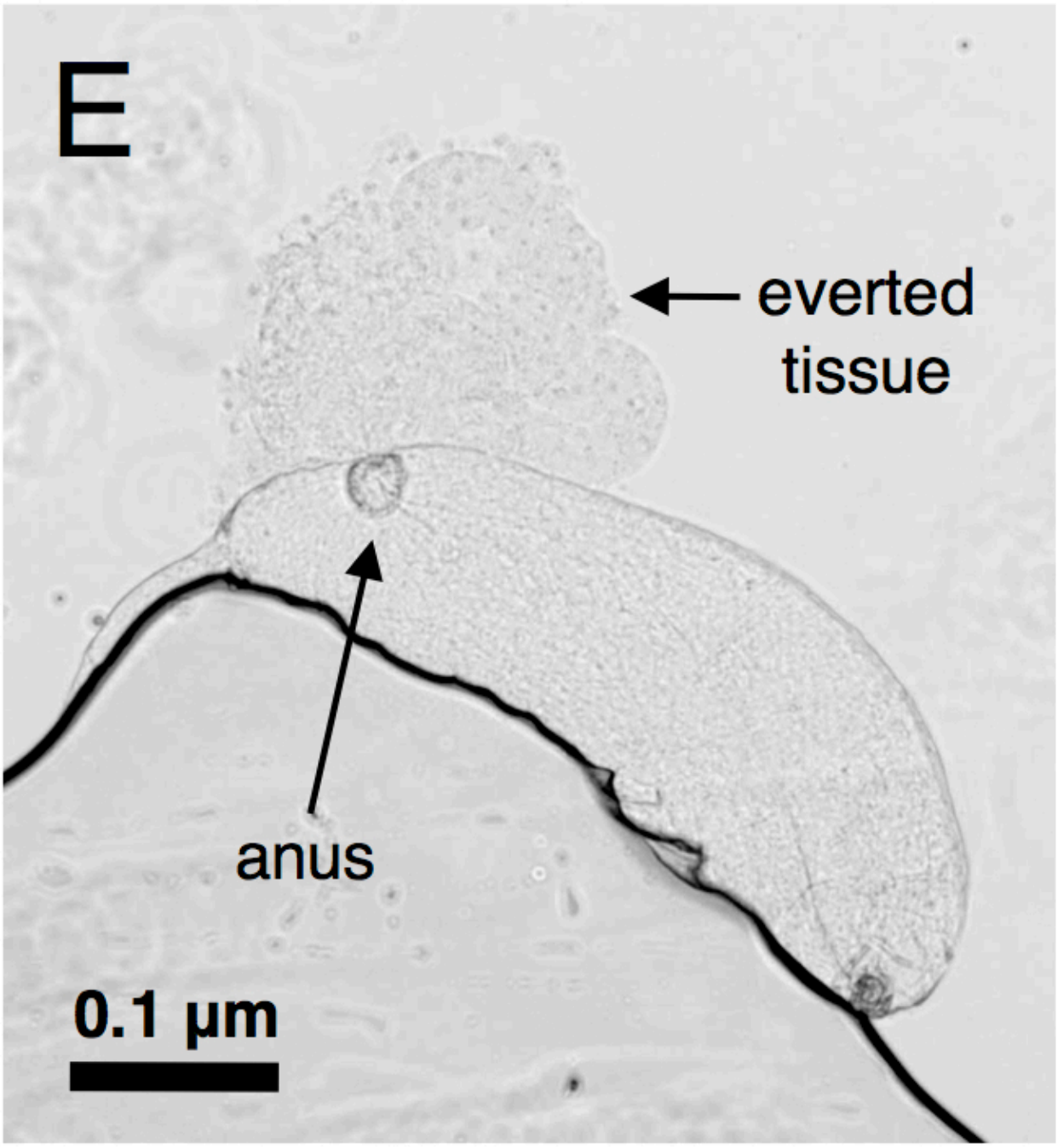
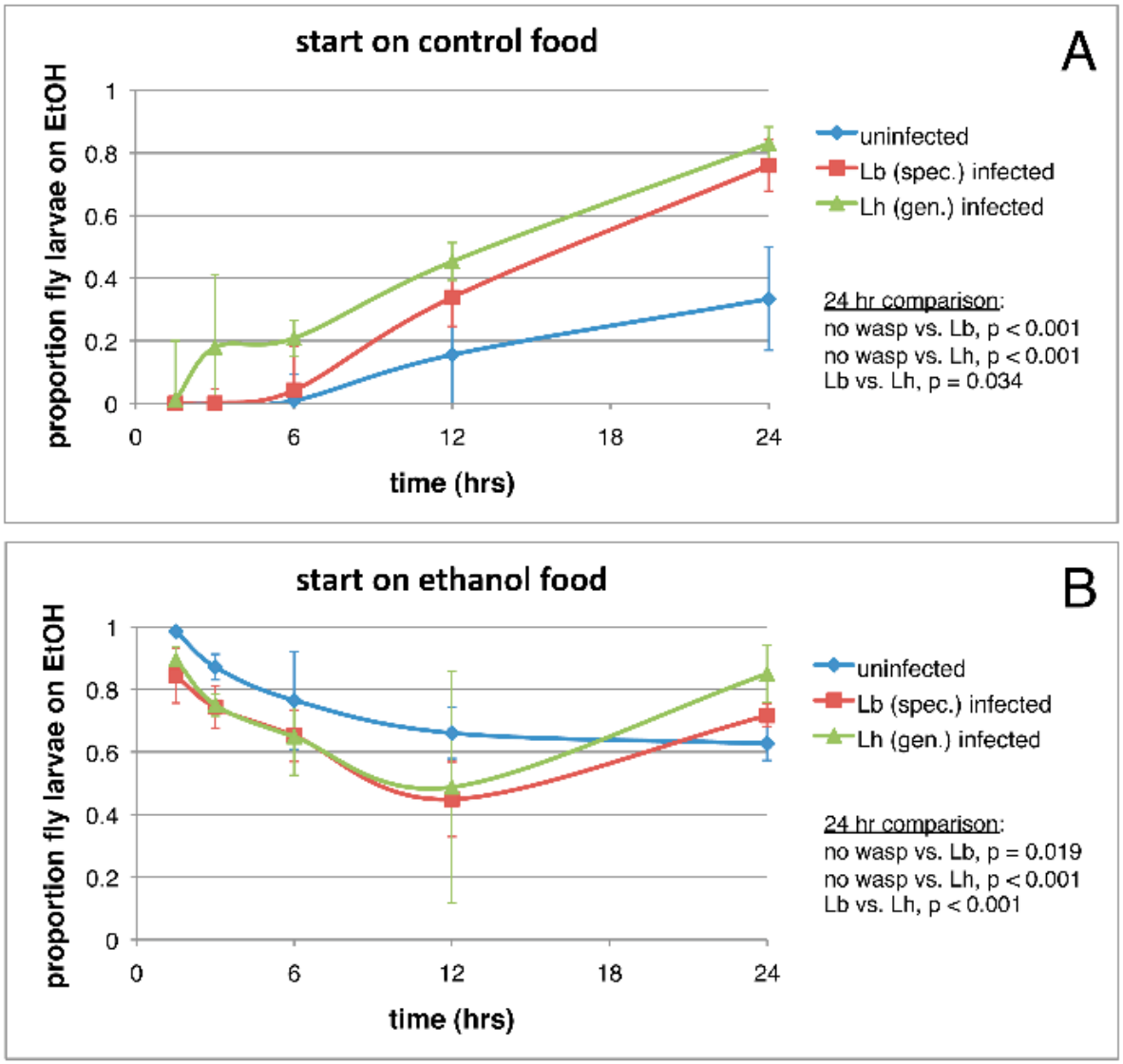
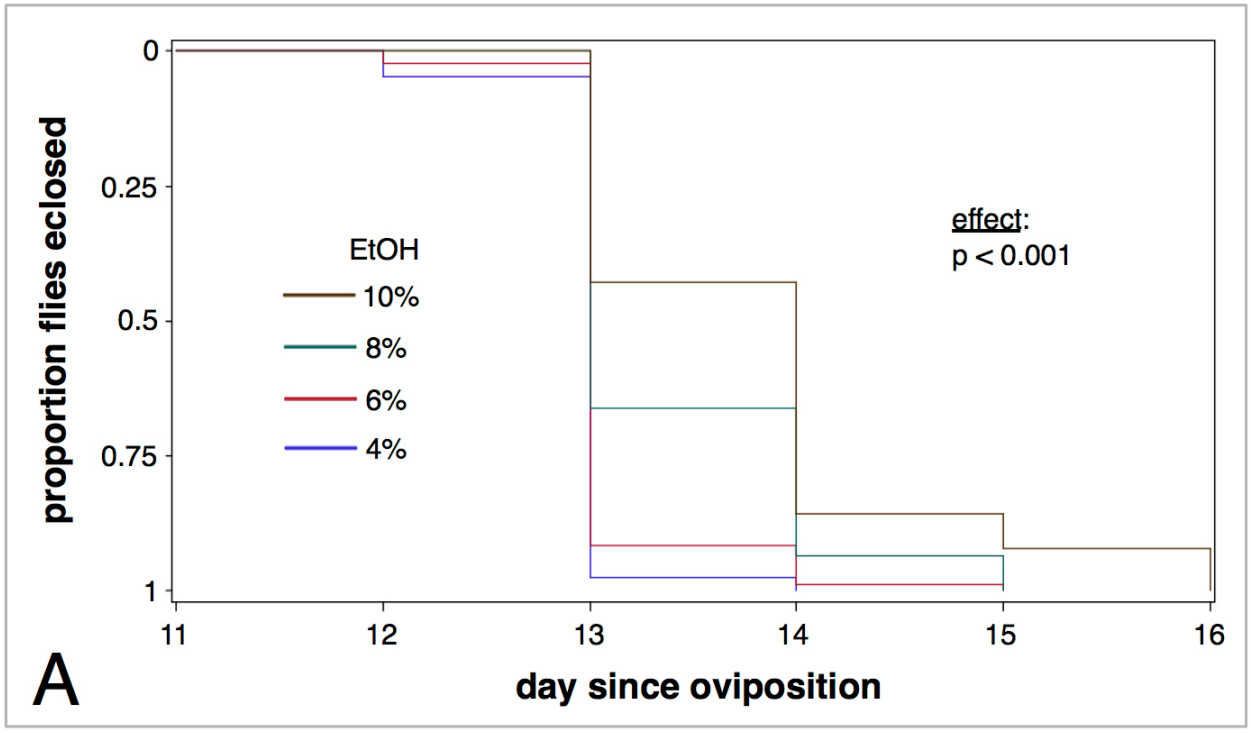


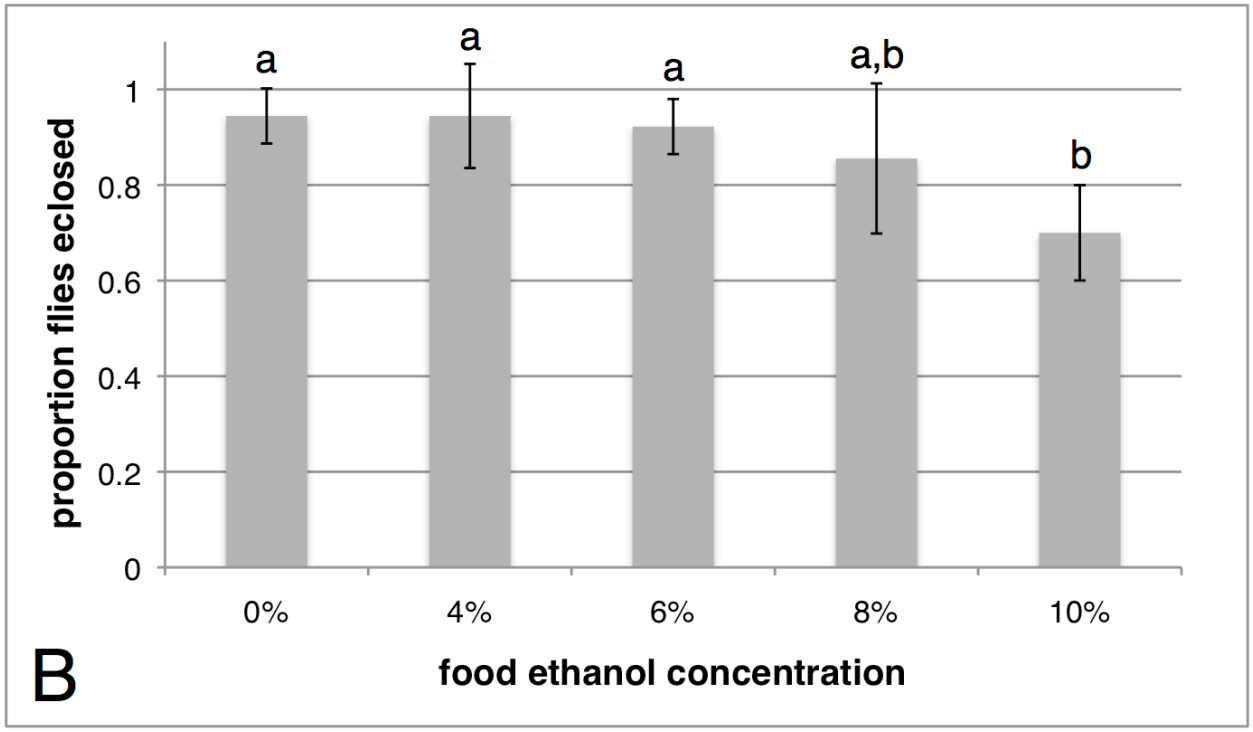
Figure S3, related to Figure 3

The effect of ethanol on fly oviposition and development. The proportion of flies eclosed over time (A) was used as a measure of the fly developmental rate. The total proportion of flies that eclosed (B) was used as a measure of fly survival. Error bars represent 95% confidence intervals across three dish replicates. Significance groups are indicated by lower case letters.


E
everted
tissue
anus
0.1 µm


start on control food
A
proportion fly larvae on EtOH
1
0.8
0.6
0.4
0.2
0
0
6
12
18
24
time (hrs)
uninfected
Lb (spec.) infected
Lh (gen.) infected
24 hr comparison:
no wasp vs. Lb, p < 0.001
no wasp vs. Lh, p < 0.001
Lb vs. Lh, p = 0.034
start on ethanol food
B
proportion fly larvae on EtOH
1
0.8
0.6
0.4
0.2
0
0
6
12
18
24
time (hrs)
uninfected
Lb (spec.) infected
Lh (gen.) infected
24 hr comparison:
no wasp vs. Lb, p = 0.019
no wasp vs. Lh, p < 0.001
Lb vs. Lh, p < 0.001

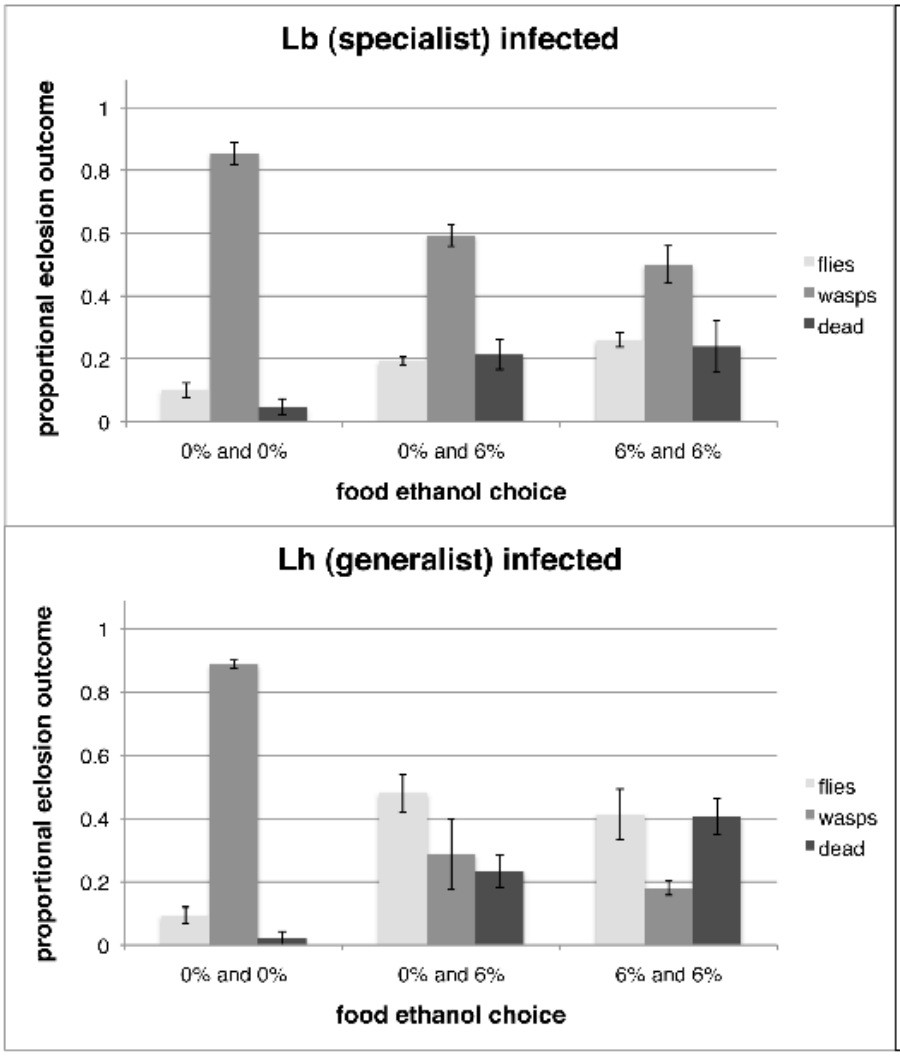




Experimental Procedures

Adult ethanol resistance

We used ACS/USP grade 95% ethanol (#111000190, Pharmco-AAPER), which contains less than 0.001% methanol, for all experiments. Batches of ten female flies and wasps were collected 3-5 days post-eclosion and aspirated into food dishes containing 0, 4, 6, 8, and



effect on fly eclosion:
wasp, $p < 0.001$

comparison:
0+0 vs. 0+6, $p < 0.001$
0+0 vs. 6+6, $p < 0.001$
0+6 vs. 6+6, $p = 1.000$

effect on wasp eclosion:
wasp, $p < 0.001$

comparison:
0+0 vs. 0+6, $p < 0.001$
0+0 vs. 6+6, $p < 0.001$
0+6 vs. 6+6, $p = 0.010$

effect on death:
wasp, $p = 0.034$

comparison:
0+0 vs. 0+6, $p < 0.001$
0+0 vs. 6+6, $p < 0.001$
0+6 vs. 6+6, $p = 0.006$

