2019

Insects and the Evolution of Developmental Pathways: Functional Analysis of Genetic Sex Determination Mechanisms in Oncopeltus fasciatus

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Honors Thesis 2019

Colby College Department of Biology

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Functional Analysis of Genetic Sex Determination Mechanisms in *Oncopeltus fasciatus*

Honors Thesis

Presented to

The Faculty of the Department of Biology

Colby College

In partial fulfillment of the requirements for the

Degree of Bachelor of Arts with Honors

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Waterville, ME

May 20, 2019

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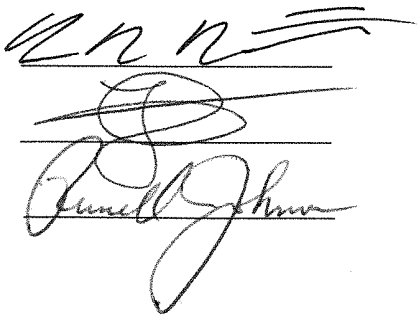

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ABSTRACT

Natural selection alters genetic pathways over evolutionary time. However, different pathways have been shown to maintain mutations at varying rates, leading to different levels of conservation across developmental pathways. Sex determination pathways, in particular, show vast diversity across animals despite the fundamental conservation of biological sex itself. Here, I investigated the sex determination pathway of the hemimetabolous insect, *Oncopeltus fasciatus*. The large milkweed bug, *O. fasciatus,* is part of the order Hemiptera, which provides a relevant outgroup study to other insects in which sex determination has been studied to date. I studied three sex determination genes, *intersex, fruitless,* and three paralogs of *doublesex* via a set of phylogenetic sequence comparisons, qRT-PCR gene expression studies, and RNAi functional analyses. I report that somatic sex differentiation requires these genes in a tissue-specific manner. Additionally, the *doublesex* locus might be less conserved than *intersex* and *fruitless. intersex* and all three *doublesex* paralogs appear to be moderately expressed during key time periods of sex determination in *O. fasciatus* specimens. Finally, I find that *intersex, fruitless,* and *doublesex-c* (or potentially all three *doublesex* paralogs in males) are required for the determination and development of two sexual dimorphisms in *O. fasciatus* in tissue-specific combinations. All three target genes appear to be required for sex determination and development in both sexes of *O. fasciatus.* These results indicate novel functions and/or locus evolution of *intersex, fruitless,* and *doublesex* in *O. fasciatus* compared to other insects, indicating evolution of the sex determination pathway within the insect lineage.

ACKNOWLEDGEMENTS

This project would not exist without the support of a myriad of people or without the necessary funding.

The funding of this research has been supported by the Colby College Division of Natural Sciences, an Institutional Development Award from the National institute of General Medical Sciences of the National Institute of Health (Grant Number: P20GM0103423), and by the grant IOS-1350207 from the National Science Foundation.

Professor David Angelini had the conception and vision of this project. His genuine enthusiasm for research and teaching fueled my dedication to studying evolution and development and kept me engaged over the years. As a mentor, Dave has taught me ownership over my learning and my work by giving me an intricate balance of guidance and independence. Through engaged discussions of data and excited planning of new experiments, he continues to remind me of the curiosity and wonder at the root of scientific questions - and to enjoy the process of investigation. I look up to him on an academic and also a human level.

Professors Russell Johnson and Johanna van Oers have provided invaluable guidance along the way of completing this project. They have been kind and patient while holding me to a high standard, which ensured I did my best work in a sustainable and supported way. Their intentional presence throughout this process taught me the importance of thoughtful feedback and genuine care for one's colleagues. I so value the time and energy they invested into my growth this past year and throughout my time at Colby. I feel lucky to know them.

Devin O'Brien, Lyndell Bade, and Josh Steele have been tremendously supportive in my writing of this thesis. They have gifted me their time in the lab, their mental energy in discussing data and drafts, and their emotional support in checking in with me throughout the weeks. They

have helped me find a community in Colby's Biology Department and the Angelini lab. I hope to continue finding such wonderful, honest people in future scientific communities I may enter.

Mara Laslo, Zhoufan Zhang, and Jane Lee contributed to the data collection and analysis of this project in tangible ways. They performed some RNAi knockdowns, measurements, and helped with data analysis. Mara Laslo, in particular, laid a lot of the foundation of this project. Science is always collaborative and this project is also theirs.

Finally, the rest of the Angelini lab members, many other students, Professors and staff at Colby College, and my other friends and family have contributed to my growth as a scientist and a person over the years. They are my community and I love them.

Indeed, this project would not exist without these people. I would not be the scientist or person I am without these people. Or without this project. I am tremendously grateful for the experience, for the lessons, and for the relationships. I can only hope to carry everything I have received forward in the future.

CHAPTER ONE

INTRODUCTION

1.1 Sex Determination and the Evolution of Developmental Pathways

The presence of biologically different sexes is nearly ubiquitous in nature. In fact, plants and animals show a variety of sexes as well as somatic and germline sexual dimorphisms. In sexually dimorphic species, the presence of different sexes is crucial to reproduction within that species. Sexual reproduction is thought to benefit organisms because the mixing of genotypes increases genetic variation and thus adaptability to the environment (Cho et al., 2007; Herpin and Schartl, 2015; Kobayashi et al., 2018; Kopp, 2012; Schütt and Nöthiger, 2000; Stern, 2011). The presence of a sex phenotype within an individual must be genetically or environmentally determined, developed, and maintained by that organism. This process of sex determination is developmentally fundamental to all sexually dimorphic organisms (Schütt and Nöthiger, 2000; Stern, 2011). Thus, its study is an integral part of investigating organismal development and evolution.

Development and evolution are linked in the sense that 1) the evolution of developmental pathways allows for the origin and fixation of novel traits within species, and 2) the position of a gene within developmental pathways or networks poses certain limitations on the "evolvability" of the gene (Stern, 2011). First, the action of evolutionary processes, such as natural selection, on developmental pathways allows for change through random mutations of specific proteins within a pathway. Changes in survival and reproductive success in accordance with novel phenotypes may lead to the fixation of mutations, and thus novel phenotypes, within populations. Eventually, these evolutionary processes can even lead to the divergence of two species if enough novel mutations have occurred and become fixed in separate populations (Stern, 2011; Waddington,

1959). The proteins involved in developmental pathways are significantly altered by natural selection. This can occur through direct and indirect effects: a gene sequence may directly acquire a mutation causing it to change slightly in interaction with other genes and thus in function. Alternatively, enhancers or silencers of another gene could acquire mutations and thus indirectly change the time-, tissue-, or sex-specific expression of the focal gene. Overall, mutations in certain genes lead to the development of individuals where some novel phenotypes are more viable or successful than others. Thus, not every new mutation will become fixed and some mutations are less likely to become fixed than others.

This idea has been developed by several authors, including David Stern and Conrad Waddington, who described a sort of interaction between developmental pathways and evolutionary processes (Stern, 2011; Waddington, 1959). Waddington (1959) introduced the concept of "canalization" to developmental biology. His "canalization landscape" is an analogy to describe the differentiation of a cell based on both genetic "underpinnings" and environment. According to Waddington, the differentiation of a cell is analogous to a ball rolling down a landscape: it will follow the path of least resistance. The ball will be least "stable" at peaks in the landscape and most "stable" in valleys. This relates to cell differentiation in the sense that the "landscape" is made of the specific alleles the cell contains. Here, the peaks and valleys are particular phenotypes as determined by the underlying genetic networks of the cell. The cell will differentiate down the predetermined path of least resistance given by its particular set of alleles and expressed genes. However, with environmental variation, the specific peaks and valleys are varied. A certain set of environmental conditions will favor a particular phenotype. Hence, the specific environmental conditions a cell (or an organism) is exposed to may change the expression and interactions of certain genes in such a way as to direct the cell's differentiation

down a slightly different path: the path of least resistance given the new set of environmental conditions (Waddington, 1959). This "funneling" of developmental trajectories based on cellular and organismal environments describes the regulation of genes and thus developmental processes to yield phenotypes most suitable for the conditions under which an organism develops. The idea of the "canalization landscape" also explains why more extreme degrees of phenotypic variation tend to be observed under more extreme environmental conditions (Stern, 2011; Waddington, 1959).

Moreover, the position of genes close to "peaks" or "valleys" within Waddington's epigenetic landscape will impact their effect on differentiation and development. A gene far upstream in development will more dramatically affect the developmental path taken by a cell or an organism. A gene further downstream, on the other hand, will have a less extreme, and more specific, effect on development. Read: a gene that determines a "peak" may plunge a cell down a certain developmental trajectory while genes that make up the "valleys" may just specify the particular occupation within that developmental valley. This is to say, a change in gene regulation at the top of developmental cascades will more dramatically alter organismal phenotypes than a change in gene regulation downstream in the cascade.

According to David Stern (2011), this positioning of genes up- or downstream in their developmental pathways affects how strongly they are affected by natural selection. Stern describes that a detrimental mutation in an upstream gene will be much less likely to become fixed than a detrimental mutation in a downstream gene. This is mainly due to the pleiotropic and epistatic effects mutations have on other developmental pathways. More generally, depending on their involvement in other pathways and necessity for the expression of other genes, certain genes will be more likely to maintain mutations than others (Stern, 2011).

Waddington and Stern describe how developmental gene interactions may influence the fixation of mutations and, thus, affect the specifics of evolutionary change to a species' genome. These effects will mostly be seen in negative rather than positive selection. This is because probabilistically, null-mutations are more likely to occur than gain-of-function mutations (Stern, 2011). Hence, the developmental positioning of genes will be most effective at eliminating mutations in genes upstream in developmental pathways where pleiotropic and epistatic effects are strong. This will lead to differing degrees of variation across loci over evolutionary time, creating more or less evolutionarily "conserved" points in developmental pathways. Hence, the genetic makeup of a pathway will affect the level of conservation within it, and certain genes in a pathway may be more conserved than others depending on their involvement in other gene networks of the organism (Stern, 2011; Waddington, 1959).

Most developmental pathways show some differences among phyla, and sometimes even between species. However, in general fundamental pathways appear to be rather conserved. This allows researchers to compare, for example, the Epidermal Growth Factor (EGF) pathway, which generally stimulates and controls growth of an organism, in flies with that in mice effectively and meaningfully (Herpin and Schartl, 2015; Schütt and Nöthiger, 2000; Stern, 2011; Waddington, 1959). Indeed, the EGF pathway is strongly conserved between mice and flies, relying on mostly the same genes and gene interactions (Lusk et al., 2017).

Sex determination pathways are part of the fundamental developmental cascade of sexually dimorphic organisms. They have the ability to regulate other developmental pathways and significantly impact the development of germline and somatic characteristics of an individual (Kobayashi et al., 2018; Rice et al., 2019; Stern, 2011). Therefore, it seems likely that sex determination pathways might be well conserved over evolutionary lineages, as is seen for

other developmental pathways. However, sex determination occurs in a variety of ways across animal lineages. Pathways that determine and orchestrate sex development range from entirely genetic to environmental, and involve various different key proteins and protein interactions. The high levels of diversity in the mechanisms by which animals determine, develop, and maintain sex phenotypes is intriguing given the fact that the presence of sexes themselves is such a universal trait (Beckers et al., 2017; Cho et al., 2007; Devi and Shyamala, 2013; Garrett-engele et al., 2002; Kijimoto et al., 2012; Kobayashi et al., 2018; Ruiz et al., 2015; Schütt and Nöthiger, 2000; Shukla and Palli, 2012; Zhuo et al., 2018). Elucidating how and why sex determination pathways show such low levels of conservation, despite their fundamental role in early development, is an important problem in the study of molecular evolution and organismal development. Moreover, furthering our understanding of the specific similarities and differences in sex determination pathways across animals will expand our understanding of molecular relatedness between different animals and help scientists understand the applicability of results in one model organism to another.

To date, little is known about the specifics of sex determination pathways in many organisms. Most sex determination studies in animals have been conducted on mammals, particularly humans and mice, and *Drosophila melanogaster* (Herpin and Schartl, 2015; Manolakou et al., 2006). In many other animals sex determination pathways remain widely understudied in both breadth and depth. In no other animal do we understand the specifics of the sex determination cascade as well as we do in *D. melanogaster* and mammals. Therefore, more detailed studies of sex determination in previously unstudied species are needed to provide a more representative picture of the diversity of sex determination mechanisms that exist among animal lineages.

In particular, insects offer an easily accessible, highly variable, and agriculturally relevant group of organisms to the study of sex determination. Many insect species are easily bred in the laboratory, manipulated genetically, and include many understudied species (Beckers et al., 2017; Cho et al., 2007; Devi and Shyamala, 2013; Garrett-engele et al., 2002; Kijimoto et al., 2012; Kobayashi et al., 2018; Ruiz et al., 2015; Schütt and Nöthiger, 2000; Shukla and Palli, 2012; Zhuo et al., 2018). To the extent that they have been examined, insect sex determination pathways have been shown to vary greatly, especially in their downstream factors. The regulators at the top of the sex determination cascade typically show higher levels of evolutionary conservation (Cho et al., 2007; Herpin and Schartl, 2015; Kijimoto et al., 2012; Schütt and Nöthiger, 2000). The variation in downstream components of the sex determination cascade among insect lineages indicates different evolutionary histories between different parts of the pathway and shows that certain parts of the pathway appear to be more conserved than others. The specifics of variation in downstream genes of insect sex determination pathways remains to be fully elucidated in most insect groups. Overall, investigating the sex determination of outgroup species relative to mammals and *D. melanogaster*, such as many non-dipteran insects, may contribute greatly to our knowledge of the genetic orchestration of sex development at a molecular level.

1.2 Sex Determination in *Drosophila melanogaster* and other Insects

Drosophila melanogaster is the only insect for which we have a detailed description of the sex determination cascade (Fig.1). Most other studies on insects investigate sex determination in comparison to the pathway in *D. melanogaster*, showing some intriguing differences as well as similarities of certain genes in the network (Fig.1; Beckers et al., 2017;

Cho et al., 2007; Devi and Shyamala, 2013; Garrett-engele et al., 2002; Kijimoto et al., 2012; Kobayashi et al., 2018; Ruiz et al., 2015; Schütt and Nöthiger, 2000; Shukla and Palli, 2012; Zhuo et al., 2018). *Drosophila melanogaster* determines sex chromosomally; the autosome-to-sex-chromosome ratio determines genetic sex in a X-dosage-dependent manner. Fruit flies with more than one X-chromosome will become females, while flies with only one X-chromosome will become males due to insufficiently high transcription of female-inducing genes (Schütt and Nöthiger, 2000). With a high enough X ratio, *Sex-lethal* (*Sxl*) is transcribed and activates *transformer* (*tra*), which facilitates the female-specific splicing of *doublesex* (*dsx*). The female-specific isoform of *dsx* (*dsxF*) is a transcription factor that acts as the master regulator of the downstream sex determination cascade in the fruit fly and regulates downstream genes to induce female-specific development. In female fruit flies, *dsxF* requires the cofactor *intersex* (*ix*). The protein encoded by *ix* is part of the mediator complex in transcription and is required for female-specific development. Male fruit flies, on the other hand, lack the activation of *Sxl* due to their lower X-chromosome-to-autosome ratio and do not activate *tra.* As a result, the default, male-specific splicing isoform of *dsx* (*dsxM*) is transcribed and leads to male-specific development. Male fruit flies do not rely on an interaction of Dsx and Ix to facilitate male-specific development. However, another gene, *fruitless* (*fru*), encodes a transcription factor that is especially important in male-specific brain development in *D. melanogaster*. Male flies lacking *fru* activity are unable to produce the male courtship song and are unable to successfully mate with females (Fig.1; Garrett-engele et al., 2002; Schütt and Nöthiger, 2000).

