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LEPTOPILINA HETEROTOMA RESPONDS TO A CHANGE IN HOST SPECIES WITH SPECIFIC ALTERED PROTEIN EXPRESSION

DAKOTA FUSS

34 Pages

Leptopilina heterotoma is a generalist endoparasitoid wasp that parasitizes *Drosophila spp.* worldwide. When *L. heterotoma* oviposits within the prey larvae it also injects venom to suppress the melanotic encapsulation immune response of the host. Without the venom to protect the egg(s) from host immunity, the parasitism would result in failure. *L. heterotoma* venom is composed of proteins, non-protein compounds, and virus-like particles (VLPs), of which proteins make up the majority of the venom and are the main determining factor conferring venom function (Goecks et al. 2013, Heavner et al. 2013, Moreau and Asgari 2015, Small et al. 2012). Previous studies focus on adaptation of the parasitoid venom. Plastic responses and subsequent adaptations come as a result of environmental changes, the most notable of which is access to different hosts. However, these previous studies fail to take into account the plastic response of the parasitoid. In order to remedy this knowledge gap, an initial generation of *L. heterotoma* was reared on *Drosophila melanogaster.* Then the initial generation was used to establish 3 replicates reared on *Drosophila melanogaster* and 3 replicates reared on *Drosophila yakuba*. Each generation's venom was analyzed for alterations in venom protein production, venom content, and relative protein abundance. Total venom protein was quantified using a Bradford Assay, and variations in venom protein content and

relative abundance was analyzed using SDS-PAGE gels, gradient gels, ImageJ, and SAS analytics software. It was found that the total protein quantity produced was not significantly affected by the change in host availability but specific proteins within bands 11, 3, 2, and 8 were significantly impacted by the change in host availability. This is indicative that *L. heterotoma*'s plastic response strategy to a new host is to change the quantity of specific proteins rather than increase the overall quantity of proteins.

KEYWORDS: Oviposit, Biological Control, Integrated Pest Management (IPM), Ectoparasitoid, Endoparasitoid

LEPTOPILINA HETEROTOMA RESPONDS TO A CHANGE IN HOST SPECIES WITH SPECIFIC ALTERED PROTEIN EXPRESSION

DAKOTA FUSS

A Thesis Submitted in Partial
Fulfillment of the Requirements
for the Degree of

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LEPTOPILINA HETEROTOMA RESPONDS TO A CHANGE IN HOST SPECIES WITH SPECIFIC ALTERED PROTEIN EXPRESSION

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D. F.

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INTRODUCTION

Background and Life History

Parasitoids are arthropods with a unique lifestyle wherein it is an obligate parasite for the first portion of its life and ultimately causes the death of its host as it matures (Moreau and Asgari 2015, Mrinalini and Werren 2015, Small et al. 2012). This unique lifestyle is found among the *Insecta,* but most commonly within the *Hymenoptera* and *Diptera* orders. The parasitoids parasitize arthropod hosts by injecting the host with the egg or eggs and some will also inject venom to facilitate a good environment for the egg(s). The venom protects the egg(s) from the host immune system and can regulate host metabolism to facilitate a properly nutritious environment for the young. Thus, they function well as natural agents of prey population management (Colmenarez et al. 2018, Moreau and Asgari 2015, Mrinalini and Werren 2015, Small et al. 2012).

Parasitoid wasps are an important natural agent of biological control. Biological control is the method by which a species population size is kept in check by natural antagonists such as predators, parasitoids, hyperparasitoids, and pathogens (Colmenarez et al. 2018, Pijnakker et al. 2020, Schulz et al. 2019). This also allows them to act as a buffer against invasive species from invading and establishing (Kremen and Merenlender 2018, Moreau and Asgari 2015, Phalan et al. 2011, Schulz et al. 2019). Parasitoids oviposit within or onto the prey organism, and their eggs will hatch, grow, and eventually consume and kill the host. Those young that are deposited within the host are called endoparasitoids and those young that are deposited outside or on the host are called ectoparasitoids. In this way they have a unique life history

where the young are parasites on their host until they are ready to mature into adults. At that time, the young will consume and kill the host to emerge as autonomous adults (Moreau and Asgari 2015, Mrinalini and Werren 2015).

Parasitoids are capable of infecting hosts at just about every stage of their life. Most commonly, parasitoids will either infect larvae or pupae, but there are few that infect eggs or adults (Moreau and Asgari 2015, Mrinalini and Werren 2015, Weinersmith et al. 2017). When ovipositing their eggs, many parasitoid wasp species will also inject the host with venom. The venom is a mixture of proteins and non-protein compounds whose function is primarily to suppress the host immune system but is also capable of performing other functions such as paralyzing the host, interrupting development, enhancing activity of polydnaviruses (PDVs), castration, and anti-microbial activity (Colmenarez et al. 2018, Goecks et al. 2013, Heavner et al. 2013, Moreau and Asgari 2015, Mrinalini and Werren 2015). The range of functions performed by the venom are determined by its contents. Proteins make up the majority of the contents of the venom, and therefore largely determine the function of the venom (Goecks et al. 2013, Moreau and Asgari 2015, Mrinalini and Werren 2015). These proteins are produced in glandular epithelial cells that line the inner walls of the venom glands. The venom proteins are then moved to the venom reservoir where they are stored for later use (Mrinalini and Werren 2015). Some parasitoid wasp venoms also contain PDVs or other virus-like particles (VLPs). The functions of PDVs and VLPs can vary among different parasitoids and can share functions with other proteins in the venom or have a synergistic effect on venom protein function. PDVs and VLPs are produced in an accessory gland followed by storage in the venom reservoir (Heavner et al. 2013, Mrinalini and Werren 2015, Rizki and Rizki 1990, Wey et al. 2020).

Venom function is imperative in the successful parasitization and subsequent development of the egg. Ectoparasitoids mainly utilize their venom to paralyze the host, induce lethargy, prevent further development, and may also induce certain behaviors such as grooming. The paralyzing effect prevents the eggs and young from being knocked or thrown off of the host. Other functions like lethargy, preventing development, and induction of behaviors all work to do the same and prevent the eggs or young from being removed from the host. This lifestyle is classified as idiobiont because the host is prevented from further development (Moreau and Asgari 2015, Mrinalini and Werren 2015). Endoparasitoids mainly utilize their venom to suppress the host's immune system, but also rely on virus-like particles (VLPs) to divert host physiological defenses away from the parasitoid. These parasitoids follow a koinobiont lifestyle because the host is allowed to continue to develop while the parasitoid within the host continues to feed and grow (Moreau and Asgari 2015, Mrinalini and Werren 2015, Rizki and Rizki 1990, Salazar-Jaramillo et al. 2014). For endoparasitoids, their lifestyle comes with challenges in facing the host immune response. The major immune response threat to the parasitoid is melanotic encapsulation, in which the lamellocytes of the host envelop the parasitoid, cutting off the parasitoid's access to the nutrient rich hemolymph of the host. This is followed by melanization of the parasitoid to kill it. However, the parasitoid's venom functions to suppress the immune response and protect the egg. If the host immune system fails to eliminate the parasitoid, then the parasitoid will hatch and grow until reaching maturity. If the host is successful in encapsulating and melanizing the parasitoid, then the host will proceed to grow and mature into an adult fly as normal. However, the tradeoff for the immune response utilized often results in a shorter life span and decreased fecundity (Moreau and Asgari 2015,

Mrinalini and Werren 2015, Rizki and Rizki 1990, Salazar-Jaramillo et al. 2014). Parasitoids are quite successful at diverting or suppressing the immune response threat posed by the host. Therefore, they are an important and common agent of biological control.