Chapter 3

High Hemocyte Load Is Associated With Increased Resistance Against Parasitoids in *Drosophila suzukii*, A Relative of *D. melanogaster*

Figures

Figure 1

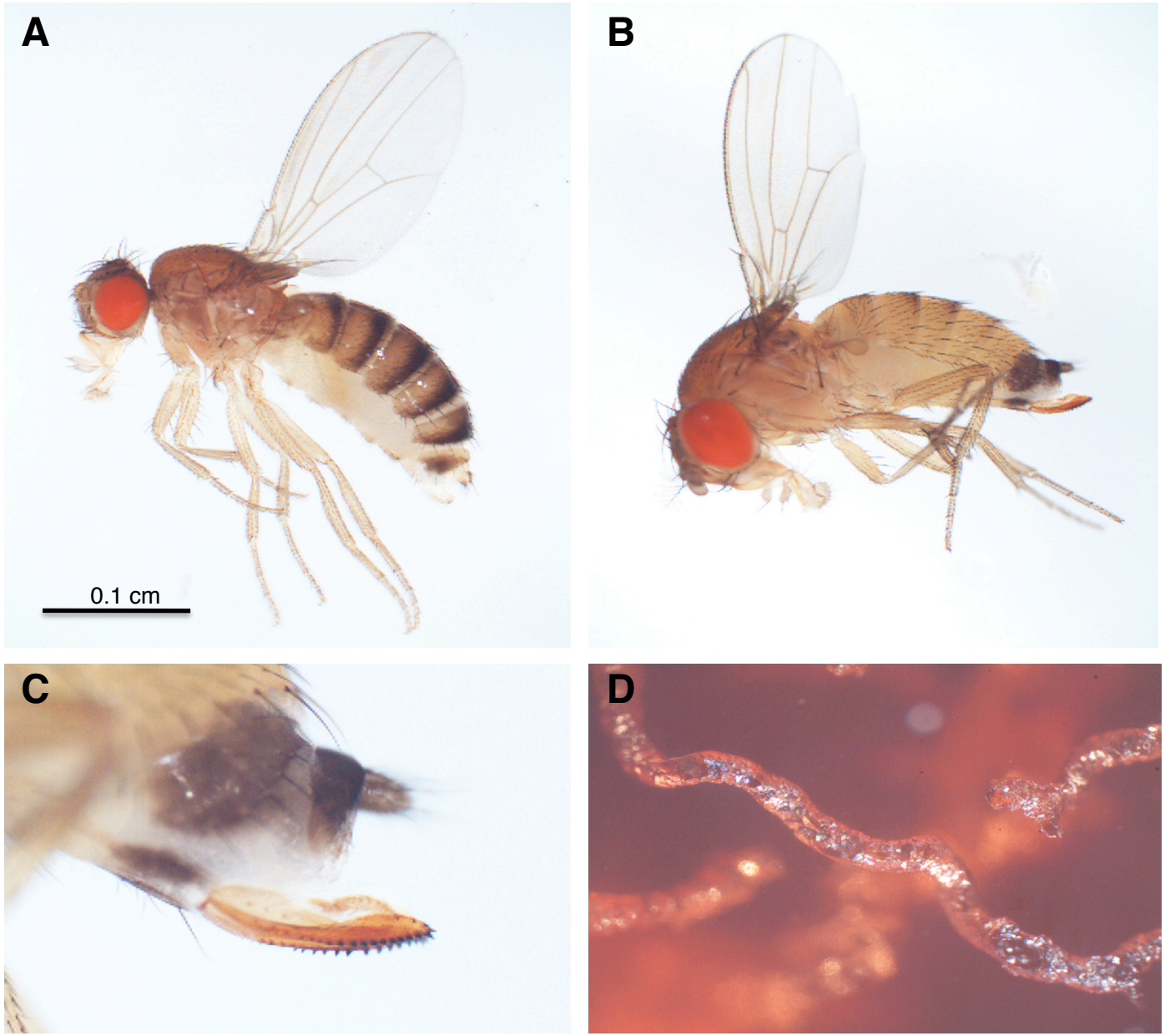
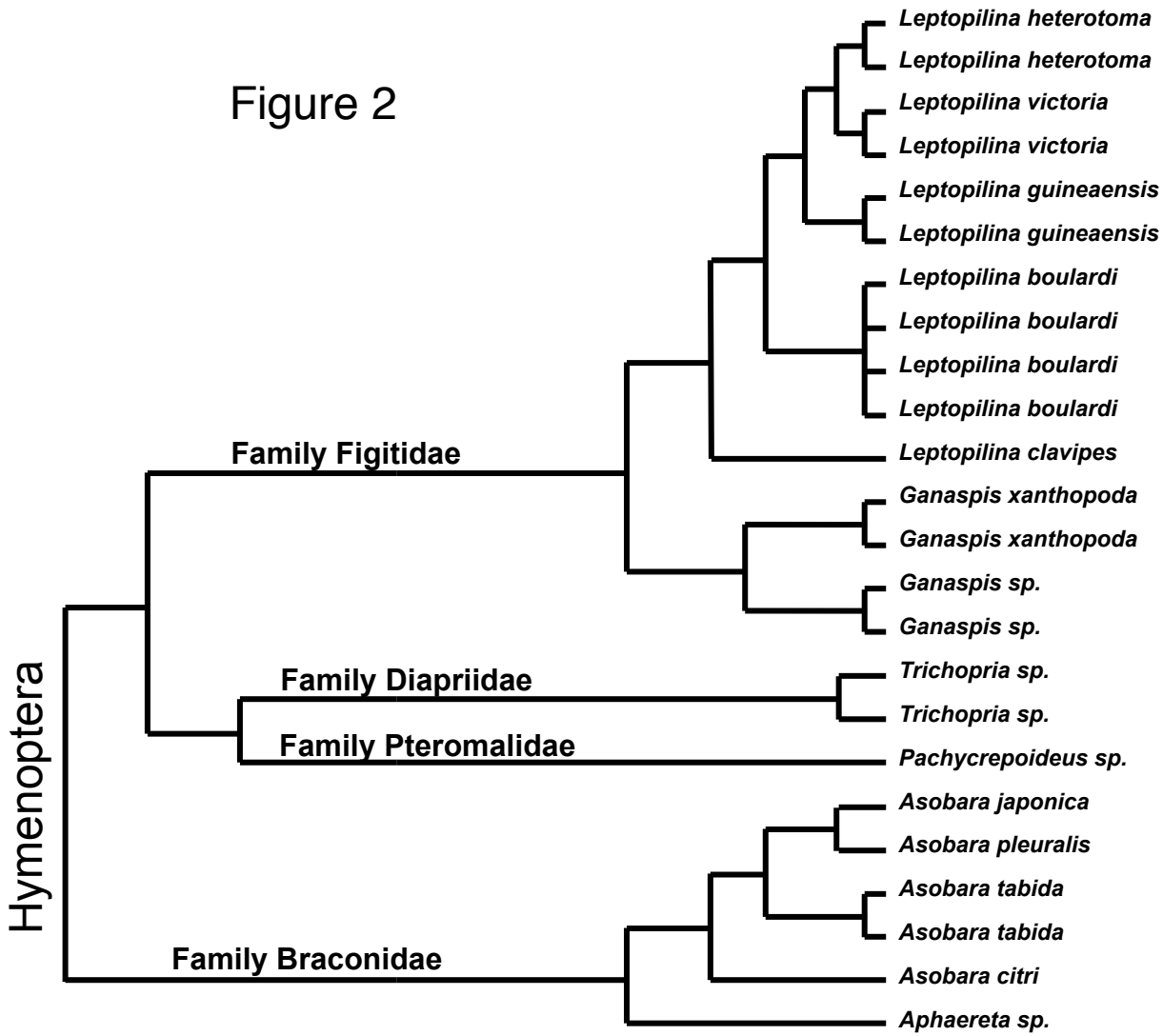


Figure 2



Strain	Place Collected	Date Collected
Lh14	Winters, California	2002
LhSw	Uppsala, Sweden	2007
LvHaw	Kaimuki, Oahu, Hawaii	2009
LvPhil	Consolacion, Cebu, Philippines	2007
LgG500	Yaounde, Cameroon	1998
LgG510	False Bay, South Africa	1999
LbFr	Marseilles, France	2007
LbG486	Brazzaville, Congo	1978
LbKen	Nairobi, Kenya	2009
Lb17	Winters, California	2002
Lclav	Heerenbergh, Netherlands	2000
GxHaw	Kaimuki, Oahu, Hawaii	2009
GxUg	Masindi, Uganda	2010
GFl	Homestead, Florida	2008
GHaw	Kaimuki, Oahu, Hawaii	2009
TriCal	Winters, California	2002
TriFr	Marseilles, France	2007
Pachy	Atlanta, Georgia	2008
Ajap	Tokyo, Japan	1995
Apleu	Manado, Sulawesi, Indonesia	2005
AtFr	Sospel, France	>20 years ago
AtSw	Uppsala, Sweden	2007
Acit	Lamto, Ivory Coast	1995
Aphae	Atlanta, Georgia	2009

Figure 3

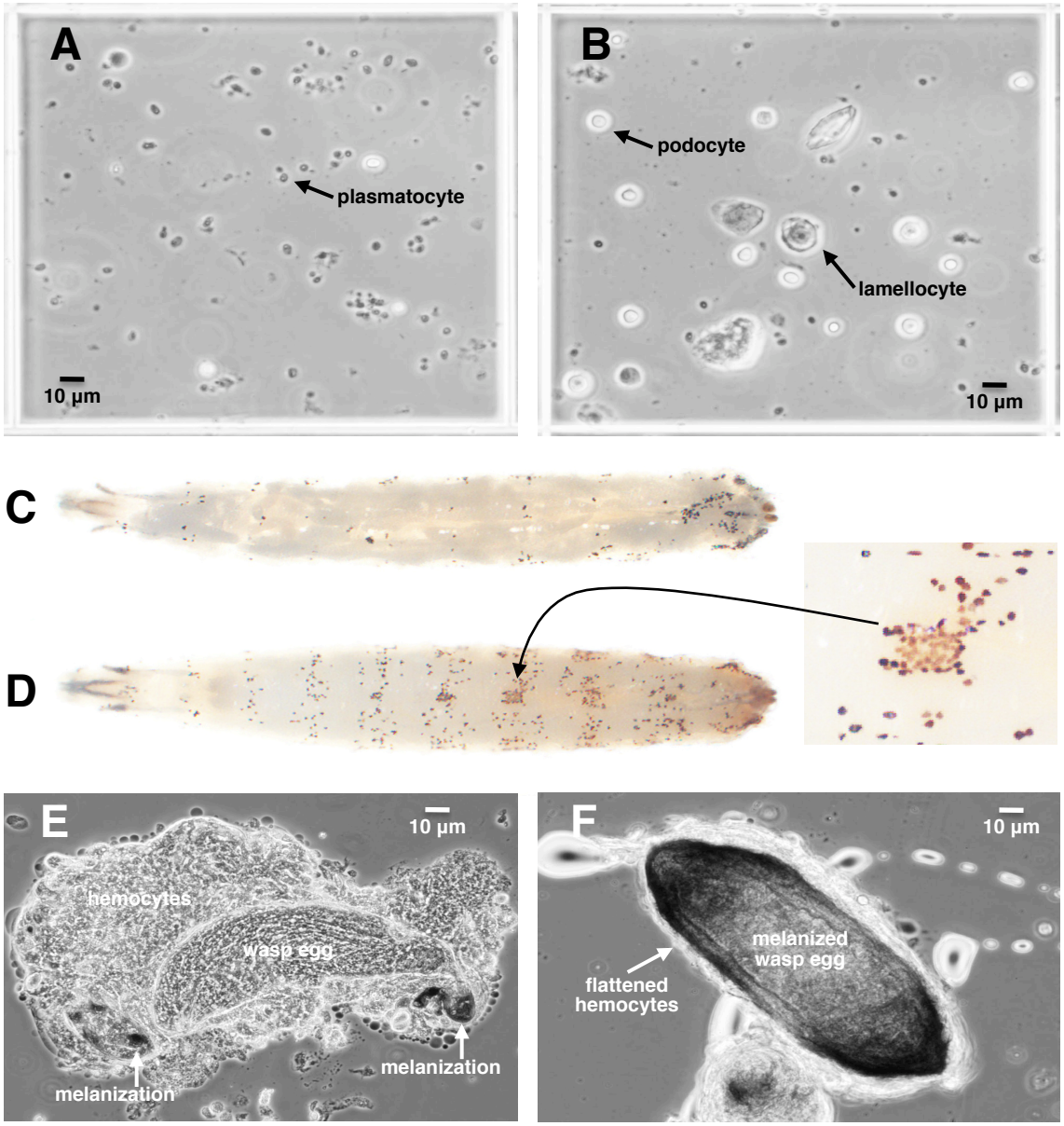
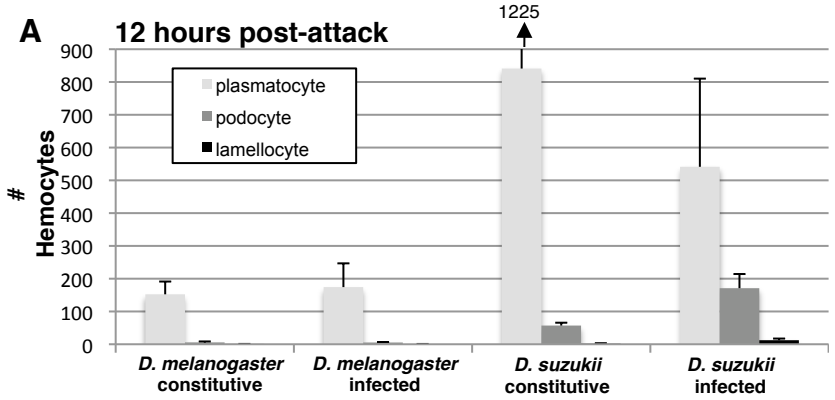

A
plasmatocyte
10 µm
B
podocyte
lamellocyte
10 µm
C
D
E
10 µm
hemocytes
wasp egg
melanization
melanization
F
10 µm
melanized
wasp egg
flattened
hemocytes

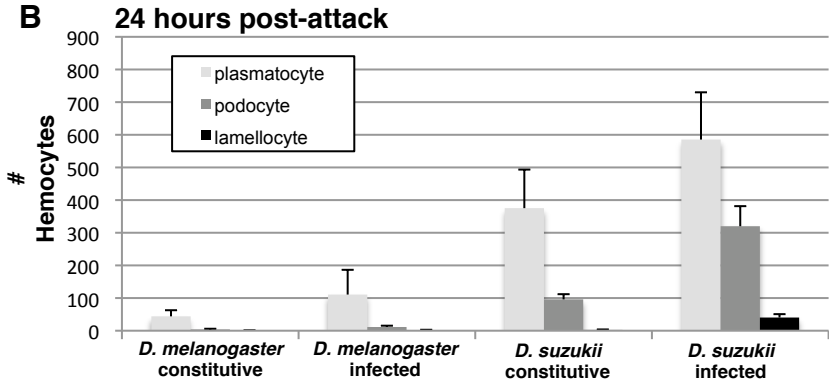
Figure 4



effect on plasmatocytes:
species, $p < 0.001$
infection, $p = 0.443$
interaction, $p = 0.142$

effect on podocytes:
species, $p < 0.001$
infection, $p = 0.132$
interaction, $p = 0.004$