In other insects the details of their sex determination cascades are still not as well-understood as in *D. melanogaster*, but different degrees of variance are seen across the pathway. Most upstream activators, including *Sxl*, are either still unknown or, to the best of our

knowledge, not conserved. *dsx* and *tra/tra2,* on the other hand, appear to be widely conserved across insect lineages (Cho et al., 2007; Schütt and Nöthiger, 2000). Particularly, *dsx* appears to be the conserved master regulator of sex determination in most insects for which the pathway has been studied. There is, however, striking variance in the downstream players of the sex determination cascade, such as factors like *ix* and *fru.* Indeed, *dsx* and *tra/tra2* appear to be among the few conserved points in the sex determination pathway across insects (Cho et al., 2007; Schütt and Nöthiger, 2000).

Another conserved feature of sex determination is alternative splicing. Key genes, such as *dsx*, are alternatively spliced to create female- and male-specific isoforms, which carry out the sex-specific functions needed for sexually dimorphic development. This appears to be a conserved mechanism for determining and developing sex in insects (Baker, 1998; Cho et al., 2007; Salz, 2011; Schütt and Nöthiger, 2000). This implies that regulation of alternative splicing is a key aspect of many sex determination pathways and could be at the root of the evolution of such pathways. Indeed, the evolution of splicing isoforms has been implicated in sunflower domestication. During domestication, the abundance of various splicing isoforms in sunflowers appeared to change significantly, which indicates that the regulation of alternative splicing is altered by natural selection. This indicates, that not necessarily a gene itself must change in sequence, but that mutations in factors that regulate their splicing might be just as effective at leading to the emergence of novel phenotypes (Smith et al., 2018). Overall, it appears that insect sex determination pathways are conserved in their use of *dsx* as a master regulator and alternative splicing as a key mechanism to carry out sex-specific development. Most other points of the sex determination cascade appear to be only mildly conserved, resulting in an intriguing amount of variation across insect lineages.

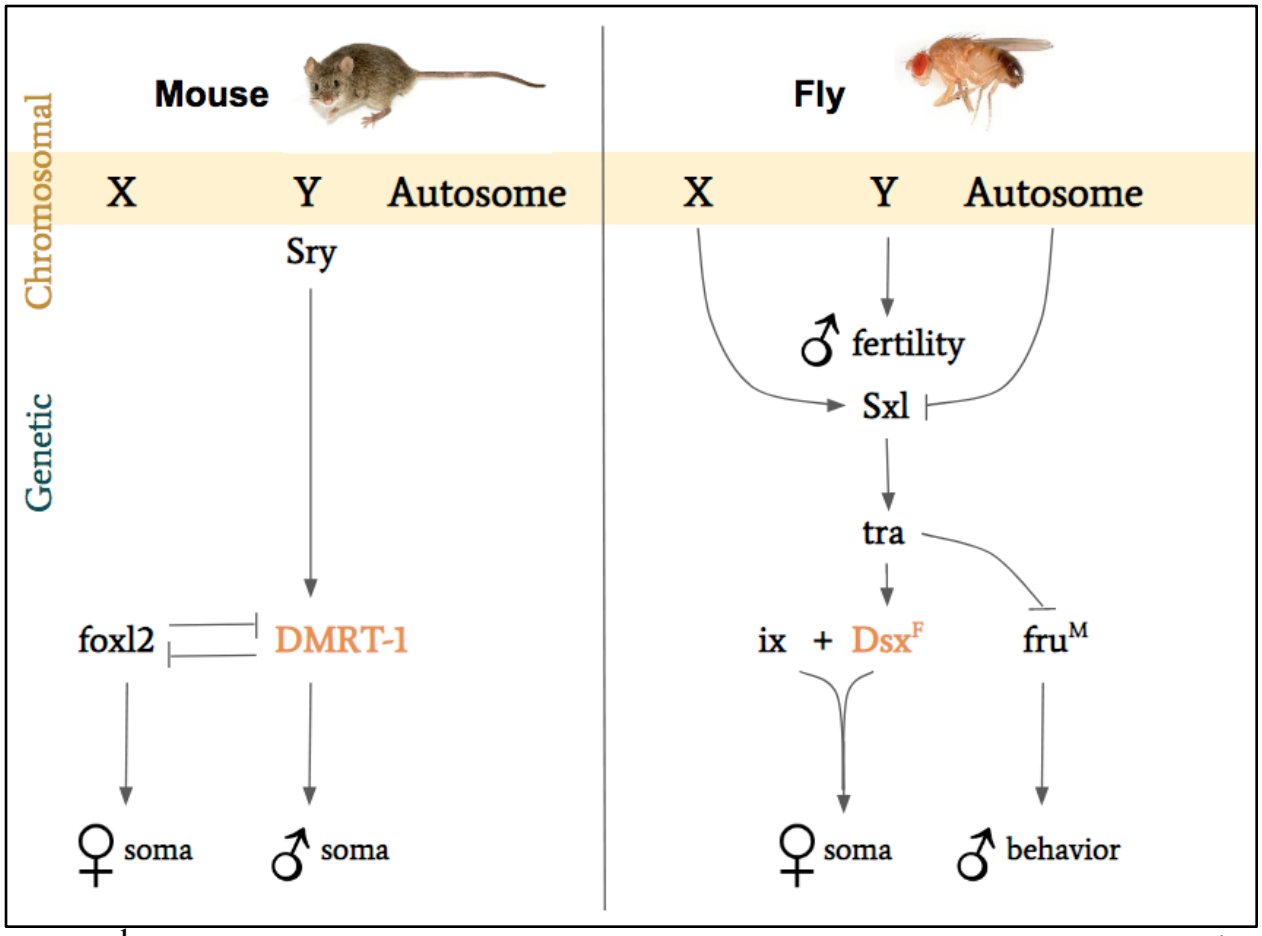

Figure 1[1]. Sex determination cascade in *Drosophila melanogaster* and *Mus musculus.* Sex is determined genetically via a Y-chromosome in *M. musculus* and an X-chromosome to autosome ratio in *D. melanogaster*. The only homologous protein between the mammal and fruit fly pathway is the DMRT-1/Dsx transcription factor, which serves as the master regulator of sex determination in both cascades. The sex determination of *D. melanogaster* is best known among the insects and often used as a starting point for investigation of sex determination pathways in other species. In *D. melanogaster*, a high enough X-chromosome ratio activates *Sxl* transcription, which activates *tra* and thereby induces female-specific splicing of *dsx*. DsxF acts as the master regulator of the downstream sex determination cascade and regulates target genes to induce female-specific germline and somatic development. With only one X-chromosome, *Sxl* and *tra* are not activated and DsxM is transcribed. DsxM leads to male-specific development of the organism. DsxF requires the cofactor *ix* while DsxM does not, but *fru* is required by male fruit flies for wild-type male-specific brain development.

[1] *D. melanogaster* images from David R. Angelini. *M. musculus* images from https://www.flickr.com/photos/dullhunk/7095792663.

1.3 *Oncopeltus fasciatus* as an Outgroup Organism in Sex Determination Studies

Non-dipteran species are of particular interest in the study of insect sex determination since they serve as outgroups to *D. melanogaster.* The order Hemiptera, the true bugs, are nested in the middle of the insect phylogeny and offer an interesting group of organisms to study. *Oncopeltus fasciatus,* the large milkweed bug is part of the Hemiptera, more specifically the sub-order Heteroptera. In addition, *O. fasciatus* is a hemimetabolous (not fully metamorphosing) insect. To date, sex determination pathways of holometabolous (fully metamorphosing) insects only have been studied to a limited extent. On the contrary, only one hemimetabolous insect, the brown planthopper, *Nilaparvata lugens*, has been studied (Cho et al., 2007; Liu et al., 2009; Zhuo et al., 2018). Hence, studying the sex determination of another hemimetabolous Heteroptera, *O. fasciatus,* will increase the possibility of orthology studies within and across insect lineages. Additionally, holometabolous insects are more derived in their early development than hemimetabola and thus studying hemimetabolous insects in more detail might be useful in elucidating more ancestral versions of the insect sex determination pathway.

Finally, better understanding the developmental pathways fundamental to organismal development may have medical applications and inform future research on disease pathways or key genes relevant to mammals and human health. Alternatively, many true bugs are crop pests and have impacts on human food production. Understanding the sex determination and life cycles of these insects may open new doors to pest prevention and elimination. For example, disrupting the life cycles of true bugs via the sex determination pathway could effectively fight their presence as crop pests. This would circumvent current problems with insects evolving insecticide resistance (Aidley, 1976).

Overall, *Oncopeltus fasciatus* is a useful model organism to the study sex determination due to its central position within the insect phylogeny as a hemimetabolous Heteropteran. Additionally, it has been bred in laboratories for decades for use as a genetic model organism (Aspiras et al., 2011; Liu et al., 2009). Its genome and several transcriptome sequences are available and many molecular genetic techniques have been developed for it. For example, RNA interference (RNAi) can be used to investigate loss-of-function phenotypes in *O. fasciatus* for developmental genetic studies (Aspiras et al., 2011; Liu et al., 2009). Thus, *O. fasciatus* makes an excellent model organism for the study of insect sex determination. It will provide valuable data as an outgroup to *D. melanogaster* and as a comparison to another hemimetabolous insect, *N. lugens.*

1.4 Sexual Dimorphisms of *Oncopeltus fasciatus*

In this study I focused on the determination for and development of somatic, morphological sexual dimorphisms in *O. fasciatus*. The large milkweed bug shows two known sexual dimorphisms in adults: distinct genitalia and abdominal sternites (Fig.2). The genitalia of the large milkweed bug are the most posterior appendages (Aspiras et al. 2011). Females have an ovipositor, with which they lay eggs into milkweed plants (Fig.2a). Male milkweed bugs have a pair of claspers, with which they hold onto the female during copulation (Fig.2a). The female ovipositor is generally two- to three-times the length of the male claspers and made up of four distinct structures, called valvulae (Fig.2a). It folds out at the most posterior end of their abdomen and is generally longer than it is wide. The claspers of males tend to be shorter than the ovipositors of females and sit at the top of the male genital capsule. They are slightly curved inward and can move independently from one another (Aspiras et al., 2011). Additionally, the

second abdominal sternite of *O. fasciatus* is also sexually dimorphic. Female milkweed bugs have a projection on their second abdominal sternite which points towards their posterior abdomen and causes the boundary of the sternite to form a triangular projection (Fig.2b). Males, on the other hand, lack this projection. The boundary to their second abdominal sternite is fairly linear and smooth (Fig.2b). Finally, the head shape of some bugs, including the soapberry bug *Jadera haematoloma*, is sexually dimorphic (D.R. Angelini, unpublished data). It is unknown whether the head of milkweed bugs is sexually dimorphic.

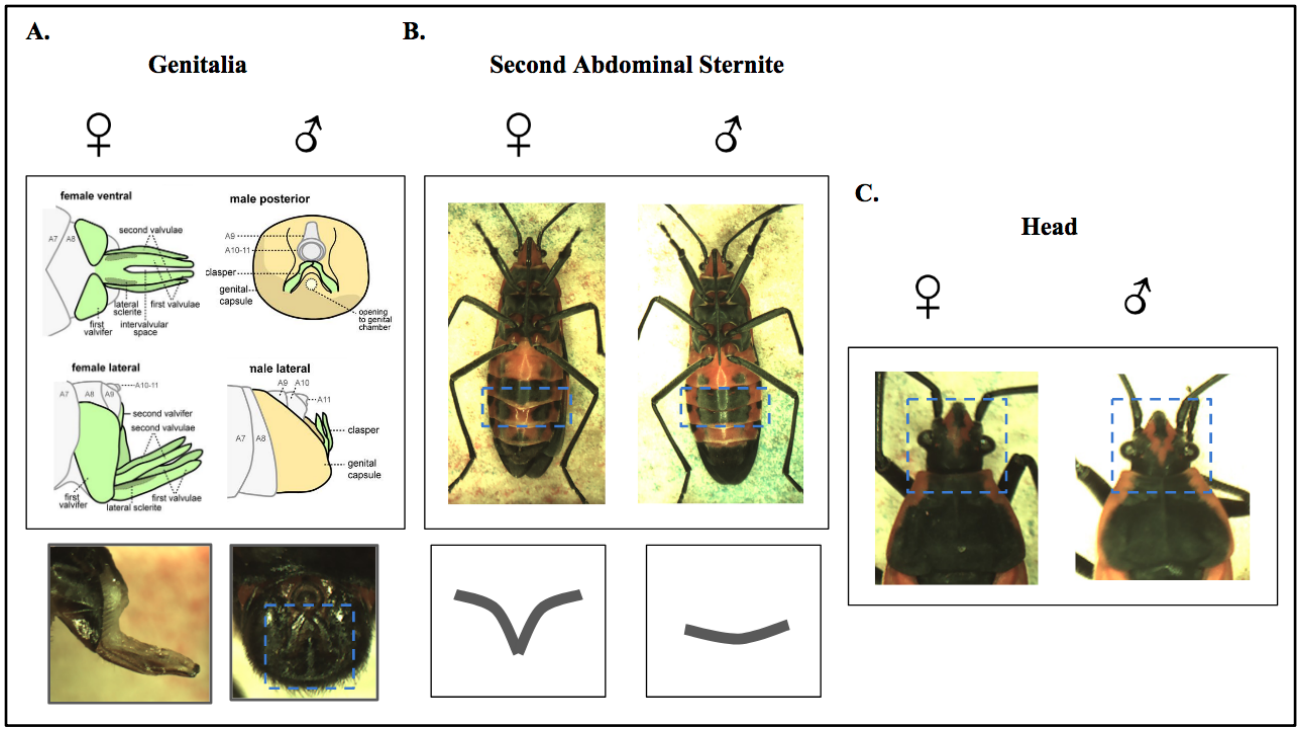

Figure 2. Sexually dimorphic anatomy of *O. fasciatus.* The large milkweed bug has two known somatic, sexually dimorphic structures: the genitalia and the process of the second abdominal sternite. **(a)**[2] The genitalia of *O. fasciatus* are their most posterior appendages and sexually dimorphic. Female milkweed bugs have an ovipositor with which they lay eggs while males have claspers with which they hold on to females during copulation. **(b)** Female *O. fasciatus* individuals have a V-shaped projection of their second abdominal sternite, while males lack curvature of their sternite. **(c)** The head shape of the red shouldered soapberry bug, *Jadera haematoloma,* is sexually dimorphic and, thus, that of *O. fasciatus* may be as well.

[2] Diagram from Figure One from: Aspiras, A.C., Smith, F.W., Angelini, D.R., 2011. Sex-specific gene interactions in the patterning of insect genitalia. Dev. Biol. 360, 369–380.

1.5 Studying the Sex Determination of *Oncopeltus fasciatus*

My interest in the sex determination pathway of the large milkweed bug first arose as part of an appendage patterning experiment carried out by previous members of the Angelini lab. Ariel Aspiras discovered that *ix* RNAi knockdowns significantly impaired the development of genitalia, the most posterior appendages, in both *O. fasciatus* females and males. Additionally, the *ix* knockdowns resulted in both female and male bugs that resembled an intersex phenotype, indicating impaired sex determination and development. This was intriguing due to its difference to the function of *ix* in *D. melanogaster* where *ix* is only implicated in female-specific sex determination, but not in that of males (Aspiras et al., 2011; Garrett-engele et al., 2002; Schütt and Nöthiger, 2000). These results implied that the sex determination cascade in *O. fasciatus* was different than that of *D. melanogaster* and that investigating it further could reveal greater diversity among the sex determination mechanisms of insects. Therefore, members of the Angelini lab went on to identify various target genes that might be involved in the sex determination of the large milkweed bug, including *ix*, *fru*, *dsx,* and various dosage-compensation genes (Laslo, 2013). Dave Angelini, Mara Laslo, and Zhoufan Zhang further identified which of these genes were present in the milkweed bug genome. Ultimately, *ix*, *fru,* and *dsx* were identified as the main genes of interest due to their interactions and relatively well-described roles in sex determination in *Drosophila*. Mara Laslo and Dave Angelini identified the presence of each gene in *O. fasciatus* by aligning the *O. fasciatus* genome and transcriptome to the gene sequences from *D. melanogaster*. They found one ortholog for *ix* and *fru*, but three orthologs of *dsx*, named *dsx-a*, *dsx-b*, and *dsx-c*, indicating at least two duplication events (Laslo, 2013). Interestingly, the presence of more than one ortholog to *D. melanogaster* has been

observed for *doublesex* of many holo- and hemimetabolous insects (Devi and Shyamala, 2013; Schütt and Nöthiger, 2000; Zhuo et al., 2018).