Parasitoids in Pest Management

Parasitoids have been utilized as a population control agent as far back as 1927, with the use of the specialist parasitoid *Encarsia formosa* to control the pest whitefly *Trialeurodes vaporariorum* in greenhouses (Pijnakker et al. 2020). Since that time parasitoids have become the most common agent used in biological control efforts worldwide (Colmenarez et al. 2018, Moreau and Asgari 2015, Mrinalini and Werren 2015). Biological control, as well as chemical control and mechanical control, are control methods commonly used to manage invasive species and pest populations. In order to implement these methods of control most efficiently and safely, use of them follows the integrated pest management (IPM) approach. This approach relies on the use of multiple control methods for the most effective and least hazardous results (Colmenarez et al. 2018, Moreau and Asgari 2015, Mrinalini and Werren 2015, Rizki and Rizki 1990, Salazar-Jaramillo et al. 2014, Schulz et al. 2019).

As a biological control agent, the use of parasitoids presents several challenges. One such challenge is the potential for the introduced parasitoid to have non-target effects. Primarily, the non-target effects involve the parasitization of non-target species present in the environment. This would result in a decreased effect on the target species than expected and increasing the threat to native species. This could also lead to the adaptation of the parasitoid to primarily target non-target species rather than the target species. This is dependent on the

circumstances of the population abundance of potential prey species, suitability as a host, and the introduced parasitoids plastic response to a new host. However, the risk of the introduced parasitoid adapting to non-target species is a dangerous outcome that has neglected to take into account the introduced parasitoids plastic response to a new environment and potential hosts (Colmenarez et al. 2018, Kremen and Merenlender 2018, Schulz et al. 2019). Adaptation is the fundamental genetic change of an organism to adjust to a novel environment or environmental conditions. Plasticity is the ability of a phenotype to generate an adaptive response to environmental challenges or changes (Acasuso-Rivero et al. 2019, Gao et al. 2018). Another challenge is that the introduced parasitoid must be capable of successfully parasitizing the target organism and be capable of surviving in the environment it will be introduced to. As part of surviving in the introduced environment it will also face predators and climates that are likely novel to it. If it is successfully introduced and becomes established, then it can be a successful agent of biological control of the target organism. The utilization of foreign parasitoids in this manner has been taking into account the immediate inter-environmental interactions and adaptation over time when implementing this method without understanding the underlying mechanisms of plasticity that are taking place to reach those successful adaptations.

When encountering changing conditions, organisms must have a plastic response to survive and proliferate. The strategies used under the biological control method utilize foreign organisms to control the target population, and by design that organism is being thrust into a novel environment with different potential hosts available in addition to the target species. Knowing how the plastic response of the introduced parasitoid wasp occurs in response to such

variations in host availability can alleviate this naivety. It will also provide assistance in the process of applying and evaluating the organism and the environment involved in biological control management decisions.

Host-Parasitoid System

The host-parasitoid system used in this experiment is between the generalist parasitoid *Leptopilina heterotoma* and the host flies *Drosophila melanogaster* and *Drosophila yakuba. L. heterotoma* is an endoparasitoid wasp that infects early stages of *Drosophila spp.* larvae. This hymenopteran parasitoid is a generalist, meaning it can successfully parasitize a wide range of *Drosophila spp.* (Heavner et al 2013, Moreau and Asgari 2015, Salazar-Jaramillo et al. 2014, Small et al 2012, Wey et al. 2020). It prefers to infect larvae during the first two instars of larval development. This is when the host immune system is most vulnerable and can be more easily manipulated (Moreau and Asgari 2015). When the egg is oviposited within the host fly, it also injects venom. The venom of *L. heterotoma* takes the approach of generic destruction by destroying host hemocytes, thereby preventing any immune response from mounting against the egg (Dziedzic 2016, Goecks et al. 2013, Heavner et al. 2013, Labrosse et al. 2005, Rizki and Rizki 1984, 1990, and 1994, Small et al. 2012, Salazar-Jaramillo et al. 2014, Wey et al. 2020). Upon infection, the *Drosophila* host fly mounts an encapsulation immune response to eliminate the parasitoid. The process is initiated by identification of the egg being a foreign object. Then the process of encapsulation occurs where the parasitoid becomes enveloped with multiple layers of specialized hemocytes. Hemocytes are blood cells found throughout the hemolymph of insects. The first layer is composed of plasmatocytes that recognize the egg as a foreign object. Plasmatocytes are one subtype of hemocytes and are analogous to vertebrate

macrophages and play roles in immunity such as chemotaxis, phagocytosis, and melanotic encapsulation. Then lamellocytes make up the remaining layers that envelop the egg. Lamellocytes are hemocytes with sticky surfaces that envelop the foreign object identified in melanotic encapsulation (DeFilippo and Beck 2018, Goecks et al. 2013, Mrinalini and Werren 2015, Tang 2009). Once the parasitoid is successfully enveloped by lamellocytes, there are a cascade of proteolytic reactions leading to the production of melanin. This process includes the upregulation of enzymes necessary in the pathway of melanin production such as dihydropteridine reductase (DHPR), GTP cyclohydrolase (GCH), tyrosine hydroxylase (TH), dopa decarboxylase (DDC), as well as serine protease which cleaves pro-phenoloxidase (PPO) to phenol oxidase (PO). The activity of serine protease is restricted by serpins except at sites of injury or presence of foreign objects to maintain localized activity (DeFilippo and Beck 2018, Tang 2009). The intermediate quinones and reactive oxygen intermediates generated during melanization kill the parasitoid (DeFilippo and Beck 2018, Tang 2009). This process is referred to as melanotic encapsulation. While this strategy is widely used by many insects, there is variation in immune systems between species that provide novel challenges to potential parasitoid invaders (DeFilippo and Beck 2018, Moreau and Asgari 2015, Mrinalini and Werren 2015, Salazar-Jaramillo et al 2014, Tang 2009).

L. heterotoma venom combats this immune response through a wholesale destructive strategy. First, the venom contains serpin-like proteins whose role is to prevent the activation of serine protease thereby preventing the production of melanin and toxic oxygen radicals. Then, *L. heterotoma* utilizes Rho GTPase Activating Proteins (RhoGAPs). Although their function in parasitization is not yet established, RhoGAPs participate in cell migration, survival, adhesion,

polarity, transcription regulation, cytokinesis and vesicle trafficking in organisms ranging from bacteria to mammals (Huang et al. 2021, Labrosse et al. 2005, Moreau and Asgari 2015, Rizki and Rizki 1984, 1990, and 1994). Evidence suggests that the RhoGAP function in parasitoid venom is to alter the cytoskeleton cell morphology and adhesion abilities of lamellocytes. In the *D. melanogaster – L. heterotoma* interaction, RhoGAPs from *L. heterotoma* infection have been connected to *D. melanogaster* lamellocyte shape alterations to bipolar shapes and loss of adhesive capabilities (Labrosse et al. 2005, Moreau and Asgari 2015, Rizki and Rizki 1984, 1990, and 1994). Thus, RhoGAPs play a major role in the suppression of encapsulation by reducing or eliminating adhesion capabilities of lamellocytes. Finally, the destruction of lamellocytes is carried out by the VLPs. VLPs have been found to have lamellolytic functions, however the mechanism by which this is performed is not known. The VLP attaches to the surface of the lamellocyte where it then enters the cell. Once inside it can be found free floating in the cytoplasm, but will generally remain near the periphery of the lamellocyte rather than in the central region where the nucleus is located. Ultimately the VLPs reduce the concentration of lamellocytes present in the hemolymph (Baryshnikova et al. 2010, Dziedzic 2016, Goecks et al. 2013, Huang et al. 2021, Labrosse et al. 2005, Moreau and Asgari 2015, Mrinalini and Werren 2015, Rizki and Rizki 1984, 1990, and 1994, Tong et al. 2001)

In addition to suppressing the host immune system, there are enzymes and other molecules present that regulate host metabolism. *L. heterotoma* venom was found to include enzymes involved in glycolysis including fructose-bisphosphate aldolase, pyruvate kinase, enolase, and phosphoglycerate kinase (Goecks et al 2013, Moreau and Asgari 2015, Mrinalini and Werren 2015). Due to the importance of their venom in the successful parasitization of the

host for immune system suppression and facilitating a nutritious environment via regulating host metabolism, any plastic response and subsequent adaptation is likely to take place in their venom.