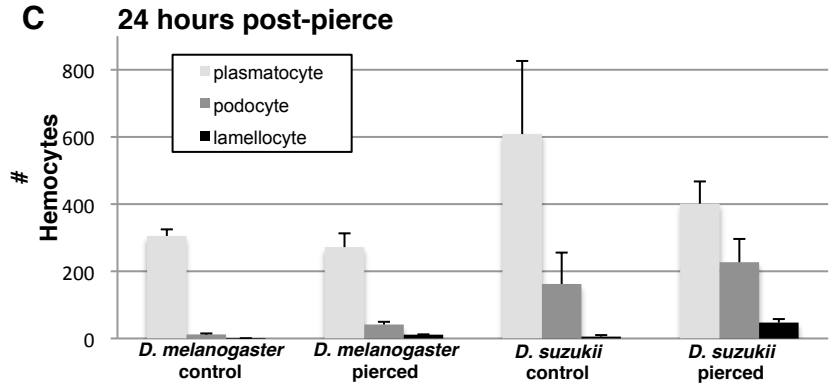
effect on lamellocytes:
species, $p < 0.001$
infection, $p = 0.034$
interaction, $p = 0.014$



effect on plasmatocytes:
species, $p < 0.001$
infection, $p = 0.335$
interaction, $p = 0.250$

effect on podocytes:
species, $p < 0.001$
infection, $p = 0.107$
interaction, $p = 0.664$

effect on lamellocytes:
species, $p < 0.001$
infection, $p < 0.001$
interaction, $p = 0.025$

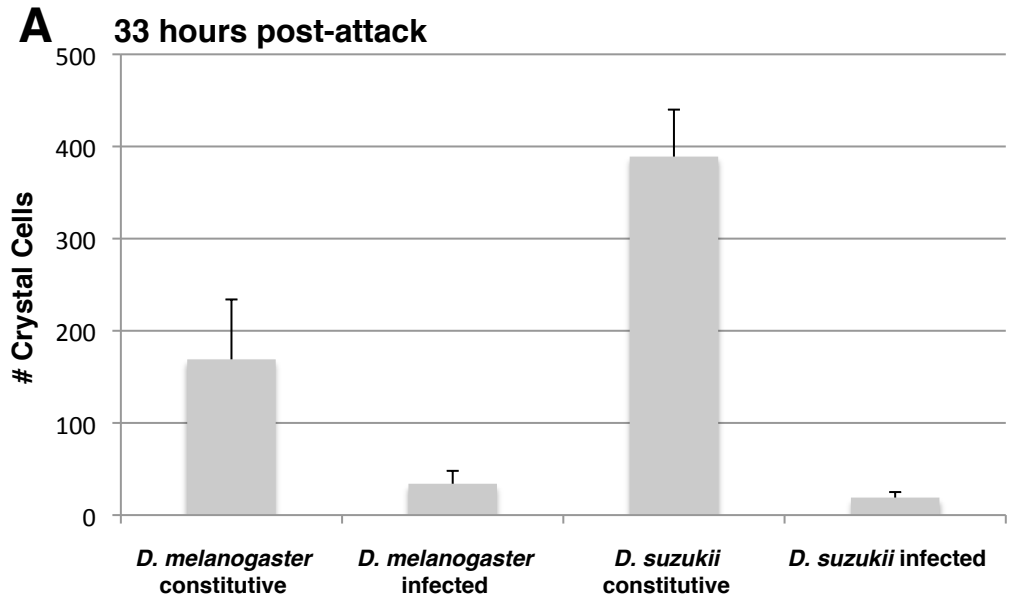


effect on plasmatocytes:
species, $p < 0.001$
pierce, $p = 0.061$
interaction, $p = 0.090$

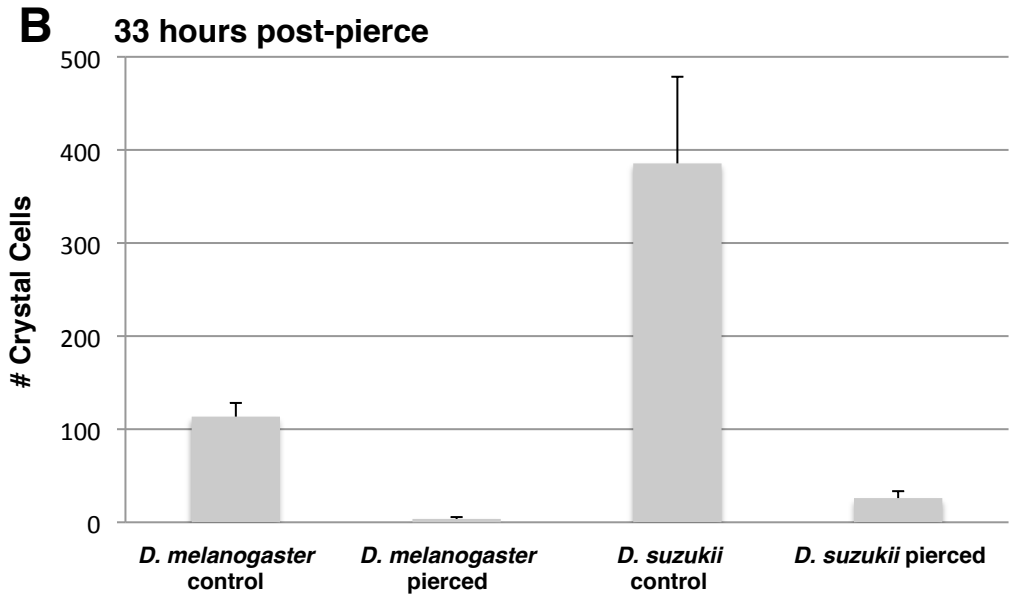
effect on podocytes:
species, $p < 0.001$
pierce, $p = 0.375$
interaction, $p = 0.008$

effect on lamellocytes:
species, $p = 0.013$
pierce, $p < 0.001$
interaction, $p = 0.113$

Figure 5

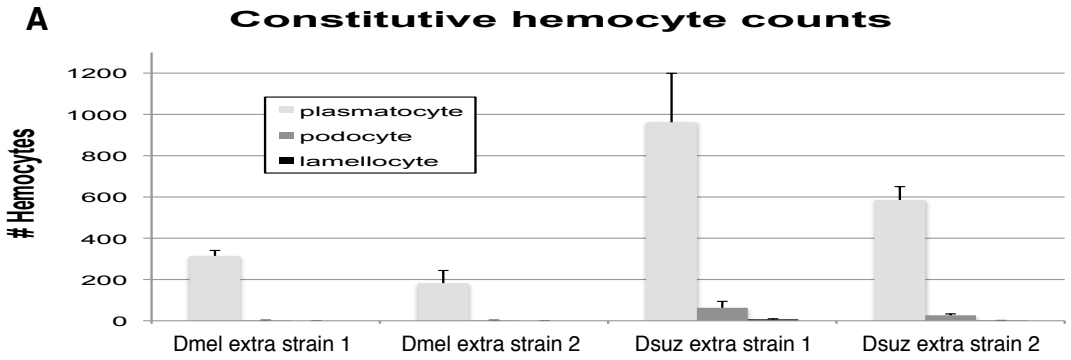


effect on crystal cells:
species, $p = 0.240$
infection, $p < 0.001$
interaction, $p < 0.001$

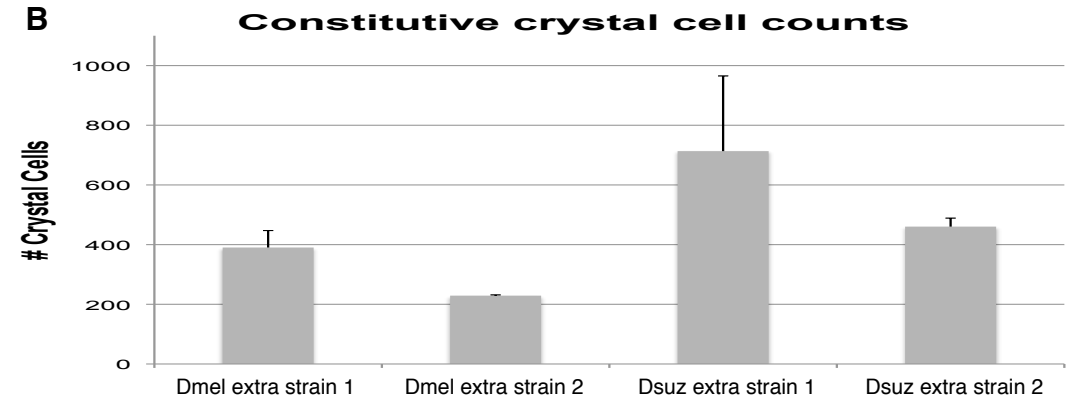


effect on crystal cells:
species, $p = 0.042$
pierce, $p < 0.001$
interaction, $p = 0.020$

Figure 6



effect of species:
plasmatocyte, $p < 0.001$
podocyte, $p < 0.001$
lamellocyte, $p = 0.989$



effect of species:
crystal cell, $p < 0.001$

Figure 7

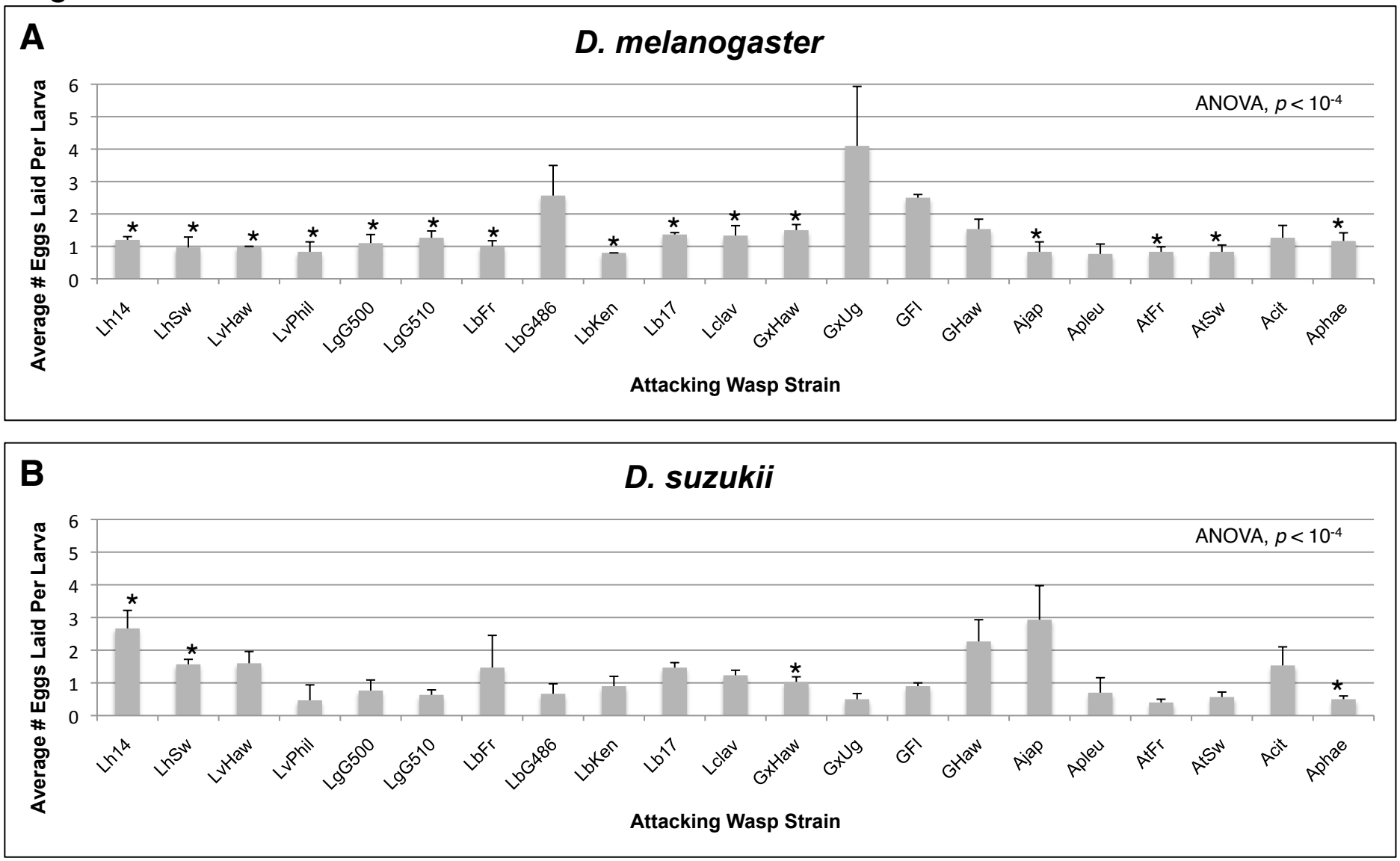

A
D. melanogaster
Average # Eggs Laid Per Larva
ANOVA, $p < 10^{-4}$
Lh14
LhSw
LvHaw
LvPhil
LgG500
LgG510
LbFr
LbG486
LbKen
Lb17
Lclav
GxHaw
GxUg
GFl
GHaw
Ajap
Apleu
AtFr
AtSw
Acit
Aphae
Attacking Wasp Strain
B
D. suzukii
Average # Eggs Laid Per Larva
ANOVA, $p < 10^{-4}$
Attacking Wasp Strain

Figure 8

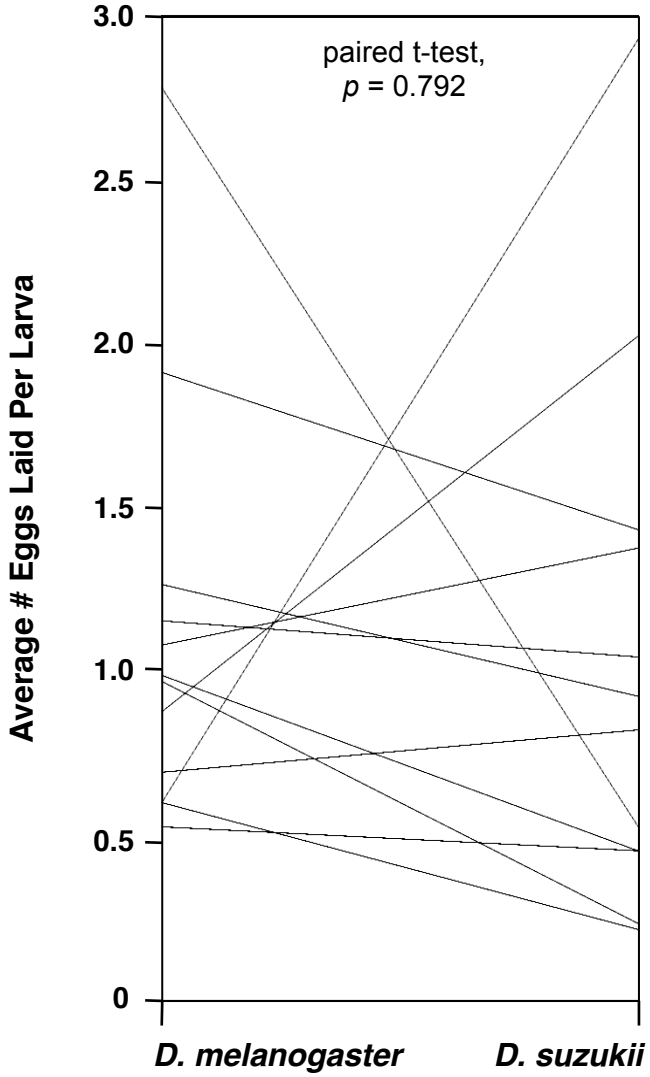
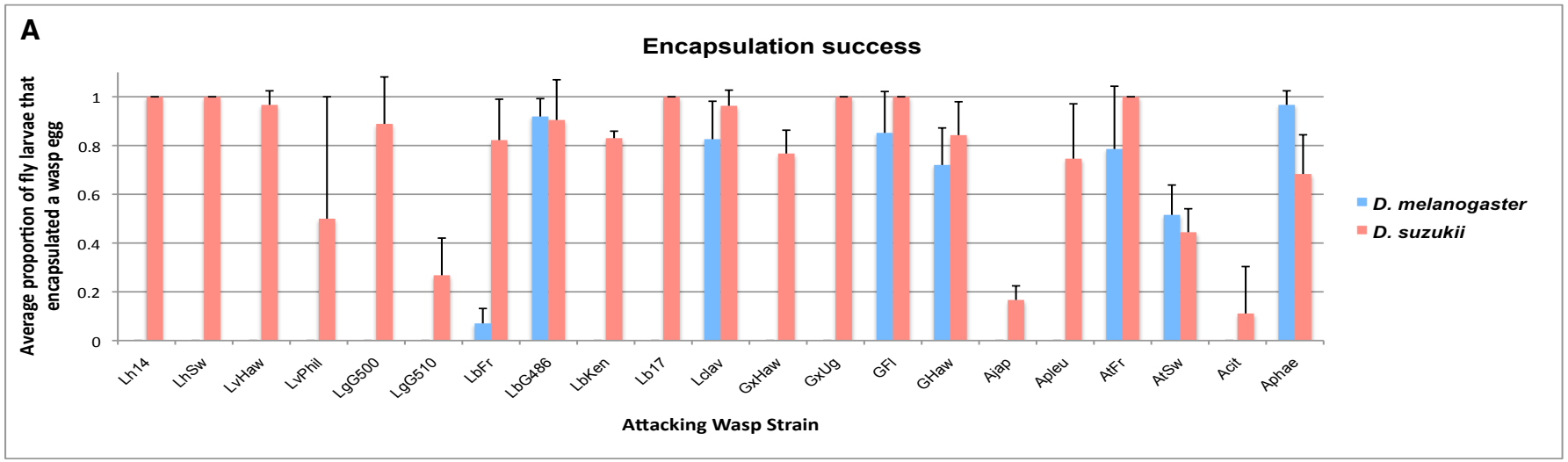

paired t-test,
p = 0.792
Average # Eggs Laid Per Larva
3.0
2.5
2.0
1.5
1.0
0.5
0
D. melanogaster
D. suzukii

