ABSTRACT

TAYLOR, SALLY VANN. Effects of Imidacloprid on Two Parasitoids, *Toxoneuron nigriceps* and *Campoletis sonorensis*, of the Tobacco Budworm, *Heliothis virescens*, and High-throughput Sequencing of the Tobacco Thrips, *Frankliniella fusca*. (Under the direction of Dr. Clyde E. Sorenson and Dr. R. Michael Roe.)

Toxoneuron nigriceps (Viereck) (Hymenoptera, Braconidae) and *Campoletis sonorensis* (Cameron) (Hymenoptera, Ichneumonidae) are parasitoids of the larval stage of the tobacco budworm *Heliothis virescens* (Fabricius) (Lepidoptera, Noctuidae). They species are valuable biological tools in controlling tobacco budworm populations in Southeastern US agricultural production systems. We calculated the topical LD50 of imidacloprid to *C. sonorensis* and to *T. nigriceps* to estimate the toxicity of imidacloprid to these beneficial species. The parasitoids in our study did not have a similar response to topical imidacloprid exposure. The topical LD50 value of imidacloprid for adult *T. nigriceps* was estimated to be 366-fold higher compared to the topical LD50 value for adult *C. sonorensis*. Field and greenhouse trials conducted from 2011-2014 compared parasitism rates and life span of parasitoids that developed inside *H. virescens* larvae which fed on tobacco plant tissue treated with and without imidacloprid. Residues of imidacloprid and its metabolites were detectable in the bodies of *H. virescens* larvae fed treated plants and *T. nigriceps* larvae and adults from *H. virescens* larvae fed treated plants. The two parasitoids in our study did not have similar responses to larvae fed with this insecticide-treated plant tissue. *T. nigriceps* had reduced parasitism and adult lifespan, but *C. sonorensis* parasitism rate and life span were unaffected. These findings suggest that there are ecological effects of imidacloprid use on multiple trophic levels, and that insecticide use may differentially affect natural enemies

with similar feeding niches.

The tobacco thrips, *Frankliniella fusca* (Hinds) (Thysanoptera, Thripidae), is a major agricultural pest in the southeastern United States for which there are increasingly limited management options. The identification of genes essential to growth, development and homeostasis of this insect provides potential targets for RNA interference (RNAi) transgene therapies. We sequenced whole-body transcriptomes of larval and adult *F. fusca* using Illumina® high-throughput technology. The resulting 236 million 100 base pair reads were assembled, batch BLASTed, mapped, and annotated into over 25 thousand contiguous sequences per life stage, to be further analyzed to identify function. To illustrate the depth of contig information in our global, putative functional analysis, messages involved in the RNAi pathway, hormonal regulation of development, steps in juvenile hormone (JH) biosynthesis and degradation, known commercial insecticide targets, and enzymes involved in insecticide detoxification were characterized. We have identified putative proteins associated with reproduction in larval thrips, illustrating a need for species-specific and life stage-specific sequencing across insect taxa. The use of whole-body insects for sequencing yielded messages produced by bacteria, fungi and viruses associated with insects and plant hosts. Our data provide a critical first step in understanding the unique physiology of *F. fusca* and potential new approaches for their control.

Effects of Imidacloprid on Two Parasitoids, *Toxoneuron nigriceps* and *Campoletis sonorensis*, of the Tobacco Budworm, *Heliothis virescens*, and High-throughput Sequencing of the Tobacco Thrips, *Frankliniella fusca*

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BIOGRAPHY

Sally Vann Taylor was born in Fayetteville, North Carolina on 18th November 1980. She was raised by four wonderful parents. She graduated from North Carolina State University with a Bachelor of Arts in Anthropology. She was admitted to the PhD program in Entomology under the direction of Dr. Clyde E. Sorenson and Dr. R. Michael Roe in 2011.

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CHAPTER 1: TOPICAL LD50 OF IMIDACLOPRID TO WASP PARASITOIDS OF THE TOBACCO BUDWORM, *HELIOTHIS VIRESCENS*

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The tobacco budworm, *Heliothis virescens*, is a highly fecund and highly mobile agricultural pest that exploits a wide variety of cultivated crops and weed hosts in North America (Fitt 1989). This species is a chronic economic pest of tobacco, soybeans, peanut, and non-transgenic cotton in the southeastern United States (Fitt 1989). *H. virescens* females show a strong preference for depositing eggs on tobacco over other cultivated crops in North Carolina (Abney et al. 2007) and can reduce crop yield and leaf quality when infesting tobacco in high numbers early in the growing season. The potential for three generations of *H. virescens* to complete development within a single tobacco growing season indicates that tobacco is a refuge for, and a source of, populations in other crops (Abney et al. 2007).

Effective chemical management of *H. virescens* in tobacco is complicated by several constraints. Larvae feed inside the bud of the tobacco plant, where insecticidal sprays may not fully penetrate and where the rapid growth of new plant material quickly provides an untreated food supply (Burrack 2011). This location on the plant, and multiple moth flights within a single growing season, contribute to repeated insecticide applications. *H. virescens* populations have demonstrated insecticide resistance from heavy and repeated insecticide exposure; to date populations have evolved resistance to most conventional classes of insecticides in the field and to multiple strains of *Bacillus thuringiensis* toxins in the laboratory (Sparks 1981; Sparks et al. 1993; Gould et al. 1992).

Naturally occurring enemies play a significant role in reducing *H. virescens* populations below damaging levels (Rabb 1971). This control is best sustained in the absence of insecticides, which disproportionately harm natural enemies (Lewis and Brazzel 1968; Reed and Pawar 1982; King and Coleman 1989; Croft 1990). *Campoletis sonorensis*

and *Toxoneuron nigriceps* (Figure 1.1) are common parasitoids of *H. virescens* in multiple cropping systems within the southeastern United States. The parasitism of budworm larvae by *C. sonorensis* or by *T. nigriceps*, and in some cases by *T. nigriceps* alone, can result in budworm mortality greater than 90% in tobacco and cotton fields (Chamberlin and Tenhet 1926; Grayson 1944; Lewis and Brazzel 1968; Lewis et al. 1970; Danks et al. 1979).

C. sonorensis is a generalist and capable of utilizing over 20 species of noctuid hosts (Lingren et al. 1970). *T. nigriceps* is a specialist and completes development only in *H. virescens* and the closely-related *Heliothis subflexa* (Chamberlin and Tenhet 1926; Grayson 1944; Lewis et al. 1967). Females of both species prefer to lay eggs in third instar larvae, though the larger-bodied *T. nigriceps* may utilize larvae of any age (Lewis et al 1970; Gunasena et al. 1989; Hu and Vinson 2000; De Moraes and Mescher 2005). Successful parasitism of a *H. virescens* larva by either *C. sonorensis* or *T. nigriceps* results in reduced feeding and ultimately death of the host (Lingren et al. 1970; Vinson and Barras 1970; Vinson 1972).

The neonicotinoid insecticide imidacloprid (Bayer CropScience, RTP, North Carolina) is heavily used in a variety of agricultural systems due to its versatility in application, efficacy against many piercing-sucking pests, and low mammalian toxicity (Tomizawa and Casida 2005; Jeschke and Nauen 2008; Jeschke et al. 2010). Imidacloprid has a high affinity for insect nicotinic acetylcholine receptors that, unlike those in mammals, are found in high densities exclusively in the central nervous system (Matsuda et al. 2001; Tomizawa and Casida 2003). Imidacloprid provides a valuable tool in insecticide resistance management because its target site specificity and novel activity compared to other heavily-

used classes of synthetic insecticides (Nauen et al. 1998). These attributes contribute to an estimated agricultural use of over 1.5 million pounds a year in the United States (USGS 2011).

Widespread and heavy use of imidacloprid has raised ecological and environmental concerns. Surface water contamination has been detected in US streams and rivers, likely from agricultural runoff (Starner and Goh 2012; Hladik et al. 2014). There are concerns with its potential to contaminate groundwater through soil leaching (Gupta et al. 2001) and its long-term persistence in soils (Oi 1999; Sarkar et al. 2001). Harm to non-target wildlife is considered likely (Goulson 2013), and imidacloprid has been implicated either directly or indirectly in decreasing populations of insectivorous birds (Hallmann et al. 2014; Lopez-Antia et al. 2013).

Direct and sublethal toxicities of imidacloprid to beneficial arthropods are well documented. Acute toxicity of imidacloprid has been estimated for pollinators (Suchail et al. 1999; Nauen et al. 2001) and arthropod predators (Mizell and Sconyers 1992; Kunkel et al. 1999; Elzen 2001; Kunkel et al. 2001). Sublethal effects on learning behaviors, foraging and reproduction following the chronic oral exposure of imidacloprid in honeybees have been demonstrated (Bortolotti et al. 2003; Decourtye et al. 2004; Yang et al. 2008; Whitehorn et al. 2012). Insects residing or feeding on plants treated systemically with imidacloprid have been intoxicated by the translocation of imidacloprid and/or its metabolites to pollen, nectar or leaf pores (Smith and Krischik 1999; Prabhaker et al. 2011).

Endoparasitoids inhabit a relatively unique position in that they have the potential to receive doses of insecticides in four ways: exposure to spray applications, tactile contact

with residues, feeding on contaminated materials and through the body of the host insect (Langley and Stark 1996; Kramarz and Stark 2003). Parasitoids are frequently more susceptible to insecticides than their host due to metabolic and physiological differences (Croft 1990).