Hypothesis and Rationale

In one experiment on artificial selection, *L. heterotoma* was raised on the invasive species *Drosophila suzukii*. After five generations of artificial selection for killing rate, the killing capability significantly increased (Kruitwagen et al. 2021). This method of artificial selection assists with the methods of biological control utilizing parasitoid wasps to manage invasive species by becoming better parasitoids of the target, but once released to the environment this artificially selected line may prove insufficient or potentially dangerous. This is because the artificial selection utilized in the *D. suzukii* experiment showed that in the lab the parasitoid can be artificially selected for a desired trait such as killing rate. However, upon release, the parasitoid wasp will run into an environment full of variables not present in a lab environment. In the environment in which they are released, a high killing rate at the expense of other traits may prove disadvantageous for survival, or the killing rate is extremely high for the target but abysmally low for other available prey and is unsustainable. It is also possible the killing rate makes them extremely efficient hunters of multiple non-target species that will then be strongly reduced or driven to local extinction by the parasitoid. Thus, knowing how they plastically respond to such environmental changes will assist in the process of applying and evaluating the organism and the environment involved in the biological control management decisions. To remedy this, I studied the plasticity of *L. heterotoma* venom raised on *D. yakuba* from wasps originally reared on *D. melanogaster. D. melanogaster* is a well-studied model

organism that is readily available and is used to set the baseline for the other host. *D. yakuba* immune strategies are capable of successfully resisting a wider range of parasitoids and are more successful than *D. melanogaster* at overcoming parasitization by parasitoids despite their close relation to each other (Salazar-Jaramillo et al. 2014).

Although *D. melanogaster* and *D. yakuba* are closely related and utilize the same melanotic encapsulation strategy, there are variations in their immune response. How *L. heterotoma* will react and ultimately adapt will vary as a result of those immune strategy variations. Therefore, when *L. heterotoma* that has been raised on *D. melanogaster* is raised on other hosts, there will be a plastic response in subsequent generations' venom protein content. I hypothesize that *L. heterotoma* reared on *D. melanogaster* and subjected to a change of host to *D. yakuba* wielding a more successful immune response will result in higher protein production, altered venom content, and changes in relative protein abundance.

METHODS AND MATERIALS

Stock and Experimental Group Maintenance

Fly and wasp stocks are maintained within fly vials on molasses fly food. Lab stocks of *L. heterotoma* have been raised on *D. melanogaster* up to this point, so the initial generation of *L. heterotoma* that will be used as founders for the rest of the wasps in the experiment were reared on *D. melanogaster*. Three independent populations from this initial generation were then established for each host used, *D. melanogaster* and *D. yakuba*. When setting up each infection for each replicate population, the host flies are transferred to a new vial for 24 hours to lay eggs. Then they are removed from the vials and replaced with 5-7 female and 2-3 male *L.*

heterotoma. The *L. heterotoma* are knocked out with carbon dioxide (CO_2) during the transfer. For each generation, including the initial generation, 20 female wasps were harvested for dissection and analysis.

Experimental Design

To identify how the generalist parasitoid wasp *L. heterotoma* responds to environmental change in the form of host variation, I reared an initial generation of *L. heterotoma* on *D. melanogaster*. Then, 20 female wasps are harvested for dissection and venom sample collection, while 3 replicate populations are begun per host, which include *D. melanogaster* and *D. yakuba*. These replicate infection populations were started with 7 female and 3 male *L. heterotoma* each. The 20 harvested female wasps have their venom sacs dissected out, the venom isolated, purified, and then stored at 4°C. The venom sample is then analyzed using an SDS-PAGE gel and ImageJ for protein banding pattern variations and banding intensity variations. The venom sample was also analyzed with a Bradford Assay to measure overall venom protein production. This is done for each replicate of venom sample. Each subsequent generation per replicate had 20 female wasps harvested for dissection and venom sample collection and set up the infection for the next generation. This process repeats until the 6th generation is reached. Upon reaching the 6th generation, all replicates are then returned to being reared on *D. melanogaster* to verify plasticity of changes found.

Wasp Dissection and Venom Purification

Harvested females are knocked out with CO_2 and transferred to a dissection well filled with 70% ethanol. Then they are transferred to a holding well filled with phosphate buffer

solution (PBS). For dissection, one at a time will be moved to another well filled with PBS. Using a dissecting scope and fine tipped forceps the ovipositor and venom sac are removed and placed in an Eppendorf tube filled with 150µL PBS. The Eppendorf tubes are maintained on ice. Upon completion of all dissections the venom is then centrifuged at 4°C at 14.0 RPM for 5 minutes. Then the venom has 1.6µL Protease Inhibitor added to it and homogenized using a homogenizer for 20 seconds. The venom is then recentrifuged at 4°C at 12.0 RPM for 10 minutes. Finally, the venom is pipetted out, transferred to a new Eppendorf tube, and leave behind the debris pellet to be disposed of. The resulting venom is stored at 4°C.

Venom Protein Production Measurements

A Bradford Assay was performed to measure venom protein production. First, 5µL of the venom sample is added to 15µL of PEB+ and vortexed. The vortexed sample is then added to 980µL of Bradford reagent and vortexed once more. The Bradford reagent is a dye that binds to proteins present within the sample. Then a sample of BSA and PEB+ is prepared, then 20µL of the prepared BSA and PEB+ is added to 980µL Bradford reagent. This sample is the standardized sample that all venom samples are compared to and utilized to measure the amount of venom proteins produced. The venom and standardized samples are then incubated at room temperature (21°C) for 10 minutes. This allows time for the Bradford reagent to bind to the proteins present in the sample. The samples will then be analyzed using the spectrophotometer set to absorbance 595nm. The standardized sample produces a standard curve that will be used to estimate the amount of protein in the venom samples.

SDS-PAGE Gel Electrophoresis and Imaging

SDS-PAGE Gel Electrophoresis was performed using a 9% resolving gel and a 4-20% gradient resolving gel. Gradient gels can further separate bands of similar sizes and allow for more distinct differentiation of bands that are very close to or otherwise are indiscernible from one another. Venom samples are prepared by mixing 15µL venom with 15µL 2X sample buffer. Protein ladder and venom samples are then loaded into the gel's wells and placed in the electrophoresis chamber for one and a half hours at 40mA per gel. Gels are removed from the electrophoresis chamber and then stained with blazin bright overnight. The blazin bright stains the proteins in the gel, allowing for easy viewing of proteins under the UV transilluminator. The gel is then imaged using the UV transilluminator with the ethidium bromide filter active.

SDS-PAGE Gel and Venom Protein Production Analysis

SDS-PAGE gels were analyzed using ImageJ for each venom sample by plotting band intensity. This is done by sectioning off each band and measuring the area under the curve to obtain band intensity. The plots are used to identify the intensity of each band to measure individual band venom production. The values of intensity as well as overall venom protein production are standardized and statistical analysis is done using SAS analytics software. Inspection of the banding pattern with the assistance of ImageJ identifies the addition or absence of protein bands to indicate protein modification changes. Figure 1 shows how sectioning of the bands was performed for analysis and band differentiation. The boxes are how ImageJ sections off target areas for analysis. The gel without the boxes is the same gel as the one with them, but allows for a better visual of the bands than when the boxes are present.

