A FUNCTIONAL GENETIC STUDY OF SEXUAL DIMORPHISM AND BEHAVIORAL ECOLOGY IN *Chrysomya rufifacies* (DIPTERA: CALLIPHORIDAE)

A Dissertation

by

MEAGHAN LEIGH PIMSLER

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Chair of Committee,	Jeffery K. Tomberlin
Co-Chair of Committee,	Aaron M. Tarone
Committee Members,	Micky D. Eubanks
	Ginger E. Carney
	Mariana Mateos
Head of Department,	David W. Ragsdale

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ABSTRACT

Chrysomya rufifacies Macquart (Diptera: Calliphoridae) is a blow fly of medical, veterinary, and forensic relevance. This species is native to the Australo-Pacific region and invasive in the New World. A unique characteristic of this species is that it lacks obvious sex chromosomes and possesses a poorly understood monogenic sex determination system in which females produce all male or female clutches. This species also engages in facultative predation on other blow fly species, but the proximate causes of this behavior remain unstudied. The unusual sex determination mechanism employed by this species was leveraged to study sexually dimorphic patterns of gene expression throughout immature development, expression of genes between sexes and sex-producers, and to evaluate the genes correlated with predation.

The exact mechanism leading to monogenic sex determination in *C. rufifacies* was not identified in this work, though many genes were differentially expressed between females relative to the sex of their offspring. Gene expression results, coupled with the observation that sex ratios deviate from a binomial distribution, suggest there may be neurological, physiological, or behavioral differences between female-types. Three specific genetic hypotheses related to the primary signal establishing sex determination in this species are raised by this work: *transformer*, *Sex-lethal*, *daughterless*. Genetic tools are now available to investigate differential expression and the effect of these genes on sexual development, but do not preclude the possibility of a gene unique to this species or non-coding RNA in directing sexual fate as hypothesized in other taxa.

The results show that sexual dimorphism in gene expression can be observed at all

stages of development, with a female bias in differentially expressed nodes in egg and adult stages and male bias in the larval and pupal stages. Male up-regulated genes tended to be those related to neurogenesis and behavior, whereas female enriched genes were involved in metabolic processes, muscle development, and oogenesis.

Several genes were differentially expressed between actively predating and non-predating individuals, including those involved in growth regulation, response to starvation, dehydration, and neurogenesis. There was also weak evidence of sexual-dimorphism in predation rates and gene expression.

DEDICATION

This work is dedicated to all of the people who think that they must give up on their dreams because they made mistakes in the past. If I can accomplish my dreams, so can you.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Broad strokes

"I don't know the question, but sex is definitely the answer."

— Woody Allen

Conflict between and within the sexes has significantly influenced great swathes of human culture. Much art, literature, and music have been dedicated to philosophical discussions on this emotional and carnal topic. Examples include the best-selling self-help book Men are from Mars, Women are from Venus [1] and the vast majority of country music songs (75% of Hank Williams' songs deal with romantic relationships and broken hearts) [2]. Developmental biologists, behavioral ecologists and microbiologists, amongst others, have long been interested in determining the evolution, ecology, and consequence of the differences between the sexes.

My goal for this chapter is to explore the role of sex genetically, behaviorally, and ecologically as related to my research topic. I will begin by discussing sexual conflict. I then will explore sex determination and sexual dimorphism. I will also discuss patchy ephemeral resource ecology, a system that has begun to garner attention for both basic and applied researchers. Finally, I will synthesize these concepts in discussing a non-model organism I focus on in my research, *Chrysomya rufifacies* Macquart (Diptera: Calliphoridae).

Sexual conflict

"Competition is inevitable, but combat is optional."

— *Max Lucade*

Early on, sexual conflict was broadly defined by biologists as, "differences in the evolutionary interests between males and females" [3]. Classical studies in sexual conflict focused on traits directly related to reproduction, such as number of mates, frequency and duration of mating, timing of oviposition, use of sperm, parental investments, and nuptial gifts to name a few [4-9]. More recent studies in sexual conflict have begun to investigate sexual conflict at a genetic level and identify genes under divergent selection between the sexes [10]. The traits that might be responding to sexual conflict could be involved in sex determination or the production and maintenance of physiological, morphological, or behaviorally sexually dimorphic traits [11-15]. This genetic conflict can be described as intergenomic or intragenomic conflict [16] and can also be broken down into interlocus and intralocus categories [17, 18]. In some cases, it can be difficult to differentiate between these cases due to various selective pressures on complex pleiotropic traits.

Sex determination

"Men and women may speak the same language, but we interpret words differently."

— *Pamela Cummins*

Biologists, comedians, and philosophers are amongst some of the professions that have considered the ramifications of sexual reproduction, and also the requirement for and distinctions between sexes [1, 19]. Public health outreach agendas are centered on

some of the disadvantages to sexual reproduction, both psychologically and physically (diseases) [20, 21], and many of these dangers are present throughout the animal kingdom [22, 23]. Despite these widespread consequences, sexual reproduction remains the most common form of reproduction amongst multicellular organisms. There are two related hypotheses behind the ubiquity and advantages of sexual reproduction [24]. First, that recombination will increase inherent genetic variability within a species and therefore enable enhanced plasticity in response to unpredictable biotic and abiotic factors in the environment [24]. Second, that recombination increases the rate at which detrimental mutations are purged from the genome and beneficial mutations are incorporated [24]. Mathematical modeling, experiments in yeast and several *Drosophila* species (Diptera: Drosophilidae), and comparisons between sexually and asexually reproducing populations of water fleas (*Daphnia pulex* Leydig, Cladocera: Daphniidae) have certainly supported the contention that sexual recombination increases the ability of a population to adapt to changing environmental conditions, removes deleterious mutations, and preserves advantageous mutations [25-30]. For instance, asexual lineages of *D. pulex* exhibit higher rates of amino acid substitution when compared to sexually reproducing lineages [29]. Work in other species have demonstrated that amino acid substitutions are neutral or positive in 27-29% of cases [31], which suggests that reproductive strategies that increase the probability of non-synonymous mutations are likely to have a net deleterious effect on the population.

However, differentiation is generally required for sexual reproduction to occur. The classic sex determination system requires inherent plasticity and bi-stability [32], as

different transcripts of the same gene may be differentially expressed between the sexes. While the genetic cascades leading to sex determination have been identified in some species, and a catalogue of the variety and ecological consequences of sexual dimorphism have been investigated, the mechanisms that give rise to these traits are poorly understood. Integration of these avenues of research with sexual conflict theory may provide valuable insights into the evolutionary processes that govern sexual reproduction and sexual dimorphism.

Sex determination systems

Sexual reproduction can be distinguished into two separate classes. Monoecious species (those with simultaneous hermaphrodites), individuals serve both male and female function, produce micro- and macro-gametes respectively [33]. Furthermore, though mating with another conspecific is not required for conception [34], sexual reproduction can accelerate the onset of oviposition, increase reproductive output, and prevent inbreeding depression [35, 36].

The other class of sexual reproduction is multiecious (also known as gonochoristic) in which species have at least two distinct sexes [37]. Dioecious species are a subgroup of multiecious that have only two sexes. Within this group, there is evidence of at least seven different mechanisms which all have the same outcome- production of at least two "sexes". The best-known system is male heterogamety, in which females are XX and males are XY for the sex determining chromosomes. The default sex is generally female, and the production of male individuals is due to dominant male determiners, usually present on the Y chromosome, such as in humans, mice [38] and the blow fly *Lucilia*

sericata Meigen (Diptera: Calliphoridae) [39]. Male heterogamety can also function through a genic balance between the number of X chromosomes and autosomes maintained through dosage compensation, such as has been experimentally determined in *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) [40] and *Caenorhabditis elegans* Maupas (Rhabditida: Rhabditidae) [41]. Female heterogamety (females are ZW, males are ZZ) is also observed through the action of dominant female determiners [42] and possibly genic balance, though there is conflicting molecular evidence [43].

Other systems rely upon maternal factors. In environmental sex determination observed in many reptiles [44] and fish [45], environmental factors such as temperature determine the sex of the developing offspring; mothers can adjust the sex-ratio with selective oviposition. In some insects (Hemiptera), at least one of the paternally donated sex chromosomes is destroyed in the embryo due to maternal factors regulated by the temperature at which the female developed [46]. In haplodiploid species, mothers determine the sex of their offspring through selective fertilization. In this system**,** females have a diploid complement of chromosomes and males are haploid [47]; more specifically, in this system heterozygosity at the *complementary sex determiner* (*csd*) locus leads to a female fate, and homozygosity or hemizygosity leads to males. Monogenic sex determination, in which a single gene in the mother controls sex determination in the developing offspring, appears to be the mechanism in the sister species of blow fly (Diptera: Calliphoridae) *Chrysomya rufifacies* Macquart and *Chrysomya albiceps* Wiedemann [48]. Polygenic sex determination is a system in which multiple genes control sex determination, though no single gene is wholly responsible

for sex determination. Though there is tenuous evidence for this system in the European Sea Bass, *Dicentrarchus labrax* L. (Perciformes: Moronidae) [49], it is still hypothetical at this point.

Sex ratios and sex determination

Though there are many different sex determination systems in the animal kingdom as explored above, wild populations will often be found in a 50:50 male to female sex ratio [37, 50]. The relatively even sex ratios in adults persist despite the ability of mothers to adjust the sex of their offspring through selective fertilization, oviposition site choices, and age-related adjustment of care investment in response to environmental conditions [44, 51, 52]. Sir Ronald A. Fisher was the first to explore this concept through mathematical modeling, and other researchers have expended upon his ideas to incorporate newer avenues of research [53, 54]. Fisher's Principle relies upon the effect of frequency-dependent selection, and argues that the fitness advantage associated with producing one sex is highest when that sex is less frequent in the population. Fisher's Principle can be extended to other phenotypes through the concept of evolutionary stable strategies initially proposed by Maynard Smith and Price [55]. Essentially, though there may be factors influencing sex-ratio investment in particular individuals or populations, these biotic and abiotic factors interact to produce a dynamic sex-ratio equilibrium of even numbers of adult males and females over time [54]. There are many exceptions to this relatively even split, most notably in eusocial hymenoptera and parthenogenetically reproducing organisms such as aphids (Homoptera: Aphididae) and some katydids (Orthoptera: Tettigoniidae) [56, 57], but these are termed "extraordinary" systems when

considered in the context of all insect and animal populations which have been studied up to this point [58]. However, this is still a fruitful and controversial area of research that modern tools and technologies are finally helping researchers to investigate empirically rather than theoretically.

Genetic mechanisms of sex determination

While researchers have identified many sex determination systems, they have yet to reveal the genetic basis and mechanisms of action in most species. However, sex-determination hierarchies have been well characterized in three model organisms; the fruit fly *D. melanogaster*, the nematode *C. elegans*, and the house mouse *Mus musculus* L. (Rodentia: Muridae).

Decades of research in *D. melanogaster* have revealed a complex hierarchy of sex-determination genes, an understanding of their modes of action and interaction, and some of the consequences of mutations at these loci [59]. Though the exact mechanism has not been elucidated, a consequence of the X-chromosome to autosome ratio is sex-specific splicing of the primary signal *Sex-lethal* (*Sxl*). This splicing leads to the production of a functional RNA-binding protein in females (SXL^F) and an inactive protein in males due to the inclusion of an exon with a precocious stop codon [60]. This protein maintains its own expression through positive auto-regulation via splice-sites within the pre-mRNA [61]. SXL^F also controls the splicing of the next step in the sex-determination cascade, *transformer* (*tra*), yielding functional female (TRA^F) and non-functional male (TRA^M) proteins [62]. TRA^F is also positive auto-regulatory, controls splicing of *doublesex* (*dsx*) into active male (DSX^M) or female (DSX^F) forms [63], and

contributes to the production of male-specific transcripts of *fruitless* (*fru*) [64]. The regulation of downstream gene expression by DSX^F in females and DSX^M and FRU^M in males gives rise to most of the physiological, biochemical, and behavioral sexual dimorphism in this species. Notably, all of the genes in this central pathway are transcription factors.

Unlike *Drosophila*, *C. elegans* has a specialized mode of reproduction, in which there are simultaneous hermaphrodites capable of inter-breeding and selfing, and males that cannot reproduce on their own [65]. Similar to *D. melanogaster*, initial cues for sex determination begins with a genic balance system, with individuals with two X chromosomes becoming hermaphrodites and individuals with a single X chromosome becoming males [66]. The system in *C. elegans* is more complicated than that of *D. melanogaster*, and involves multiple cascades and signaling pathways. These pathways converge upon the *transformer-1* (*tra-1*) gene [67], which is sex-specifically spliced into the active and robust protein TRA-1 in hermaphrodites and the easily degraded and inactive form TRA-1A in males [68]. This gene is homologous to cell-to-cell signaling genes from the hedgehog-signaling pathway in both *D. melanogaster* and in mammals [69]. Two genes homologous with *Drosophila dsx*, *male-abnormal-3* (*mab-3*), and *doublesex/male abnormal 3 domain family 3* (*dmd-3*), are the downstream targets of TRA-1 and lead to differentiation [70].

Compared to the systems outlined above, mammal sex determination is dependent upon the dominant male determiner *Sry* located on the Y chromosome [71]. Work in mice has shown that expression of the SRY protein in the bipotential gonadal tissue

leads to differentiation into testes, rather than ovaries, in genetically male zygotes [72]. The SRY protein acts with a nuclear receptor, Steroidogenic Factor 1 (SF-1), through an upstream *cis*-enhancer to regulate expression of *SRY-box 9* (*Sox9*). A homolog of *Sox9*, *Sox100B*, is required for proper testicular development in *Drosophila* [73], suggesting that this gene may be conserved in function across taxa. Compared to the nematodes and flies however, mammalian sexual dimorphism is caused by the response of other tissues to steroidal hormones, testosterone in males and oestrogen in females, produced in the gonads [74].

Model organisms might only be a model for themselves

Research in other flies has suggested that the sex-determination system identified in *Drosophila* is somewhat distinct in the order [75, 76]. One common feature to other dipteran systems is that *tra* is the basal signal, rather than *Sxl*, and *Sxl* is not somatically sex-specifically spliced [39, 75-77]. The "standard" system in blow flies such as *L. sericata*, the true fruit flies (Diptera: Tephritidae), or the common house fly *Musca domestica* L. (Diptera: Muscidae) is one of heteromorphic sex chromosomes and a presumed dominant male determiner (*M*) on the Y chromosome [39, 78-81]. The presence of this *M* factor in the zygotic genome is presumed to cause that individual to develop into a fertile male.

Musca domestica employs the largest variety of sex determination mechanisms known in the flies [82-84]. In some populations, sex appears to be determined by the maternal genotype, with females heterozygous for a dominant arrhenogenic allele (*Ag*) having only male offspring [84]. Therefore, in populations with this allele, there are two

types of females: male-only producing females, and females that have bigenic (both male and female) offspring. The presence of maternally derived female form of *M. domestica tra* (*Mdtra*) appears to be required for initialization of the auto-regulatory feedback loop that leads to female development [83]. In *Ag* mutants, maternal *Mdtra* is not found in the eggs, though whether this is due to a mutation in *Mdtra* itself or instead in some transport function is still unclear.

Other factors affecting sex determination

As mentioned previously, one genetic trait predicted to be under strong sexual conflict is cytoplasmically inherited features (i.e., mitochondria and endosymbionts) that are transmitted maternally via female gametes (eggs) [85]. Endosymbionts in particular can often have strong effects in sex determination [86] and alter operational sex ratios [16, 87], in extreme cases leading to the production of functional females from genotypically male offspring [88, 89]. In comparison, genes present on the autosomes are generally predicted to be under selection to balance the sex ratio [53]. There is broad evidence of conflict between cytoplasmically and autosomally inherited genes [90-92].

Another mechanism that may lead to abnormal sex-determination or sex-ratios of offspring are sex-ratio distorters, such as X and Y drivers, which affect meiosis or gamete investment such that the only functional gametes are those which carry a 'selfish gene' [93, 94]. In many cases, though local populations exhibit relatively even sex ratio, population crosses demonstrate evidence of complex antagonistic coevolution of sex-ratio distorters [95]. In other systems, the genes that are obscured by opposing genetic elements are dominant sex determiners, and it is only through the presence of both

elements that an even sex ratio is maintained in natural populations [92]. A few genes have been implicated as being a part of this process in *D. melanogaster* (examples include *Stellate* [96] and *sting* [97]). Further work is necessary to identify levels of conservation in genetic pathways and functions across taxa. Additional research is also needed to characterize the genetic elements involved in sex determination and their patterns of interaction in other organisms with different systems of sex determination. Beyond understanding the ultimate cause of sex determination, a better understanding of the way these pathways give rise to sexually dimorphic traits will enhance biological theory.

Sexual dimorphism

The development of sexual dimorphism is dependent upon sex-limited genetic expression, and a conserved feature of this process is the synergism between the final products of the sex-determination systems and non-sex-specific transcription factors [74]. Sexually dimorphic traits generally fall into one of three categories. Primary sex traits are those that are directly related to sexual reproduction, and include both anatomical and physiological factors [74]. Secondary sex traits are those that increase reproductive success in the individual expressing them, and include any modification to morphology or behavior [74]. This category can include courtship behavior, color, body size, or flamboyant body parts. The third category, ecological sex traits, are those that differ between the sexes and lead to niche divergence [98]. These categories, the genetic mechanisms that contribute to their development and the intersexual conflict affecting their maintenance and evolution are described individually in greater detail below.

Though the exact mechanisms by which all of these differences arose have not yet been discovered, researchers have begun to elucidate some of these processes.

Primary sex traits

The first step in understanding sexual dimorphism is to examine the primary sexual characteristics that function directly in reproduction, including differentiation of copulatory organs and gamete maturation. Potential sexual conflict and fitness consequences of simultaneous development of male and female gonads is avoided by systems that incorporate positive auto-regulation of one pathway and suppression of the other, and sex-linked expression is a conserved characteristic of sexually dimorphic traits.

Much work related to the genetic regulation of the development of primary sex traits has been done in *D. melanogaster*. Sex-determination genes regulate the timing and pattern of development of the adult genital structures [99]. The genital imaginal disc contains precursors to both male and female genitalia, and repression of the male organs requires expression of the female forms of *tra* and *dsx* until the end of the third larval instar [99, 100]. Repression occurs through isoform-specific DSX mediation of the cell-to-cell signaling pathways, with continuous DSX^F expression required for the proper development of female tissues and repression and resorption of male tissues, and DSX^M plays a similar role in the development of male traits [100]. Therefore, each sex-specifically expressed isoform plays a positive auto-regulatory role in the development of its sex-specific traits and repressive role in the development of traits of the other sex.

In *C. elegans* there are many physical traits that differ between the sexes, and the shape of the tail is directly related to reproduction. Hermaphrodites have a whip-like tail and male tails are blunted and possess copulatory organs [70]. The decreased activity of the TRA-1 isoform in males due to sex-limited gene expression leads to increased expression of *dmd-1* and *mab-3*, and these genes function in cell fusion and retraction to shape the male tail [101]. Sex-limited expression of many genes responsible for differentiation and sexual dimorphism is likely due to the upstream CRE binding sites of the functional TRA-1A isoform in hermaphrodites with repressor function [67, 101-103].

In mammals, many sexually dimorphic characters arise as a function of hormones produced in the gonads. The "default" sex of mammals is female, and it is the presence of the dominant male determiner *Sry* on the Y-chromosome that directs bipotential gonad development [72] and male development. In mouse embyros, expression of SRY in Sertoli cell precursors causes an up-regulation of *Sox9*, a gene required for normal testicular development [73]. The binding of both SRY and the general binding factor SF1, found in the same form in both sexes, to an upstream CRE is required for increased expression of *Sox9* [104]. Furthermore, *Sox9* contains a DNA-binding domain, and acts in a positive auto-regulatory fashion to maintain gonadal fate [105]. The gonads then secrete sex-specific hormones that interact with cognate steroid receptors on target cells to direct growth and differentiation, through DNA-binding domains and CRE binding [106, 107]. For example, up-regulation of *Sox9* leads to the production of Anti-Müllerian

Hormone by Sertoli cells in the testis of humans, which suppresses the development of fallopian tube precursor cells [105].

Secondary sex traits

Secondary sex traits are more variable in their control and development, though many play a role in mate preference. In some species, the mechanisms of genetic control of sexually dimorphic traits such as cuticle pigmentation, initiation of courtship behavior, mating frequency, or traits under sexual selection has been studied extensively in *Drosophila* [108, 109]. Genetic intersexual conflict plays a much bigger role in the evolution and maintenance of these traits, though in some cases intersexual conflict has been limited through the modification or mutation of *cis*-regulatory element(s) (CRE) binding sites to sex-specific function. We are limited in our understanding of the genetic mechanisms leading to most of these traits, however.

One sexually dimorphic trait that has received extensive treatment is cuticle pigmentation in *D. melanogaster,* which is known to affect mating preferences [110]. The genes *bric-a-brac1* (*bab1*) and *bric-a-brac2* (*bab2*) act as dominant pigmentation repressors, and expression of these genes demonstrates an inverse pattern of expression to that of the melanic pigmentation pattern [110]. Expression of these genes is regulated by two upstream CRE's. One of these genes is bound in both sexes by monomorphic expression of Abdominal-B (AbdB) protein, and the other by sex-specific isoforms of DSX. Binding of this CRE by DSX^F activates *bab*, while binding with DSX^M represses expression [109].

Another secondary sexual trait in *D. melanogaster* that has received attention is the development, evolution, and function of the genetics of seminal fluid proteins in mediating female mating latency, longevity, sperm use, and oviposition [7]. Using artificial selection, researchers demonstrated that males adapted to conditions of high male-male competition evolved seminal fluid proteins that increased their fitness to the detriment of their mates; females that mated with these males were less receptive to subsequent matings, laid eggs sooner, and died younger [111-113]. Females from these same selection lines were less sensitive to the effects of these proteins than females evolved in populations of low male-male competition, including surviving 13% longer when controlling for number of matings [114]. These studies demonstrate that operational sex ratios can play an important role in the local evolution of secondary traits, and the presence of genetic elements that distort sex ratios can have a significant effect on the development of these traits [115].

Sex-linked expression of sexually dimorphic traits was postulated to reduce intralocus conflict, and this sex-linkage could lead to more rapid speciation [116]. However, a trend that is emerging from artificial selection studies is that the effects of selection on sex-linked traits are generally not limited only to the sex of interest as would be predicted [117, 118]. For example, in selection lines where males were selected for larger mandible size, females also exhibited larger mandibles and a constellation of other morphological features to support them. Expression of this phenotype had a negative effect on female fitness, specifically decreased fecundity [119]. Intralocus sexual conflict can also decrease the benefits of selective mating [120].

Because of this, more research is clearly required to elucidate the conservation, relative strengths, reciprocal effects, and mechanisms sexual selection, sexual dimorphism, and sexual conflict.

Unlike the *Drosophila* examples discussed, the development of secondary sexual traits in vertebrates, such as deer antlers, bird wattles, and mammary tissue development, was assumed to be due to gonadally-secreted sex hormones [74]. In humans, the large increase in secretion of testosterone and oestrogen at the onset of puberty [121] leads to the maturation of gonads and development of secondary sexual characteristics such as mammary tissues in woman and facial hair on men.

Recent work with gynandromorph chickens suggests that the assumption that only hormones are responsible for secondary sexually dimorphic traits may not be true, at least in birds [122, 123]. Gynandromorphy is a departure from properly functioning sexual dimorphism in which an individual expresses both male and female secondary sexual traits[50]. Sexual fate of individual cells is determined by the interaction of gonadally-secreted hormones and somatic cell genetic identity; therefore, despite the circulating presence of male hormones, ZW cells will maintain their female fate. However, interactions with normal male and females demonstrated that these birds self-identified as male and tried to initiate mating though their advances were unwanted and their fertility uncertain [122].

Sex on the brain

As the gynandromorph example illustrates, the proper combination of morphology and behavior is required for successful breeding. Production of these behavior patterns is

generally governed by sexually dimorphic brain development and function as determined by genetics. Determining the anatomical basis for sexually dimorphic behavior in fruit flies has been a fertile ground for research. Beginning in the 1970's, different parts of the brain were discovered to be responsible for various courtship and mating behaviors in males [124]. The neural cluster P1 is present only in males and is primarily responsible for courtship behavior [125]. Proper neural differentiation of this cluster (number of neurons and their connections) is directly determined by products of sex-determination pathway genes, including *dsx* and *fru* [126, 127]. Furthermore, DSX^F in normal females induces programmed cell death, and artificial induction of male transcripts causes females to court other females [125, 128, 129].

The genetic mechanisms governing the neural dimorphism that results in sexually distinct behaviors have not been well elucidated in other organisms. However, work in mammals has shown that the presence of sex hormones is important in sex-appropriate development and function [130-132]. Work in this field has supported the hypothesized default female state of mammals, as certain co-activators are required for successful development of male brain morphology and reproductive behavior in rats [133]. However, not all traits are this simple, and total brain morphology is the product of complex cellular multi-signaling processes [134].

Some behavioral differences between the sexes are not directly related to courtship and mating behaviors. Researchers have long been interested in the possibility of conflict between males and females when reproduction is not their primary focus. Of particular interest is the possibility of ecological dimorphism, or niche divergence, between the

sexes[135]. Studies have not only demonstrated that there are differences between the sexes as adults in terms of nutritional requirements [136], but that there are also differences between gravid/nursing and non-gravid females [137], and that these variable nutritional requirements can shape foraging behavior in early stages of development [138, 139]. Based on studies in mammals, birds, raptors, and reptiles, it appears that sexual dimorphism precedes niche divergence in most cases, though evidence is not overwhelming [98, 135, 138, 140-143]. Certainly, morphological differences can lead to dimorphic swimming ability and foraging site and diet divergence in marine birds [141]. However, work in northern map turtles, *Graptemys geographica* Leuseur (Testudines: Emydidae), demonstrated that sexual dimorphism in diet or habitat was observed in rivers but not lakes, suggesting that there may be plasticity in sexual dimorphism [140]. The importance of ecological and sexual factors in structuring sexual dimorphism has been observed in other taxa in other traits [142, 144], and highlights the value of holistic studies to try to differentiate between these factors. Furthermore, this complexity significantly increases the challenge in trying to apply mathematical models to predict foraging behavior, as it is difficult to model temporal-, sexual-, and condition-dependent foraging in Optimal Foraging Theory or other such ecological models [143]. Another limitation is that it is not yet known what genetic mechanisms give rise to these physiological and behavioral traits, and these may be difficult to identify due to pleiotropic and polygenic traits [117].

Patchy ephemeral resource ecology

"When we die, our bodies become the grass, and the antelope eat the grass and so, we are all connected in the great circle of life."

— Mufasa

Patchy ephemeral resources are a class of resources that contain high quality nutrients, are unevenly and somewhat randomly distributed in space, and only exist for a short time [145]. Fruit, dung and carrion are examples [146], and competition is expected to be fierce for such materials as their occurrence is unpredictable and typically short-lived [147]. Carrion is an especially tractable system in which to study patchy ephemeral resource ecology as it does not rely on acts of nature or seasonal plant and fungal growth, and under some conditions the patch can pass from new (fresh decay) to unusable (skeletonization) in a matter of days [148]. For example, larval bow flies can consume up to 75% of the soft tissue of elephant carcasses in 7 days [149]. As a result, carrion-breeding organisms have long been predicted to be under intense competitive pressures [150].

Interspecific competition

There are a variety of strategies organisms employ to address interspecific competitive pressures. In the carrion system, many blow fly species have adapted a primary colonizer strategy- they arrive first, and so their offspring are generally the largest and most vigorous on the remains and are therefore able to exclude competing species [148]. Their colonization patterns may be related to different responses to stimuli, faster flight capabilities, or greater dispersal distances [151]. Other species are

secondary colonizers, and their offspring are seen on the remains 24 to 48 hours after the primary colonizers have arrived [152].

Despite differences in arrival times, all blow fly larvae, like many Diptera, have a pair of scraping mouth hooks [153], and feed by secreting salivary gland products and excreting proteolytic enzymes [154] onto the resource, scraping off the top surface, and then ingesting the partially digested slurry [155, 156]. As both solitary blow flies and those in small groups have drastically reduced fitness [157], the combined efforts of hundreds or thousands of larvae feeding at once is hypothesized to provide a fitness advantage [155]. However, as smaller, more fragile blow fly maggots have weaker mouth hooks [158] and a smaller volume of excretion/secretions, feeding with larger, older maggots may allow them to ingest more nutrients than they have actually contributed to releasing.

Facultative predation

Three species of blow fly are known to be facultative predators: *Chrysomya rufifacies*, *Ch. albiceps*, and *Ch. bezziana* Villeneuve [150, 156]. They actively feed on the carrion source but will switch and consume other larvae on the resource in the third and sometimes second instars [159]. There are advantages and disadvantages to facultative predation. Time and energy spent hunting and subduing a prey item is time not spent consuming the resource [160]. However, if an attack is successful, which may not always be the case, a competitor has been removed and the energy and nutrients they have sequestered internally have been transferred to the predator [161].

Whether there is preferential prey selection relative to same versus other species, or sex-dependent predation rates is not known. Laboratory research has shown that *C. rufifacies* larvae will feed on heterospecifics and conspecifics [162, 163]. From a purely objective standpoint, any competition, even with siblings, can reduce the fitness of an individual. Evolution by natural selection relies upon the heritability of traits that increase an organism's fitness in the context of a variety of abiotic and biotic factors. Generally, this heritability is thought to be the result of the genetic code of the organism, though there is literature on the effect of both genotype and environment on physiological, morphological, and ethological phenotype expression [164-169]. Research has related genotype and gene expression to behavior such as feeding and locomotion in other Diptera [170-172]. Next-generation sequencing techniques and the budding field of transcriptomics might be useful for identifying genes or gene products correlated with predation behavior.

Potential for sexual conflict

There are major challenges when investigating behavioral plasticity, sexual dimorphism, and potential ecological divergence and sexual conflict in immature organisms, especially in immature insects. Firstly, in ethological work in insects, distinguishing between males and females in immature stages is not possible as there does not appear to exist any overt sexual dimorphism at this stage, though literature on the subject is lacking. Secondly, in sexual conflict work, immatures are not thought to experience divergent selection, at least not until the insects are nearly adults, as the selection is thought to act on adult structures and behaviors [58]. Thirdly, asking more

subtle ecological questions in field situations can be challenging, as the spatial and temporal scales required may be prohibitively expensive, or due to the fact that it is difficult to control environmental variables.

Conflicts of interest between males and females might be a force selecting for specific feeding behaviors [10], and nutrition acquired in the immature stages may be important for later egg development [173]. However, it is not yet clear how important nutrients obtained during the larval stage may be in oogenesis in the resulting adult and whether this may impact nutrient source shifts in immature insects. Furthermore, studies on sexual conflict have been restricted to interactions between mating adults [18], or adults and their offspring [174]. There has yet to be any research into sexual conflict between immature insects.

Chrysomya rufifacies

"I am weird, and you know what? That's OK. So are most interesting people."

— Em Bailey

The hairy maggot blow fly, *Chrysomya rufifacies,* is a tropical species native to the Orient and Australia [175]. This species was first detected in the New World in Brazil in the 1978 and has since spread throughout the Americas [176]. This species has been known to produce myiasis in humans and animals [56, 177], but is generally researched as an important species in carrion decomposition in both its native and invasive ranges [159, 163, 175, 178]. Females produce unisexual offspring, and several attempts have been made at understanding the mechanism of sex determination in this species [48, 178-182]. Females are known to produce an average of 210 eggs per clutch [180]. *Ch.*

rufifacies first instar larvae feed solely on decaying animal tissues. Second instar larvae are primarily necrophagous, but they have been documented to predate upon other species. Third instar larvae are necrophagous, but also engage in facultative predation and cannibalism [159, 163]. This predatory behavior has the potential for ecological ramifications in the invasive territories including driving the local extinction of native fauna, altering attraction and colonization patterns of other blow flies [152, 183], and changing the predation patterns of beetle species [159].

The sex-determination pathways in *Drosophila* [37] and other Diptera have been thoroughly investigated [39, 82, 83, 184, 185]. Though maternal products can be an important part of the gene cascade [186], *Ch. rufifacies* is unique in that it appears that due to an ancestral loss of a primary zygotic sex-determination gene, maternal products became the initial signal [75]. Further investigation of the sex-determination pathways in this species will benefit from some baseline genetic data, especially if researchers hope to use transgenic methods to modify the sex-determination pathways of these insects in a Sterile Insect Technique control program [187]. Finally, studies on the ecology of this species in the context of sexual conflict, behavior, nutrition, and other fields will require a method to differentiate between the three classes that is easier to employ that that which is currently available.

Chrysomya rufifacies sex determination

Common characteristics of sex-determination in Diptera include amphogenic sex determination, in which males are heterogametic. In these systems, females have bigenic (mixed male and female) clutches and zygotic-genome determined sex [188]. In

comparison, *Ch. rufifacies* and its closest sibling species *Ch. albiceps* Wiedemann [189] both exhibit monogenic sex determination with maternally-determined sex, single sex offspring clutches [178, 180] and homomorphic sex chromosomes [48, 181, 182, 190]. Previous research has demonstrated that females produce single sex clutches, a trait independent of environmental factors such as diet, season, and temperature experienced by the mother [180], and that female-producing females produce an approximately even ratio of male-producers and female-producers [191]. Based on mating studies, ovary and pole-cell transplantation, and patterns of protein expression, female-producing (thelygenic) females are heterozygous for a dominant female-determiner (F) that causes them to produce a sex-determination signal in the germ line during gametogenesis [179, 182, 192, 193]. Furthermore, according to this hypothesis, male-producing (arrhenogenic) females and males are homozygous f/f at this same locus.

Based on available evidence, differences in *transformer* alleles or the presence of an endosymbiont could be responsible for monogeny in *Ch. rufifacies.* Recent advances have highlighted the importance of *tra* and *transformer-2* (*tra2*) homologs in the sex-determination mechanisms of a diverse array of arthropods, including bees, wasps, *Daphnia*, shrimp, and beetles [79, 81, 83, 194-200]. The proteins encoded by these two genes are RNA-binding proteins [201], and homology is inferred based on predicted amino acid sequence similarity in the functional regions. A *tra* homolog was identified in a *L. sericata* transcriptome using *D. melanogaster* sequences [202] and *tra* also seems to play a crucial role in *M. domestica* sex-determination. At present, there is no

indication that there are any endosymbionts which affect sex determination in any flies in the family Calliphoridae [203] .

Chrysomya rufifacies gene expression

Research has shown that there are sex specific differences in gene expression in Diptera, for example *Sxl* in *D. melanogaster* [40] and *tra* in *L. sericata* [39]. As there are no known morphological differences between males and females in the immature stages (sex chromosomes are homomorphic in *Ch. rufifacies* [204]) genetic markers allowing for quick sex-specific screening of larvae will be very useful in investigating behavioral differences between the three classes, males, arrhenogenic females, and thelygenic females, and concurrent differences in gene expression. As males and females are fairly easy to distinguish in the adult stage [205], and it is the phenotype of the mother (sex of the offspring) that separates females into two distinct classes, utilization of adults to identify genetic markers of the three classes is the simplest approach. Kirchhoff and Schroeren [179] demonstrated differences in protein patterns of the ovaries of thelygenic and arrhenogenic female *Ch. rufifacies*, which suggests that there is likely to be correlative differences in gene expression between these two classes of females. Genetic research until this point has failed to identify a specific genetic element that may be function in sex determination or sexual dimorphism in *Ch. rufifacies* [204, 206-208].

Researchers have identified temporal patterns of gene expression throughout immature development in arthropods generally [209], and Diptera [210-212] specifically, and there is a wealth of research demonstrating sex-specific patterns of gene expression [108, 213-216]. Furthermore, some researchers have observed that male blow

flies complete immature development at a faster pace than females in the same cohort under the same conditions [217]. Though there is a well-annotated and manually curated database, "FlyBase" [218], of gene expression profiles throughout development in *Drosophila*, the only genetic tools available for *Ch. rufifacies* are cytochrome oxidase I sequences for identification purposes, and the sequence of the *Sex-lethal* gene [206, 219, 220]. A greater breadth of temporal gene expression profiles across Diptera will help researchers to better understand the evolution of developmental pathways and patterns of conservation. Further development of sex-specific differences in temporal gene expression patterns should also prove informative.

Chrysomya rufifacies behavioral ecology

Genetics have been demonstrated to be crucial to understanding biochemistry [221, 222], morphology [223], and physiology [224]. Researchers have also worked on correlating genetics and behavior, and the growing body of literature in behavioral genetics has begun to lay informative and interesting groundwork [172, 225]. Research on a variety of Diptera has identified the role that genetics can play in learning, in the context of conditional responses to visual, olfactory, and other stimuli [226]. In 2006, researchers used microarrays and artificial selection to investigate the genetic underpinnings of aggressive behavior in *Drosophila*; in fact, they found that a single locus that was significantly correlated with increased fighting frequency [227]. Another study in honeybees has suggested that there is a high degree of conservation with *Drosophila* sequences in genes that have expression profiles that can be used to predict the behavior of an individual honeybee [228]. Finally, behavior is plastic, as is

expression of the genes that contribute to it, and can be conditional based on environmental stimuli such as temperature [229], experience [230], parasitism [231], or gustatory signals [232].

As previously mentioned, *Ch. rufifacies* is one of a few blow fly species demonstrated in the literature to be facultatively predacious, though it is not clear with what frequency this species exhibits predatory behavior. Studies on this behavior in this species have not relied upon observations under naturally occurring conditions [159]. Finally, as studies have also demonstrated that these larvae are cannibalistic [163], there is a possibility that larval sex ratios and adult sex ratios are not the same. More research and new tools are required for investigations in the field on this subject, however.

***Chrysomya rufifacies*, more than a pretty face**

"It takes more than just a good looking body.

You've got to have the heart and soul to go with it."

— Lee Haney

Research on *Ch. rufifacies* will prove useful in a number of different fields, including but not limited to: sexual conflict, population genetics, molecular ecology, behavioral ecology, sex determination, veterinary entomology, competition research, and the study of invasive species. Molecular tools will allow for field based behavior studies coupled with molecular identification of different sexes or sex producers. A better understanding of the sex-determination system will allow researchers to develop control programs of this myiasis causer by using transgenics to engineer sex-specific condition-dependent lethality [39]. Also, as this fly is invasive to the New World, application of these

markers to study these flies in their native ranges may yield interesting insights into the evolution and adaptation of facultative predation and cannibalism, as well as this unique method of sex determination. Studies in this system offer an opportunity to investigate a unique sex-determination system, the molecular basis of a complex ethology, and the possibility of sexual conflict manifesting in immature insects as larvae are genetically destined to be males, arrhenogenic (male-producing) females, or thelygenic (female-producing) females.

As previously noted, a great deal of research into sex determination of other Diptera has been conducted [82, 83, 233-236], and there are a number of theories regarding the evolution of sex-determination pathways and primitive primary signals [75, 76, 237, 238]. It has recently been suggested that *transformer* is the basal gene governing sexual differentiation [199]. There is also evidence that maternal products can be required for proper functioning of sex-determination pathways [239], which leaves open the possibility of loss of a primary signal and promotion of the maternal products to the primary signal as has been demonstrated in one experimental population of *Musca domestica* [84]. A better understanding of the these pathways in insects other than *Drosophila* will lend to a greater understanding of the processes governing the evolution of these pathways.

Experiments in the field of sexual conflict have traditionally focused on the interactions of adults in the context of mating. However, as the definition is broad and focuses on conflicts at the genetic level between male and female fitness optima [19], it is possible that these conflicts could occur in immature insects. Sex, as governed by sex-

determination genes, is important to the ecological tradeoffs within a species. *Ch. rufifacies* offers a tractable system in which to study the ecological tradeoffs among and between the sexes and different sex producers.

Research into the genetic basis of behaviors has only just begun. Advances in sequencing technologies have opened the door to this avenue of investigation. There is evidence already that single-loci allelic differences can affect larval feeding behavior in *Drosophila* [170, 171]. A single gene may not correlate with behavior as complex as food source shift, but such ethological changes could be due to differential gene expression or splicing rather than allelic differences. Correlation of gene expression with predation behavior will enhance understanding of the molecular basis for shifts in behavior.

Questions

"A wise man can learn more from a foolish question

than a fool can learn from a wise answer."

— Bruce Lee

The overarching goal of this research is to determine the extent and pattern of sexual dimorphism in *Chrysomya rufifacies*, especially given the unusual sex-determination mechanism employed. First, I will investigate the sex-determination system with *de novo* transcriptomics to determine whether the sex-determination cascade genes used by *Ch. rufifacies* share homology with those from other species. I hypothesize that there will be several gene expression differences between thelygenic and arrhenogenic females. I further hypothesize that *Ch. rufifacies* sex-determination cascade genes will share

sequence homology with other organisms, but that *transformer* will be broken or "non-functional" in the eggs of one of the female adult types. Second, I will use *de novo* transcriptomics to investigate sexually-dimorphic patterns of gene expression throughout immature development. I hypothesize that there will be many similarities in gene expression patterns with those discovered in *D. melanogaster,* and also that the number of genes or transcripts with are sex-specifically expressed will increase throughout development. Finally, the facultative predation of *Ch. rufifacies* is understudied, and it is still not clear how frequently this behavior is expressed in the wild nor whether males and females are equally as likely to engage in acts of predation. I hypothesize that females are more likely to engage in predation, but that there will not be sex-specific predation gene expression (i.e. the same genes will be used for predation in both males and females).

Applications

"The usefulness of science is sometimes exaggerated."

— Richard Dawkins

Identification of the genes correlated with predation behavior will make it possible to do more detailed fieldwork in behavioral ecology. Sampling of wild populations of *Ch. rufifacies* will enable population studies to predict the frequency of this behavior in nature. It will also generate candidate genes for investigating this behavior in other species, to determine if there is conservation in genetics associated with facultative predation. Finally, as markers for sex determination will have been identified in the first

objective, it will be possible to ascertain if the sex-determination markers segregate by expression of the behavior.

Estimating the age of third instar larvae and pupae submitted as evidence in forensic entomology analyses is difficult. Current methods rely on prediction of age based on length or weight correlated with prevailing weather conditions or imaginal disc development. Tarone et. al. (2007) demonstrated that is possible to predict the age of blow fly eggs by correlating age with gene expression [212]. It may improve time of colonization (TOC) estimates [240] if gene expression can also be correlated with life stage information in *Ch. rufifacies*, as this species is growing in importance in forensic investigations since its introduction to the Americas in the 1970's [175]. Transcripts that are uniquely or differentially expressed at different time points in the pupal stage can be used to develop tools to predict the age of pupae and improve accuracy in estimating insect age in forensic entomological investigations.

Finally, there is a great deal of interest in the development of antibiotics based on naturally produced compounds in saprophagous insects [241, 242], and so information on sequences of genes of interest will be invaluable. It has become clear that pathogenic microbes are not only able to quickly develop resistance to antibiotics, but that they are able to transfer these resistance genes to other bacteria [243]. Traditional antibiotics research has relied upon additions and improvements to known methods of action of antibiotics, but experts in the field no longer consider that a fruitful direction of research. There is a clear need for new sources of antibiotics [244], and endogenously produced antimicrobial peptides of carrion breeding Diptera are already proving to be an

interesting pool of potential new compounds [245-247]. Additional genetic information coupled with advances in computational biology will allow researchers to find genes with predicted functions based on homology with known defensins within this dataset.

CHAPTER II

A FUNCTIONAL GENETIC EVALUATION OF SEX DETERMINATION IN *Chrysomya rufifacies*

Introduction

Sexual reproduction remains the most common form of reproduction amongst multicellular organisms despite its potential costs with differentiation into two different sexes is required for sexual reproduction to occur in most multicellular organisms [248, 249]. Therefore, in an effort to understand the maintenance of sexual reproduction, sex determination has been extensively studied in a variety of organisms [39, 43, 59, 71, 82]. A common characteristic of sex determination in flies, nematodes, and mice, appears to be differential splicing of a single gene that causes the developing organism to use one of two paths of differentiation and development, male or female [40, 43, 69-71]. Generally, differential splicing of a single primary signal into either a male or female form sets the stage for further gene cascades that result in sexual dimorphism. Beyond this general pattern of differential splicing, a variety of sex-determination systems are employed by animals and many of them can be observed within Diptera. Common characteristics of sex-determination in Diptera include amphogenic sex determination, in which males are heterogametic. In these systems, females have bigenic (mixed male and female) clutches and zygotic-genome determined sex [188].