Studies estimating the toxicity of imidacloprid to adult parasitoids report a range of responses. Cohen et al. 1996 estimated the LC50 of *Aphelinus mali* (Haldeman) (Hymenoptera, Aphelinidae) at under 1/2000 of the recommended field rate (350 ppm) following 24 hours of residue exposure; Preetha et al. 2010 estimated the LD50 of *Chelonus blackburni* Cameron (Hymenoptera, Braconidae) at the recommended field rate (25 g a.s./ha) following 24 hours of residue exposure. Intoxication from imidacloprid has also been observed in adult parasitoids that fed on treated plants (Krischik et al. 2007; Paine et al. 2011). Lethal and sublethal effects from imidacloprid can reduce the ability of parasitoids to provide biological control (Stapel et al. 2000; Rogers and Potter 2003; Tran et al. 2004). However, imidacloprid does not affect the performance of the parasitoid in some agricultural systems (Bethke and Redak 1997).

There is a rare opportunity for examining potential tritrophic effects of imidacloprid in tobacco production. Imidacloprid is used for the management of thrips, aphids, and flea beetles, but it has no insecticidal activity against *H. virescens* larvae. Because these larvae are not affected by imidacloprid treatments, they present an opportunity to study the effects of imidacloprid to parasitoids without host mortality. It is possible that non-target effects of imidacloprid, if detected, could alter the ability of *C. sonorensis* and *T. nigriceps* to provide biological control. Adults and free-living immature stages of both parasitoids can encounter

spray applications; adults and pupae of *T. nigriceps* can be exposed either in pupation or following adult emergence in contaminated soils. Additionally, immature stages of both parasitoids can be exposed to imidacloprid and/or its metabolites inside the body of the host if budworm larvae sequester quantities of this toxicant. To determine the potential impacts of exposure, we estimated the acute contact toxicity of imidacloprid to adults of the parasitoids *C. sonorensis* and *T. nigriceps*.

Materials and Methods

Insects. Laboratory colonies of *C. sonorensis* and *T. nigriceps* were established in 2011 from adult females collected from tobacco fields in Rocky Mount and Kinston, North Carolina. Parasitoids were cultured in *H. virescens* larvae from a laboratory colony maintained at North Carolina State University, Raleigh, North Carolina. Adult parasitoids were kept in 29.8cm x 29.8cm Bugdorm® cages (Bioquip, Rancho Dominguez, CA), fed honey (North Carolina State University Apiculture, Raleigh, NC), provided with water, and maintained at 25 ± 1° C, ≥ 70% relative humidity, 17 hours light: 10 hours dark, and a ratio of 3:1 female to male.

Adult insects included in assays were used within the first 5 days of emergence and had access pre and post-exposure to uncontaminated food and water. Approximately equal numbers of male and female insects were tested for each species. Female and male *C. sonorensis* were housed in separate containers following exposure because of an observed increase in mortality of the intoxicated females when the sexes were combined. There was no such observation with *T. nigriceps* and the sexes were housed together following

exposure.

Insecticidal materials. Industrial grade (≥ 98% purity) imidacloprid was provided by Bayer CropScience (RTP, North Carolina). Test dilutions of imidacloprid and acetone were prepared immediately before use (≤ 1 hour) and not stored or reused in subsequent assays. Compounds were hand-shaken for ≥ 30 seconds immediately before dose administration. Tested compounds consisted of a stock solution with 4 serial dilutions and a base-only control. A series of exploratory experiments was used to determine the optimal range of concentrations for each species prior to testing.

Topical toxicity. Insects were anesthetized for 10-20 seconds with CO_2 and doses were applied to the pronotum with a Hamilton repeating syringe as described in Stark et al. 1995. A 0.5-ul dose was used for *C. sonorensis* while a 1.0-ul dose was used for *T. nigriceps* because of the difference in body size between the adults of these species. Doses were allowed to dry and insects were returned to cages. Mortality was observed at 12 hour increments for 48 hours. A minimum of 10 insects was used at each dose in each assay, and the assays were replicated at least 3 times using separate generations of insects.

Analysis. Cumulative mortality of each treatment was used to determine the topical LD50 dose with 95% confidence limits. Dose-response relationships were estimated using the probit analysis procedure from SAS version 9.3 (SAS Institute, Cary, NC). Abbot's formula was used to correct for mortality in the control treatment for *C. sonorensis* (Fleming and Retnakaran 1985). There was no mortality in the control treatment for *T. nigriceps*.

Results

We found that *C. sonorensis* and *T. nigriceps* responded differently to topical imidacloprid exposure (Table 1.1). Imidacloprid is 59-fold more toxic to *C. sonorensis* compared to *T. nigriceps* when toxicity is expressed in terms of micrograms of imidacloprid per gram of insect body weight. This ratio translates into a 366-fold increase in toxicity to the smaller-bodied *C. sonorensis* when the LD50 is expressed in terms of the number of micrograms of imidacloprid per individual insect. Female *C. sonorensis* were more tolerant of exposure than males. The opposite trend was observed in *T. nigriceps* where females were slightly more susceptible than males, but females of this species showed a larger variability in dose-response.

Discussion

Two parasitoid species that inhabit the same agricultural environments and utilize the same host display dissimilar acute responses to topical imidacloprid exposure. Imidacloprid is more toxic when applied topically to the generalist parasitoid *C. sonorensis,* compared to the specialist parasitoid *T. nigriceps*. Additionally, the increase in susceptibility of *C. sonorensis* is further magnified by its smaller size. Susceptibility decreased with body weight for *C. sonorensis,* and females of this species are more tolerant of exposure than males, perhaps due their larger body size or different physiology. *T. nigriceps* females are slightly more susceptible despite being larger-sized than males.

It is possible that *T. nigriceps* better survives contact with imidacloprid because its life history preadapts it to nicotine exposure. The quality of food plants, including the

presence of allelochemicals like nicotine, can select for the better adapted parasitoid in intrinsic competition (Harvey et al. 2013). Long-term exposure to nicotine leads to physiological adaptions in parasitoids based on historical observations of *Apanteles congregatus* (Hymenoptera, Braconidae) and its host *Manduca sexta* (Linnaeus) (Lepidoptera, Sphingidae) in tobacco cropping systems (Thurston and Fox 1972). Radiolabeled nicotine in the diet of *M. sexta* and *Spodidoptera frugiperda* (Lepidoptera; Noctuidae) can transfer to the tissues of their parasitoids. (Barbosa et al. 1986). Further, nicotine has fewer detrimental effects on the survival and development of *Cotesia congregata* (Say) (Hymenoptera, Braconidae) compared to *Hyposoter annulipes* (Cresson) (Hymenoptera: Ichneumonidae) because its host, *M. sexta*, routinely feeds on tobacco (Barbosa et al. 1986). There is no empirical evidence that nicotine transfers to *H. virescens* parasitoids from the host. However, nicotine toxicity increased in *H. virescens* parasitized by *C. sonorensis* compared to non-parasitized larvae, suggesting some interaction between nicotine and parasitoids occurs (Gunasena et al. 1990). Nicotine and imidacloprid share a mode of action (Tomizawa & Casida 2003); and nicotine tolerance may confer cross-resistance to imidacloprid (Devine et al. 1996).

Insects used in this study were from laboratory colonies established from adult females collected in tobacco fields located in Eastern North Carolina, a region with a long history of flue-cured tobacco production. Because multiple generations of *H. virescens* occur in tobacco (Abney 2007), and cotton transformed to express *Bt* toxins does not provide a suitable alternative habitat (Tabashnik 2008), a parasitoid that tolerates nicotine containing host gains a competitive advantage over those that do not. *T. nigriceps*, as a specialist, is

under more selection pressure to adapt to homogenous agricultural habitats than *C. sonorensis*, a generalist that can utilize a wider variety of hosts and thereby encounters a more heterogeneous environment (Kassen 2002). These parasitoids provide a unique opportunity to test if a long-term association with plant-produced alkaloids influences toxicity and how life-history traits influence intrinsic competition between parasitoids.

The compatibility of chemical and biological control methods is an important consideration in insect pest management. It is possible for insecticides to have additive, neutral or destructive effects on biological control of insect pests by altering interactions between beneficial arthropods (Croft and Brown 1975; Fagan et al. 1998; Straub et al. 2008). Our data add to research showing that imidacloprid can differentially affect natural enemies on a species-specific basis, supporting the need to study how insecticides impact the range of species found in diverse agricultural systems (Stark et al. 1995).

Additional information is needed to accurately characterize the risk of imidacloprid to these parasitoids. Risk analysis is based not only on the inherent hazard of insecticidal material, but on the nature of and opportunity for exposure to it; thus, insecticide formulations are an important consideration when determining effects on non-target populations (Horn 1988; Stark et al. 1995). It is possible for adult parasitoids to encounter spray applications of imidacloprid in an agricultural environment, but this is not the most common application method for imidacloprid in agricultural systems. Parasitoids, like predators, can also be exposed by feeding on hosts contaminated with imidacloprid (Croft and Brown 1975). The more robust *T. nigriceps* better survives topical encounters with imidacloprid applications than *C. sonorensis*, but questions remain on how these parasitoid

species will respond to different routes of exposure. Another important consideration is determining if imidacloprid use affects the rate of successful parasitism of *H. virescens* by parasitoids in the field in light of the demonstrated toxicity presented here. In order to better predict how imidacloprid will affect *H. virescens* parasitism, we will have to further evaluate different routes of potential parasitoid exposure.

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Tables

Table 1.1 Topical toxicity of imidacloprid to *C. sonorensis* and *T. nigriceps*.