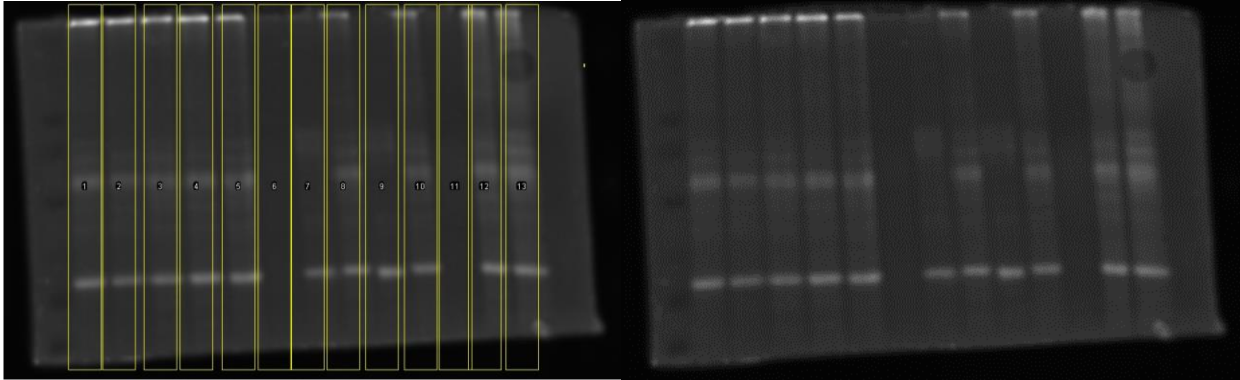

Figure 1. ImageJ Gel Sectioning and Analysis. Both gels above are the same except that the left image of the gel includes the boxes used to section off target areas for analysis. Each box encompasses a lane within the gel. Then the output provides the intensity of all bands within that established region. When collecting and interpreting this data both versions of the imaged gel above for all gels was necessary for intensity and visual examination.

SAS Statistical Analysis

The following statistical analysis was performed using SAS analytics software. The hypotheses and predictions analyzed here were that rearing *L. heterotoma* on *D. yakuba* would result in increased overall venom protein production and would result in increased band intensity. First, to test the effect of the host change on overall venom protein production I tested the covariate of generation to see if it would be prudent to perform an ANCOVA. Since the covariate of generation was not found to be significantly related to the standardized absorbance, instead an ANOVA was performed on the data. The raw data did not meet the assumptions of the test and the standardized absorbance was log transformed, after which the data met the assumptions to perform the ANOVA. The assumption of homogeneity of variance and normal distribution of data was visually determined by inspecting the residuals. Brown and Forsythe's test for homogeneity of variance also supported the data meeting the assumption of homogeneity of variance.

To test the band intensity obtained from the SDS-PAGE gels and ImageJ, I began by determining if I should perform an ANCOVA. The covariate of generation was found to be significant in both hosts, therefore I determined that I would perform an ANCOVA. Next, I tested the interaction effect between generation and host to determine whether or not the slope of the relationship between generation and intensity was the same for each band. I determined that the slopes are equal. Therefore, I removed the interaction from the model and tested the assumptions of homogeneity of variance and normal distribution of residuals for the ANCOVA. To determine if the homogeneity of variance and normal distribution of residuals assumptions were met, I visually inspected the Q-Q Plot of residuals and the histogram of the residuals respectively. After square root transforming the data to meet the assumptions, I then ran the ANCOVA. P-value results from this ANCOVA were evaluated by adjusting the critical p-value using the sequential Bonferroni method, which conserves more power and is less conservative than the Bonferroni correction. In this method all p-values from the tests are ranked from smallest to largest. Then starting with the first ranked (smallest p-value) the experimental p-value (0.05) is corrected using the following formula: $P_C < P_E/(S-(K-1))$. P_C is the corrected p-value. P_E is the initial experimental p-value of 0.05. S is the total number of tests, which in this case is 14. K is the rank of the p-value being evaluated. Then, the observed p-value will be evaluated by the P_C calculated for it as the new critical p-value.

A couple of follow up tests were performed on the band intensity data. The first was to perform the same test but first separating the tests by host. This will allow me to test the individual effect of generation on each host individually. The ANCOVA for each host (*D. melanogaster* and *D. yakuba*) were performed following the same steps as previously described

including the square root transformation. The p-values were evaluated following the same sequential Bonferroni correction as previously described. The other follow up test was to view the intensity of each band as a percentage of the whole present for each individual replicate. To evaluate this data, a Kruskal-Wallis test was performed.

RESULTS

Venom Protein Production

Once the Bradford Assay had been performed and the raw data absorbances had been collected, all absorbances were standardized using the standard curve to allow for ease of comparison. Analysis of the absorbances began by determining if performing an ANCOVA was the correct course of action by first testing if the covariate generation was significantly related to the standardized absorbance for either host. For neither host, *D. melanogaster* and *D. yakuba*, was the covariate generation significantly related to the standardized absorbance ($F_{1,24}$=3.49, p=0.0747 and $F_{1,17}$=0.03, p=0.8645 respectively). Therefore, an ANCOVA was not necessary, and an ANOVA was performed instead. The assumptions of an ANOVA are: all observations are random and independent, the variance of all groups is the same (homogeneity of variance), the residuals are normally distributed, and the effects of being in a group are additive and constant. The experimental design ensured that all observations are random and independent from each other and interaction effects for being in a group are additive and constant. In SAS I tested the homogeneity of variance and residuals being normally distributed assumptions by visual inspection of the residuals by predicted scatter plot and histogram respectively. Figure 2 shows the histogram and scatter plot of the residuals from the

standardized absorbances used to test the assumptions. While the scatter plot for homogeneity of variance meets the assumption, the distribution of the residuals does not. The Brown and Forsythe's test for homogeneity of variance further supports that the data meets the assumption of homogeneity of variance ($F_{1,41}$=1.02, p=0.3192). Therefore, the standardized absorbance was log transformed. Doing so resulted in both graphs meeting the assumption and again the Brown and Forsythe test supported that the data meets the assumption of homogeneity of variance ($F_{1,41}$=0.31, p=0.582)(Figure 3). Now that the data meets the assumptions for the test, the ANOVA was then performed. The result of the ANOVA determined that the change in host did not affect the overall production quantity of venom proteins ($F_{1,41}$=0.29, p=0.5909). Figure 4 shows the average overall protein quantity for each host across all generations. Generation 0 is the founding generation and generation 9 is the *D. yakuba* wasps reintroduced to *D. melanogaster*. Throughout the generations the overall protein quantity produced by the wasps of either host did not waver significantly from each other.

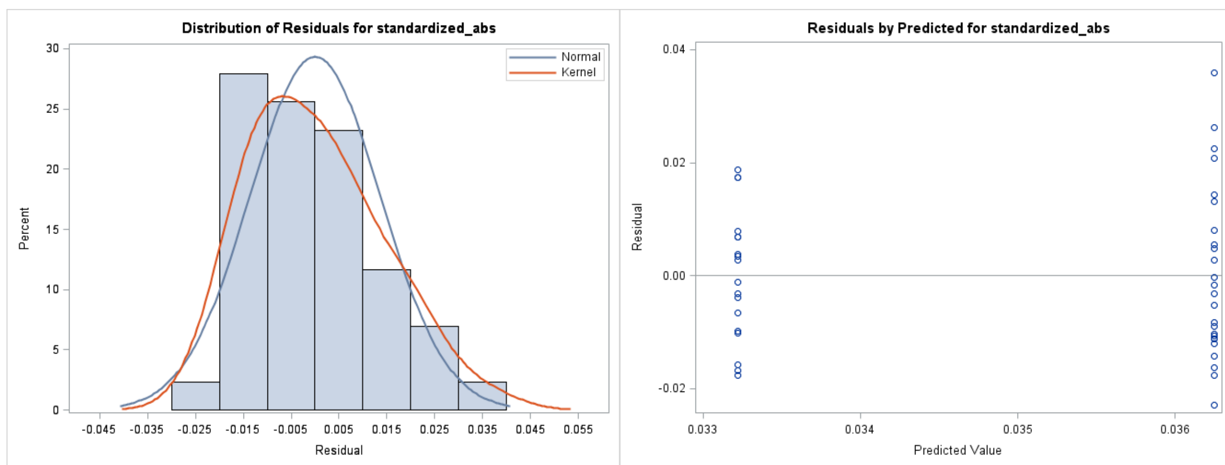

Figure 2. Overall Protein Production Test of Assumptions. The histogram (left) is the graph visually inspected for the assumption of normal distribution of the residuals. The left leaning state of the distribution does not meet the assumption. The scatter plot (right) is the graph visually inspected for the assumption of homogeneity of variance. The variances are quite similar and is further supported by the Brown and Forsythe's test for homogeneity of variance ($F_{1,41}$=1.02, p=0.3192).