1.5.1 intersex, fruitless, and doublesex in O. fasciatus

The gene *intersex* (*ix*) is a cofactor to *doublesex* (*dsx*) that is required for wild-type sex determination and development in *D. melanogaster.* This is due to the requirement of *intersex* for wild-type function of the female *dsx* splicing isoform (DsxF) (Garrett-engele et al., 2002; Schütt and Nöthiger, 2000). *ix* is part of the mediator complex active during transcription and has been shown to be required for wild-type genitalia development in both females and males in *O. fasciatus,* indicating an evolution of its function within insect evolutionary lineages. *ix* appears to be present as a single copy in most insect genomes and specifically interacts with the C-terminus of DsxF (Aspiras et al., 2011; Garrett-engele et al., 2002; Kopp, 2012; Schütt and Nöthiger, 2000). Thereby, *ix* appears to be able to regulate transcription without having transcription factor ability or a DNA binding domain itself.

The gene *fruitless* (*fru*) encodes a transcription factor that directly alters the expression of its target genes. It encodes various isoforms of the Fru protein, which have been implicated in the development of sexually dimorphic neuronal structures in *D. melanogaster* (Baker, 1998; Schütt and Nöthiger, 2000). Similarly to *dsx, fru* orchesterates sex-specific development via alternative splicing, where sex-specific splicing isoforms regulate the downstream cascade for the respective sex. Additionally, some isoforms appear to be tissue-specific. As aforementioned, *fru* has been particularly implicated in the development of male-specific neuronal structures. However, its function is less well understood in female *D. melanogaster*, but it is likely also involved in the development of anatomical structures important for female courtship behavior and response to

the courtship advances of males (Baker, 1998; Schütt and Nöthiger, 2000). No mammalian homologs of Fru have been found thus far, but it appears that *fru* is important in the sex determination and development of many insects, where it also seems to be particularly implicated in behavior. *fru* expression has been measured in the heads of insects, which indicates expression in the brain and further supports the hypothesis that this gene is required for sexually dimorphic behaviors (Baker, 1998; Laslo, 2013; Schütt and Nöthiger, 2000).

The gene *doublesex* (*dsx*), like *fruitless,* encodes a transcription factor protein. *dsx* is the homolog to DMRT proteins in mammals, has been identified as the master regulator of sex determination in most insects, and acts as such by regulating downstream sex-specific genes (Kopp, 2012; Schütt and Nöthiger, 2000). Previous members of the Angelini lab identified three orthologs to *D. melanogaster doublesex* in *O. fasciatus* (Fig.3). It is unclear at this point whether all paralogs are functional and whether they carry out the same or different functions in sex determination. It appears that the ortholog *dsx-c* most closely resembles the *D. melanogaster doublesex* gene and hence might be the functional paralog. Similarly, other insects tend to have one paralog among the duplications that most closely fits in with the "true" *doublesex* genes as identified by alignment to *D. melanogaster* (Laslo, 2013; Zhou et al., 2018). Moreover, Angelini lab members identified many splicing isoforms of each of the three *dsx* paralogs from an exhaustive Rapid Amplification of cDNA Ends (RACE) screen of cDNA from female and male bugs of juvenile and adult stages. Some of these splicing isoforms appear to be sex-specific and may be crucial for sex-specific development. Each paralog and splicing isoform contains the DNA binding domain, which allows for transcription factor activities of the protein. However, other domains of the protein vary quite strongly between the different paralogs and splicing isoforms. *dsx-a* appears to have three splicing isoforms, including one shorter and two longer

isoforms (Fig.3). All three isoforms vary in the particular exons included, which might allow them to carry out slightly different functions depending on tissue, developmental stage, and sex. *dsx-b* also has three splicing isoforms that vary in the particular exons included (Fig.3). Finally, *dsx-c* has five splicing isoforms (Fig.3). Every splicing isoform contains the DNA binding domain while many of the other exons are variably included in the splicing isoforms. Hence, it is likely that all splicing isoforms act by regulating gene transcription. They might do so in slightly different ways to effect sex-specific gene expression and development.

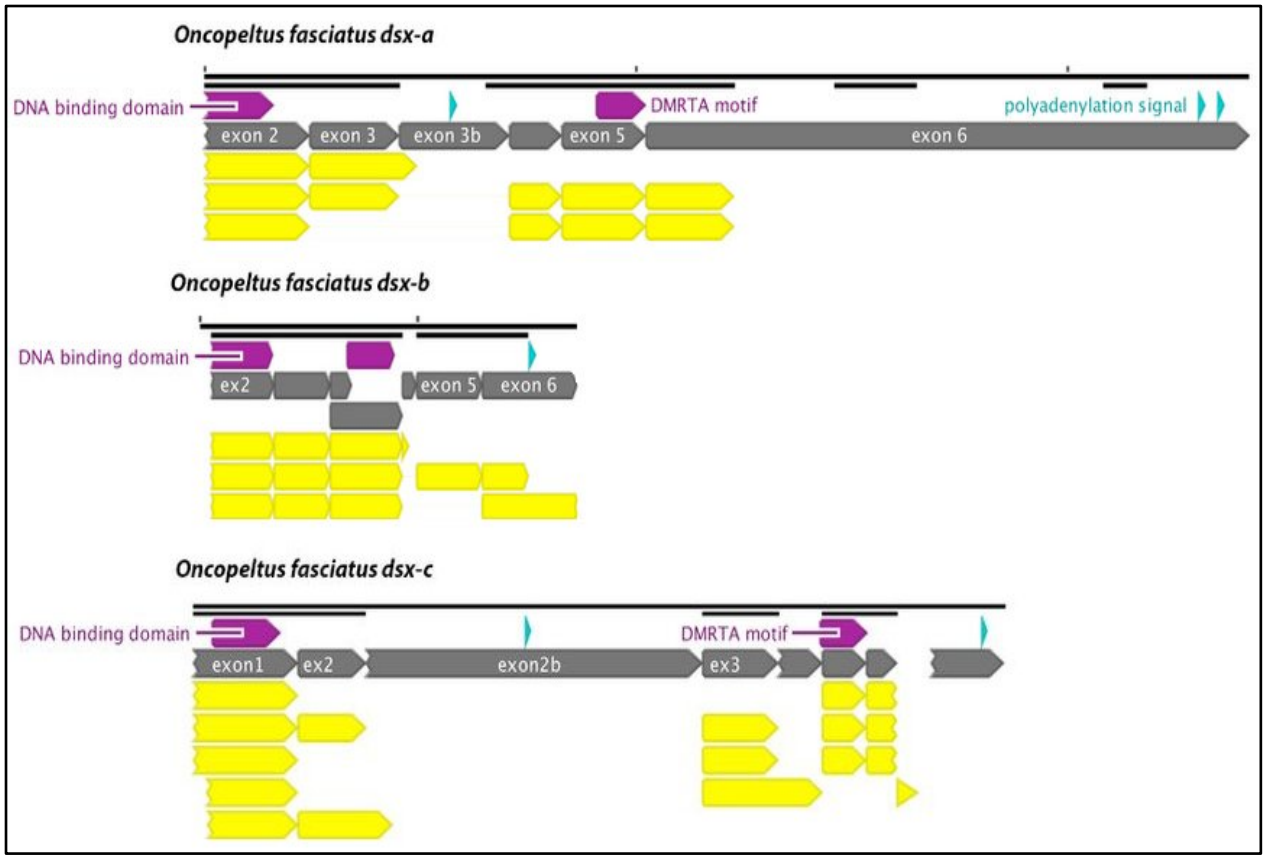

Figure 3. *O. fasciatus* splicing isoforms of the *dsx* paralogs identified via orthology to *dsx* in *D. melanogaster.* The transcription factor *dsx* has three paralogs in *O. fasciatus* and *dsx-c* appears most similar to the *D. melanogaster* gene. Each of the paralogs has various splicing isoforms, which could have sex-specific, and potentially tissue- and time- specific functions during sex determination and development. Each paralog and splicing isoform contains the DNA binding domain while other exons vary in isoform-inclusion.

Previous members of the Angelini lab began RNAi studies of *ix* and *fru*, knocking down both genes early during bug development. Both *ix* and *fru* knockdowns produced similar adult phenotypes and appeared to be impaired in their sex development. Generally, both knockdowns appeared intersex-like in body morphology and genitalia development. Additionally, the genitalia of both females and males were significantly misshapen and shorter than in wildtype bugs for both knockdowns (Laslo, 2013). This implied that both *ix* and *fru* were important in the sex determination and development of female and male *O. fasciatus* and should be further investigated. Larger sample sizes for RNAi studies and quantitative Real-Time-PCR (qRT-PCR) studies of gene interactions were needed to investigate the function and timing of both *ix* and *fru* in the sex determination of *O. fasciatus.* Moreover, all three *dsx* paralogs needed to be further investigated given that studying them via RNAi had proved difficult. Surprisingly, knockdowns of neither *dsx-a* or *dsx-b* paralogs in *O. fasciatus* appeared to produce non-wildtype specimens. Finally, *dsx-c* knockdowns did not appear to alter phenotypes either, but appeared to yield high lethality of specimens, particularly males. However, the *ix, fru,* and *dsx* RNAi studies needed significant further work and refinement. Sample sizes were low in number and RNAi experiments focused only on the effects of each knockdown on the genitalia and not on the sternite sexual dimorphism. Very few knockdowns of each *dsx* paralog individually and no double or triple knockdowns had been performed. The possibility of redundancy between the three *dsx* paralogs had not at all been investigated. Thus, as part of this project I further investigated the role of *ix, fru*, and *dsx* in the sex determination of *O. fasciatus*. I chose to focus on extending the studies of *ix, fru,* and *dsx*, for which only few knockdowns had been conducted. Additionally, I decided to investigate the sex- and time-specific expression of the *dsx* orthologs and *ix* using quantitative qRT-PCR. I hoped that this would complement the functional RNAi

studies by elucidating not only what functions these genes serve during development in *O. fasciatus*, but also when and in what sexes they are expressed.

Here I present the results of my senior honors thesis where I conducted a combination of orthology, qRT-PCR, and RNAi studies of the roles of *ix*, *fru*, and the *dsx* paralogs in the sex determination of *O. fasciatus*. First, I conducted an orthology study of *ix, fru*, and the *dsx* paralog sequences in *O. fasciatus* as compared to the sequences of orthologs in other holometabolous insects and the hemimetabolous insect *N. lugens.* The various orthologous *ix, fru,* and *dsx* sequences in Holometabola and *N. lugens* were used to compare sequence conservation across Holometabola and Hemiptera; this was also conducted within Hemiptera to investigate the rate of sequence alteration of the gene within insects.

Next, to gain information about the expression of *ix*, *fru*, and the *dsx* paralogs as indicators about their potential functions during sex determination and development, I conducted qRT-PCR studies to measure their sex-, age-, and tissue-specific expression in the large milkweed bug. Due to the lack of *fru*-specific probes and primers for qRT-PCR, I measured only the expression levels of *ix, dsx-a, dsx-b,* and *dsx-c* in female and male large milkweed bugs at various developmental time points.

Finally, to complement the expression pattern data obtained from qRT-PCR studies and investigate the specific functions of the three candidate genes in sex determination, I carried out a series of RNAi knockdowns of *ix*, *fru*, and the *dsx* paralogs. I injected *O. fasciatus* specimens with dsRNA for *ix*, *fru,* and the *dsx* paralogs and used the obtained loss-of-function phenotypes to infer possible function of the respective target genes during sex determination. To carry out these experiments, I conducted single-paralog knockdowns as well as a triple knockdown for all

three *dsx* paralogs at once via paralog-specific primers to investigate possible redundancy in function of the three paralogs.

Overall, I attempted to gain an understanding of the conservation in orthology to other species of these target genes, their expression during milkweed bug sex determination and development, and their particular functions during and effects on sex determination via this set of experiments. I hoped to gain a better understanding of milkweed bug sex determination pathways and how they have been altered by natural selection over evolutionary time. Here, I report the implication of *ix, fru,* and the *dsx* paralogs in the sex determination cascade of *O. fasciatus.* My data suggest that the *dsx* locus shows accelerated orthology changes compared to *ix* and *fru.* Additionally, *ix, dsx-a, dsx-b* and *dsx-c* appear to be expressed throughout early developmental stages into adulthood. Finally, *ix, fru,* and *dsx-c* (or potentially all three paralogs in males) appear to serve tissue-specific functions in regulating genitalia and sternite development in the large milkweed bug.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Lab rearing of *O. fasciatus* populations

Oncopeltus fasciatus have been raised in the Angelini lab at Colby College since 2012 and have been used for various developmental genetic experiments. The lab population used for the experiments in this project was raised in 3.38 L aquariums at room temperature, provided excess sunflower seeds for food and excess filtered water to drink. Water was changed every week and cages changed every two to three weeks depending on cleanliness. The milkweed bugs were given teased cotton balls in order to simulate natural milkweed coma ("fluff"), where they lay their eggs in nature (Liu et al., 2009). Adults and juveniles were kept in the same cages and cages were split and dead bugs removed as needed to maintain the population. Bugs used in RNAi experiments were removed and put into separate treatment-specific cages, which were kept in the same manner as described above.

2.2 Phylogenetic study of *ix, fru,* and *dsx* sequence orthology between *O. fasciatus* and other Hemiptera as well as Holometabola

To investigate the molecular conservation of *ix*, *fru*, and the *dsx* paralogs across different insects, I conducted a phylogenetic study of the protein sequences. Professor Angelini and I obtained the transcriptomes and genomes of as many Hemiptera as published or available pre-publishing. We obtained protein sequences from the gene sequences and identified all orthologs to the original *D. melanogaster ix, fru,* and *doublesex* sequences via BLAST alignments for each organism. This allowed us to create a phylogenetic tree based on the similarity of protein sequences of the three genes. Sequences were aligned using ClustalW in Geneious (release 11)

and exported as a PHYLIP format alignment. Sequence names were manually edited to conform to a 9-character identifier. Phylogenies were inferred using RAxML version 8.2.11 on Colby College's natural science computing cluster (NSCC). RAxML was run on multiple processors using a BLOSUM62 rate matrix modeled with a gamma distribution. The program was called from a screen on NSCC's node 26, as "`mpirun -np 30 raxml-mpi -d -f a -x 123 -p 123 -# autoMRE -m PROTGAMMABLOSUM62 -s X.phy -n X`" where "`X`" stands in for the gene-alignment name.

2.3 qRT-PCR studies of *ix, dsx-a, dsx-b,* and *dsx-c* expression during *O. fasciatus* development

2.3.1 O. fasciatus specimens RNA extractions

To investigate the age-, sex-, and tissue-specific expression of *ix* and the *dsx* paralogs, I conducted a set of qRT-PCR studies. I collected bugs of the 4th (L4) and 5th (L5) instars, as well as first, second, and third day adults (d1-A, d2-A, d3-A respectively), for RNA extraction and qRT-PCR analysis of gene expression. During collection, I attempted to agitate the bugs as little as possible to not artificially alter their gene expression. After collection, I flash-froze and then homogenized the specimens. Next, I extracted their total RNA using the Maxwell 16 LEV Tissue RNA Kit following the kit protocol exactly (Promega). I conducted all preparatory work on ice and as efficiently as possible to prevent RNA degradation. Extracted RNA was stored at -80 °C before analysis and was checked for concentration before further handling to ensure success.