Species	N	mean body weight, mg	LD50 (95% C.I.), ug/g	LD50 (95% C.I.), ug/insect	
C. sonorensis	360	2.66	0.84 (0.73-0.98)	0.00224	(.00193-.00262)
Males	180	2.19	0.83 (0.70-1.00)	0.00182	(.00153-.00219)
Females	180	3.35	0.93 (0.72-1.36)	0.00313	(.00242-.00457)
T. nigriceps	180	16.47	49.84 (43.41-62.66)	0.8209	(.7150-1.032)
Males	90	15.21	50.78 (42.36-67.78)	0.7723	(.6443-1.031)
Females	90	17.46	50.60 (40.68-108.36)	0.8834	(.7102-1.892)

Figures

Figure 1.1 Adult females of *C. sonorensis* (left) and *T. nigriceps* (right).

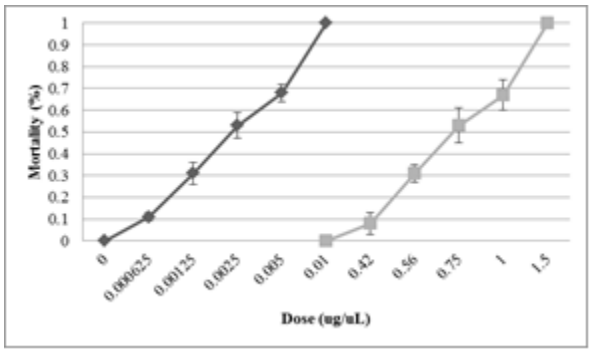

Figure 1.2 Dose-response relationship resulting from contact exposure to imidacloprid. *Campoletis sonorensis* (♦) and *Toxoneuron nigriceps* (■) mortality observed at 48 hrs after the topical application of imidacloprid at the specified dose. The lack of error bars indicates SD=0.

CHAPTER 2: THE USE OF SYSTEMIC IMIDACLOPRID AFFECTS INTERGUILD PARASITOIDS DIFFERENTLY

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Systemic insecticide applications have a low risk of topical exposure to natural enemy populations because toxicants are transported inside of treated plant tissue (Croft and Brown 1975). However, there is a potential for food-chain effects when predators feed on contaminated prey (Price 1997). Toxicants that transfer in this manner, including insecticides, affect the interactions between plants, herbivores and predators (Kiritani and Kawahara 1973).

Insect parasitoids consume the body of their host and may be exposed to intoxicated prey. Parasitoid's physiological and metabolic traits often make them more susceptible to toxicants than their host (Croft 1990). A tomato alkaloid, α-Tomatine, incorporated into the diet of *Helicoverpa zea* (Boddie) larvae decreases the survival and health of its parasitoid *Hyposoter exiguae* (Viereck) despite a tolerance of this toxin in the host (Campbell and Duffey 1979). Similarly, nicotine in the diet of *Manduca sexta* (Linnaeus) and *Spodoptera frugiperda* (J. E. Smith) transfers to parasitoid tissues and negatively affect survival and development of parasitoids *Cotesia congregata* (Say) and *Hyposoter annulipes* (Cresson) (Barbosa et al. 1985). Parasitized hosts may also respond differently to toxicants compared to non-parasitized hosts; nicotine is lethal in lower levels to *Heliothis virescens* larvae that are parasitized by *C. sonorenis* compared to non-parasitized larvae (Gunasena et al. 1990). In some cases, toxicants enhance the performance of parasitoids. *Bacillus thuringiensis* toxins administered in sublethal doses can aid parasitoids by lengthening the amount of time hosts are vulnerable to attack and/or increasing the length of time the host spends in a susceptible stage (Johnson and Gould 1992).

Parasitoid species that coexist in the same environment and on the same resources

compete to discover and exploit hosts (Taylor 1988). *Campoletis sonorensis* and *Toxoneuron nigriceps* are solitary endoparasitoids of *H. virescens* larvae (Figure 2.1). They occur in overlapping regions of the southeastern United States and are found in cotton and tobacco cropping systems as well as non-crop hosts of *H. virescens*. Adult females of both species prefer to lay eggs in larvae in the third instar (Lewis et al. 1970; Gunasena et al. 1989; Hu and Vinson 2000; De Moraes and Mescher 2005). *C. sonorensis* is a generalist capable of utilizing over 20 species of Noctuid hosts (Lingren et al. 1970). The host stops growing and feeding almost immediately following parasitism by *C. sonorensis* (Vinson et al. 1979). *T. nigriceps* is a specialist and only completes development in *H. virescens* and *Heliothis subflexa* (Chamberlin and Tenhet 1926; Grayson 1944; Lewis et al. 1970). These hosts continue to eat and digest plant tissue, at a reduced rate, for up to 6 days following parasitism by *T. nigriceps* (Guillot and Vinson 1973). Parasitoids play a significant role in biological control of *H. virescens* populations (Rabb 1971). The percent of larvae parasitized by *T. nigriceps* or *C. sonorensis* has been reported between 50% and 100% in SE cropping systems (Chamberlin and Tenhet 1926; Grayson 1944; Lewis and Brazzel 1968; Lewis et al. 1970; Danks et al. 1979).

Imidacloprid (Bayer CropScience) is a widely-used agricultural insecticide that comes in a variety of formulations, is effective against piercing-sucking pests, and has low mammalian toxicity (Tomizawa and Casida 2003; Jeschke and Nauen 2008; Jeschke et al. 2010). Imidacloprid has the same target as nicotine within the insect nervous system (Tomizawa & Casida 2003), yet it varies in its toxicity to tobacco-feeding insects. Imidacloprid is used for management of thrips, aphids and flea beetles in flue-cured tobacco,

but it has no insecticidal activity against lepidopteran pests, including *H. virescens*.

Imidacloprid can have lethal and sublethal effects on parasitoids exposed to environmental residues or to contaminated food sources (Stapel et al. 2000; Rogers and Potter 2003; Tran et al. 2004; Krischik et al. 2007; Paine et al. 2011). However, imidacloprid use is compatible with some host/parasitoid systems (Bethke and Redak 1997). Since *H. virescens* larval mortality is not affected by imidacloprid treatments, the tobacco cropping system presents the opportunity to study how larval parasitoids respond to imidacloprid in the diet of their hosts. In this study, we determined whether imidacloprid was present in the body of larval *H. virescens*, whether imidacloprid and/or its metabolites transferred from a host larva to its parasitoid, and determined any effects on the performance of parasitoids.

Materials and Methods

Field trials. Field trials were conducted in 2012-2013 at the Lower Coastal Plains Research Station in Lenoir County, North Carolina, and the Upper Coastal Plains Research Station in Edgecombe County, North Carolina, to measure the rate of *H. virescens* parasitism in imidacloprid treated and untreated tobacco plants. For all analyses, data for both years and locations were pooled.

Flue-cured tobacco seedlings (*Nicotiana tabacum* L, var. NC71), were grown in the greenhouse, following organically acceptable production methods. In 2012, transplants were set on April 18 at the Lenoir County site and on April 30 at the Edgecombe County site. In 2013, transplants were set on April 24 at the Lenoir County site and on April 26 at the Edgecombe County site. Standard agronomic practices for tobacco production in North

Carolina were used in all field trials (Fisher et al. 2007).

Untreated plants were compared to systemic, soil applications of imidacloprid (Admire Pro, Bayer CropScience, Morrisville, NC). Imidacloprid was applied at the recommended field rate for tobacco (23.65 ml/1000 plants) using two methods: as a greenhouse tray drench followed by an immediate wash-off with clean water or in transplant water at planting. Imidacloprid applied in the greenhouse was applied $\leq$ 2 days prior to transplanting. Treatments were arranged in a randomized complete block design and replicated four times each. Plots consisted of eight 50-foot rows of 20-25 plants per row.

H. virescens larvae were censused twice weekly from prior to the initiation of the adult *H. virescens* moth flight, as measured by the appearance of adult moths and eggs in the field, until tobacco plants flowered. Larvae were collected when they attained a size corresponding to $\geq$ late 3rd stadium as measured by a gauge of head capsule size (Johnson and Gould, 1992) and a ratio of head capsule size to body size. Larvae were placed on artificial diet and observed daily for parasitism. Successful parasitism was defined by emergence of a live wasp larva.

A subset of the *H. virescens* larvae collected at 60 (±7) days following planting date and the *T. nigriceps* that emerged from this same cohort were randomly selected for measurement of imidacloprid residues. Pesticides were extracted using methods established for parasitoids and host larvae (Dhammi 2010). *H. virescens* larvae were starved for $\geq$ 1 day prior to residue testing to minimize the amount of plant tissue in the digestive tract. *T. nigriceps* larvae were tested $\leq$ 1 day following emergence from host. *T. nigriceps* adults were tested $\leq$ 1 day following emergence. Insects were rinsed 3 times in distilled water and

dried with clean sterile Kim Wipe® lab tissues. Larvae were homogenized in a 1:1 ratio with distilled water and centrifuged at 13,000 rpm for 2 minutes and 40µl of the resulting supernatant was added to 100µl of distilled water. The same procedure was used for *H. virescens* and *T. nigriceps* larvae, and two *T. nigriceps* adults were used in each sample. Imidacloprid concentrations were measured using the QuantiPlate ELISA kit (ENVIROLOGIX, Portland, Me) following manufacturer's instructions. There is cross-reactivity of the ELISA kit with other neonicotinoid insecticides (thiamethoxam, thiacloprid, clothianidin and acetamiprid) and imidacloprid metabolites (imidacloprid olefin, desnitro imidacloprid and imidacloprid urea). Imidacloprid concentrations detected by ELISA are likely to be from applied treatments; however, residues should be understood to potentially include imidacloprid metabolites and cross-reacting neonicotinoids. Residues for *C. sonorensis* were below the limit of quantification (0.3 ppb).