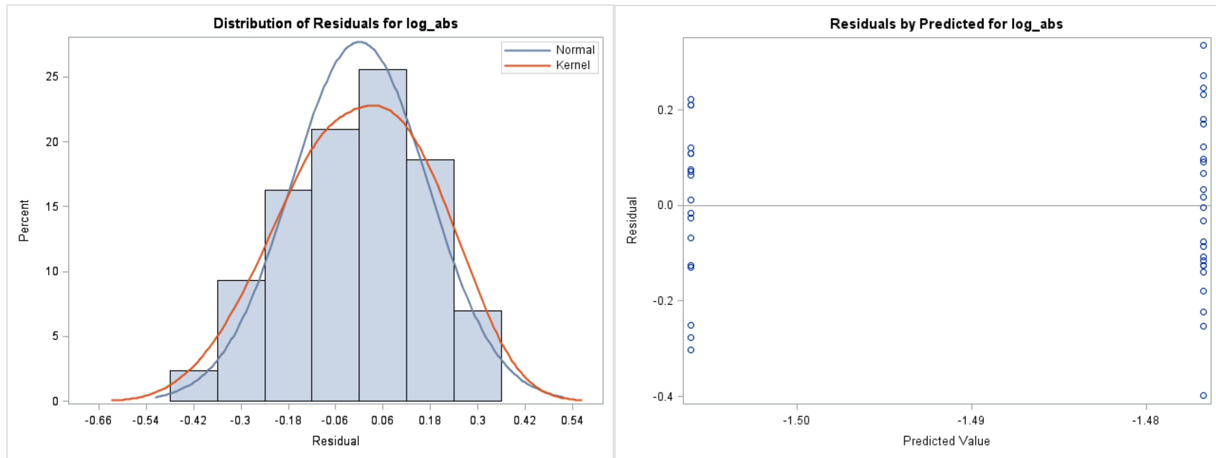

Figure 3. Log Transformed Overall Protein Production Test of Assumptions. The histogram (left) is the graph visually inspected for the assumption of normal distribution of the residuals and determined that it meets the assumption. The scatter plot (right) is the graph visually inspected for the assumption of homogeneity of variance. The variances are quite similar and is further supported by the Brown and Forsythe's test for homogeneity of variance ($F_{1,41}$=0.31, p=0.582).

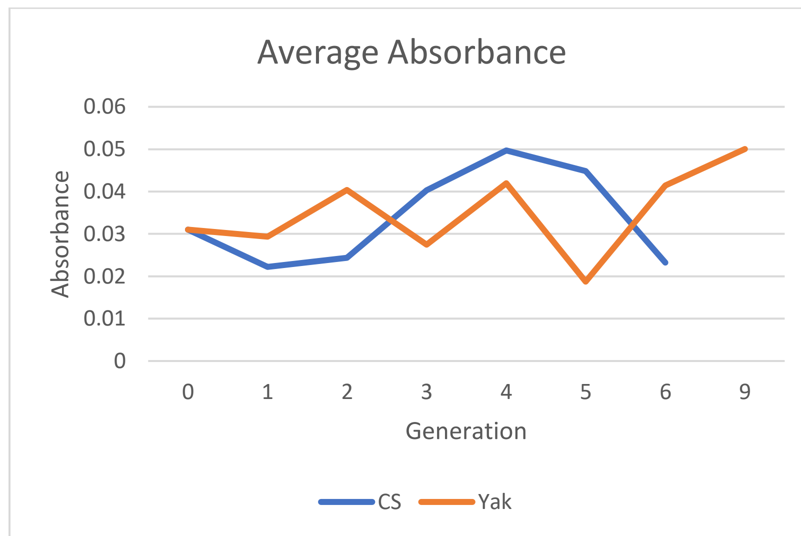

Figure 4. Average Overall Protein Quantity Across Generations. The graph shows *D. melanogaster* (CS) and *D. yakuba* (Yak) average overall protein production across all generations. Generation 0 is the founding generation from which all subsequent lines were founded. Generation 9 is the generation that each *D. yakuba* line was reintroduced to and reared on *D. melanogaster*.

SDS-PAGE Gel Electrophoresis

As each generation of wasp eclosed and the venom samples were collected, the venom was put into a gel for visualization of individual protein bands. Figure 5 includes the gels that had all replicates run on them. Figure 6 shows the gel with the best visually apparent bands

among each generation for each host. However, all ImageJ data collection and SAS analysis was performed from data collected from the original gels in figure 5.

Once each gel was completed the image of the gel was opened in ImageJ, sectioned off, and all band intensities were recorded. Table 1 shows the summary of each band's approximate size and whether or not it was present or absent in each of the host groups. All bands were found in both hosts except for band 14 which appeared in *D. melanogaster* generation 3 and 4 only. The band intensities were then standardized the same as the absorbances from the Bradford assay. Following the same process, I first tested generation as the covariate for its effect on each host for band intensity, and I found that it had a significant effect on band intensity in both *D. melanogaster* and *D. yakuba* ($F_{1,106}$=11.4, p=0.001 and $F_{1,70}$=8.36, p=0.0051 respectively). Therefore, it was determined that I would run an ANCOVA. The next step was to determine if the slope of the relationship between the covariate generation and intensity are the same for each group to determine if I will do the equal slope or unequal slope ANCOVA. The slope of the interaction between covariate generation and host was found to be equal. Therefore, I will perform an equal slope ANCOVA without the interaction effect ($F_{1,177}$=0.17, p=0.6829). ANCOVA uses the same assumptions as ANOVA and so I am testing them in the same fashion as before. The initial data failed to meet the assumption for homogeneity of variance, but the distribution of residuals was normally distributed (Figure 7). To correct for this the standardized intensity was square root transformed. This resulted in meeting the assumptions for homogeneity of variance and normal distribution of residuals (Figure 8).