2.3.2 Dual-label qRT-PCR studies of gene expression during O. fasciatus development

I performed qRT-PCR on the collected milkweed bug specimens of the L4, L5, d1-A, d2-A, and d3-A stages to investigate the expression of *ix*, *dsx-a, dsx-b,* and *dsx-c* during each developmental stage. To do so I used primers specific for *ix*, *dsx-a*, *dsx-b*, and *dsx-c* to target each gene of interest and dual-labeled probes for amplification. Primers were specific for the DNA-binding domain of each *dsx* paralog, which allowed me to target all splicing isoforms. Unfortunately, we were unable to obtain probes and primers for *fru*. I used *Elongation Factor 1-α* (*EF1-α*) as an endogenous reference gene to quantify baseline levels of gene expression. *EF1-α* appeared to be the best choice for a reference gene due to its fairly consistent expression during development (Su et al. 2011). cDNA was generated from 1µg total RNA using the iScript cDNA kit (BioRad) with a poly-T primer. I carried out the qRT-PCR run in 96-well plates on a BioRad CFX-1000 thermocycler using iQ Supermix (BioRad). Samples were run as technical triplicates. In cases were a group of samples needed to be compared, but spanned multiple plates, two samples were included as both plates for interplate calibration. The analysis of qRT-PCR data was carried out using Excel and R. The expression of each gene was normalized to *EF1-α* expression.

2.4 RNAi studies of *ix, fru, dsx-a, dsx-b,* and *dsx-c* function in *O. fasciatus* sex determination and development

To investigate the functions of *ix*, *fru*, and the *dsx* paralogs during sex determination in *O. fasciatus,* I conducted a set of RNA interference (RNAi) experiments to infer the function of the five target genes from loss-of-function phenotypes. RNAi is a molecular technique that uses a cell-endogenous defense mechanism to knock down the expression of target genes. To inhibit

gene expression, double-stranded RNA (dsRNA) is transcribed from a fragment of the target gene and introduced into the cell or organism in which gene expression is to be inhibited. The cell recognizes the dsRNA and interprets it as viral DNA. The cytosolic dsRNA is then processed by Dicer and assembled in a RISC complex, which targets complementary mRNAs to be degraded. (NCBI, 2019). The phenotypes resulting from such experiments often are used to deduce what function those genes are performing under wildtype conditions. Thus, I inferred the function of *ix, fru,* and the *dsx* paralogs in sex determination from loss-of-function phenotypes. To ensure that RNAi knockdowns were effective at decreasing expression of the target genes, I verified them using qRT-PCR to measure gene expression of the respective target gene(s). This was especially important to do since individual bugs sometimes escape RNAi treatment. I used treatment with *Ampicillin Resistance* (*AmpR*) dsRNA to serve as a negative control. The *AmpR* gene is not present in the *O. fasciatus* genome and thus appeared to be a good choice of control treatment for factors such as injury of the bug specimens during injection.

2.4.1 Double-stranded RNA synthesis

I synthesized double-stranded RNA (dsRNA) fragments for each of the candidate genes in order to conduct RNAi experiments. To do so, I amplified template DNA from either a cloned or synthetically generated fragment of each gene using primers with a 5' T7 RNA polymerase promoter sequence. From the template DNA, I synthesized dsRNA fragments for each gene using the MegaScript Transcription Kit (Thermo Fisher Scientific). I precipitated and purified the dsRNA using ammonium acetate and ethanol. I measured the concentrations of the synthesized dsRNA using a NanoDrop spectrophotometer (Thermo Fisher Scientific) and stored the dsRNA at -20 °C.

I mixed a desired injection concentration ranging from 1.0 µg/µL to 6.0 µg/µL of each dsRNA sample with 0.05 % McCormick green food coloring to create an injection solution for each target gene. I also added 0.01 mM $NaPO_4$ and 5 mM KCl to buffer the injection solution. I stored the injection solutions at -20 °C.

2.4.2 Injection of O. fasciatus

Oncopeltus fasciatus first show sexually dimorphic characteristics when they enter the fifth instar (L5); in all previous instars it is not possible to externally distinguish females from males. Hence, I conducted RNAi knockdowns at the fourth instar since we suspected sex determination pathways to be active during this developmental time point. Moreover, earlier knockdowns were not possible because third instar (L3) *O. fasciatus* specimens do not survive injections.

I injected L4 specimens with dsRNA for *ix, fru, dsx-a, dsx-b,* and *dsx-c* individually. Additionally I carried out a triple knockdown of all three *dsx* paralogs at the same time to check for redundant functions of the *dsx* genes. The triple knockdown injection solution was prepared in the same manner as the single knockdowns but from two separate DNA templates. First, dsRNA for the DNA-binding domain of each paralog was synthesized individually and then an equimolar solution mixed of all three of them. Secondly, a synthetic gene fragment of the *dsx-a, -b,* and *-c* DNA binding domains was used as a template for dsRNA synthesis, yielding a long dsRNA product targeting all three genes. Finally, I performed a dsRNA injection for the *Ampicillin Resistance* (*AmpR*) gene to serve as a negative control since the *AmpR* gene is not present in the *O. fasciatus* genome. All injections and injection concentrations are listed in Table 1.

I carried out injections using a micro-capillary needle, which we pulled in the laboratory using a Sutter Instruments pipette puller. I used a syringe to draw up 6 μL of the buffered and diluted injection solution for the respective gene of interest. I anaesthetized bug specimens using a CO_2-pad which was placed under a light microscope and injected the L4 specimens on the pad under the microscope with about 0.5 μL per bug a the right side of their abdomen on the first or second abdominal sternite. After injecting them, I placed the specimens into a cage used to rear the milkweed bugs in the lab with plenty of food and water. I examined their phenotypes once they had reached adulthood. I reared the injected specimens as described above and allowed them to fully develop into adult milkweed bugs. At adulthood, I measured their sexually dimorphic structures, including the genitalia, second abdominal sternite, and head, to investigate their sex determination and development phenotypes.

Table 1. dsRNA injections performed on *O. fasciatus.* The following injections were performed on L4 *O. fasciatus* specimens to investigate the roles of *intersex* (*ix*)*, fruitless* (*fru*)*,* and *doublesex* (*dsx*) in sex determination. Concentrations were altered to lower lethality levels while still obtaining knockdown phenotypes. *Ampicillin Resistance* knockdown was used as a negative control. Treatment numbers, RNAi validation inclusion, and lethality are also given.

gene targeted	injection concentration (µg/µL)	treatment number	included in RNAi validation?	lethality (%)
ix	1.0	352-353, 299-300	Y (352, 353)	54 %
	1.5	291-292	N	61 %
	2.5	257	N	100 %
fru	1.5	422-425	N	33 %
dsx-a	2.5	395	N	0 %
	3.5	419	Y	42 %
dsx-b	2.5	396	N	0 %
	3.5	420	Y	53 %
dsx-c	2.5	258-259, 261-262, 266, 290, 293, 325, 329, 331, 333, 348-349, 354, 397	Y (262, 329, 348)	32 %
dsx-abc, one dsRNA	1.0	417	N	15 %
	2.5	416	N	15 %
	5.0	414	N	50 %
	6.0	421	Y	50 %
dsx-abc equimolar mix	1.17	398, 399	N	0 %
	2.4	413	Y	49 %
Ampicillin Resistance	2.5	265, 324	Y	34 %

2.4.3 Imaging of O. fasciatus RNAi specimens for morphological phenotype analysis

I used a light microscope connected to a camera to photograph *O. fasciatus* specimens. I imaged the bugs dorsally, ventrally, and their genitalia using the software MoticImagesPlus2.0 live imaging.

2.4.4 Verification of RNAi treatments and study of sex determination gene interactions via qRT-PCR measurements of gene expression

I used qRT-PCR studies to verify the functioning of the RNAi technique and to gain information about the interactions of *ix* and the *dsx* paralogs. To do so, I collected first-day adults of each RNAi treatment group to measure their expression of the RNAi target genes. First-day adults are fairly similar in their gene expression to bugs during the last developmental stages and, thus, I used these bugs for validation of the RNAi treatments. I collected first-day adults without agitating them as to not alter gene expression and euthanized them by placing them at -80 °C for fifteen minutes. Next, I decapitated them and placed them in RNAlater, fully letting the RNAlater solution penetrate the tissue. Those specimens were stored at -80 °C until I extracted total RNA in the same manner as described above for qRT-PCR experiments. Next, I synthesized cDNA for qRT-PCR experiments. Both the head and the body of the bug specimens were included in the tissue from which RNA was extracted and cDNA synthesized. I used the same probes, primers, and procedure as described above for qRT-PCR experiments to measure gene expression of each RNAi target gene. By measuring expression of the knocked down genes, I was able to both verify whether RNAi treatments were effectively decreasing target gene expression and also gain information about gene interactions. I was able to do the latter by

measuring the expression of, for example, *ix* in a *dsx-c* knockdown to see if *dsx-c* was involved in regulating *ix* expression. Other gene interactions were investigated in an analogous way.

2.5 Morphological analysis of RNAi phenotypes

2.5.1 Analysis of O. fasciatus specimens genitalia

To investigate the effects of RNAi treatment on sexually dimorphic development in *O. fasciatus*, I measured the absolute length of their genitalia. I imaged the genitalia of RNAi specimens and recorded their length using ImageJ (Rasband, 1997-2018). Using the line-tool, I traced out the ovipositor for females and one of the claspers for males (Fig.4a). Next, I exported the the length of the structure in pixel numbers and converted them to metric measures of length by scaling based on the magnification used to take the picture. This allowed me to compare genitalia length across different RNAi treatment groups and between sexes to investigate effects on genitalia development.

2.5.2. Analysis of O. fasciatus abdominal sternite curvature

I measured curvature of the abdominal sternite to investigate the effects of *ix, fru,* and *dsx-a, dsx-b, dsx-c,* and *dsx-abc* knockdowns on development of this sexually dimorphic structure (Fig.4b). To quantify curvature, Professor Angelini and I conducted a landmark-based geometric morphometric (GMM) analysis. Using ImageJ, I placed nine points along the posterior edge of the second abdominal sternite (Rasband, 1997-2018). I placed points 1 and 9 always on the lateral edge of the abdomen. I always placed point 5 at the midline of the sternite, directly on the tip of the projection when present. The rest of the points were evenly spaced along the edge of the sternite to trace its arch across the abdomen (Fig.4b). Next, I copied the coordinates of the

nine points into an Excel spreadsheet. A custom R script was used to convert the cartesian coordinates into TPS format (Rohlf, 2015).

To analyze abdominal curvature, we imported the point coordinates into R using the package "`geomorph`" (Adams et al., 2013). I kept points 1, 5 and 9 as fixed landmarks, but allowed the other points to slide in such a way as to be equally spaced along the the curve they define. Specimens were then aligned using Generalized Procrustes Analysis (GPA), sliding the semilandmarks based on minimizing bending energy. To analyse and compare curvatures, we used a linear regression model on the Procrustes-aligned coordinates. We added a line of best fit to the nine points tracing the sternite arch and calculated its residuals. Here, larger residual absolute values indicated a higher amount of curvature in the sternite and, thus, a more characteristically female projection.

2.5.3 Analysis of O. fasciatus head shape

The head of the soapberry bug, *Jadera haematoloma,* is sexually dimorphic in its shape (D.R. Angelini, unpublished data). Males have a shorter, wider head compared to females. To investigate whether this trait is also sexually dimorphic in *O. fasciatus* we recorded and compared head shape of unmanipulated and RNAi specimens from this experiment. I took dorsal images of the bugs and we conducted a similar morphometric analysis as we did to investigate sternite curvature. Again, I placed nine points, tracing the shape of the head of a bug specimen. The points were placed at the posterior, lateral and anterior edges of each eye, at each antennal joint, and at the anterior tip of the head (Fig.4c). These point placements had previously been shown in *J. haematoloma* to be sufficient for detecting sexual dimorphisms. Thus, we suspected they would be a good choice of landmarks for analyzing RNAi treatment effects on head shapes

in *O. fasciatus.* I exported the point coordinates into R and aligned them using the `geomorph` function for GPA. To determine whether a sexual dimorphism in head shape was present in *O. fasciatus,* I compared the head shapes between female and male bugs using Procrustes ANOVA with permutation as implemented in the "`geomorph`" function "`procD.lm`" (Adams et al. 2013).

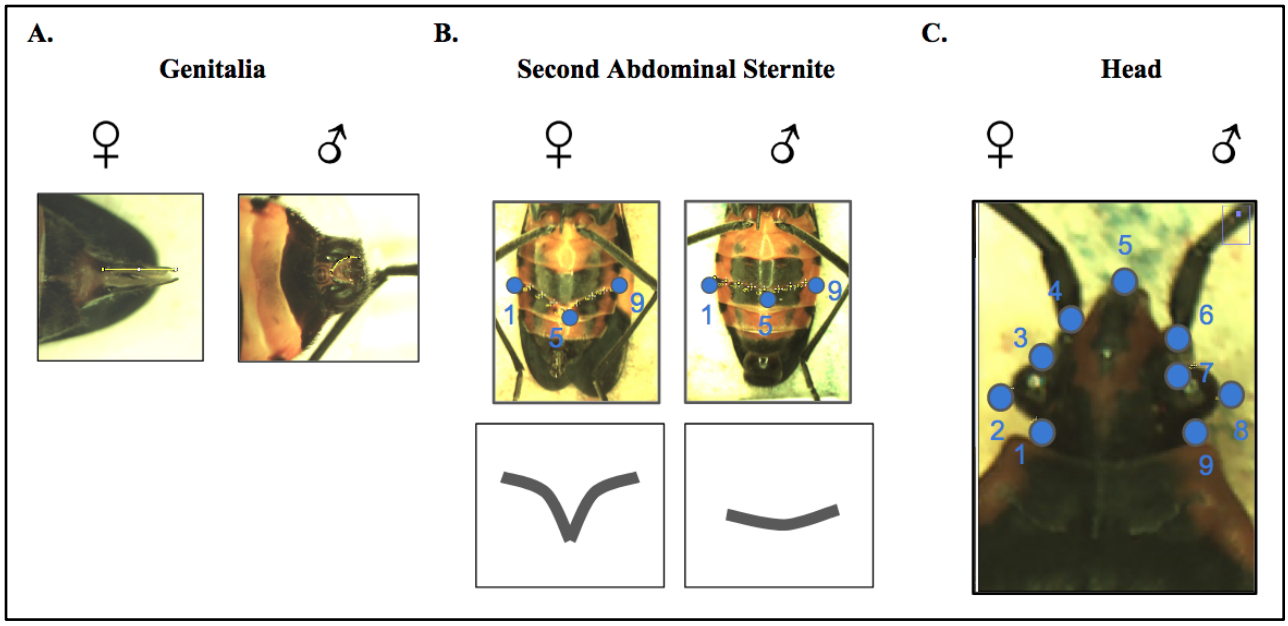

Figure 4. Sexually dimorphic genitalia, second abdominal sternites, and heads were measured in *O. fasciatus* to evaluate RNAi knockdown phenotypes. (a) Genitalia length was measured in females and males using raw pixel documentation via ImageJ. Raw pixel values were converted to length and used to evaluate genitalia phenotypes of RNAi gene knockdowns. **(b)** Curvature of the second abdominal sternite process was quantified by placing nine points in ImageJ. The sternite shape was analyzed using the geomorph package in R to evaluate RNAi effects on sternite curvature. **(c)** Head shape of female and male *O. fasciatus* specimens was measured by placing nine points around the head followed by GMM analysis in R.

2.6 Statistical Analyses

RNAi is expected to produce a skewed distribution of phenotypic effects, with strong effects in some specimens and others having mild or no obvious effects. Individual bugs can escape RNAi treatments and thus not receive the full gene knockdown. Therefore, analyses were performed using nonparametric statistical methods. I conducted the Kruskal-Wallis analysis of variance to detect overall effects, using a post-hoc one-sided Wilcoxon rank sum test to detect significant differences between individual treatment groups and controls. In cases where multiple tests were performed on the same dataset, P-values were adjusted with FDR correction.

Additionally, as aforementioned, a Procrustes ANOVA with permutation as implemented in the "`geomorph`" function "`procD.lm`" in R was used to compare head shapes between *O. fasciatus* specimens (Adams et al. 2013).