Adult female parasitoids emerging from the remaining *H. virescens* larvae collected at 60 (±7) days following the planting date were used to assess parasitoid longevity. Parasitoids were kept in individual pint-sized Bugdorm® cages (Bioquip, Rancho Dominguez, CA), fed honey, provided with water and maintained at 25 ± 1° C, ≥ 70% relative humidity, 17 hours light: 10 hours dark. Wasps were observed daily for mortality.

Greenhouse trials. Greenhouse trials to measure the rate of *H. virescens* parasitism in treated and untreated tobacco plants were conducted in 2011-2013. Parasitoids from laboratory colonies of *C. sonorensis* and *T. nigriceps* established from wasps collected in tobacco fields in Rocky Mount and Kinston, North Carolina in 2011 were used for all experiments. *H. virescens* larvae used in all trials were obtained from research colonies

maintained at North Carolina State University, Raleigh, North Carolina. Adult parasitoids were kept in 29.8cm x 29.8cm bugdorm® cages (Bioquip, Rancho Dominguez, CA), fed honey (North Carolina State University Apiculture, Raleigh, NC) at approximately a 1:1 female to male ratio, provided with water and maintained at 25 ± 1° C, ≥ 70% relative humidity, 17 hours light; 10 hours dark.

Flue-cured tobacco seedlings (*Nicotiana tabacum* L, var. NC71) were grown under approved organic production methods in the greenhouse and transferred to 3-gallon plastic pots when 15-25cm tall. Plants treated with imidacloprid applied as a systemic soil-drench (Admire Pro, Bayer CropScience, Morrisville, NC) at the rate of 0.192 LB A/A (simulating 23.65ml/1000 plants at 6,700 plants per acre) were compared to untreated plants. Insecticide solutions were applied in 118ml of water as a transplant water drench to the crown of the seedlings at planting. Treatments were arranged in a randomized complete-block design. The mean greenhouse temperature was maintained at ≥ 25°C. Plants were watered 3-4 times a week as needed and not to overflowing.

Individual neonate *H. virescens* larvae were placed by hand on plants ≥ 30 days following transplant to simulate field infestation timing. Plants were covered with organza bags to prevent movement of larvae. Second instars were collected after 1 week and parasitized by wasps in the laboratory. Wasp ovipositor insertion into larvae was observed to ensure parasitism was attempted. Larvae were immediately returned to the plants of their origin and allowed to continue development. Larvae were observed daily for 7 days for those parasitized by *C. sonorensis* and 10 days for those parasitized by *T. nigriceps*. A successful parasitism event was defined by emergence of a live wasp larva. The trial was replicated 4

times with different plants and different generations of insects.

Imidacloprid residue concentrations in parasitized *H. virescens* larvae were measured during 2013-2014. Plants were maintained, treated and arranged as described above. Imidacloprid applied as a systemic soil-drench at two rates, the label rate and twice the label rate (23.65ml/1000 plants and 47.3ml/1000 plants), were compared to untreated plants. Rates were selected to test the relationship between the rates of imidacloprid applied to the plant with the residues in the larvae. *H. virescens* larvae were placed on the plants, confined with bags and parasitized by wasps as above. Larvae were removed for residue testing prior to parasitoid emergence at 6 days. The trial was replicated 3 times with different generations of insects.

Data analysis. Data were analyzed using SAS version 9.3 (SAS Institute, Cary, NC). Field trial data for parasitism rates were log transformed to decrease skew and meet assumptions for statistical analysis. Explanatory variables included collection date, number of days between planting and the collection date, and replication and environment, which included both location and year; the variables environment and replication were treated as random. Data were subjected to analysis of variance (ANOVA) using the GLIMMIX procedure and a binomial response distribution. Data from greenhouse trials were analyzed via the MIXED procedure. Means were separated using the Tukey-Kramer method.

Results

Field trials. The two parasitoids in our study did not have similar responses to larvae fed imidacloprid-treated plant tissue. The rate of parasitism by *T. nigriceps* was significantly

higher in *H. virescens* collected from untreated plants compared to *H. virescens* from plants treated with imidacloprid applied in the greenhouse and in transplant water, but there were no differences between the two imidacloprid applications (Table 2.1; df=2, 268: F=9.56: P<0.0001). There were no treatment differences in parasitism by *C. sonorensis* (Table 2.1; df=2, 268: F=0.08: p=0.9301).

Imidacloprid residues were detected in *H. virescens* larvae, *T. nigriceps* larvae and *T. nigriceps* adults (Table 2.2). Residue levels differed significantly by treatment and were significantly lower in the control compared to imidacloprid applied in the greenhouse or as a transplant water drench [*H. virescens* larvae (df=2, 57: F=512.88: P<0.0001), *T. nigriceps* larvae (df=2, 120: F=15.99: P<0.0001) and *T. nigriceps* adults (df=2, 108: F=8.06: P=0.0005)]. There were no significant differences between the two imidacloprid applications.

The average lifespan of *T. nigriceps* adult females differed significantly between treatments (Table 2.3; df=2, 31: F=16.04: p=<0.0001). The average lifespan for *T. nigriceps* females was significantly longer in the control compared to either imidacloprid treatment, but there were no differences between the two imidacloprid applications. Insect weight also significantly influence survival, with larger insects having longer lifespans (df=5, 31: F=3.50: p=0.0127). There were no treatment differences in *C. sonorensis* lifespans (Table 2.3; df=2, 7: F=0.70: p=0.5279).

Greenhouse trials. The average percentage parasitism by *T. nigriceps* was significantly higher in untreated plants compared to the imidacloprid treatment (Table 2.4; df=1, 7: F=8.84: P=0.0249). There were no treatment differences in the average percentages

of successful parasitism by *C. sonorensis* (df=1, 7: F=1.08: P=0.3395).

Imidacloprid residues were detected in parasitized *H. virescens* larvae (Table 2.5). Residue levels differed between imidacloprid rates (df=2,108: F=288.31: P<0.0001) and parasitoid species (df=2,108: F=162.04: P<0.0001). The interaction of the imidacloprid rate and the parasitoid species was also significant (df=2,108: F=35.32: P<0.0001). Imidacloprid residues in parasitized larvae were higher when a higher rate of imidacloprid was applied to the plant, and imidacloprid residues were higher in larvae parasitized by *T. nigriceps* than by *C. sonorensis*.

Discussion

Our results demonstrate that two parasitoid species that inhabit the same agricultural environments and utilize the same host are exposed to imidacloprid during their development. However, they differ in amount of, and response to, this exposure. The specialist parasitoid *T. nigriceps* is exposed to imidacloprid residues in higher concentrations, and the successful parasitism of *H. virescens* larvae is reduced. The generalist parasitoid *C. sonorensis* is exposed to imidacloprid residues in lower concentrations, and the successful parasitism of *H. virescens* larvae is unaffected. Previously, we showed that *C. sonorensis* adults are more susceptible than *T. nigriceps* adults to topical imidacloprid exposure (Taylor 2015); and, based on this toxicity, *T. nigriceps* would be expected to be less affected by imidacloprid in the field. The specialist *T. nigriceps* is under higher selection pressure to adapt to toxins, like nicotine, in the host food plant compared with the generalist *C. sonorensis* (Kassen 2002; Harvey et al. 2013); and imidacloprid and nicotine share a mode of

action (Tomizawa & Casida 2003). However, increasing the concentration of nicotine in the host diet negatively affects parasitoid survival and development in species preadapted to their host feeding on tobacco (Barbosa et al. 1986). Imidacloprid residues are present in the hosts of *T. nigriceps* at a 4-fold increase compared to the hosts of *C. sonorensis* when their hosts are fed on imidacloprid-treated tobacco; this may overwhelm the metabolic capacities of *T. nigriceps* larvae and cause differential mortality between these parasitoids. Differences in imidacloprid toxicity between adult and immature parasitoids and between parasitoid species are possible; this phenomenon has previously-observed with other insecticidal classes (Schuster 1994). Our findings strongly support the need to evaluate both species-specific and life stage specific toxicity in beneficial insects (Croft 1990).

The compatibility of insecticides and beneficial insects is an important consideration in integrated pest management. Insecticides that destroy natural enemies can be causal agents in pest resurgences and secondary pest outbreaks (Metcalf 1980). Here we report an instance of an insecticide that decreases the percentage of parasitoids that survive to parasitize following generations of a pest. The economic value of these species to biological control has not been determined. Imidacloprid is commonly used as a systemic application in alternate *H. virescens* host habitats, such as cotton and peanut. It is possible that populations of *H. virescens* will increasingly reach damaging levels from the reduction in successful parasitism by *T. nigriceps*. Conversely, *H. virescens* populations may remain stable or decrease if larger populations of *C. sonorensis*, and a higher rate of total parasitism by this species, result from reduced competition with *T. nigriceps*.

The successful exploitation of a host by a parasitoid relies on four events: the female

parasitoid finding the host habitat, the female parasitoid finding the host, the female parasitoid accepting the host, and the host being sufficient to sustain the immature parasitoid through its development (Doutt 1959). The presence of imidacloprid reduces host finding in other host-parasitoid systems by altering plant volatiles (Stelinski et al. 2006), or by causing irritation or locomotive depression in female wasps (Tran et al. 2004). Effects on host finding, host acceptance and/or host suitability could be responsible for the observed decrease in *H. virescens* parasitism by *T. nigriceps* in the imidacloprid-treated test plots. In our greenhouse trials, where insertion of the parasitoid's ovipositor into the host larva was observed, the cause of the observed decrease in successful parasitism by *T. nigriceps* is limited to a decrease in host suitability for the larval parasitoid.