The most thoroughly studied sex-determination system in Diptera is that of *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) [40, 59, 60, 186, 233]. In common with most other Diptera, *D. melanogaster* has an X:Y chromosome system in

which females are XX and males are XY. However, in *D. melanogaster* the ratio of X-chromosomes to autosomes, rather than a dominant male determiner on the Y-chromosome, leads to sexual differentiation [40, 59]. Individuals with a 1:1 ratio of X chromosomes to autosomes (XX individuals) produce transcripts of the gene *Sex-lethal* (*DmSxl*) in the female form [40, 59-61, 236]. This female form codes for a complete protein, SXL^F. In males, differential splicing leads to the inclusion of an exon with a precocious stop codon, making the male splice form of the protein inactive. The SXL^F protein has three primary functions. First, it sets up an automatic feedback loop that causes the female-specific form of *Sxl* to continue to be produced. Second, it directs the splicing of the downstream gene *transformer* (*Dmtra*); the presence of SXL^F leads to the production of female transcripts of *tra* (tra^f). Third, *Sxl* functions in dosage compensation, a related process that is required for viable male offspring in this species; males have half the number of copies of the X-chromosome as that of females, so up-regulation of X-linked genes is required [40, 59, 250, 251].

Research in other dipterans has suggested that the sex-determination system identified in *Drosophila* is moderately distinct in the order in terms of the splicing cascade [75, 76]. One common feature to other dipteran systems is that *tra* is the basal signal, rather than *Sxl*, and *Sxl* is not somatically sex-specifically spliced [39, 75-77]. The "standard" system in blow flies, such as *L. sericata* Meigen (Diptera: Calliphoridae) or the common house fly *Musca domestica* L. (Diptera: Muscidae), is one of heteromorphic sex chromosomes and a presumed dominant male determiner (*M*) on the

Y chromosome [39, 78, 79]. The presence of this *M* factor in the zygotic genome is presumed to cause development into a fertile male.

Musca domestica employs several varieties of sex-determination mechanisms [82-84]. As with many flies, most populations of *M. domestica* exhibit a typical XY sex-determination system [239]. In some populations, however, sex appears to be determined by the maternal genotype, with females heterozygous for a dominant arrhenogenic allele (*Ag*) having only male offspring [84]. Therefore, populations with this allele have two types of females: male-only producing females, and females that have bigenic (both male and female) offspring. Furthermore, maternally-derived female form of *M. domestica tra* ($Mdtra^F$) is required for the initialization of auto-regulatory feedback loops that lead to female development [83]. In *Ag* mutants, maternal $Mdtra^F$ is not found in the eggs, though whether this is due to a mutation in *Mdtra* itself or instead in some transport function is still unclear.

In comparison, *Chrysomya rufifacies* Macquart (Diptera: Calliphoridae) and its closest sibling species *C. albiceps* Wiedemann [189] exhibit only monogenic sex determination with maternally-determined sex, single sex offspring clutches [178, 180] and homomorphic sex chromosomes [48, 181, 182, 190]. Previous research demonstrated that females produce single sex clutches, a trait independent of environmental factors such as diet, season, and temperature [180], and thelygenic (female offspring only) females produce an approximately even ratio of arrhenogenic (male-producers) and thelygenic females [191]. Results from mating studies [180], ovary and pole-cell transplantation [48, 192, 193], and patterns of protein expression [179]

have shown that female-producing females are heterozygous for a dominant female-determiner (F) that causes them to produce a sex-determining factor in the germ line during gametogenesis. Furthermore, according to this hypothesis, arrhenogenic females and males are homozygous f/f at this same locus.

Based on available evidence, differences in *tra* alleles, the presence of an endosymbiont, or the *D. melanogaster* maternal effect gene *daughterless* (*da*) could be responsible for monogeny in *C. rufifacies.* Recent advances have highlighted the importance of *tra* and *transformer-2* (*tra2*) homologs in the sex-determination mechanisms of a diverse array of arthropods, including bees and wasps (Hymenoptera) [199], water fleas (Cladocaera) [200], shrimp (Decapoda) [196], and beetles (Coleoptera). While there is evidence of rapid divergence in the sequences of the RNA-binding proteins encoded by these two genes, homology is inferred based on predicted amino acid sequence similarity in the functional regions [201]. The gene *transformer* was identified in a *L. sericata* transcriptome using *D. melanogaster* sequence homology [202] and *tra* plays a crucial role in *M. domestica* sex-determination. Furthermore, *transformer* has been sequenced in four separate species of blow fly thus far [39, 79]. At present, there is no indication of any endosymbionts that affect sex determination in any flies in the family Calliphoridae [203]. Previous work demonstrated that a *da* probe based on *D. melanogaster* sequences hybridized to the area of the 5th chromosome predicted to contain the putative maternal effect gene, though no further work has been done to determine whether *da* has the same functional effect in *C. rufifacies* [204].

There are many theories regarding the nature and identity of the proximate mechanism underlying monogenic sex determination in *C. rufifacies* (see Scott et al. [252] for a review). But, the lack of genetic tools historically made more detailed investigations challenging. The purpose of this work is to develop genetic tools that will be useful in investigating sex determination and other traits in this species.

Materials and methods

Quantification of sex ratios in wild populations on human cadavers

Immature *C. rufifacies* were collected from human cadaver donations at the Forensic Anthropology Research Facility at Texas State University in San Marcos, Texas as approved by the Institutional Biosafety Committee, Texas A&M University, College Station, Texas. Approximately 100 dipteran larvae from at least one maggot mass on the remains were collected between 9 am and 2 pm on 6 August 2013 and five dates between 6 June and 8 September of 2014. An effort was made to collect only *C. rufifacies*. Larvae were collected, placed on approximately 50 g of raw beef liver on vermiculite in a 500 mL sealed plastic container and transported to the Forensic Laboratory for Investigative Entomological Sciences Facility at Texas A&M University in College Station, Texas. These larvae and the liver they were feeding on were transferred to a 35 mL opaque plastic cup in a 1.1 L glass canning jar with approximately 400 mL of vermiculite and maintained at approximately 28°C for a 16:8 light:dark (L:D) photoperiod. Additional liver was supplied as needed. These individuals were permitted to eclose to adulthood to determine their sex and species [253, 254]. Sex ratios were analyzed in R 3.1.3 according to methods discussed in Hardy [255] to

determine whether samples had a sex-ratio significantly different than 0.5 (z-test on proportions), whether this distribution of sex-ratios was likely to have come from a binomial distribution (goodness of fit) and a Marascuilo procedure for large-sample multiple comparisons to test for significant pairwise differences between samples [256].

Colony maintenance

Chrysomya rufifacies larvae were collected from vertebrate carrion in College Station, Texas, USA between May and September of 2011 and eclosed adults were identified morphologically [253, 254]. Adult flies were released into a 30 x 30 x 30 cm BugDorm 1 plastic cage (MegaView Science, Taiwan) and allowed to interbreed to found the laboratory colony. The colony was provided with fresh deionized water and refined sugar *ad libitum*, as well as fresh beef liver blood daily as a protein source for oogenesis. Flies were maintained at 28°C for a 16:8 L:D.

Gene expression sample collection

For each sample, a single male and female *C. rufifacies* were isolated together in a 1.1 L glass canning jar with approximately 100 g oplayground sand, a Wype-All on the top to prevent escape but allow air flow, refined sugar and water *ad libitum*, and a 10 mL glass beaker filled with one Kim-wipe®, and approximately 1 mL of fresh beef liver blood. An additional 1 mL of blood was added each following day up until the 6th day post eclosion. The protein source was then excluded for 24 hours. Beginning on the 7th day post-eclosion, twice each day, a 35 mL plastic cup with approximately 25 g of fresh beef liver covered with a moistened Kim-wipe® was introduced to the jar as an oviposition medium for a four hour window. Once eggs were observed, the two adults

were collected and flash frozen for subsequent RNA extraction and analysis after the progeny had eclosed and the progenitor female had been phenotyped. This was repeated over seven generations in a single incubator until at least four females of each type (thelygenic and arrhenogenic) had been collected.

RNA preparation

Four thelygenic females and their mates, and four arrhenogenic females and their mates, were collected for RNA sequencing. RNA was extracted in a two-step process. The first, whole RNA was extracted using TriReagent (Sigma-Aldrich Corp., St. Louis, Missouri) preparation according to manufacturer's protocols and eluted in a 1:100 μL dilution of mixture SUPERase•In ™ (Invitrogen, Life Technologies Incorporated, Grand Island, New York) and DNase/RNase/Nucleotide-free water. This whole RNA was then purified using a Qiagen RNeasy Micro Kit and on-column DNase treatment following manufacturer protocols (Qiagen Inc., Valencia, California). RNA was eluted again into a fresh 1:100 mixture of SUPERase•In and DNase/RNase/Nucleotide-free water and stored at -80°C until sequencing.

RNA sequencing

RNA was sequenced on an Illumina Hi-Seq 2000 (Illumina, Inc., San Diego, California) following manufacturer protocols regarding library preparation at the University of North Carolina at Chapel Hill High-Throughput Sequencing Facility as in Sze et al. [202]. All sequencing was 100bp paired-end reads. Each female (4 thelygenic females and 4 arrhenogenic females) was sequenced on her own lane. The mates of the thelygenic females (N = 4) were each sequenced on their own lanes, while the mates of

the arrhenogenic females (N=4) were multiplexed on a single lane. The thelygenic females and their mates were all run on one flow cell, while the arrhenogenic females and their mates were sequenced in a separate run on a different flow cell. One pair of adults was also sequenced at 50 bp paired end.

Transcriptome assembly

Prior to assembly, reads underwent trimming and quality control. First, reads were filtered to remove all sequences that contained adaptor sequences and known contaminants as defined by Illumina. Second, all reads containing the following sequences (or their reverse complements) were removed: "GATCGGAAGAGCACACGTCTGAACTCCAGTCACTGACCAATCTCGTATGC", "GATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCG", "GATCGGAAGAGCACACGTCTGAACTCCAGTCACACAGTGATCTCGTATGC", "GATCGGAAGAGCACACGTCTGAACTCCAGTCACTAGCTTATCTCGTATGC". Third, each read was trimmed to remove all bases including and after the first position that had a quality score of 15 or less.

The transcriptome was assembled with 2.1 x 10^9 100bp paired-end reads following [202] under a variety of k-mer (k) and k-mer coverage (c) parameters. Briefly, assemblies were generated with the ASplice algorithm on the Whole Systems Genome Initiative (WSGI) computing cluster (wsgi-hpc.tamu.edu). Reads were included in the assembly based on the following criteria. Firstly, the trimmed length of the read was the same size or larger than the k-mer used in the assembly. Second, reads containing FastQC-identified overrepresented sequence patterns of 50 bp in length were not

expected to affect assemblies based on coverage cutoff [257]. These assemblies were then analyzed to identify potential alternative splicing patterns. This program assembles reads into splicing graphs, rather than predicted transcripts, similar to SOAPdenovo2 [258]. Briefly, the program produces an output of nodes connected together by edges. Nodes are sections of unambiguously aligned k-mers, and edges are the connections between nodes in alternatively spliced transcripts. The ASplice algorithm can be found at http://faculty.cse.tamu.edu/shsze/ASplice/.

Once the assembly was completed for a given parameter pair, count of reads from each library that aligned to the transcriptome was calculated for each node. The nodes of the transcriptome were then compared against known *D. melanogaster* proteins using a translated Basic Local Alignment Search Tool (BLAST) search [259]. For each node, only the top BLAST hit with an *E*-value below 10^{-7} was considered.

To augment the assembly prior to gene expression analyses, additional RNA-seq reads sequenced on a Hi-seq at the Texas A&M Genomics facility (50-bp paired end) were included though these were not included in analyses. Additional sequence information from other life stages were not included in this transcriptome assembly as previous work by other researchers demonstrated that additional data can lead to impaired analysis in groups of interest [260].

Global transcriptome analysis

Twenty-four preliminary assemblies were created from a range of k-mer sizes (21, 25, 31, 35, 41, and 45) and a range of coverage cutoffs (50, 100, 200, and 500). Exploratory plots of various summary statistics (N50, number of unique *D.*

melanogaster based annotations, etc) were made in R 3.1.3. Preliminary statistical analyses of differential gene expression were done in R with DESeq [261]. A false discovery rate was applied by only considering nodes with p-adjusted < 0.05.

To test for sexually dimorphic gene expression patterns, a comparison between male and female libraries was conducted. These differentially expressed nodes were plotted as a heatmap of normalized expression per library, and as raw count of nodes, genes, and transcripts per assembly. To test for differences between females based on the sex of their offspring, a nested analysis was used. First nodes that demonstrated significantly different of expression between males mated with thelygenic females and males mated with arrhenogenic females were identified and excluded. The remaining nodes were then analyzed for differential expression between female types. The classes identified through this analysis were: male biased, female biased, female-producing female biased and male-producing female biased. A Levene's Test for homogenity of variances [262] using the lawstat package [263] in R was used to determine whether there were significant differences in the variance of expression of significantly sexually dimorphically expressed nodes between males and females, thelygenic females and arrhenogenic females, or average female variance taking female phenotype into consideration.

Preliminary analyses of these assemblies led to the selection of 25_200, 21_200, and 21_500 as the best assemblies for further analysis with DESeq2 [264]. This was based on the following criteria: 25_200 had the highest number of genes to the lowest number of nodes and assembled *dsx* into a single splicing graph with three nodes; 21_200 and 25_500 each had a high ratio of number annotated genes to number of nodes.

Sex-specific gene transcriptome subsets

Information in other species was leveraged to select a subset of splicing graphs from each assembly that had at least one node with ontology to sex determination and sexual dimorphism related genes known from *D. melanogaster*, *Cochliomyia macellaria* Fabricius (Diptera: Calliphoridae), *Co. hominivorax* Coquerel, *L. sericata* and *L. cuprina* Wiedemann (Table 1); accession numbers: FJ461621.1, JX315620.1, JX315619.1, JX315618.1, FJ461619.2, FJ461620.1, FJ462786.1, FJ462785.1, GU784832.1, GU784833.1, GU784834.1, S79722.1, AF234184.1, AF234183.1). These *a priori* assemblies were analyzed with DESeq2 in the same way as the global assembly analyses.

Table 1. Known *D. melanogaster* dosage compensation, sex-determination, and sexual dimorphism regulating genes that were compared to the *de novo C. rufifacies* transcriptomes generated from adult whole-body RNA-Seq. Items in bold are those which had a homolog in at least one assembly. A consensus of 14 of these homologs were differentially expressed, with 1 gene showing consistent male bias in expression (*Crfru* indicated with the Ω symbol) and the rest female-biased in expression (all indicated with the † symbol).

CG Number	Abbr.	Gene	CG Number	Abbr.	Gene
CG1849	*run*	*runt*	**CG3496**	***vir***	***virilizer †***
CG1007	***emc***	***extra macrochaetae***	CG3827	*sc*	*scute*
CG10128	***tra2***	***transformer 2 †***	CG43770	*Sxl*	*Sex lethal*
CG10385	***msl-1***	***male-specific lethal 1 †***	**CG4528**	***snf***	***sans fille †***
CG11094	***dsx***	***doublesex***	CG4694	*her*	*hermaphrodite*
CG11680	***mle***	***maleless †***	**CG5102**	***da***	***daughterless †***
CG11853	*to*	*takeout*	CG5993	*os*	*outstreched*
CG12399	*Mad*	*Mother against decepentaplegic*	**CG6315**	***fl(2)d***	***female lethal d †***
CG13201	***ix***	***intersex†***	**CG7015**	***Unr***	***Upstream of N-ras***
CG14307	***fru***	***fruitless Ω***	**CG8384**	***gro***	***groucho †***
CG1641	*sisA*	*sisterless A*	**CG8599**	***Su(var)3-7***	***Suppressor of variegation 3-7 †***
CG16724	*tra*	*transformer*	**CG8631**	***msl-3***	***male-specific lethal 3 †***
CG17820	*fit*	*female-specific independent of transformer*	CG8704	*dpn*	*deadpan*
CG3025	***mof***	***males absent on the first †***	**CG9148**	***scf***	***supercoiling factor***
CG3241	***msl-2***	***male-specific lethal 2***	CG9019	*dsf*	*dissatisfaction*
CG33261	***Trl***	***Trithorax-like †***	CG12399	*Mad*	*Mother against decepentaplegic*

Gene ontology analyses

Differentially expressed genes annotated with *D. melanogaster* were analyzed for gene ontology (male biased, female biased, male-producer biased, and female-producer biased), to understand patterns of gene expression differentiation using the open-source database Generic Gene Ontology (GO) Term Finder from the Lewis-Sigler Institute for Integrative Genomics at Princeton University [265] and Reduce and Visualize Gene Ontology (REVIGO) [266]. Treemaps were generated in R. Further annotation of function was done using the list of genes resulting from a FlyBase query of the terms "testis", "spermatogenesis", "spermatocyte", "spermatozoan", "oogenesis", "oocyte", "ovary", and "ovariole". To avoid repetition, lists were prioritized in the following manner: oogenesis>ovary>oocyte>ovariole and spermatogenesis>testis>spermatocyte>spermatozoan, with genes under the higher order terms removed from lower order term gene lists. The ovariole gene list did not contain any unique terms.

Results

Sex ratios in the wild

A total of 5,133 larvae collected from 15 donations survived to pupation (Table 2). Of these, 3,926 were identified as *C. rufifacies*, the rest being *Co. macellaria*. Overall, 49 *C. rufifacies* individuals pupated but did not eclose, and 1,779 females and 2,098 males were collected. Less than 50 adult *C. rufifacies* were collected from three donations and were excluded from the rest of the analysis. Overall, the sex ratio deviated significantly from 50:50 (Z-test for proportions, $p = 0.00244$). However, the sex ratio

was only significantly different from 0.5 on two of the 12 donations considered here (Table 2). The distribution of sex ratios was non-normal, demonstrating a right skew (Figure 1), and the probability of this distribution of sex ratios coming from a binomial distribution with a 50:50 chance is less than 10^{-14}. *C. rufifacies* samples collected from D33-2014, D35-2014, and D38-2014 had sex ratios most similar to each other and most distinct from other samples collected (Figure 2).

RNA sequencing

Approximately 4.21 x 10^9 reads were generated in this experiment; 3.78 x 10^9 100bp paired end reads and 4.27 x 10^8 50bp paired end reads. The average number of reads per individual of the 100bp was 2.36 x 10^8 reads. After trimming, average read length was 91 bp, though the average library size remained the same. A total of 362 Gbp of sequence data was used for the transcriptome assemblies.

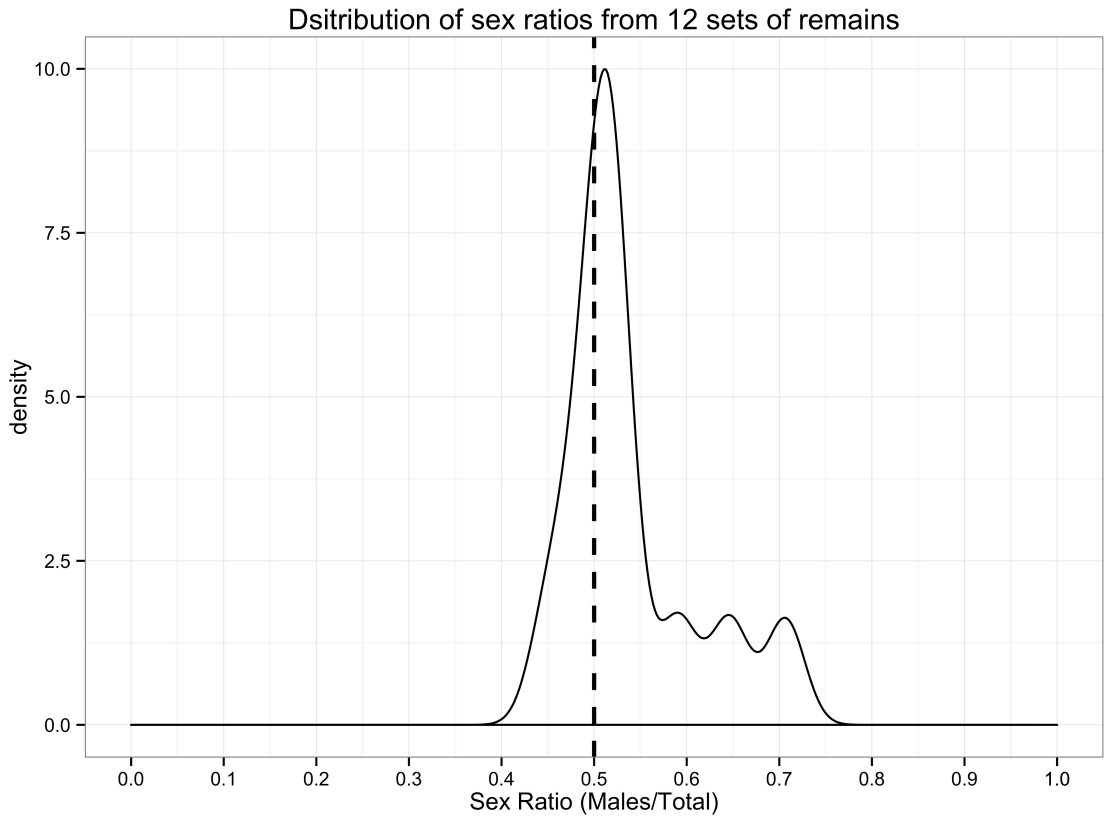


Figure 1. Distribution of sex ratios of *C. rufifacies* collected from human remains. This is a density plot of the distribution of sex ratios of collected *C. rufifacies* larvae from 12 sets of human remains at the Forensic Anthropology Research Facility at Texas State University, San Marcos, Texas.

Table 2.Quantification of sex ratios of *C. rufifacies* on human remains. This table summarizes the sex-ratio analyses of *C. rufifacies* collected from human remains at the Forensic Anthropology Research Facility at Texas State University in San Marcos, Texas. Columns from left to right: Date of collection (Collection Date), donation ID (Donation), date of placement (Placement Date), number of *Co. macellaria* that survived to adulthood (*Co. macellaria*), number of *C. rufifacies* female male, individuals which pupated but did not eclose (NA), sex ratio of surviving adults (Males/Total), and whether the sex ratio deviated significantly from 0.5 (NA: not applicable; -: not significant; *: $p<0.05$; **:$p<10^{-5}$; ***: $p< 10^{-10}$). An empty cell indicates a value of 0.

Collection Date	Donation	Placement date	*Co. macellaria*	*C. rufifacies*				
				Female	Male	NA	Ratio	
13-Aug-13	D43-2013	29-Aug-14		126	115	9	48%	-
6-Jun-14	D23-2014	21-May-14	500		1			NA
	D24-2014	20-May-14	150					NA
	D26-2014	28-May-14	525	36	52		59%	-
3-Jul-14	D28-2014	18-Jun-14		138	144	1	51%	-
	D30-2014	20-Jun-14		15	12			NA
	D32-2014	24-Jun-14		65	75		54%	-
	D33-2014	30-Jun-14		44	106	2	71%	**
17-Jul-14	D35-2014	14-Jul-14	3	231	422	7	65%	***
11-Aug-14	D38-2014	5-Aug-14		535	574	14	52%	-
	D40-2014	6-Aug-14		125	123	9	50%	-
8-Sep-14	D43-2014	29-Aug-14		241	260	5	52%	-
	D44-2014	1-Sep-14		99	99	0	50%	-
	D45-2014	2-Sep-14		72	59	2	45%	-
	D46-2014	2-Sep-14	29	52	56	0	52%	-

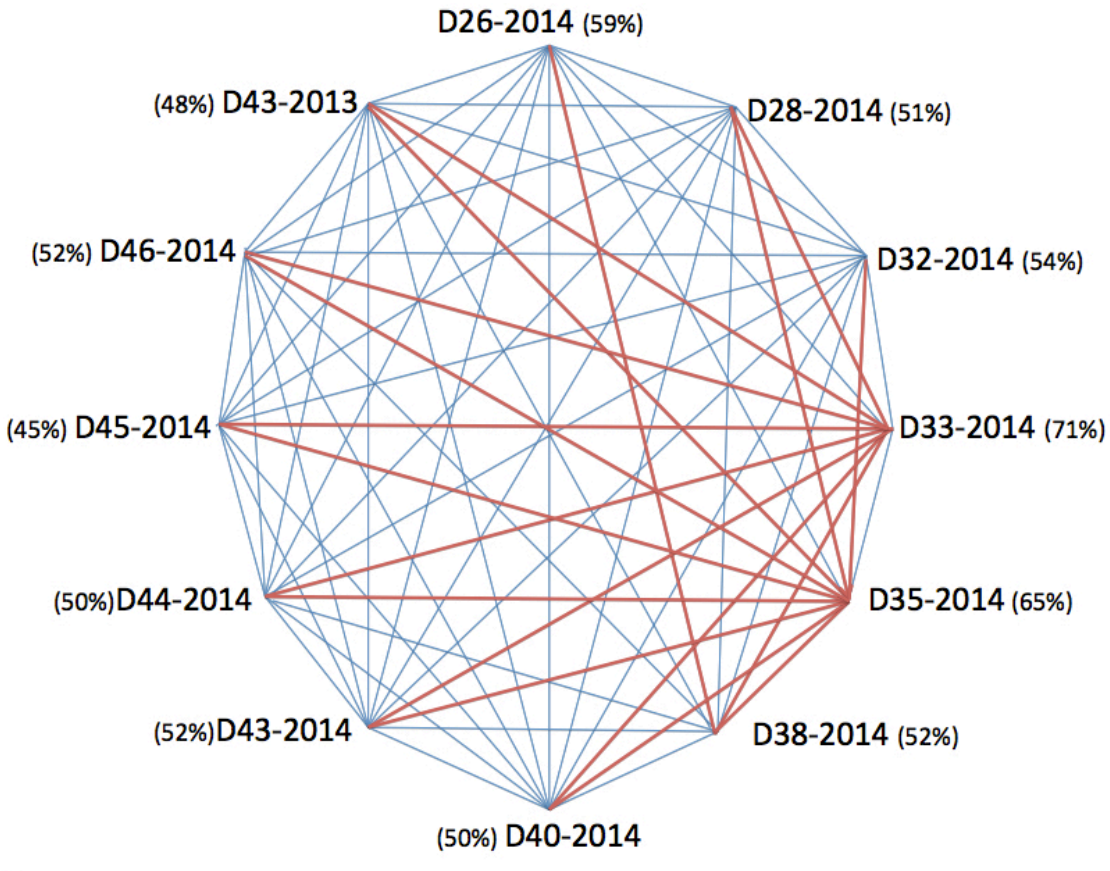


Figure 2. Marascuilo analysis of differences in sex ratios between the *C. rufifacies* samples collected from 12 sets of human remains. This figure summarizes the pairwise comparisons of sex:ratio of surviving *C. rufifacies* collected from human remains. Significant differences between samples ($p<0.05$) indicated with thick red lines, no significant differences in thin blue lines. Sex ratio of the sample indicated parenthetically.

Transcriptome assembly

Twenty-four preliminary assemblies were created from a range of k-mer sizes (21, 25, 31, 35, 41, and 45) and a range of coverage cutoffs (50, 100, 200, and 500). Assemblies with a coverage cutoff of 50 yielded the most nodes, with decreasing overall numbers of nodes with increasing coverage cutoff (Table 3, Figure 3). Within a given

coverage cutoff, at a kmer of 21 the number of nodes is initially low, peaks at 25 or 31, and then drops off again with increasing kmer size. Decreasing coverage cutoff led to an increase in all statistics except N50. There was a general pattern of a peak within a coverage cutoff at a kmer of 31 (coverage 50 and 100) or 25 (200 and 500) for total number of nodes, N50, number of splicing graphs with more than one node, and average number of nodes per splicing graphs. The number of total number of splicing graphs was highest at a kmer of size 21 (by between 25 and 50%) and increased with increasing kmer size. The maximum number of nodes assembled into a single splicing graph (the tangle) was variable and lacked a discernable pattern. Annotations of genes and transcripts with *D. melanogaster* sequences decreased in an approximately linear fashion with increasing assembly stringency (Figure 4).

Sexual dimorphism in gene expression

Preliminary analyses

Many genes demonstrated sexual dimorphism in expression. The total number of genes and transcripts in each assembly decreased slightly within a coverage cutoff with increasing k-mer size (Figure 4). Although k-mer size 21 had the lowest number of differentially expressed nodes within a given coverage cutoff (Figure 5A), the highest number of sexually dimorphically expressed genes occurred in k-mers size of 21 (Figure 5B). On average, DEseq2 predicted five times as many nodes to be differentially expressed between males and females as DESeq, and an average of 94% of nodes predicted to be differentially expressed by the earlier version were also significant in the updated analytical package.

Table 3. General *de novo Chrysomya rufifacies* transcriptome assembly statistics. This table summarizes descriptive statistics of the 24 *de novo* transcriptomes assembled from whole-body RNA-Seq of adult *C. rufifacies.* Columns from left to right: coverage cutoff (c), k-mer used in assembly (k), total number of nodes assembled in the transcriptome (total nodes), total number of splicing graphs assembled (splicing graphs), number of unique *D. melanogaster* BLAST gene hits (Genes), number of unique *D. melanogaster* BLAST transcript hits (Tx), total number of splicing graphs with more than one node (>1 SG), the average number of nodes per splicing graph (Ave nodes/SG), the n50 of the assembly (n50), number of nodes in the tangle (max nodes), and an identifier for the tangle (MaxNode ID). The top row is the most conservative assembly and assemblies decrease in stringency with increasing row number.

c	k	Total Nodes	Splicing graphs	Genes	Tx	>1 SG	Ave nodes/SG	N50	Max nodes	MaxNode ID
500	**Ave**	**80306.17**	**31664.17**	**7864.67**	**9309.33**	**5584.17**	**2.56**	**829.00**	**458.17**	
	SD	***6985.30***	***3402.20***	***136.90***	***241.03***	***455.71***	***0.33***	***106.94***	***203.72***	
	45	71352	31507	7692	9072	4944	2.26464	819	623	NODE_302849
	41	73414	31313	7745	9115	5226	2.34452	856	149	NODE_66
	35	78863	29990	7835	9220	5731	2.62964	885	718	NODE_783458
	31	83961	29257	7886	9291	6049	2.86977	909	402	NODE_924603
	25	89111	29566	7976	9433	6084	3.01397	885	350	NODE_388162
	21	85136	38352	8054	9725	5471	2.21986	620	507	NODE_307571
200	**Ave**	**163047.67**	**40685.83**	**8501.83**	**10287.67**	**9568.83**	**4.16**	**616.67**	**716.17**	
	SD	***19584.21***	***8351.37***	***105.69***	***217.86***	***946.13***	***0.99***	***75.80***	***285.89***	
	45	147936	38464	8367	10091	8967	3.84609	640	338	NODE_492720
	41	157962	37196	8420	10119	9577	4.24675	652	471	NODE_919244
	35	172352	36477	8481	10202	10169	4.72495	657	822	NODE_1857484
	31	181845	36088	8507	10246	10455	5.03893	654	1134	NODE_1978575
	25	183512	38266	8576	10393	10256	4.79569	634	852	NODE_1595366
	21	134679	57624	8660	10675	7989	2.3372	463	680	NODE_1272962

Table 3. Continued

c	k	Total Nodes	Splicing graphs	Genes	Tx	>1 SG	Ave nodes/SG	N50	Max nodes	MaxNode ID
100	**Ave**	**265443.83**	**51702.17**	**8913.67**	**10983.83**	**12709.67**	**5.46**	**433.00**	**2476.83**	
	SD	***46204.59***	***12910.60***	***96.10***	***169.97***	***1555.06***	***1.61***	***33.52***	***909.90***	
	45	252053	46114	8781	10812	12291	5.46587	443	2844	NODE_284012
	41	268747	45798	8840	10860	12930	5.86809	445	2759	NODE_1704199
	35	290965	45228	8900	10908	13652	6.43329	450	3345	NODE_3218435
	31	305218	45189	8925	10970	13891	6.75425	452	3209	NODE_1872506
	25	295835	50097	8995	11078	13713	5.90524	443	1581	NODE_2110522
	21	179845	77787	9041	11275	9781	2.31202	365	1123	NODE_2318039
50	**Ave**	**406297.50**	**67967.67**	**9243.83**	**11504.00**	**15962.17**	**6.43**	**316.50**	**2223.33**	
	SD	***87542.53***	***17575.90***	***77.06***	***119.16***	***2316.51***	***2.12***	***13.72***	***773.32***	
	45	402356	59779	9131	11382	15496	6.73072	315	1399	NODE_5262076
	41	425353	59329	9179	11404	16346	7.16939	319	1673	NODE_5497406
	35	460666	58768	9239	11459	17296	7.83872	322	3537	NODE_5809403
	31	475798	58816	9272	11497	17619	8.0896	326	2401	NODE_60125
	25	438037	68011	9317	11584	17484	6.44068	327	2514	NODE_3795952
	21	235575	103103	9325	11698	11532	2.28485	290	1816	NODE_5256812

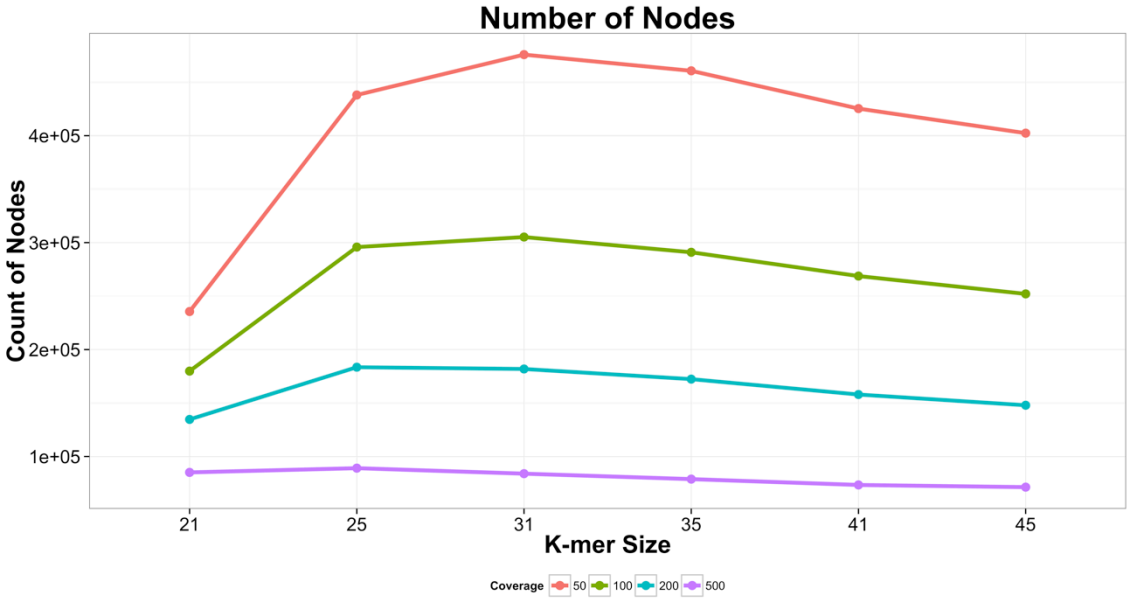


Figure 3. **Number of nodes in all 24 *C. rufifacies* transcriptome assemblies.** This is a line plot of number of nodes (y-axis) per k-mer size (x-axis), given a particular coverage cutoff for the assembly (red = 50, green = 100, blue = 200, and purple = 500).

Sexual dimorphism in adult gene expression

Selection of nodes based on a p-adjusted FDR cutoff of 0.05 demonstrated that an average 32% of the total nodes were differentially expressed between males and females, and these nodes were distributed 45:55 between males and females (Table 4, Figure 6). Genes annotated with *D. melanogaster* and significantly differentially expressed between males and females made up 65% of all annotated genes and of these 61% were up-regulated in females and 39% were up-regulated in males. Differentially expressed transcripts (63% of all transcripts) were less sex-biased, with 58% up regulated in females and 42% up-regulated in males. Differentially expressed genes were enriched in females compared to males, but males appeared to do more differential splicing. Furthermore, female variance in expression was higher than male variance in all significantly differentially expressed genes (two sided T-test, $p < 2.2 \times 10^{-16}$). Furthermore, though taking the phenotype of the female into consideration decreased the test-statistic by two fold, female variation in expression was still significantly higher than male variance (two sided T-test, $p < 2.2 \times 10^{-16}$). Interestingly, arrhenogenic female variance in expression was higher than thelygenic female variance across all this same set of nodes (two-sided T-test, $= 2.025 \times 10^{-9}$).

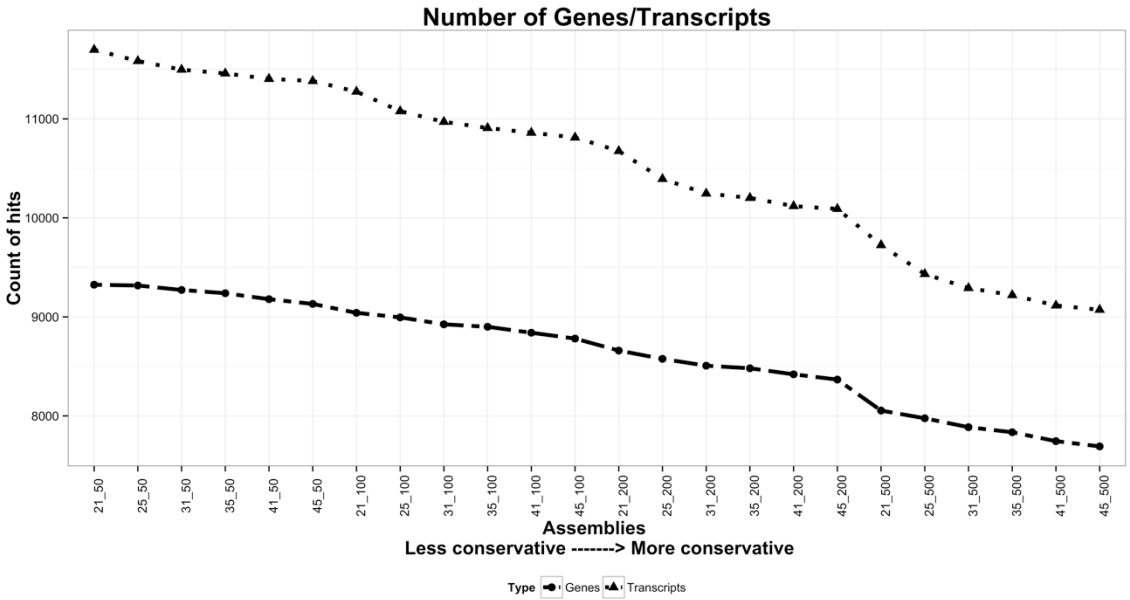


Figure 4. Unique annotations of *C. rufifacies* transcriptome assemblies with *D. melanogaster* genes and transcripts. Assemblies are on the x-axis, ordered from "least conservative" to "most conservative" by coverage cutoff and then k-mer size (50 c < 100 c, 21_50< 25_50). A count of total BLAST hits to *D. melanogaster* is on the y-axis, with genes plotted as circles and dashed lines and transcripts as dotted triangles.

Female-biased genes were involved in over 600 biological processes, two fifths of which were directly related to RNA-production and processing (Figure 7). The other largest clusters of female-biased genes were related to cellular metabolism, microtubule organization, and oogenesis and reproduction. Approximately half of these female-biased genes function intracellularly in either RNA or small molecule binding. Male biased genes were involved in approximately 200 biological processes, with approximately one third of those processes related to phototransduction, vision, and detection of external stimuli (Figure 8). Other processes which were significantly

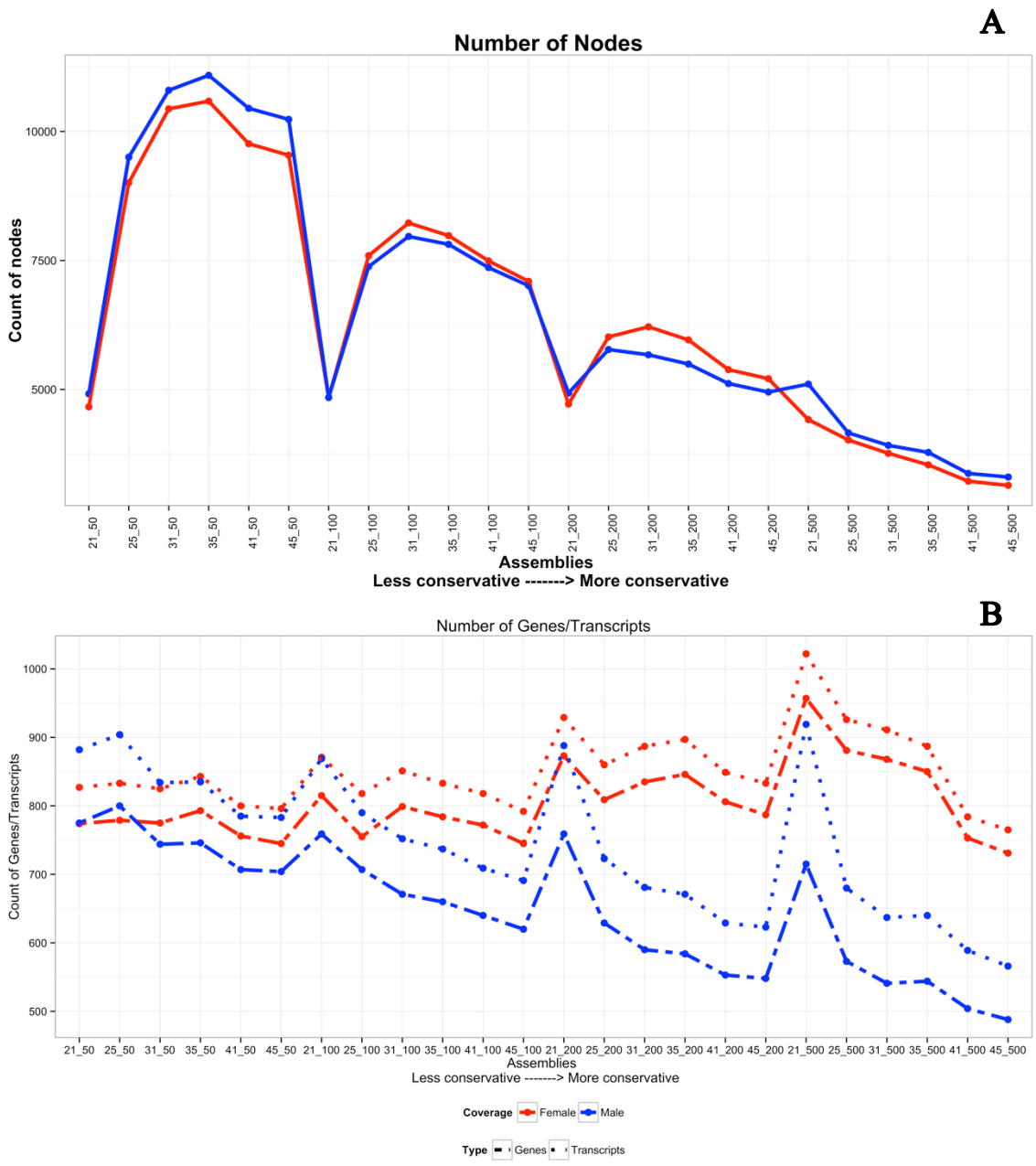


Figure 5. Preliminary analysis to compare male and female gene expression in *C. rufifacies*. For both graphs, assemblies ordered from least conservative to most conservative on the x-axis, count on the y-axis. Females are indicated in red and males in blue. A) Nodes that were differentially expressed between males (blue) and females (red) at a p-adjusted level of 0.05. B) *Drosophila melanogaster* gene (dashed line) and transcript (dotted line) hits in the differentially expressed nodes.