CHAPTER THREE

RESULTS AND DISCUSSION

3.1 Phylogenetic study of *ix, fru,* and *dsx* sequence

To investigate the conservation of the *ix, fru*, and *dsx-a, -b,* and *-c* sequences, I obtained the amino-acid sequence of each gene of interest from a number of insect species from the NCBI protein database and aligned them using ClustalW. I used the protein sequences to investigate conservancy of the three target genes because at these large evolutionary distances we expect nucleotide sequences to be saturated with mutations at synonymous sites. To compare the amino-acid sequences of *ix, fru,* and *dsx,* I used maximum likelihood phylogenetic analysis to infer a tree with bootstrap resampling to estimate confidence in the relationships suggested in the tree.

3.1.1 The intersex protein tree recapitulates species relationships

The phylogenetic tree obtained from aligning *ix* sequences appears to recapitulate the species phylogeny (Fig.5). There is only a single ortholog of *ix* in *O. fasciatus.* There is also a single copy of the gene in *D. melanogaster* and other insects that have been examined. Changes in the protein sequence of *intersex* might occur to a similar degree as the baseline mutation rate over time. These data do not suggest accelerated evolution of the *ix* sequence as compared to genes of other pathways. However, given the fact that the genes are different in sequence due to background evolutionary processes, they could still be performing different specific functions in *O. fasciatus* and *D. melanogaster.* This could be the case due to randomly acquired mutations that slightly change the Ix protein structure and thus also the specific interactions and actions of the *ix* gene. Moreover, mutations in gene regulatory elements could also be causing changes in expression of the *ix* gene during developmental time and in various tissues.

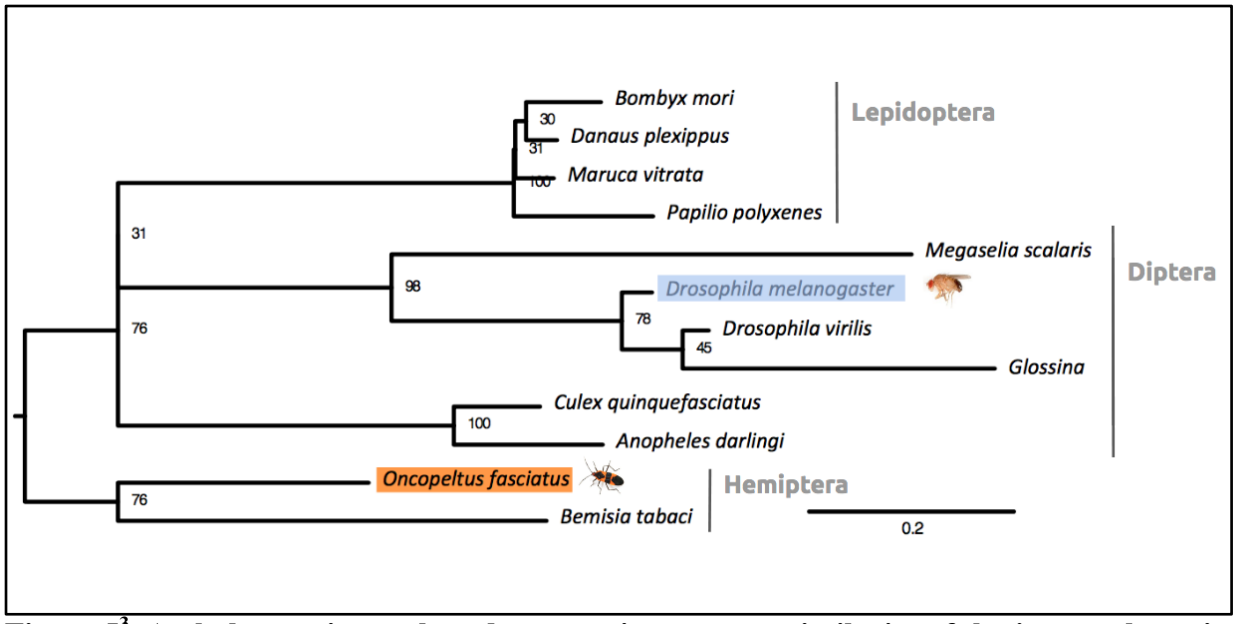

Figure 5[3]. A phylogenetic tree based on protein-sequence similarity of the *intersex* locus in insects recapitulates general phylogeny of the compared species. The Ix sequence between different insects appears to not be altered at a higher rate than explained by baseline, random evolutionary processes. Numbers at the nodes of the phylogenetic tree indicate bootstrap values.

[3] *O. fasciatus* and *D. melanogaster* images from David R. Angelini.

3.1.2 The fruitless protein tree recapitulates species relationships

Similarly to *intersex*, the phylogeny created from *fruitless* protein-sequence alignments in various insects appears to recapitulate species relationships (Fig.6). This, again, implies that the *fru* sequence itself might not be prone to accelerated evolution. Thus, it might be more conserved than the sequence of other genes involved in sex determination pathways and might carry out functions similar to those of the Fru protein in *D. melanogaster.* However, this cannot be confirmed without functional experiments and more statistically rigorous evolutionary sequence analyses. Just like *ix, fru* may exhibit gene interactions or expression patterns that are different from *fru* in *D. melanogaster.* Alternatively, novel gene regulatory elements rather than the protein-coding sequence could also cause *fru* to perform novel functions in *O. fasciatus.*

Figure 6[4]. A phylogenetic tree based on protein-sequence similarity of the *fruitless* locus in insects recapitulates general phylogeny of the compared species. The Fru sequence between different insects appears to not be altered at a higher rate than explained by baseline, random evolutionary processes. Numbers at the nodes of the phylogenetic tree indicate bootstrap values.

[4] *O. fasciatus* and *D. melanogaster* images from David R. Angelini.

3.1.3 Phylogeny of doublesex proteins shows clustering of orthologs

To investigate the relationship of *doublesex* genes, I created a phylogenetic tree from the protein sequences of *dsx* orthologs of diverse insects. Several insects have more than one *doublesex* ortholog, compared to the single sequence of *D. melanogaster.* Thus, orthologous sequences were aligned to the *D. melanogaster* sequence, as well as one another (Fig.7).

doublesex orthologs of different insect species appear to be more similar to orthologs in other insects than to *dsx* copies within the same organism. For example, each of the three *dsx* orthologs found in *O. fasciatus* appears to be more similar to orthologs in other insects than to the other two paralogs within the *O. fasciatus* genome. This is apparent in the clustering of particular *dsx* ortholog groups. There appears to be one "*dsx*-clade" which contains *dsx* orthologs most similar in protein-sequence to the eponymous *D. melanogaster* sequence. This clade includes the *dsx-c* paralog of *O. fasciatus* and both *dsx* and *dsx-like-1* of *N. lugens.* Additionally, the *dsx-a* and *dsx-b* paralogs in *O. fasciatus* appear to fall within two other *dsx* clades across insects and only show similarity to one of the other two *dsx* orthologs of *N. lugens.*

Hence, overall *dsx* orthologs appear to differ in sequence conservation from *dsx* orthologs in *N. lugens,* the only other hemimetabolous insect in which sex determination genes have been studied (Zhou et a., 2018). Moreover, the *dsx-c* ortholog of *O. fasciatus* appears most similar to the *D. melanogaster dsx* locus in protein-sequence.

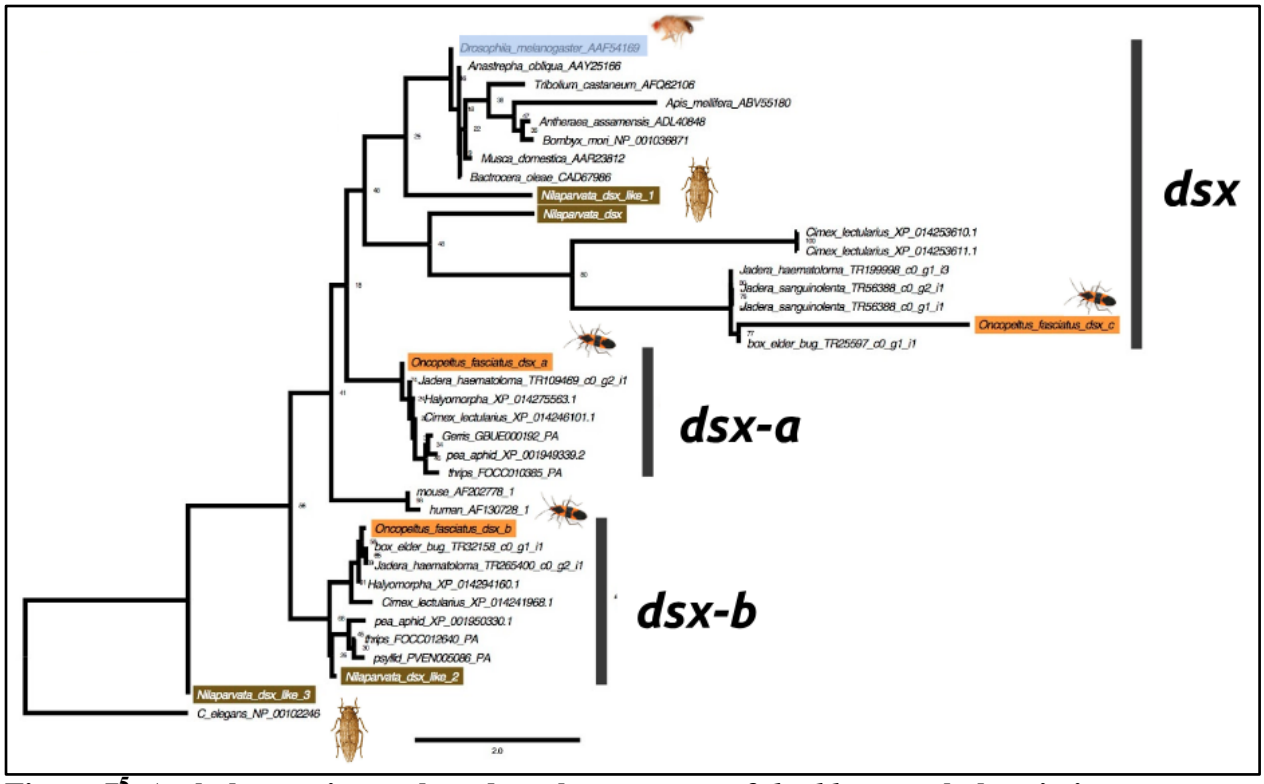

Figure 7[5]. A phylogenetic tree based on the sequence of *doublesex* orthologs in insects shows locus-specific differences in sequence that do not recapitulate general species phylogeny. The *dsx-c* ortholog of *O. fasciatus* appears most similar to the *dsx* sequence of *D. melanogaster* while *dsx-a* and *dsx-b* fall within ortholog-clusters of other insects. They appear to be more similar to other *dsx-a*-like and *dsx-b*-like orthologs than one another and those of *N. lugens*. The *dsx* orthologs in *O. fasciatus* appear more similar in protein-sequence to the *dsx* sequences of holometabolous insects than the one other thus-far-studied hemimetabolous insect, *N. lugens.* Numbers at the nodes of the phylogenetic tree indicate bootstrap values.

[5] *O. fasciatus,* and *D. melanogaster* images from David R. Angelini. *N. lugens* images from http://www.ces.csiro.au/aicn/name_c/a_605.htm.

Overall, these results indicate that the *doublesex* gene might be more apt to retain mutations in its sequence than both *ix* and *fru*. This is shown by various duplication events of the gene within the insect lineage and by lower sequence conservation than in *ix* and *fru* between and within hemi- and holometabolous insects. All three genes might well be changing in their developmental functions across insects, but *dsx* orthologs appear to alter in sequence in an accelerated fashion. This could suggest faster and more drastic changes of *dsx* function in insects. However, these orthology studies do not take into account the possibility of mutations in enhancers and silencers of these genes and thus cannot account for the full range of possibilities of how these genes might be changing in function over evolutionary time. Functional studies of sex determination genes are needed to investigate and compare their specific roles during development.

3.2 Increased *ix, dsx-a, dsx-b,* and *dsx-c* expression during the fifth instar and adulthood in both female and male bugs

The expression of the three *dsx* orthologs and *ix* was measured in female and male *O. fasciatus* specimens during the fourth and fifth instar, as well as adulthood, in order to investigate time- and sex-specific expression of the four target genes. The normalized expression of *ix, dsx-a*, *dsx-b,* and *dsx-c* appears to increase during the fifth instar compared to the expression of each gene during the fourth instar. The expression of all four genes appears to remain at a similar level in adulthood compared to the fifth instar (Fig.8). For *ix, dsx-a,* and *dsx-b* expression appears to be slightly higher in females than males during the fifth instar. This is reversed for *dsx-c.* During adulthood, the expression of all four genes appears to be more widely spread between individual bugs than during the fourth and fifth instars, but generally the

expression of all four genes appears to be preserved at levels similar to those in the fifth instar during adulthood. Overall, *ix, dsx-a, dsx-b,* and *dsx-c* all appear to be moderately expressed during the fifth instar and adulthood. Generally, *dsx-b* and *dsx-c* show higher levels of expression throughout developmental stages and in both sexes than *dsx-a. ix* shows the highest levels of expression throughout developmental stages and in both sexes of all four genes. We were unable to detect statistically significant differences in gene expression of all four target genes. This is likely due to the small sample size of this data set (n = 2 - 9 samples per stage and sex).

To test for co-expression of the four genes with one another, a spearman's rank correlation test was run between normalized expression values of all four genes. All four genes showed strong correlations with one another in their expression patterns (spearman's rank correlation test, rho > 0.50). Hence, these data show association patterns between the expression of all four genes *ix, dsx-a, dsx-b,* and *dsx-c*. In particular *dsx-a, dsx-b* and *dsx-c* are correlated with one another in their expression (rho > 0.60). *ix* is most strongly associated in expression with *dsx-c* (rho = 0.60) and slightly less-so with *dsx-a* (rho = 0.54) and *dsx-b* (r = 0.55).

These results indicate that all four genes are expressed in both female and male bugs. However, these experiments were not carried out using isoform-specific primers. Therefore it can only be concluded that some isoform of each gene is expressed during the fifth instar and adulthood in *O. fasciatus*. Sex-, tissue, and time-specific isoforms might be carrying out specific functions during sex determination and development. The expression of such isoforms could be investigated in the future using primers with the necessary specificity in a similarly set up qRT-PCR experiment.

The detected expression correlation between the *dsx* paralogs and *ix* does not necessarily imply that the four genes interact or require one another for expression, but it might suggest that they are carrying out developmental functions during the same time period.