The effect of imidacloprid on *T. nigriceps* parasitism is likely the result of a decrease in host suitability. Furthermore, the differences between how *T. nigriceps* and *C. sonorensis* manipulate host feeding may be responsible for the differences in their toxicant exposure and the resulting differences in parasitism success. *H. virescens* larval growth and feeding cease almost immediately following parasitism by *C. sonorensis* (Vinson et al. 1979), but larvae parasitized by *T. nigriceps* continue to ingest insecticide contaminated leaf tissue. Food also travels slower through the digestive tracts of these parasitized larvae (Guillot and Vinson 1973). Since toxicology is influenced by rates of penetration and excretion, increased duration of feeding on treated materials and/or an increased residence time of treated materials in the digestive tract may cause increased imidacloprid residues in the host body. These increased residues of imidacloprid may be linked to increased mortality of *T. nigriceps*.

Residues of imidacloprid were detected in insects collected from untreated field plots, although the levels of these residues were much lower than those observed in treated plants. This may be the result of the carry-over of imidacloprid from agricultural usage in previous years, since residues of imidacloprid were found in the water supply at both research stations (unpublished data). There is some cross-reactivity of the ELISA kit with other neonicotinoid insecticides (thiamethoxam, thiacloprid, clothianidin and acetamiprid) and imidacloprid metabolites (imidacloprid olefin, desnitro imidacloprid and imidacloprid urea). It is also possible that there is a matrix effect when hemolymph is insufficiently diluted that can falsely indicate higher imidacloprid concentrations (Dhammi 2010). However, when imidacloprid rates applied to the plants were increased, there was a corresponding increase in the imidacloprid concentrations in herbivores and in their parasitoids, validating the use of this method for comparative, if not quantitative, purposes.

The movement and fate of pesticides released into the environment is of great concern to the health of its inhabitants. *A priori* knowledge tells us that individual organisms that encounter toxic materials suffer metabolic stress from this exposure (Calow 1990). Here we provide empirical evidence that imidacloprid is capable of moving through trophic levels of the agroecosystem, and that this movement causes deleterious effects on some, but not all, beneficial species. The long-term consequences on the population dynamics of this predator-prey system, if any, are unknown.

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Tables

Table 2.1 ANOVA results: the mean percentage of field-collected *H. virescens* larvae parasitized by *C. sonorensis* and *T. nigriceps* over both years and locations.

Treatment	Total no. larvae	% Parasitism (±SEM)	
		C. sonorensis	*T. nigriceps*
untreated	1005	11.43 (3.4)	47.94 (3.6)*
imidacloprid (23.65ml/1000 plants) - greenhouse	1109	5.84 (1.5)	30.27 (3.4)
imidacloprid (23.56ml/1000 plants) - transplant	1192	7.24 (1.8)	28.38 (4.9)

*denotes means significantly different within a column at α=.05 (PROC GLIMMIX) (SAS Version 9.3, SAS Institute, Cary, NC).

Table 2.2 ANOVA results: the mean concentration of neonicotinoids and metabolites (in ppb) in field-collected insects over both years and locations.

Species (life stage)	Total no. individuals	Concentration (ppb) (±SEM)		
		untreated	imidacloprid (23.65ml/1000 plants) greenhouse	imidacloprid (23.65ml/1000 plants) transplant
H. virescens (larvae)	63	0.69 (0.07)*	7.66 (0.02)	7.91 (0.01)
T. nigriceps (larvae)	126	0.09 (0.02)*	0.55 (0.07)	0.70 (0.06)
T. nigriceps (adults)	114	0.22 (0.01)*	0.31 (0.03)	0.37 (0.03)

*denotes means significantly different within a row at α=.05 (PROC GLIMMIX) (SAS version 9.3, SAS Institute, Cary, NC)

Table 2.3 ANOVA results: the mean lifespan (in days) of adult female *T. nigriceps* and *C. sonorensis* that emerged from field-collected *H. virescens* over both years and locations.

Species	Total no. individuals	Life span (days) (±SEM)		
		untreated	imidacloprid (23.65ml/1000 plants) greenhouse	imidacloprid (23.65ml/1000 plants) transplant
T. nigriceps	34	39.8 (3.9)*	27.9 (2.8)	28.3 (2.8)
C. sonorensis	10	35.7 (8.0)	24.6 (4.6)	34.3 (6.6)

*denotes means significantly different within a row at α=.05 (PROC GLIMMIX) (SAS version 9.3, SAS Institute, Cary, NC)

Table 2.4 ANOVA results: the mean percentage of *H. virescens* larvae that were parasitized by *T. nigriceps* and *C. sonorensis* with parasitoid emergence in greenhouse trials.

Species	Treatment	Percent parasitism (±SEM)	P-value
T. nigriceps	untreated	62.27 (5.2)*	0.0249
	imidacloprid (23.65ml/1000 plants)	37.00 (6.6)	
C. sonorensis	untreated	48.75 (10.6)	0.3395
	imidacloprid (23.65ml/1000 plants)	35.25 (7.0)	

*denotes means significantly different at α=.05 (PROC MIXED) (SAS version 9.3, SAS Institute, Cary, NC)

Table 2.5 ANOVA results: the mean concentration of neonicotinoids and metabolites (ppb) in parasitized *H. virescens* larvae in the greenhouse.

Species	Total no. individuals	Concentration (ppb) (±SEM)		
		untreated	imidacloprid 23.65ml/1000 plants	imidacloprid 47.3ml/1000 plants
T. nigriceps	52	0.338 (.07)*	8.268 (.54) *†	12.961 (.25)*†
C. sonorensis	64	0.246 (.02) *	2.251 (.38) *†	6.958 (.58)*†

*denotes means significantly different within a row at α=.05 (PROC MIXED) (SAS version 9.3, SAS Institute, Cary, NC)

†denotes means significantly different within a column at α=.05 (PROC MIXED) (SAS version 9.3, SAS Institute, Cary, NC)

Figures

Figure 2.1 Adult females of *C. sonorensis* (left) and *T. nigriceps* (right).

CHAPTER 3: HIGH-THROUGHPUT SEQUENCING PROVIDES INSIGHTS INTO THE BIOLOGY AND POTENTIAL FOR GENETIC CONTROL OF TOBACCO THRIPS

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Introduction

The tobacco thrips, *Frankliniella fusca* (Hinds), is a polyphagous agricultural pest that exploits a variety of cultivated crops and weeds (Kahn et al. 2005). Thrips damage cotton and peanuts through feeding and are major vectors of phytopathogenic viruses and bacteria. The small size (~ 1 mm long) and long, fringed wings of *F. fusca* adults aid in their dispersal by long distance and localized wind currents. *F. fusca* is historically distributed in North America east of the Rocky Mountains. The recent spread of this species, perhaps due to global trade, has been documented as far as Japan (Nakao et al. 2011). *F. fusca* populations have the capacity to adapt to local conditions and plant hosts (Mound and Teulon 1995), have a 16 to 25 day generation time, are multivoltine, and can reproduce parthenogenetically. *F. fusca* females lay eggs inside of plant tissue and immature thrips have two feeding larval stadia followed by two non-feeding stadia - the pre-pupal and pupal instars - followed by the adult stage. *F. fusca* has demonstrated pesticide resistance in response to high insecticide selection pressure (Mound and Teulon 1995).

Thrips consistently present the largest economic threat to cotton production in the Southeast and Mid-south regions (Bacheler 2012; Akin et al. 2010) of the United States. *F. fusca* is the most abundant species present throughout most of the U. S. cotton belt (Reed et al. 2010). Direct feeding on seedling cotton can result in malformed leaves, stunted plants, delayed maturity and yield reduction. Thrips are often expensive and difficult to control, requiring the use of both insecticidal seed treatments and foliar sprays (Toews et al. 2012). The phasing out of aldicarb and the now singular reliance of cotton growers on neonicotinoid seed treatments, against which the tobacco thrips has now developed resistance on cotton in

the Mid-south and Midwest, necessitates the discovery of alternative methods of control.

Thrips are the only known vectors of tospoviruses, the sole plant-infecting genus in the family *Bunyaviridae*. A single member of this genus, the tomato spotted wilt virus (TSWV), is responsible for approximately 1 billion dollars in crop damage worldwide (Prins and Goldbach 1998). *F. fusca* can transmit TSWV and impatiens necrotic spot virus (Prins and Goldbach 1998) as well as the bacterial agent responsible for center rot of onion (Gitaitis et al. 2003). *F. fusca* is the primary vector of TSWV, which presents an economic threat to tobacco, peanut, pepper and tomato crops (Groves et al. 2003), in the southeastern United States. TSWV can only be acquired by immature thrips and is only transmitted by adults, which remain infectious for life. TSWV is a propagative virus that must be actively transported across cellular membranes and circulated in the insect's hemocoel (Andret-Link and Fuchs 2005). Research into potential virus receptors for TSWV has identified candidates located in the midgut (Bandla et al. 1998). Thrips species vary in their capacity to transmit viruses and may gain or lose the capacity to vector virus isolates over time (Wijkamp et al. 1995).