Table 4. Sexually dimorphic patterns of gene expression in three *de novo C. rufifacies* transcriptome assemblies generated from adult whole-body RNA-Seq. Columns from left to right: coverage cutoff (C), k-mer used in assembly (k), nodes, genes, transcripts, and sex-determination/sexual-dimorphism related genes. Within each column it is subdivided by: total number of (col name) in the assembly (Tot), that are differentially expressed (Diff), the percent of which are up-regulated in males (M) or females (F).

k	*C*	*Nodes*				*Genes*				*Transcripts*				*Sex Determination*			
		Tot	***Diff***	***M***	***F***	***Tot***	***Diff***	***M***	***F***	***Tot***	***Diff***	***M***	***F***	***Tot***	***Diff***	***M***	***F***
21	**500**	85136	33372	15074	18298	8054	5358	2044	3314	9725	6235	2599	3636	18	15	2	13
	200	134679	47754	21554	26200	8660	5804	2333	3471	10675	6876	2993	3883	19	20	3	17
25	**200**	183512	53934	23731	30203	8576	5593	2107	3486	10393	6505	2665	3840	19	18	1	17

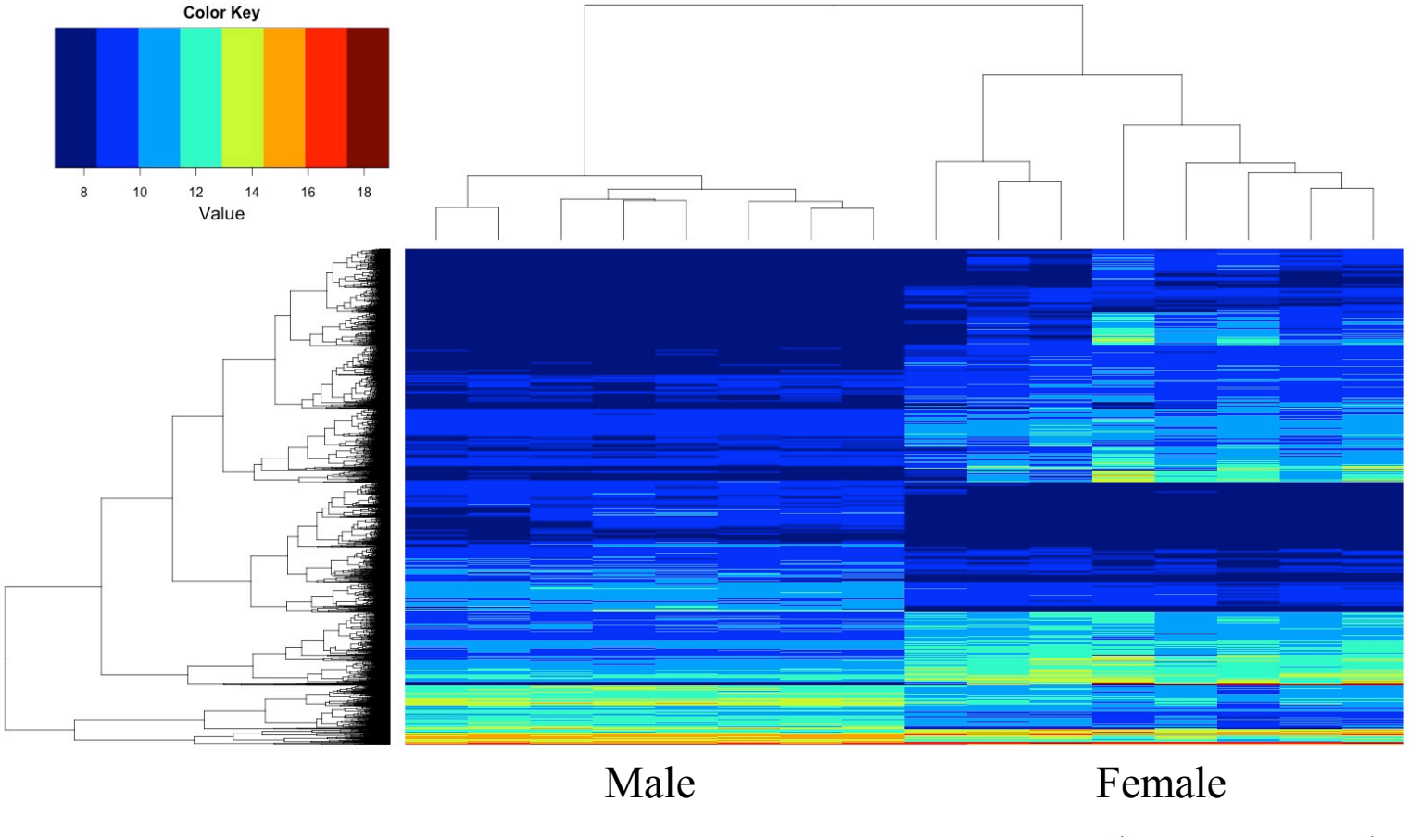


Figure 6 Heatmap of sexually dimorphically expressed nodes in *C. rufifacies* (p-adjusted < 0.00005). Nodes are on rows, libraries in columns, reciprocally clustered. Males are in one strongly supported cluster on the left and females in a strongly supported cluster on the right.

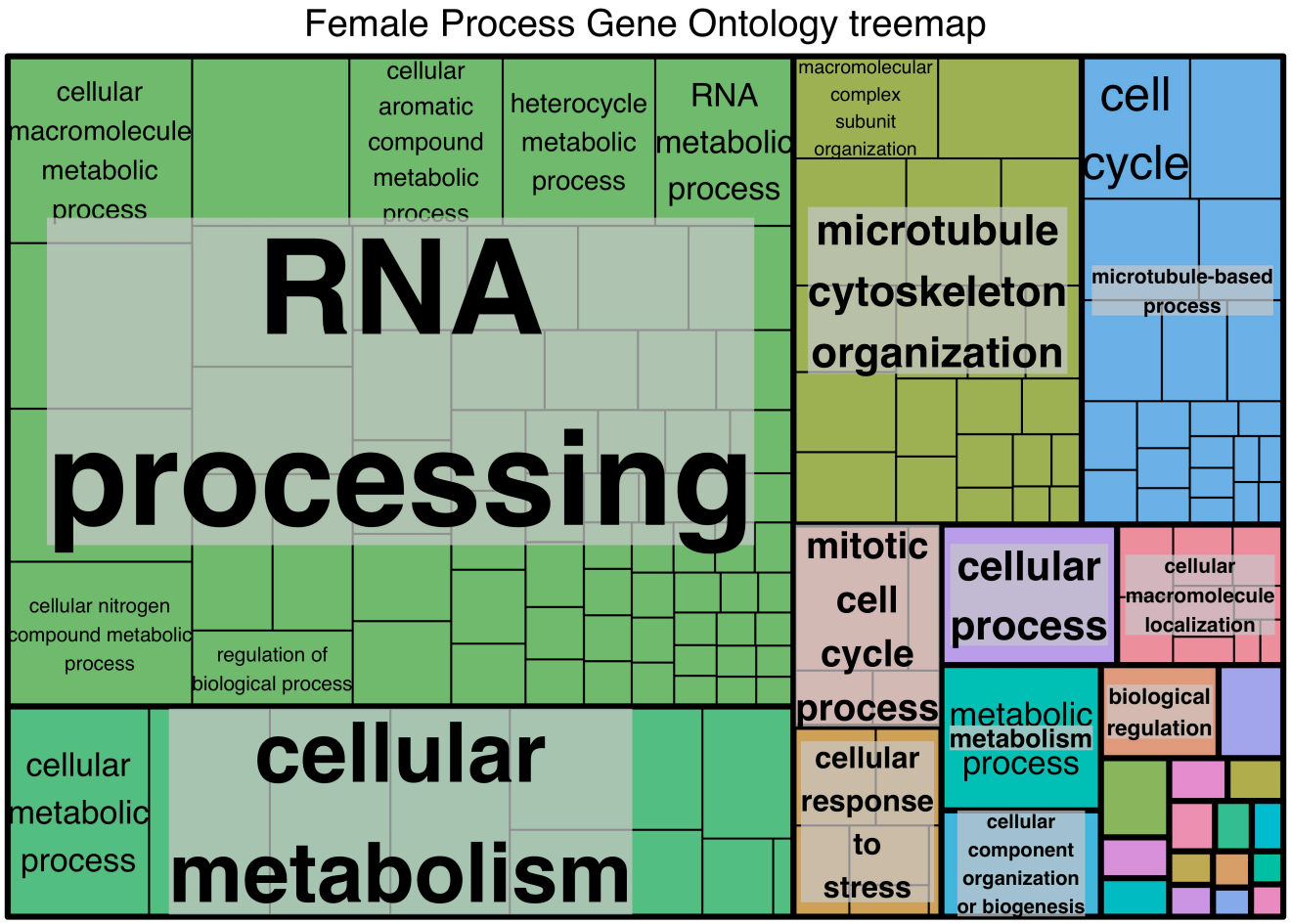


Figure 7. Treemap of process gene ontology for genes significantly up-regulated in adult female *C. rufifacies*. The size of the box indicates the percentage of all enriched genes with that annotation grouped by general processes (color and grey background text).

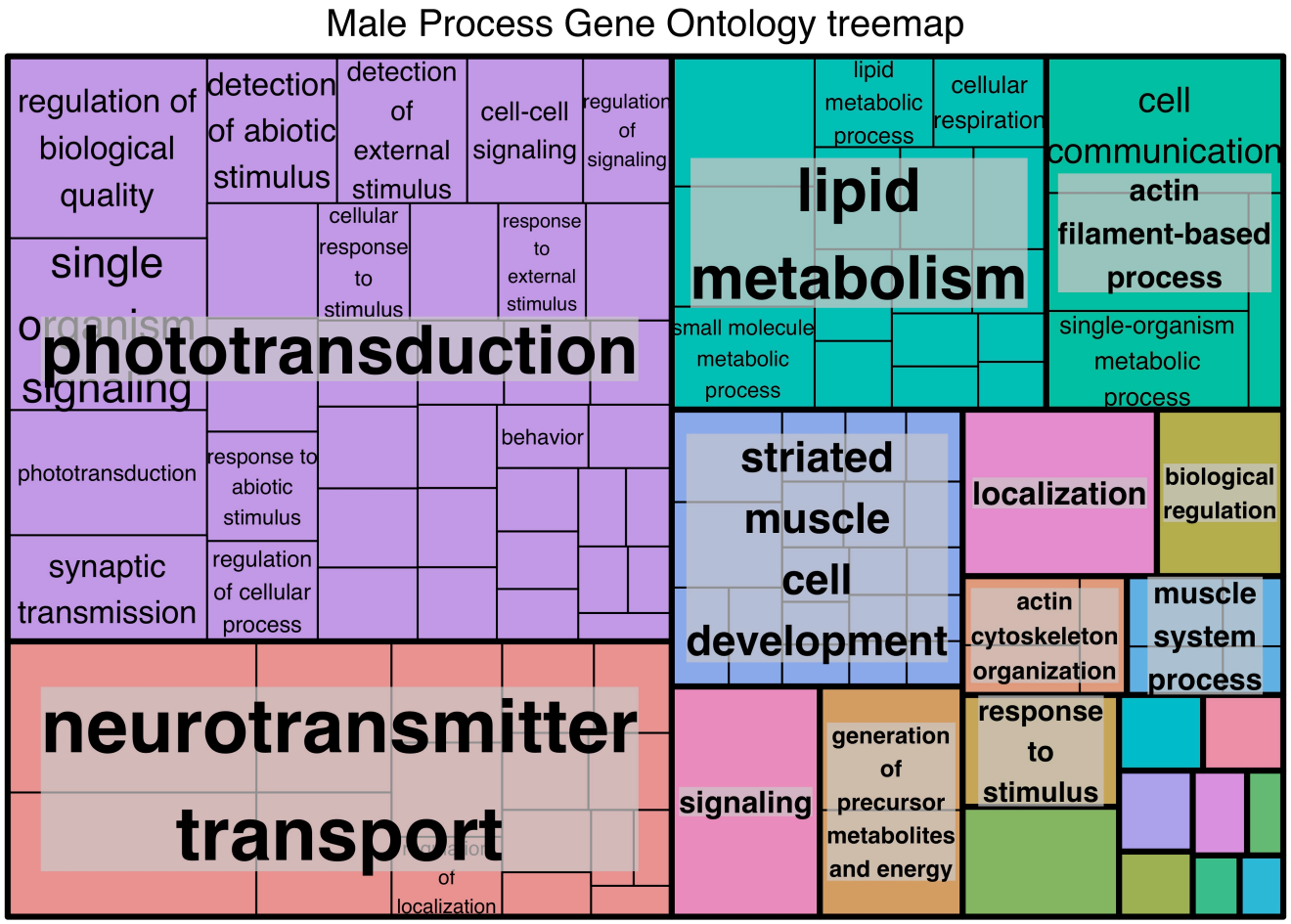


Figure 8. Treemap of process gene ontology for genes significantly up-regulated in adult male *C. rufifacies*. The size of the box indicates the percentage of all enriched genes with that annotation grouped by general processes (color and grey background text).

up-regulated in males included lipid metabolism, general neurotransmitter transport, and sarcomere and muscle development and function. Despite different levels of fragmentation in the three assemblies considered here (21_200, 21_500, and 25_200), there was consensus on the function and processes differentially expressed between adult males and females.

Of 36 genes known to function in sex determination and sexual dimorphism development in *D. melanogaster*, 19 homologs were identified in the 24 different assemblies (Table 4). Although there was variation between assemblies, all assemblies demonstrated significant dimorphism in expression in at least 14 of these genes (Table 1). The gene *fruitless* (*Crfru*) was the only gene consistently up-regulated in males relative to females, though *extra macrochaetae* (*Cremc*) and one node of *Trithorax-like* (*CrTrl*) were also male-biased in expression in some assemblies. In comparison, 13 genes were consistently up-regulated in females (Table 1), and an additional four were female biased in expression in at least two assemblies (*Crdsx*, *Crmsl2*, *CrUnr*, and *Crscf*). Further analysis of genes which have been demonstrated to be differentially expressed in gonads or somatic tissues of *D. melanogaster* demonstrated that many of the sexually dimorphically expressed genes exhibit consistent patterns (Table 5).

Table 5. Summary of patterns of enrichment of *C. rufifacies* homologs of sex-specific germ-line and somatic tissue genes identified in *D. melanogaster*. Number of homologs to *D. melanogaster* genes known to function or be expressed in (columns from left to right): germ-line tissues (oogenesis, ovaries, oocytes, spermatogenesis, testis, spermatocytes, and spermatozoa) in female and male *D. melanogaster* found to be differentially expressed between females or males, or thelygenic or arrhenogenic female *C. rufifacies* in three assemblies, and a count of somatic tissue specific male or female *D. melanogaster* genes enriched in two or more assemblies. An empty cell indicates a value of 0.

	Female				**Male**					**Thelygenic**					**Arrhenogenic**				
	Germ				**Germ**					**Germ**			**Soma**		**Germ**			**Soma**	
	Oogenesis	**Ovary**	**Oocyte**	**Soma**	**Spermatogenesis**	**Testis**	**Spermatocyte**	**Spermatozoa**	**Soma**	**Oogenesis**	**Ovary**	**Oocyte**	**Female**	**Male**	**Oogenesis**	**Ovary**	**Oocyte**	**Female**	**Male**
21_500	345	86	91		14	2	2	126		1					16	11	8		
21_200	116	33	44	460	36	3	12	154	109	1	1			1	19	3	6	33	7
25_200	359	88	97		19	3	2	160		1	2				34	11	12		

Table 6 Thelygenic versus arrhenogenic differential expression in *de novo Chrysomya rufifacies* transcriptome. Columns from left to right: coverage cutoff (C), k-mer used in assembly (k), nodes, genes, transcripts, and sex-determination/sexual-dimorphism related genes. Within each column it is subdivided by: total number of (col name) in the assembly that are differentially expressed (Diff), the percent of which are up-regulated in thelygenic (T) or arrhenogenic (A) females.

k	*C*	*Nodes*				*Genes*				*Transcripts*			
		Total	*Diff*	*T*	*A*	*Total*	*Diff*	*T*	*A*	*Total*	*Diff*	*T*	*A*
21	**500**	85136	2957	2024	933	8054	263	15	248	9725	267	15	252
	200	134679	5184	2249	1835	8660	314	19	295	10675	317	20	297
25	**200**	183512	10964	6960	4004	8576	608	27	581	10393	622	27	595

Thelygenic versus arrhenogenic females

After excluding nodes that were differentially expressed between males based on the female type they mated with, between 3 and 6% of all assembled nodes were differentially expressed between thelygenic and arrhenogenic females (Table 6). On average, the number of nodes differentially expressed between female producer types was found to be an order of magnitude lower than the number of differences between males and females. Between 55 and 70% of the differentially expressed nodes were thelygenic female biased in expression. In contrast, between 94 and 96% of the *D. melanogaster* annotated genes and transcripts were up-regulated in arrhenogenic females (Table 6). Though the number of genes or transcripts differentially expressed between males and females did not vary much between the three assemblies assessed here (Table 4), there was a two-fold increase in the number of differentially expressed genes between thelygenic and arrhenogenic females with an increase in k-mer length from 21 to 25 (Table 6).

Most of the splicing graphs up-regulated in thelygenic females could not be annotated with *D. melanogaster* sequences under the present criteria, though 14 *D. melanogaster* homologs were identified. The gene *CrCyp4d14* was up-regulated in thelygenic females in all three assemblies assessed. The other genes up-regulated in thelygenic females in two or more assemblies were not well studied in *D. melanogaster*, although five are known to have some RNA or DNA binding activity and one has been implicated in female meiosis chromosome segregation in *D. melanogaster* (Table 7). Additionally, across all three assemblies considered here six genes specific to female germ-line tissues were enriched in thelygenic females (Table 5), with *longitudinals lacking* (*Crlola*) being differentially expressed in two of three assemblies. In contrast, most of the splicing graphs up-regulated in arrhenogenic females demonstrated homology with at least one *D. melanogaster* gene or transcript. Despite the two-fold increase in number of differentially expressed genes, there was conservation in the gene ontology of these genes across the three assemblies. Genes involved in the processes of translation, cellular metabolism, and establishment of localization made up three fourths of these differentially expressed genes (Figure 9). In comparison to thelygenic females an average of 40 genes with female germ-line tissue effects were up-regulated in arrhenogenic females (), with 12 significantly up-regulated in all assemblies (Table 8).

Many of the nodes differentially expressed between thelygenic and arrhenogenic females were also statistically significantly differentially expressed between males and females (Table 9, Figure 10). For example, NODE_42844 in the 25_200 assembly is a single-node splicing graph of 852 bp that demonstrates significant homology ($E < 10^{-155}$)

with the *D. melanogaster* gene CG9246 and was significantly differentially expressed between males and females, and thelygenic and arrhenogenic females (Figure 11). An average of 14% of thelygenically enriched nodes were also differentially expressed between males and females, with only an average of 22% of these thelygenic-biased nodes also exhibiting female-biased in expression (Table 9). Genes up-regulated in males and thelygenic females included *CrCyp4d14*, *Common Dpr-interacting protein* (*CrcDIP*), *Larval serum protein 1 β* (*CrLsp1β*), and *Outer segment 1* (*CrOseg1*). In comparison, an average of 29% of arrhenogenically-biased nodes were sexually dimorphically expressed, and an average of 85% of these nodes were up regulated in females. Genes up-regulated in females and arrhenogenic females were involved in establishment of localization, translation, and membrane organization. Of the 1995 genes known to be differentially expressed between males and females in adult somatic tissues in *D. melanogaster*, a single gene, *ER degradation enhancer, mannosidase alpha-like 1* (*Edem1*) was enriched in at least two assemblies in thelygenic females, and this gene has been shown to be up-regulated in male *D. melanogaster* (Table 5). In contrast, a total of 40 of these somatically sexually dimorphic genes were up-regulated in arrhenogenic females, though seven of these genes have a male biased expression pattern.

Sex-specific analyses

These annotated, smaller assemblies were comprised of all splicing graphs with at least one node with sequence homology to a known sex-determination or sexual dimorphism regulating gene in *D. melanogaster* (Table 1) and sex-determination genes

Table 7. Genes significantly up regulated in thelygenic female *C. rufifacies.* Genes identified in two or more assemblies as being significantly up regulated in thelygenic females. Columns from left: Name of the gene in *D. melanogaster*, abbreviation (if applicable), number of assemblies with differential expression, and notes regarding the function or expression pattern in *D. melanogaster*.

Name	Abbr.	#	Notes
Cyp4d14	*Cyp4d14*	3	Located in the adult digestive system and larval mid gut [267, 268]
Common Dpr-interacting protein	*cDIP*	2	Excreted extracellularly and highly expressed in CNS and interacts with 19 different proteins in extracellular interactome [269]
CG11854		2	Structurally similar to *takeout*; high expression in third instar larvae, some low expression in adult males [267]
longitudinals lacking	*lola*	2	Involved in neurogenesis; intra-male aggressive behavior; positive regulation of DNA transcription [270, 271]
CG14763		2	Dyeinin ATP-ase mediated microtubule based movement [272]
Cyp4d20	*Cyp4d20*	2	Membrane bound; Detected in adult fat body [267]
Esterase P	*Est-P*	2	Carboxylic hydrolase ester activity in third instar larval bodies [273]
ATP synthase, subunit C	*ATPsynC*	2	H-exporting ATPase activity; high expression in a life stages [267, 274]
CG18596		2	Predicted RNA-binding/processing activity most highly expressed in ovaries [267]
α-Esterase-2	*α-Est2*	2	Very high expression in fat body and spermathecae (virgin and mated) [267]
Larval serum protein 1 β	*Lsp1β*	2	High in larvae and larval fat body, with nutrient reservoir activity and deficient ovary development and function in mutants [275, 276]
Ribosomal protein L7	*RpL7*	2	mRNA binding /translation; mitosis; important for pupariation [277, 278]
Outer segment 1	*Oseg1*	2	Cilium assembly and sensory perception of mechanical stimuli [279]
Replication factor C subunit 4	*RfC4*	2	Female meiosis chromosome segregation [280]

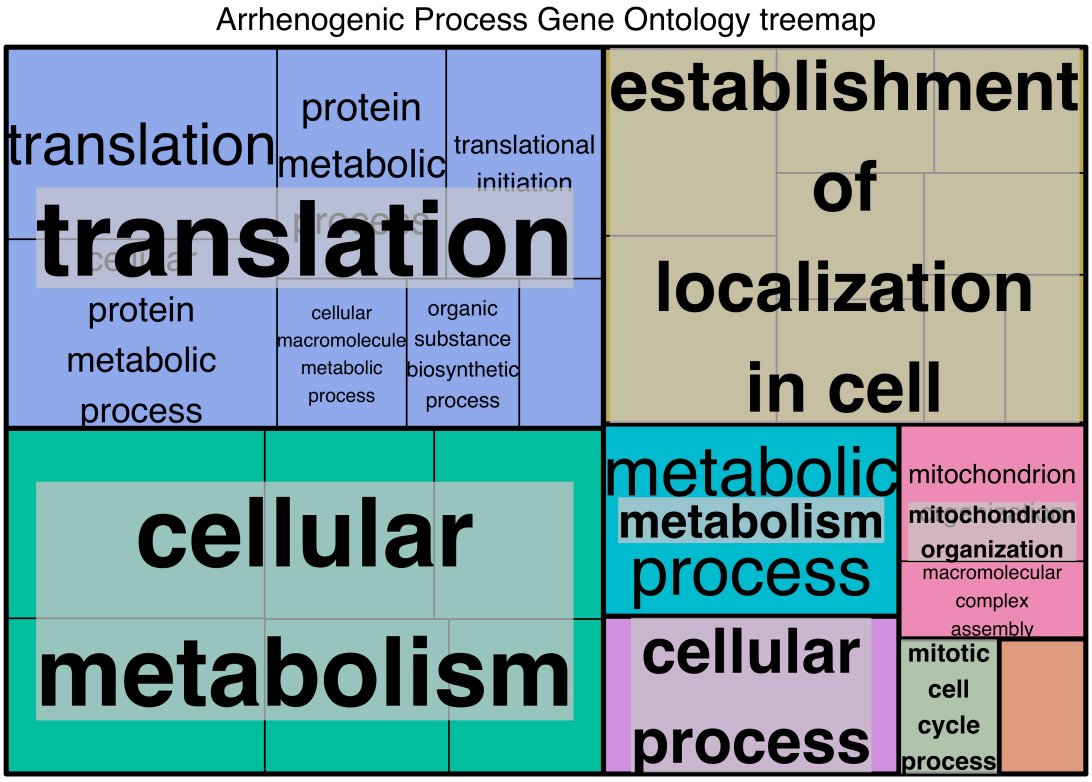


Figure 9. Treemap of process gene ontology of arrhenogenic up-regulated genes in *C. rufifacies*. The size of the box indicates the percentage of all enriched genes with that annotation grouped by general processes (color and grey background text).

Table 8. Homologs of *D. melanogaster* female germ-line tissue genes up-regulated in arrhenogenic female *C. rufifacies* in all three assemblies. Columns from left to right: CG number in *D. melanogaster*, name of the gene (if available), abbreviated gene name.

CG	Gene	Abbr.
CG9078	*infertile crescent*	*ifc*
CG8882	*Trip1*	*Trip1*
CG2674	*S-adenosylmethionine Synthetase*	*Sam-S*
CG6174	*Actin-related protein 1*	*Arp1*
CG8269	*Dynamitin*	*Dmn*
CG7266	*Ecdysone-induced protein 28/29kD*	*Eip71CD*
CG9680	*Dead box protein 73D*	*Dbp73D*
CG5119		*pAbp*
CG9710	*nudC*	*nudC*
CG14548	*Enhancer of split mβ, helix-loop-helix*	*E(spl)mβ-HLH*
CG3637	*Cortactin*	*Cortactin*
CG3664	*Rab5*	*Rab5*

Table 9. Summary table of patterns of differential expression shared between males and females and thelygenic and arrhenogenic females in *C. rufifacies*. Columns from left to right: Type (nodes, genes, or transcripts), assembly, count of enriched in males (M) or females (F) in sexual dimorphism analysis or thelygenic (T) or arrhenogenic (A) females in the female type analysis, count of items enriched in males and thelygenic (T) or arrhenogenic (A) females, and count of items enriched in females and thelygenic (T) or arrhenogenic (A) females.

Type	Assembly	Count				Male Enriched		Female Enriched	
		M	F	T	A	T	A	T	A
Nodes	21_500	15074	18298	2024	933	247	46	59	238
	21_200	21554	26200	2249	1835	275	80	90	434
	25_200	23731	30203	6960	4004	653	161	210	965
Genes	21_500	2044	3314	15	248	2	23	2	97
	21_200	2333	3471	19	295	2	30	3	117
	25_200	2107	3486	27	581	5	30	0	181
Transcripts	21_500	2599	3636	15	252	2	23	2	98
	21_200	2993	3883	20	297	2	30	3	118
	25_200	2665	3840	27	595	5	30	0	182

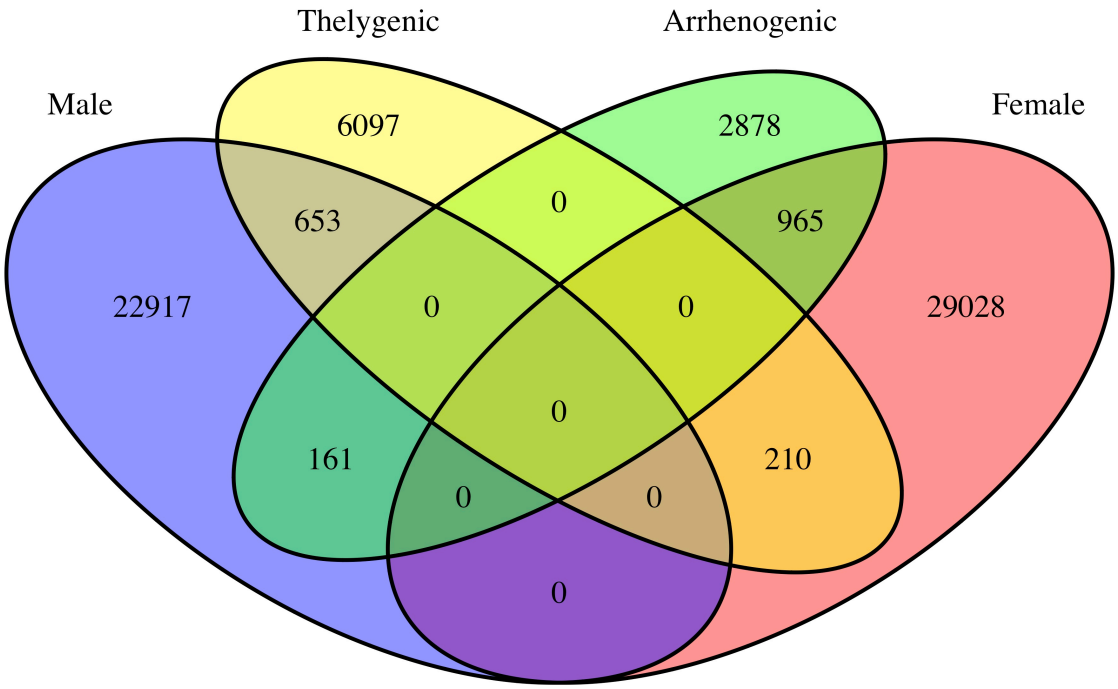


Figure 10. Venn diagram of overlap of nodes from the 25_200 assembly differentially expressed in *C. rufifacies* between males and females (blue and red) and thelygenic and arrhenogenic females (yellow and green) in separate analyses.

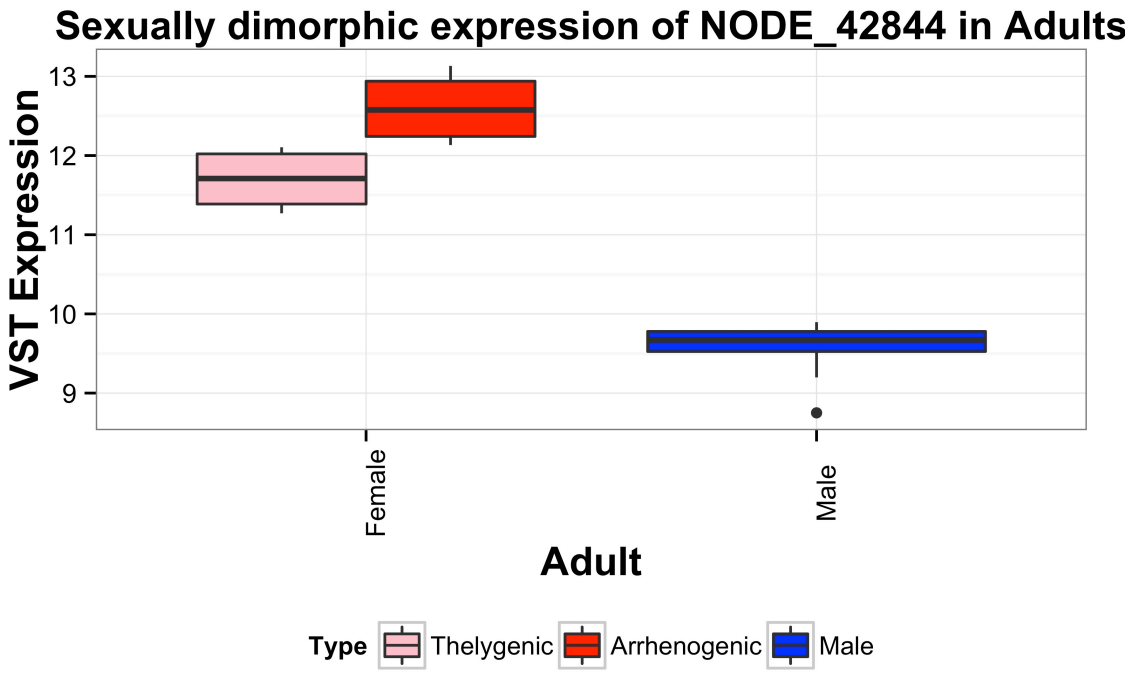


Figure 11 Boxplots of expression of NODE_42844, *Cr*CG9246. This is a boxplot of normalized expression of thelygenic females (pink), arrhenogenic females (red) and males (blue) for NODE_42844, which shares significant sequence homology with *D. melanogaster* CG9246.

known from other blow flies. These subset assemblies varied in size between 42 nodes and 12,837 nodes (Table 10). In comparing the transcripts that were detected in the subset assemblies, as well as the differentially expressed genes, only one assembly (45_500) failed to detect any genes or transcripts. A total of 23 genes had homology in at least one assembly (Table 11). In each assembly, several sequential nodes in a single splicing graph were assembled which demonstrated 99% sequence identity across 1057 bp to the 1335 bp previously sequenced of *CrSxl* (Accession #S79722) [206].

Table 10 Subset assembly statistics. This table summarizes descriptive statistics and analyses of the splicing graphs of 24 *de novo C. rufifacies* transcriptomes with at least one node with significant sequence homology to genes known to function in sex-determination in *D. melanogaster* or blow flies. Columns from left to right: coverage cutoff (C), k-mer used in assembly (k), nodes, genes, transcripts, and sex-determination/sexual-dimorphism related genes. Within each column it is subdivided by: total number of (col name) in the assembly that are differentially expressed (Diff), the percent of which are up-regulated in thelygenic (T) or arrhenogenic (A) females. An empty cell indicates a value of 0.

		Nodes					Genes					Transcripts				
C	k	All	F	M	T	A	All	F	M	T	A	All	F	M	T	A
500	45	1167														
	41	696	154	127		1	18	10	6			19	10	6		
	35	1233	237	180	4	8	17	10	6			19	10	6		
	31	1455	237	202	2	7	17	13	5			18	14	5		
	25	735	143	127	6	7	18	14	4			20	16	4		
	21	119	31	15	4		18	7	4			20	7	4		
200	45	1685	309	214	23	4	18	12	7			23	12	10		
	41	2372	447	285	20	10	20	10	7			25	10	10		
	35	3438	587	381	37	17	20	14	7		1	25	14	10		1
	31	6013	971	615	106	69	19	11	7			25	11	10		
	25	834	133	104	17	11	18	10	6			24	10	7		
	21	200	31	33	5	4	18	4	3			21	4	4		
100	45	5809	1066	835	144	82	19	16	9			23	17	10		
	41	4164	772	548	49	19	21	11	8			25	11	11		
	35	6508	1060	758	70	39	21	15	8		1	25	15	11		1
	31	8652	1322	896	188	129	21	14	7	2	1	27	14	10	2	1
	25	1046	197	154	15	9	19	13	7	1		23	14	8	1	
	21	42	10	5			19	4	3			21	4	4		
50	45	7503	1106	815	153	77	0	15	9	1		0	15	11	1	
	41	8322	1398	948	189	75	22	13	9			27	13	12		
	35	9333	1417	929	207	100	17	14	10	2	1	19	14	13	2	1
	31	12837	1916	1433	304	168	21	13	8	1	2	26	14	10	1	2
	25	1559	270	167	1		18	13	7			20	14	8		
	21	51	16	6			19	7	4			21	7	4		

Table 11 Summary of differential expression by gene in sex-specific assemblies. This table summarizes descriptive statistics and analyses of the splicing graphs of 24 *de novo C. rufifacies* transcriptomes with at least one node with significant sequence homology to genes known to function in sex-determination in *D. melanogaster* or blow flies. Columns from left to right: Gene or accession number (Gene), transcript name (Transcript), count of the number of assemblies in which a particular transcript was detected (ALL) or up-regulated in which it was up-regulated in males (M) or females (F), or thelygenic females (T) or arrhenogenic females (A), and percentage of up-regulated gene subsets in which was transcript was detected. Percentages greater than 75% for males and females (blue and pink respectively), both sexes (yellow) are shown in bold. Transcripts up regulated in any thelygenic (purple) and arrhenogenic (green) are also shown in bold.

		Count					% of total			
Gene	Transcript	ALL	M	F	T	A	M	F	T	A
CG11680	*mle-RC*	23	0	2	0	0	0%	9%	0%	0%
CG12399	*Mad-RB*	23	0	21	0	0	0%	**91%**	0%	0%
CG14307	*fru-RD*	20	5	16	1	0	25%	**80%**	**5%**	0%
	fru-RK	14	8	1	0	0	57%	7%	0%	0%
	fru-RL	15	15	0	0	0	**100%**	0%	0%	0%
	fru-RN	18	17	0	0	0	**94%**	0%	0%	0%
CG1641	*sisA-RA*	9	0	0	0	0	0%	0%	0%	0%
CG1849	*run-RA*	6	6	0	0	0	**100%**	0%	0%	0%
CG33261	*Trl-RB*	20	18	4	0	0	**90%**	20%	0%	0%
	Trl-RE	23	0	5	0	0	0%	22%	0%	0%
	Trl-RF	18	16	17	3	5	**89%**	**94%**	**17%**	**28%**
CG3496	*vir-RA*	23	0	12	0	0	0%	52%	0%	0%
CG4528	*snf-RA*	21	0	20	0	0	0%	**95%**	0%	0%
CG6315	*fl(2)d-RA*	22	8	10	0	1	36%	45%	0%	**5%**
CG7015	*Unr-RB*	23	17	18	1	0	**74%**	**78%**	**4%**	0%
CG8384	*gro-RC*	23	2	16	0	0	9%	70%	0%	0%
CG9019	*dsf*	9	0	8	0	0	0%	**89%**	0%	0%
gb|AF234183.1	*L. cuprina_Sxl*1	23	0	21	0	0	0%	**91%**	0%	0%
gb|AF234184.1|	*L. cuprina _Sxl*2	23	0	21	0	0	0%	**91%**	0%	0%
gb|FJ461619.2|	*L. cuprina tra*	18	0	0	0	0	0%	0%	0%	0%
gb|FJ461620.1|	*L. cuprina tra*2	23	0	23	0	0	0%	**100%**	0%	0%
gb|GU784833.1|	*L. cuprina dsx*M	23	23	0	0	0	**100%**	0%	0%	0%
gb|GU784834.1|	*L. cuprina dsx*F	23	16	22	0	0	**70%**	**96%**	0%	0%
gb|JX315618.1|	*Co. hominivorax tra*	23	20	2	0	0	**87%**	9%	0%	0%
gb|JX315619.1|	*Co. macellaria tra*	17	9	0	1	0	53%	0%	**6%**	0%
gb|JX315620.1|	*L. sericata tra*	21	5	7	0	0	24%	33%	0%	0%
gb|S79722.1|	*C. rufifacies Sxl*	23	0	23	0	0	0%	**100%**	0%	0%

Sexual dimorphism in expression in sex-specific assemblies

On average, 30% of the nodes were differentially expressed between males and females (Table 10). A total of 12 genes with 15 transcripts demonstrated up-regulation in only one sex (Table 11). Homologs which were up-regulated only in females included several nodes which had homology to *L. cuprina Sxl* or *C. rufifacies Sxl* and *L. cuprina tra2*. Homologs to two isoforms of *fru* and the male form of *L. cuprina dsx* were up-regulated only in males. An additional eight genes with ten transcripts demonstrated mixed differential expression with up regulation in both sexes in one or more assembly (Table 11), and these included *Trl-PF*, *Unr*, and the female isoform of *L. cuprina dsx*. Several different nodes in each assembly were annotated with the same gene but demonstrated significantly different patterns of expression.

Differential expression between thelygenic and arrhenogenic females

Differences between females were an order of magnitude lower than differences between the sexes, with an average of 2% of the nodes in these assemblies exhibiting significant differential expression (Table 10). Nodes with homology to three genes, *fru-RD*, *Unr,* and *Co. macellaria tra* were significantly up-regulated in only thelygenic females and the gene *fl(2)d* was up regulated in only arrhenogenic females in at least one assembly. Two different nodes which both had homology to *Trl-PF* were differentially expressed between female types, mirroring the pattern for this transcript observed in comparisons between males and females, and these nodes were all in assemblies with a k-mer of 31 or 35. Many of the nodes differentially expressed between males and females in these subset assemblies were differentially between thelygenic and

arrhenogenic females and demonstrated a similar pattern as the global transcriptome analysis of thelygenic:male and arrhenogenic:female pairing.

Discussion and conclusions

A total of 433 Gb of mRNA sequence was used to generate 24 *de novo* transcriptomes under a combination of hash lengths (k-mer) and coverage cutoffs (c) to investigate sexual dimorphism in adult *C. rufifacies* flies and differences in gene expression between thelygenic and arrhenogenic females. This is four orders of magnitude greater than the volume of sequence data used to investigate sexual dimorphism in gene expression in the western terrestrial garter snake *Thamnophis elegans* Baird and Girard (Squamata: Colubridae) and five orders of magnitude greater that used in investigation of the developmental patterns of gene expression in the blow fly *L. sericata* [202, 281]. The deep coverage in the present work, and results in *D. melanogaster* that demonstrated sex-biased expression of the rare transcripts *dsx* and *fru*, suggests that the present analyses should be sensitive even to low-abundance transcripts [282]. Furthermore while very little genetic data is currently available for *C. rufifacies*, the assembled *CrSxl* here shared 99% identity with the previously published sequence [206]. Between 9,300 and 7,600 genes were annotated through sequence homology with *D. melanogaster*, lower than the average of 12,000 unique *D. melanogaster* hits annotated in *L. sericata* [202]. This is not unexpected as the current work only included adult mRNA samples whereas libraries throughout development were included in the *de novo* transcriptome of *L. sericata*, and agrees with transcriptome work with adult somatic *D. melanogaster* tissues [282].

Broad agreement in gene ontology was observed between assemblies overall, between differentially expressed genes across assemblies, and between both analytical packages. More than five times more nodes were predicted to be differentially expressed with DESeq2 than DEseq. This is likely due to two important updates in DESeq2 to address type-I error control in a manner that was less costly in terms of statistical power [264]. First, differential expression is calculated on zero-centered normalized values in DESeq2, intended to improve detection and statistical validity of differential expression in genes with low copy number or with high variance in expression [264]. Secondly, the updated algorithm incorporates new methods for automatic outlier detection and handling using Cook's distance. Therefore, analyses with DESeq2 are more sensitive to detection of differential expression in lowly expressed genes and less sensitive to the effect of outliers.

In the present work, sex-biased expression was observed in 65% of all annotated genes. This is congruent with the results of microarray work in *D. melanogaster* and its sibling species *D. simulans* Sturtevant, which demonstrated that between 1/2 and 1/3 of genes demonstrated patterns of sex-biased expression [283-285]. Transcriptome work to identify sexually dimorphic patterns of gene expression in *D. melanogaster* assembled approximately 9000 genes and identified 1,381 genes as being differentially expressed between males and females, with 40% demonstrating a female bias in expression [282]. This study specifically targeted somatic tissues, however, and several other studies have shown that expression and splicing is highly tissue specific and that germ-line tissues represent a large proportion of whole body differential expression [285-287].

Comparative work in *D. melanogaster* and *D. simulans* Sturtevant identified 2,418 genes differentially expressed between males and females in both species, with 62% of these dimorphically expressed genes demonstrating a female bias [284]. This pattern is conserved in the present work, with 61% of all differentially expressed genes showing a female bias in expression. Though male biased expression in *C. rufifacies* was only observed in 39% of genes, 42% of the differentially expressed transcripts were enriched in male libraries. Given that the present *de novo* transcriptome used whole-body mRNA, these results are consistent with findings in *D. melanogaster* which demonstrated that testes-specific splicing events made up 2/3 of male splicing events, while ovary-specific splicing occurred in only 1/2 of female splicing events [287]. Additional work on the precision of sex-specific splicing in *D. melanogaster* showed that while female splice forms of many genes can be detected in males, male splices are rarely expressed in females [288].