Figure 9


A
Encapsulation success
Average proportion of fly larvae that encapsulated a wasp egg
0
0.2
0.4
0.6
0.8
1
Lh14
LhSw
LvHaw
LvPhil
LgG500
LgG510
LbFr
LbG486
LbKen
Lb17
Lclav
GxHaw
GxUg
GFl
GHaw
Ajap
Apleu
AtFr
AtSw
Acit
Aphae
Attacking Wasp Strain
D. melanogaster
D. suzukii

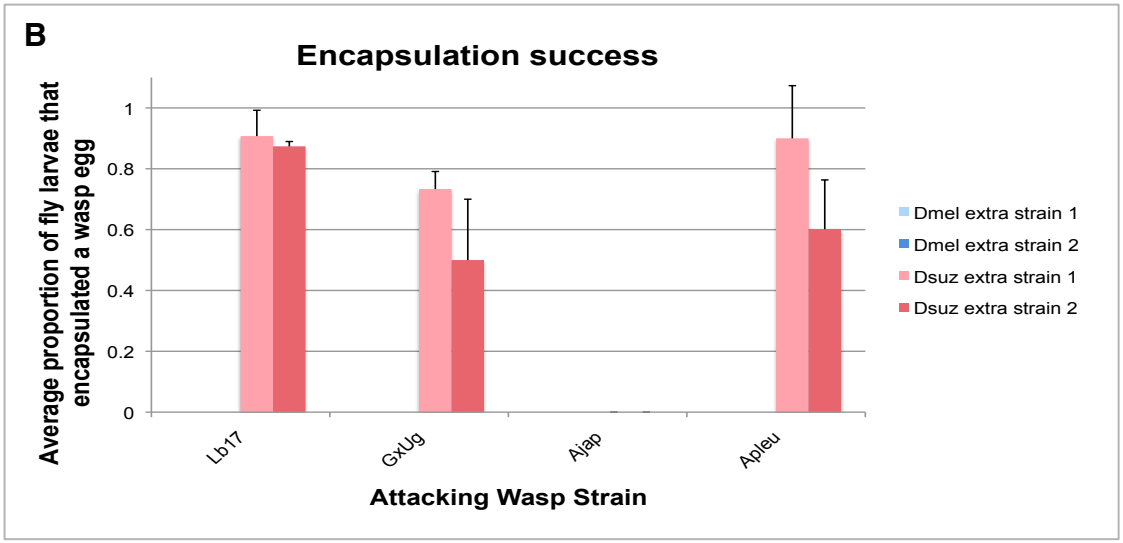

B
Encapsulation success
Average proportion of fly larvae that encapsulated a wasp egg
0
0.2
0.4
0.6
0.8
1
Lb17
GxUg
Ajap
Apleu
Attacking Wasp Strain
Dmel extra strain 1
Dmel extra strain 2
Dsuz extra strain 1
Dsuz extra strain 2

Figure 10

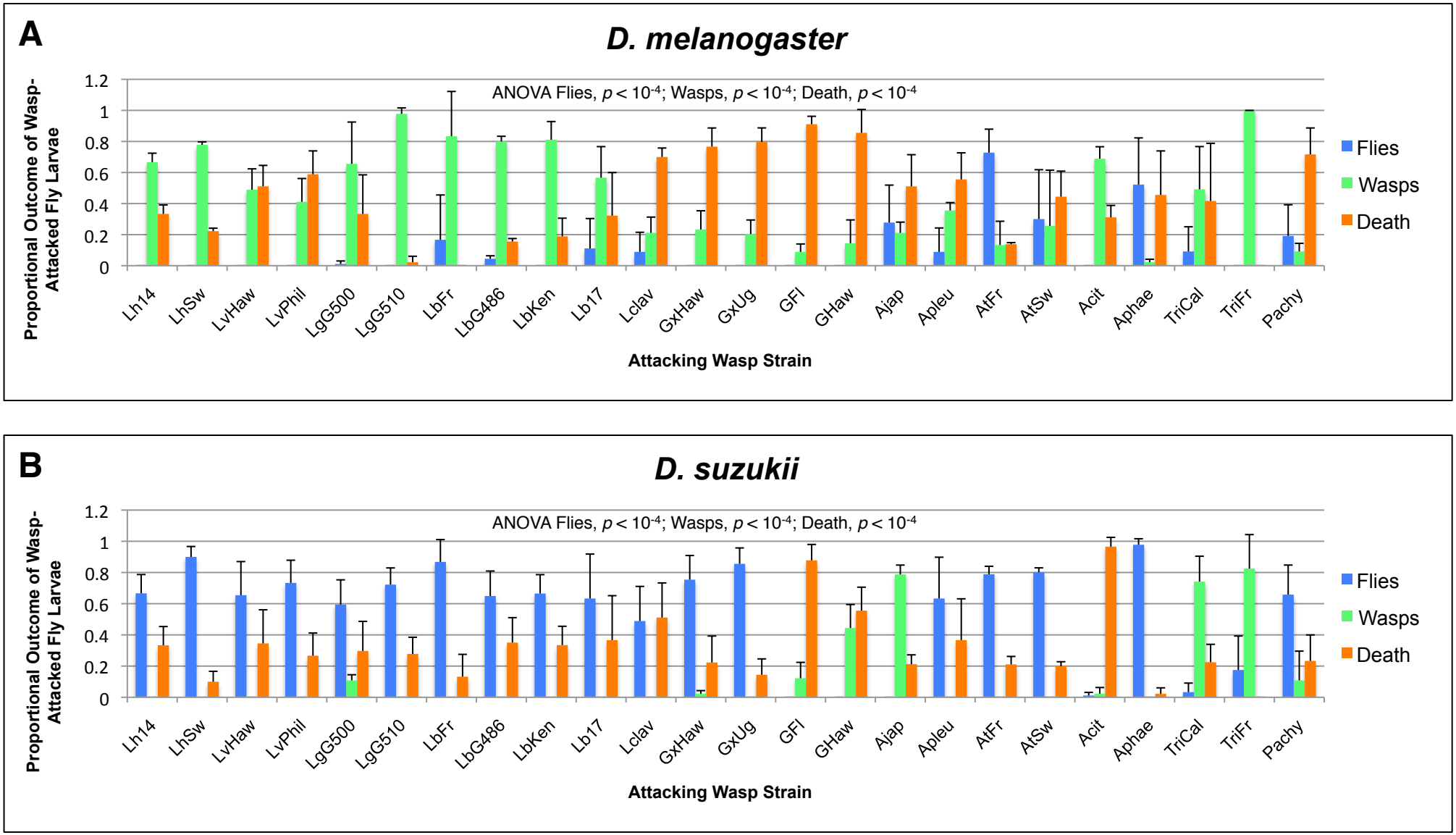

A
D. melanogaster
ANOVA Flies, p < 10-4; Wasps, p < 10-4; Death, p < 10-4
Proportional Outcome of Wasp-Attacked Fly Larvae
1.2
1
0.8
0.6
0.4
0.2
0
Lh14
LhSw
LvHaw
LvPhil
LgG500
LgG510
LbFr
LbG486
LbKen
Lb17
Lclav
GxHaw
GxUg
GFl
GHaw
Ajap
Apleu
AtFr
AtSw
Acit
Aphae
TriCal
TriFr
Pachy
Attacking Wasp Strain
Flies
Wasps
Death
B
D. suzukii
ANOVA Flies, p < 10-4; Wasps, p < 10-4; Death, p < 10-4
Proportional Outcome of Wasp-Attacked Fly Larvae
1.2
1
0.8
0.6
0.4
0.2
0
Lh14
LhSw
LvHaw
LvPhil
LgG500
LgG510
LbFr
LbG486
LbKen
Lb17
Lclav
GxHaw
GxUg
GFl
GHaw
Ajap
Apleu
AtFr
AtSw
Acit
Aphae
TriCal
TriFr
Pachy
Attacking Wasp Strain
Flies
Wasps
Death

Figure 11

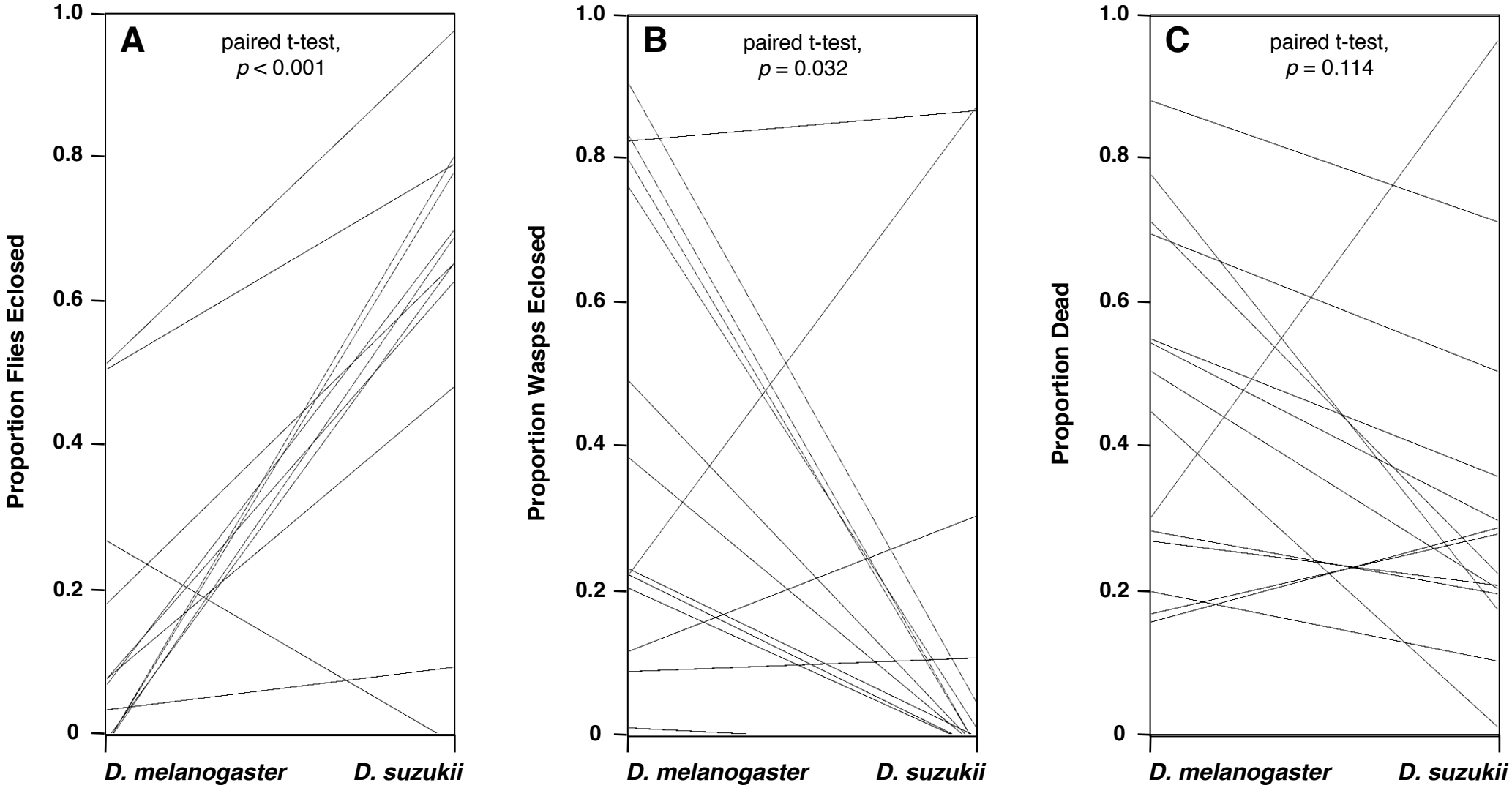

A
paired t-test,
p < 0.001
Proportion Flies Eclosed
1.0
0.8
0.6
0.4
0.2
0
D. melanogaster
D. suzukii
B
paired t-test,
p = 0.032
Proportion Wasps Eclosed
1.0
0.8
0.6
0.4
0.2
0
D. melanogaster
D. suzukii
C
paired t-test,
p = 0.114
Proportion Dead
1.0
0.8
0.6
0.4
0.2
0
D. melanogaster
D. suzukii

Figure 12

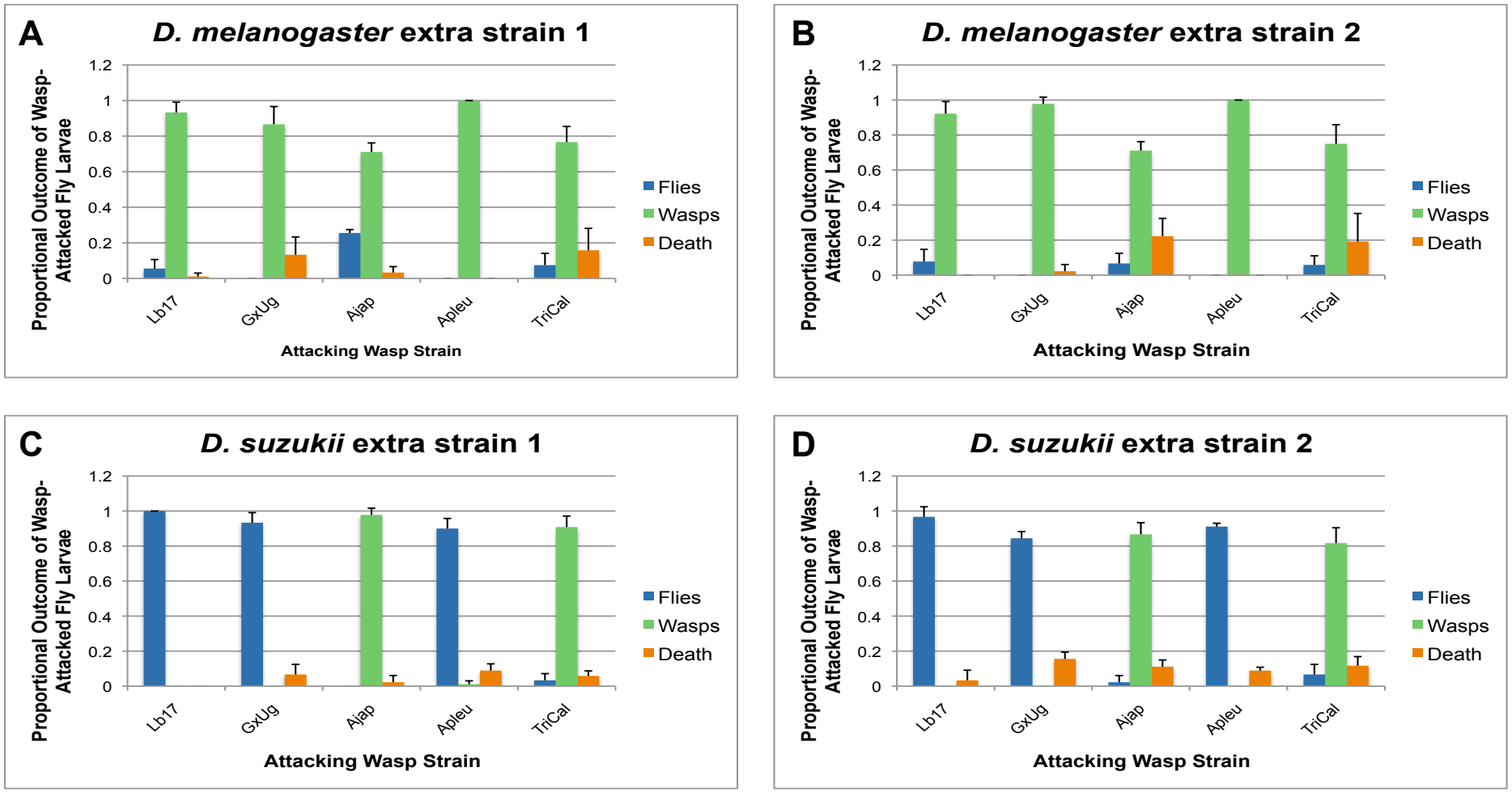

A
D. melanogaster extra strain 1
B
D. melanogaster extra strain 2
C
D. suzukii extra strain 1
D
D. suzukii extra strain 2
Proportional Outcome of Wasp-Attacked Fly Larvae
1.2
1
0.8
0.6
0.4
0.2
0
Lb17
GxUg
Ajap
Apleu
TriCal
Attacking Wasp Strain
Flies
Wasps
Death