One prospect for developing transgenes to thrips may lie in the use of RNA interference (RNAi) to suppress or silence critical genes essential to the physiology of the pest organism (Gordon and Waterhouse 2007). RNAi disrupts protein synthesis in the cytoplasm of target organisms at the gene transcript, or messenger RNA (mRNA), level (Huvenne and Smagghe 2010). The agent of RNAi is small interfering RNA (siRNA), a double stranded RNA (dsRNA) molecule with complementary nucleotide sequences to target mRNA. Molecules of siRNA interact with complementary mRNA and prevent the

translation of proteins; siRNA can be introduced directly to the insect or result from cleavage of dsRNA inside the cell.

Several labs have used RNAi to cause varying levels of gene suppression in targeted insects fed or injected with dsRNA or siRNA. Expression levels of hemipteran salivary enzymes in the tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae) (Walker and Allen 2010); trehalose phosphate synthase in the brown planthopper, *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae) (Chen et al. 2010); and actin in the glassy-winged sharpshooter, *Homalodisca vitripennis* (Germar) (Hemiptera: Cicadellidae) (Rosa et al. 2010) have been decreased. Expression levels of Lepidopteran JH (juvenile hormone) regulating neuropeptides in the fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) (Griebler et al. 2008); acetylcholinesterase in the cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) (Kumar et al. 2009); and chitin synthase in the beet armyworm, *Spodoptera exigua* (Hübner) (Tian et al. 2009) have been reduced. The efficacy of transforming plants to express dsRNAs for pest control has been demonstrated. Corn (*Zea mays* L.) has been transformed with transgenes expressing dsRNA that reduce expression of a target gene in the western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) (Baum et al. 2007); and its performance has been tested and evaluated in field trials (Friday et al. 2014). A reduction in the weight of *H. armigera* larvae resulted when they were fed leaves of cotton transformed to express dsRNA targeting a P450 that metabolizes gossypol, a phenolic secondary metabolite in cotton with insect antifeedant activity (Mao et al. 2011).

A first step in the development of an RNAi-based insect control method is to identify

potential pest genes that can be targeted with dsRNA or siRNA. Our objective was to use Illumina® sequencing technology to sequence, for the first time, the transcriptome of *F. fusca* and then to use bioinformatics to analyze the resulting sequences and identify targets for RNAi. This work can also be used to develop a global approach for the evaluation and diagnosis of thrips resistance to chemical insecticides. Other uses for this technology are the global identification of RNA viruses, leads to a better understanding of plant disease transmission and mechanisms in thrips for the maintenance and transmission processes, and the study of the physiology and ecology of this important pest.

Materials and Methods

Insects. *F. fusca* 1st and 2nd stadium larvae and adults were obtained from the laboratory of G. G. Kennedy at North Carolina State University, Raleigh, NC. The colony was originally collected in 1997 from peanut at the Peanut Belt Research Station in Lewiston, North Carolina. Thrips were kept in 1-gallon plastic tubs covered with one layer of cheesecloth, fed on pole beans, *Phaseolus vulgaris*, and maintained at 25 ± 1° C, 55-60% relative humidity, 14 hours light: 10 hours dark until needed for RNA extraction. Six hours before extraction, beans were removed from the rearing containers. This starvation period allowed the insects to clear their digestive system of plant material (as confirmed by gut observation across the insect cuticle). Adult thrips were used $\leq$ 1 week post-emergence from the pupal exuvium. Thrips larvae in the 1st and 2nd feeding stadia were used $\leq$ 4 days following emergence from the egg.

Total RNA isolation. Whole live thrips were flash frozen with liquid nitrogen and

then ground to a fine powder in liquid nitrogen with a mortar and pestle. Lysis buffer (600µl) was added to this powder just after grinding. Total RNA was extracted using the RNAeasy Mini Kit® (QIAGEN) from animal tissue following the manufacture's protocol. Total RNA samples were taken from 5 different generations of adult thrips and 5 different generations of larval thrips; there were 200 individual insects used per generation. The Agilent 2100 Bioanalyzer® was used to estimate the quantity and quality of RNA from 5ul of each sample at a concentration of 50-200 ng/ul. The 5 adult samples were combined into 1 sample and the 5 larval samples were combined into 1 sample.

cDNA library preparation and Illumina® sequencing. Two cDNA libraries, one adult library and one larval library, were prepared from the RNA samples. The cDNA libraries were prepared from 5 µg of total RNA following vendor recommendations from the Illumina® TruSeq RNA™ Sample Preparation v2 Guide (Illumina, Inc., San Diego, CA). The adult cDNA sample was loaded onto two lanes of an Illumina® eight-lane flow cell and the larval cDNA sample was loaded onto two separate lanes of the same eight-lane flow cell; 2 lanes were used per life stage for a combined 4 lanes total. High-throughput sequencing was performed on the Illumina Genome Analyzer IIx®. Sample preparation and sequencing were performed by the Genome Sciences Laboratory (North Carolina State University, Raleigh, NC).

Bioinformatics. Reads were trimmed for a minimum Phred score of Q20, sequence adapters were removed, and reads shorter than 25 nucleotides were discarded (FASTX-Toolkit, Hannon Lab, available from http://hannonlab.cshl.edu/fastx_toolkit/). Trinity software (20 August 2013 release; www.trinityrnaseq.sourceforge.net) was used to assemble

the reads into contigs (contiguous nucleotide sequences) with a k-mer length of 25 (Haas and Zody 2010). Blast2GO® software (Conesa et al. 2005) was used to align, map and annotate the contigs. Blast2GO analysis was conducted in Jun. 2014. For the alignment step, the contigs were translated to proteins in all six reading frames and compared to the GenBank nr (non-redundant) protein database using the BLASTx (Basic Local Alignment Search Tool) algorithm with E-value cut-off set at E^{-3} (10^{-3}). BLAST hits (thrips query contigs with database-sequence matches where E-value $\leq E^{-3}$) were mapped and annotated with GO (Gene Ontology) terms. GO terms assign the translated query sequences to categories of putative protein function (GO level 2 functional categories) on the basis of sequence and functional conservation among organisms represented in publicly-accessible protein/gene-product sequence databases (Gene Ontology Consortium; Ashburner et al. 2000). Nucleotide sequences were translated and open reading frames were identified (ExPASy, Swiss Institute of Biotechnology, available from http://web.expasy.org/translate/). Putative protein sequences were aligned to top hits using EMBOSS Needle (The European Molecular Biology Open Software Suite, available from http://www.ebi.ac.uk/Tools/psa/emboss_needle/) for pairwise alignments and ClustalW2 for multiple-sequence alignments (Analysis Tool Web Services from the EMBL-EBI, available from http://www.ebi.ac.uk/Tools/msa/clustalw2/). Conserved domains were identified using the NCBI Conserved Domain Database (Marchler-Bauer et al. 2015).

Results

Sequencing of reads & assembly of contigs. The Illumina Genome Analyzer IIx®

sequencer produced 23.6 gigabases of sequence data distributed across a total of 236 million 101-base reads (105 million adult and 131 million larval). Approximately 55% of these reads have a minimum quality score of Q20 across the entire length of the read and 85% of the bases among all reads had a minimum quality score of Q30. Reads were assembled into 244,746 contiguous sequences (contigs) (116,719 adult and 128,027 larval). Contig lengths ranged from 101 bps (base pairs) to greater than 9,000 bps, with an average read length of 563 bps for adult assembly; while contigs lengths ranged from 101 bps to greater than 10,000 bps with an average read length of 645 bps, for the larval assembly (Figure 3.1).

Global analysis of contigs. Assembled contigs were batched BLASTed, mapped and annotated with Blast2GO software. Contigs with BLAST hits numbered 76,697 (36,054 adult and 40,643 larval). As expected, the greatest numbers of hits were associated with insects where the genome is available. Contigs annotated with Gene Ontology terms numbered 56,418 (26,927 adult and 29,491 larval). The data distribution of the assembled reads for both life stages are shown in Figure 3.2. The vast majority of the annotated contigs were associated in the order of highest to lowest number of GO assignments to binding, catalytic activity, transporter activity, structural molecular activity and molecular transducer activity (Figure 3.3).

Annotation of contigs of specific functional interest. To demonstrate some of the specific content of the contigs obtained, a few messages associated with the RNAi pathway, insecticide mode of action, xenobiotic metabolizing enzymes, insect growth and development, and the microbiome are briefly characterized. Future study efforts will address how these sequences can be used in pest management for *F. fusca*.

RNA interference (RNAi) pathway components. We sequenced transcripts from adult and larval *F. fusca* with homology (E-value $\leq E^{-3}$) to proteins involved in regulation of gene expression by RNA silencing (Table 3.1). A comprehensive list of these sequences is shown in Table 3.1 and the total number of sequences by life stage is shown in Table 3.2. Insects have distinct proteins for microRNA (miRNA) and siRNA pathways, and the number and composition of these proteins vary by taxa (Forstemann et al. 2007; Jaubert-Possamai et al. 2007). The miRNA pathway is responsible for responding to endogenous sources of dsRNA and targets those mRNA sequences that are mostly complementary, but have some incomplete base pairing; the siRNA or RNAi pathway responds to exogenous sources of dsRNA and targets only complementary mRNA sequences (Tang 2005).

F. fusca transcriptomes contained putative Argonaute (AGO) and Dicer (DCR) proteins with homology (E-value $\leq E^{-3}$) to those responsible for siRNA and miRNA pathways in other insects; including *Drosophila* proteins AGO1, AGO2, DCR-1 and DCR-2 (Jaubert et al. 2007). Dicer proteins, endonucleases in the RNase III family, produce miRNAs and siRNAs through cleavage of dsRNA (Ketting et al. 2001). Argonaute proteins use miRNAs or siRNAs produced by Dicer to recognize and prevent translation of corresponding mRNA sequences (Carmell et al. 2002). The Argonaute protein family, containing both Ago and Piwi subgroups, is characterized by the N terminal domain of variable length, the central PAZ (Piwi-Argonaute-Zwille) domain, the mid domain and the RNase H-like C-terminal Piwi domain (Zha et al. 2012). Figure 3.4 shows a multiple sequence alignment of the deduced amino acid sequences of putative *F. fusca* argonaute-2 proteins sequenced from adult and larval *F. fusca* with the top hit from *Tribolium castaneum*

(NP_001107828.1). Conserved domains PLNO3202, DUF1785, PAZ_argonaute_like and Piwi_ago-like are indicated.