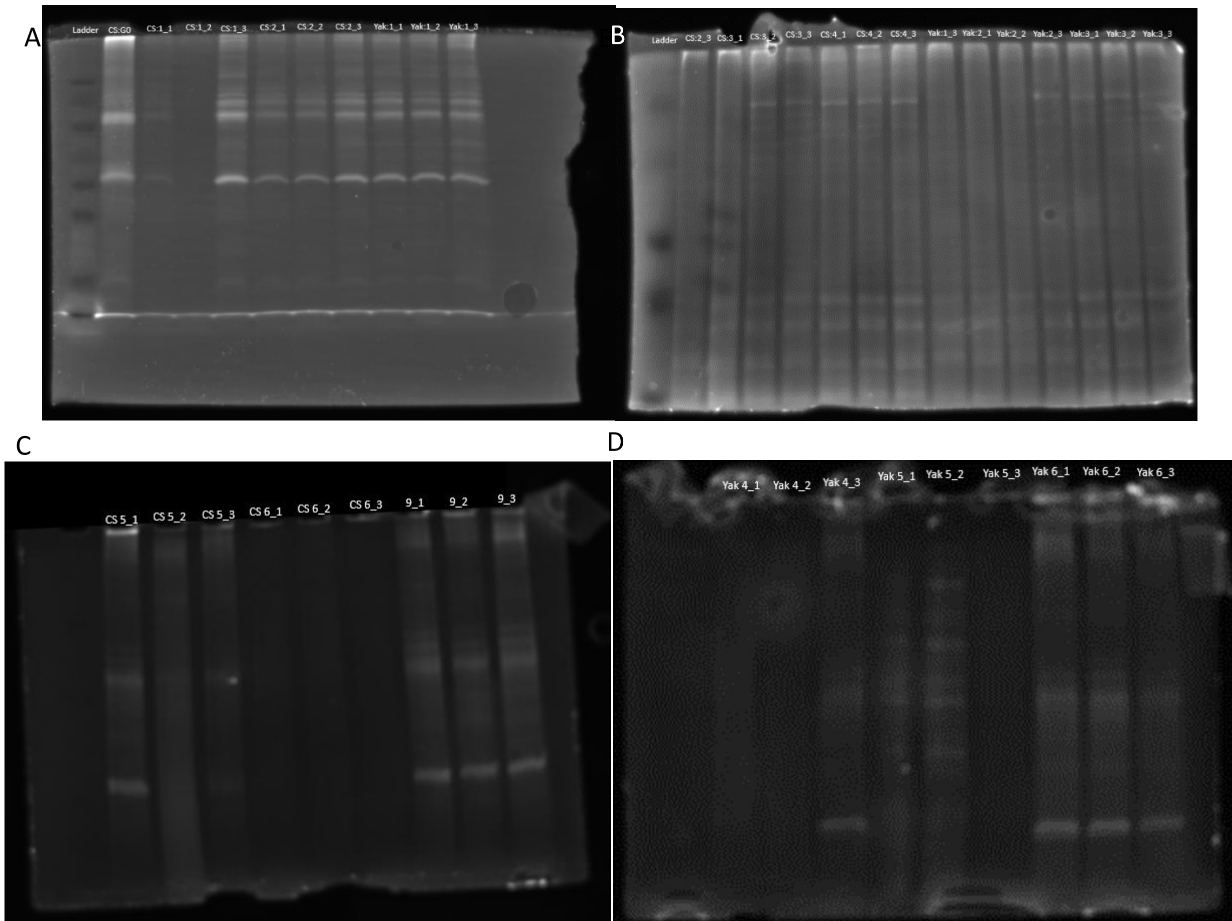

Figure 5. Gels of All Replicates for *D. melanogaster* (CS) and *D. yakuba* (Yak). These gels are the gels used for data collection and subsequent analysis of banding pattern and intensity. Labels indicate fly species, generation, and replicate. (A) This gel includes the founding generation, CS generation 1 and 2, and Yak generation 1. (B) This gel includes CS generation 3 and 4 and Yak generation 2 and 3. (C) This gel includes CS generation 5, 6, and 9 (Yak reintroduced to CS). (D) This gel includes Yak generation 4, 5, and 6.

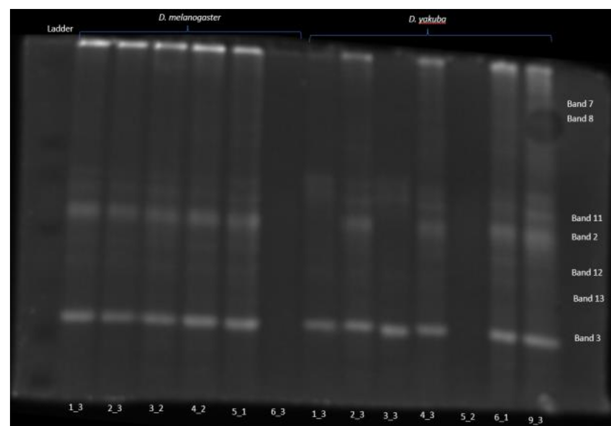

Figure 6. Gel Consisting of the Best Visually Apparent Replicates. The above gel includes the replicate from each generation and host that had the most visually apparent bands for ease of comparison. The bands noted on the right side of the gel indicate which bands they represent on the gel. The species of each lane is labeled across the top of the gel and the generation_replicate is labeled across the bottom of the gel.

Now that the assumptions for the test are met, the ANCOVA was performed. The ANCOVA was run and subdivided to test each band identified. The results from each band for covariate generation and main effect of host are presented in Table 2. After performing the sequential Bonferroni correction to evaluate the observed p-values, it was determined that generation had a significant effect on band intensity of bands 11 and 2 ($F_{1,22}$=32, p<0.0001 and $F_{1,18}$=12.28, p=0.0029 respectively).

This raised the question of how the effect of generation had on either host. So, two ANOVAs were performed to assess the effect of generation on each band for separated by host. P-values were evaluated after a sequential Bonferroni correction. The generation effect for *L. heterotoma* raised on *D. melanogaster* had no significant effect for band 11 nor 2 ($F_{1,12}$=10.94, p=0.007 and $F_{1,11}$=4.46, p=0.0609 respectively). The generation effect for *L. heterotoma* raised on *D. yakuba* had a significant effect for band 11, 2, and 3 ($F_{1,9}$=69.03, p<0.0001; $F_{1,6}$=36.43, p=0.0018; $F_{1,4}$=12.09, p=0.0401 respectively).

These results raised the question of how the overall venom protein quantity could be unaffected if the intensity of the bands were being affected, which would indicate changes in specific protein production at the loss of others. So, the intensities of each band present in each replicate were calculated as a percent of all intensity and bands present for that replicate. This percent data was then used to test if the change in host affected the percent makeup of each band in relation to all venom protein produced. For this test, a Kruskal-wallis test was performed and only band 8 was found to be significantly impacted by the change in host (X^2=4.2667, p=0.0389).

Band ID	Size (kD)	CS	Yak
1	>250	y	y
2	120	y	y
3	55	y	y
4	25	y	y
5	10	y	y
6	>250	y	y
7	>250	y	y
8	>250	y	y
9	250	y	y
10	170	y	y
11	140	y	y
12	85	y	y
13	70	y	y
14	>250	y	n

Table 1. Presence/Absence Table. Each band is identified by an arbitrarily determined number ID and those bands are sized and noted whether or not they appear in any generation or replicate within either host. CS is *D. melanogaster* and Yak is *D. yakuba*.

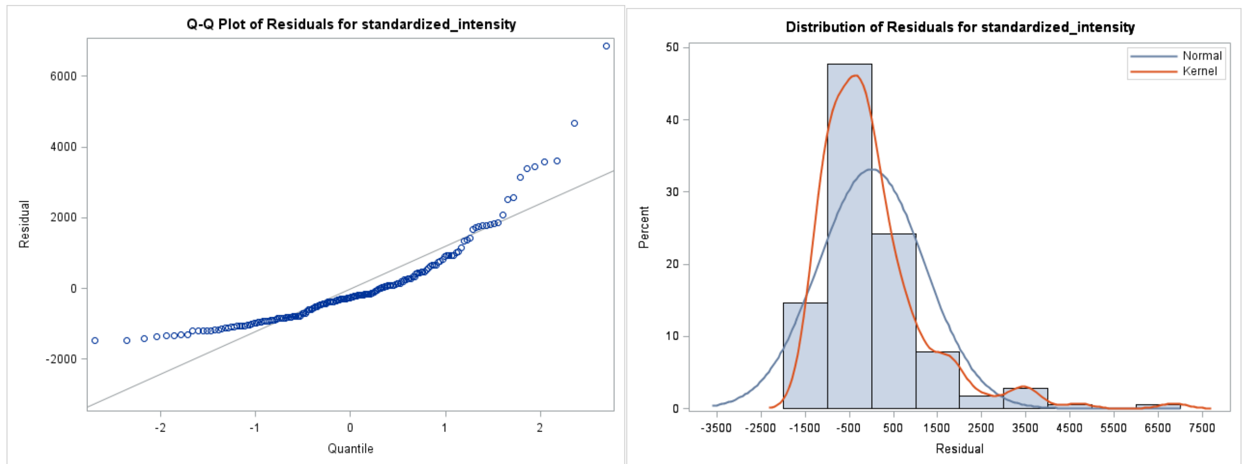

Figure 7. Band Intensity Test of Assumptions. The histogram (right) is the graph visually inspected for the assumption of normal distribution of the residuals and is normally distributed. The Q-Q plot (left) is the graph visually inspected for the assumption of homogeneity of variance. The significant bending of the residuals away from the line indicates that the data does not meet the assumption for homogeneity of variance.