Figure 13

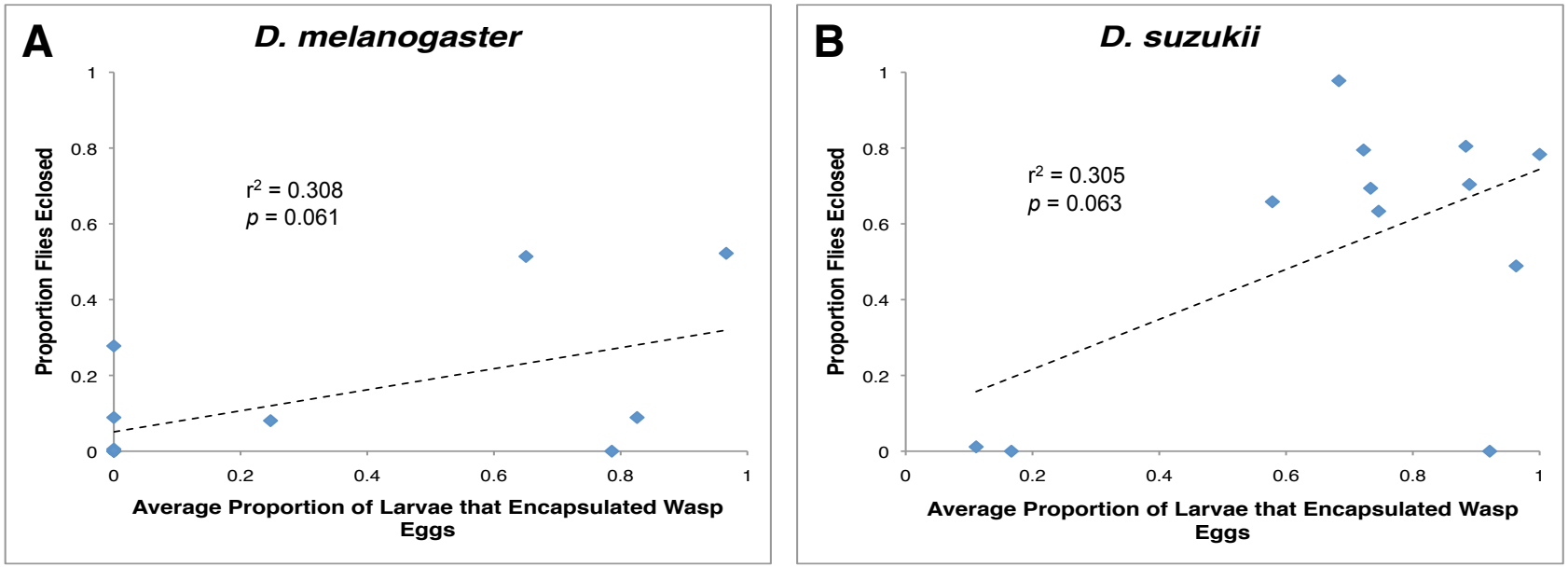
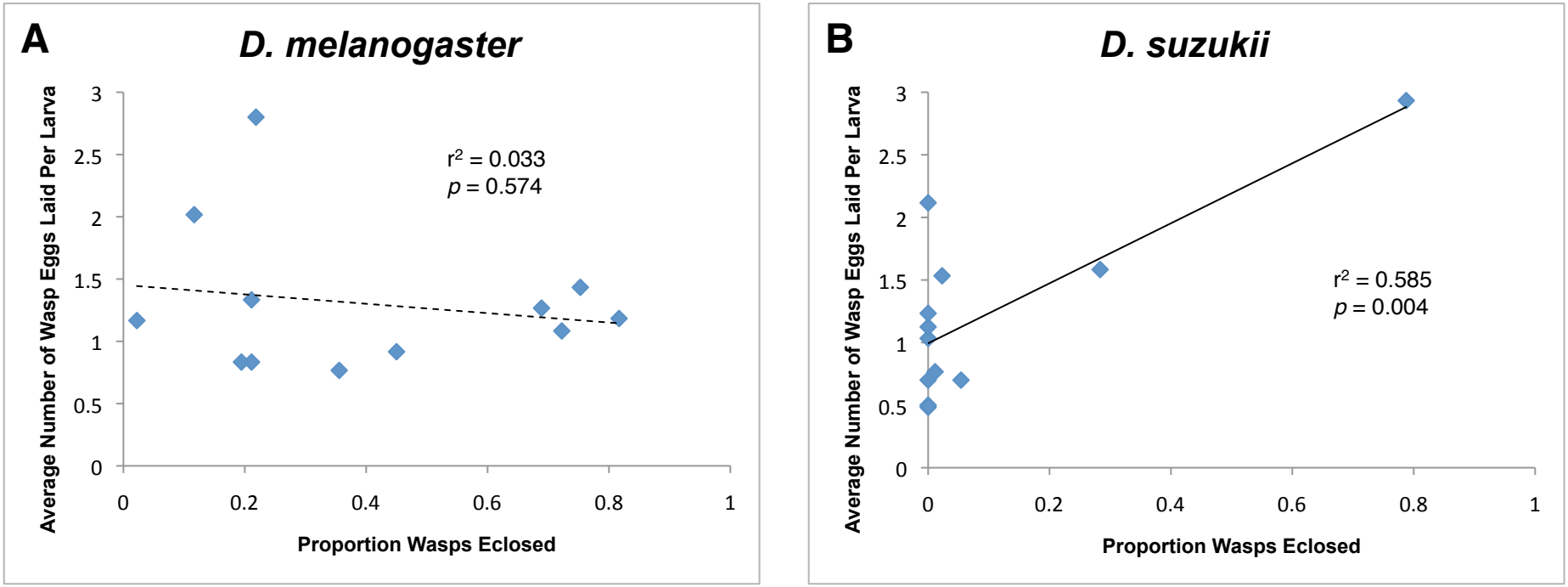

A
D. melanogaster
Proportion Flies Eclosed
r2 = 0.308
p = 0.061
Average Proportion of Larvae that Encapsulated Wasp Eggs
B
D. suzukii
Proportion Flies Eclosed
r2 = 0.305
p = 0.063
Average Proportion of Larvae that Encapsulated Wasp Eggs

Figure 14


A
D. melanogaster
Average Number of Wasp Eggs Laid Per Larva
Proportion Wasps Eclosed
r² = 0.033
p = 0.574
0
0.5
1
1.5
2
2.5
3
0
0.2
0.4
0.6
0.8
1
B
D. suzukii
Average Number of Wasp Eggs Laid Per Larva
Proportion Wasps Eclosed
r² = 0.585
p = 0.004

Chapter 4

A role for nematocytes in the cellular encapsulation response mounted against parasitic wasps by the Drosophilid *Zaprionus indianus*.

Figures

Figure 1

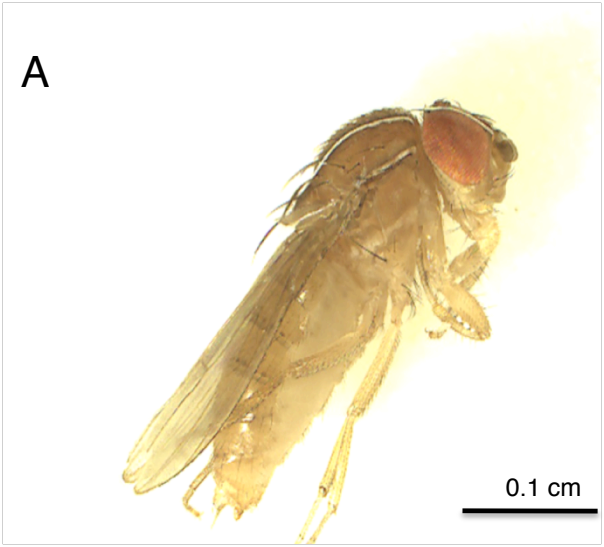
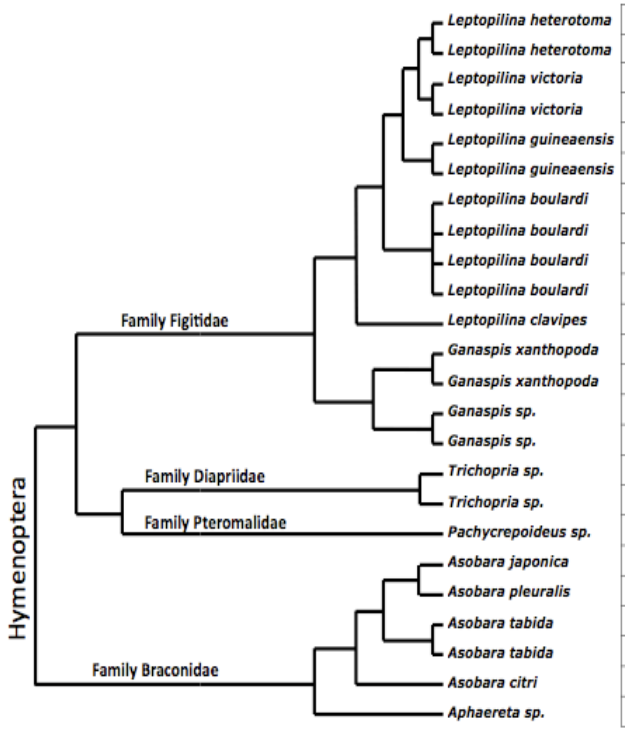
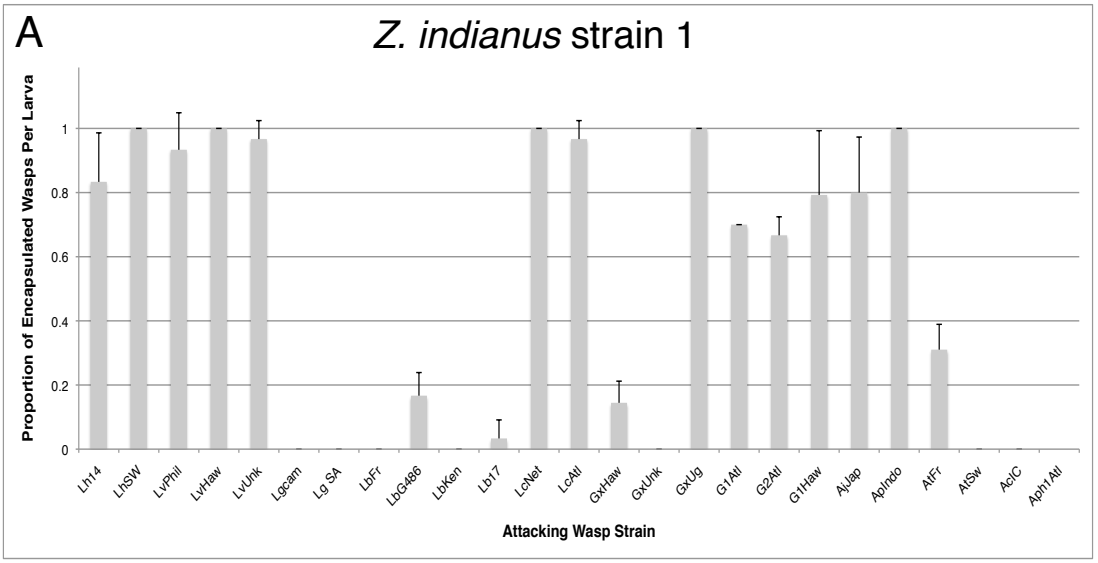


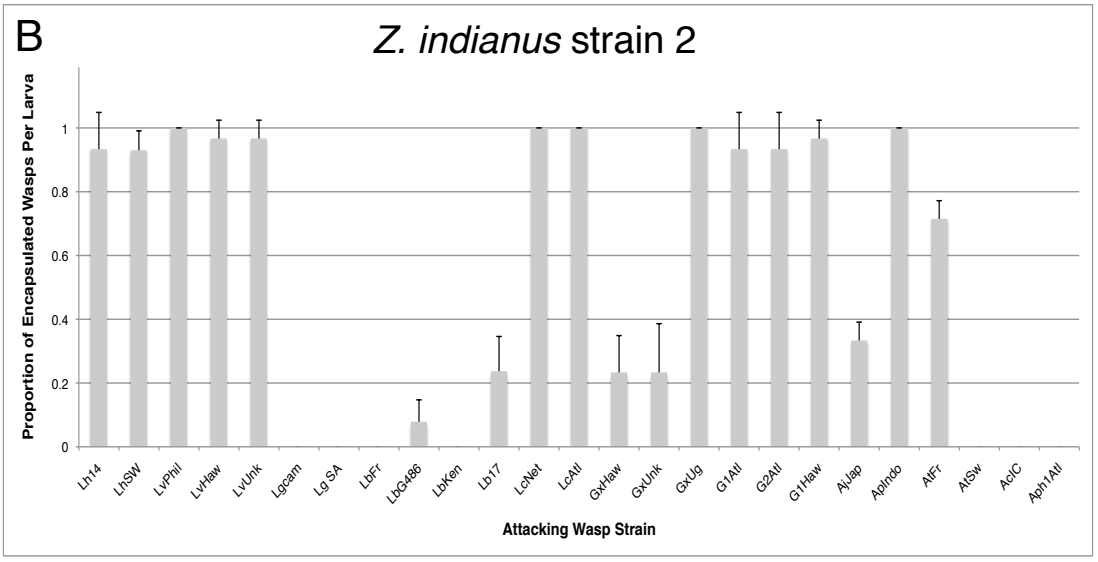
Figure 2



	Strain	Place Collected	Date Collected
Leptopilina heterotoma	Lh14	Winters, California	2002
Leptopilina heterotoma	LhSw	Uppsala, Sweden	2007
Leptopilina victoria	LvHaw	Kaimuki, Oahu, Hawaii	2009
Leptopilina victoria	LvPhil	Consolacion, Cebu, Philippines	2007
Leptopilina guineaensis	LgG500	Yaounde, Cameroon	1998
Leptopilina guineaensis	LgG510	False Bay, South Africa	1999
Leptopilina boulardi	LbFr	Marseilles, France	2007
Leptopilina boulardi	LbG486	Brazzaville, Congo	1978
Leptopilina boulardi	LbKen	Nairobi, Kenya	2009
Leptopilina boulardi	Lb17	Winters, California	2002
Leptopilina clavipes	Lclav	Heerenbergh, Netherlands	2000
Ganaspis xanthopoda	GxHaw	Kaimuki, Oahu, Hawaii	2009
Ganaspis xanthopoda	GxUg	Masindi, Uganda	2010
Ganaspis sp.	GFl	Homestead, Florida	2008
Ganaspis sp.	GHaw	Kaimuki, Oahu, Hawaii	2009
Trichopria sp.	TriCal	Winters, California	2002
Trichopria sp.	TriFr	Marseilles, France	2007
Pachycrepoideus sp.	Pachy	Atlanta, Georgia	2008
Asobara japonica	Ajap	Tokyo, Japan	1995
Asobara pleuralis	Apleu	Manado, Sulawesi, Indonesia	2005
Asobara tabida	AtFr	Sospel, France	>20 years ago
Asobara tabida	AtSw	Uppsala, Sweden	2007
Asobara citri	Acit	Lamto, Ivory Coast	1995
Aphaereta sp.	Aphae	Atlanta, Georgia	2009

Figure 3


A
Z. indianus strain 1
Proportion of Encapsulated Wasps Per Larva
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
Lg SA
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AcIC
Aph1Atl
Attacking Wasp Strain


B
Z. indianus strain 2
Proportion of Encapsulated Wasps Per Larva
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
Lg SA
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AcIC
Aph1Atl
Attacking Wasp Strain