Transmembrane protein SID-1 is responsible for spreading the RNAi response among neighboring cells (systemic RNAi) in *Caenorhabditis elegans* (Winston et al. 2002). Because a systemic RNAi response would increase the efficacy of insecticidal dsRNA compounds, the presence and function of SID-1 proteins are being investigated in insects (Price and Gatehouse 2008). Insect cells expressing a sid-1 gene from *C. elegans* are more efficient at the uptake of dsRNA from a medium (Kobayashi 2012). The expression level of a SID-1 homolog in grasshopper tissues has been linked with the systemic RNAi response (Dong and Friedrich 2005). However, the role of sid-1 genes in the systemic RNAi response has not been empirically-determined in *Tribolium* (Tomoyasu et al. 2008). *F. fusca* transcriptomes contain sequences (adult contig 27876 and larval contig 31274) that have homology (e-value = 0.0 and >60% similarity) to SID-1 proteins in other insects (Table 3.1) and contain conserved protein domain family SID-1_RNA_chan; a requirement for systemic RNAi. Insecticidal uses of dsRNA for *F. fusca* hold promise if there is definitive connection between systemic RNAi and SID-1.

Insecticide mode of action. Table 3.3 contains sequences with homology (e-value = 0.0) to proteins that are known targets of current commercial insecticides, including acetylcholine receptors, acetylcholine esterase, sodium channels, gamma-aminobutyric acid (GABA) channels and ryanodine receptors. The cholinergic system is the primary target of many insecticide classes, including neonicotinoids, spinosad, organophosphates, and carbamates. Multiple transcripts with homology (e-value = 0.0) to insect acetylcholine,

acetylcholinesterase and acetylcholine receptors were identified in *F. fusca* (Table 3.4). A putative nicotinic acetylcholine receptor subunit (adult contig 30107) with an e-value of 0.0 was explored to identify its conserved domains. Figure 3.5 shows this contig aligned with the top hit from *Ctenocephalides felis* with conserved extracellular loops and transmembrane helices identified (Marshall et al. 1990; Dederer et al. 2011).

Xenobiotic metabolizing enzymes. Insecticide resistance management is of vital importance in the control of *F. fusca*. Reported instances of populations that demonstrate neonicotinoid-resistance are increasing in Southeastern and Mid-south cotton growing regions of the US (Martin 2015). Increased enzymatic activity is the most common mechanism of resistance found in insects, though target-site insensitivity is also common (Scott 1999). It is possible to investigate both mechanisms by using RNA-sequencing to compare population-level differences in gene expression levels and nucleotide sequences.

A large number of putative xenobiotic enzymes that in general can be responsible for insecticide resistance were located in *F. fusca* libraries. Table 3.5 shows examples of sequences that have matches (E-value $\leq E^{-3}$) to proteins in the available, public database. Cytochrome p450 monoxygenases are responsible for metabolism of insecticides and plant toxins. Aphids that overexpress a cytochrome p450 enzyme as a result of a mutation to a promotor sequence are resistant to nicotine, a condition that may preadapt populations to neonicotinoid resistance (Bass et al. 2013). RNAi was used to knock down expression of a NADPH-cytochrome reductase in *Cimex lectularius*, thereby increasing its pyrethroid susceptibility (Zhu et al. 2012). Figure 3.6 shows a putative *F. fusca* cytochrome p450 (adult contig 3422 and larval contig 19647) with an e-value of 0.0 aligned with the sequence from

this protein (AFD50507.1). Conserved domains CYPOR, SDR super family, CysJ and Flavodoxin_1; binding motifs for FAD, NADPH and phosphate; and catalytic residues that were identified in the *F. fusca* contigs are indicated (Marchler-Bauer 2015). There were a total of 348 adult sequences and 360 larval sequences with homology (E-value ≤ E-3) to xenobiotic metabolizing enzymes found in the GenBank nr (non-redundant) protein database (Table 3.6).

Growth and Developmental hormones. Table 3.7 shows examples of contigs for putative hormone receptors sequenced in our *F. fusca* cDNA libraries. The neuropeptide allatostatin is produced in the frontal ganglion and although it may have several functions in insects, most research has been on its role in the regulation of JH biosynthesis (Stay and Tobe 2007); JH is responsible for many aspects of insect development including metamorphosis, reproduction, diapause, migration, color, and much more (Riddiford 2007). Eclosion hormone is another neuropeptide involved in the fundamental process of molting in insects (Ewer et al. 2007). In general, discovery of these relatively small neuropeptides, which are typically in low abundance, especially in a whole body transcriptome, is a demonstration of the power of Illumina® sequencing. Deep sequencing of many short reads with this technology will aid in the discovery of important regulatory elements associated with insect endocrinology.

JH biosynthesis in insects occurs in a pair of endocrine organs, the corpora allata (Schooley and Baker 1985). Table 3.7 demonstrates how deep sequencing was successful in obtaining at the least the putative partial sequence of some of the enzymes in this pathway in *F. fusca* and indicates if sequences were not obtained. Farnesyl pyrophosphate synthase, for

example, is involved in the final stages of JH III biosynthesis prior to the JH III branch, and JH acid methyl transferase is found in the JH branch and is responsible for the addition of a methyl group to farnesoic acid to produce methyl farnesoate, the next to last step before the synthesis of JH III by the addition of a C10,11 epoxide (Schooley and Baker 1985). Regulation of JH titer in insects is unique compared to other animals since two processes are involved: biosynthesis, as just discussed, and dynamic changes in JH degradation (de Kort and Granger 1998). Table 3.7 shows two of the most important enzymes in the degradation process that were putatively sequenced from *F. fusca*: (i) JH epoxide hydrolase, which converts the epoxide of JH to a diol; and (ii) JH esterase, which removes the JH methyl ester, producing JH acid (Schooley and Baker 1985).

Vitellogenin (Vg) is an egg yolk precursor that, in general, shows expression specific to sex, tissue-type and developmental stage (Valle 1993). Vg is synthesized in fat bodies and ovarian follicles of female insects and transported into eggs by receptor-mediated endocytosis. Synthesis of Vg begins in pupal or adult stages of most insects (Pereira and De Bianchi 1983). The silk moth *Bombyx mori*, a species which does not feed as pupae or adults, begins Vg synthesis during the final larval molt and continues synthesis through pupation (Lin et al. 2013; Yang et al. 2014). Adult (contig 32814) and larval (contig 339075) *F. fusca* have putative transcripts for Vg and vitellogenin receptor proteins (VgR). VgR contigs contain multiple Low Density Lipoprotein Receptor Class A (LDLa) conserved domains, a protein domain associated with endocytosis of lipoproteins (Marchler-Bauer A et al. 2015). The VgR contigs with the lowest e-value from adult and larval *F. fusca* are shown in Figure 3.7 aligned with the top hit from *Periplaneta americana* (BAC02725.2) with

conserved domains and cysteine residues indicated. However, the identity of these proteins as insect Vg and VgR, opposed to other carrier and receptor proteins, remains unclear. Thrips have a unique developmental pathway with characteristics of both hemimetabolous and holometabolous insects. Thrips are considered a hemimetabolous sister-group to Hemiptera; however, they have two non-feeding life stages, the propupa and pupa, between larval and adult stages. It is highly unlikely that larval samples were contaminated by adult tissues during or prior to RNA extraction since thrips are easily visually and temporally separated by life stage (Lowry et al. 1992). It is possible that fast-developing larval thrips could molt to the propupal stage immediately prior to or during the time of RNA extraction. It is of further scientific value to explore when the transcription and expression of hormones occur to better understand, and perhaps interrupt or interfere with, development and reproduction in *F. fusca*.

Microbiome. RNA-sequencing of *F. fusca* revealed putative protein messages produced by its microbiome. Whole body sequencing has been shown to enable exploration of microorganisms that live within insects (Zhang et al. 2010). Contigs from the *F. fusca* libraries were identified that had best matches in the non-redundant protein database to *Wolbachia pipientis* and other genera of bacteria (Table 3.8), yeast-like symbionts, and plant-infecting viruses.

Wolbachia, a genus of bacteria closely-associated with insect and nematode species, has the capacity to influence host reproductive systems and confer protection against invading viruses (Hedges et al. 2008). It is possible to use *Wolbachia* gene sequences, like those sequenced from *F. fusca*, to determine insect phylogeny (Zhou et al. 1998) or provide

alternative methods of biological control (Werren 1997). Contigs in the *F. fusca* libraries had homology (E-value ≤ E^{-3}) to *Wolbachia* proteins found in *Nasonia vitripennis* and *Culex quinquefasciatus*. There were additional contigs from *F. fusca* libraries with hits to other bacterial genera including *Streptococcus*, *Candidatus*, *Escherichia*, *Curvibacter* and *Rickettsia*.