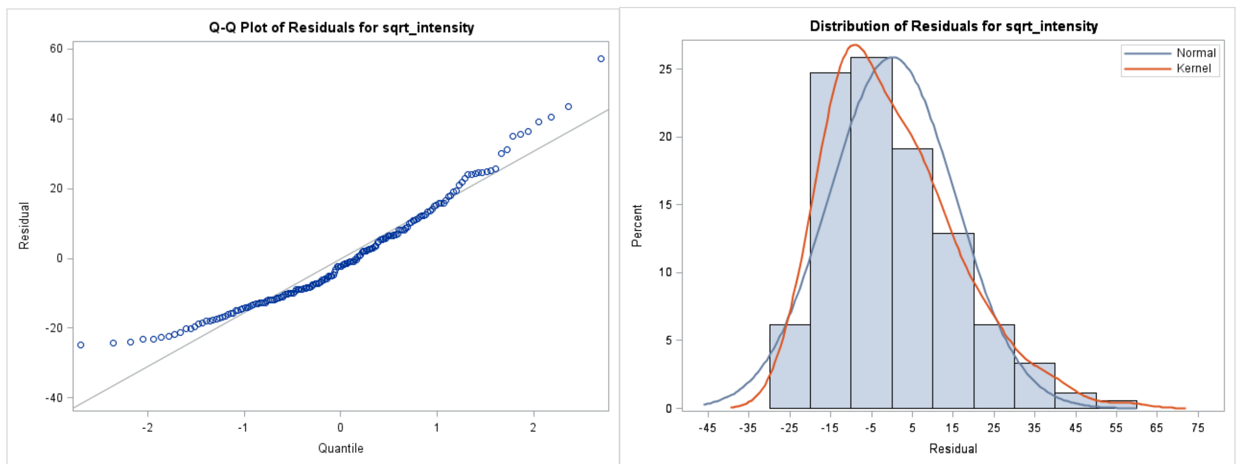

Figure 8. Square Root Transformed Band Intensity Test of Assumptions. The histogram (right) is the graph visually inspected for the assumption of normal distribution of the residuals and is normally distributed. The Q-Q plot (left) is the graph visually inspected for the assumption of homogeneity of variance. The slight bending of the residuals away from the line is within acceptable range to determine that the data meets the assumption for the homogeneity of variance.

DISCUSSION

Venom Production Analysis

I predicted that *L. heterotoma* reared on *D. melanogaster* and then changed to be reared on *D. yakuba* would result in an increase in the overall quantity of venom proteins being

produced. This was a likely result as *L. heterotoma* as a generalist utilizes a strategy of generic destruction by destroying host hemocytes (Dziedzic 2016, Goecks et al. 2013, Heavner et al. 2013, Labrosse et al. 2005, Rizki and Rizki 1984, 1990, and 1994, Small et al. 2012, Salazar-Jaramillo et al. 2014, Wey et al. 2020), and coming into contact with a new host might have *L. heterotoma* respond by increasing the venom protein production as a plastic response to this new host. However, I found that the total venom protein quantity being produced was not altered as a result of being reared on a different species of host (*D. yakuba*). The average absorbance of those reared on *D. melanogaster* and *D. yakuba* do not differ significantly from each other despite the strange up and down pattern seen in the *D. yakuba* reared wasps. This could be due to the fact that *D. melanogaster* and *D. yakuba* are of similar size and therefore provide a similar amount of resources for *L. heterotoma*. If this is the case, then producing more proteins would be counterproductive as doing so would only waste resources that could be used elsewhere. The extra proteins produced would not have anything to act on and would remain unused. On the other hand, this could indicate that the overall amount of protein produced does not mean that *L. heterotoma* will be more successful at parasitization. Instead, it would be more effective to make a specific change to a few important proteins. The gels performed provide this more in-depth look into the specific protein effects.

SDS-PAGE Gel Electrophoresis

I predicted that *L. heterotoma* reared on *D. melanogaster* and then changed to be reared on *D. yakuba* would result in the increase of protein band intensity and banding pattern. Within the first 3 generations of being reared on *D. yakuba* the *L. heterotoma* saw band 2, a

major band in all *D. melanogaster* generations, in generation 2, 3, and 4 vanish completely. In generation 2 and 3 in the *D. yakuba* group band 7 became duller and band 8 vanished

	Generation		Host	
Band ID	F Statistic	P Value	F Statistic	P value
1	$F_{1,18}$=0.07	0.792	$F_{1,18}$=0.95	0.3449
2	$F_{1,18}$=12.28	0.0029	$F_{1,18}$=0.45	0.5126
3	$F_{1,15}$=8.95	0.0104	$F_{1,15}$=0.00	0.9969
4	$F_{1,7}$=5.02	0.0752	$F_{1,7}$=0.74	0.4301
5	$F_{1,9}$=0.00	0.9989	$F_{1,9}$=1.52	0.2573
6	$F_{1,14}$=1.26	0.2843	$F_{1,14}$=0.11	0.743
7	$F_{1,4}$=0.01	0.9219	$F_{1,4}$=1.44	0.3525
8	$F_{1,8}$=0.38	0.5625	$F_{1,8}$=2.68	0.1527
9	$F_{1,17}$=0.53	0.4772	$F_{1,17}$=1.38	0.2576
10	$F_{1,21}$=2.54	0.1274	$F_{1,21}$=0.24	0.6329
11	$F_{1,22}$=32	<0.0001	$F_{1,22}$=2.25	0.1491
12	$F_{1,7}$=0.33	0.5924	$F_{1,7}$=0.01	0.9112
13	-	-	-	-
14	$F_{1,3}$=4.12	0.1794	-	-

Table 2. ANCOVA Results. Each band is associated with the covariate generation and main effect host F and p values. After performing the sequential Bonferroni correction only band 2 and 11 had significant effect on their intensity by generation.

completely. In generation 5 and 6 of the *D. yakuba* group, bands 12 and 13 became more prominent. Finally, when reintroduced to *D. melanogaster* the previously described differences reversed back to the same as they are in the control group (*D. melanogaster* reared) except bands 12 and 13 remain more prominent (Figure 5 and 6).

The protein band intensity statistical analysis found some unexpected results. The initial ANCOVA found that host did not have an impact on the band intensity, but the covariate of generation did. This could indicate that there could have been other selective pressures at play that could have impacted the result, or could be as a result of genetic drift, or due to the large number of tests performed could have been due to increased potential of type I errors. I performed the sequential Bonferroni correction to compensate for this potential pitfall and still found that band 11 and 2 were significant. To get a better understanding of this result, I plotted each band's intensity across generations for each replicate (Figure 9). Band 11 for the *D. yakuba* replicates follows a downward trend while the *D. melanogaster* replicates decrease initially, but after 3 generations the intensity increases in the following generation. After the fourth generation, band 11 did not appear in subsequent *D. melanogaster* generations. However, the decrease in band 11 intensity is much faster in *D.* melanogaster than in *D. yakuba.* The selective pressures associated with *D. yakuba* resulted in the decrease in production of proteins in band 11 initially and decreased at a slower rate than *D. melanogaster* (Figure 9A). The difference in band 2 banding patterns follow a similar decrease in intensity between the *D. melanogaster* and *D. yakuba* reared *L. heterotoma,* while *D. yakuba* keeps a higher concentration of band 2 proteins. In the first 2 generations, *D. melanogaster* intensity nosedived while *D. yakuba* maintained more than three times the amount of intensity as *D. melanogaster* in the first

generation. However, in subsequent generations *D. melanogaster* wasps continued to decrease but *D. yakuba* wasps lost band 2 in generations 2, 3, and 4. This would represent a plastic response where band 2 is lost in *D. yakuba* wasps and reappears later in generation 5 and 6 and is kept when reintroduced to *D. melanogaster* (Figure 9B).

Then to answer the question of generation's effect on each host separate from the other on each band, I performed the same analysis but with *D. melanogaster and D. yakuba* separately. This resulted in generation having no significant impact on *D. melanogaster* band intensities, but it was significantly impacting *D. yakuba* band 11, 3 and 2 intensities. Since the effect of generation is only significantly impacting the *D. yakuba* reared wasps it would indicate that there is some selective pressure being applied only to that group. As the only difference between the two groups is the host, I can conclude that the difference in host is responsible for this intensity variation. Also, because the variation for these band's intensity and banding pattern is primarily found in the first 3 generations of *D. yakuba*, and when reintroduced to *D. melanogaster* these changes reversed to match those of *D. melanogaster* reared *L. heterotoma*, then I can conclude that the effects seen in band 11, 3, and 2 are as a result of their plastic response to being reared on a new host (Figure 9A, B, C).