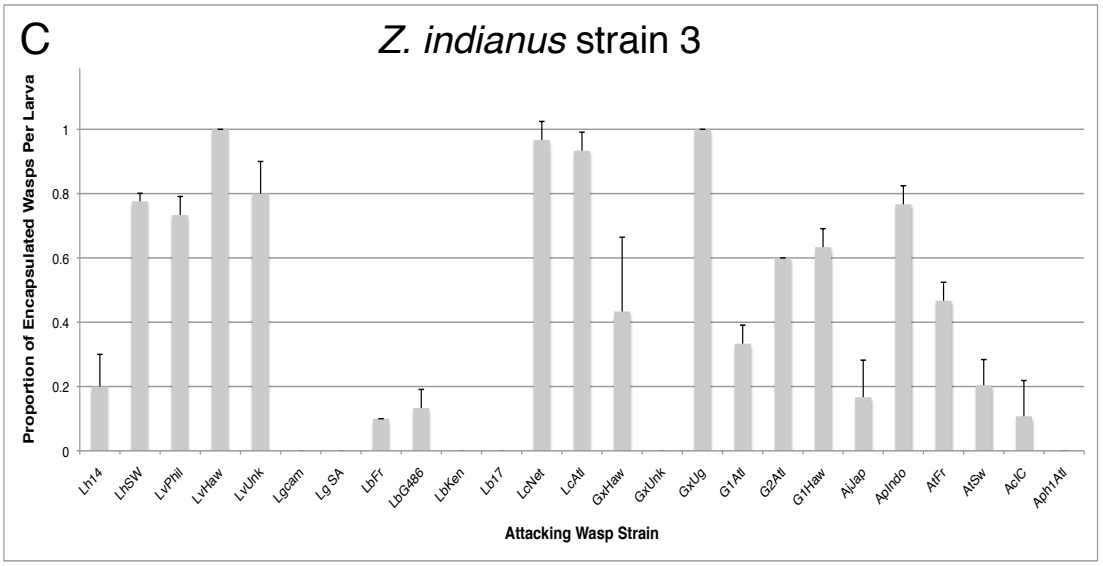

C
Z. indianus strain 3
Proportion of Encapsulated Wasps Per Larva
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
Lg SA
LbFr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
ApIndo
AtFr
AtSw
AcIC
Aph1Atl
Attacking Wasp Strain

Figure 4

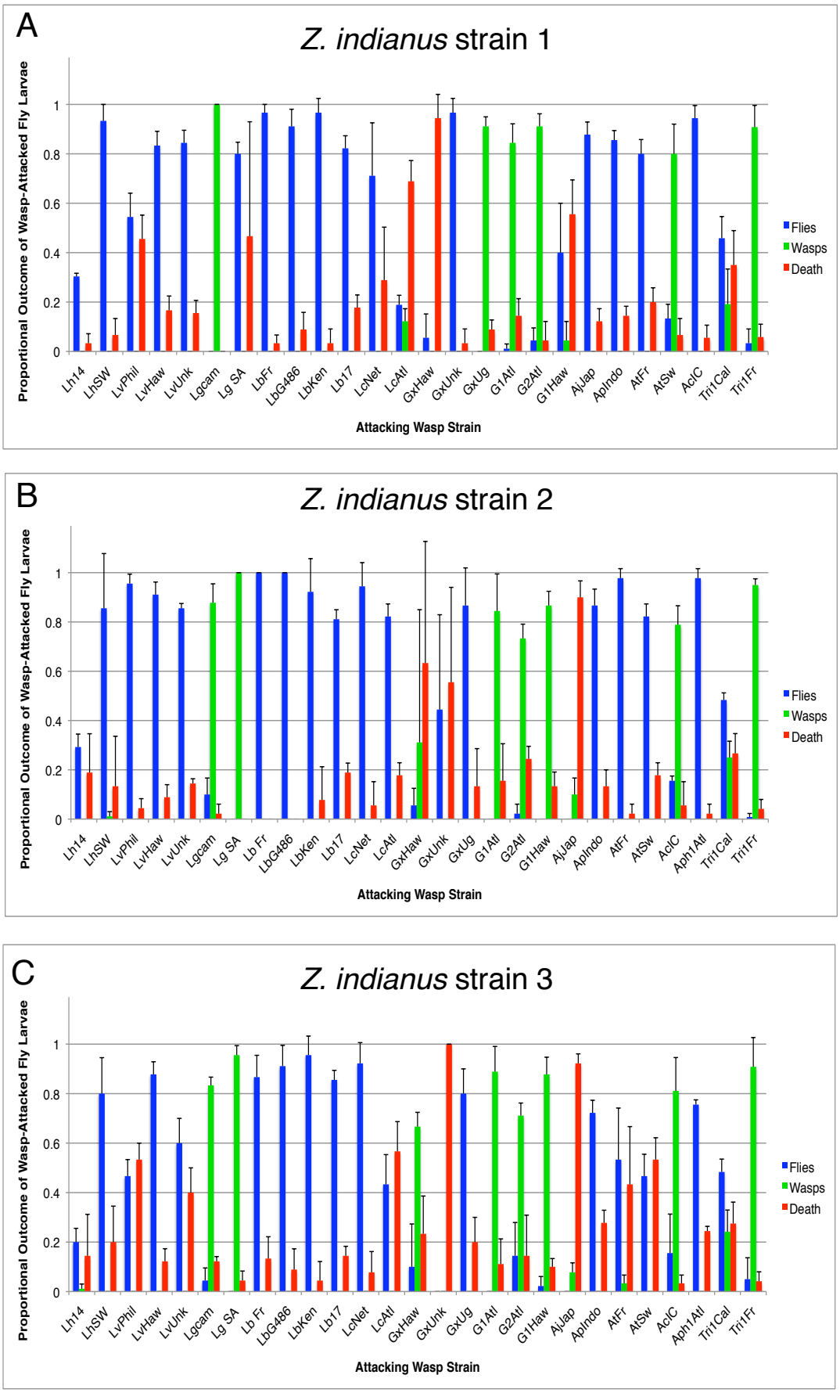

A
Z. indianus strain 1
B
Z. indianus strain 2
C
Z. indianus strain 3
Proportional Outcome of Wasp-Attacked Fly Larvae
0
0.2
0.4
0.6
0.8
1
Lh14
LhSW
LvPhil
LvHaw
LvUnk
Lgcam
Lg SA
Lb Fr
LbG486
LbKen
Lb17
LcNet
LcAtl
GxHaw
GxUnk
GxUg
G1Atl
G2Atl
G1Haw
AjJap
AplndoAtFr
AtSw
AclC
Aph1Atl
Tri1Cal
Tri1Fr
Attacking Wasp Strain
Flies
Wasps
Death

Figure 5

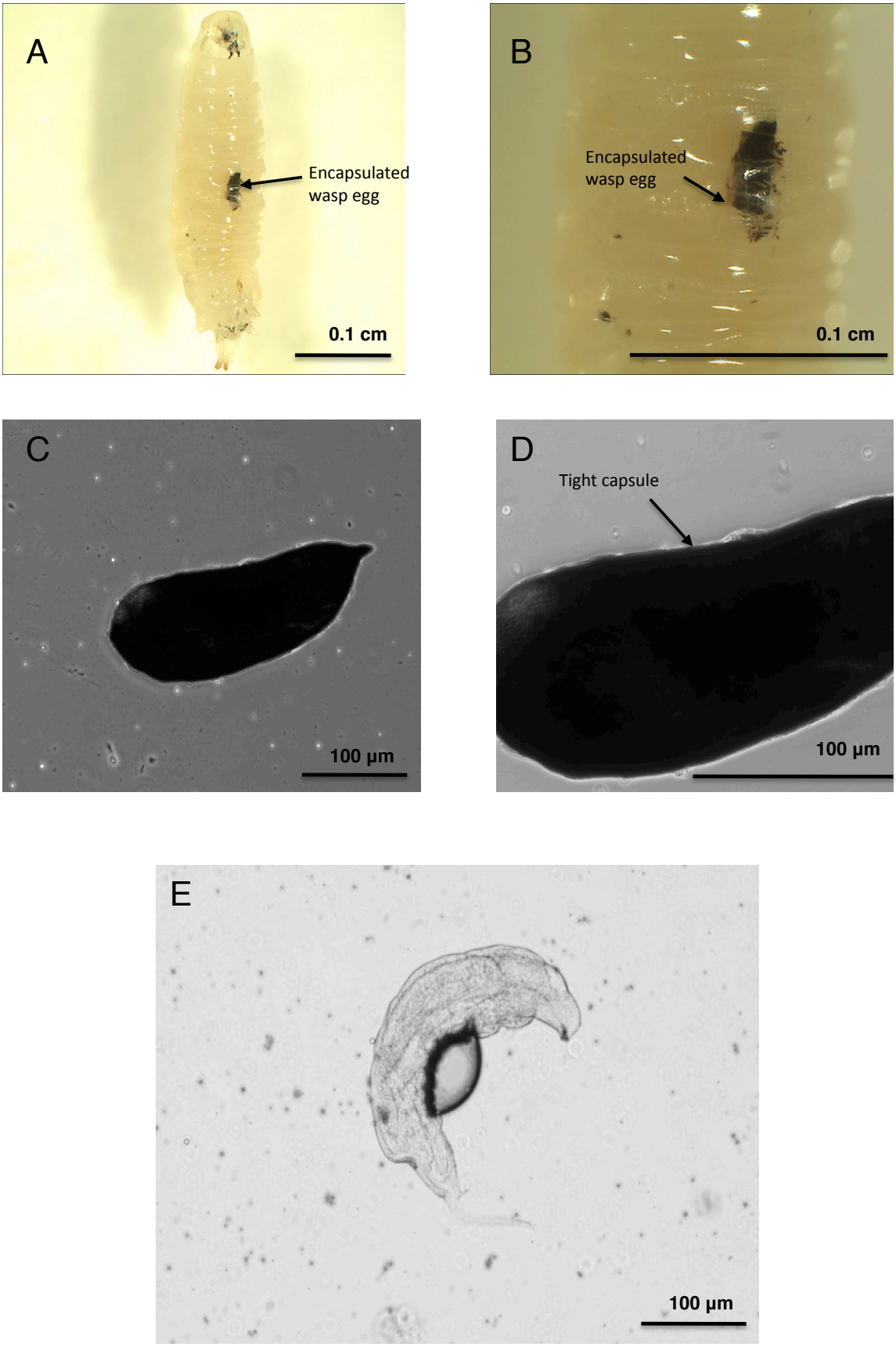

A
Encapsulated
wasp egg
0.1 cm
B
Encapsulated
wasp egg
0.1 cm
C
100 µm
D
Tight capsule
100 µm
E
100 µm

Figure 6

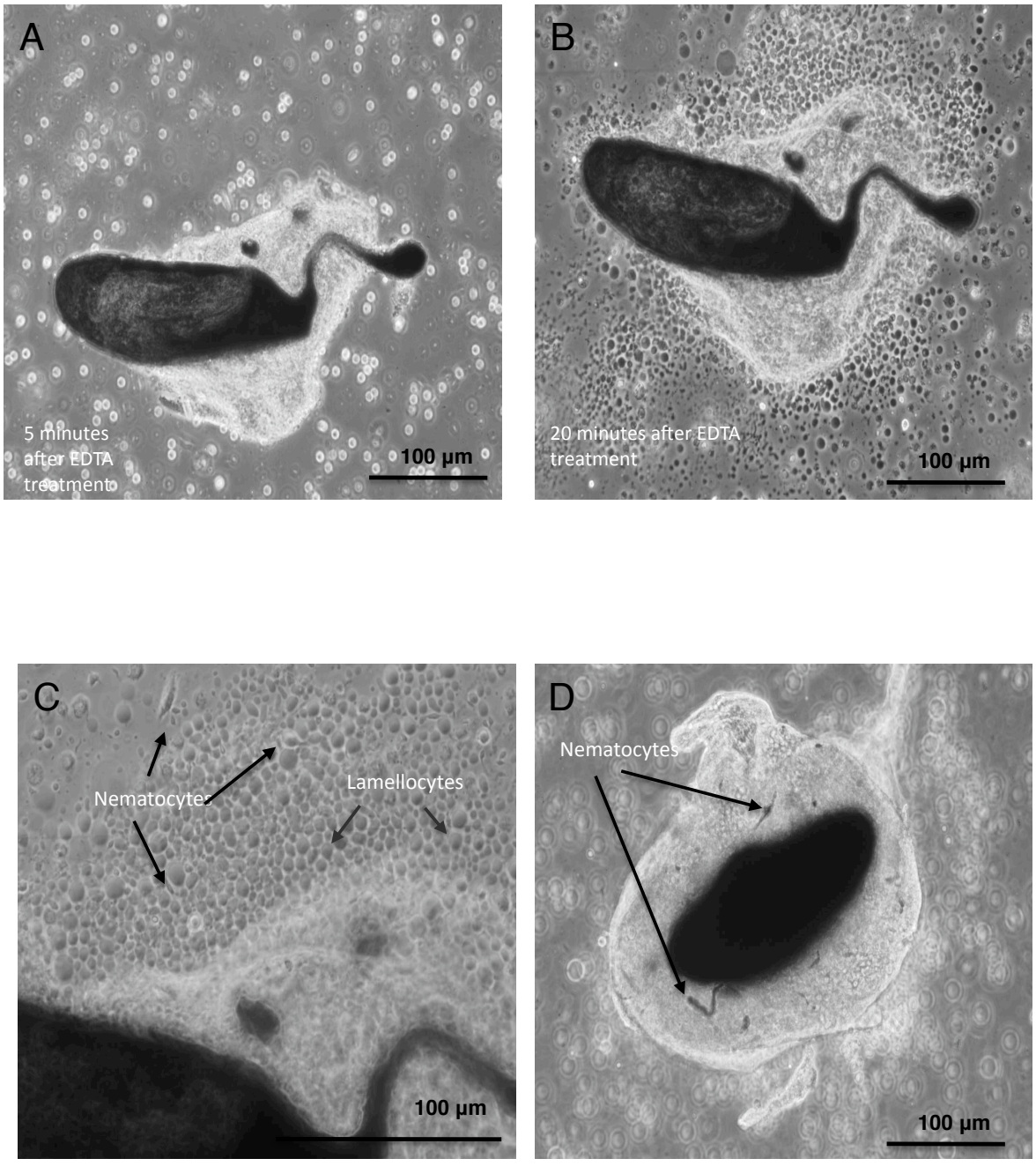

A
5 minutes after EDTA treatment
100 µm
B
20 minutes after EDTA treatment
100 µm
C
Nematocytes
Lamellocytes
100 µm
D
Nematocytes
100 µm