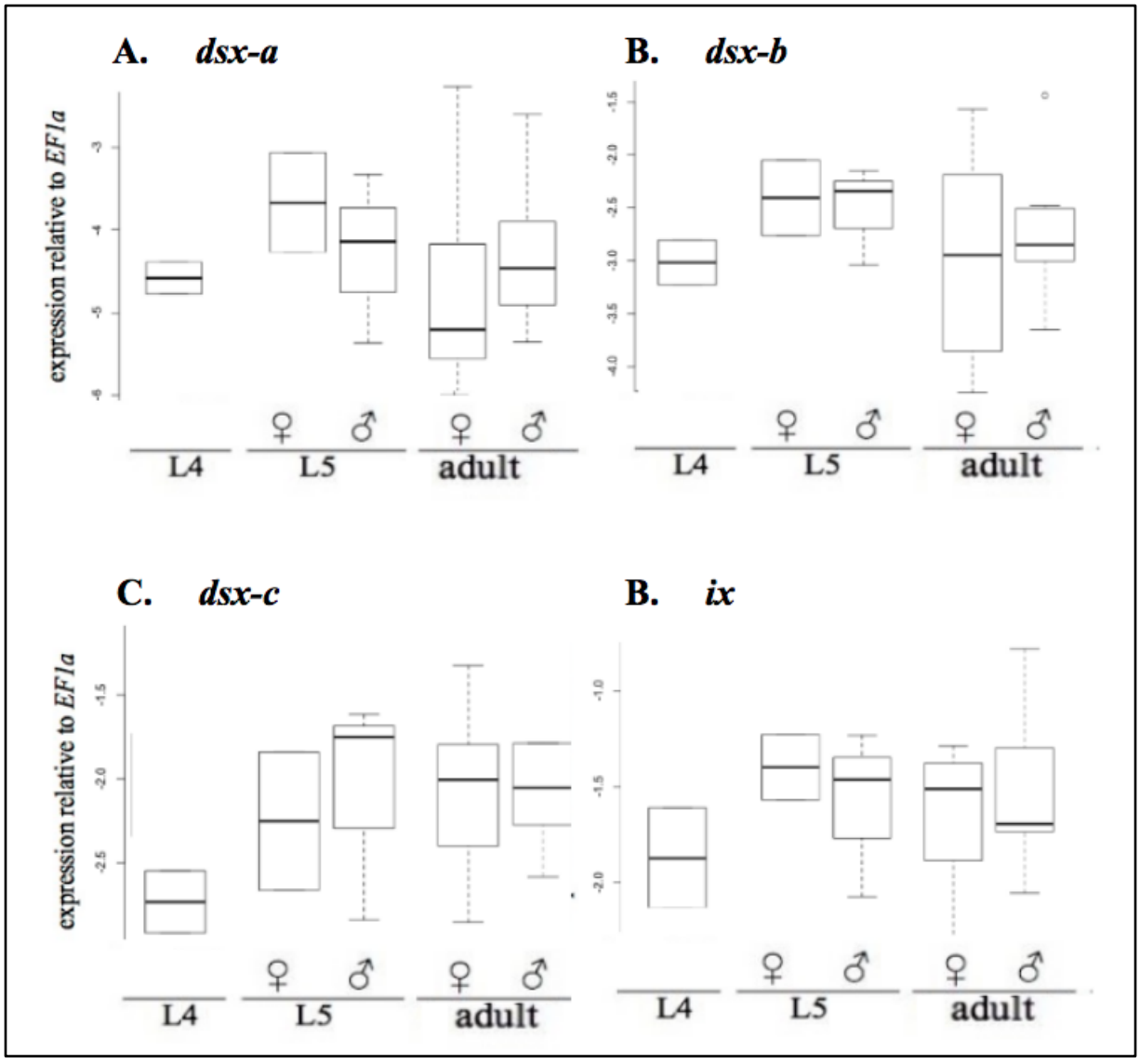

Figure 8. qRT-PCR analysis of *ix, dsx-a, dsx-b,* and *dsx-c* expression shows moderate expression of all four target genes during the fifth instar and adulthood in both sexes. Slightly higher *ix, dsx-a,* and *dsx-b* expression was detected in female than male *O. fasciatus* specimens during the fifth instar. No statistical significance was detected (n = 2 - 9 samples per stage and sex). All genes are correlated in expression (spearman's rank correlation test, rho > 0.5).

3.3 RNA interference effects on development of sexually dimorphic structures

To investigate the specific function of *ix*, *fru*, and the three *dsx* orthologs I conducted RNAi knockdowns to investigate loss-of-function phenotypes associated with each target gene. Additionally, I carried out a *dsx-abc* triple knockdown to check for the possibility of functional redundancy of the *dsx* paralogs. I measured the effect of each knockdown on both genitalia and sternite development, which are the two external sexually dimorphic structures in *O. fasciatus.*

Additionally, I examined head shape in *O. fasciatus*, testing for sexual dimorphism in this species, because another heteropteran, *Jadera haematoloma*, shows sexual dimorphism in head shape (D.R. Angelini, unpublished data). However, after measuring and analyzing head shapes of unmanipulated female and male *O. fasciatus* specimens, I discovered that head shape does not appear to significantly differ based on sex (Procrustes ANOVA with permutation, 1000 iterations, $F(1,40) = 0.88104$, $p = 0.195$). Hence, head shape measurements and results were excluded from the rest of this thesis.

3.3.1 RNAi effects on genitalia development in female and male O. fasciatus

Both *ix* and *fru* significantly shortened ovipositors and claspers in female and male *O. fasciatus* specimens, respectively (Fig.9; Wilcoxon rank sum test, W = 1952.5, W = 568, W = 1068, W = 260 respectively, $p < 0.0001$ for all treatments in both sexes). Among the *dsx* treatments, only the *dsx-c* knockdown produced significantly shorter ovipositors in female *O. fasciatus* specimens (W = 1281, $p < 0.0001$), but not the *dsx-a, dsx-b,* or *dsx-abc* treatment (Fig.9a; W = 171.5, W = 220.5, W = 1022 respectively, $p > 0.05$ for all three treatments. Additionally, the *dsx-c* knockdown appeared to shorten ovipositors less dramatically than *ix* and *fru*, which appeared to have a similar effect (Fig.9a, Fig.11a). Additionally, the *dsx-abc*

knockdown appeared to produce a greater spread of ovipositor lengths than did *dsx-a* or *dsx-b* knockdowns (Fig.9a) In males, on the other hand, the *dsx-abc* triple knockdown did appear to significantly shorten claspers ($W = 819$, $p = 0.0051$). Additionally, while it did not significantly shorten clasper length, the *dsx-c* knockdown in male *O. fasciatus* specimens appeared to create two clusters of clasper length: one cluster which coincided with the control treatment clasper lengths and another one that appeared more similar to the clasper lengths of *fru* knockdowns (Fig.9b). This looked similar to the *dsx-abc* results in both females and males with the exception that the *dsx-abc* knockdowns produced more specimens with genitalia of an intermediate length rather than two distinct clusters (Fig.9).

These results implicate *ix* and *fru* in ovipositor and clasper development. RNAi targeting both genes produced specimens with significantly shortened genitalia in both females and males. This developmental function could be carried out by female- or male-specific splicing isoforms of one or both genes given their expression in both sexes as shown by the qRT-PCR results (Fig.8). Additionally, it appears that either *dsx-c* is implicated in the genitalia development of both sexes or that *dsx-c* is required in females, but all three *dsx* paralogs, redundantly, in males. While the *dsx-c* knockdown only significantly reduced ovipositor length, the triple knockdown significantly reduced clasper length. Additionally, as aforementioned, the *dsx-c* knockdown in males appeared to produce two clusters of clasper phenotypes: one that grouped with the control phenotypes and one that grouped with the *fru* knockdown phenotypes (Fig.9b). The grouping of the *dsx-c*- and *dsx-abc*-injected males is likely an artefact of some of the *dsx-c* and *dsx-abc* knockdown specimens escaping the RNAi treatment and hence showing a wildtype phenotype due to insufficient knockdown of the gene. In this case, *dsx-c* could be implicated in genitalia development for both females and males as shown by impaired genitalia development for some

bugs of the *dsx-c* and *dsx-abc* knockdown in both sexes. Alternatively, *dsx-c* could be implicated in female genitalia development but not in that of males. In this case, the significantly shortened claspers produced by the *dsx-abc* triple knockdown in males could imply a redundant function of all three *dsx* paralogs in clasper development, where the respective other two paralogs compensate in function for the one knocked down in the single knockdowns. Both of these possibilities should be further investigated by validating RNAi knockdowns, especially of both phenotype clusters for the *dsx-c* knockdown in males.

Overall, it appears that both *ix* and *fru* are needed for genitalia development in both sexes. *dsx-c* appears to be required for ovipositor development in females, albeit less fundamentally than *ix* and *fru*. Finally, either *dsx-c* or all three *dsx* paralogs appear to be required for clasper development in males.

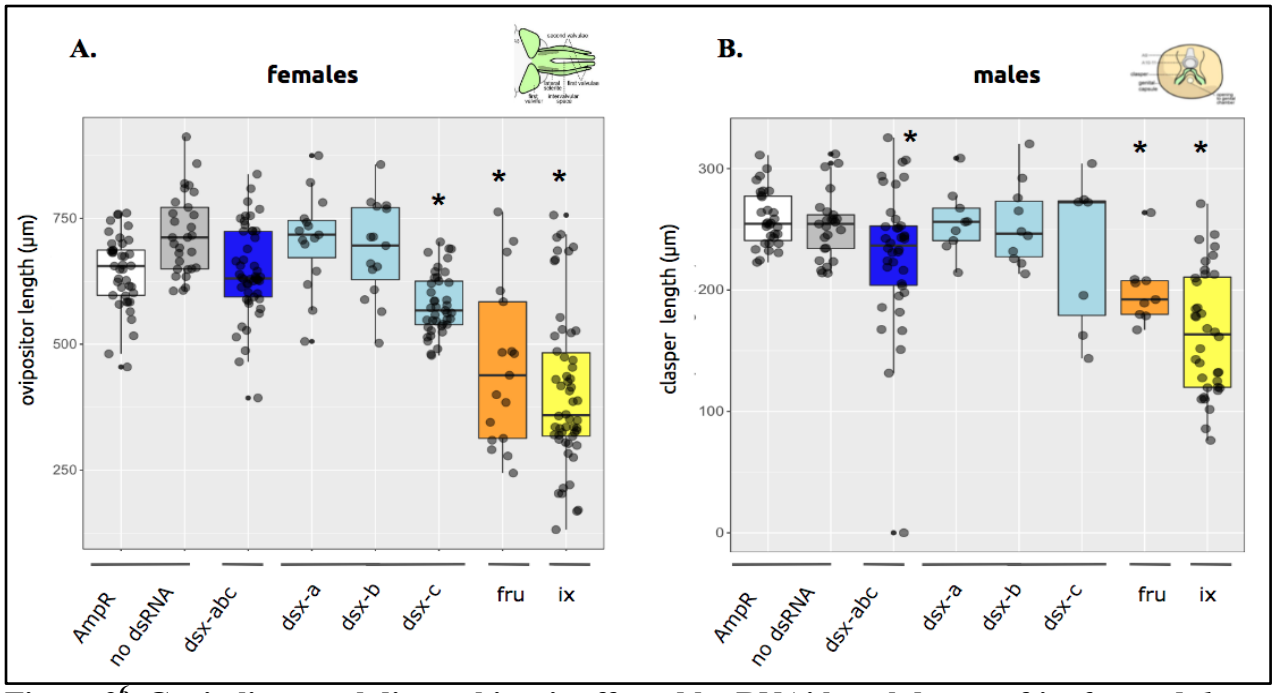

Figure 9[6]. Genitalia sexual dimorphism is affected by RNAi knockdowns of *ix, fru,* and *dsx-a, -b,* and *-c* in a sex-specific manner. (a) Compared to the *AmpR* control*, ix*, *fru, and dsx-c* knockdowns significantly shortened ovipositor length in female *O. fasciatus* specimens (Wilcoxon rank sum test, W = 1952.5, W = 568, W = 1281 respectively, $p < 0.0001$ for all three treatments). The *dsx-abc* knockdown appeared to produce a large spread of ovipositor length in female specimens. **(b)** *ix, fru,* and *dsx-abc* significantly shortened claspers compared to the *AmpR* control in male *O. fasciatus* specimens (W = 1068, W = 260 respectively, $p < 0.0001$ for *ix* and *fru,* W = 819, $p = 0.0051$ for *dsx-abc*). *dsx-c* produced two clusters of clasper lengths in males. One cluster appeared to fall within the control group while the other cluster appeared to fall within the *fru* knockdown.

[6] Diagram of ovipositors and claspers from Figure One from: Aspiras, A.C., Smith, F.W., Angelini, D.R., 2011. Sex-specific gene interactions in the patterning of insect genitalia. Dev. Biol. 360, 369–380.

3.3.2 RNAi effects on sternite curvature in female and male O. fasciatus

In order to investigate the effects of *ix, fru, dsx-a, dsx-b,* and *dsx-c* knockdowns on another sexually dimorphic structure, I measured RNAi knockdown phenotypes of the second abdominal sternite. Curvature was quantified as a measure of sex phenotype, where higher values of curvature imply a more "female" and lower values of curvature a more "male" phenotype. *fru* and *dsx-c* RNAi in female bugs led to significantly less curved sternites than the *AmpR* control group (Fig.10; Wilcoxon rank sum test, W = 428, $p = 0.018$, and W = 1821, $p = 0.018$ respectively). *O. fasciatus* females injected with *fru* and *dsx-c* dsRNA appeared to occupy an intersex region of morphospace, showing slightly lower curvature than control females, but more curvature than control males on average. In males, *dsx-c* and *dsx-abc* knockdowns led to significant increases in curvature (W = 238, $p = 0.020$, and W = 156, $p < 0.001$ respectively). Male bugs injected with dsRNA for *dsx-c* and *dsx-abc* either underwent full sex-reversal, showing fully "female-like" curvature, or occupied an intersex space with a curvature value between the average for control females and males (Fig.10). *fru* knockdowns did not significantly alter sternite curvature in male *O. fasciatus* specimens (W = 283, $p > 0.05$).

Therefore, it appears that *fru* is required for development of female-specific curvature of the second abdominal sternite in *O. fasciatus.* On the contrary, *fru* did not appear to be required for milkweed bugs to maintain L4-like lack of curvatures in male adults. *dsx-c*, which produced intersex phenotypes in both sexes, might be required for the maintenance of the respective curvature phenotypes in both female and male *O. fasciatus* once specified by *fru*. Alternatively, sex-specific splicing isoforms of *dsx-c* could facilitate the curvature development for each respective sex where only females also require the action of *fru.* This is a possibility since RNAi

targeting the common DNA-binding domain of *dsx-c* might knock down transcript isoforms in both sexes that are in fact sex-specific.

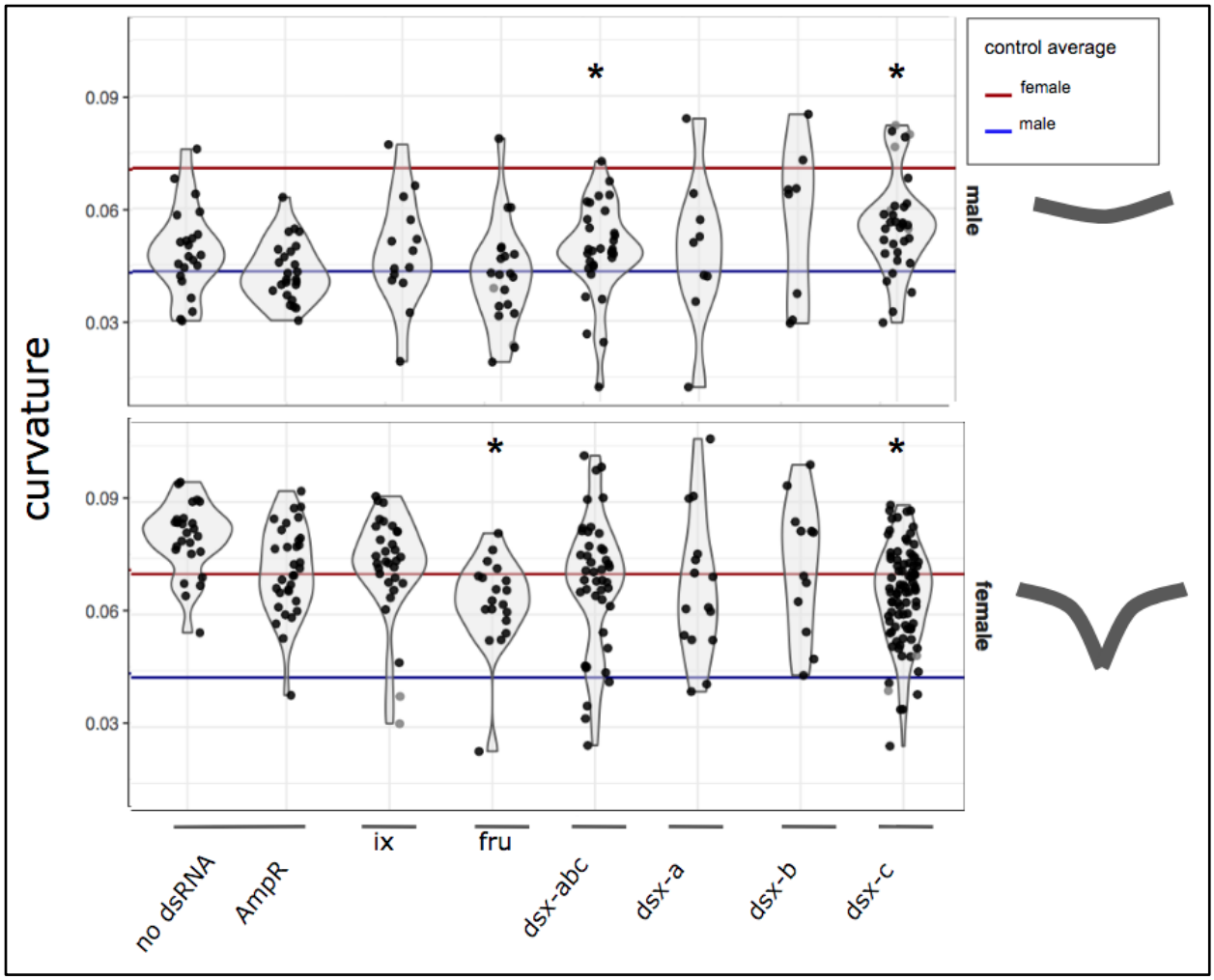

Figure 10. Sternite sexual dimorphism is affected by RNAi knockdowns of *ix, fru,* and *dsx-a, -b,* and *-c* in a sex-specific manner. Females: Only *fru* and *dsx-c* significantly decreased sternite curvature of female *O. fasciatus* (Wilcoxon rank sum test, W = 428, $p = 0.018$, and W = 1821, $p = 0.018$ respectively). Female *O. fasciatus* injected with *fru* or *dsx-c* dsRNA showed significantly reduced sternite curvature and their phenotype resembled an intermediate curvature value characteristic of an intersex phenotype. All other knockdowns were not significantly different from the *AmpR* controls. **Males:** Only *dsx-c* and *dsx-abc* significantly increased sternite curvature of male *O. fasciatus* specimens (W = 238, $p = 0.020$, and W = 156, $p < 0.001$ respectively). *O. fasciatus* specimens injected with *dsx-abc* or *dsx-c* dsRNA showed either full sex-reversal phenotypes and displayed female-like curvature values or showed curvature values between the averages of control females and males. All other knockdowns were not significantly different from the *AmpR* controls.