Finally, when looking at the band intensities as a percentage of all protein for that replicate, it was found that band 8 was significantly impacted by the change in host. While the intensity values of band 8 across generations may not have been significant there was a significant change in the proportion of band 8 that made up the total venom protein produced (Figure 9D). As a percentage of the total venom protein produced band 8 maintained about a 4% makeup of the *D. melanogaster* reared while the *D.* yakuba reared wasps had band 8

makeup 12.2% in generation 3 and 26.7% in generation 5. In addition to band 11, 3, and 2 as previously found, band 8 also is affected by the change in host.

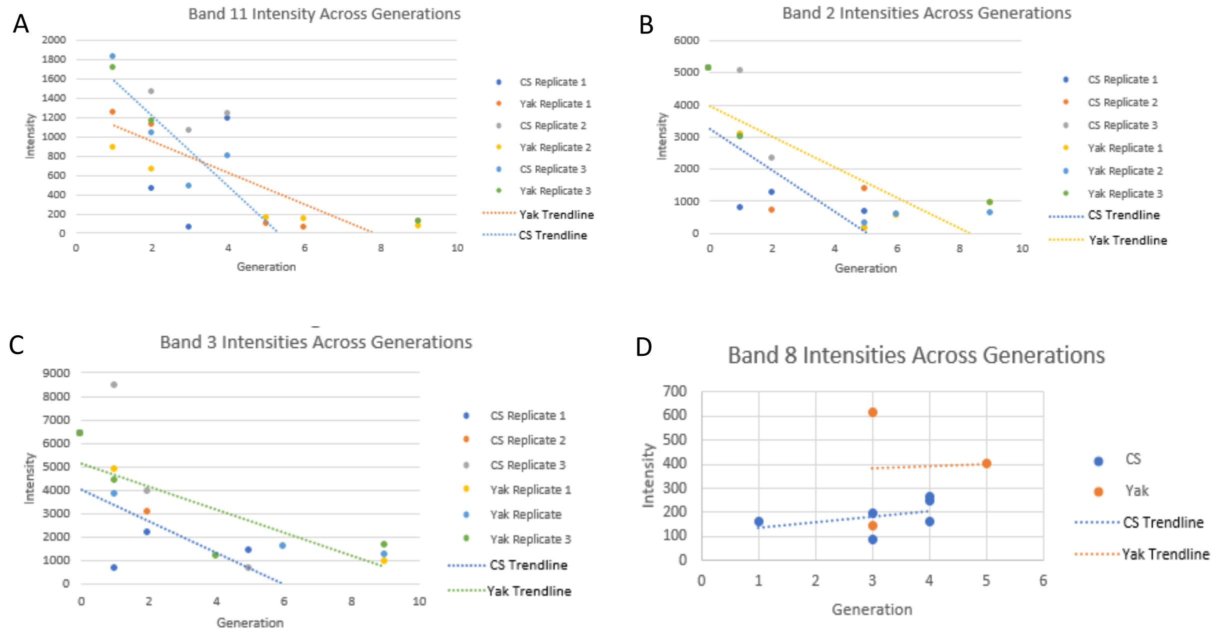

Figure 9. Intensity Patterns Across Generations of Bands 11, 2, 3, and 8. (A) Band 11 intensity pattern across generations for each replicate of *D. melanogaster* and *D. yakuba*. *D. yakuba* and *D. melanogaster* follow a downward slope (-163.77 and -367.38 respectively) but *D. melanogaster* intensity falls much faster than *D. yakuba*. (B) Band 2 intensity pattern across generations for each replicate of *D. melanogaster* and *D. yakuba*. Both follow a negative slope (-648.2 and -490.97 respectively) but *D. yakuba* maintains a higher level of band 2 proteins in the first 2 generations than *D. melanogaster* does despite the fact that band 2 did not appear in *D. yakuba* generation 2, 3, and 4. (C) Band 3 intensity pattern across generations for each replicate of *D. melanogaster* and *D. yakuba*. Both follow a negative slope (-664.93 and -487.68 respectively) but *D. yakuba* decreased at a slower rate leveling out around intensity 1500. (D) Band 8 intensity pattern across generations for each host. Band 8 appeared 6 times in *D. melanogaster* reared *L. heterotoma* while band 8 appeared on those reared on *D. yakuba* only 3 times. It is difficult to draw conclusions from so little data but both host groups, *D. melanogaster* and *D. yakuba*, had a slightly positive slope (24.24 and 10.761 respectively) and *D. yakuba* had a significantly higher intensity than *D. melanogaster* (X^2=4.2667, p=0.0389).

CONCLUSION AND FUTURE DIRECTIONS

Parasitoid wasps are the most common biological control agents used worldwide against arthropod pests and invasive species. Yet, the studies concerning their future viability and immediate viability for implementation as a biological control agent has remained isolated

to adaptation studies. However, when released into the wild they will encounter numerous environmental conditions and organisms not found in the lab that could easily change the viability of a biological control agent previously deemed viable. To remedy this gap in knowledge I set out to understand the plastic responses of a parasitoid biological control agent currently being used and researched as a control agent for the pest *D. suzukii*, *Leptopilina heterotoma* (Kruitwagen et al. 2021 and Quicray et al. 2022). *L. heterotoma* was reared on *D. melanogaster* and subsequent lines were reared on *D. melanogaster* or *D. yakuba*. As a result of this change in available hosts for infection, the venom of the wasps resulted in significant changes to specific proteins found in bands 11, 3, 2, and 8. Band 11 decreased, bands 3 and 2 kept a higher amount of those proteins before declining after 3 generations, and band 8 made up a significantly higher proportion of *L. heterotoma*'s total venom protein than the control group reared on *D. melanogaster*.

The study performed laid the groundwork to establish that how a change of host even as closely related to each other as *D. melanogaster* and *D. yakuba* are, can impact the specific protein production and overall composition within the *L. heterotoma* venom. The next steps to this study are to determine what proteins bands 11, 3, 2, and 8 represent and what their functions are. Doing so will provide the information to better understand how they plastically respond to overcome the challenge of preying upon a new host. Doing so would then better prepare those who wish to use *L. heterotoma* and other parasitoid wasps as biological control agents.

To do so, a follow up to this experiment would be to begin with a sucrose gradient to get a breakdown of the protein bands, identifying how much of that protein is present at that

band size, and reconfirm the results presented in this paper. Then the gels can be fractioned out at bands 11, 3, 2, and 8 and ran through mass spectrometry to determine what proteins are present there. The fractions would cover the size of >250kD, 140kD, 55kD, and 120kD (Table 1). Following this and potentially determining the function of these proteins, the structures of the proteins could be predicted to confirm the function or if unknown then the predicted protein structures can be used to predict the function of those proteins.

Something that this study did not include was to encompass a wider range of possibilities the *L. heterotoma* may encounter in the wild. *D. yakuba* is just one example of a host that has a better resistance to parasitoids and higher encapsulation rate. Therefore, to cover a wider range of parasitoid resistances and encapsulation rates I would suggest expanding the experiment to include two other hosts, *Drosophila simulans* and *Drosophila ananassae,* which are more distantly related to *D. melanogaster* than *D. yakuba* is. *D. simulans* also has a better resistance to parasitoids than *D. melanogaster* as well as higher encapsulation rate. *D. ananassae*, however, has less resistance to parasitoids and has a lower encapsulation rate than *D. melanogaster*. This would provide a wider range of immune systems that the parasitoid would have to contend with. I would also suggest expanding the experiment to include another parasitoid of these *Drosophila spp.*, *Leptopilina boulardi*. *L. boulardi* should be included because it is a specialist on certain *Drosophila spp.* Understanding how the specialist versus the generalist plastically responds to new hosts could make choosing specialists as a biological control agent more possible as their inherent limitation to certain species makes them an important potential agent to control the target population without the risk of off-target effects.

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