Figure 7

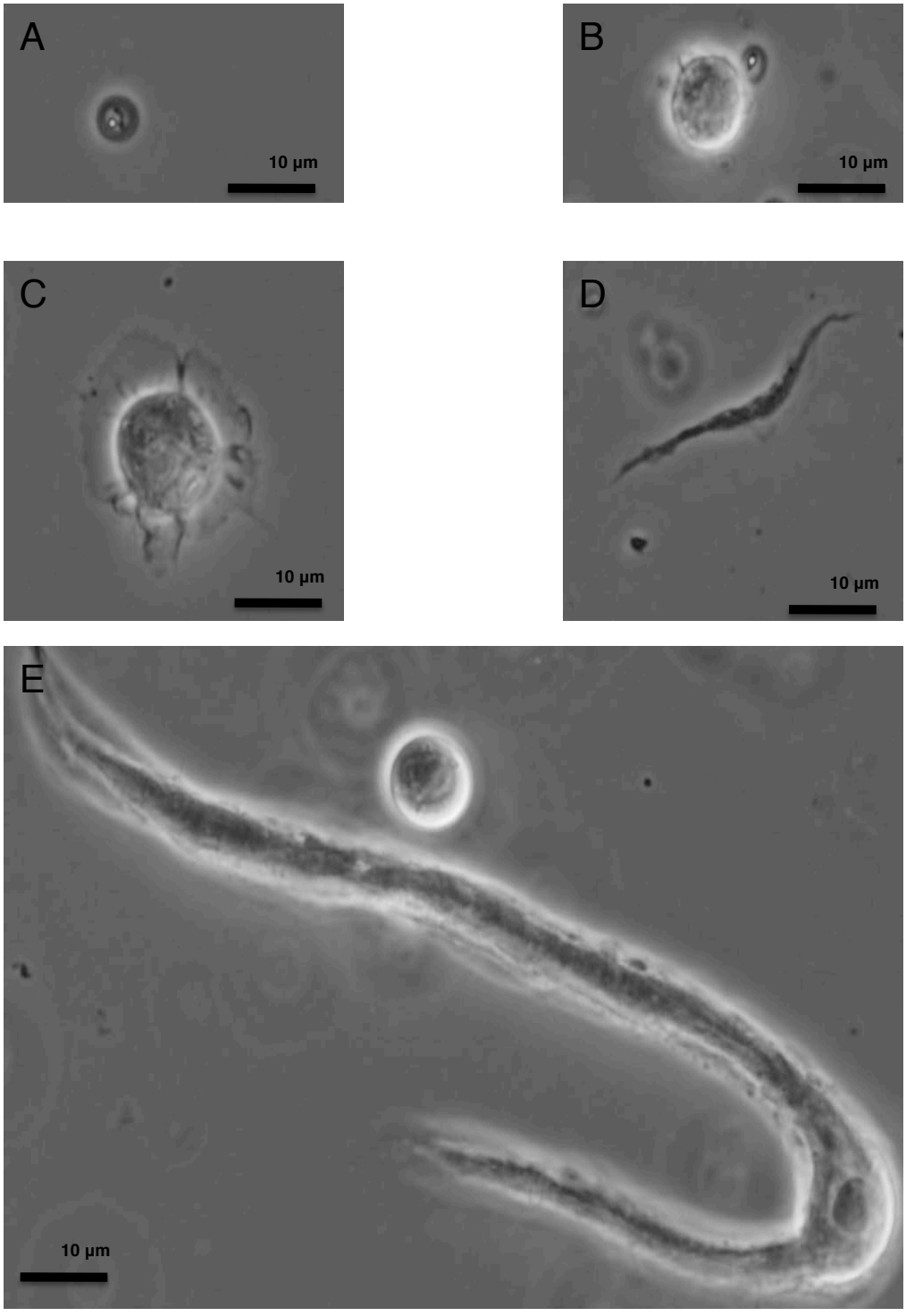


Figure 8

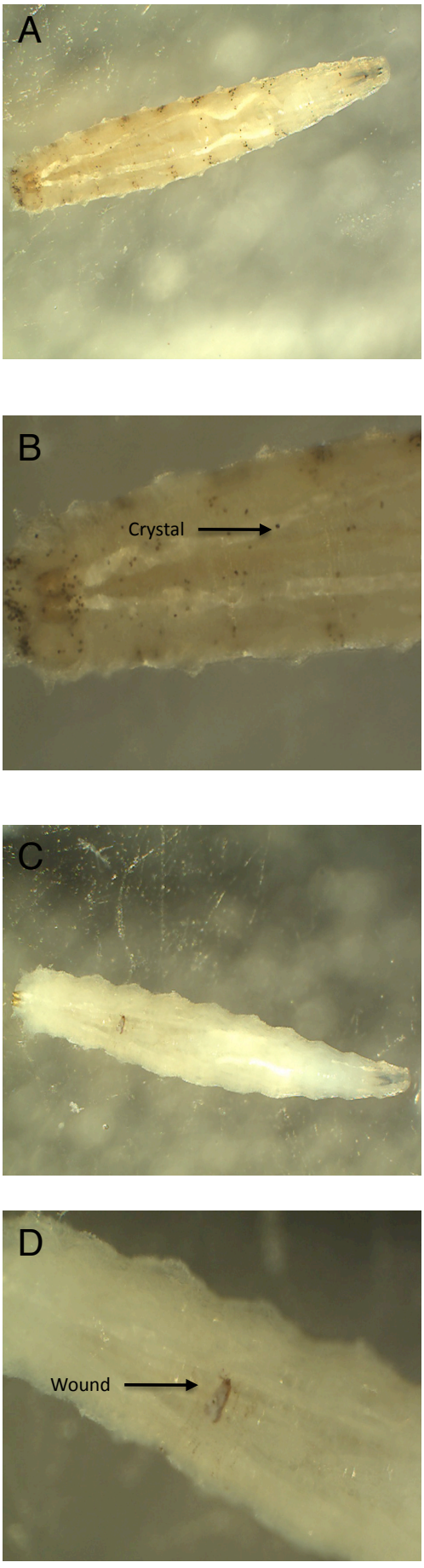


Figure 9

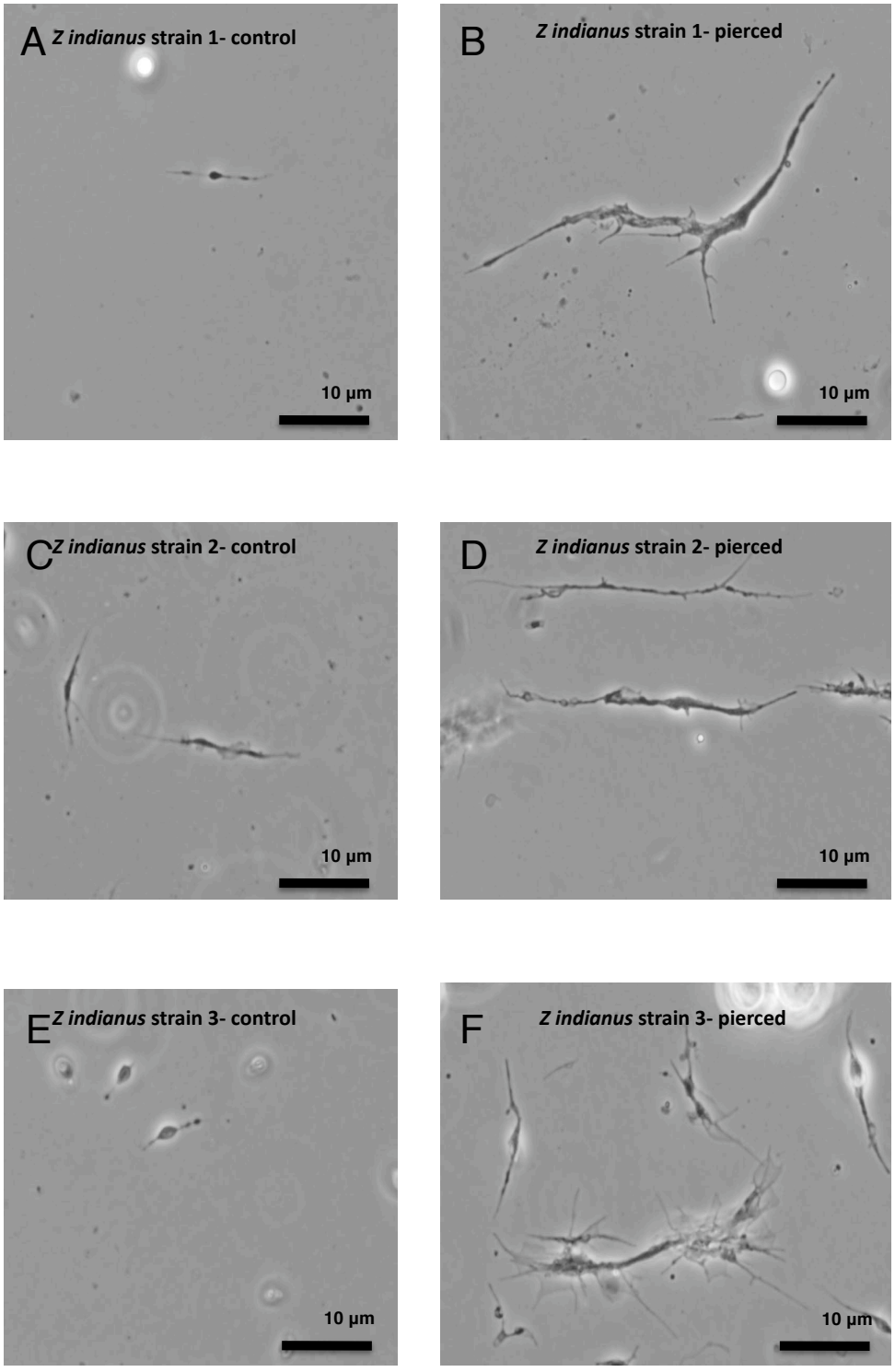
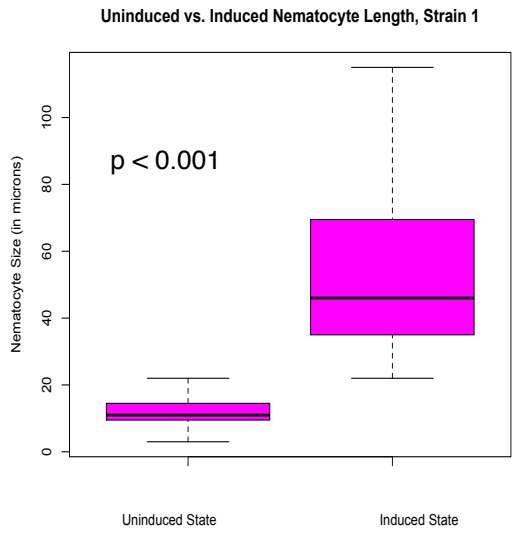
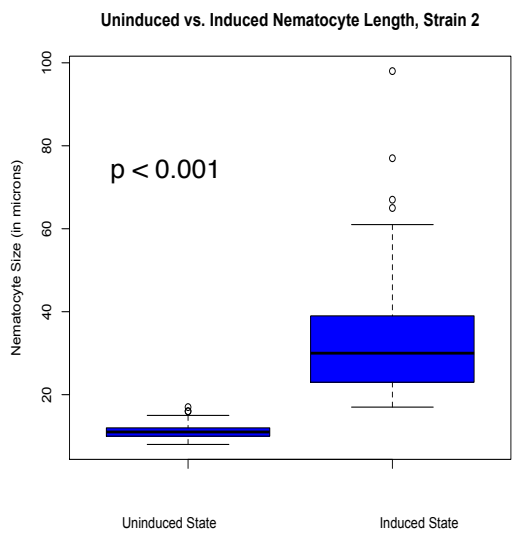

A
Z indianus strain 1- control
10 µm
B
Z indianus strain 1- pierced
10 µm
C
Z indianus strain 2- control
10 µm
D
Z indianus strain 2- pierced
10 µm
E
Z indianus strain 3- control
10 µm
F
Z indianus strain 3- pierced
10 µm

Figure 10

A



B



C

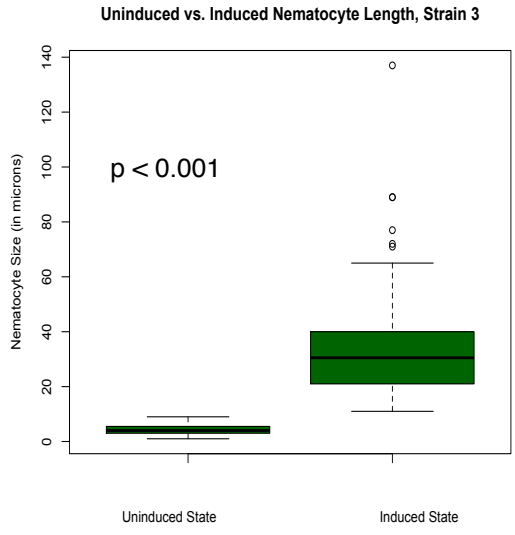


Figure 11

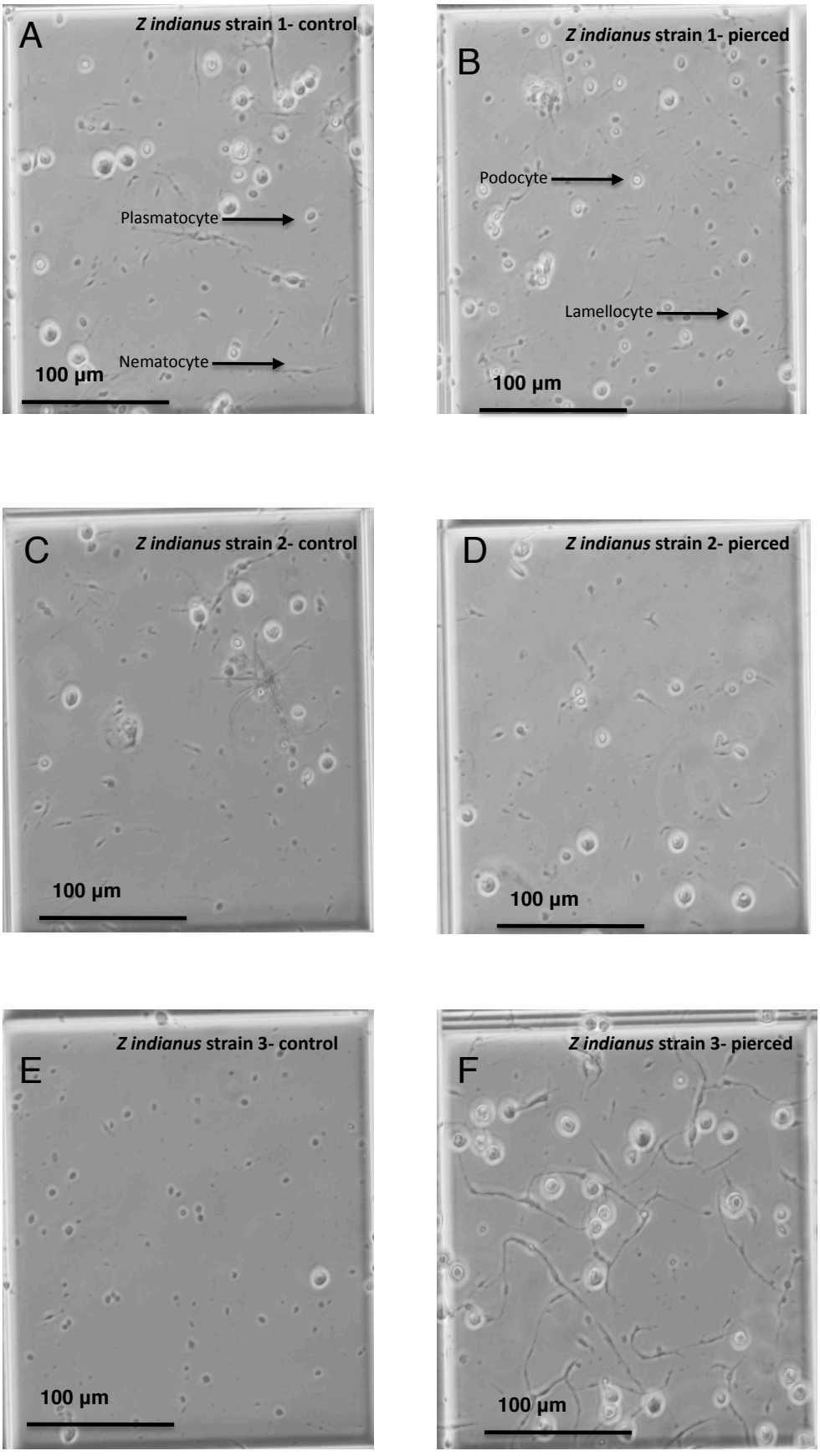
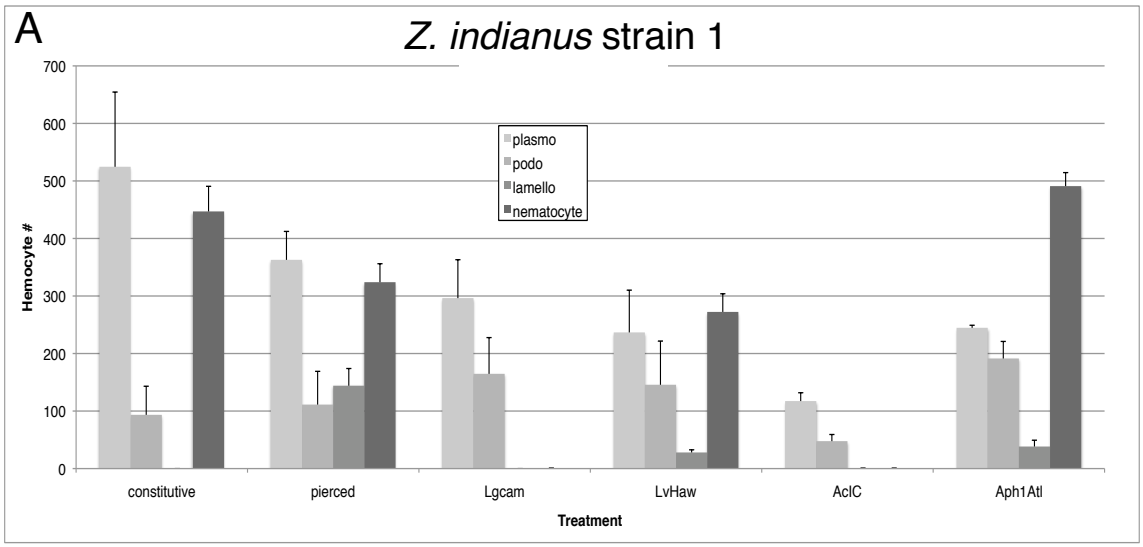
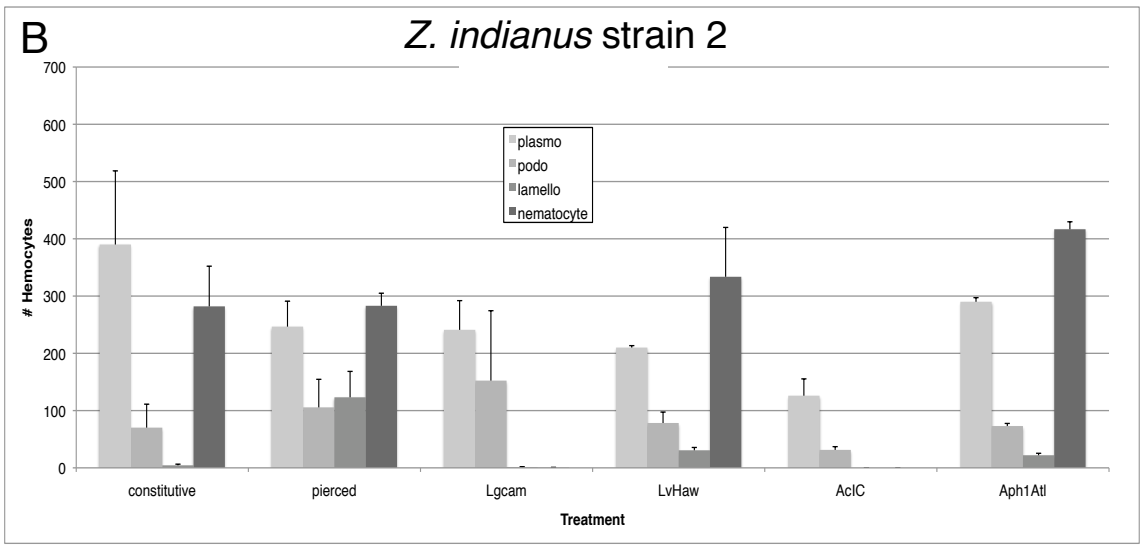