The observed patterns of gene ontology enrichment in males and females of *C. rufifacies* is consistent with findings in *D. melanogaster* and *M. domestica* [285]. The most highly overrepresented GO process group in females was related to RNA processing including translation and transcription, consistent with the importance of maternal RNA and protein contributions to the early phases of embryonic development prior to the initiation of zygotic transcription (for a review see Gandolfi and Gandolfi [289]). The enrichment of genes involved in metabolism, microtubule cytoskeleton organization and processes, and ribosomal components in female-biased gene sets is also in line with results from *D. melanogaster* [285]. The significant up-regulation of genes

related to photo-transduction, striated muscle development, and neurotransmitter transport and signaling in males has also been observed in other species of flies [285]. It has been shown that male Calliphoridae, Sarcophagidae, and Muscidae flies are better at tracking and intercepting small moving targets, and this is likely due to the fact that males catch their mates in flight [290-293]. Enrichment of genes related to precursor metabolins, dynein-associated ATPase activity, and cytoskeleton organization in males is also consistent with previous research as these are consistent with the motile nature of sperm [285].

Several homologs of genes related to sex-determination and sexual dimorphism were identified in the previous work and demonstrated patterns of expression consistent with those observed in other species of flies. Across all assemblies, the RL and RN transcripts of *fru* were up-regulated in males while the RD transcript was up-regulated in females. In *Drosophila*, sexual dimorphism in the TRA protein controls splicing of *fru* into male or female forms, though FRU^{M} (the male form of the protein) is the only isoform known to direct physiological and behavioral sexual dimorphism as well as alter sexual orientation [294-296]. Nodes with orthology to *L. cuprina dsx* were differentially expressed, with the male isoforms only up-regulated in males. The observation that female-form transcripts were also detected in males is consistent with observations on the precision of sex-biased splicing in *D. melanogaster* [288]. One surprising finding was the consistent female enrichment of *CrSxl*, given that previous research in this species, *M. domestica,* and *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) found that *Sxl* was conserved in both sequence and structure relative to *D.* melanogaster but

was not differentially expressed or spliced [77, 206, 297]. In *D. melanogaster*, it was shown that inclusion of the male-specific exon of *Sxl* was the most reliably sexually dimorphically expressed splice site in the *D. melanogaster* transcriptome [288]. Furthermore, female enrichment of *Crmsl2* was unexpected, as this gene is an important part of dosage compensation in male *D. melanogaster*,s and expression in females results in death [298]. The gene *da* has been shown to regulate expression of *Sxl* in *D. melanogaster* [299], and the finding that *Crda* is also sexually dimorphically expressed suggests that discounting the importance of this gene in monogenic sex determination may have been premature [252]. Another interesting finding was the differential expression of *CrUnr*, which as *Unr* functions to repress expression of *msl2*, another gene known to function in dosage compensation, in *D. melanogaster* [300]. However, as *C. rufifacies* does not have heteromorphic sex chromosomes, it is unlikely that dosage compensation would function in this species the same way it does in flies with X:Y sex-determination systems [181, 182]. Comparative work in *Drosophila* suggests that sex-biased gene expression can be labile and patterns of expression of some genes can be opposite in sister species, and there is evidence that the most rapid evolving genes in *Drosophila* are likely to be under sex-dependent selection [284, 301-303]. Further work characterizing the sequence, function, and location of these genes in *C. rufifacies* is needed. Once the genome becomes available it will be possible to evaluate whether and how dosage compensation may be functioning in this species and more effectively compare evolutionary patterns in this species to other species. It is also curious that females exhibited significantly greater variance in expression than males and that

arrhenogenic females exhibit greater variance in expression than thelygenic females. This is because the current hypothesis is that thelygenic females are heterozygous for the maternal effect factor, while arrhenogenic females and males are homozygous [181, 182, 207]. The current results suggest that additional work is needed to confirm whether this is truly the case.

Many genes were differentially expressed between thelygenic and arrhenogenic females in the present work. Most of the genes enriched in thelygenic females have an unknown function in *D. melanogaster*, though a core, consistently enriched set are known to be involved in neurogenesis and RNA/DNA binding [267, 269-271, 277, 278]. Furthermore, despite the fact that more nodes were up-regulated in thelygenic females, and average of 95% of the annotated genes differentially expressed between types of females exhibited an arrhenogenic bias in expression. Homologs of some of these thelygenic:male genes, such as *Cr*CG9246, have been demonstrated to have neurological effects in *D. melanogaster*. In comparison, many of these arrhenogenic genes are involved in female reproductive processes, as evidenced by the gene ontology of the full set, overlap shared with female-biased expression patterns globally, and enrichment of female germ-line related genes. Additionally, thelygenic biased nodes and genes exhibited a 4-fold increase in male-biased patterns relative to female up-regulated genes compared, while arrhenogenic females exhibited a 5-fold increase in female-biased genes relative to male enrichment patterns. It is unclear why so many genes are differentially expressed, but it raises the possibility of neurological, physiological, or behavioral differentiation of females related to the sex of their offspring.

Analysis of the sex-ratios of *C. rufifacies* larvae collected from human remains suggests that the unusual sex-determination mechanism of this species may have affect immature intersexual conflict. It does appear that the distribution of males and females in space and time does not conform to a binomial distribution, though more sampling, sampling at other facilities, or from non-human remains is still needed. This, however, raises the possibility of large amplitude variation in sex ratio in wild adult populations, which could lead to increased intrasexual competition for mates or oviposition resources. Additional research, utilizing genetic markers of thelygeny or arrhenogeny will enable future work to investigate whether these females have different preferences or behaviors, whether these preferences effect the fitness of their offspring, and perhaps help up understand the selective forces generating these differences.

While the exact genetic mechanism responsible for monogeny in this species is still uncertain, several reasonable targets for further work and the genetic tools to do it are now available. Of specific interest is the characterization and investigation of the nodes which had significant homology to *Co. hominivorax tra*, as this was up-regulated in thelygenic females. Work on arrhenogenic *M. domestica* has shown that females with the *Ag* mutation fail to include a female form of *Mdtra* in their eggs, leading to default male splicing of *tra* and commitment of genetically female offspring to male development [83, 84]. However, given the differential expression of genes related to egg provisioning between types of *C. rufifacies* females it is also possible that it is a mutation in this pathway, and not of a core sex-determination cascade gene, that is responsible for monogeny in this species. Once *Crtra* has been fully sequenced, it will

be possible to look at whether key differences in the structure and sequence of this gene contribute to the curious reproductive biology of this species. Another interesting target is *CrSxl*, as this gene was significantly enriched in females relative to males, contrary to previous work in this species.

There are several interesting implications of this work and the molecular tools generated in this work could prove useful in a number of different fields. First, despite a lack of sex chromosomes in *C. rufifacies*, broad patterns of genetic sexual differentiation in adults appear to be conserved with other flies [285]. Comparative transcriptomic analysis including these data will help researchers understand evolutionary relationships and the selective forces shaping speciation, sexual selection, and the maintenance of sexual dimorphism [304-306]. Secondly, this and other species of blow fly are important in medical and veterinary entomology as they cause myiasis in both man and animal around the world [307-311]. The first successful application of the sterile insect control technique was accomplished in the primary screwworm, *Co. hominivorax*, through the release of sterile, irradiated males [312, 313]. A better understanding of the sex-determination system could allow researchers to develop control programs myiasis-causing species by using transgenics to engineer sex-specific condition-dependent lethality [39, 314, 315]. Furthermore, the results here suggest that there may be neurological differences between females based on the sex of their future offspring, and this could have an impact on the implementation of control strategies. Finally, it would be unwise to discount the importance of the un-annotated transcripts up regulated in thelygenic females and additional work is needed to characterize the effect of these

genes using techniques such as RNAi or transgenic work using the CRISPR/cas system [316, 317].

In conclusion, the *de novo* transcriptomic analyses presented here demonstrates several conserved patterns of sexually dimorphic gene expression consistent with findings in other species of fly, despite the unusual mode of sex determination employed by *C. rufifacies*. Though the exact gene cascade leading to sexual differentiation in this species is not yet clear, this work has generated a catalog of genetic tools which can be used to investigate specific hypotheses including *transformer, Sex-lethal*, and *doublesex*. However, the results do not exclude the possibility that noncoding RNA or mutations in ovarian transport mechanisms are responsible for monogeny in *C. rufifacies*. Another interesting result is the possibility of additional differentiation between females beyond the sex of their offspring, and this warrants further investigation.

CHAPTER III

SEXUALLY DIMORPHIC PATTERNS OF IMMATURE *Chrysomya rufifacies* GENE EXPRESSION

Introduction

Sexual dimorphism is most easily and often studied in adult insects, as it is adults which possess gonads and genitalia [318]. Sexual dimorphism has been observed in many adult traits, including morphology, behavior, ecological niche differentiation, and gene expression [301, 319-321]. Many detailed studies and some meta-analyses have been done to categorize sexual size dimorphism and determine the mechanisms which lead to it [322, 323].

The development and maintenance of sexually dimorphic traits is dependent upon sex-limited genetic expression, controlled by the final products of the sex-determination gene cascade [74]. Decades of research in *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) have revealed a complex hierarchy of sex-determination genes, an understanding of their modes of action and interaction, and some of the consequences of mutations at these loci [59]. In *D*. melanogaster, sex-specific splicing of the primary signal *Sex-lethal* (*Sxl*) leads to the production of a functional protein in females and an inactive protein in males [60]. The functional female form maintains its own expression through positive auto-regulation and directs the splicing of *transformer* (*tra*) to yield functional female (TRA^F) and non-functional male proteins [61, 62]. TRA^F is also positive auto-regulatory, controls splicing of *doublesex* (*dsx*) into active male (dsx^M) or female (dsx^F) forms and contributes to the production of male-specific transcripts of

fruitless (*fru*). The regulation of downstream gene expression by DSX^F in females and DSX^M and FRU^M in males gives rise to most of the physiological, biochemical, and behavioral sexual dimorphism in this species [324, 325]. Notably, these genes in the central sex-determination pathway of *D. melanogaster* are transcription factors which direct splicing and gene expression of downstream genes and, in some cases, positive feedback loops of self-regulation [61, 63].

Research in other dipteran families suggests that the sex-determination system identified in *Drosophila* is distinct within the order [75, 76]. One common feature to other dipteran systems is that *tra* is the basal signal, rather than *Sxl*, and *Sxl* is not somatically sex-specifically spliced [39, 75-77]. The "standard" system in blow flies such as *Lucilia sericata* Meigen (Diptera: Calliphoridae), true fruit flies such as *Bactrocera oleae* Rossi (Diptera: Tephritidae), or the common house fly *Musca domestica* L. (Diptera: Muscidae) is one of heteromorphic sex chromosomes and a presumed dominant male determiner (*M*) on the Y chromosome which directs sex-specific splicing of *tra* and therefore *dsx* [39, 78-81].

Work in adults to identify the causes and consequences of sexual dimorphism has identified three general categories of sexual dimorphism. Primary sex traits are those that are directly related to sexual reproduction, and include both anatomical and physiological factors, such as gonads or hormones [74, 326]. Secondary sex traits are those that increase reproductive success in the individual expressing them, and include any modification to morphology or behavior such as elaborate ornamentation [74, 327]. The third category, ecological sex traits, are those that differ between the sexes and lead

to niche divergence [98]. However, differentiating between males and females in immature insects can be very challenging. Early taxonomic work across several orders of holometabolous insects demonstrated that obvious sexual differentiation in immature insects was most often observed in pupae (especially Coleoptera and Lepidoptera) when in evidence at all [318]. Though there are cases wherein larvae and even eggs can be differentiated by sex based on external characteristics [318], they are not common.

The difficulty in identifying immature insects of a particular sex has made it challenging to study sexual dimorphism in gene expression across developmental stages [328]. Further complicating this issue is that while overt sexual differentiation is not observed until late third instar, sexual fate is determined early in embryonic development by synergism between maternally donated factors and the zygotic genome [299]. The limited work which has been done in *D. melanogaster* to study global sexual dimorphism in patterns of gene expression in immatures has therefore relied primarily on inbred lines and/or sex-lethal mutations [286, 328]. However, some work has identified male and female-specific gene expression in wild type pupae, as it is possible to identify an individual's sex using developing adult features such as sex-combs [328]. Furthermore, while it is possible to differentiate between male and female fated immatures in most species of fly though karyotyping or detection of y-linked markers, it is a labor intensive process [329-331].

The hairy maggot blow fly, *Chrysmoya rufifacies* Macquart (Diptera: Calliphoridae), has an unusual sex-determination mechanism (monogeny) which makes it uniquely well suited to the study of sexual dimorphism across all life stages. In this species, females

have single-sex offspring clutches, with thelygenic females hypothesized to be heterozygote dominant for a factor which they incorporate into their eggs that causes their offpsring to develop into fertile females, independent of the zygotic genome [181, 192]. Arrhenogenic females and males are hypothesized to be homozygous recessive for this same factor. Though it is possible to use genome size to distinguish between male and female larvae in other Calliphoridae, *C. rufifacies* has homomorphic sex chromosomes and so this technique does not work in this species [190]. Furthermore, this unusual sex-determination mechanism makes the development of inbred lines challenging, as full siblings cannot be mated.

Chrysomya rufifacies, like other blow flies, is also important in medical/veterinary and forensic entomology as it can cause myiasis in man and animals [307], and is also frequently observed during forensic entomology research and casework [308, 332-334]. Current control strategies for blow flies rely upon the release of irradiated sterile males [335], but researchers are interested in finding transgenic methods for control [336]. Forensic entomologists have long valued genetic techniques for identification [337, 338] but are now also becoming interested in the use of genetic markers to estimate insect age [202, 212, 339]. Futhermore, though sexual dimorphism in development rates has long been appreciated in other Diptera [340-342], it has only recently gained attention in forensic entomology [343].

The purpose of this work is to leverage the monogenic sex determination of *C. rufifacies* to study sexual dimorphism in wild-type flies throughout the life history. First, the possibility of sexual dimorphism was assessed in a simple phenotype: development

rate. Next sexual dimorphism was assesed in a more subtle phenotype: gene expression, through the application of next generations sequencing and *de novo* transcriptomics.

Materials and methods

Colony maintenance

Larvae of *C. rufifacies* were collected from numerous carcasses in College Station, Texas, USA between May and September of 2011 and eclosed adults were identified morphologically [253, 254]. Adult flies were released into a BugDorm 1 plastic cage (MegaView Science, Taiwan) and allowed to interbreed to found the laboratory colony. The colony was provided with fresh deionized water and refined sugar *ad libitum*, as well as fresh beef liver blood daily as a protein source for oogenesis. Flies were maintained at 28°C for a 16:8 light:dark (L:D) photoperiod.

Sexually dimorphic development

To collect *C. rufifacies* larvae of a known age, flies in the colony were allowed access to an oviposition substrate of fresh beef liver in a 32.5 mL opaque plastic cup covered with a KimWipe® (Kimberly-Clark, Irving, Texas) moistened with deionized water for a three-hour window. After oviposition, the eggs were placed in a Percival model I-36LLVL Incubator (Percival Scientific, Perry, Iowa) at 30°C, 75% relative humidity (RH), and a 12:12 L:D. After hatching, aliquots of 100 first instars were then transferred by paintbrush to 50 g of fresh beef liver in a 32.5 mL opaque plastic cup covered with a moistened KimWipe® in a 1.1 L canning jar with approximately 100 grams of playground sand and a Wype-All on the top to prevent escape but allow air flow. This method was replicated three times per egg collection, with a total of three

biological replicates. These rearing jars were then placed in a Percival model I-36LLVL Incubator (Percival Scientific, Perry, Iowa) at 30°C, 75% RH, and a 12:12 LD. This was repeated 4 times for at total of 1,200 larvae in 12 jars.

Observations were made every three hours beginning 138 hours after oviposition. All individuals from each jar observed to pupate at the time of observation was placed in a labeled, capped 30mL plastic cup. Observations continued after pupation, and the sex of each eclosed individual was recorded. This process continued until no eclosion had been observed for four days, after which daily observations were made for two weeks in which no flies eclosed. Data were analyzed in R 3.1.3 [344] to assess survival and sex ratios. Sexually dimorphic development rates for egg to pupation, egg to eclosion, and pupation to eclosion was assessed in R using a random effects least squared regression model, where replicate (egg collection time) and trial (aliquots of 100 larvae) were treated as random effects.

Gene expression sample collection

For each sample, a single male and female *C. rufifacies* were isolated together in a 1.1 L canning jar with approximately 100 g of playground sand, a Wype-All on the top to prevent escape but allow air flow, and refined sugar and water *ad libitum* and a 10 mL glass beaker filled with one Kim-wipe® and approximately 1 mL of fresh beef liver blood. These were kept in the incubator conditions previously mentioned. An additional 1 mL of blood was added each following day up until the 6th day post eclosion. The protein source was then excluded for 24 hours. Beginning on the 7th day post-eclosion, twice each day, a 35mL plastic cup with approximately 25 g of fresh beef liver covered

with a moistened Kim-wipe® was introduced to the jar as an oviposition medium for four hours. If a female oviposited during this time, the females were removed and flash frozen for later RNA extraction, and the progeny were allowed to develop under the same conditions listed above in a separate incubator. From a total of six different females per sex of offspring for each stage, the following samples were collected: ~100 eggs (max of 4-hours-old), ~100 first instars(12 hours post oviposition), ~10 second instars (24 hours post oviposition), 2 third instars (36 hours post oviposition), early pupal development (0-1 into pupation), mid-pupal development (2-3 days into pupation), or late pupal development (4-5 days into pupation). All samples were flash frozen and stored at -80°C until RNA extraction.

RNA preparation

RNA was extracted via TriReagent preparation according to manufacturer's protocols. Briefly, one sample (ie. ~100 eggs, single pupa) was macerated in 1mL of cold TriReagent (Sigma-Aldrich Corp., St. Louis, Missouri) in a 1.5 mL RNAse-free microfuge tube. Following this, 50 mL of ice-cold BAN reagent (Molecular Research Center, Inc., Cincinnati, Ohio) was added and the solution was vigorously mixed. Next, the tubes were spun at 14,000 G at 4°C for 15 minutes to isolate the RNA from the DNA and proteins. Approximately 500 µL of the top, clear layer was carefully removed via pipet and added to 500 µL of ice-cold 100% isopropanol. The tubes were mixed via inversion three times and allowed to rest on ice for 10 minutes to precipitate the RNA. The precipitate was then centrifuged at 14,000 G at 4°C for 15 minutes. The supernatant was completely removed, 1 mL of cold 70% ethanol was used to wash the RNA pellet,

and then the pellet centrifuged at 4°C for 5 minutes at 14,000 G. The ethanol was eluted, and any remaining ethanol was allowed to evaporate completely. The RNA was then dissolved in a 100 µL mixture of 99 µL of DNase/RNase/Nucleotide-free water and 1 µL of SUPERase•In ™ (Invitrogen, Life Technologies Incorporated, Grand Island, New York).

The extracted RNA was further purified using a Qiagen RNeasy Micro Kit and on-column DNase treatment following manufacturer protocols (Qiagen Inc., Valencia, California). Sample concentration and quality and control were assessed with NanoDrop {need to check on the model #} (Thermo Fisher Scientific Inc., Wilmington, Delaware) and an Agilent 2100 BioAnalyzer (Agilent Technologies Inc., Santa Clara, California). Two samples per sex and stage were pooled based on total RNA concentration into a single library, for three libraries per sex and stage. The exceptions was the third instar samples which were collected to study both immature gene expression (this work) and the molecular ecology of predation (a separate analysis). Libraries were prepared under standard protocols. In total, 66 libraries were sequenced on three separate RNA HiSeq flow cells. The libraries for the following stages were prepared as 100bp paired end reads: adult, third instar, and mid pupal development. The rest of the libraries were prepared as 100 bp single-end reads.

Transcriptome assembly

Prior to assembly, reads underwent trimming and quality control: reads were filtered to remove all sequences that contained adaptor sequences and known contaminants as defined by Illumina. The transcriptome was assembled with all 66 RNASeq libraries

following [202] under a variety of k-mer (k) and k-mer coverage (c) parameters. Briefly, assemblies were generated with the ASplice algorithm on the Whole Systems Genome Initiative (WSGI) computing cluster (wsgi-hpc.tamu.edu). These assemblies were then analyzed to identify potential alternative splicing patterns. This program assembles reads into splicing graphs, rather than predicted transcripts, similar to SOAPdenovo2 [258]. Briefly, the program produces an output of nodes connected together by edges. Nodes are sections of unambiguously aligned k-mers, and edges are the connections between nodes in alternatively spliced transcripts. The ASplice algorithm can be found at http://faculty.cse.tamu.edu/shsze/ASplice/.

Once the assembly was completed for a given parameter pair, the absolute count of reads which mapped to each nodes was calculated for each library. Transcriptome nodes were compared against known *D. melanogaster* proteins using a translated Basic Local Alignment Search Tool (BLAST) search [259]. For each node, only the top BLAST hit with an *E*-value below 10^{-7} was considered.

Selection of assembly for analyses

Twenty-four preliminary assemblies were created from a range of k-mer sizes (21, 25, 31, 35, 41, and 45) and a range of coverage cutoffs (50, 100, 200, and 500). Preliminary analyses of these assemblies were conducted to assess quality and completeness. The assembly with a k-mer of 31 and a coverage cutoff of 100 (31_100) was selected as the best candidate for analysis as it optimized completeness (high number of nodes, low number of single node splicing graphs) and quality (high N50, high number of *D. melanogaster* genes and transcripts detected, small "tangle") (Figure

12). The “tangle” is a feature of all of these assemblies, and represents a large group of nodes computationally predicted to be transcribed together which cannot be separated based on read alignment, and is the splicing graph in the assembly with the maximum number of nodes.

Transcriptome analysis

Analyses were done in R (version 3.1.3) using the DESeq2 package [264]. Several analyses to assess sexually dimorphic patterns of gene expression were conducted. First, identification of nodes that only showed expression in one life stage (counts of zero in all other libraries). Second, identification of genes demonstrating sexually dimorphic expression in eggs, across all larval samples, per instar (first, second, and third), across all pupal samples, and per pupal time point (early, mid, and late). Third, genes with significant differential expression between life stage within all of the female samples or all of the male samples were identified. Finally, fourth, identification of genes with sexually dimorphic patterns of gene expression across all life stages using a likelihood ratio test (LRT) comparing models of expression including a sex by stage interaction term (alternative hypothesis) with a null model without the interaction term. For all analyses, only nodes with a p-adjusted < 0.05 were considered for gene ontology analysis; p-adjusted values are automatically calculated by the DESeq2 package as a false discovery rate correction measure. Heatmaps with the GA package [345] and clustering analyses with the pvclust package [346] were generated in R using variance stabilizing transformation (VST) data generated by the DESeq2 package using a p-adjusted cutoff of < 0.05 unless otherwise indicated. Boxplots for individual nodes were

done in R with the ggplot2 package [347]. Variance was calculated for each sex:stage differentially expressed node for each sex within each stage (i.e. “female eggs”, “male eggs”, “female first instar”, “male first instar”), and then average female and average male variances were calculated. Average female and male variances per node were then compared using Levene’s Test for homogenity of variances [262] using the lawstat package [263] in R.

Gene ontology

Differentially expressed or unique node lists were queried against *D. melanogaster* genes for putative annotation as this is the closest model organism with the most complete gene ontology information [348]. The resultant gene lists were assessed for gene ontology using the Generic GO Term Finder and Generic GO Term Mapper online tools with FlyBase annotation at the Lewis-Sigler Institute for Integrative Genomics (http://go.princeton.edu/). These gene ontologies were only conducted based on sequence similarity with *D. melanogaster*, therefore, therefore many “unknowns” did not have homology under the annotation methodology described above. Gene ontology was visualized with REVIGO [266] and the treemap package in R [349].

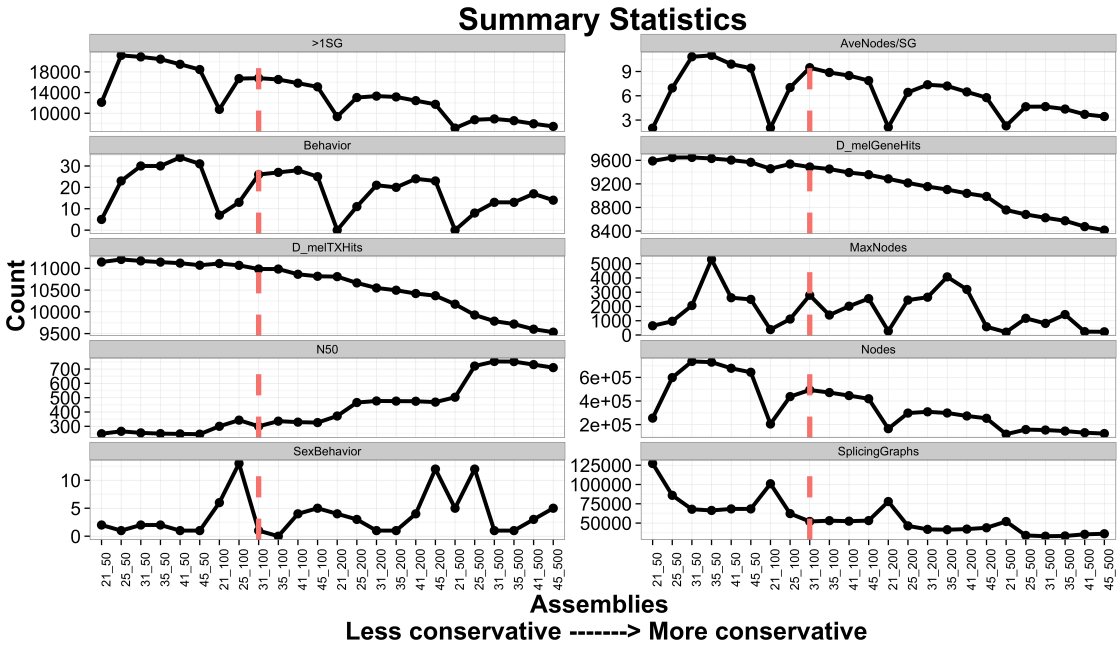


Figure 12. Descriptive statistics of all 24 *C. rufifacies* immature transcriptome assemblies assembled from a range of k-mer sized (21, 25, 31, 35, 41, and 45) and coverage cutoffs (50, 100, 200, 500). X-axes are assemblies ordered from least conservative (left) to most conservative (right) based on increasing k-mer size within increasing coverage cutoff. All y-axes are count. Left to right, top row to bottom: Number of splicing graphs with more than one node (>1SG), average number of nodes per splicing graph (AveNodes/SG), number of unique *D. melanogaster* gene hits (D_melGeneHits), number of unique *D. melanogaster* transcript hits (D_melTXHits), maximum number of nodes in a splicing graph (MaxNodes), N50, number of nodes assembled (Nodes), number of splicing graphs assembled (Splicing Graphs). The 31_100 (k-mer = 31, coverage = 100) assembly is indicated with the dashed red vertical lines.

Nodes of particular interest which did not show any homology with *D. melanogaster* sequences were annotated by predicting transcripts that contained those nodes from the whole splicing graph containing that node and comparing the sequence against the National Center for Biotechnology Information protein and nucleotide sequence databases using the BLAST algorithm [350]. Only hits with an *E*-value below 10^{-7} were

considered. Further annotation of function was done using the list of genes resulting from a FlyBase query of the terms “testis”, “spermatogenesis”, “spermatocyte”, “spermatozoan”, “oogenesis”, “oocyte”, “ovary”, and “ovariole”. To avoid repetition, lists were prioritized in the following manner: oogenesis>ovary>oocyte>ovariole and spermatogenesis>testis>spermatocyte>spermatozoan, with genes under the higher order terms removed from lower order term gene lists. The ovariole gene list did not contain any unique terms. To assess sex-specific somatic gene expression, the list of genes found to be differentially expressed in males and females in Lebo et al. [328] were compared to those genes found to be differentially expressed in this work.

Clustering

To understand underlying structure and correlation in gene expression patterns from the genes sexually dimorphically expressed throughout the life stages, expression was averaged within each sex and stage (eg. female eggs, male eggs) for all nodes with a LRT p-adjusted value of 5×10^{-5} (9311 nodes, see Results). To analyze patterns of gene expression throughout development in females or males alone, p-adjusted cutoffs of 5×10^{-15} and 5×10^{-21} were used to identify the top ~10,000 differentially expressed nodes. These data were analyzed in Cluster 3.0 [351, 352] using hierarchical clustering on average Pearson correlation. All clusters that had a correlation of 0.8 or greater and a minimum of 100 nodes were analyzed further to assess patterns of gene ontology (as described above). The clustering of libraries based on the significant results of analyses of sexual dimorphism in each stage was done using hierarchical clustering of libraries based on VST expression with average centered correlation and bootstrap support (1,000

iterations) with the pvclust package in R. Heatmaps for tables based on these clusters were created in R with the GA package on averaged expression per library type (eg. "egg" in sex-specific analyses, “female first instar” for sex:stage interaction analysis) within that cluster.

Results

Sexual dimorphism in development

A total of 937 flies survived to eclosion, with only one of four replicates of having significantly higher than average survival (replicate B). All replicates had sex ratios that were significantly male skewed (t-test, $p < 0.0001$), with replicates A and B, and C and D not being significantly different from each other (t-test, $p > 0.05$). Female overall immature development from oviposition to eclosion was nine hours slower than males (Figure 13) (REML, $p < 0.0001$), with a five hour difference in oviposition to pupation time (REML, $p < 0.0001$) and a four hour difference in pupation to eclosion times (REML, $p < 0.0001$).

Sequencing and transcriptome assembly

A total of 66 RNAseq libraries were sequenced, 33 male and 33 female. Average read length after trimming and quality control was 86.3 bp. There was an average of 6.3 x 10^7 reads per library, for a total of 68.5 Tbp of sequence data. These were assembled into 24 *de novo* transcriptome assemblies based on a range of k-mer sizes (21, 25, 31, 35, 41, and 45) and coverage cut-offs (50, 100, 200, and 500) (Table S1). On average, 50% of the reads mapped to the transcriptome.

Stage by stage differential expression

Egg

There were 57 nodes that only exhibited expression in the egg stage (Table 12), and of these homology to a single *D. melanogaster* gene, *slow as molasses* (*Crslam*), was detected. The rest have unknown annotation. An additional two genes were shared with adults (*Ribosomal protein L4* and *Heat shock protein cognate 1*), not exhibiting expression in any other stages. Analysis of sexually dimorphism patterns of expression in the egg stage across all nodes assembled identified a female bias in expression, with 166 of the 197 differentially expressed nodes exhibiting up-regulation in female fated eggs (Figure 14). None of these had homology with any known *D. melanogaster* sequences.

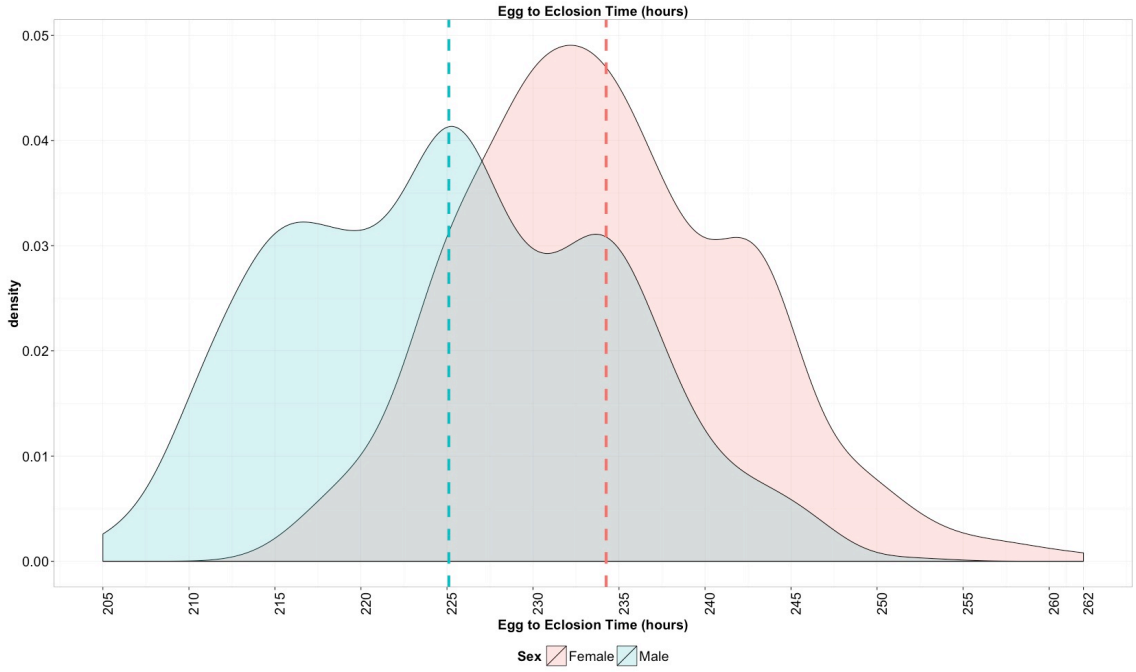


Figure 13. Density plot of sexual dimorphism in egg to eclosion development rate for *C. rufifacies* at 30°C. This is a density plot of number of hours from oviposition to eclosion (x-axis) for male (blue) and female (red) *C. rufifacies* reared at 30°C, 12:12 LD. Vertical lines indicate the average egg to eclosion time for each sex.

Table 12. Summary of stage-by-stage sexually dimorphic expression analyses in *C. rufifacies*. Summary of results of analysis of sexual dimorphism in gene expression within stages of development of *C. rufifacies* using DESeq2 on the 31_200 *de novo* **tr**anscriptome. Columns from left to right: stage of interest (by stage, across all instars, within a single instar, across all pupal time points, and within a single time point) (Stage), comparison (uniquely expressed in that stage only, expressed only in this stage and adults, significant interaction terms, up-regulated in females, and up-regulated in males) (Comparison), count of nodes (Nodes), number of *D. melanogaster* gene hits (Genes), number of *D. melanogaster* transcript hits (Transcripts), and differentially expressed sex determination related gene (if any) (SD).

Stage		Comparison	Nodes	Genes	Transcripts	SD
Adult		Unique	22732	420	424	
Egg		Unique	57	1	1	
		Shared with adults	325	2	2	
		Male	31	0	0	
		Female	166	0	0	
Larvae	***All Instars***	Unique	462	1	1	
		Shared with adults	2028	37	37	
		Sex: Stage	2945	132	138	
		Male	45	2	2	
		Female	7	1	1	
	First instar	Unique	0	0	0	
		Shared with adults	104	1	1	
		Male	3136	312	331	
		Female	901	25	25	
	Second Instar	Unique	6	0	0	
		Shared with adults	124	8	8	
		Male	1279	70	91	
		Female	646	27	27	
	Third Instar	Unique	216	1	1	
		Shared with adults	647	13	13	
		Male	935	70	72	
		Female	867	54	54	*dsx*

Table 12. Continued.

	Stage	Comparison	Nodes	Genes	Transcripts	SD
Pupae	***All Time Points***	Unique	1082	0	0	
		Shared with adults	7918	101	105	
		Sex: Stage	8999	69	70	*fl(2)d*
		Male	2730	350	363	
		Female	232	8	9	*dsx*
	Early Pupal	Unique	24	0	0	
		Shared with adults	218	2	2	
		Male	2253	308	318	
		Female	667	11	11	*dsx*
	Mid-Pupal	Unique	335	0	0	
		Shared with adults	1713	9	9	
		Male	14587	314	325	
		Female	1461	19	19	*dsx*
	Late Pupa;	Unique	4	0	0	
		Shared with adults	870	35	37	
		Male	2295	299	309	
		Female	785	13	13	*dsx*

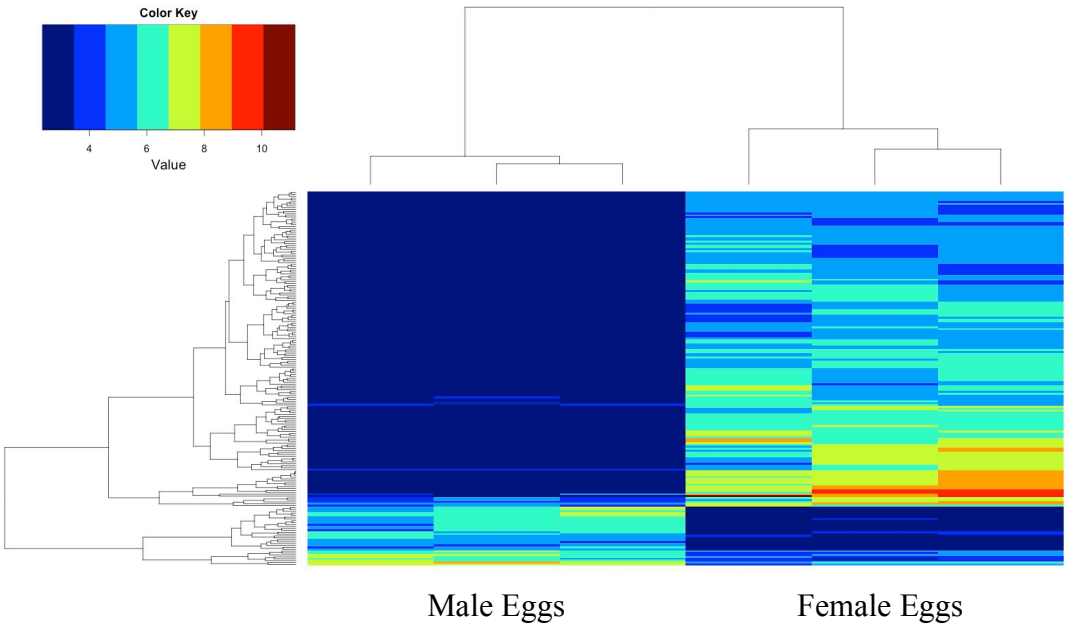


Figure 14. Heatmap of differentially expressed nodes in eggs of *C. rufifacies*. This is a heatmap of nodes differentially expressed between male-fated eggs and female-fated eggs of *C. rufifacies*, with nodes on the rows and libraries in the columns. Rows and columns are reciprocally hierarchically clustered based on correlation in expression patterns.

Larvae

More nodes were found to be unique to larvae rather than eggs, with 462 nodes expressed only in larvae (Table 12). Of these nodes, the only gene that could be annotated with *D. melanogaster* was *δTrypsin* (*CrδTry*). An additional 37 genes were shared with adults, and all together these genes are involved in mitotic spindle elongation, translation, biosynthesis, microtubule based processes, cellular component organization, and metabolism. Most of these genes have multiple functions, primarily as either structural components of ribosomes or in the activity of structural molecules.

Differential expression was male skewed, with one gene showing up-regulation in females (*CrTweedleF*) and two unknown genes in males (CrCG15014 and CrCG11892). The differential expression occurred primarily as a result of significant up-regulation in the third instar males relative to third instar females (Figure 15A) and all other instars, though first instar males also clustered significantly relative to first instar females and all second instars (Figure 15B). Although many nodes demonstrated significant sex by stage interactions, they were found mathematically to fall into four clusters (Figure 16). The first included genes which were highly expressed in the first and second instars, and female third instars exhibited lower expression than male third instars. Cluster L1 genes were primarily components of synapses. The second cluster followed a similar pattern, but these were up-regulated in females relative to males in the third instar. Cluster L2 genes were also primarily involved in transmembrane transportation, though it is not known whether they are also involved in neural signaling. The third cluster demonstrated equally high expression in males and females in the third and second instars, with up-regulation in males relative to females in the first instar. Cluster L3 was comprised of genes that function in lipid transportation. Finally, nodes with a similar pattern, but up-regulation in females relative to males, made up the fourth cluster. Cluster L4 was made up of genes involved in hemostasis and dopamine and L-DOPA monoxygenase activity.

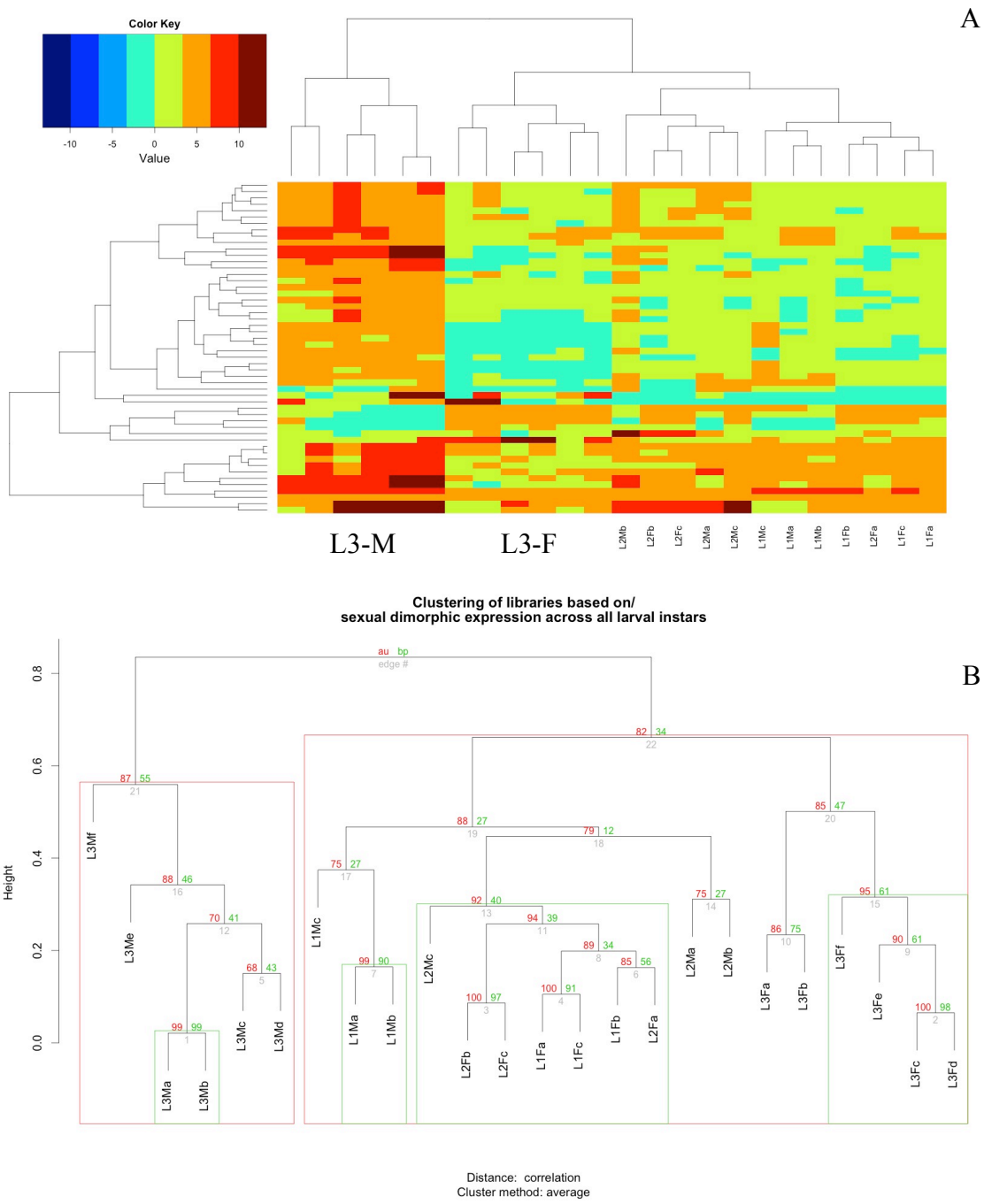


Figure 15. Differential expression between males and female *C. rufifacies* larvae. These figures summarize patterns of differential expression between male and female *C. rufifacies* larvae across all instars. A: Heatmap of nodes with significant differential expression, with nodes on the rows and libraries in the columns. Rows and columns are reciprocally hierarchically clustered by expression. B: Bootstrapped hierarchical clustering of libraries based on expression levels of differentially expressed genes with values of approximately unbiased (red numbers) and bootstrap probability (green numbers) support using pvclust, with clusters with AU correlations ≥0.8 (red boxes) and ≥0.9 (green boxes).

First instars

Nothing was found to be uniquely expressed in first instars (Table 12), however this stage did share expression of a single unknown gene (CrCG6295) with adults that was not found in other libraries. Dimorphically expressed nodes (n= 4,037) were significantly male skewed in expression, and 312 genes were found to be up-regulated in males while only 25 were found to be up-regulated in female first instars (Figure 17). Male up-regulated genes were primarily involved in metabolic processes, especially lipid metabolism (Figure 17B) and were primarily components of peroxisomes. Female up-regulated genes, on the other hand, were important in muscular differentiation and organization as components of the sarcomere.