3.3.3 RNAi produced different combinations of impaired genitalia and/or sternite development

RNAi knockdowns of *ix, fru, dsx-a, dsx-b,* and *dsx-c* produced a variety of sex phenotypes where *ix, fru,* and *dsx-c* appeared to be required for ovipositor development. Alternatively, *ix, fru,* and either *dsx-c* or all three *dsx* paralogs appeared to be required for clasper development (Fig.9). On the other hand, *dsx-c* and *fru* appeared to be required for the acquisition and maintenance of female sternite curvature while only *dsx-c* appeared to be required for that of males (Fig.10). Qualitative analyses of the loss-of-function phenoyotes from RNAi experiments seemed to further demonstrate these effects of *ix, fru,* and *dsx-c. ix* and *fru* knockdowns produced ovipositors that appeared to be significantly shorter, stubbier, and malformed compared to wildtype ovipositors or those of *AmpR* dsRNA-injected milkweed bugs. Additionally, these ovipositors took on a "clasper-like" shape, acquiring curvature and pointy ends (Fig.11a). Interestingly, while *dsx-c* knockdowns did also significantly shorten ovipositors, they appeared to do so less dramatically than *ix* and *fru* knockdowns and did not lead ovipositors to resemble claspers (Fig.11a, Fig.12a). Additionally, *ix* knockdowns showed female-characteristic curved abdominal sternites while *fru* knockdowns did not. *dsx-c* knockdowns appeared to produce female specimens with significantly more male-like sternite curvatures (less curved; Fig.11a). Similarly, *ix* and *fru* knockdowns in males led to *O. fasciatus* specimens with no or severely shortened claspers, while these knockdowns maintained the male-like absence of sternite curvature. In contrast, *dsx-c* and *dsx-abc* knockdowns increased sternite curvature in male bugs in addition to shortening the claspers (Fig.11b).

Hence, these results implicate *ix, fru,* and *dsx-c* in tissue- and sex-specific functions during sex determination of *O. fasciatus. ix* promotes genitalia development in both females and males, but does not affect sternite curvature (Fig.9, Fig.10, Fig.11, Fig.13). *fru* is also required

for genitalia development, but is necessary only in females for the development of abdominal curvature (Fig.9, Fig.10, Fig.11, Fig.13). Finally, *dsx-c* is implicated in female ovipositor development and might also control clasper development. Since *dsx-c* knockdowns shortened ovipositors less severely than *ix* and *fru* knockdowns, *dsx-c* might be performing a more specific function in ovipositor development once their presence has already been specified by another gene. Alternatively, all three *dsx* paralogs could be required for clasper development. Finally, *dsx-c* might be needed for the maintenance of both female and male curvatures (Fig.9, Fig.10, Fig.11, Fig.13). It might be preventing the bugs from entering an intersex curvature morphospace once either female- or male curvature has developed.

Overall, it appears that both *fru* and *dsx* are implicated in genitalia development and curvature development in *O. fasciatus*, where each gene has a sex-specific function in one of those two tissues. *ix,* on the other hand, appears to only be required in genitalia development in *O. fasciatus*, but not in sternite development. These results show that genes may act to control the development of different somatic sexual dimorphisms by acting in tissue-specific as well as sex-specific manners.

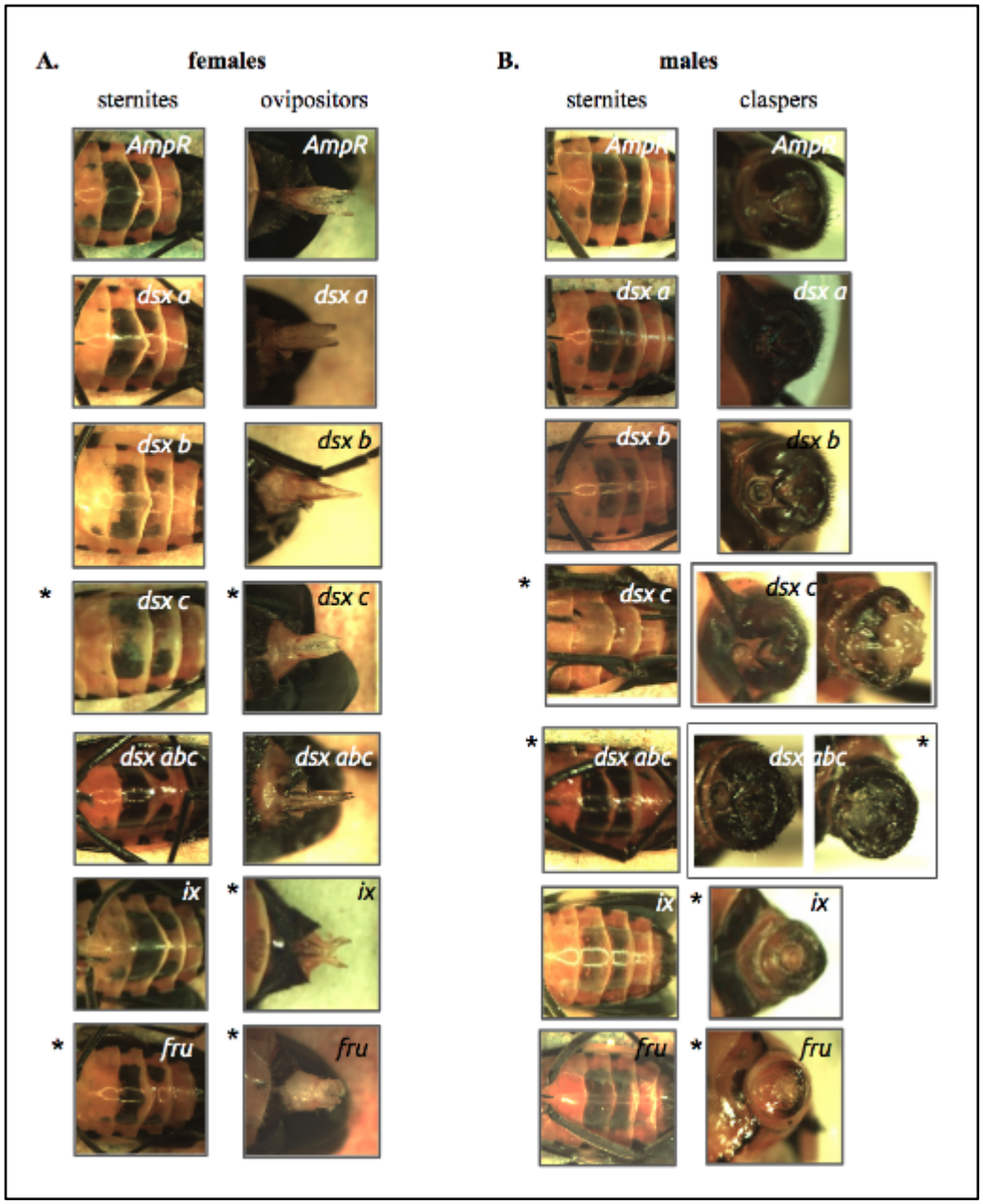

Figure 11. Qualitative analysis of *ix, fru,* and *dsx* paralog RNAi knockdowns demonstrates tissue- and sex-specificity of target genes. (a) Female bugs appear to require *ix*, *fru,* and *dsx-c* for genitalia development and *dsx-c* and *fru* for sternite development. *ix, fru,* and *dsx-c* led to shortened ovipositors while only *fru* and *dsx-c* also led to loss of female-characteristic curvature. Additionally, *ix* and *fru* knockdowns more severely shortened ovipositors than *dsx-c.* **(b)** Male *O. fasciatus* specimens appear to require *ix, fru*, and either *dsx-c* or all three *dsx* paralogs for genitalia development but only *dsx-c* for that of sternites. *ix, fru,* and *dsx-abc* knockdowns produced individuals with a complete loss or significant shortening of claspers while only *dsx-c* and *dsx-abc* knockdowns also produced males with acquired sternite curvature.

3.4 qRT-PCR examining gene interactions and RNAi validation

To validate the RNAi treatments and to investigate interactions of *ix* and the *dsx* paralogs, I used qRT-PCR to measure *ix, dsx-a, dsx-b,* and *dsx-c* expression in RNAi treatments targeting these genes (Fig.12). *ix* mRNA levels were significantly lowered in the *ix* RNAi knockdown (Wilcoxon rank sum test, W = 36, $p = 0.001$), validating this knockdown. *ix* mRNA levels were not significantly lowered in any other dsRNA treatments, indicating no interactions with other genes in which *ix* expression might be activated by any of the *dsx* paralogs.

Several of the samples, including the *AmpR* dsRNA controls, had low expression of *dsx-a* and *dsx-b*, indistinguishable from the no-RT controls (Fig.12a,b). This fact makes inferences for these genes impossible, but accounts for the high variance in *dsx-a* and *dsx-b* expression (Fig.12a,b). Hence, different control individuals are needed to draw inferences about the expression of these two genes. *dsx-b* expression appeared to be increased in the *dsx-a, dsx-b, dsx-c,* and *dsx-abc* knockdowns (Fig.12b). This could be due to the qRT-PCR probe binding the dsRNA injected into the bug at a complementary sequence for the *dsx* paralogs. Alternatively, it could also be due to an endogenous amplification that is a part of the RNAi mechanism of the cell. In some cases, dsRNA treatments have been shown to increase mRNA levels for the knocked down gene because the cell is trying upregulate the gene's expression in an attempt to compensate for the knockdown (Yoshinori Tomoyasu, personal communication). More qRT-PCR studies for validation, with higher sample sizes, should be conducted to further investigate the validity of our *dsx-a* and *dsx-b* knockdowns.

dsx-c mRNA levels were significantly lower in the *dsx-abc* knockdown, but not in the *dsx-c* knockdown (Fig.12c; Wilcoxon rank sum test, W = 50, $p = 0.016$). This appears to validate the *dsx-abc* knockdown for *dsx-c*, but not *dsx-a* and *dsx-b.* Additionally, *dsx-c* mRNA levels

appeared to be lower in the *dsx-a, dsx-b,* and *ix* knockdowns, though these results were not statistically significant (Fig.12c). This could imply interactions between *ix, dsx-a, dsx-b,* and *dsx-c*, where the expression of *dsx-a, dsx-b,* and *ix* might be needed to activate *dsx-c* transcription.

Overall, our RNAi phenotypes for *ix* and *dsx-c* in the triple knockdown can be validated. Given that obvious phenotypes were observed in RNAi treatments for *ix, fru,* and *dsx-c* and those knockdowns could be validated, it appears that conclusions can be drawn from those data. However, *dsx-a* and *dsx-b* inferences should only be drawn with caution.

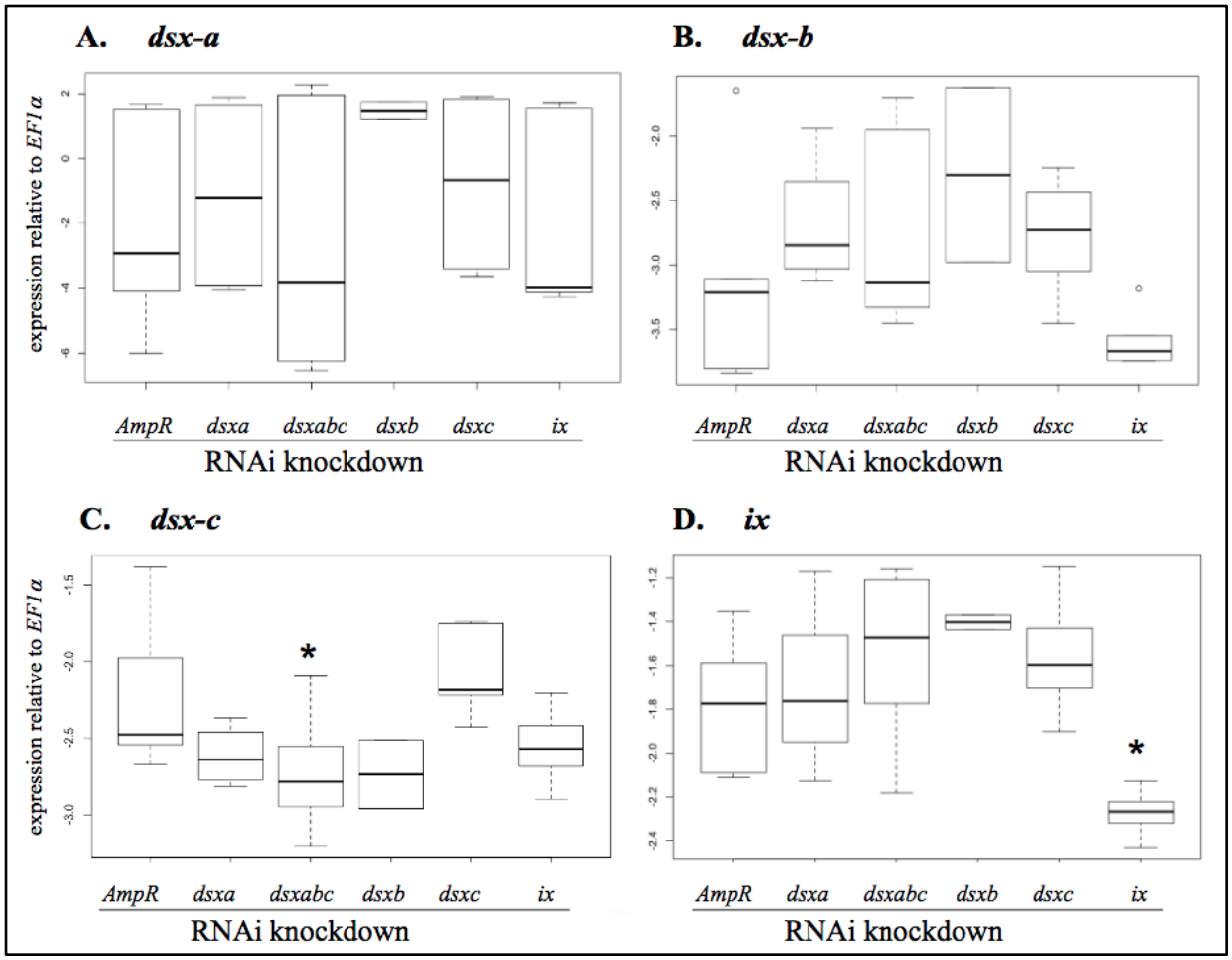

Figure 12. qRT-PCR expression measurements of *ix*, *dsx-a*, *dsx-b*, and *dsx-c* show decreased *ix* and *dsx-c* expression in *ix* and *dsx-abc* knockdowns respectively. (a) Expression measurements of *dsx-a* showed a large spread of expression in all knockdowns except *dsx-b*. Low samples were likely equivalent to no expression of *dsx-a* in those samples. **(b)** Expression measurements of *dsx-b* showed a considerable spread of expression in all knockdowns except *ix*. Low samples were likely equivalent to no expression of *dsx-a* in those samples. Expression of *dsx-b* was increased in *dsx-a, dsx-b, dsx-c* and *dsx-abc* knockdowns, though these results were not statistically significant. **(c)** *dsx-c* expression was decreased in the *dsx-a, dsx-b,* and *dsx-abc* knockdowns, though the result was only significant for the *dsx-abc* knockdown (Wilcoxon rank sum test, W = 50, $p = 0.016$). *dsx-c* expression was increased in the *dsx-c* knockdown though this result was not statistically significant. **(d)** *ix* expression was significantly decreased in the *ix* knockdown (W = 36, $p = 0.001$) but not in any of the other knockdowns.