A
Z indianus strain 1- control
Plasmatocyte
Nematocyte
100 µm
B
Z indianus strain 1- pierced
Podocyte
Lamellocyte
100 µm
C
Z indianus strain 2- control
100 µm
D
Z indianus strain 2- pierced
100 µm
E
Z indianus strain 3- control
100 µm
F
Z indianus strain 3- pierced
100 µm

Figure 12


A
Z. indianus strain 1
Hemocyte #
700
600
500
400
300
200
100
0
plasmo
podo
lamello
nematocyte
constitutive
pierced
Lgcam
LvHaw
AcIC
Aph1Atl
Treatment


B
Z. indianus strain 2
Hemocytes
700
600
500
400
300
200
100
0
plasmo
podo
lamello
nematocyte
constitutive
pierced
Lgcam
LvHaw
AcIC
Aph1Atl
Treatment

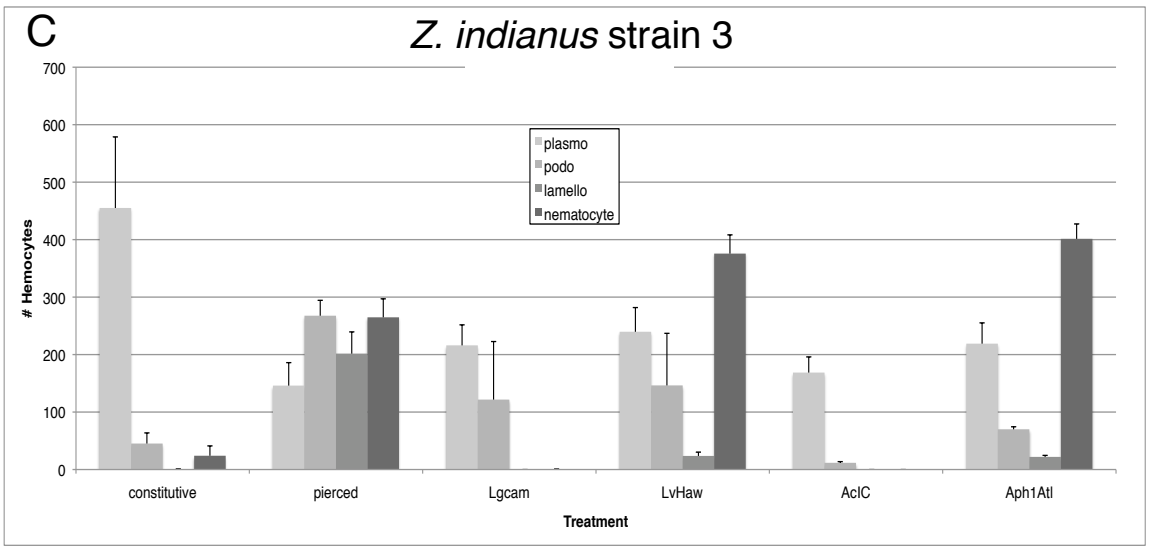

C
Z. indianus strain 3
Hemocytes
700
600
500
400
300
200
100
0
plasmo
podo
lamello
nematocyte
constitutive
pierced
Lgcam
LvHaw
AcIC
Aph1Atl
Treatment

Figure 13

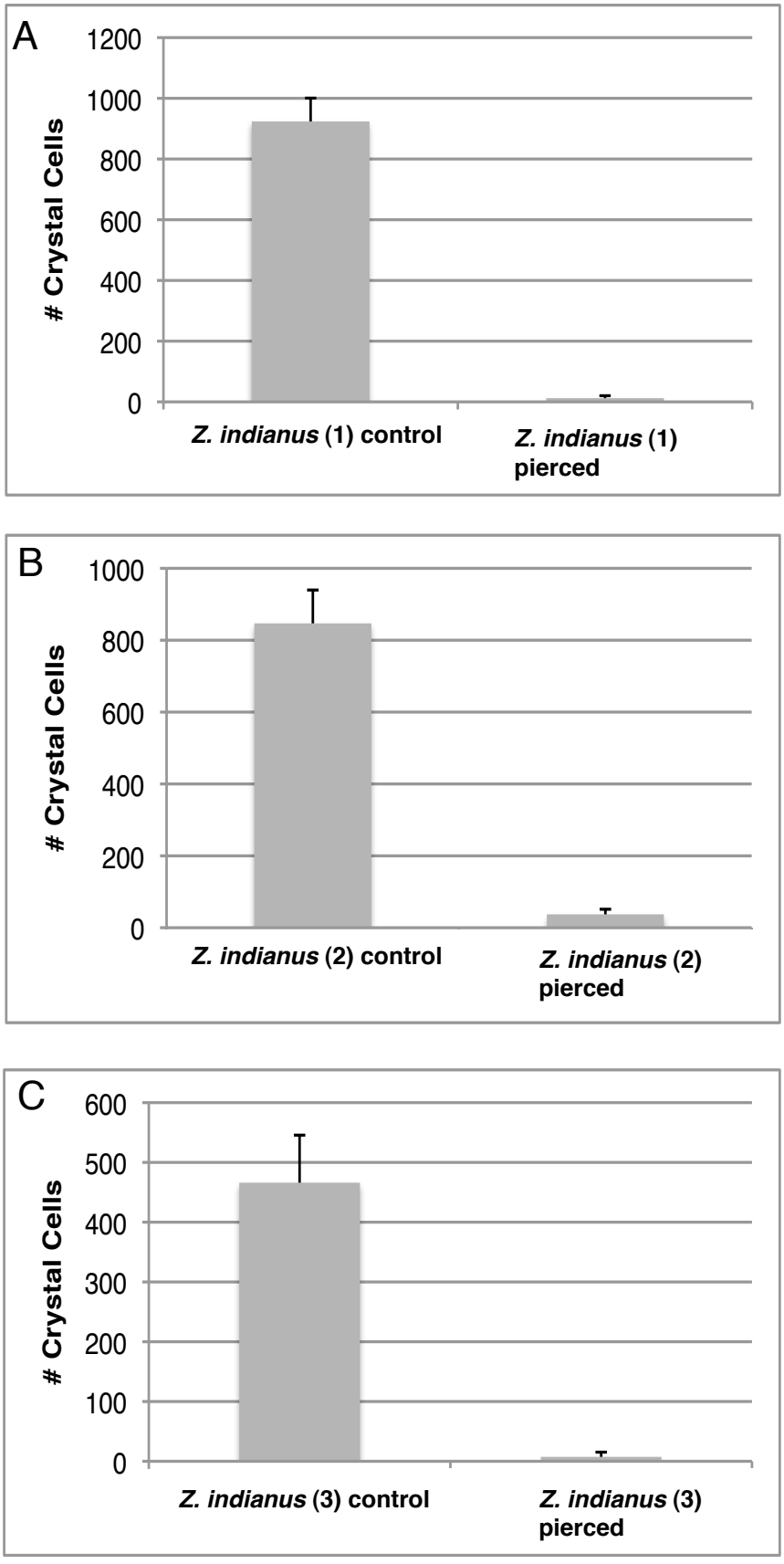
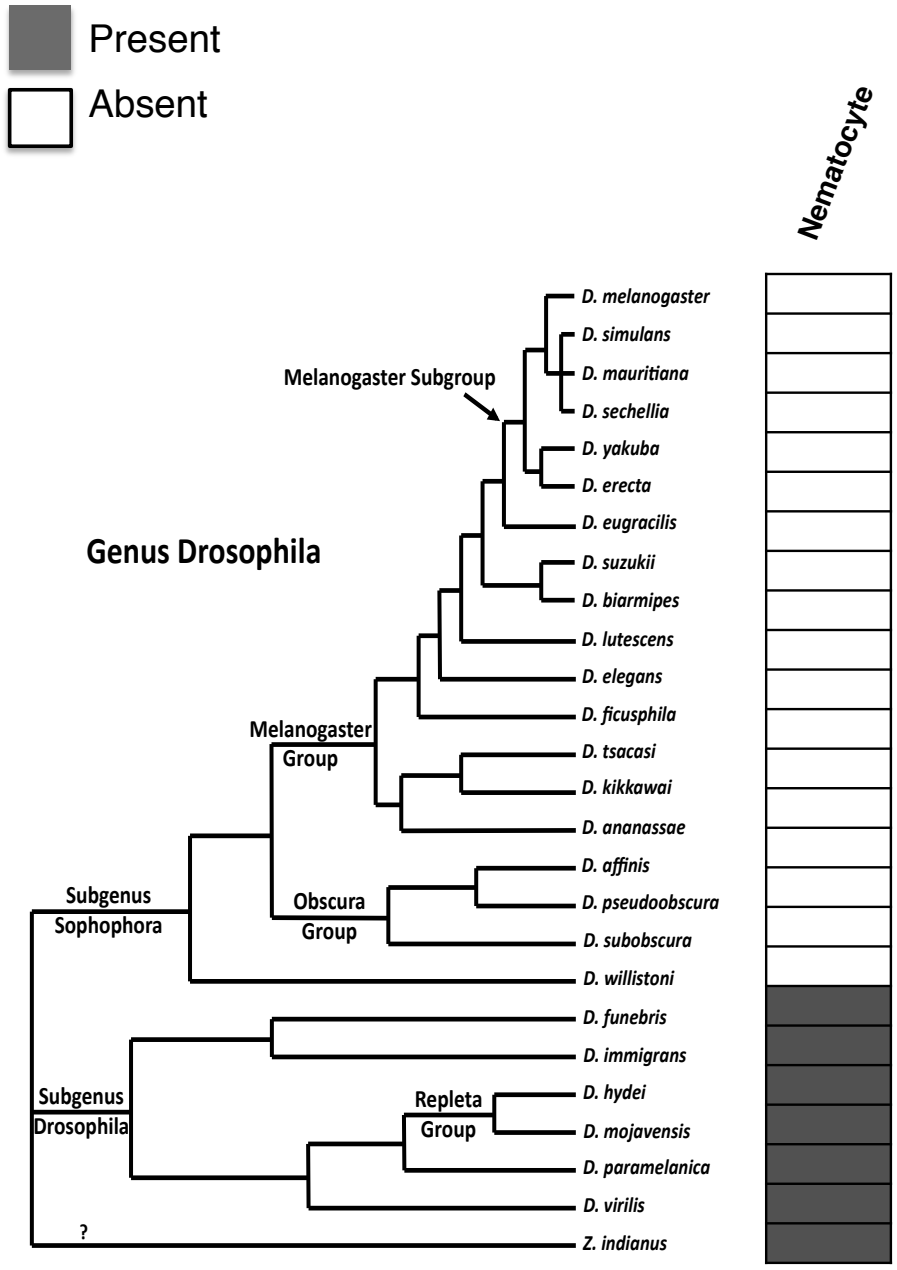
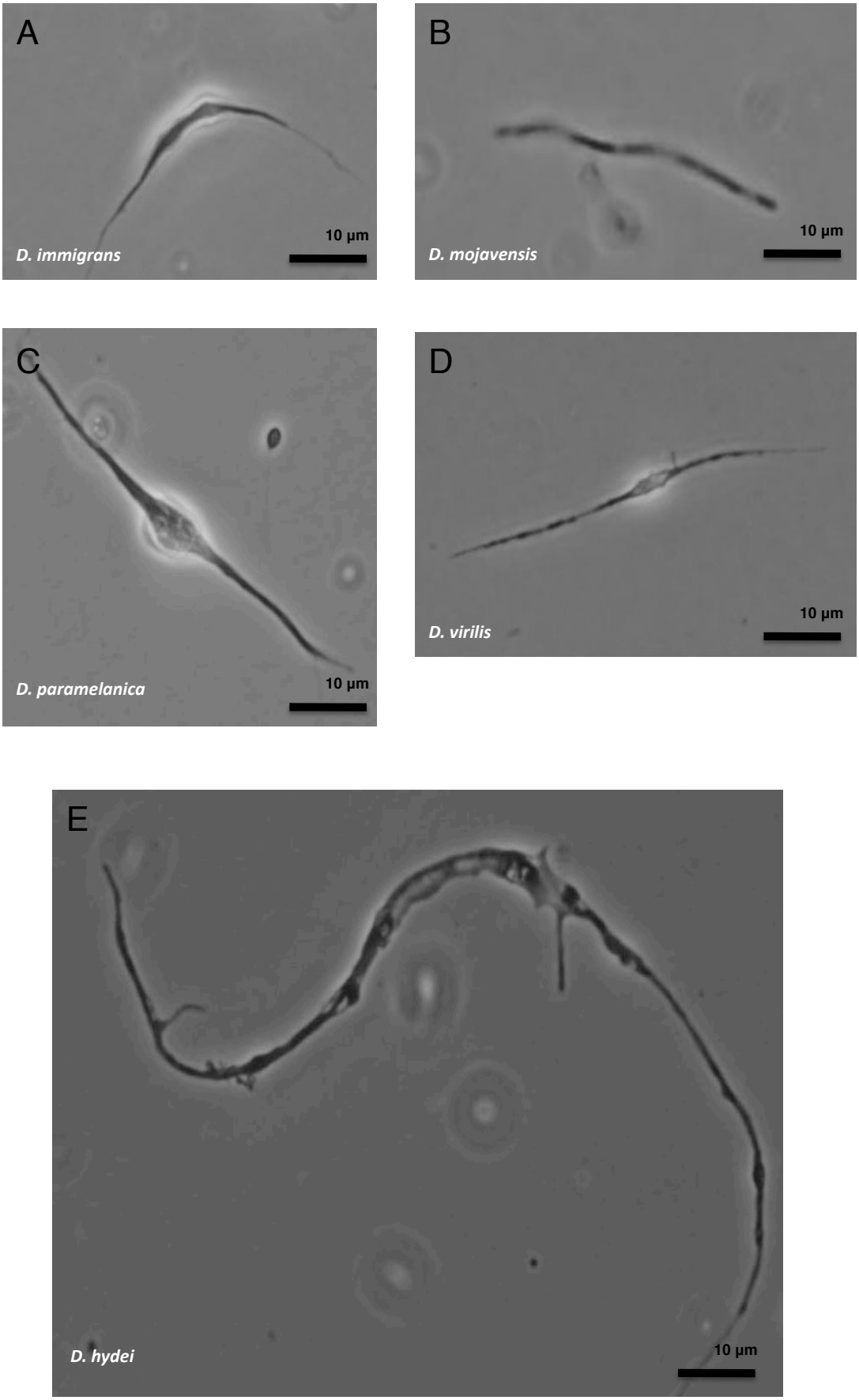

A
1200
1000
800
600
400
200
0
Crystal Cells
Z. indianus (1) control
Z. indianus (1) pierced
B
1000
800
600
400
200
0
Crystal Cells
Z. indianus (2) control
Z. indianus (2) pierced
C
600
500
400
300
200
100
0
Crystal Cells
Z. indianus (3) control
Z. indianus (3) pierced

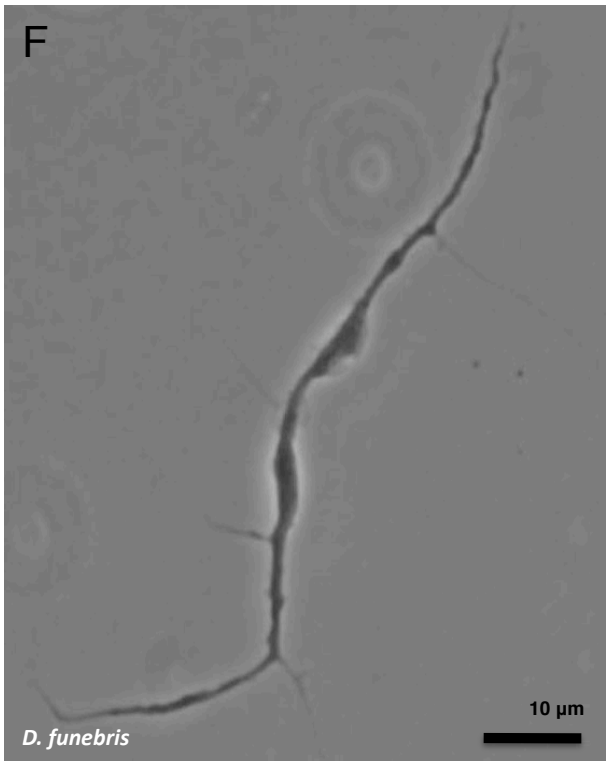
Figure 14



Adapted from Markow and O'Grady 2006, Drosophila: A Guide to Species Identification and Use.

Figure 15


A
10 µm
D. immigrans
B
10 µm
D. mojavensis
C
D. paramelanica
10 µm
D
10 µm
D. virilis
E
D. hydei
10 µm


F
10 µm
D. funebris