Second instars

Although 6 nodes were found to be uniquely expressed in second instars (Table 12), none here homologous with any *D. melanogaster* genes. Of the eight genes shared only with adults, five are known to function in small molecule binding and the functions of the remaining three are unknown. Fewer nodes were detected to be sexually dimorphically expressed in the second instar (n = 1,925) than in first instar samples, and only 29 of these were shared between first and second instar (19 in males, 12 in females). Significant differential expression demonstrated a male skew in second instars as well (Figure 18A). Male up-regulated gene with a known function were primarily involved in the synthesis and production of chitinous cuticle (Figure 18B), while those up-regulated in female second instars were involved in nitrogen transport and muscular tissue.

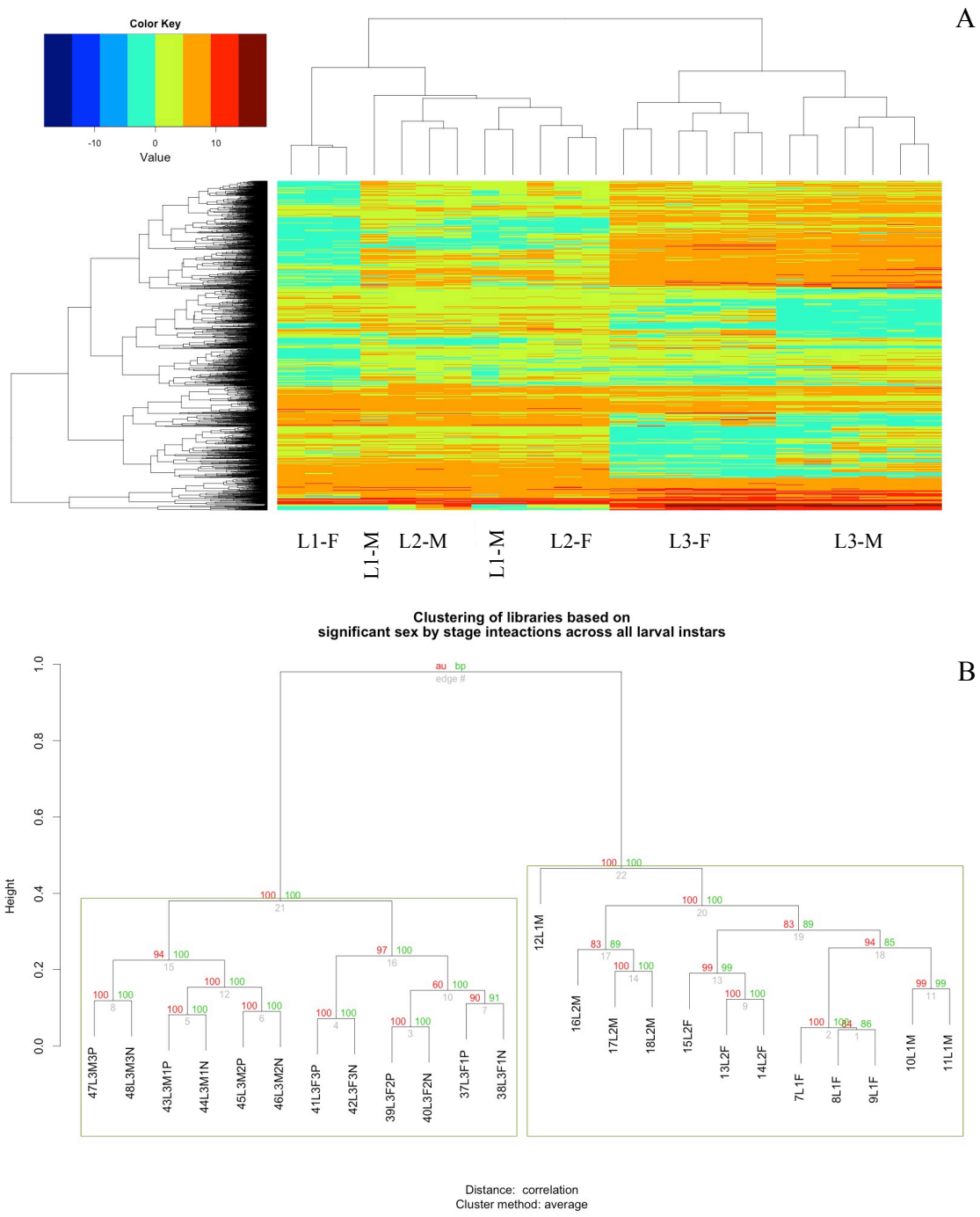


Figure 16. Sexually dimorphic differential expression in *C. rufifacies* larvae. These figures summarize results of genes that have significant sex: stage interaction in expression patterns across all three larval instars. A: Heatmap of nodes with significant sex and stage interactions models all three larval instars, with nodes on the rows and libraries in the columns. Rows and columns are reciprocally hierarchically clustered by similarity in expression. B: Bootstrapped hierarchical clustering of libraries based on expression levels of differentially expressed genes with values of approximately unbiased (red numbers) and bootstrap probability (green numbers) support using pvclust, with clusters with AU correlations ≥0.8 (red boxes) and ≥0.9 (green boxes).

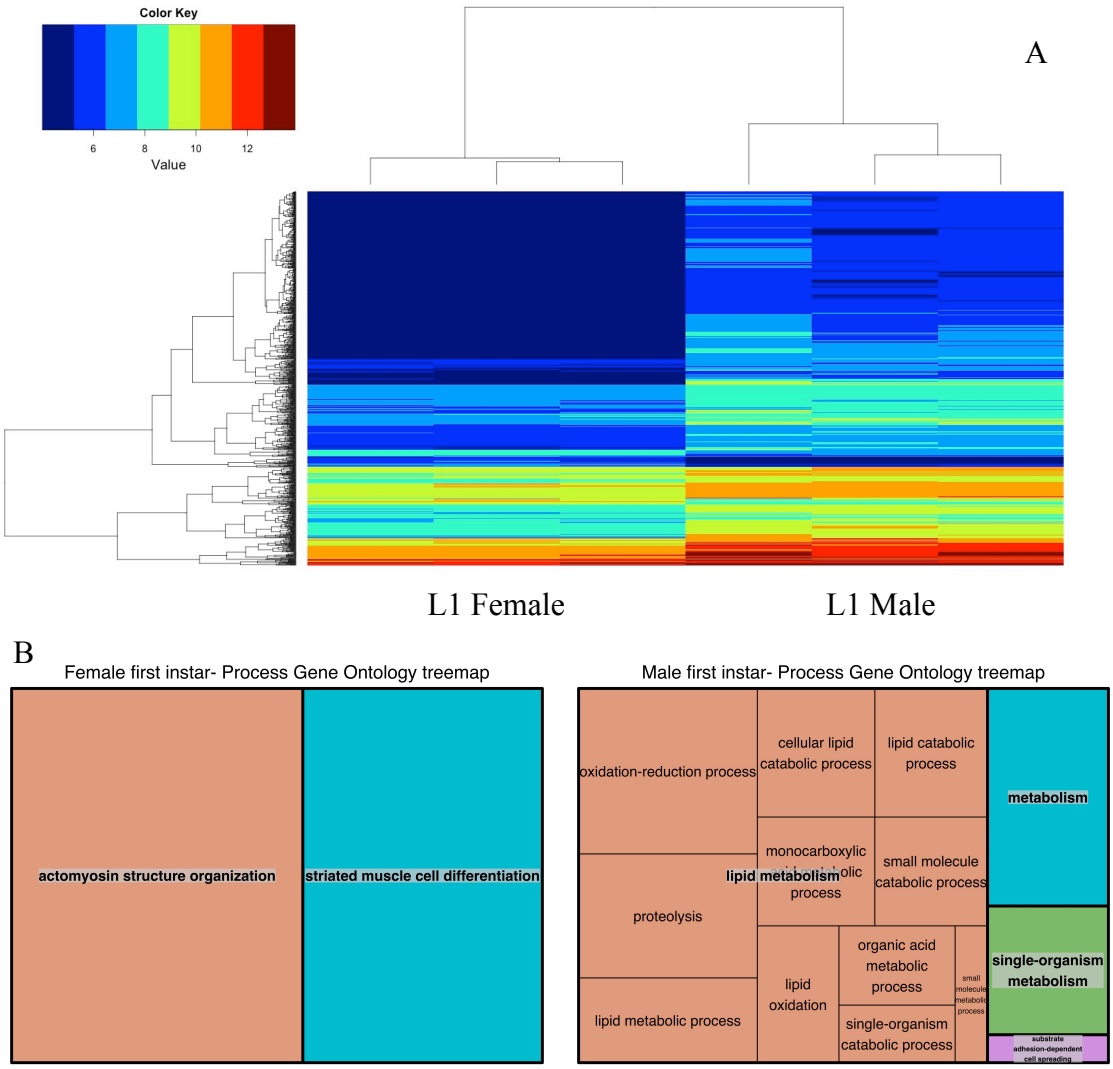


Figure 17. Sexually dimorphic differential expression in first instar *C. rufifacies*. These figures summarize the results of differential expression analyses between male and female first instars. A: Heatmap of nodes with significant differential expression between males and females, with nodes on the rows and libraries in the columns. Rows and columns are reciprocally hierarchically clustered based on similarity in expression patterms. B: Treemaps of gene ontology of male up-regulated (left) and female up-regulated (right) homologs to *D. melanogaster*. The size of the box indicates the percentage of all enriched genes with that annotation grouped by general processes (color and grey background text).

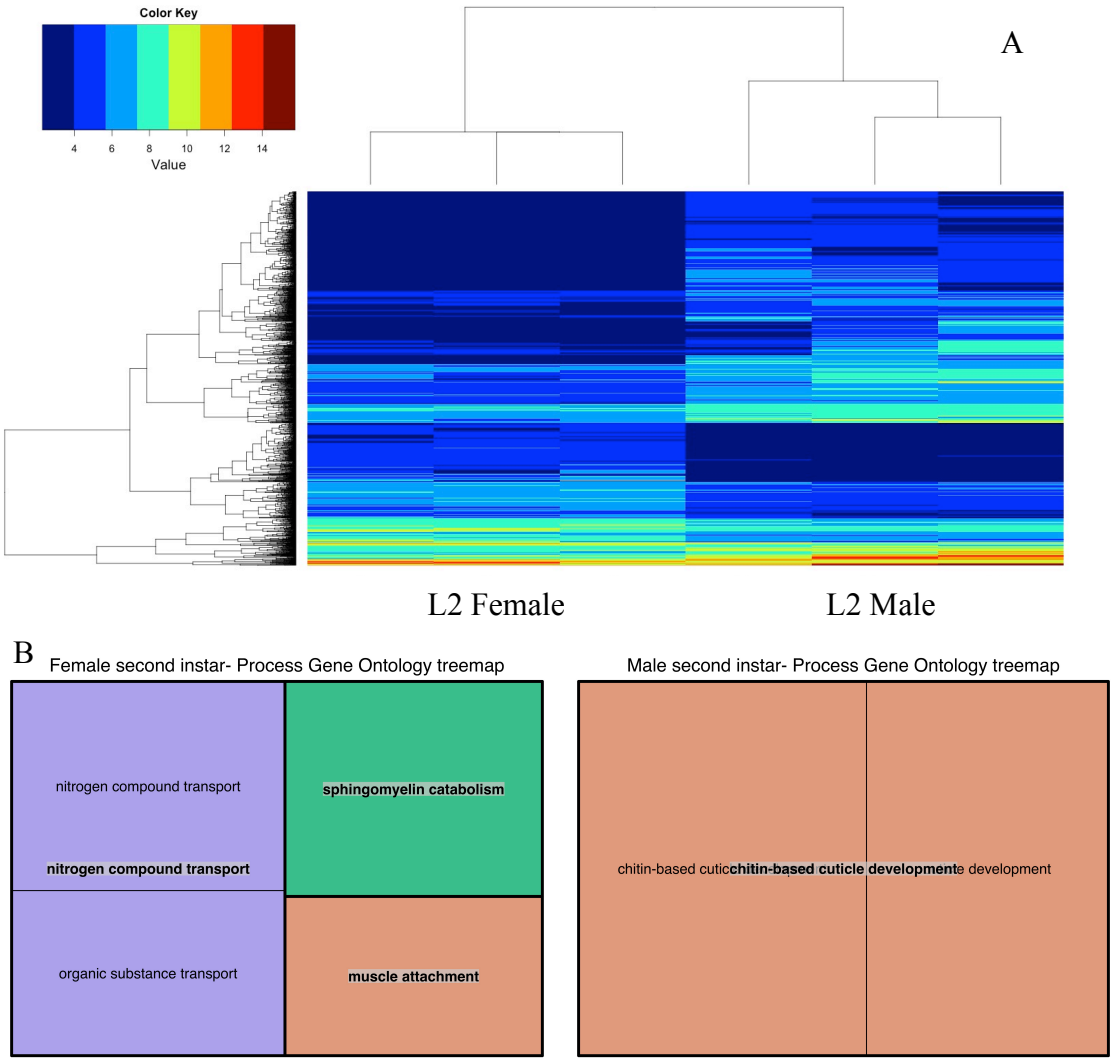


Figure 18. **Sexually dimorphic differential expression in second instar *C. rufifacies*.** These figures summarize the results of differential expression analyses between male and female second instars. A: Heatmap of nodes with significant differential expression between males and females, with nodes on the rows and libraries in the columns. Rows and columns are reciprocally hierarchically clustered based on similarities in expression patterns. B: Treemaps of gene ontology of male up-regulated (left) and female up-regulated (right) homologs to *D. melanogaster*. The size of the box indicates the percentage of all enriched genes with that annotation grouped by general processes (color and grey background text).

Third instars

216 uniquely expressed nodes were found in the third instar libraries (Table 12), although the only gene annotated with *D. melanogaster* was *CrδTry*. Twelve genes were shared with adults, and these are involved in translation and cytoskeletal and cellular component organization primarily as part of ribosomes. The sexually dimorphically expressed nodes were more evenly split between males and females (52:48) (Figure 19), with some highly up-regulated in both sexes (albeit higher in males) and others with a more sex-specific pattern of expression. Male up-regulated genes functioned primarily in membrane transport, especially as components of synaptic membranes. Female up-regulated genes functioned in single-organism metabolism as ion and metal binding compounds for oxidoreductase and catalytic activity functions.

Pupae

Over 1,000 nodes were found to be unique to the pupal stages, however none had homology to *Dr. melanogaster* (Table 12). Nearly 100 genes were shared with adults, and functioned in metabolism and cytoskeleton organization as part of microtubule associated complexes. Many nodes were dimorphically expressed between males and females across all pupal stages (Figure 20A), and 92% of these were up-regulated in males. The male-biased genes were involved in various precursor metabolite and energy production. In comparison, the function and gene ontology of the female up-regulated genes are unknown, with the exception of *doublesex* (*Crdsx*).

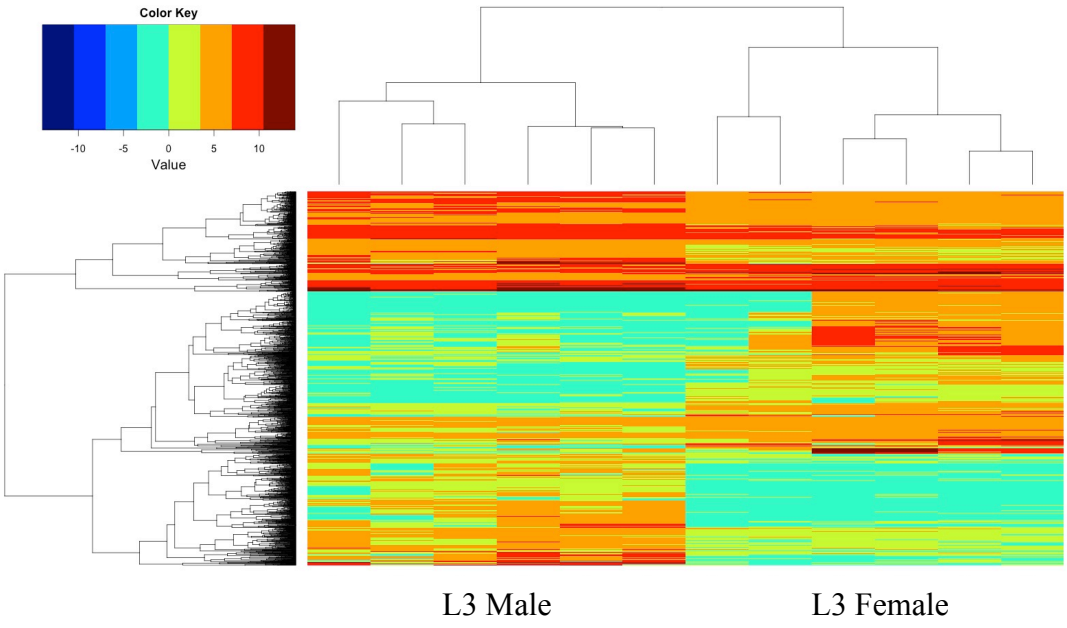


Figure 19. Sexually dimorphic differential expression in third instars *C. rufifacies*. These figures summarize the results of differential expression analyses between male and female third instars. Heatmap of nodes with significant differential expression between males and females, with nodes on the rows and libraries in the columns. Rows and columns are reciprocally hierarchically clustered based on similarities in expression patterns.

Two general patterns were significant with sex and pupal time-point interactions (Figure 20B). The first demonstrated high expression early in the pupal stage, high expression in females and low expression in males mid-pupal development, and low expression late in the pupal stage. The second pattern was that of low expression in females and high expression in males early in pupation, and high expression in the mid and late pupal stages. These all had unknown gene ontology and function with the exception of *female lethal d* (*Crfl(2)d*).

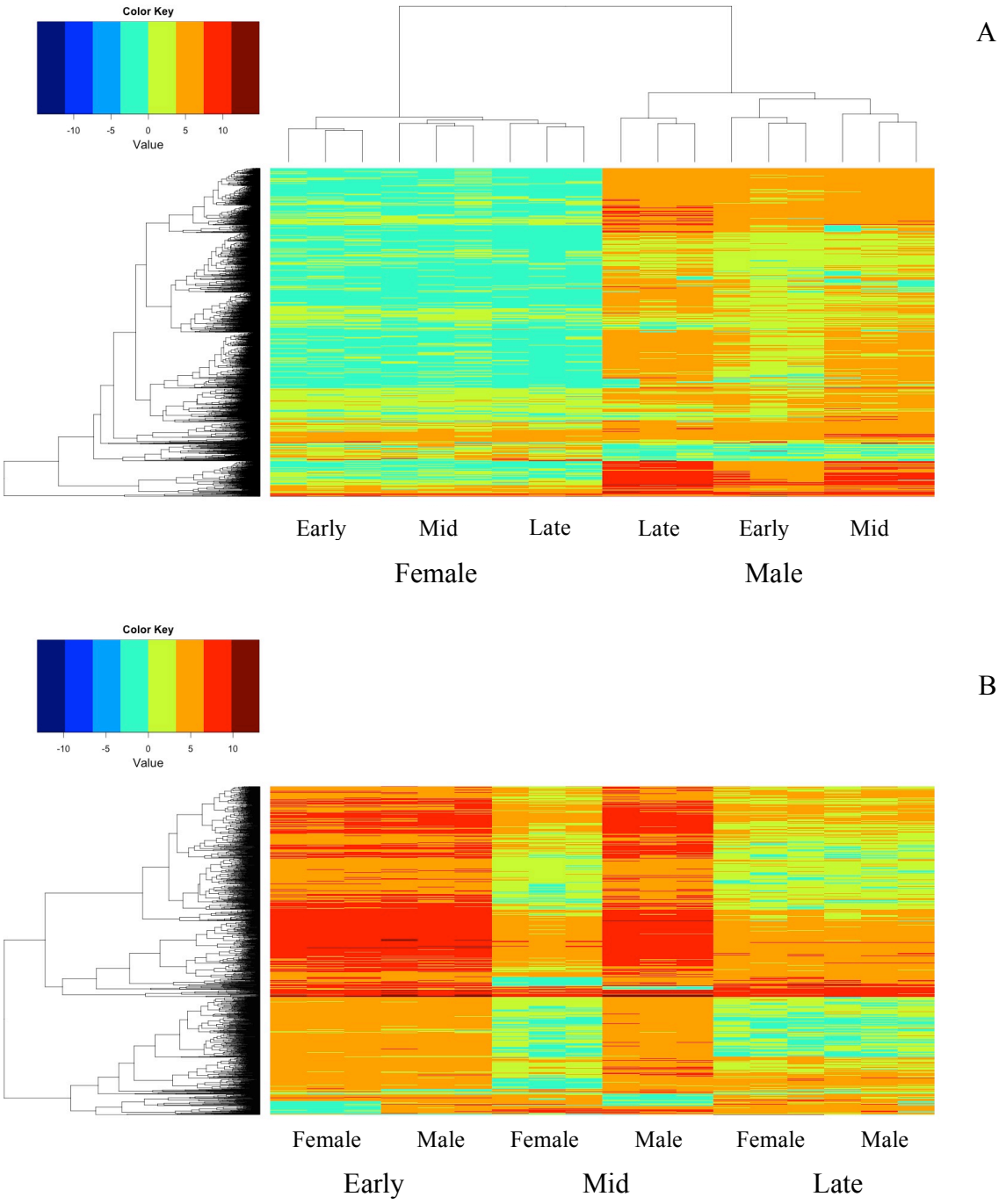


Figure 20. Sexually dimorphic differential expression across all pupal time points in *C. rufifacies*. These figures summarize the results of differential expression analyses between males and females throughout pupal development. A: Heatmap of nodes with significant differential expression between males and females, with nodes on the rows and libraries in the columns. Rows and columns are reciprocally hierarchically clustered based on similarities in expression patterns B: Heatmap of nodes with p-adjusted values of the model with the interaction between sex and pupal time point ≤ 0.00005, with nodes on the rows and libraries in the columns. Rows are hierarchically clustered based on similarities in expression patterns.

Early pupal development

No unique nodes expressed early in the pupal stage had any homology with *D. melanogaster* (Table 12). Of the two genes unique to the early pupal stage and adults, only one, *Juvenile hormone esterase* (*CrJhe*), is well studied while the other (CrCG12539) is predicted to function in ecdysteriod metabolic processes. Of the sexually dimorphically expressed nodes early in the pupal stage, 77% were significantly up-regulated in males. These were involved in metabolite and energy precursor catabolism and pyridine and purine-containing compound metabolism. Only *Dmdsx* has been studied of the genes homologous to those up-regulated in females.

Mid-pupal development

Nodes unique to the mid-pupal development also were unable to annotated with *D. melanogaster*. Nine genes were unique to the mid-pupal development and adults and these were involved in carbohydrate metabolic processes such as alphagucosidase activity and ATP-ase activities related to transmembrane ionic movement. Sexual dimorphism was most pronounced in this part of pupal development (Table 12), with over 90% of the 16,000 differentially expressed nodes being up-regulated in males (Figure 21). The gene ontology of the genes with female-biased expression is largely unknown, though *Dmdsx* is well characterized and another (*Neuroglian*) is known to be important in neural development in *D. melanogaster*. Genes up-regulated in males are involved in many different functions, though mostly these genes were involved in metabolic processes, microtubule movement, or were important for sperm development and proper motility.

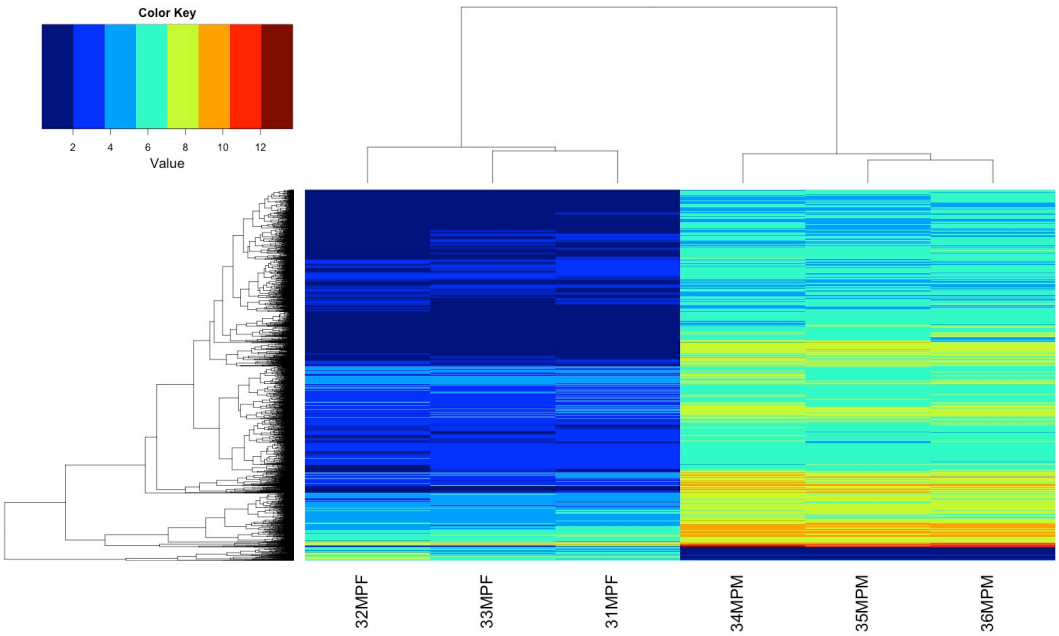


Figure 21. Sexually dimorphic differential expression mid-pupal development in *C. rufifacies*. This figure summarizes the results of differential expression analyses between males and females mid-pupal development and are limited to nodes are with a p-adjusted < 0.00005. Heatmap of nodes with significant differential expression between males and females, with nodes on the rows and libraries in the columns. Rows and columns are reciprocally hierarchically clustered by similarity in expression patterns.

Late pupal development

As with the other pupal time-points, nodes uniquely expressed late in pupal development did not demonstrate homology with *D. melanogaster* genes. Late pupal development shared the most unique expression with adults, and these genes were involved in many different processes (Figure 22), although in *D. melanogaster* microtubule based processes are significantly related to spermatogenesis. Nearly 13,000 fewer nodes were found to be differentially expressed between males and females late in pupal development compared to mid-pupal development, and only 74% of these were

male-biased in expression (Figure 23A). However, 95% of annotated dimorphically expressed genes were up-regulated in males. Furthermore, many of these genes demonstrated a pattern high expression in one sex and little to no expression in the other sex (Figure 23B). Overall, as with results in other stages, gene ontology for female-biased genes was limited, demonstrating mainly extracellular metallocarboxypeptidase activity. In comparison, male-up-regulated genes were involved in diverse precursor metabolite biosynthesis, hydrogen-ion gated transmembrane transport, and spermatogenesis.

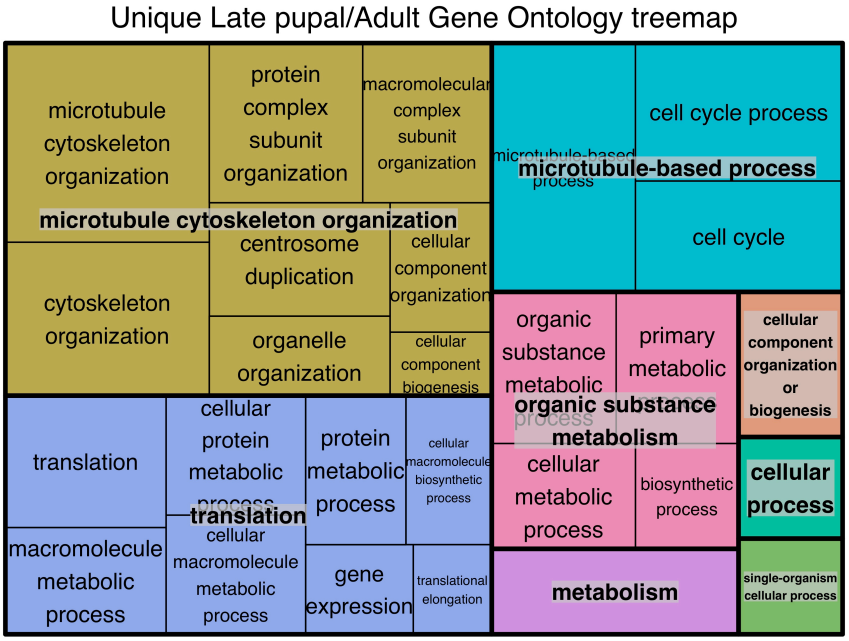


Figure 22. Treemap of gene ontology of uniquely expressed late in the pupal stage and in adults in *C. rufifacies*. The size of the box indicates the percentage of all enriched genes with that annotation grouped by general processes (color and grey background text).

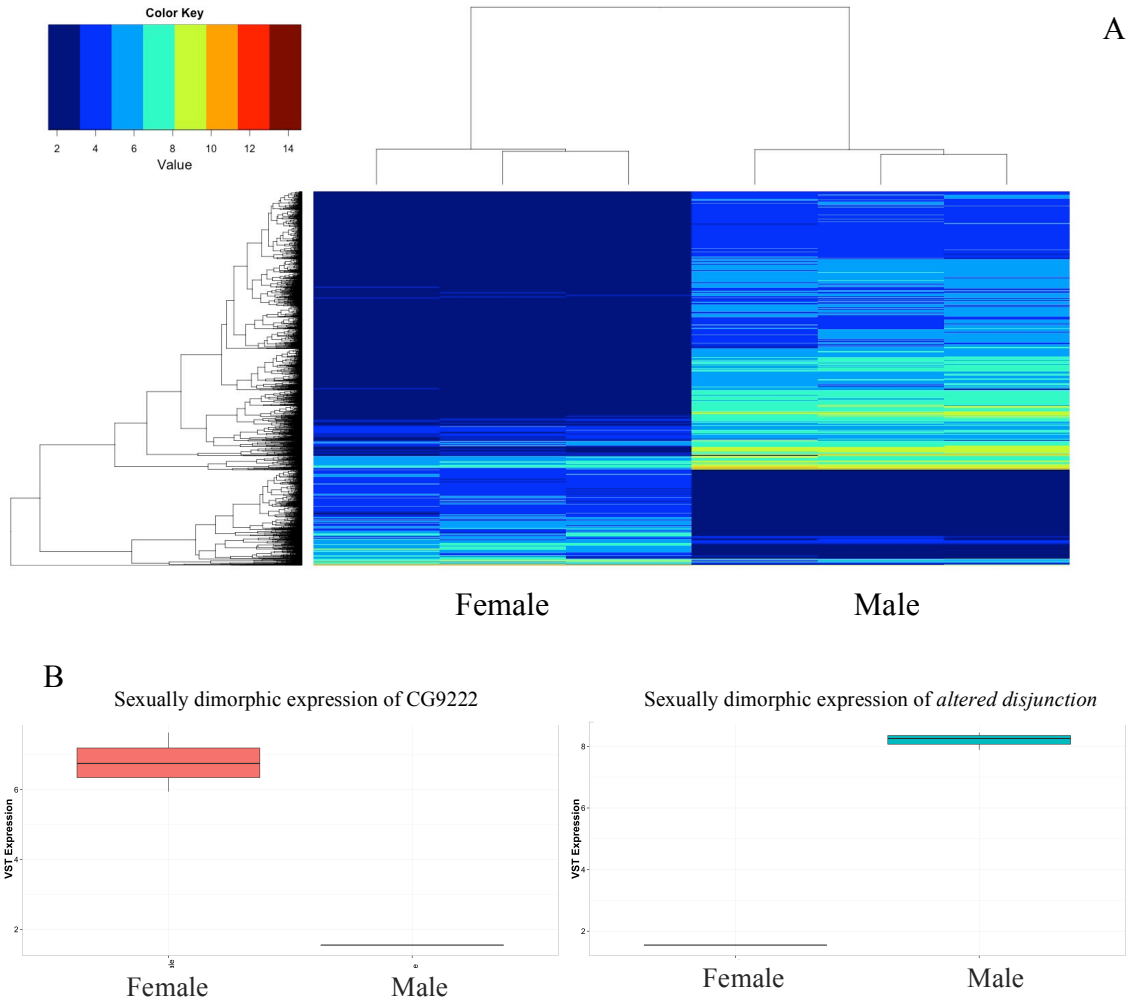


Figure 23. Sexually dimorphic differential expression in late pupal development in *C. rufifacies*. This figure summarizes the results of differential expression analyses between males and females mid-pupal development.A: Heatmap of nodes with significant differential expression between males and females, with nodes on the rows and libraries in the columns. Rows and columns are reciprocally hierarchically clustered based on similarity in expression patterns. B: Representative boxplots of expression of female up-regulated (left) and male up-regulated (right) genes, color-coded by sex (female = red, male = blue).

Sex specific developmental gene expression

Female libraries only

There were many nodes unique to female libraries, however, only a single gene *CrδTry*, could be annotated with *D. melanogaster* (Table 13). These unique nodes demonstrated three major patterns of expression. The first pattern was of increased expression in all stages except in adults. The second pattern was of increased expression in all stages except the mid-pupal time point. The third pattern was that of increased expression in all stages except third instars.

A total of 6,862 genes with *D. melanogaster* homology were differentially expressed across life stages in females, including at least one transcript of 22 different sex-determination and sexual-dimorphism related genes (Table 13). Approximately 1,470 genes were differentially expressed between life stages in females at the more stringent cutoff, and these fell into 16 clusters (Table 13). Four sex-related gene homologs exhibited differential expression throughout development in females at this cutoff: *C. rufifacies runt* (*Crrun*), *virilizer* (*Crvir*), *fruitless* (*Crfru*), *deadpan* (*Crdpn*), and *Trithorax-like* (*CrTrl*). All of these genes were significantly up-regulated in eggs and more or less down-regulated through the rest of development (with the exception of *Crdpn* which was up-regulated throughout pupal development).

The largest cluster (Cluster F6, 329 genes) demonstrated significant down-regulation in eggs, and to a lesser extent early in pupation and in adults, with up-regulation in other stages and time points. This cluster contained *Crfru*, and was comprised of genes involved in sulfur compound metabolism, muscle cell development and organization,

organonitrogen compound catabolism, and chitinase activity (Figure 24). Two clusters (Clusters F8 and F9) exhibited very low expression in eggs and adults, and moderate expression in all other libraries. Genes in Cluster F8 were especially up-regulated in all larval and pupal stages, and were primarily involved in chitin metabolism and development, in part as constituents of the peritrophic membrane. On the other hand, expression was lower early and in the middle of pupal development in Cluster F9, and expression was increased in the second and third larval instars. These genes were involved in cellular lipid and juvenile hormone hydrolase activity. In *D. melanogaster*, one of the component genes (*Juvenile hormone epoxide hydrolase 3*) is highly expressed early in embryonic development and in both adult and larval midguts [267].

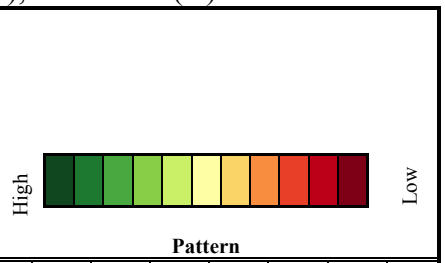
Gene expression was most distinctly divergent between the egg stage and the third instar samples, as there were no clusters in which these samples had similar expression. The first and second larval instars had similar patterns of expression across clusters, though the degree of up- or down-regulation was not always the same. Expression across pupal development was similar across most of the clusters, with early pupal development expression differing in degree from the later stages of development in 8 clusters.

Table 13. Female specific developmental patterns in gene expression in *C. rufifacies*. Columns from left to right: Type (Unique to females, or differentially expressed), level of significance (p-adjusted cut-off or cluster), number of nodes, number of *D. melanogaster* gene hits, number of *D. melanogaster* transcript hits, *D. melanogaster* sex-related genes hits (count, shared with males, unique to females), and pattern of expression in eggs (E), first instar (L1), second instar (L2), third instar (L3), early in pupation (EP), mid pupation (MP), late in pupation (LP), and adults (A).

Type	Significance	Nodes	Genes	Transcripts	Sex Determination: Count	Sex Determination: Shared	Sex Determination: Unique	Pattern: E	L1	L2	L3	EP	M	LP	A
	Unique	340	1	1	0										
Stage	**p < 0.05**	204430	6862	7821	22	*gro*, *emc*, *Trl*, *Unr*, *run*, *fl(2)d*, *tra2*, *fru*, *mle*, *da*,*snf*, *vir, mof*, *Su(var)3-7*, *dsx*, *sc*, *msl-3*, *os*, *msl-1*, *sis*, *ix*, *dpn*									
	p < 5e-15	9789	1469	1609	5	*run*, *fru*, *dpn*, and *vir*	*Trl*								
	F1	155	42	46	0										
	F2	156	42	43	1	*dpn*									
	F3	530	114	115	3	*run*, *Trl*, *vir*									
	F4	576	126	127	0										
	F5	661	136	146	0										
	F6	2057	329	388	1	*fru*									
	F7	763	130	140	0										
	F8	253	31	34	0										
	F9	270	38	40	0										
	F10	1156	220	244	0										
	F11	159	26	26	0										
	F12	764	86	88	0										
	F13	766	141	144	0										
	F14	123	12	12	0										
	F15	667	78	80	0										
	F16	403	32	33	0										

Table 14. Male specific developmental patterns in gene expression in *C. rufifacies*. Columns from left to right: Type (Unique to males or differentially expressed by developmental stage), level of significance (p-adjusted cut-off or cluster), number of nodes, number of *D. melanogaster* gene hits, number of *D. melanogaster* transcript hits, *D. melanogaster* sex-related genes hits (count, shared with females, unique to males), and pattern of expression in eggs (E), first instar (L1), second instar (L2), third instar (L3), early in pupation (EP), mid pupation (MP), late in pupation (LP), and adults (A).

Type	Sig	Nodes	Genes	Transcripts	Sex Determination Count	Shared	Unique	Pattern
Unique		756	15	17	0			
Stage	**< 0.05**	217556	7387	8404	24	*gro*, *emc*, *Trl*, *Unr*, *run*, *fl(2)d*, *tra2*, *fru*, *mle*, *da*, *snf*, *vir*, *mof*, *Su(var)3-7*, *dsx*, *sc*, *msl-3*, *os*, *msl-1*, *sis*, *ix*, *dpn*	*scf*, *msl2*	
	< 5e-21	9547	1979	2128	9	*run*, *fru*, *dpn*, and *vir*	*gro*, *fl(2)d*, *da*, *snf*, *Su(var)3-7*	
	M1	2043	382	444	1	*fru*		
	M2	405	51	55	0			
	M3	481	78	84	0			
	M4	373	69	74	0			
	M5	283	67	73	0			
	M6	312	30	31	0			
	M7	1324	268	278	0			
	M8	521	76	78	0			
	M9	209	16	16	0			
	M10	493	177	189	3	*gro*, *run*, *fl(2)d*		
	M11	774	230	241	3	*gro*, *da*, *dpn*		
	M12	1564	417	431	3	*snf*, *vir*, *Su(var)3-7*		
	M13	495	194	195	0			



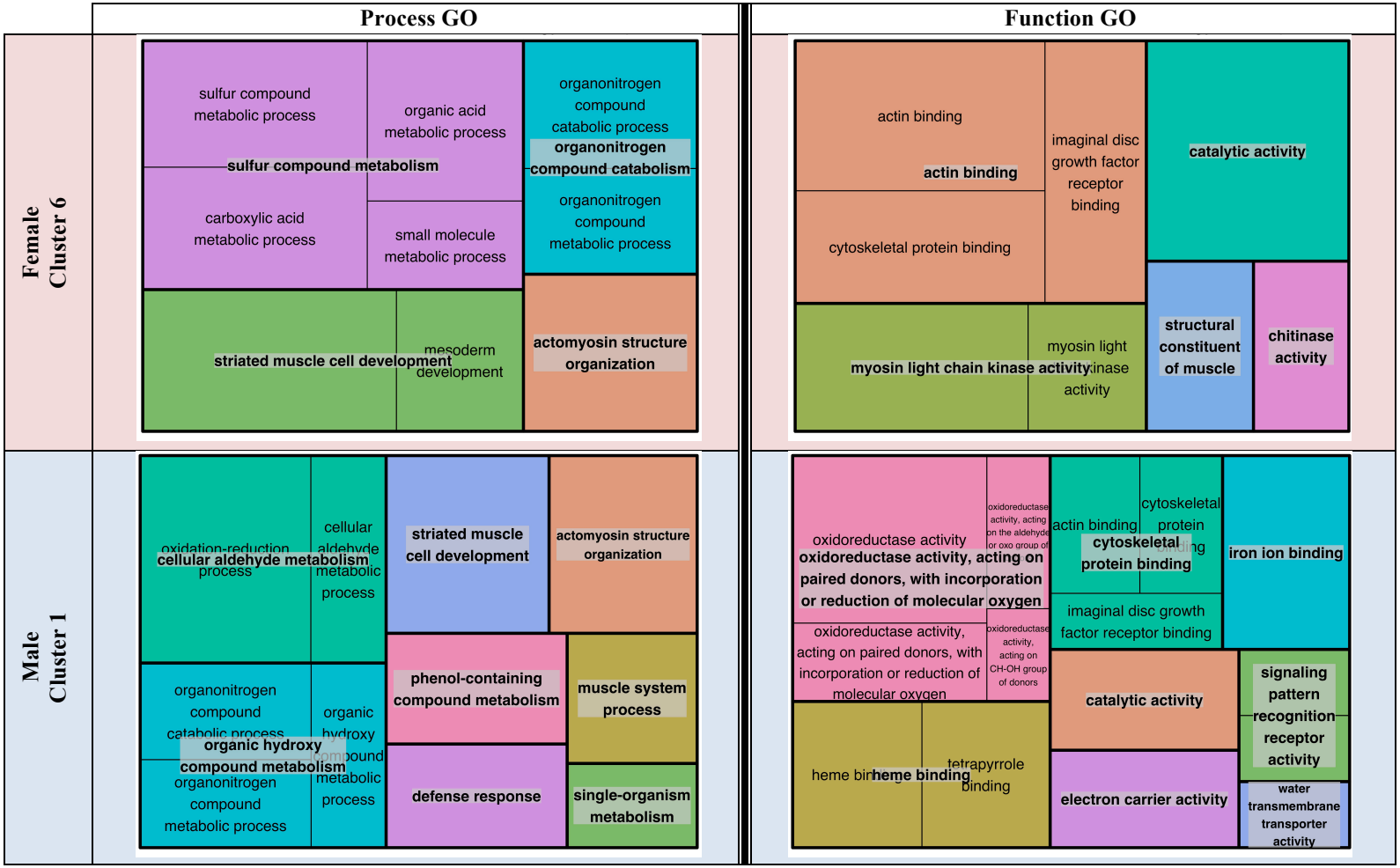


Figure 24. Treemaps of gene ontology of *Crfru* containing clusters demonstrating differentially expressed throughout development in both sexes in *C. rufifacies*. The size of the box indicates the percentage of all enriched genes with that annotation grouped by general processes (color and grey background text). Top: Female Cluster 6 process (left) and function (right) GO treemaps (red shaded cells). Bottom: Male Cluster 1 process (left) and function (right) GO treemap (blue shaded cells).