CHAPTER FOUR

CONCLUSIONS AND FUTURE DIRECTIONS

4.1 The Sex Determination Cascade of *O. fasciatus*

The experiments conducted as part of this project suggest that *ix, fru*, and *dsx* serve different functions within the insect evolutionary lineage. All three genes might be altered in sequence and function by natural selection, while accelerated sequence alterations appear to be present in *dsx* but not *ix* and *fru*. While both *dsx-a* and *dsx-b* appear to be expressed during development of *O. fasciatus,* their RNAi knockdowns did not produce phenotypes significantly different from the wildtype. Additionally, these knockdowns could not yet be validated. Hence, it is unclear whether these two *dsx* paralogs serve a function during the development of *O. fasciatus* or whether they are transcribed and then degraded without playing a significant developmental role.

Moreover, it appears that *dsx* orthologs differ within Hemiptera and between the two studied hemimetabolous insects. This further suggests the rapid evolution of insect sex determination genes, especially *doublesex* orthologs, and might imply their greater tolerance of mutations and functional changes compared to genes of other developmental pathways. This would explain the vast diversity observed in sex determination mechanisms across insects. Additionally, this notion is supported by the fact that both *ix* and *fru* appear to serve different functions in the sexually dimorphic development of *O. fasciatus* and *D. melanogaster.* In *D. melanogaster ix* is only required by females for wildtype genitalia development. However, our RNAi results suggest that both females and males require *ix* for genitalia development in *O. fasciatus.* Additionally, *fru* appears to be a transcription factor particularly involved in the development of male-specific neurons in *D. melanogaster*, but seems to also be implicated in the

genitalia development of both female and male large milkweed bugs. This suggests changes in the function of *ix* and *fru* within the insect lineage. Interestingly, the role of *dsx-c* in *O. fasciatus* appears not to be that of a sole master regulator of sex determination as it has been labeled in other insects. Rather, *dsx-c*, together with *ix* and *fru*, appears to help facilitate tissue-specific development of sexual dimorphism. *dsx-c*, in particular, seems to be required for the wildtype development of female and male genitalia in *O. fasciatus,* as well as for preventing the intersex sternite phenotypes of both females and males. Alternatively, all three *dsx* paralogs could be required for male genitalia development.

Overall, our results might suggest the presence of female- and male-specific splicing isoforms of *ix, fru*, and *dsx-c* that might carry out tissue- and sex-specific functions. *ix* and *fru* could both simply promote the development of genitalia while the specific determination of claspers versus ovipositors may be specified for by another gene, such as, for example, *dsx-c* versus that of all three *dsx* paralogs. On the other hand, sex specific splicing isoforms of *ix* and *fru* might be, in themselves, initiating the development of sex-specific genitalia. This is the same for *dsx-c*'s and *fru*'s implications in sternite curvature determination and development. Either the presence of a female- and male-specific isoform of *fru* and *dsx-c* could lead to the curvature development of the respective sternite. Or alternatively, the absence of a male-specific *fru* isoform could lead to curvature acquisition of the sternite only in the female via female-specific *fru* expression. At this point, our qRT-PCR and RNAi results are unable to provide isoform-specific information, but future experiments should use isoform-specific primers to investigate the time-, sex-, and tissue-specific expression of *ix, fru,* and *dsx-c* isoforms in order to further elucidate how alternative splicing might be implicated in the sex determination cascade of *O. fasciatus.* Alternative splicing has been implicated in the sex determination pathways of many

animals. Moreover, the regulation of alternative splicing has been shown to be altered by natural selection and could be a key mechanism by which sex determination pathways change in animals (Baker, 1998; Cho et al., 2007; Salz, 2011; Smith et al., 2018). Hence, the future investigation of its implication in the sex determination cascade of *O. fasciatus* will be crucial in further investigating how it might contribute to the evolution and regulation of sex determination pathways in animals.

Thus, while we have not found one master regulator of sex determination in *O. fasciatus*, we have shown differing functions of *ix* and *fru* in *O. fasciatus* compared to *D. melanogaster,* tissue-specific control of dimorphisms by *ix* and *dsx-c*, and a potential implication of alternative splicing in the sex determination cascade of *O. fasciatus.* The sex determination cascades of other insects should be investigated in more detail to allow for more orthology studies to further our understanding of the evolution of sex determination mechanisms in animals.

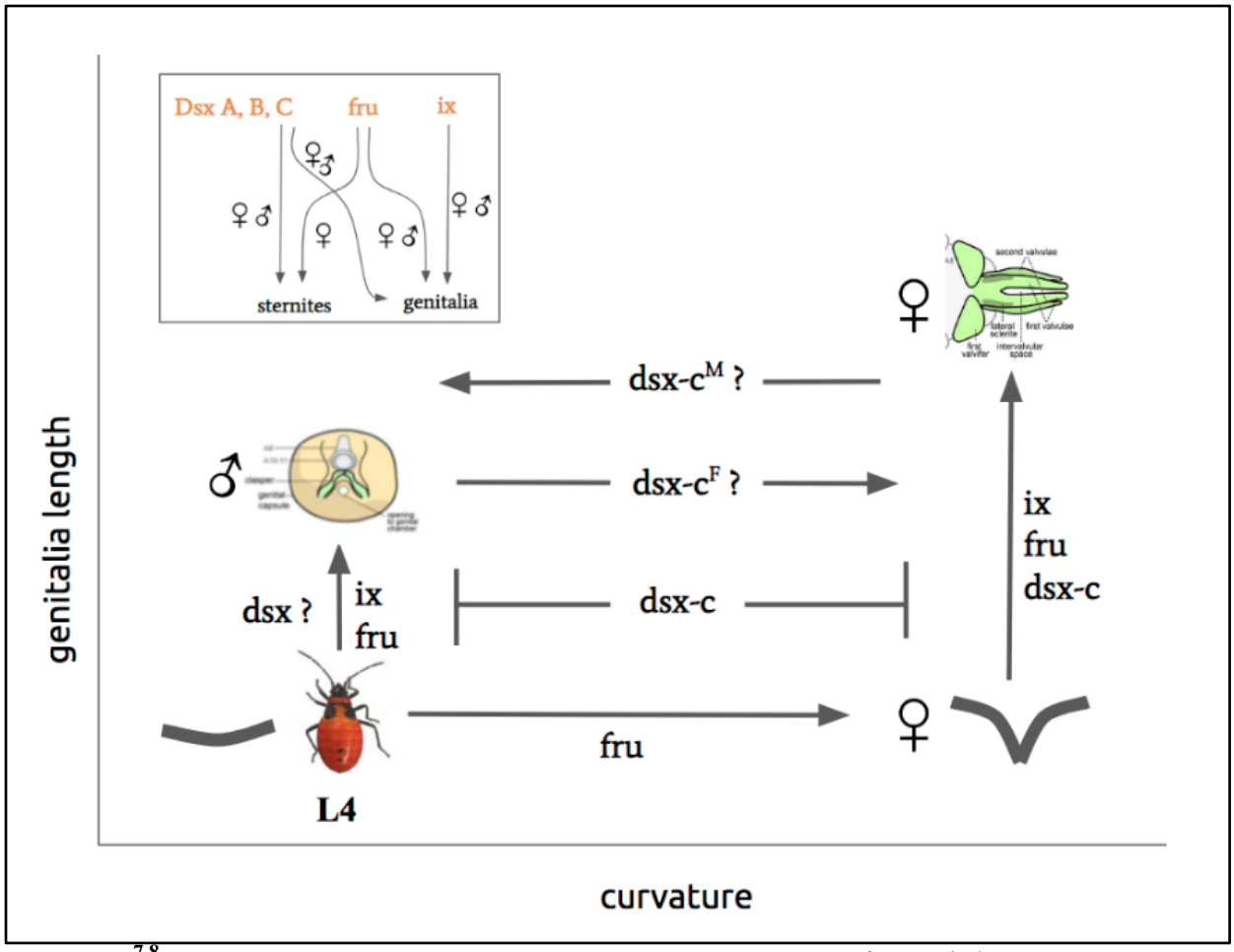

Figure 13[7,8]. The Sex Determination Cascade of *O. fasciatus.* *ix, fru,* and *dsx-c* appear to initiate genitalia development in female and male milkweed bugs. However, all three *dsx* paralogs might be serving this function redundantly only in males. This could be achieved by sex-specific splicing isoforms of *ix, fru*, and *dsc-c* (or all *dsx* paralogs in males). *fru* appears to initiate female-specific curvature development while *dsx-c* appears to prevent the acquisition of intersex curvature in both sexes after initial specification. This might be achieved via sex-specific splicing isoforms of *dsx-c* that serve to maintain a phenotype already specified for by a previous genetic event.

[7] Diagram of ovipositors and claspers from Figure One from: Aspiras, A.C., Smith, F.W., Angelini, D.R., 2011. Sex-specific gene interactions in the patterning of insect genitalia. Dev. Biol. 360, 369–380.

[8] *O. fasciatus* L4 image from Figure Three from: Pacheco, P.F., Fernandes, C.P., Xavier, A., Santos, M.G., Mexas, R., Ratcliffe, N.A., Gonzalez, M.S., Mello, B., Feder, D., 2013. Laboratory evaluation of the effects of *Manilkara subsericea* (Mart.) Dubard extracts and triterpenes on the development of *Dysdercus peruvianus* and *Oncopeltus fasciatus.* Pest Manag. Sci. 69, 292–301. https://doi.org/10.1002/ps.3388

4.2 Future Directions

Additional experiments remain to be done to further expand the data collected and analyzed as part of this project. Firstly, qRT-PCR studies should be further conducted to increase the sample size to allow for conclusions with more statistical rigor. Moreover, qRT-PCR studies with exon-specific primers should be done to investigate the expression of *ix* and *dsx-a, dsx-b,* and *dsx-c* splicing isoforms during *O. fasciatus* development. These experiments should include the investigation of isoform expression in different tissues. Finally, *fru* should be added to all of the aforementioned qRT-PCR experiments to better understand its expression patterns during the development of *O. fasciatus*.

Additionally, qRT-PCR validations of the *dsx-a, dsx-b,* and *dsx-c* knockdowns should be continued. The sample sizes for these experiments should be increased. Finally, it might be worth re-designing probes and primers for *dsx-a* and *dsx-b.*

Next, RNAi experiments should be followed up with further *dsx-c* knockdowns. Particularly sample sizes for male specimens should be increased to investigate the clustering of phenotypes within this treatment group. This should be done to confirm or reject the possibility that *dsx-c* is, in fact, individually required for clasper development as opposed to all *dsx* paralogs in redundant fashion. Moreover, maternal RNAi knockdowns should be conducted for all investigated genes to better elucidate the functions of *ix, fru*, and the *dsx* paralogs during earlier stages of juvenile development. Specimens treated with RNAi before the fourth instar tend not to be viable, but maternal knockdowns have been shown to effectively reduce the expression of target genes in offspring.

Finally, two hox genes should be added to this analysis to better understand the relationship between sex determination and body-plan patterning. *Abdominal-A* (*AbdA*) controls

the development of the body segment that includes the second abdominal sternite and previous *AbdA* RNAi knockdowns have been shown to reduce sex-specific sternite curvature (Angelini et al. 2005). It is likely that *AbdA* knockdowns will not significantly affect genitalia development since this hox gene does not specify the most posterior body segment. *Abdominal-B* (*AbdB*), the hox gene which specifies the most posterior segment in *O. fasciatus*, should also be knocked down to investigate its effect on both genitalia and sternite development (Angelini et al. 2005). Additionally, both hox genes could be investigated for expression during development and for interactions with the target genes of sex determination via qRT-PCR.

Overall, these follow-up experiments should allow us to better investigate the specifics of the *O. fasciatus* sex determination cascade, especially with regard to alternative splicing, activities of the target genes during early development, and interactions with other developmental pathways. Holistically, these experiments will contribute to our knowledge of insect sex determination mechanisms and their changes during insect evolution.

4.3 Evolution of the *O. fasciatus* Sex Determination Pathway

Sex determination pathways evolve rapidly and differ among animals, especially insects. Additionally, it appears that changes in alternative splicing, the specific interactions of genes, and the expression patterns of certain genes are mechanisms by which these pathways change (Smith, 2028; Stern, 2011). Overall, this fits with Waddington's and Stern's conception of evolution and development as interacting processes, in which the underlying developmental genetic network biases what evolutionary changes are more likely to become fixed. *dsx-c*, for example, might occupy a place in the developmental network that tolerates mutations without quickly leading to lethal or detrimental effects. This could explain its accelerated sequence

evolution, the frequency of duplication events compared to *ix* and *fru*, and its less dramatic effect on female genitalia in *O. fasciatus*. *ix* and *fru*, on the other hand, might be more susceptible to change in function due to mutations in enhancers or silencers of the two genes, preserving the protein sequence of each gene. These results could suggest that *dsx* is, in fact, more downstream in the sex determination cascade of *O. fasciatus* than *ix* and *fru.*

This phenomenon can be conceptualized by Waddington's canalization landscape where genetic "peaks and valleys" affect an organism's phenotype and bias acquisition of new mutations in certain genes. Stern even goes so far as to argue that by better understanding developmental networks we might become able to predict which evolutionary changes to an organism's genome might be most likely to occur given certain evolutionary pressures. This project offers relevant insight into how the genes of sex determination are altered during evolution in different ways. Further studies of fundamental developmental pathways and their differences between animals will broaden our perspective of how genetic networks bias evolutionary outcomes and how evolutionary processes lead to the alteration of those genetic networks. Overall, such experiments might help us better understand how and why different animals often show various genetic mechanisms to achieve similar developmental outcomes. In fact, they might just help us better explain the genetic and phenotypic diversity we observe in the world around us.

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APPENDIX

Appendix A. Raw Data and R-Scripts

The raw data collected and analyzed as part of this project are available for download at Colby College's "digital commons" (https://digitalcommons.colby.edu/honorstheses/) in a folder titled "jjust_honors_2019_supplementary". All sequence alignments, and qRT-PCR and RNAi datasets are available as they were imported into R. In addition, the R-scripts that were used for analysis are provided as well. A text file that describes each file's contents is included in the folder available for download.