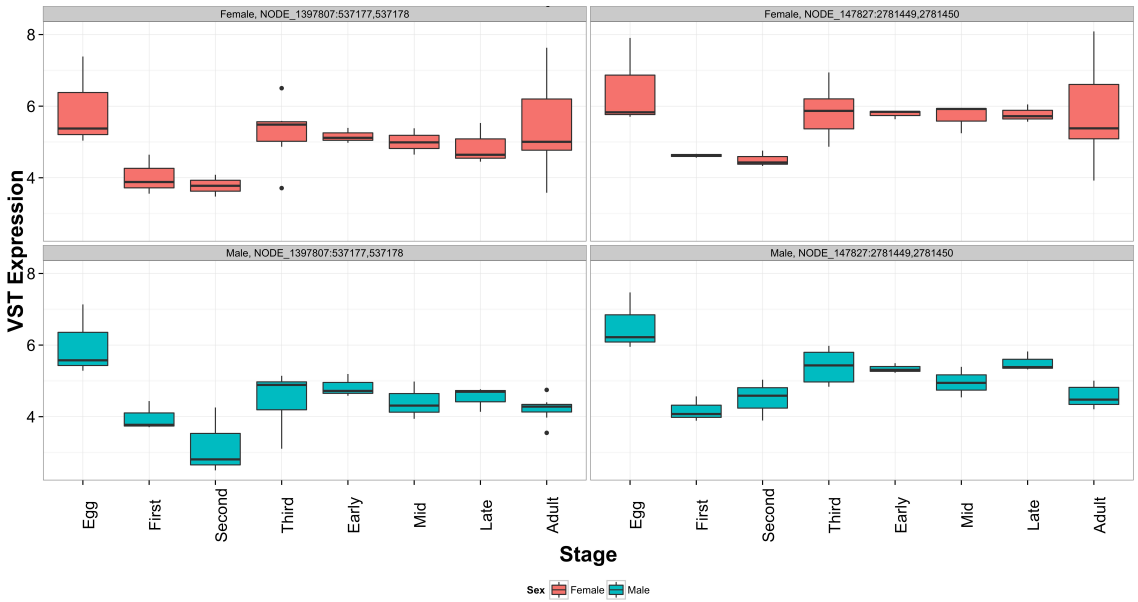


Figure 25 Ontological expression of two nodes of *Crmsl-2* in male and female *C. rufifacies* throughout development. Representative boxplots of expression of two nodes (columns) of *Crmsl-2*, color-coded by sex (female=red, male=blue).

Male libraries only

Nearly twice as many elements were uniquely expressed in male samples than female samples, with homology to 15 different *D. melanogaster* genes. All of the unnamed genes demonstrate very high expression in males, in testis in particular. These unique nodes fell into three clusters based on expression pattern: (i) high expression in all stages except adults, (ii) mid-pupal stage, or (iii) high expression in eggs, first and second instars and low expression beginning in the third instar.

There were approximately 7,400 genes differentially expressed between stages in males (Table 14). In addition to the 22 sex-determination and sexual-dimorphism related *D. melanogaster* homologs found to be differentially expressed throughout development

in females, males also exhibited differential expression in *C. rufifacies male sex lethal 2* (*Crmsl2*) and *supercoiling factor* (*Crscf*). This difference in significance appears to be due to decreased variation in expression in males in many life stages (Figure 25). The 1,979 differentially expressed nodes at the more stringent cutoff fell into 13 clusters. The largest cluster in males (Cluster M1, 382 genes) also included *Crfru* (Table 14). This cluster demonstrated highest expression in the third instar, late in pupal development, and in adults. However, compared to the largest cluster in females, in males this group of genes demonstrated low expression in the middle of pupal development and high expression in adults. *Chfru* was expressed in a similar manner in both sexes (Figure 26), however again females demonstrated high variation in expression than males. This largest male cluster included all of the processes in the *Chfru* cluster of females except sulfur-containing compound metabolism (Figure 24). Furthermore, this cluster was not involved in chitinase activity. Instead, cellular aldehyde and phenol-containing compound metabolism, as well as various ion binding and trans-membrane transporter functions were included in this cluster in males, likely due to neurological patterning development in males.

The next two largest clusters in males (Clusters M7 and M11, 268 and 230 genes respectively) had divergent patterns of expression (Table 14). Cluster M7 demonstrated high expression in all three larval instars and to a lesser extent adult, and low expression in eggs and throughout pupal development. These genes were predominantly involved in active transmembrane transport, proteolysis, and metabolism as part of vacuoles and the peritrophic membrane. These genes are therefore likely to be important in digestion and

movement. In contrast, Cluster M11 demonstrated high expression in eggs and to a lesser extent early in pupal development, while expression was low in adults and larvae, especially in the second and third instars. Furthermore, Cluster M11 contained three sex-determination and dosage-compensation related genes: *C. rufifacies groucho* (*Crgro*), *daughterless* (*Crda*), and *deadpan* (*Crdpn*). The genes in Cluster M11 were predominantly related to cell fate commitment, mitotic cell cycle processes, and nuclear division, with protein and transcription factor binding activity predominantly localized in the nucleus.

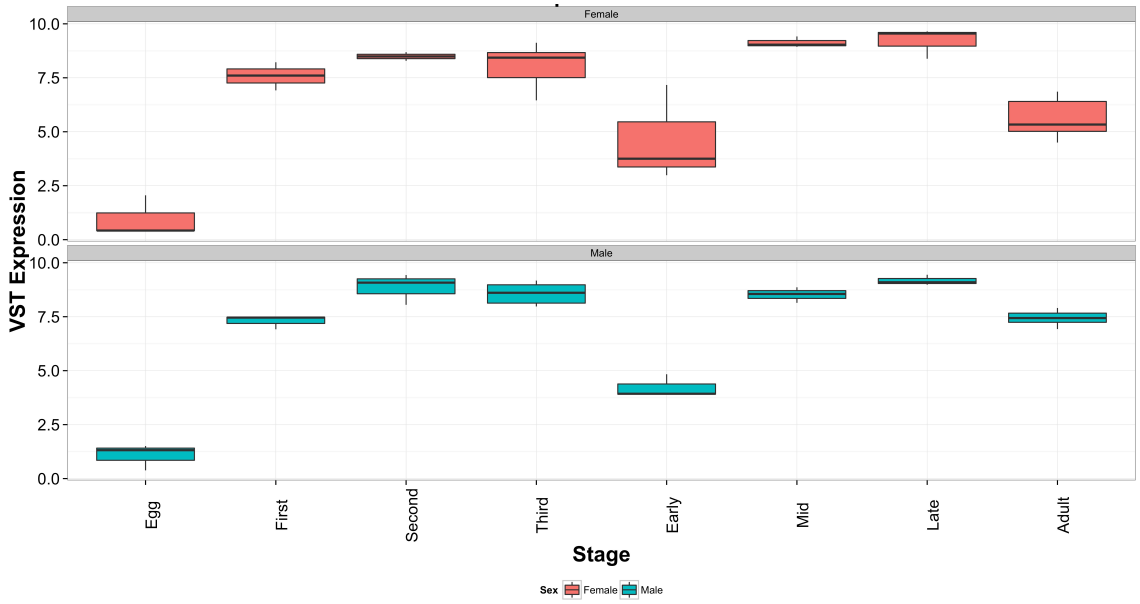


Figure 26. Ontological expression of *Crfru* in male and female *C. rufifacies* throughout development. Representative boxplots of expression of *Crfru*, color-coded by sex (female=red, male=blue).

As with females, there were no clusters in which eggs and third instars had similar expression. The first and second larval instars had similar patterns of expression across clusters, except for Cluster M9. Expression across pupal development was divergent in direction in two clusters (Clusters M3 and M6), but generally there was more variation in expression during pupal development in males than females.

Sexual dimorphism in gene expression

Significant sex-by-stage interaction models were found for 3812 genes, including eight sex determination and dosage compensation genes (Table 15). Of these, nearly 1,400 were significant at a p-adjusted cut-off of 0.00005 or less and fell into 13 clusters. On On average, female variance over significantly differentially expressed nodes was higher than male variance ($T=42.8352$, $p < 10^{-16}$) (Figure 25, Figure 26, Figure 27). Three sex determination genes were significantly dimorphically expressed at this threshold: *C. rufifacies transformer2* (*Crtra2*), *sans fille* (*Crsnf*), and *males absent on the first* (*Crmof*) (Table 15, Figure 27). These genes were in clusters (Clusters 1 and 5) with high expression in eggs (male and females) and adult females (Figure 27), though *Crmof* exhibited a high expression in early pupae relative to *Crsnf*.

The largest cluster (Cluster 6), demonstrated the same level of expression in eggs, first and second instars, and throughout pupal development (Table 15). These genes were more down-regulated in males than females in the third larval instar, and exhibited up-regulation in females and down-regulation in males in adults. These genes were predominantly involved in RNA transcription and binding, translation, and neurogenesis.

Table 15. Sex by stage interaction patterns in developmental gene expression in *C. rufifacies*. Columns from left to right: Level of significance (p-adjusted cut-off) (Sig), cluster, number of nodes, number of *D. melanogaster* gene hits, number of *D. melanogaster* transcript hits, *D. melanogaster* sex-related genes hits, and pattern of expression in females (F) and males (M) in eggs (E), first instar (L1), second instar (L2), third instar (L3), early in pupation (EP), mid pupation (MP), late in pupation (LP), and adults (A).

Sig	Cluster	Nodes	Genes	Tx	SD genes	Pattern: High ... Low															
< 0.05	All	55375	3812	4273	*run, tra2, fru, dpn, snf, mof, Su(var)3-7, msl-3, Sxl*	E		L1		L2		L3		EP		MP		LP		A	
						F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M
< 0.00005	All	9311	1397	1501	*tra2, snf, mof*																
	1	282	41	41	*tra2*																
	2	1102	103	109																	
	3	246	32	32																	
	4	269	4	5																	
	5	833	114	115	*snf, mof*																
	6	1452	223	227																	
	7	222	23	24																	
	8	106	13	13																	
	9	102	19	20																	
	10	224	32	41																	
	11	324	53	63																	
	12	380	75	89																	
	13	786	207	225																	
	14	331	131	134																	

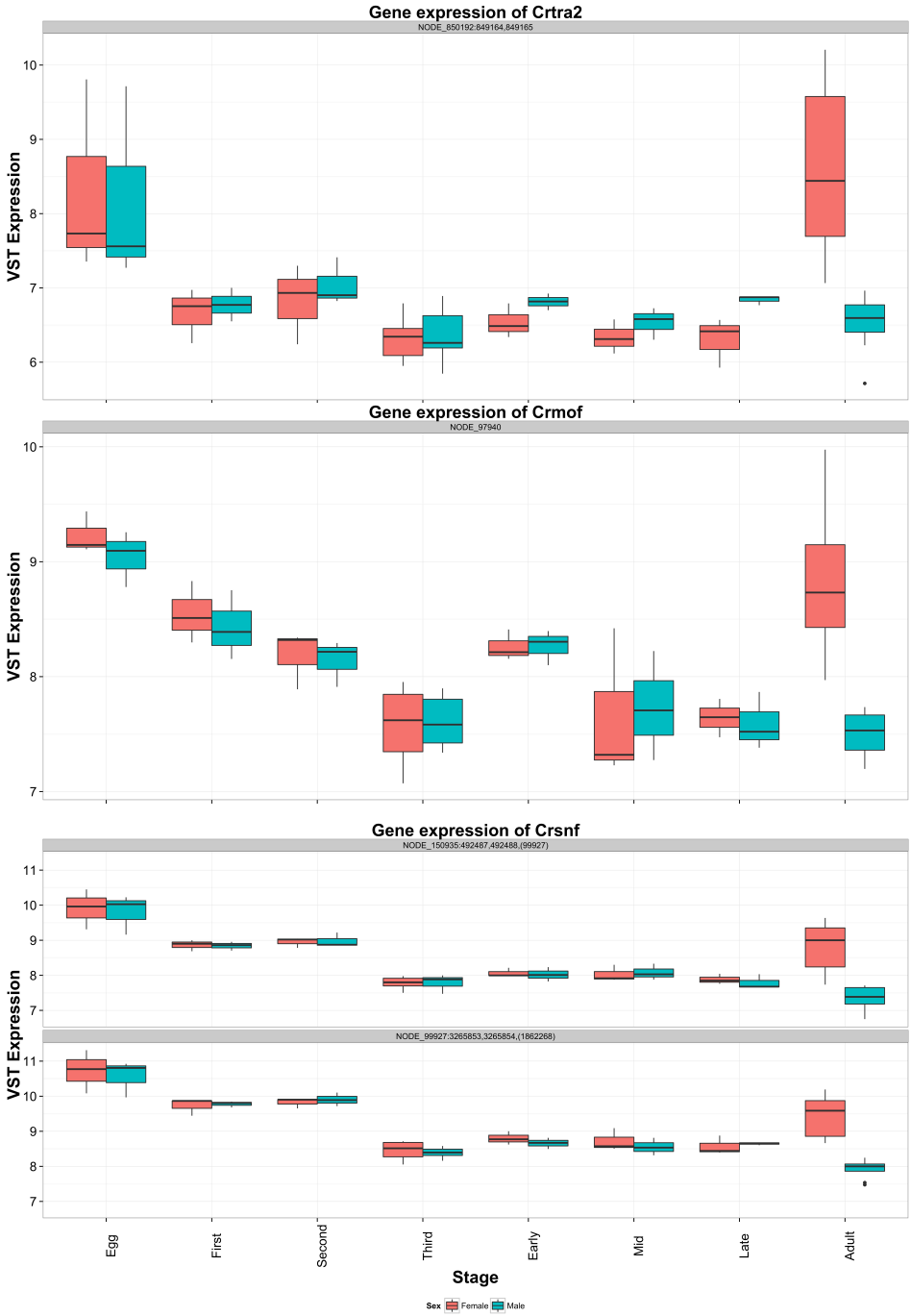


Figure 27. Boxplots of expression of three sex-determination homologs in *C. rufifacies* throughout development. Expression throughout development (left to right: egg to adult) split by sex (female=red, male=blue) for *Crtra2* (top), *Crmof* (middle), and two nodes of *Crsnf* (bottom).

Two clusters demonstrated significant differential expression was in the pupal stages (Clusters 4 and 14) (Table 15). Males and females expressed the genes in Cluster 4 in opposite directions in the middle of pupal development, and this was where dimorphism in expression was most extreme. There were only 4 genes in Cluster 4: *C. rufifacies CG10417* (*CrCG10147*), *Chromatin assembly factor 1 subunit* (*CrCaf1*), *Deoxyuridine triphosphatase* (*CrdUTPase*), and *Receptor component protein* (*CrRcp*). These four genes are all known to be highly up-regulated in larval CNS and ovaries in *D. melanogaster*. Cluster 14 exhibited down regulation in all samples except males throughout pupal development and in the adult stage. The gene ontology of the 131 genes in the cluster is not well understood, though they are known or predicted to function in various glycolytic, nucleotide metabolism, and ATP-mediated transport capacities.

Germ-line and somatic-tissue associated gene expression

A total of 1,876 genes were related to *D. melanogaster* male and female germ-line gene expression with 119 shared genes between male and female germ tissue gene lists. All of the terms associated with "ovariole" were also associated with "oogenesis", "ovary", or "oocyte". Gene ontological analysis of the male germ-line genes (821 total) indicated that these were involved in spermatogenesis, purine ribonucleoside metabolism, chromosome organization, regulation of cell differentiation, and were mostly in microtubule associated complexes. A similar analysis of the female germ-line related genes (1,174 total) were involved in reproduction and oogenesis processes, through protein and mRNA binding associated with chromosomes.

When these lists of germ-line related genes were compared to the 14 clusters identified to have significant ontological sexual dimorphism in expression, five clusters were identified (Table 16). Female germ-line related genes, especially those involved in oogenesis, were found primarily in Clusters 1, 2, 5, and 6 and were similar in value across these clusters. The genes in Cluster 1 demonstrated a pattern of high expression in eggs (regardless of sex) and adult females, and low expression elsewhere. None of the genes in Cluster 1 were differentially expressed between male and female fated eggs. The three other clusters demonstrated high expression in other stages as well. In comparison, 50 of the genes in Cluster 14 are known to be expressed in spermatozoa. No other cluster exhibited such high expression of male-germ line-related genes.

Of the 258 somatic sex-specifically expressed genes identified in *D. melanogaster*, 162 were differentially expressed throughout development in the male-specific and female-specific analysis. However, all but six of these were differentially expressed in both males and females throughout development. The following exhibited differential expression in females but not in males: *yellow-emperor* (*Crymp*), *HEM-protein* (*CrHem*), CrCG12290, CrCG8549, CrCG8813, and CG9330. Only 17 genes previously identified as demonstrating somatic, sex based expression in *D. melanogaster* were detected as having significant developmental sexual dimorphism in expression in this work (Table 16), including *CrSxl*.

Table 16. Significant sexual dimorphically pressed germ-line related genes in *C. rufifacies*. Number of homologs to *D. melanogaster* genes known to function or be expressed in (columns from left to right): germ-line tissues (oogenesis, ovaries, oocytes, spermatogenesis, testis, spermatocytes, and spermatozoa) or somatic tissues in females and males found to be differentially expressed in *C. rufifacies* in each cluster. An empty cell indicates a value of 0.

	Female				Male				
	Germ				Germ				
	Oogenesis	Ovary	Oocyte	Soma	Spermatogenesis	Testis	Spermatocyte	Spermatozoan	Soma
Cluster1	2	2	1	*smg*	3		1	2	
Cluster2	22	7	7		6		2	5	*SKIP*, *MBD-like*
Cluster3	2	3					1	1	
Cluster4									
Cluster5	23	2	1	*Sxl*, CG6961	1	1	1	3	*Tango4*
Cluster6	22		1				1	7	*wds*, *RAD23*
Cluster7	6	2	1		2			1	
Cluster8	1								
Cluster9		1						1	
Cluster10		1	1					2	
Cluster11	2			*Zasp66*				2	
Cluster12	8		4		2		1	3	
Cluster13	6	1	4		1	1		6	CG4238
Cluster14		2	1	*ymp,* CG8813, CG14540	1			5	*S-Lap1*, *mtsh,* CG9975*,* CG16935

Analysis of Crdsx, Crtra, Crtra2 and CrSxl

The *Crdsx* was annotated with *D. melanogaster* sequences and one splice variant demonstrated a pattern of significant up-regulation in females relative to males beginning in the third instars and continuing though the pupal stage and into adults. These nodes with *Dmdsx* homology were in a single splicing graph with significant (E value < 10^{-7}) homology to *Lucilia cuprina* Weidemann (Diptera: Calliphoridae) (GU784832, GU784833, and GU784834) and *M. domestica* (AY41853 and AY41854) *dsx* sequences in the NCBI databases. Three of these nodes demonstrated sexually dimorphic expression throughout development, two with up-regulation in females and one with up-regulation in males (Figure 28).

Chtra could not be annotated with *D. melanogaster.* Two nodes were found to be significantly up-regulated in males in each larval instar individually, both with high expression in males which dropped slightly with increasing age and low and more variable expression in females. These nodes were in a splicing graph that had significant (E value < 10^{-7}) homology to blow fly *tra* sequences in the NCBI databases, specifically the male splice variants of *L. cuprina.* Further analysis of this splicing graph with all life stages identified seven nodes that were up-regulated in males and nine which were up-regulated in females (Figure 29).

A single node was annotated with *D. melanogaster tra2*, and this node demonstrated a significant sex by stage interaction in expression (Table 15). Highest expression was observed in the egg stage in both sexes and in adult females (Figure 27). Sexual dimorphism in expression began in the pupal stage, with higher expression in male

pupae than female pupae. In the adult stage, however, female expression was significantly higher than male expression, and demonstrated a higher variance in expression.

CrSxl assembled in this work was identified based on homology with *D. melanogaster.* Several nodes were observed to sexually dimorphically differentially expressed throughout immature development (Figure 30). The *CrSxl* sequence assembled here, specifically including differentially expressed nodes, had 99% identity across 1120 bases that aligned with the 1,355 bp *CrSxl* sequence previously published, accession number: S79722.1 [206].

Discussion and conclusions

The *de novo* transcriptome analysis presented in this paper has generated a suite of genetic tools for a relatively unstudied non-model fly and insights into sexual dimorphism in gene expression throughout development. Prior to this work, six sequences were known for genes outside of the mitochondrial genome and various genes frequently used in genetic barcoding and phylogenetics (e.g. cytochrome oxidase I and II, the rDNA gene, and intergenic spacer regions) [353-355]. In this work I have putatively identified more than 9,400 homologs to *D. melanogaster* genes based on sequence identity, and further characterized expression of these genes throughout development in individuals.

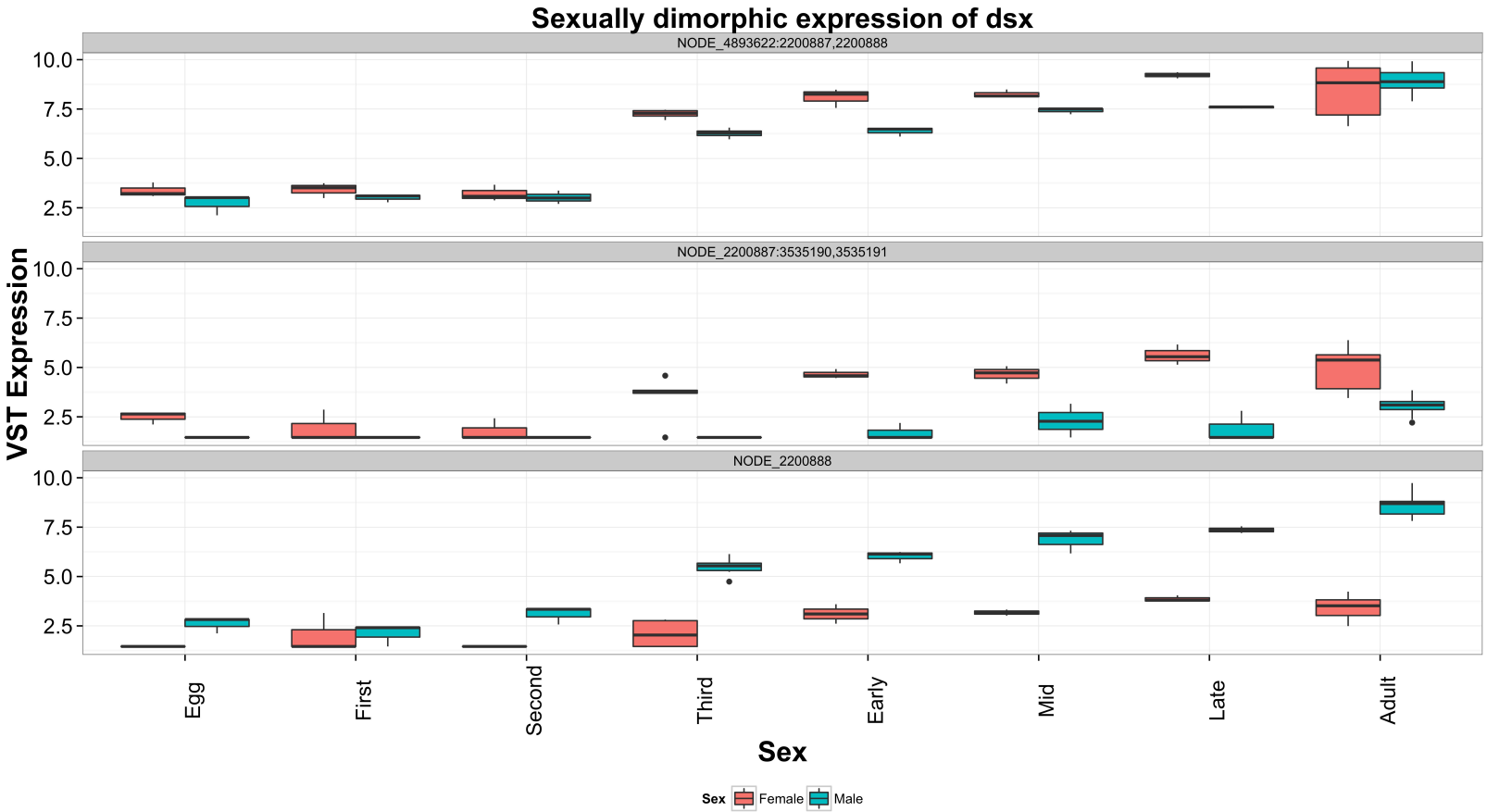


Figure 28. Sexually dimorphic differential expression across all life stages of putative *C. rufifacies doublesex*. Boxplots of expression across all life stages of two female nodes (op and middle) and one male node (bottom), separated by sex (female=red, male= blue).

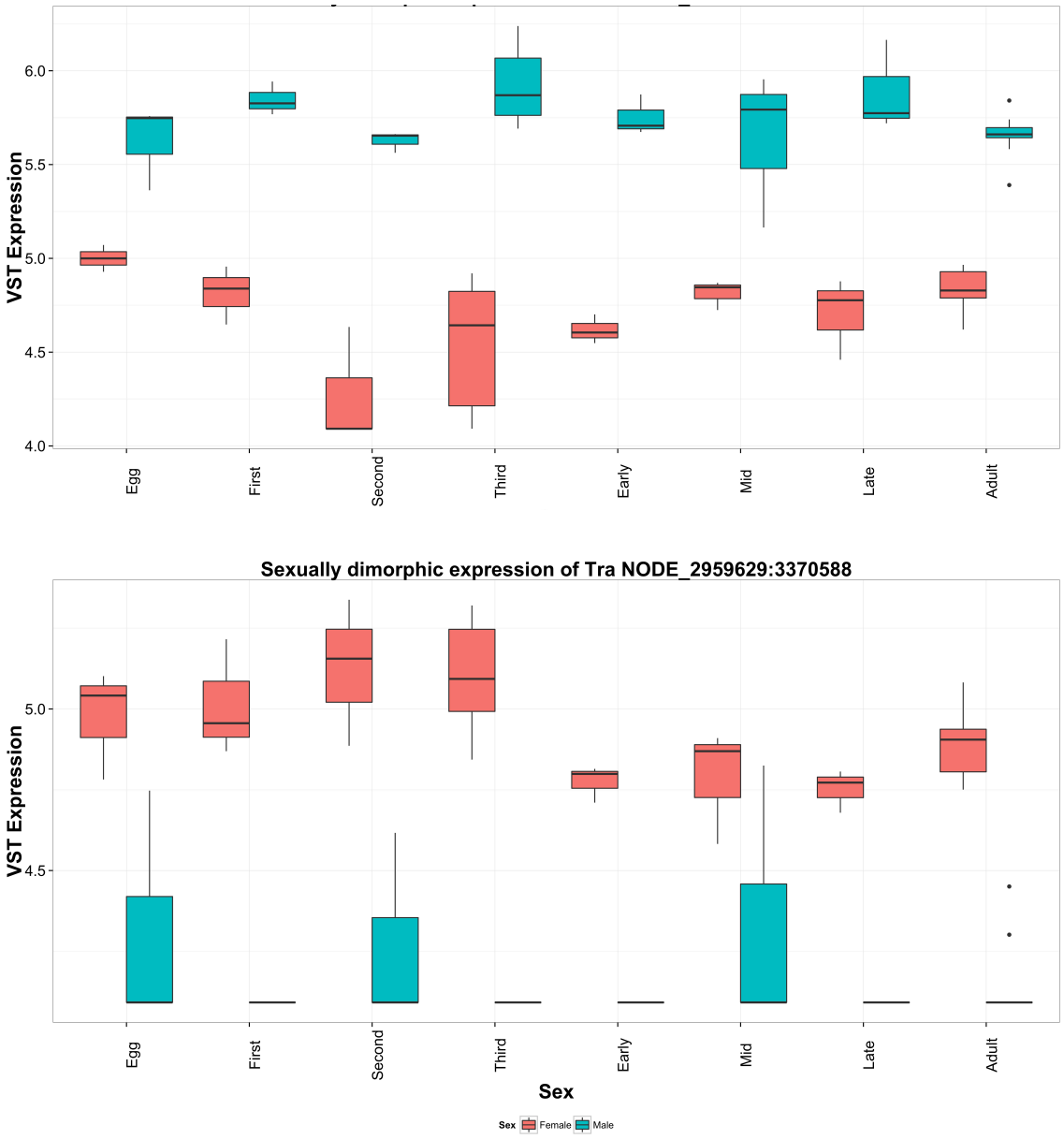


Figure 29. Sexually dimorphic differential expression across all life stages of putative *C. rufifacies transformer*. A) Boxplots of expression across all life stages of a male node (NODE_4655090:3521834), separated by sex (female=red, male= blue). B) Boxplots of expression across all life stages of a female node (NODE__2959629:3370588**)**, separated by sex (female=red, male= blue).

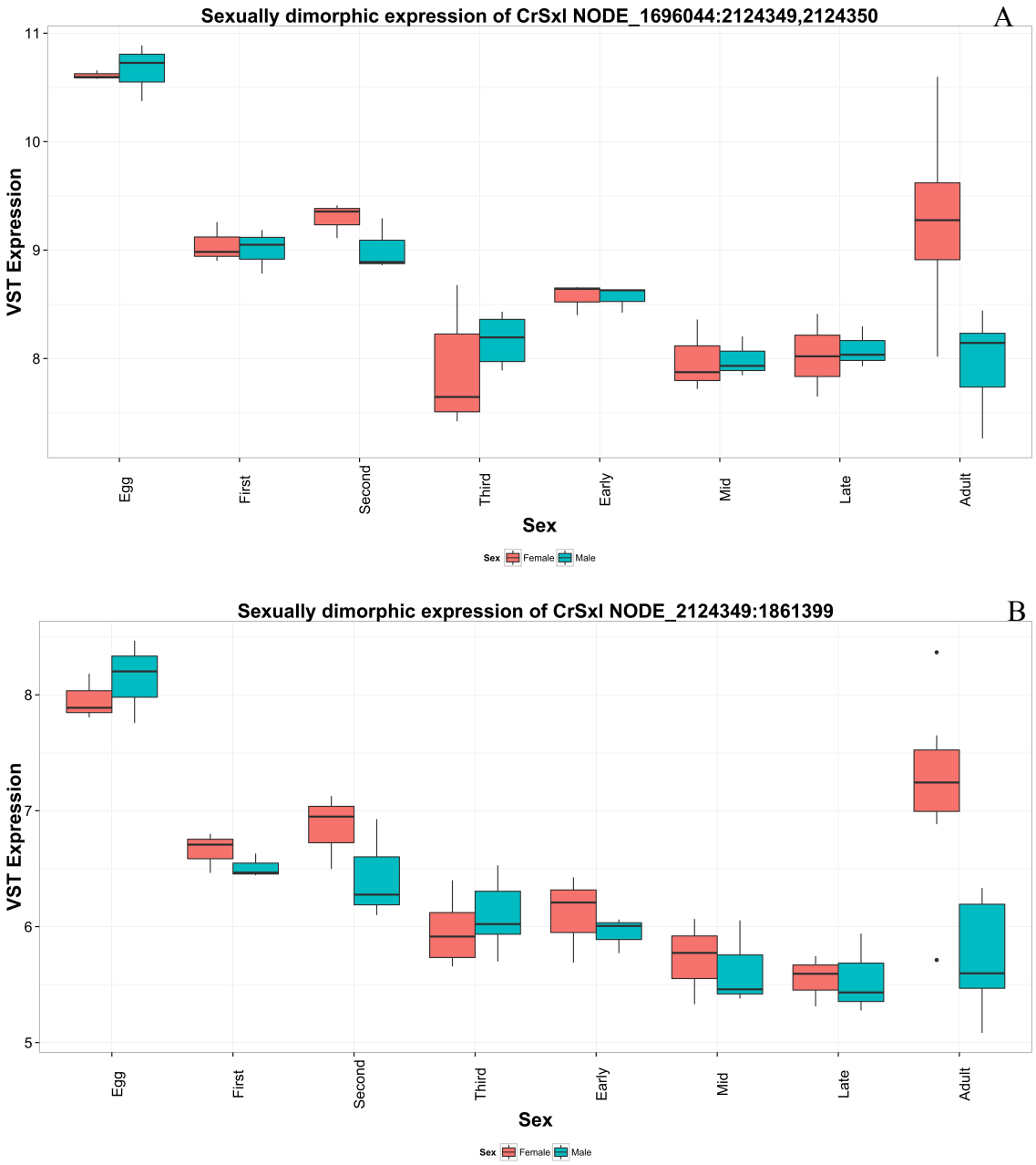


Figure 30. Sexually dimorphic differential expression across all life stages of two sequential *C. rufifacies Sex-lethal* nodes. A) Boxplots of expression across all life stages separated by sex (female=red, male= blue). B) Boxplots of expression across all life stages separated by sex (female=red, male= blue).

Many genes exhibited patterns of expression consistent with *D. melanogaster.* For example, the gene *slam* demonstrates highest expression in *D. melanogaster* embryos in the first four hours after fertilization, with low expression in larvae or pupae [267, 286] and is important for cleavage and polarized membrane growth during cleavage in the *D. melanogaster* embryo [356]. Consistent with *D. melanogaster, Crslam* was found to be uniquely expressed in the egg stage. Another gene that demonstrated conservation in its expression pattern was *CrδTry*, a gene unique to the larval stage. In *D. melanogaster*, this gene is expressed in all instars and new white prepupae at moderate to high levels [267]. This gene is homologous to part of a family of four trypsin-like genes whose products were shown to be a major component of *L. cuprina* larval digestive enzymes [357]. Finally, *CrJhe* was found to be uniquely expressed early in pupal development and in adults. This is consistent with the pattern of expression of *DmJhe,* which is highest in 12 hour white prepupae and decreases throughout pupal development [267, 358]. This gene is important in the regulation of juvenile hormone, a hormone which plays an important role in the timing and proper development of insects [358-360].

This work represents the first analysis of sex-specific global gene expression patterns throughout all stages of immature development in a wild-type fly. Statistically significant dimorphism in gene expression between males and females was observed in all stages of development. In eggs, differentially expressed nodes demonstrated a female skew of 85%. Although none of these differentially expressed nodes were present in Cluster 1 which likely contains the maternal contributions to eggs, differential expression could be due to a combination of zygotic transcription and maternal factors. Specifically, the

unusual sex-determination mechanism in this fly, with females that have all male or female offspring, has been shown to lead to differences in maternal contributions between male and female-fated eggs [179]. Further work is required to determine the source of these differences in gene expression between male and female-fated eggs.

While differential expression between males and females was heavily female skewed in eggs, dimorphically expressed genes were male biased in all other life stages. On average, differential expression of nodes and annotated genes were 77% and 84% male biased. Across larval development, female up-regulation occurred primarily in genes related to muscle development and differentiation. While dimorphism in gene expression of those related to synaptic development and function were up-regulated in males in the third instar, components of the dopaminergic pathways were up-regulated in females relative to males in the first instar. Given the dimorphism in development rate between males and females, it is possible that these differences in larval gene expression are different developmental ages between the sexes though they are the same chronological age. Additional work to examine gene expression, neurological connections, and musculature at several time points throughout larval development would help to differentiate between these possibilities.

In sex-specific developmental gene expression pattern analyses, 6% more nodes and 8% more genes were differentially expressed across male development than female development. Though approximately the same number of nodes were analyzed in the cluster analysis for each sex, the p-adjusted cutoff for males was 10^{-6} lower than that used for female samples, suggesting that gene expression in males during is highly dynamic

and changes are extreme in comparison to females. Furthermore, the finding that female gene expression variance is higher than males suggests that the inability to control for female type in the immature samples may have introduced an additional source of variation in the female libraries, and that there may be differences in gene expression between thelygenic and arrhenogenic females. In conjunction with these sex-specific findings, many genes demonstrated significant sex by stage interactions in expression. These differences were observed primarily in the pupal stage. Expression of these genes appears in the similar sex-dependent manner in *D. melanogaster*. The gene DmCG9222 has been shown to demonstrate expression beginning in the larval stage, with high expression in males and testis and no expression in females [267], and in this work it was found to be expressed in male pupae but not female pupae. Another gene up-regulated in male pupae and adults was *Succinyl coenzyme A synthetase α subunit* (*CrScsα*), which demonstrates significant up-regulation in male adult brains relative to female adult brains [267]. Additional work in *D. melanogaster* with neural stem cells has shown that *DmScsα* functions in neurogenesis [361], though research is needed to determine whether this gene has a sexually dimorphism effect on neurogenesis. In comparison, *altered disjunction* (*Dmald*) functions in female meisosis chromosome segregation [362] and demonstrates high expression in ovaries in females, specifically affecting the 4^{th} and sex chromosomes. Expression of *Crald* was high in female pupae and low in male pupae, though this species is known to exhibit heteromorphic sex chromosomes so the effect of this gene on female meiosis in *C. rufifacies* warrants more work [204]. Results of these

analyses point towards muscular and neurological divergence between males and females.

Excluding eggs, 77% of nodes and 84% of up-regulated genes demonstrated male bias in expression. Two specific genes were shown to be up-regulated in males across development relative to females: *C. rufifacies male sex lethal 2* (*Crmsl2*) and *supercoiling factor* (*Crscf*) differentially expressed in males across development. Interestingly, both of these genes regulate dosage compensation in male *D. melanogaster* [298, 363]. Dosage compensation is the mechanism by which males, with one X chromosome, and females, with two, produce equivalent activity per cell of X-linked gene products [364]. However, as *C. rufifacies* does not possess heteromorphic sex chromosome [182, 204], such significant up-regulation in males may be a sign of misapplied dosage compensation, as it appears that males are dosage compensating without the heteromorphic sex chromosomes which would make that necessary. When a genome for this species becomes available, it will be possible to determine how these differentially expressed genes localize and whether this supports the possibility of inappropriate or broken dosage compensation in this species.

Previous similar work on large scale developmental gene expression patterns focused on sexual dimorphism is limited [286, 328]. Both studies used microarrays to study gene expression in mutants and wild type *D. melanogaster* to identify somatic, germ line, and developmental patterns. Genes were identified germ-line tissue specific and somatic in expression through the use of *tudor* mutants and testes specific analysis [286]. However as it was not known whether the immature stages, specifically eggs and larvae, were male

or female, and patterns of gene expression on these somatic genes in immature stages could be affected by the sex of the offspring. Furthermore, the authors suggested that the genes identified as sex-specifically expressed in somatic tissues were likely to be involved in adult differentiation.

Additional work by Lebo et al. [328] on temporal expression patterns of these and additional genes in male and female pupae resulted in a list of 258 genes exhibited sex-specific somatic expression in *D. melanogaster*. A total of 17 of these genes were found to exhibit statistically significant sexual dimorphism in expression in *C. rufifacies*, including *CrSxl*. In the present work, dimorphically expressed genes in the pupal stage were ~95% male biased in expression across all time points, in comparison to the average approximately 50:50 split observed across all pupal time points until male enrichment occurs at 96 hours in *D. melanogaster* [328]. The increased duration of pupation in *C. rufifacies* compared to *D. melanogaster* does not account for the significant enrichment in early stages of metamorphosis, and further supports the hypothesis that dosage compensation may be spuriously engaged.

Sexually dimorphic expression in male and female fated eggs has been assessed in three species of tephritid fruit flies [330, 331, 365]. Only one of these studies used wild-type flies [365], and both studies in *Bactrocera* spp. embryos relied upon detection of a previously identified Y-linked DNA marker in *B. jarvisi* Tryon (Diptera: Tephritidae) to phenotype the individuals prior to gene expression based on extracted mRNA. Furthermore, one of the studies used hybrid introgression to retain this Y-linked marker against the genetic background of another species, *B. tryoni* Froggatt [330]. Other work

in *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) relied upon subtractive hybridization and a maternal-effect RNAi transgenic strain that produces all-male offspring, independent of the zygotic genome, to assess embryonic sexual dimorphism in gene expression [331]. Morrow et al. [330] demonstrated that onset of differential splicing of *tra* and *dsx* occurred five to six hours into embryonic development. In contrast, differential expression of male and female nodes of *Crtra* and *Crdsx* was detected in the present work in embryos no more than four hours old, though this may be due to increased development rate due to the higher rearing temperature. None of the studies were able to identify a Y-linked transcript, and suggested that the male determiner M predicted to be on the Y-chromosome may be a non-coding RNA.

In this work I identified sequences of the three canonical sex-determination genes, *CrSxl, Crtra,* and *Crdsx*. Though research on sex determination in *C. rufifacies* has been ongoing since the 1930's, only one gene, *CrSxl*, in the canonical *D. melanogaster* sex-determination hierarchy has been sequenced [206]. Though my predicted *CrSxl* sequence shares 99% sequence identity with the established gene sequence, my results contradict these and other previous findings in that *CrSxl* was significantly sex-specifically expressed [206, 252]. The other two genes demonstrate temporal expression patterns consistent with *D. melanogaster*, with high expression of *Crtra* in eggs and early immature stages, and high expression of *Crdsx* beginning in the 3rd instar and continuing into the adult stages. Work in Drosophilidae, Tephritidae, and Muscidae flies have identified *tra2* as an important component in functional sex-determination, as wild-type *tra2* is needed for proper female-specific splicing of *dsx* [366-368]. However, other work

has shown that coding region for this gene is identical in males and females in somatic tissues in *Drosophila*, *Ceratitis*, and *Anastrepha* (Diptera: Tephritidae) flies [367, 369]. Notably, the germ-line of *D. melanogaster* demonstrates sex-specific splicing of *tra2* and this is required for proper testes development [370]. Previous work in other species of flies has not identified a significant difference in expression between males and females in whole body tissues, though it is enriched in ovaries relative to testes in tephritid and muscid flies [367, 368, 371]. In the present work, *Crtra2* demonstrated highly significant ($p<10^{-5}$) sexual dimorphism in expression, with high expression in eggs and adult females relative to all other life stages, and slight but significant increase in expression in male pupae relative to female pupae. Further work to fully sequence these four genes is necessary in order to understand if and how these may function in monogenic sex determination in *C. rufifacies*.

In addition to addressing issues in many basic biological fields, the findings of this work are also important in the fields of medical/veterinary and forensic entomology as previously mentioned above [310, 333, 372, 373]. *Chrysomya rufifacies* can be an important agricultural and medical pest internationally as either a primary or secondary agent of myiasis [308, 310]. Current control strategies in for myiasis causing blow flies rely upon the release of sterile males [335], and sequence and timing information related to sexual-dimorphism in expression and development offer potential targets for transgenic control strategies [336, 374]. Furthermore, this work provides limited but empirical evidence of sexual dimorphism in development rate of a forensically relevant fly. As developmental data sets used in forensic entomology to estimate blow fly age

based do not currently take sex into account [375, 376], it is possible that sex-specific development data could help improve estimate precision. There is evidence that several genetic tests, including genome sizing and PCR of sex-specific splices of mRNA, may be sufficient for differentiation between male and female larvae [190]. Finally, this work provides genetic data which can be used to develop gene expression profiles for use in differentiating between behavioral and developmental landmarks which are difficult to differentiate between morphologically [377]. For example, *C. rufifacies* is known to pupate directly on, under, or in the larval feeding substrate, making differentiating between feeding and post-feeding larvae challenging [377]. This work has identified genes, such as *CrJhe*, which may be useful in quantitatively differentiating between these two states. Similarly, many of the genes in this work observed to exhibit both temporal and sexually dimorphic patterns of gene expression throughout metamorphosis can be used to develop quantitative estimates of insect age such as has been done with *L. cuprina* eggs and *Calliphora vicina* Robineau-Desvoidy (Diptera: Calliphoridae) pupae [212, 339], instead of relying upon technically challenging and qualitative measures [378].

In summary, sexual dimorphism in gene expression occurs throughout all stages of *C. rufifacies* development, including eggs, and one phenotype that results from this is more rapid development in males than females. The timing of expression *tra* early in development, starting with eggs, with significant dimorphism in expression of *dsx* beginning in the third instar is consistent with patterns identified in other flies. Some progress has been made in understanding a few of the genetic mechanisms underlying the

unusual sex-determination mechanism evidenced in this species. Finally, it appears based on this work that males in this species continue to exhibit dosage compensation up-regulation of gene expression, though there is no evidence of sexually dimorphic sex chromosomes. Future work to sequence the genome of this species and study patterns of gene expression is still necessary to understand the implication and interspecific applicability of findings in this species.

CHAPTER IV

INVESTIGATION OF SEXUAL DIMORPHISM OF BEHAVIOR AND GENE EXPRESSION IN PREDATION IN *Chrysomya rufifacies*

Introduction

Sexual dimorphism across the animal kingdom has fueled a multitude of behavioral, physiological, and ecological studies [98, 125, 135, 144, 217, 379]. Sexually dimorphic traits generally fall into one of three categories [74]. Primary sex traits are those that are directly related to sexual reproduction, and include both anatomical and physiological factors [74]. Secondary sex traits are those that increase reproductive success in the individual expressing them and include any modification to morphology or behavior. Examples include color, body size, flamboyant body parts, or courtship behavior [142, 321, 327]. Ecological sex traits are those that differ between the sexes and can lead to sexual conflict or niche divergence [135].

Sexual conflict was initially broadly defined as "differences in the evolutionary interests between males and females" [3]. Classical studies in sexual conflict focused on traits directly related to reproduction, such as number of mates, frequency and duration of mating, timing of oviposition, use of sperm, parental investments, nuptial gifts [4-9]. More recent studies in sexual conflict have begun to investigate sexual conflict on a broader scale and to identify genes responsible for traits under divergent selection between the sexes [10]. The traits that might be responding to sexual conflict could be involved in sex determination or the production and maintenance of physiological, morphological, or behaviorally sexually dimorphic traits [11-15]. To further complicate

the issue, there may also be trans-generational sexual conflict, in which maternal decision can affect the ratio of male to female eggs [52, 380] or the survival of a specific sex [381].

Behavioral differences between the sexes do not have to be directly related to courtship or mating behaviors. In particular, ecological dimorphism, or niche divergence, between the sexes is a possibility [135]. Studies have not only demonstrated that there are differences between the sexes as adults in terms of nutritional requirements [136], but that there are also differences between gravid/nursing and non-gravid females [137], and that these variable nutritional requirements can shape foraging behavior in early stages of development [138, 139]. Based on studies in mammals, birds, and reptiles, it appears that sexual dimorphism precedes niche divergence in most cases, though evidence is not overwhelming [98, 135, 138, 140-143]. Certainly, morphological differences can lead to dimorphic swimming ability, foraging site, and diet divergence in marine birds [141]. However, work in northern map turtles, *Graptemys geographica* Leuseur (Testudines: Emydidae), indicates that ecological niche divergence is context dependent, as sexual dimorphism in diet preference or habitat choice was observed in rivers but not lakes [140]. The importance of ecological and sexual factors in structuring sexual dimorphism has been observed in other taxa in other traits [142, 144], and highlights the value of holistic studies to differentiate between these factors. Furthermore, this complexity significantly increases the challenge in trying to apply mathematical models to predict foraging behavior, as it is difficult to model temporal-, sexual-, and condition-dependent foraging in Optimal Foraging Theory or other such ecological models [143].

There are major challenges when investigating behavioral plasticity, sexual dimorphism, and potential ecological divergence and sexual conflict in immature organisms, especially in immature insects. Firstly, in ethological work in insects, distinguishing between males and females in immature stages, as their overt sexual dimorphism at this stage is rare is typically not possible [318]. Secondly, in sexual conflict work it has long been assumed that immatures are not experiencing divergent selection, as selection was thought to act on adult structures and mature behaviors, though this is changing [382]. Thirdly, it can be extremely challenging to ask more subtle ecological questions in field situations due to the large number of uncontrolled variables.

Conflicts of interest between males and females might be a force selecting for specific feeding behaviors [10], and immature nutrition may be important for later egg development in resulting adults [173]. However, it is not clear how often larvally-obtained nutrients affect oogenesis in resultant adults and whether this may impact nutrient source shifts in immature insects. Furthermore, studies on sexual conflict have been restricted to interactions between mating adults [18], or adults and their offspring [174]. There has yet to be any research into sexual conflict between immature insects.

Chrysomya rufifacies Macquart (Diptera: Calliphoridae) and its closest sibling species *C. albiceps* Wiedemann [189] both exhibit monogenic sex determination with single sex offspring clutches and homomorphic sex chromosomes [48, 181, 182]. Furthermore, both are facultative predators, though it is not clear with what frequency they exhibit this behavior. Previous studies on this behavior by these species have not relied upon observations under naturally occurring conditions [383]. Finally, as studies

have also demonstrated that these larvae are cannibalistic [163], there is a possibility that larval sex ratios and adult sex ratios are not the same. Studies in this system offer an opportunity to investigate a unique sex-determination system, the molecular basis of a complex ethology, and the possibility of sexual conflict manifesting in immature insects (as larvae are genetically destined to be males, male producing females, or female producing females). The purpose of this study is to determine the frequency of predation, genetic markers of predation, and if there is a sex bias in the behavior or gene expression. The results of this work will help generate specific testable hypotheses regarding the proximate causes of predation, the impact of supplemental food availability, and the possibility of sexually dimorphism in the nutritional ecology in *C. rufifacies*.

Materials and methods

Colony maintenance

Chrysomya rufifacies larvae were collected from numerous carcasses in College Station, Texas, USA between May and September of 2011 and eclosed adults were identified morphologically [253, 254]. Adult flies were released into a BugDorm 1 plastic cage (MegaView Science, Taiwan) and allowed to interbreed to found the laboratory colony. The colony was provided with fresh deionized water and refined sugar ad libitum, as well as fresh beef liver blood daily as a protein source for oogenesis. Flies were maintained at 28°C for a 16:8 light:dark (L:D) photoperiod.

Predation assay preparation

To collect *C. rufifacies* larvae of a known age, colonies were allowed access to an oviposition substrate of fresh beef liver in a 32.5 mL opaque plastic cup covered with a

KimWipe® (Kimberly-Clark, Irving, Texas) moistened with deionized water for a three-hour window. After oviposition, the eggs were placed in a Percival model I-36LLVL Incubator (Percival Scientific, Perry, Iowa) at 30°C, 75% relative humidity (RH), and a 12:12 L:D. After hatching, aliquots of 100 first instars were then transferred by paintbrush to 50 g of fresh beef liver in a 32.5 mL opaque plastic cup covered with a moistened KimWipe® in a 1.1 L canning jar with approximately 100 g of playground sand and a Wype-All on the top to prevent escape but allow air flow. This was replicated three times per egg collection, with a total of three biological replicates. These rearing jars were then placed in a Percival model I-36LLVL Incubator (Percival Scientific, Perry, Iowa) at 30°C, 75% RH, and a 12:12 L:D photoperiod.

Laboratory colonies of *Co. macellaria* Fabricius (Diptera: Calliphoridae) established in Owings et al. [384] were used to supply prey larvae, as *Co. macellaria* is a native blow fly species preyed upon by *C. rufifacies* in the field in the United States [383]. To collect *Co. macellaria*, laboratory colonies were allowed access to an oviposition substrate of fresh beef liver in a 32.5 mL opaque plastic cup covered with a KimWipe® moistened with deionized water for a 24 hour period. After this, aliquots of ~300 larvae were transferred to 50 g of beef liver. These larvae were maintained in rearing jars as above in a Percival model I-36LLVL Incubator.

Predation assay

The predation assays were initiated 96 hours post oviposition of *C. rufifacies*. A single predation arena consisted of one third instar *C. rufifacies* and one third instar *Co. macellaria* sealed in an empty 30mL plastic cup. This was repeated 30 times for each trial

(three generations). Each trial also included a set of 30 controls, consisting of a single *Ch. rufifacies* isolated in an empty 30mL plastic cup. The predation assays were kept in the Percival model I-36LLVL Incubator under conditions previously described for two weeks. One additional replicate without controls was also conducted.

After two weeks, the results were tabulated. Each individual arena was assessed for: survival of predator, sex of predator (if adult), survival of prey, sex of prey (if adult), and level of prey consumption. Prey consumption level was categorized as: no consumption (whole, dead prey or prey adults eclosed), partial consumption (prey larvae partially consumed), and total consumption (prey appears absent, or the empty cuticle could be identified) (Figure 31).

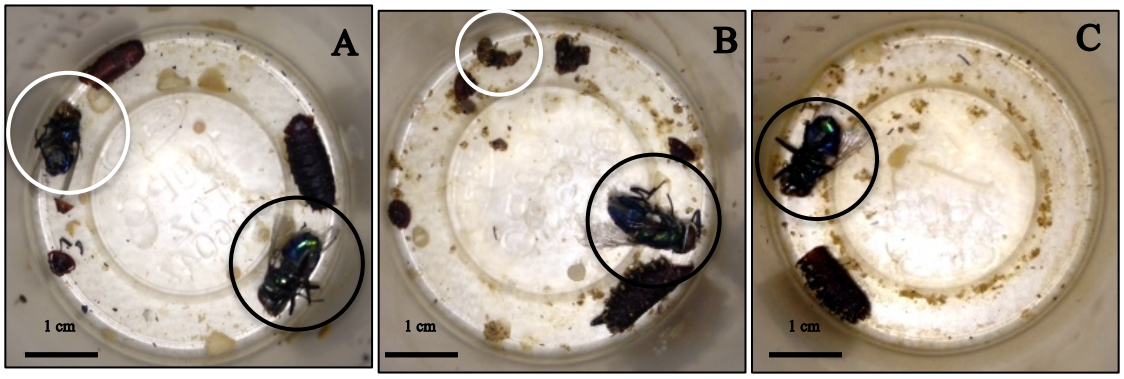


Figure 31. Prey consumption level of *Co. macellaria* by *C. rufifacies* categorization. These are images representative of prey consumption level categorization. Black circles indicates *C. rufifacies*, and white circles indicate *Co. macellaria*. A) No consumption- two pupal casings (one *C. rufifacies* and one *Co. macellaria*). B) Partial consumption- one pupal casing (*C. rufifacies*) and part of prey remaining. C) Total consumption- one pupal casing (*C. rufifacies*) and no evidence of prey remaining.

Data were analyzed in R 3.1.3 to assess sex ratios using Bonferroni corrected z-tests of proportions [255]. Data were analyzed in SAS ® Studio v.9.4 (SAS Institute Inc., Cary, NC, USA) to examine survival relative to supplemental food (control versus treatment, consumption versus non-consumption) and to determine whether sex affected predation level using Proc Freq and the Cochran-Mantel-Haenszel (CMH)Test and Fisher's Exact Test [385], as SAS is a powerful tool for categorical data analysis [386].

Field behavior observation

Observations of *C. rufifacies* on human cadaver donations were made at the Forensic Anthropology Research Facility at Texas State University in San Marcos, Texas as approved by the Institutional Biosafety Committee, Texas A&M University, College Station, Texas. Researchers observed all human cadavers with second and third instar *C. rufifacies* and at least one other species for a period of thirty minutes each between 0900 and 1400 hours on five dates between 6 June and 8 September of 2014. Remains were permitted to lay *in situ* and *C. rufifacies* observed actively predating were collected. These individuals were permitted to eclose to adulthood to determine their sex.

Gene expression sample collection

For each sample, a single male and female *C. rufifacies* were isolated together in a 1.1 L canning jar with approximately 100 grams of playground sand, a Wype-All on the top to prevent escape but allow air flow, and refined sugar and water ad libitum and a 10 mL glass beaker filled with one Kim-wipe® and approximately 1 mL of fresh beef liver blood. An additional 1 mL of blood was added each following day up until the 6th day post eclosion. The protein source was then excluded for 24-hour period. Beginning on the

7th day post-eclosion, twice each day, a 35 mL plastic cup with approximately 25 g fresh beef liver covered with a moistened Kim-wipe® was introduced to the jar as an oviposition medium for a four hour window. If a female oviposited during this time, the females were removed and the progeny allowed to develop.

Approximately 96 hours post oviposition, ten prey individuals in the third instar (*Co. macellaria*) were moved into a predation arena (10 cm diameter high walled container). After this, 10 *C. rufifacies* siblings were simultaneously moved into the predation arena. The larvae were observed until a single individual *C. rufifacies* attacked a *Co. macellaria* and exhibited the classic "wrap-around" (Figure 32), at which point the predator and prey were collected to an eppendorf tube and flash frozen. Two *C. rufifacies* which were not exhibiting predatory behavior were also collected at the same time, to control for environmental influences on gene expression. Samples were collected from a total of three maternal lines for each sex.

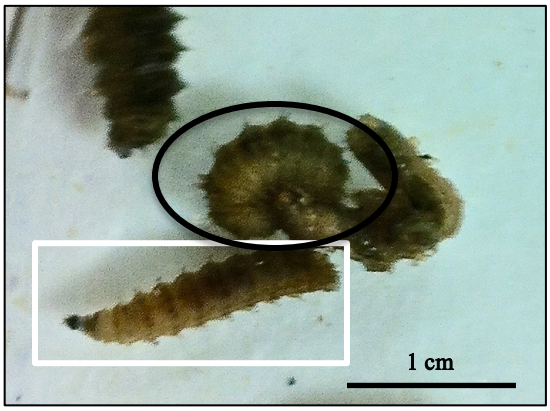


Figure 32. Classic "wrap around" behavior. When *C. rufifacies* is predating, it will wrap itself around the body of its prey item. Black oval indicates an actively predating *C. rufifacies* and the white box indicates a non-predating individual.

RNA preparation

RNA was extracted via TriReagent preparation according to manufacturer's protocols. Briefly, a single larva was macerated in 1mL of cold TriReagent (Sigma-Aldrich Corp., St. Louis, Missouri) in a 1.5 mL RNAse-free microfuge tube. Following this, 50 mL of ice-cold BAN reagent (Molecular Research Center, Inc., Cincinnati, Ohio) was added and the solution was vigorously mixed. Next, the tubes were spun at 4°C for 15 minutes at 14,000 G to separate the RNA from the DNA and proteins. Approximately 500 µL of the top, clear layer were carefully removed to prevent contamination and added to 500 µL of ice-cold 100% isopropanol. The tubes were mixed via inversion three times and allowed to rest on ice for 10 minutes to precipitate the RNA. The precipitate was then spun down at 4°C for 15 minutes at 14,000 G. The supernatant was completely removed, 1 mL of cold 70% ethanol was used to wash the RNA pellet, and then the pellet was spun down again at 14,000 G at 4°C for 5 minutes. The ethanol was eluted, and any remaining ethanol allowed to evaporate completely. The RNA was then dissolved in a 100 µL mixture of 99 µL of DNase/RNase/Nucleotide-free water and 1 µL of SUPERase•In ™ (Invitrogen, Life Technologies Incorporated, Grand Island, New York).

The extracted RNA was further purified using a Qiagen RNeasy Micro Kit and on-column DNase treatment following manufacturer protocols (Qiagen Inc., Valencia, CA, USA). Samples were then assessed for quality and concentration using a NanoDrop XXXX (NanoDrop Products, Thermo Fisher Scientific Inc., Wilmington, Delaware) and Agilent 2100 Bioanalyzer Instrument (Agilent Technologies, Inc., Santa Clara, California). The six RNA libraries were multiplexed 100 bp paired-end on two lanes of

Illumina HiSeq2500 (Illumina, Inc., San Diego, CA, USA).

Female third instars were libraries 17-22, with 17 the predator to 18's non predator, 19 (Pr) and 20 (non), 21 (pr) and 22 (non) (Table 17). Male third instar larvae were in libraries 27-32, with 27 (pr) and 28 (non), 29 (pr) and 30 (non), and 31 (pr) and 32 (non) (Table 17).

Table 17. Sibling predator and non-predator library numbers and abbreviations. Columns from left to right: sex of the individual (Sex), sibling pair number (Pair), whether predator (Predator) or non-predator (Non-Predator) sibling, library number (Library #) and name of the sample (Sample Name).

Sex	Pair	Predator		Non-Predator	
		Library #	Sample Name	Library #	Sample Name
Female	1	17	FP1	18	FN1
	2	19	FP2	20	FN2
	3	21	FP3	22	FN3
Male	1	27	MP1	28	MN1
	2	29	MP2	30	MN2
	3	31	MP3	32	MN3

Transcriptome assembly

Prior to assembly, reads underwent trimming and quality control: reads were filtered to remove all sequences that contained adaptor sequences and known contaminants as defined by Illumina. The transcriptome was assembled with reads data from all life stages of *C. rufifacies* following Sze et al [202] under a variety of k-mer (k) and k-mer coverage (c) parameters. Briefly, assemblies were generated with the ASplice algorithm on the Whole Systems Genome Initiative (WSGI) computing cluster (wsgi-hpc.tamu.edu).

These assemblies were then analyzed to identify potential alternative splicing patterns. This program assembles reads into splicing graphs, rather than predicted transcripts, similar to SOAPdenovo2 [258]. Briefly, the program produces an output of nodes connected together by edges. Nodes are sections of unambiguously aligned k-mers, and edges are the connections between nodes in alternatively spliced transcripts. The ASplice algorithm can be found at http://faculty.cse.tamu.edu/shsze/ASplice/.

Once the assembly was completed for a given parameter pair, the number of reads per third instar library that aligned was calculated for each node. The nodes of the transcriptome were then compared against known *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) proteins using a translated Basic Local Alignment Search Tool (BLAST) search [259]. For each node, only the top BLAST hit with an *E*-value below 10^{-7} was considered.

The assembly was done with additional RNA-seq data for other life stages that were not included in the following expression analyses- only the libraries for the third instar predator and non-predator individuals were considered.

Transcriptome analysis

Twenty-four preliminary assemblies were created from a range of k-mer sizes (21, 25, 31, 35, 41, and 45) and a range of coverage cutoffs (50, 100, 200, and 500). Analyses were done in R using the DESeq2 package on all assemblies [264]. Only those nodes with a p-adjusted < 0.1 were considered for further analysis. The classes of genes identified through this analysis were: predator biased, non-predator biased, female biased, male biased, and sexually dimorphic nodes. The node information was analyzed relative

to the assemblies, to determine the number of genes, and number of transcripts of those genes, that were identified as differentially expressed in each assembly.

Gene ontology

The splicing graphs containing differentially expressed nodes were compared with *D. melanogaster* to putatively identify function. If no *D. melanogaster* hits were associated with the splicing graphs, transcripts were predicted from the splicing graphs and compared against the nucleotide and protein databases, using four different algorithms at the NCBI website: blastn (nucleotide to nucleotide database), blastx (translated nucleotide to protein database), tblastx (translated nucleotide to translated nucleotide database), and comparison of translated nucleotide sequences to the conserved domain database (CDD). Only those hits with an E-value of 10^{-7} or less were considered significant.

Results

Behavior assays

A total of 276 control and 395 behavioral assays were conducted over the course of 3 and 4 generations respectively (Table 18). Overall survival was 88% with a slight but significant skew of 56% males (Z-test on proportions, $p = 0.0007$) in the surviving *C. rufifacies*. However, the sex ratio ($^{\text{Males}}/_{\text{total}}$) of each rep was significantly different than 0.5 at a Bonferroni-corrected p-value of 0.0125. There was no significant difference in sex ratios between the control and treatment groups. There was no significant difference in survival between the control and treatment groups in the first trial, but the CMH test did not detect a significant difference between trials (Breslow-Day test of homogeneity,

p=0.9501). Overall, membership within the treatment group was associated with 2.4558 increased odds of survival ($p = 0.0007$). Consumption of supplemental food did not significantly increase survival odds in the first and second trials, but the CMH tests did not detect evidence of a significant difference between replicates (0.5996). Overall, consumption of supplementary food was associated with a 0.342 increased in odds of survival ($p < 0.0001$). All individuals who engaged in partial predation survived ($n = 181$), though 17% and 14% of no predation and total predation samples, respectively, did not survive. There was a significant difference overall in survival rates between the different levels of predation ($\chi^2_{df=2}$, $p < 0.0001$), though this pattern was only significant in trials 2 and 3 ($p = 0.00127$ and $p = 0.00344$ respectively). There was no evidence for sexual dimorphism in predation rates between males and females within trials 2 and 3 ($p = 0.1211$ and $p = 0.1908$ respectively) or when taking replicate effects into account (CMH, $p = 0.7122$), though there was evidence of sexual dimorphism in predation rates in trials 1 and 4 ($p = 0.0391$ and $p = 0.0152$ respectively).

Table 18. Results of *C. rufifacies* predation on *Co. macellaria* predation assays and analysis. This is a table of the results of laboratory predation assays of a single *C. rufifacies* confined with a single *Co. macellaria*. The columns from left to right: Trial (Trial), Number of individuals in control group (N_C), Number of individuals in control group (N_T), percent male of eclosing adult *C. rufifacies* (%Male), percent of *C. rufifacies* surviving to eclosion (%Surv), p-value of Fisher's Exact Test comparing sex ratios between treatment and control ($\%Male_{CvT}$), p-value of Fisher's Exact Test comparing survival rates between treatment and control ($Surv_{CvT}$), p-value of Fisher's Exact Test comparing survival rates between consumption (Partial and Total) and no consumption (Control and None) supplemental food $Surv_{Sup}$), p-value of Fisher's Exact Test comparing survival rates by Predation level in treatment groups only ($Surv_{PredLevel}$), and p-value of Fisher's Exact Test comparing Predation levels by sex in eclosed adult flies from the treatment group only ($PredLevel_{Sex}$). The B-D row is the p-value for the Breslow-Day test for homogeneity of variances- a high p-value means that there is no statistically significant difference between replicates. The CMH row includes the p-value of global significance of the association across replicates. Cells with a - indicate values which were not calculated because they were unnecessary or could not be calculated due to lacking data or a mathematical inability to calculate values. Values in bold are those p-values which are significant at an $\alpha = 0.05$.

Trial	N_C	N_T	% Male	%Surv	$p^{\%MaleCvT}$	$p^{SurvCvT}$	$p^{SurvSup}$	$p^{SurvPredLevel}$	$p^{PredLevelSex}$
1	90	96	40%	98%	0.7643	0.6111	0.2491	0.0938	**0.0391**
2	89	89	35%	85%	0.7327	**0.0352**	**0.0118**	**0.0034**	0.1211
3	97	119	90%	80%	0.4516	**0.0173**	**0.0063**	**0.0013**	0.1908
4	0	91	63%	96%	-	-	-	0.2191	**0.0152**
B-D	-	-	-	-	0.6098	0.9501	0.5996	-	-
CMH	-	-	-	-	0.7812	**0.0007**	**<0.0001**	0.1722	0.7122
Overall	276	395	56%	88%	0.3697	**0.0017**	**0.0003**	**<0.0001**	**0.0375**

Field predation

A total of 13 human cadavers were observed, though predators were only observed on a single donation (D45-2014). From this donation, only ten individuals were observed to predate in the half hour window. All of these individuals were collected from the maggot mass under the head, though there were larvae under the whole body and observations were also made at the genital region and the left foot. Of these predators, eight of 10 were male. However, maggot mass under the head, groin, and leg were 54% male (n = 146), 46% male (n = 146) and 52% male (n = 310) respectively. Overall, the population of *C. rufifacies* from this donation was 51% male. None of the masses had a sex ratio that was statistically different from 0.5, though the sex ratio of the predators was statistically significantly higher than 0.5 ($p = 0.0289$). The sex ratio of the predators was not significantly different than that of the mass under the head or from the overall sampled population.

Transcriptome assembly

A total of 66 RNAseq libraries were sequenced (33 male and 33 female throughout all of *C. rufifacies* development). There was an average of 6.3×10^7 reads per library at an average length (post-trimming) of 86.3 bp. A total of 68.5 Tbp of sequence data was assembled into 24 *de novo* transcriptome assemblies based on a range of k-mer sizes (21, 25, 31, 35, 41, and 45) and coverage cut-offs (50, 100, 200, and 500) (Table S1). On average, 50% of the reads mapped to the transcriptome. Assemblies with a coverage cutoff of 50 yielded the most nodes, with decreasing overall numbers of nodes with increasing coverage cutoff (Table 19, Figure 33A). Within a given coverage cutoff, at a

k-mer of 21 the number of nodes is initially low, peaks at 31 (25 at a coverage of 500), and then drops off again with increasing hash length. The average number of nodes per splicing graph and the count of splicing graphs with more than more node followed a similar pattern as total number of nodes (Table 19). The number of nodes in the largest splicing graph in each assembly did not follow a consistent pattern. In contrast, N50 increased with increasing coverage, though within a coverage cutoff N50 was initially low, peaked at 25 or 31, and then dropped off with increasing k-mer (Table 19). The number of unique hits to *D. melanogaster* genes and transcripts was highest at the lowest coverage cutoff, and dropped off with increasing k-mer and coverage cutoff (Table 19, Figure 33B).

After trimming, the 12 third instar predator and non-predator libraries averaged 87.3 bp in length. The average male library was smaller than the average female library (6.85×10^7 and 6.27×10^7 reads respectively; single-tailed T-test $p = 0.04203$), though there was no difference in average size between predator and non-predatory libraries (two tailed T-test; $p = 0.8707$).

Table 19 General *de novo Chrysomya rufifacies* transcriptome statistics. Columns (left to right): coverage cutoff (c), hash-length (k), total number of nodes assembled (Total Nodes), total number of splicing graphs (Splicing Graphs), count of unique D. melanogaster gene alignments (Genes), count of unique D. melanogaster transcript alignments (Tx), number of splicing graphs with more than one node (>1 SG), average number of nodes per splicing graph (Ave nodes/SG), N50 of the assembly (n50), maximum number of nodes in a single splicing graph (max nodes), and identifier node of maximum splicing graph (MaxNode ID).

n	k	Total Nodes	Splicing graphs	Genes	Tx	>1 SG	Ave nodes/SG	% Map	N50	Max nodes	MaxNode ID
500	**Ave**	**138609.7**	**37322.5**	**8587.8**	**9790.5**	**8124.2**	**3.9**		**695.0**	**679.0**	
	SD	***16155.6***	***7314.3***	***127.7***	***234.5***	***729.1***	***0.9***		***95.6***	***540.8***	
	45	124248	36163	8415	9533	7450	3.43677		710	225	NODE_326028
	41	131279	35428	8475	9601	7966	3.70552		731	234	NODE_439033
	35	145233	33311	8574	9719	8561	4.35991		752	1433	NODE_1181639
	31	153981	33007	8625	9786	8903	4.6651		753	813	NODE_1412293
	25	158014	33983	8681	9928	8741	4.6498		721	1170	NODE_441125
	21	118903	52043	8757	10176	7124	2.28471		503	199	NODE_161227

Table 18. Continued.

n	k	Total Nodes	Splicing graphs	Gene	Tx	>1 SG	Ave nodes/SG	% Map	N50	Max nodes	MaxNode ID
200	**Ave**	**266177.3**	**48949.0**	**9131.0**	**10552.2**	**12172.5**	**5.9**		**455.8**	**2203.3**	
	SD	***53239.4***	***14308.3***	***111.1***	***162.7***	***1504.0***	***1.9***		***41.3***	***1490.7***	
	45	253409	43911	8987	10371	11726	5.77097		469	578	NODE_1234130
	41	273350	42203	9039	10421	12440	6.47703		475	3194	NODE_1002692
	35	298558	41421	9104	10497	13161	7.20789		476	4076	NODE_2577181
	31	308385	41872	9154	10549	13319	7.36495		477	2649	NODE_1513952
	25	297816	46367	9216	10665	13043	6.42302		466	2449	NODE_985147
	21	165546	77920	9286	10810	9346	2.12456		372	274	NODE_3830891
100	**Ave**	**412147.7**	**62368.5**	**9446.8**	**10971.8**	**15285.2**	**7.3**		**322.3**	**1708.5**	
	SD	***104699.2***	***19300.8***	***65.0***	***113.5***	***2314.0***	***2.7***		***18.3***	***914.2***	
	45	419136	53233	9356	10818	15112	7.87361		326	2551	NODE_3195431
	41	445956	52477	9392	10864	15819	8.49812		329	2019	NODE_4482480
	35	471573	53120	9452	10984	16533	8.8775		336	1398	NODE_990069
	31	493544	52099	9487	10985	16793	9.4732		300	2786	NODE_5088358
	25	437460	62309	9537	11069	16708	7.02082		343	1117	NODE_384398
	21	205217	100973	9457	11111	10746	2.03239		300	380	NODE_786022

Table 18. Continued.

n	k	Total Nodes	Splicing graphs	Gene	Tx	>1 SG	Ave nodes/SG	% Map	N50	Max nodes	MaxNode ID
50	**Ave**	**606519.5**	**80712.7**	**9614.3**	**11142.7**	**18768.5**	**8.3**		**251.3**	**2347.5**	
	SD	***179788.7***	***23946.3***	***33.6***	***45.2***	***3411.1***	***3.4***		***7.3***	***1654.9***	
	45	643637	68373	9566	11072	18457	9.41361		245	2497	NODE_1767513
	41	677294	68352	9604	11120	19473	9.90891		247	2613	NODE_6865467
	35	729475	66381	9631	11143	20468	10.9892		249	5299	NODE_1683802
	31	735262	67945	9649	11171	20902	10.8214		254	2067	NODE_9369794
	25	598317	85972	9647	11205	21195	6.95944		265	961	NODE_5123586
	21	255132	127253	9589	11145	12116	2.00492		248	648	NODE_5446931

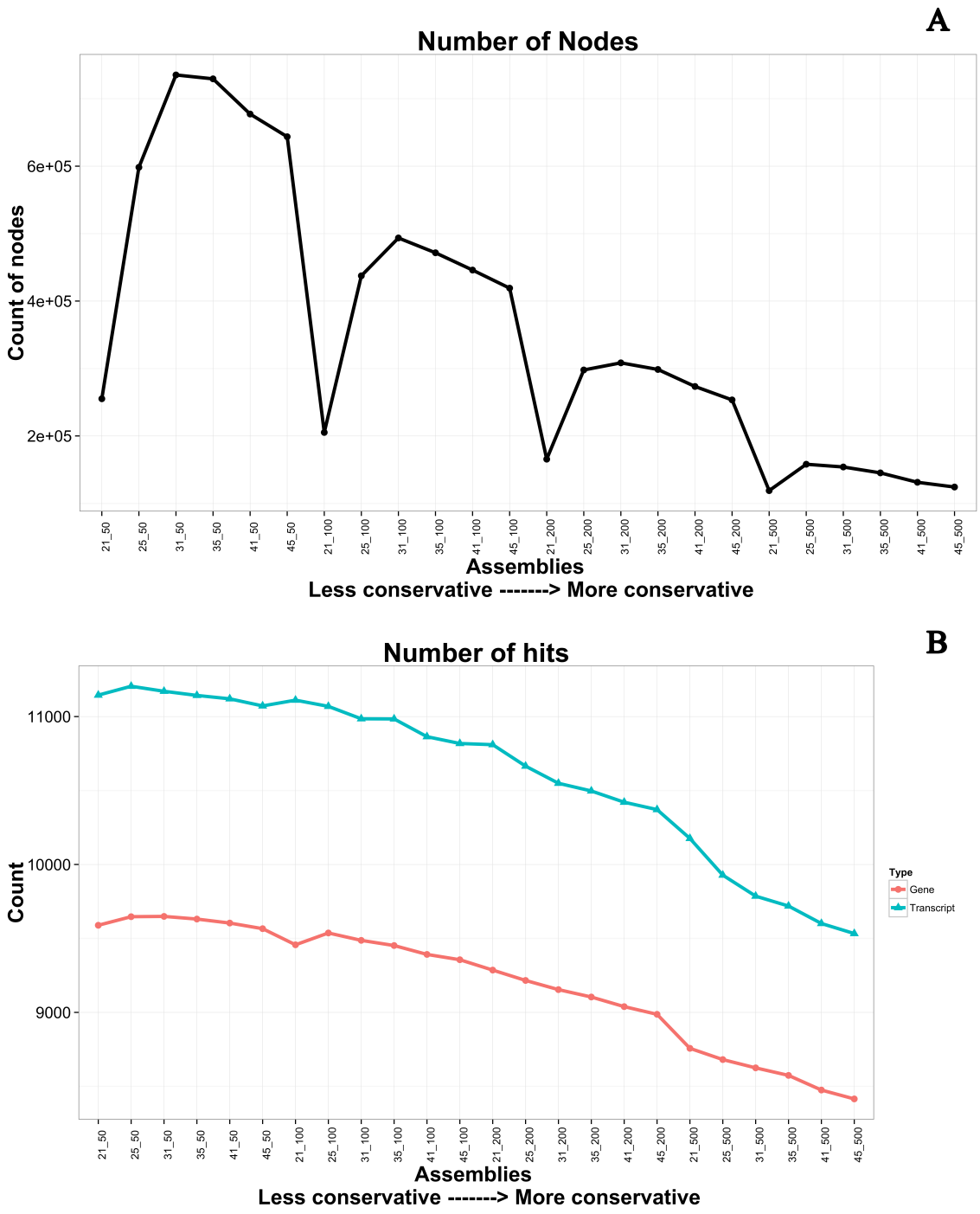


Figure 1. Descriptive figures of *de novo Chrysomya rufifacies* transcriptomes. A) Line plot of total number of nodes (y-axis) per assembly (x-axis), ordered from most conservative to most permissive. B) Line plot of count of unique hits (y-axis) of *D. melanogaster* genes (red) and transcripts (blue) per assembly (x-axis), ordered from most conservative to most permissive.

Differential expression

On average, 18.5 nodes (maximum 34, minimum 0) in 9 splicing graphs (maximum 16, minimum 0) were differentially expressed between predators and non-predators (Figure 34). These differentially expressed nodes were predominantly up-regulated in predators, relative to their non-predatory siblings (Figure 35). One female predator did not show the same pattern of gene expression as the other predator libraries. Differential expression followed a similar pattern as general assembly statistics- within a coverage cutoff, the number of nodes was low initially, peaked at 41, and then dropped off again (Figure 34). Some of these splicing graphs had no homology to any known *D. melanogaster* genes, and some were found to align to multiple genes. A total of 36 genes were annotated, with most (23) only being detected once in four or fewer assemblies (Table 20). Of the remaining 13 genes, most were detected only once per assembly- the exception was *AMP-activated protein kinase α subunit* (*CrAMPKα*), detected in two different splicing graphs in both 25_50 and 25_100.

Eleven genes were detected in seven or more assemblies as being differentially expressed. Three have not yet been named in *D. melanogaster*, though two of these had some predicted function information. DmCG5254 is predicted to have mitochondrial trans-membrane transporter function, and DmCG1336 contains a calponin-like domain. The four most frequently homologous predator up-regulated genes were *Host cell factor* (*CrHcf*), arginase (*Crarg*), *CrAMPKα*, and *silver* (*Crsvr*) (20, 18, and 16 times respectively). Only one gene, *asterix* (*Crarx*), was up-regulated in non-predators and found in more than 7 assemblies.

Table 20. Genes differentially expressed between predators and non-predators in *de novo Chrysomya rufifacies* transcriptomes. This table summarizes the results of analysis of genes differentially expressed between actively predating and non-predating *C. rufifacies* third instars across 24 *de novo* transcriptome assemblies. Columns (left to right): Up-regulated (up arrows) or down-regulated (down arrows) in predators, number of assemblies detected in (#), name of gene based on *D. melanogaster* annotation (Name), experimental molecular function(s) (Experimental), predicted molecular function(s) (Predicted), and entry in the InterPro database.

	#	Name	Experimental	Predicted	InterProt
↑	20	*Host cell factor*	chromatin binding; contributes to histone acetyltransferase activity	sequence-specific DNA binding transcription factor activity; transcription coactivator activity	Fibronectin type III; Kelch repeat type 1; Immunoglobulin-like fold; Kelch-type beta propeller; Galactose oxidase, beta-propeller
↑	20	*arginase*		arginase activity; metal ion binding	Ureohydrolase; Arginase; Ureohydrolase domain
↑	18	*AMP-activated protein kinase α subunit*	AMP-activated protein kinase activity	ATP binding; G-protein coupled receptor kinase activity; protein serine/threonine kinase activity	Protein kinase domain; Serine/threonine/dual specificity protein kinase, catalytic domain; Serine/threonine-protein kinase, active site; Protein kinase-like domain; Protein kinase, ATP binding site; KA1 domain/Ssp2 C-terminal domain; 5'-AMP-activated protein kinase catalytic subunit alpha-2
↑	16	*silver*	carboxypeptidase activity; metallocarboxypeptidase activity	metallocarboxypeptidase activity; serine-type carboxypeptidase activity; zinc ion binding	Peptidase M14, carboxypeptidase A; Carboxypeptidase-like, regulatory domain; Carboxypeptidase, regulatory domain; Peptidase M14B, caboxypeptidase D
↑	14	*Transport and Golgi organization 5*			
↓	11	*asterix*			TRM13/UPF0224 family, U11-48K-like CHHC zinc finger domain
↑	10	*glass bottom boat*		growth factor activity; transforming growth factor beta receptor binding	Transforming growth factor-beta, N-terminal; Transforming growth factor-beta, C-terminal; Transforming growth factor-beta-related; Transforming growth factor beta, conserved site; Cystine-knot cytokine

Table 19. Continued

	#	Name	Experimental	Predicted	InterProt
↑	9	CG5254		transmembrane transporter activity; tricarboxylate secondary active transmembrane transporter activity	Mitochondrial substrate/solute carrier; Mitochondrial carrier domain
↑	8	CG5273			
↑	7	CG13366			Calponin homology domain
↓	4 assemblies	CG14036(↓), CG7122(↑)			
↑	3 assemblies	CG15618, CG33178, CG6051			
↑	1 assembly each	CG10546, CG12173, CG12673, CG14478, CG1512, CG15592, CG17715, CG2146, CG30109, CG31064, CG31543, CG32694, CG4511, CG6521, CG7583, CG7595, CG8306, CG9155, CG9239, CG9985			

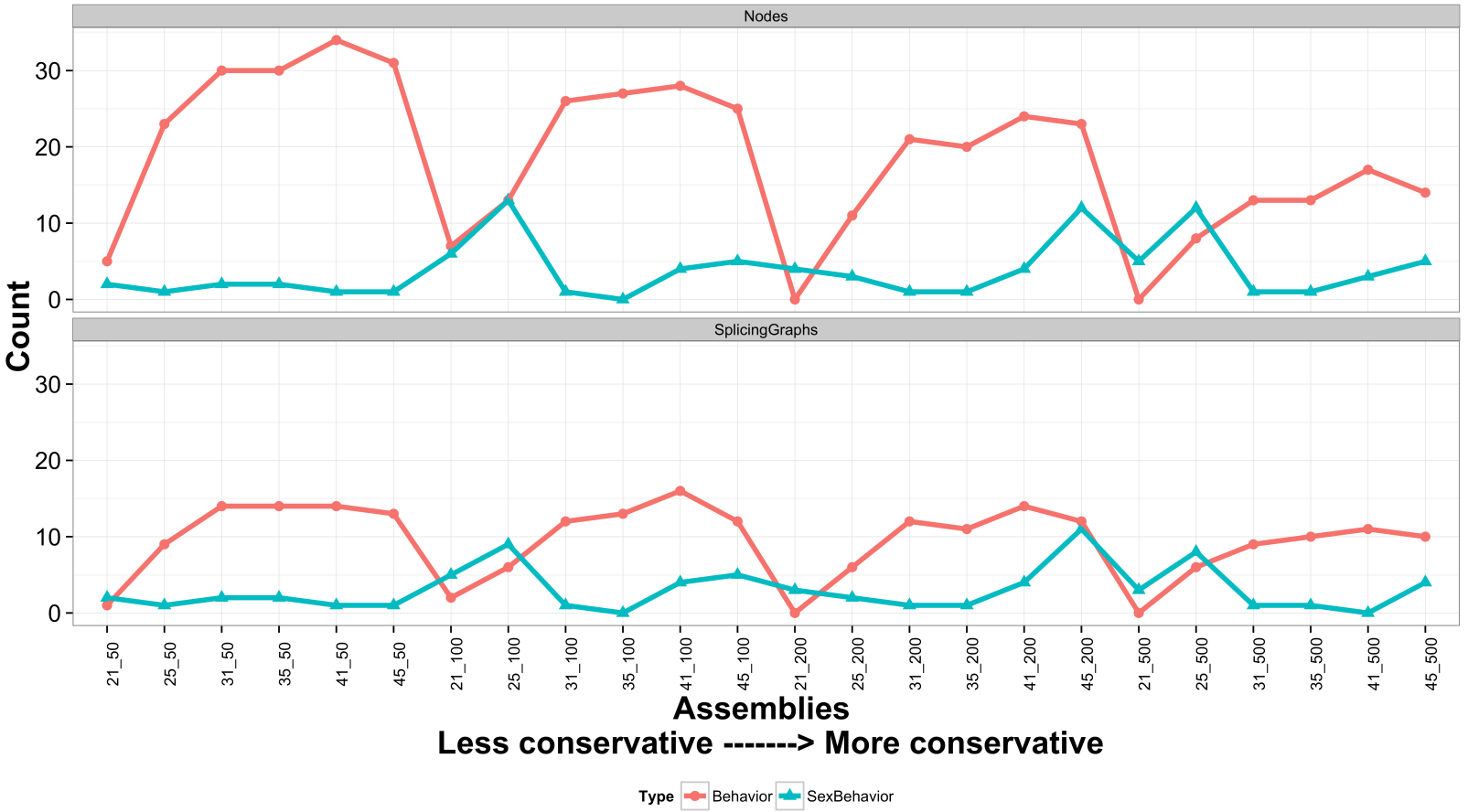


Figure 34. Differential expression between predator and non-predator third instar *Chrysomya rufifacies* in *de novo* transcriptomes. Top: Line plot of count of number of nodes (y-axis) differentially expressed by behavior (red) and sexually dimorphically (blue) per assembly (x-axis), ordered from most conservative to most permissive. Bottom: Line plot of count of number of splicing graphs (y-axis) differentially expressed by behavior (red) and sexually dimorphically (blue) per assembly (x-axis), ordered from most conservative to most permissive.

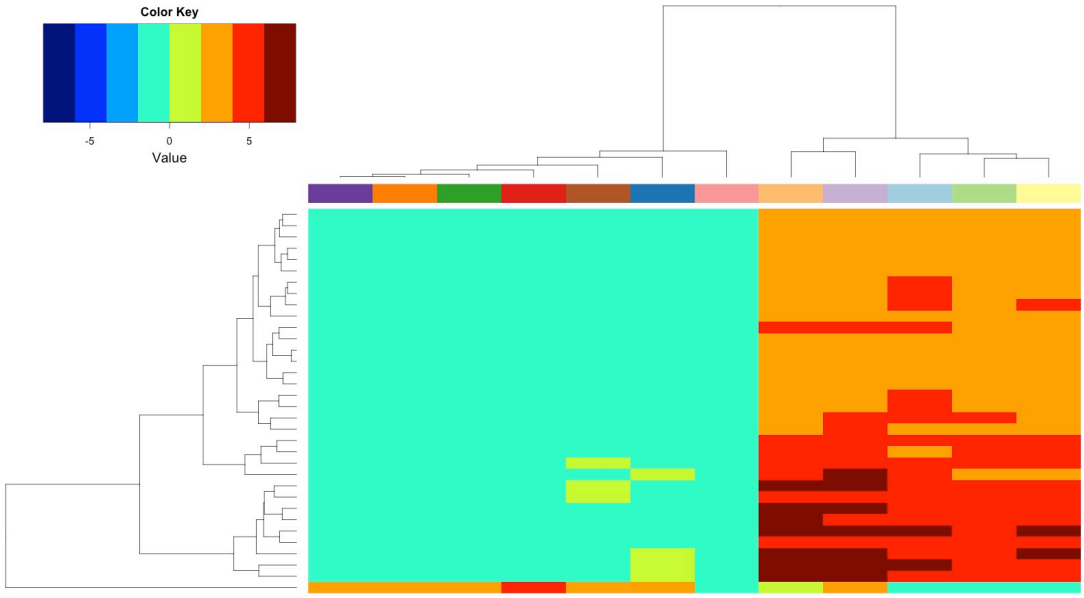


Figure 35. Heatmap of differentially expressed nodes between currently predating and non-predating third instar *Chrysomya rufifacies* larvae. This figure shows a heatmap of differentially expressed nodes (rows) in 12 different libraries (columns) of currently predating individual larvae and their non-predating siblings, reciprocally hierarchically clustered by similarity in expression pattern.

Most assemblies with statistical differential expression had at least one splicing graph which could not be annotated with *D. melanogaster*. Predicted transcripts from these splicing graphs generally did not have significant BLAST (blastn, blastx, or tblastx) hits. The exceptions were two splicing graphs in different assemblies with homologies to *Musca domestia* L. (Diptera: Muscidae) *Hcf* (XM_005187593.2), and three assemblies with homologies to *Chrysomya bezziana* Villeneuve (Diptera: Calliphoridae) *pertrophin-48 precursor* (AF139718.1) and *Lucilia cuprina* Wiedemann (Diptera: Calliphoridae) *heat shock proteins 24* and *83* (HQ609500.1 and HQ609502.1). The remaining un-

annotated predicted transcripts were compared to the conserved domain database (CDD). Annotation of these transcripts were to domains not found in insects: Nematoda phylum NADH dehydrogenase subunit 2 ($E < 10^{-10}$), EpsG family proteins related to a *Bacillus subtilis* (Cohn) (Bacillales: Bacillaceae) membrane-bound glycosyl transferase ($E < 10^{-10}$), Bateriodes phylum integral membrane protein (domain of unknown function) DUF4271 ($E < 10^{-9}$), and *Borellia* genus ORF-A ($E < 10^{-9}$).

In comparison, an average of 3.75 nodes in 3 splicing graphs were found to have sexual dimorphism in expression relative to the predation behavior, and there was no obvious pattern relative to assembly parameters (Figure 34). A total of 40 genes were sexually-dimorphically expressed by behavior, though 27 of these were detected in fewer than 7 assemblies (Table 21). The remaining three genes were all up-regulated in female predators and male non-predators, relative to male predators and female non-predators. There were significantly differentially expressed genes with the opposite pattern in 7 or more assemblies. Two of the most frequently differentially expressed genes (CrCG4847 and *CrCp1*, 10 and 8 assemblies respectively) are both cysteine peptidases. Though no experimentally determined function has been identified for CrCG8235 (7 assemblies), it is predicted to have a tRNA binding function based on sequence homology to known proteins.

Table 21. Genes sexually-dimorphically differentially expressed between predator and non-predator third instar *Chrysomya rufifacies* in *de novo* transcriptomes. This table summarizes the results of analysis of sexually dimorphic gene expression with respect to predation behavior in third instar *C. rufifacies* across 24 *de novo* transcriptome assemblies. Columns (left to right): Up-regulated in (up arrows) or down-regulated (down arrows) in predatory females and non-predatory males relative to predatory males and non-predatory females, number of assemblies detected in (#), name of gene based on *D. melanogaster* annotation (Name), experimental molecular function(s) (Experimental), predicted molecular function(s) (Predicted), and entry in the InterPro database.

	#	Name	Experimental	Predicted	InterProt
↑	10	CG4847		cysteine-type endopeptidase activity; cysteine-type peptidase activity	Cysteine peptidase, cysteine active site; Peptidase C1A, papain C-terminal; Peptidase C1A; Proteinase inhibitor I29, cathepsin propeptide; Cysteine peptidase, asparagine active site
↑	8	*Cysteine proteinase-1*	cysteine-type endopeptidase activity; peptidase activity	cysteine-type endopeptidase activity	Cysteine peptidase, cysteine active site; Peptidase C1A, papain C-terminal; Peptidase C1A; Proteinase inhibitor I29, cathepsin propeptide; Cysteine peptidase, histidine active site; Cysteine peptidase, asparagine active site
↑	7	CG8235		tRNA binding	tRNA-binding domain; Nucleic acid-binding, OB-fold
	4 assemblies	CG15118			
	3 assemblies	CG3819			
	2 assemblies	CG13138, CG14480, CG32904, CG3292, CG4139, CG5502, CG9411			
	1 assembly each	CG10737, CG10776, CG11857, CG11866, CG12025, CG12065, CG12178, CG14304, CG15817, CG17664, CG2604, CG3032, CG30377, CG31716, CG32644, CG32647, CG33113, CG3820, CG3954, CG3962, CG4019, CG42268, CG42308, CG42671, CG42678, CG6424, CG6501, CG9373			

Discussion and conclusions

The behavioral work here represents the first attempt to characterize sexual dimorphism in predation rates in blow fly larvae in both laboratory and wild populations [387, 388]. While the treatment groups had a 2.442 increased odds of survival relative to the control group, consumption (in whole or in part) of supplementary food only increased survival odds by 0.342. At this developmental stage in blow flies, there are several thresholds associated with the initiation of pupation and survival of metamorphosis such as minimum viable weight (MVW) and critical weight (CW) which may explain some of the observations of this study [389]. Specifically, some individuals in the control group may have not yet reached the MVW needed to initiate pupation whereas individuals in the treatment group that did not predate had already passed this threshold. However, the fact that 100% of the "partial consumption" group survived despite accounting for 46% of all individuals in the treatment category suggests that consumption of supplementary food alone does not account for this increased survival. In previous work, *C. rufifacies* larvae were observed to be capable of surviving to eclosion at 45% of their maximum attainable weight [390]. As larvae used in this work were reared on an excess of food [391], all eggs within a replicate were laid within three hours of each other, and all assays were done at a standardized time relative to oviposition, suggesting that general nutritional state alone is not sufficient to account for these differences in survival. Therefore, an alternative hypothesis is that supplementary food in the form of predation on other maggots satisfies some specific nutritional requirement [392, 393], and that individuals which did not meet this goal even with

predation were not viable. The replicate effects observed in this work certainly could support this hypothesis, as there are likely to be subtle nutritional or microbial community differences that may have affected both predation and survival [394, 395]. Though there was no observed difference in size of larvae within a replicate or between treatments, individual larvae were not quantitatively compared. Future work to correlate weight or length measurements to survival and predation rates may help account for some of this variation and help determine when facultative predation has the greatest impact on survival.

The monogenic sex-determination mechanism is *C. rufifacies* is still not well understood [252]. This species exhibits homomorphic sex chromosomes, and males and females are indistinguishable on the basis of genome size and karyotyping [190, 208, 396]. It has been suggested that thelygenic females are heterozygote dominant for a genetic factor which causes them to have all female offspring [181]. The arrhenogenic females and males are hypothesized to be homozygous recessive at this same locus. Despite this unusual sex-determination mechanism, the overall sex ratio of adults in this species has been expected to adhere to a 50:50 ratio based on Hardy-Weinberg equilibrium and Fischerian sex ratio expectations [53], and preliminary work supported this assertion [178]. It is not clear whether the variability in sex ratios observed in the present work is a feature of this experiment and laboratory conditions or a natural phenomenon in this species.

There was mixed evidence for sexual dimorphism in predation rates. No individual replicate provided statistical evidence for differing predation level rates between males

and females. The calculation of Cochran-Mantel-Haenszel and Breslow-Day tests statistics were complicated by: statistical blocking and the facts that 100% of the individuals which engaged in partial predation survived and that the number of males and females in each initial replicate was unknown. Therefore, though the pooled data suggests that there is some sexual-dimorphism in predation rates, this may be an artifact of the significant deviation in sex ratios from the expected value of 50:50 males and females [178]. Therefore, future work to examine sexual dimorphism in this or other behaviors in larval insects should employ methodologies that enable researchers to know the number of individuals of each sex used at the beginning of the experiment.

An attempt was made to characterize predation rates in the field of wild populations of *C. rufifacies* permitted to naturally colonize human remains. This preliminary assessment of predation in the field indicated that predation was unlikely to occur on easily visible locations on human remains, as only ten actively predating individuals were collected from a single cadaver. While all of these individuals were only collected from the maggot mass under the head, this may have been because these larvae were easier to observe. While most of these predating individuals were male, the small sample size makes extrapolation problematic. Additional fieldwork to assess predation rates is therefore needed, and molecular tools to evaluate the prevalence of predation would be useful given the difficulty observing the behavior in a natural and undisturbed context.

The *de novo* transcriptome analysis presented here provides a suite of genetic tools for the study of a complex behavior in a relatively understudied non-model fly for which limited genetic information was available [353-355]. Molecular behavioral ecology

studies in other non-model organisms using *ab initio* or *de novo* transcriptomes used between 45 Mb and 21.5 Gb of mRNA sequence data [260, 397, 398], and Chen et al. [260] found that sequencing in a single lane was sufficient for expression profiling. Therefore, the 13.8 Gb of sequence data analyzed here should be sufficient for detection of differentially expressed transcripts even at a low level.

Compared to the number of nodes and splicing graphs assembled in the *de novo* transcriptome assemblies, a low number of nodes were found to be differentially expressed between predators and non-predators. Furthermore, the *Drosophila* genes homologous to these differentially expressed splicing graphs were moderately consistent, with several identified as differentially expressed across at least one third of assemblies.

The human homolog of the gene *Hcf1* was shown to code for a protein necessary for transcription of genes of viruses in the herpes simplex family, and subsequently demonstrated to be essential for normal cell cycle functioning in vertebrate cells [399, 400]. A single *Hcf* homolog has been identified in *D. melanogaster*, and it has also been shown to be necessary for transcription activation and the regulation of cellular growth [401, 402]. Specifically, localization of *DmHcf* with histone methyl- and acetyltransfase complexes suggests that *DmHcf* regulated transcription through chromatin remodeling [403-405]. Furthermore, *D. melanogaster* mutants for this gene demonstrate 50% survival through the pupal stage, with reduced body size due, in part, to reduced cell size [267]. Additionally, this gene has been shown to be a part of regulatory networks related to osmotic stress [406]. The significant enrichment of *CrHcf* in predatory individuals

coupled with the results of previous work that showed that *C. rufifacies* only engages in cannibalism under conditions of water stress [163], suggests that predation could be a response to water stress conditions.

The gene *AMPKα* has been experimentally demonstrated to induce hypersensitivity to starvation in *Drosophila* [407], mutants being small bodied with small fat cells, low triglyceride levels, and low pupal survival, despite consuming more calories than the wild-type controls. Further work has shown that this gene functions to regulate both nutrient absorption and smooth muscle function in the gastrointestinal tract, as well as phagocytosis of *Candida albicans* (Saccharomycetales: Saccharomycetaceae) as part of the *Target of Rapamycin* (*TOR*) signaling pathway [408, 409]. One mechanism through which *AMPKα* impacts nutrition-state dependent behavior and physiology is the role it plays in proper dendrite morphogenesis and the maintenance of neural cell integrity [410]. Furthermore, work in *Drosophila* with *Ras homolog enriched in brain* (*Rheb*) demonstrated that a mutation that induced axon-misrouting and decreased phototaxis could be rescued by nutritional state or altered *AMPKα* expression [411]. Many of the mutant alleles for *AMPKα* demonstrate the greatest aberration in behavior and phenotype in the third larval instar or late second instar, and may also exhibit significant lethality beginning in the third instar and into the pupal stage [267]. Altogether, this suggests that altered expression of *AMPKα* would begin in the late second instar and become most significant in effect in the third instar, and indeed this is the stage at which predation is observed most frequently in *C. rufifacies* [307, 332]. Furthermore, predation behavior

may be due not only to altered *AMPKα* expression as a result of nutritional state, but more specifically due to differences in neuronal morphogenesis.

In *Drosophila, glass bottom boat* (*gbb*) is a growth factor that is classically identified by its significant larval mortality, abnormal gut and wing morphogenesis, transparent appearance due to fat body defects, and reduction in brain size in specific parts of the brain [412-414]. Products of this *gbb* a have been found to be located in the Golgi apparatus and endoplasmic reticulum. This gene and its homologs in mammals, *bone morphogenic protein 5/6/7/8*, are part of a conserved cell-signaling pathway known as the Bone Morphgenic Pathway (BMP) [415]. As a part of the BMP-signaling function, the product of *gbb* has been shown to be an important part of the proper function and morphology of the neuromuscular junction, and mutations at this gene can result in reduced *gbb*-derived ligand-guided neuromuscular synapse development [415, 416]. Some mutations in *gbb* result in aberrant phenotypes similar to nutrient-starved or *TOR* signaling mutants [413], and loss-of-function *Dmgbb* mutants maintain a physiological state similar to that induced by starvation in wild-type flies. The *Dmgbb* mutants did not exactly mimic gene expression induced by starvation and this appears to be due to alteration of fat body signaling, lipid transport, and sensitivity to nutritionally available lipids. Differential expression between predators and non-predators in *Crgbb* could therefore have several causes, including developmental ontogeny, neurological or muscular dimorphism, and differences in responsiveness to dietary lipids.

Another gene that was differentially expressed in this work and localized in association with the Golgi apparatus is *Tango5*, which in *Drosophila* may function

directly in membrane trafficking [417]. Information on the function of this gene in *Drosophila* is limited. Gene expression studies have shown that it is highly expressed in the digestive system of wandering third instar larvae and in new white prepupae [267]. Further, expression is high in the central nervous system and trachea of larvae but absent in these tissues in adults. The association of this gene with the Golgi paired with its high expression in the CNS and alimentary tract of larvae suggests that it plays a role in secretion, though additional research is needed.

An *arg* homolog was found to be differentially expressed in 20 of 24 assemblies. This gene is predicted to produce a protein that catabolizes arginine, though its exact function in *Drosophila* is still unknown [274, 418]. This gene, *arg*, was putatively identified as a genetic marker of aggressive *Drosophila* in transcriptional profiles via a selection experiment and microarray analysis, though further work with mutants at only the *arg* locus failed to detect a significant difference in aggression behavior. However, the microarray analyses were done with males and females, while the further work looking at the effect of a single locus only used males [419]. Furthermore, this functional work examined only the effect of altered expression at a single locus, and does not take into account the possibility of the interaction of mutations of multiple genes.

Initial research on *svr* focused on the phenotypic effect of mutations on body color, cuticular tanning, and wing morphology [420], and also demonstrated that some mutants exhibit increased N-acetyldopamine levels. Furthermore, incubation of unpigmented pharate adults with dopamine induced a phenotype similar to the *svr* mutation, consistent with the fact that N-acetyldopamine is downstream of dopamine in melanic pathways.

Later work at the *svr* locus identified six alternative splice forms and three carboxypeptidase D (CPD) domains [421], the first two of which demonstrate a preference for C-terminal Arg residues [422]. Furthermore, male adults mutants at the *svr* locus demonstrate long-term memory deficits, though this effect depends upon which of the three CPD domains is compromised [423]. Experiments in mammals have shown that CPD is primarily located in the trans-Golgi network and is involved with secretory protein processing, specifically for neuropeptides [424-426], though this gene demonstrates pleiotropic effects in *Drosophila*. Mutations in *yellow* (*y*), also in the melanic pathway downstream from dopamine, have been shown to affect memory and behavior in a sex-specific manner [420, 427, 428]. This appears to be due to the expression of the male form of *fruitless* (fru^M) and therefore the increased expression of the *y* protein in the parts of the brain necessary for the development and expression of male behaviors [428]. Future work to determine the localization of this *svr* homolog in *C. rufifacies* both between tissue types and within cells, as well as research to characterize the effect of ectopic application or induced over-expression of N-acetyldopamine and dopamine on predation behavior would be a valuable next step.

The gene *asterix* (*asx*) was the only *Drosophila* gene to have homology with nodes that were significantly up-regulated in non-predators relative to predators. The classical presentation of mutation in this gene has a phenotype of small ovaries and female sterility, though mutant males appear normal and fertile [429]. Proper functioning of *arx* is necessary for Piwi-piRNA (Piwi-interacting RNA) complex silencing of transposons in the female germ line [429, 430]. More specifically, though cells lacking expression of

arx contain the same amount of Piwi and piRNAs as normal cells, but they lack the ability to negatively regulate transposon transcription. This suggests that the product of *arx* directs the action of the Piwi-piRNA complex. The highest expression of this gene in larvae is localized to the fat body [267], though it is unknown whether this gene serves the same function in larval fat body as it does in female gonads.

Most of the differentially expressed genes, including those with unknown functions, are located on the X chromosome in *Drosophila,* several in close proximity to each other [267]. The genes *svr* and *arg* are both located near each other on the X chromosome (1B5-1B7 and 1B7-1B8 respectively), with CG13366 at 1B11-1B12. Further down, CG5254 and CG5273 are co-located at 1C4-1C4, and only 900 bp separate them in the genome. Finally, *AMKPα* is at 2A1-2A1 and *Tango5* is at 9E4-9E6. The other differentially expressed genes discussed here, *Hcf*, *arx*, *cwo*, and *gbb*, are located on 4, 3L, 3R, and 2R respectively. Though attempts have been made at identifying the sex chromosome in *C. rufifacies*, the obvious lack of heteromorphic sex chromosomes has made this work challenging [182, 208]. In light of the lack of genetic tools available for this non-model species, it not possible to asses whether these differentially expressed genes have synteny with *D. melanogaster* or can even be found on the same chromosome. However, once the genome has been sequenced it will be possible to address some of these issues.

The gene and protein interactions of some of these genes has been elucidated in *D. melanogaster*, and suggests that some of these differentially expressed genes may be co-expressed as part of specific processes. For example, *DmHcf* has been shown to both

enhance and repress the expression of *Polycomb* (*Pc*), which in turn has been shown to enhance expression of *y* [267]. Furthermore, enhanced expression of *y* suppresses expression of *svr*. This suggests the possibility that significant up-regulation of *CrHcf* could lead to enhanced expression of *Crsvr* through repression of *Cry*, though additional work is required to identify whether these genes interact in *C. rufifacies* in the same way that they interact in *D. melanogaster*.

Given the mixed support for sexual dimorphism in the predation rate assays, it was surprising to detect significant sexual dimorphism in expression relative to the predation behavior. Furthermore, these few genes only demonstrated a pattern of significant up-regulation in female predators and male non-predators. Two of these genes, *Cysteine proteinase-1* (*Cp1*) and CG4847, are both located on 2R and both produce enzymes which catalyze the hydrolysis of internal alpha-peptide bonds through a focus on cysteine residues [431]. However, this broad family of peptidases is found in many different kingdoms serving a wide variety of functions [432]. Based on localization of the gene *Cp1*, it is predicted to function as part of the immune system, specifically in the digestion of materials brought into cells through phagocytosis [433]. In adult *Drosophila*, null mutations in this gene have been shown to lead to female sterility, partial male sterility, reduction in abdominal pigmentation, and abnormal wing morphologies [434]. Two different transcripts of *Cp1* were found to be up-regulated immediately prior to salivary gland apoptosis in pupae [435].Though this gene is highly expressed throughout all life stages in both sexes, significant expression of *Cp1* in immature *Drosophila* is especially high in the digestive tract or larvae, specifically the

salivary glands and midgut [436]. Though work in other flies has suggested that cysteine peptidases are not a large part of the extra-oral excretion/secretion enzymes produced by calliphorid larvae [437] or digestive enzymes of *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) [438], further work on endogenous proteolytic activity is required to determine whether these enzymes can be found in the midgut of *C. rufifacies*.

DmCG4847, another cysteine-proteinase, demonstrates the highest level of expression in male accessory glands and very low expression in all immature stages in *D. melanogaster* [267]. This gene appears to be specifically implicated in reproductive biology in *D. melanogaster* [439, 440]. Additional work has shown that the accessory glands in males secrete an inactive form of the product of CG4847, which undergoes an activating structural change in the female to process various hormones [441]. Several other studies on accessory gland proteins has further shown that this gene is also an accessory gland protein within the *Drosophila* genus, and appears to be under positive selection [302, 442, 443]. In protein hybridization studies, the product of CG4847 interacted strongly with the product of *proliferation disruptor* (*prod*) [444], a chromatin binding protein important for mitotic chromosome condensation [445].

The other gene that demonstrated sexual dimorphism in expression are less well understood. The gene DmCG8235, a gene predicted be a part of the aminoacyl tRNA sythetase (Aats) complex [446], is highly expressed in immature *D. melanogaster* in the central nervous system of larvae and the imaginal discs of wandering third instar larvae [267]. Furthermore, this gene was seen to increase 3 fold in expression in adult *brain tumor* ($brat^{k06028}$) mutant brain tumor cells relative to wild-type cells [447], and other

mutations in *Dmbrat* have demonstrated an increases in larval body, cell, and brain size [448, 449]. Mutations in *brat* have also been suggested to affect catecholamine and therefore dopamine metabolism [450, 451]. Significantly, the product of DmCG8235 has been shown to interact strongly with the Aats of six different amino acid tRNA synthetase, including arginine tRNA [446]. Therefore, it is possible that differences in CrCG8235 expression could be due to sexually dimorphic genetic responses to altered dopamine or arginine pathways.

The finding of significantly up-regulated transcripts in predators that only demonstrated homology to microbial (and to a lesser extent, nematode) domains raises the possibility of the microbiome having an effect on this this behavior. Certainly, research on gut microbiota in a nutritional context has demonstrated that many insects rely upon these bacterial communities for improved digestion, the production of digestive enzymes, or even the synthesis of vitamins and amino acids [452-454]. It was first demonstrated that intestinal microbes stimulate larval growth using the blow fly *Calliphora vomitoria* L. (Diptera: Calliphoridae) [455], and later work in *D. melanogaster* demonstrated that microbial contributions to larval growth in Diptera are due to *TOR*-dependent nutrient-sensing growth regulation [456, 457]. Other work on the behavioral effect of bacteria on animals has demonstrated that the microbiome or even specific bacterial species can influence mating preferences, memory and learning, and feeding choices, in some cases in a sex- or diet-specific manner [458-461]. Research has only begun investigating the complex relationship between blow flies and bacteria, though it is at least clear that microbes are important for mediating the attraction of

adults in a physiologically and sexually selective manner [395, 462, 463]. More work is needed to identify the microbial species associated with *C. rufifacies*, and whether these stimulate larval growth or affect behavior, however.

The expression pattern of one of the predatory female libraries was a significant outlier in the present work. There are some possible explanations for this. Firstly, it is possible that the "wrap-around" is not always tied to predation, and that *C. rufifacies* may exhibit this behavior under other circumstances. Secondly, the behavior and/or observed patterns of gene expression may be the result of environmental effects that could have differed between and within sibling groups, such as microbes. Thirdly, the weak evidence of sexual dimorphism in the frequency of this behavior and gene expression and the fact that there are two different kinds of females (thelygenic and arrhenogenic) leave open the possibility of these two types of females having slightly different nutritional requirements or genetic thresholds. Further work is needed to investigate these, and other, hypotheses.

There are several ecological and evolutionary implications of this work. Initial research on predation and ecological interactions between *C. rufifacies* and native North American blow fly species led to concerns of extinction of endemic Calliphoridae [383]. However, 40 years after the initial invasion and 20 years after these concerns were raised, both the native and invasive species have continued to coexist [176]. Both *C. rufifacies* and *Co. macellaria* are of international forensic relevance and are known to co-occur on human and animal remains [464, 465]. There is evidence that that the relative timing of colonization is important for both species, and *C. rufifacies* have a

highest fitness when oviposition occurs within one day of *Co. macellaria* colonization [466]. Furthermore, the presence of *C. rufifacies* alters the development and behavior of co-occurring *Co. macellaria*; treatment of *Co. macellaria* larvae with *C. rufifacies* excretion/secretions caused the *Co. macellaria* larvae to pupate at a smaller size or remain in the second instar longer relative to control samples [183]. This is in concordance with observations that *Co. macellaria* was not observed on bear carcasses past the third instar feeding stage when *C. rufifacies* also colonized the remains [464]. Finally, it has been shown that diet can have a significant impact on *C. rufifacies* development so far as both species and type of vertebrate tissue consumed [467]; however, similar work looking at the developmental consequences of predation has not yet been conducted. Given that estimation of time of colonization is an important part of forensic entomology investigations, the primary colonizing species may be driven from the resource after reaching CW or MVW, and that diet can affect development, suggests that inclusion of the results of research into predation in *C. rufifiacies* could help improve both the precision and accuracy of these estimates. Genetic markers of predation represent one useful tool for future work.

Taken all together, the results presented here suggest several particular hypotheses regarding the genetic regulation of predation behavior in *C. rufifacies*. The first in that predation may be a general starvation or water stress response, or response to sub-optimal amino acid or lipid availability. These could be tested with artificial diets or application of arginine, for example. Furthermore, given the identification of several genes related to the *TOR* signaling pathway and the significant differential expression of

transcripts of a possible microbial origin, it is possible that treatment with the antibiotic rapamycin will result in a change in the frequency of predation. This work also suggests that there may be neurological differentiation between predatory and non-predatory individuals due to differential expression of genes that are specifically related to neurogenesis, transmembrane-transport at synapses and neuromuscular-junctions, and dopamine related metabolic pathways. Therefore, experiments to evaluate the effect of treatment with dopamine or dopaminergic compounds may be one interesting direction. Though there was mixed evidence of sexual dimorphism in behavior, significant patterns of sexually dimorphic gene expression were observed. Future work in *C. rufifacies* will identify whether there are other sexually dimorphic patterns in gene expression, development, or morphologies and provide additional tools to better understand the molecular behavioral ecology of this species.

CHAPTER V

DISCUSSION AND CONCLUSIONS

The big questions

"The wise man doesn't give the right answers, he poses the right questions."

— Claude Levi-Strauss

The hairy maggot blow fly, *Chrysomya rufifacies* Macquart (Diptera: Calliphoridae), is one of two blow fly species of forensic and veterinary relevance known to have a monogenic sex-determination mechanism and engage in facultative predation, though previous research has not integrated these two aspects of its biology. Despite the handful of papers that have been published since the 1930's on the subject, we still do not know what genes or genetic cascades are involved in the maternal sex-determination mechanism observed in *C. rufifacies* [48, 180-182, 192, 193, 206, 207]. Furthermore, research on global patterns of sexual dimorphism in gene expression in immature insects is limited, in part due to the difficulty required in determining the sex of dipteran larvae [318, 330, 365]. Therefore, *C. rufifacies* is a tractable system in which to ask questions regarding developmental sexual dimorphism as females produce single-sex clutches. Finally, *C. rufifacies* and its sibling species, *C. albiceps* Weidemann, are most often studied in an effort to understand the effect of facultative predation by these invasive species on the behavior, ecology, and evolution of native blow fly species [159, 183, 332, 464, 468]. However, the proximate and ultimate causes of the initiation of predation remain unknown, and it is not clear whether there is sexual dimorphism in frequency or effect. Therefore, the unusual monogenic sex-determination mechanism of *C. rufifacies*

makes it a tractable system in which to study sexual dimorphism in behavior in immature insects, and therefore the possibility of sexual conflict happening at these stages.

Summary of results

"However beautiful the strategy, you should occasionally look at the results."

— Winston Churchill

In Chapter II, I used *de novo* transcriptomics to investigate sexual dimorphism in gene expression in adult *C. rufifacies* and to identify genetic markers of thelygeny/arrhenogeny. Approximately 9,000 genes were annotated with homology with *Drosophila melanogaster* Meigen (Diptera: Drosophilidae), and assembled sequences shared 99% identity with published genetic sequences in *C. rufifacies*. Approximately 65% of the transcriptome was differentially expressed between males and females, and demonstrated a slight but significant skew of 55% female enrichment. Many dimorphically expressed genes shared homology with genes known to affect sex determination and dosage compensation in other species. The gene *doublesex* was assembled into three nodes, with one commonly expressed node and the nodes which shared homology with male or female *dsx* sequences of other species expressed appropriately. Of note, the genes *Sex-Lethal* (*CrSxl*) and *daughterless* (*Crda*) were dimorphically expressed between males and female. Though the gene *transformer* (*tra*) did not share sufficient sequence homology with *D. melanogaster* to be annotated under my criteria, several nodes had homology to published blow fly *tra* sequences. In comparison, an average of 4% of the transcriptome was differentially expressed between

thelygenic and arrhenogenic females, but most of the theylgenically up-regulated splicing graphs did not share sufficient sequence homology with *D. melanogaster* to be annotated. I also collected larvae from human remains and observed that the distribution of sex ratios of *C. rufifacies* is male skewed and does not conform to a binomial distribution.

In Chapter III, I investigated sexual dimorphism in immature *C. rufifacies* in both development and gene expression. I observed that male *C. rufifacies* develop faster than females, and eclose an average of 9 hours sooner than females of the same age, and that the observed sex ratio was slightly male skewed from laboratory colonies. Through leveraging the unusual sex-determination mechanism of this species, I generated *de novo* transcriptomes to look at sexual dimorphism in immature insects. Sexual dimorphism in gene expression was observed in all stages of development. Egg dimorphism was female biased, whereas a male bias in differential expression was observed in all larval instars and throughout pupal development. Sexual dimorphism was most pronounced mid-pupal development. Male enriched genes in the immature stages were primarily involved in neurogenesis and synaptic function in the larval stage, and spermatogenesis in the pupal stage. In comparison, female enriched genes were primarily involved in muscular development and function in the larval stage, and oogenesis in the pupal stage. Furthermore, sex-specific analysis demonstrated that males differentially express significantly more genes throughout development than females. Several genes involved in sex-determination and sexual dimorphism in other species were differentially expressed throughout development, including *CrSxl*. Again, though a *Crtra* homolog

was not annotated with *D. melanogaster* sequences, one of the most stably differentially expressed nodes was in a splicing graph that shared significant sequence homology with published blow fly *tra* sequences. These *Crtra* nodes and *Crtra* demonstrated an expression pattern consistent with *D. melanogaster*, with differential expression of *Crtra* early in development and *Crdsx* differentially expressed beginning in the third instar [267].

Finally, in Chapter IV I investigated the possibility of sexual dimorphism in gene expression and behavior as related to facultative predation. I again found that the sex-ratios produced by my laboratory colonies were male biased. I found that individuals which partially consumed a supplementary prey *Cochliomyia macellaria* Fabricius (Diptera: Calliphoridae) survived 100% of the time, and that access to supplementary food increased survival in *C. rufifacies*. There was mixed evidence of sexual dimorphism in predation rates. Several genes up-regulated in predators relative to non-predators, and primarily these were involved in growth regulation and response to starvation (*Host cell factor*, *AMP-activated protein kinase α subunit*, and *glass bottom boat*) [401, 407, 413], neurological function (*AMP-activated protein kinase α subunit, glass bottom boat*, *Transport and Golgi organization 5,* and *silver*) [267, 410, 416, 423] in *D. melanogaster*. Three markers were sexually dimorphically expressed relative to predation, primarily up-regulated in female predators and male non-predators, and are predicted to produce cysteine-proteinases (*Cr*CG4847 and *Cysteine proteinase-1*) and tRNA (*Cr*CG8235).

Synthesis

"Thinking is the hardest work there is,

which is probably the reason why so few engage in it."

— Henry Ford

There are several interesting comparisons between the different analyses discussed the previous three chapters. Firstly, despite the challenges of genomics and variety of approaches, the results of my work are similar to that of other species in terms of broad patterns of differential gene expression. Secondly, sexual dimorphism in gene expression was observed in all life stages, though the magnitude of differential expression and direction of bias changed. The genes dimorphically expressed between stages and stages and predators and non-predators suggest that dimorphism may have an effect in development and behavior. Finally, the number of genes differentially expressed between types of females and the results of the organismal work suggests several interesting hypothesis related to both sex determination and the ecology of *C. rufifacies*.

Genomics and bioinformatics in non-model organisms

There will always be challenges in genomics utilizing next generation sequencing technologies, and it is unlikely that a "magic bioinformatics bullet" will be developed. One difficulty is that assembly parameters such as the hash-length and coverage cut-off can have a significant effect on the quality and completeness of assemblies, and assembly can be memory intensive and require super-computing capabilities. Furthermore, different assembly programs produce different outputs and there is variation in performance of various assembly algorithms [202, 469-472]. Another

challenge with *de novo* transcriptomes is that it is not yet clear what metrics are best for identifying optimal transcriptome assemblies [473, 474], and annotating transcriptomes can be difficult in non-model organisms [475]. Despite these challenges, genomics remains a valuable tool for studies in both model and non-model organisms. The large volume of data generated by next-generation sequencing technologies also provides significant experimental power [282]. Given that over 300Tbp of sequence data were generated in the course of this work, suggests that detection of differential expression should be possible even for very rare transcripts. Furthermore, genomics and transcriptomics studies are a powerful tool for developing specific hypothesis related to the mechanism and causes of traits of interest.

Despite the potential pitfalls of genomics outlined, comparison of the results of my analyses with previously published work in *D. melanogaster* and other species of flies is comforting. Similar work in the fruit fly *Bactrocera dorsalis* Hendel (Diptera: Tephritidae) and the flesh fly *Sarcophaga crassipalpis* Macquart (Diptera: Sarcophagidae) annotated between ~ 7,400 and ~9,300 genes based on sequence homology to *D. melanogaster*, despite using 454-pyrosequencing for their mRNA libraries [476, 477]. Furthermore, although the mathematical nature of the analysis I employed was blind to the ontology of the assembled nodes, there was conformity in the general gene ontology of differential gene expression associated with both sexual dimorphism in adults as well as patterns of gene expression related to specific developmental stages. Differentially expressed genes between adults were female biased and comprised approximately half of the transcriptome, female biased genes exhibited a

greater variance in expression, and male biased genes exhibited a larger magnitude of fold induction [285, 478, 479].

Additional work is necessary to determine the validity of these results in *C. rufifacies* and also identify how applicable these results are to other species. One bioinformatics concern is discerning between computational artifacts and real signals, some of which can only be resolved though validation work including PCR. The large quantity of sequence data I generated necessitated the development of an assembly algorithm with efficient memory and parallelization capabilities to take advantage of available super-computing infrastructure. However, the agreement between my results and previously published work in other species using different bioinformatics tools suggests that the exact assembly algorithm or piece of analytical software used may not matter as much as experimental design. Therefore, future bioinformatics work in this species may benefit from additional number of replicates for each stage or sample of interest to increase the precision and accuracy of the differential expression patterns.

Sex determination and genetic markers

Three hypotheses regarding sex determination in *C. rufifacies* have been considered in the literature. *Sex-lethal* had been characterized in this species and lack of sexual dimorphism in expression and splicing led researchers to believe that *Sxl* was not an important part of this system [206]. Hybridization studies with a *da* probe demonstrated that a homolog to this gene was located near a sex-determination-linked chromosomal translocation [204]. However, *da* is involved in dosage compensation through interactions with *Sxl* in *D. melanogaster* [299], and so it was assumed that *da* also did

not regulate monogenic sex–determination system in *C. rufifacies* [252]. Finally, research in blow flies suggested that *tra* is the master regulator of sex determination in Calliphoridae [39], and as such it was believed that a mutation in *tra* might cause the production of single-sex clutches.

While the exact genetic mechanism responsible for the monogenic sex-determination mechanism in *C. rufifacies* has not been elucidated by the present work, several potential genetic markers have been generated. Many genes were differentially expressed between males and females. Of particular interest are the sequences of *doublesex* (*Crdsx*), *transformer* (*Crtra*), *transformer-2* (*Crtra2*), and *Sex-lethal* (*CrSxl*). These three genes were shown to be differentially expressed between males and females in all life stages. Furthermore, it was observed that the *Crtra* differential expression was significant in early in development in the egg and larval stages, while *Crdsx* nodes were significantly differentially expressed beginning in the third instar and continuing to adults. This suggests that there may be conservation in the genetic cascade leading to sexual differentiation, with *tra* acting upstream of *dsx* in the. The differential expression of *CrSxl* and *Crda* was unexpected. Previous work in *C. rufifacies* and other species has demonstrated that *Sxl* is not sex specifically spliced or expressed, and may not be a primary signal in sex determination in these species [77, 206, 297]. Work in other species has shown that the product of *tra2* is important for the maintenance of *tra*-directed splicing and autoregulation [64, 201, 480, 481], though evidence of differential expression or splicing is taxon specific. In *D. melanogaster, tra2* is important in proper splicing of *dsx* into the female form through its participation in the TRA/TRA2

spliceosome complex [481, 482], and does demonstrate some sexually dimorphic expression [483]. However, *tra2* has not been shown to be sex-specifically spliced or expressed in somatic tissues in Calliphoridae or Tephritidae [39, 367, 371].

Several potential markers of thelygenic and arrhenogenic females were also generated in this work. Particularly interesting are the nodes with homology to *fruitless* (*Crfru-RD*), *Trithorax-like* (*CrTrl-RF*), *female-lethal (2) d* (*Crfl(2)d*) and *C. rufifacies* sequence with homology to second female *Co. macellaria tra* exon, as these were differentially expressed between types of females and the homologous genes are known to affect sex-determination and viability in other species. Other interesting targets are involved in meiosis chromosome segregation such as *Replication factor C subunit 4* (*CrRfC4*) [280], enriched in thelygenic females, and maternally-deposited genes which direct transcription and translation such as *encore* (*Crenc*) [484], up-regulated in arrhenogenic females.

Taken together, future research in sex determination in *C. rufifacies* should re-examine *Sxl* and *da*, and continue to focus on *tra*. Furthermore, several genetic hypotheses regarding the maternal effect genes have been identified above, and warrant future investigation. Validation work to examine the sequence, and temporal and spatial patterns of expression of these genes is necessary. However, sequences are now available for the development of genetic markers of sex and potentially sex-producer type for testing specific hypotheses and ultimately identifying the genetic mechanisms responsible.

Sexual dimorphism, dosage compensation, and sexual conflict

Certain patterns in sexual dimorphism in gene expression were observed in both the adult-only and developmental pattern analysis. In agreement with work in other species, sexual dimorphism in gene expression can be detected in *C. rufifacies* in the egg stage [330, 365]. The present work is unique in the identification of sex-specific patterns of gene expression throughout development. The observed dimorphic gene expression patterns suggest that neurological and muscular divergence between males and females starts in the first instar. Metabolic genes are also differentially expressed between males and females, though whether this is the result of faster male development rates or sexual dimorphism in nutritional requirements remains to be tested. There is evidence from *D. melanogaster* that the *TOR* signaling pathway that regulates growth and interacts with the insulin signaling and protein metabolism pathways is also involved in sexual dimorphism in behavioral responses to nutrient availability [485-487], though most of this work was done with adult flies. Given the differential expression of genes involved in neurogenesis, signaling, and the *TOR* signaling cascade regulation of growth related to predation behavior suggests that the interaction of nutrition, behavior, and development deserves more attention in this species. There is some evidence that *C. rufifacies* is a poor competitor relative to other blow fly species [488], and adoption of facultative predation may be one strategy to reduce interspecific competition. This is supported by the assertion of some researchers that the paucity of nutritionally-explicit research on sexual reproduction and the development and maintenance of sexual dimorphism [489]. Certain hypotheses, for example the effect of dopamine, arginine, or

rapamycin on predation behavior, survival, and fitness, are one potential direction for research.

Also of interest related to sexual dimorphism in expression is the possibility of dosage compensation being in action in *C. rufifacies* despite the lack of heteromorphic sex chromosomes. Some of the genes sexually dimorphically expressed in adults included *CrSxl* and *Crmsl2*, homologs of which are known to be important components of the dosage compensation machinery in *D. melanogaster* [251, 298, 364, 490]. Dosage compensation as a method for the balancing gene dose between sex chromosomes and autosomes was first proposed by Ohno [491], and initial research in *D. melanogaster* certainly supported this hypothesis. The prevalence of dosage compensation in animals is controversial [492], however, and the shift to the increased sensitivity of RNAseq experiments over microarray experiment has not shown evidence of up-regulation in X linked protein expression [493]. However, this work also demonstrated evidence that dosage compensation may be transient, as occur in immatures but not adults, at least in *Caenorhabditis elegans* Maupas (Rhabditida: Rhabditidae). There was a conspicuous pattern of male up-regulation compared to females during immature development but not adults in the present work. Some of this may be attributable to the fact that many of the female up-regulated nodes were not annotated with *D. melanogaster* sequences under the current criteria, including female nodes in the immature analysis and thelygenic-enriched nodes in the adults. This cannot account entirely for the pattern of male-biased expression, however, as the differentially expressed nodes exhibited a significant male-bias throughout development as well. It is curious that the male-biased nodes exhibited

more sequence ontology (given the higher level of annotation) compared to the female nodes, which may reflect the maternal sex-determination system. Studies in *M. domestica*, a species which also exhibits arrhenogeny, have identified patterns of "masculinization" of third chromosome gene expression in males carrying autosomal dominant male determiner on the third chromosome [494]. Work in the zebrafish, *Danio rerio* Hamilton (Cypriniformes: Cyprinidae), a species without a described sex chromosome or genetic sex marker [495], has shown that this species also exhibits masculinization of the transcriptome, with male-enriched genes demonstrating higher magnitude fold-change than female-biased genes [496]. However, sex determination in the zebrafish is at least partially controlled by the environment [497, 498], and so it is not clear whether the similarities in male-biased expression patterns are due to the same evolutionary forces.

The importance of dosage compensation in immature insects but not adults remains an unexplored, and could be a valuable tool to leverage in transgenic insect control programs. In any case, assessment of whether dosage compensation is happening, which sex it occurs, and the significance of its timing is not possible without mapping to a reference genome. Work in the Indian meal moth, *Plodia interpunctella* Guenée (Lepidoptera: Pyralidae), used the genome of the domesticated silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae), to map reads and investigate dosage compensation [499]. However, given the uniqueness of the karyotype of *C. rufifacies*, collaboration with researchers to sequence and assemble the genome of this species is likely to be the most effective method to address the issue of dosage compensation in this species.

Sex ratios and the ecology of C. rufifacies

One of the consistent patterns throughout the whole-organism work was the observation that sex ratios, both in the laboratory and in the wild, deviate from 50:50 and demonstrate a significant male bias. While there are several potential explanations, one, which stands out, is the possibility that arrhenogenic and thelygenic females have slightly different reproductive physiologies or behaviors. Previous work did not show a significant difference in fecundity between types of females [180]. However, the subtly unequal sex ratios taken together with the fact that arrhenogenic females significantly up-regulate genes related to oogenesis and provisioning of offspring suggests that it is possible that male-producing females produce more eggs than thelygenic females. Another possibility is that thelygenic and arrhenogenic females have slightly different preferences for oviposition sites or tolerance of laboratory conditions, and as such the sampling in the present work failed to capture the full spectrum of the preferences of this species. There is evidence that *C. rufifacies* larval development is significantly affected by the species and tissue type they are feeding on, and females may be able to detect differences in oviposition resources and respond to them [467]. Prior to this work, there were only two methods to differentiate between female types: isolate a female and phenotype based on offspring sex, or use sex-determination linked eye-color mutations, though work has not been done with these mutated lines outside of the lab which created them [182]. Therefore, application of these tools to investigate whether females are physiologically capable of detecting differences in oviposition substrates, demonstrate a

preference, or have equivalent fecundities could be a valuable first step in determining the cause of the right-skewed sex-ratio distribution observed here.

Sexual conflict

It was not possible with the experimental design employed to directly test for genetic sexual conflict in *C. rufifacies* with either the gene expression or behavioral work. While there are differences in expression between males and females, the function and effect of these differences remain unknown. Furthermore, the gene expression differences between thelygenic and arrhenogenic females (as discussed above) suggest that these two types of females may respond differently to the same selective pressure. One avenue of future work could rely upon selection experiments to manipulate selective pressures on one sex but not the other, or on one type of female versus another. Altered gene expression through transgenic manipulation or RNAi could be another way to investigate genetic sexual conflict in this species.

Sex-specific differences in survival related to predation behavior, could not be answered with the experimental design employed here. Lack of knowledge regarding the initial sex-ratio distribution makes assessment of sexual dimorphism is survival rates problematic. Therefore, it is suggested that future work either starts with an equal number of male and female larvae or a known ratio of males to females. The former may be possible though the application of genetic markers for thelygeny and arrhenogeny developed in *C. rufifacies* in laboratory studies, or in species with sexually dimorphic eggs [318]. However, sub-sampling and qPCR to assess sample-level ratios of male and female markers is likely to be the most broadly applicable approach for samples of *C.*

rufifacies collected in the field. Furthermore, given that males and females develop at different rates as shown here and that cannibalism occurs in this species [163], manipulation of the starting sex ratio could have an impact on the frequency and impact of facultative predation.

Conclusion

"I am turned into a sort of machine for observing facts and grinding out conclusions."

— Charles Darwin

The work presented in this dissertation provides a foundational set of tools to test a variety of hypotheses in this and other species. Firstly, nutrition may have a sexually dimorphic impact on behavior and therefore ecological impact of a species and further work to investigate sexual dimorphism in immature insects is one way to address this. Secondly, the prevalence and transience of dosage compensation in unclear, but monogenic sex determination in this species could be a valuable instrument to examine the temporal effect of gene dose balance. Thirdly, although the exact mechanism of maternal sex determination in *C. rufifacies* remains unknown, this work has generated specific genetic sequences which will be useful in future work using RNAi and other molecular tools to identify the gene or genes responsible. Finally, the uneven sex ratios observed in this work suggest that there may be differences between thelygenic and arrhenogenic females beyond the sex of their offspring, which could have implications in the ecology of *C. rufifacies*.

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