Contigs from *F. fusca* had homology (E-value ≤ E^{-3}) to proteins from insect-vectored plant viruses. The *F. fusca* used to generate our data were from tomato spotted wilt virus (TSWV) vector populations, but they had no exposure to this virus; thus there were no TSWV sequences in our transcriptomes. Putative viral RNA-dependent RNA polymerases (RdRp) proteins were identified in *F. fusca* (Table 3.9). Adult contigs (contig 30023 and contig 28934) had hits with homology (E-value ≤ E^{-3}) with proteins from viruses in the genus *Phytoreovirus*, with the top hit to wound tumor virus (WTV) (GenBank accession no. CAA32438.1). Phytoreoviruses are double-stranded RNA viruses that infect plants and invertebrates; members of this genus include WTV, rice dwarf virus (RDV) and rice gall dwarf virus (Omura 1995). These viruses are vectored by leaf hoppers and have no association to date with thrips. Adult *F. fusca* also contain 2 putative minor core proteins (contig 69323 and 11083) and a putative mRNA capping enzyme (contig 20609) with homology (E-value ≤ E^{-3}) to proteins from phytoreoviruses. Larval contig (30026) had homology (E-value ≤ E^{-3}) to RdRp proteins from *Rhabdoviridae*, a large viral family with multiple animal and plant hosts. However, the larval contig did not share homology (E-value ≤ E^{-3}) with the putative adult RdRp contig, indicating that they may have separate origins. Viral RNA messages captured by the high-throughput sequencing of host organisms, like

those found in our *F. fusca* transcriptomes, present an intriguing area for further research.

Summary

In summary, we report here the first Illumina® transcriptome to *F. fusca*, a serious pest of agricultural in the Southeast and Midsouth regions of the US. Global analysis of these data and characterization of specific messages show that the transcriptome was successful in the identification of rare and small messages, even when the transcriptome was constructed for mRNA from a whole body homogenate. These results provide many new leads for the development of both RNAi and artificial antibody approaches of control of this important insect pest of cotton and other crops, where currently there is no commercial plant transgenic approach to thrips control. Our data can be used to compare gene expression levels between discreet thrips populations for a global evaluation of the risk of insecticide resistance. In addition, these transcriptomes provide opportunities to study the basic physiology of this pest.

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Tables

Table 3.1 Contigs sequenced from adult (D) and larval (L) *F. fusca* with homology (E-value ≤ E-3) to proteins from the GenBank nr (non-redundant) protein database involved in RNAi and/or miRNA pathways.

Protein name	Contig (D, L)	Length (bps) (D, L)	E-value (D, L)	Conserved domain(s)	Best match to NR protein database	
					Organism	Accession no.
Argonaute-1	14354 , --	1390, --	0.0, --	PAZ_argonaute_like; Piwi-like super family; DUF1785	*T. castaneum*	EFA09197.1
Protein Argonaute-2-like	31592 , 26183	3137, 3443	0.0, 0.0	Piwi_ago-like (D, L); PAZ_argonaute_like (D, L); DUF1785 (D, L) M_domain super family (L); PLN03202 (L)	*T. castaneum*	NP_001107828.1 (D, L)
Argonaute 3	27083 , 20522	1581, 2253	1.5e-116, 0.0	PAZ_piwi_like (D, L); Piwi_piwi-like_Euk (D, L)	*B. mori* (D, L)	BAF98575.1
Dicer-partial	78542 , 24396	619, 333	4.5e-87, 4.8e-29	RIBOc (D); RIBOc super family (L)	*B. germanica* (D) *A.* gossypii (L)	CAX68236.1 CCD32417.1
Endoribonuclease dicer	32160 , 28876	2573, 748	7.0e-73, 1.6e-25	DEXDc (D); RIBOc (L); DSRM super family (L)	*T. castaneum* (D) *C. floridanus* (L)	NP_001107840.1 EFN62420.1
Endoribonuclease dicer-like	30681 , 33423	2700, 828	5.8e-63, 3.2e-23	RIBOc (D); Rnc (D); RIBOc super family (D, L)	*A. echinatior* (D) *C. quinquefasciatus* (L)	EGI69620.1 XP_001844757.1
Dicer-1	75368 , 21724	624, 1627	1.1e-41, 2.0e-144	PAZ super family (D, L)	*L. migratoria* (D, L)	AFK29469.1
Dicer 2	43573 , 33432	436, 699	2.0e-29, 2.2e-6	Ribonuclease III C Terminal Domain (D, L)	*A. gambiae* (D) *B. mori* (L)	320248.4 NP_001180543.1
Sid1 transmembrane family member	27876 , 31274	2811, 2461	0.0, 1.1e-81	SID-1_RNA_chan super family (D, L)	*A. echinatior* (D) *H. saltator* (L)	EGI61710 EFN87718.1
Piwi	28314 , 22089**	3181, 3082	0.0, 0.0	Piwi_piwi-like_Euk (D, L) PAZ_piwi_like (D, L)	*A. mellifera* (D, L)	ACV84378
Piwi-like protein 1-like	28095*, --	755, --	4.3e-83, --	Piwi_piwi-like_Euk (D)	*D. plexippus*	EHJ69790.1
Protein Piwi-like	27488*, --	446, --	2.0e-19, --	RAM (D)	*B. mori*	NP_001098066
Piwi-like homolog 4	20799*, 15277	702, 1144	3.0e-6, 8.9e-7	Piwi_piwi-like_Euk (D, L)	*B. mori* (D) *T. castaneum* (L)	ABV60274 EFA07425.1

-- Indicates a homolog was not found in the corresponding library.
* Indicates multiple contigs with homology with the same protein, the contig shown has the lowest e-value.
** Indicates multiple contigs with homology with the same protein that have the same e-value, the contig shown has the greatest length.

Table 3.2 The total number of sequences from *F. fusca* with BLAST hits from the GenBank nr (non-redundant) protein database that are associated with the RNAi and miRNA pathways.

Name	No. adult sequences	No. larval sequences
Argonaute	3	3
Dicer	12	10
Piwi	5	4
Sid 1	1	2

Table 3.3 Contigs sequenced from adult (D) and larval (L) *F. fusca* with homology (E-value ≤ E-3) to proteins from the GenBank nr (non-redundant) protein database that are currently commercial insecticidal targets.

					Best match to NR protein database	
Protein name	Contig (D, L)	Length (bps) (D, L)	E-value (D, L)	Conserved domain(s)	Organism	Accession no.
Acetylcholinesterase	31613**, 25168*	2184, 1444	0.0, 0.0	Aes (D); Esterase_lipase (D, L)	*L. bostrychophila* (D) *N. cincticeps* (L)	ACN78619.1 BAI63733.1
GABA-gated chloride channel	30589 , 32210*	1585, 1104	0.0, 2.2e-147	Neuronal_acetylcholine_receptor_ subunit_alpha-7 (D) Neur_chan_LBD (D, L); Neur_chan_memb (D, L);	*S. furcifera* (D) *T. castaneum* (L)	BAL63029.1 EFA12941.1
Glutamate-gated chloride channel	24336* , 32703*	1959, 1807	0.0, 0.0	Neur_chan_LBD (D, L); Neur_chan_memb (D, L)	*L. striatella* (D, L)	AFI09244.1
Nicotinic acetylcholine receptor	30107**, 30375**	2296, 1873	0.0, 0.0	Neur_chan_LBD (D, L); Neur_chan_memb (D, L); Neuronal_ acetylcholine_receptor_ subunit_alpha-7 (D, L)	*C. felis* (D, L)	CBX19382.1
Ryanodine receptor	23491**, 33300*	2033, 7738	0.0, 0.0	RIH_assoc (D, L); RYDR_ITPR (L); RyR (L);	*H. saltator* (D) *L. striatella* (L)	EFN78897.1 AFK84959.1
Voltage-gated sodium channel	9104**, 28590*	1546, 1210	0.0, 7.3e-166	Ion_trans (D, L); Na_channel_gate super family (L); Na_channel_gate (L); MIG-14_Wnt-bd super family (L)	*A. echinatior* (D) *D. plexippus* (L)	EGI61230 EHJ74501.1

* Indicates multiple contigs with homology with the same protein, the contig shown has the lowest e-value.
** Indicates multiple contigs with homology with the same protein that have the same e-value, the contig shown has the greatest length.

Table 3.4 The total number of sequences from *F. fusca* with BLAST hits from the GenBank nr (non-redundant) protein database that are associated with insecticidal mode of actions in the insect nervous system.

Name	No. adult sequences	No. larval sequences
Acetylcholinesterase	8	10
GABA-gated chloride channel	3	2
Glutamate-gated chloride channel	4	7
Acetylcholine receptor	51	57
Ryanodine receptor	19	26
Sodium channel	28	42

Table 3.5 Contigs sequenced from larval adult (D) and larval (L) *F. fusca* with homology (E-value ≤ E-3) to proteins from the GenBank nr (non-redundant) protein database that are involved in xenobiotic metabolism.

					Best match to NR protein database	
Protein Name	Contig (D, L)	Length (bps) (D, L)	E-value (D, L)	Conserved domain(s)	Organism	Accession no.
Carboxylesterase	31375* , 25201*	1969, 2165	0.0, 2.8e-168	Aes (D); COesterase (D, L)	*L. striatella* (D) *A. echinatior* (L)	ADR73024.1 EGI68854.1
Catalase	26939* , 19412*	1881, 1842	0.0, 0.0	catalase_clade_3 (D, L); KatE (D, L)	*S. gregaria* (D, L)	AEV89764.1
Cytochrome p450	28270**, 19647**	3422, 3087	0.0, 0.0	CYPOR (D); Flavodoxin_1 (D); CysJ (D); p450 (L)	*C. lectularius* (D) *F. occidentalis* (L)	AFD50507.1 AED99066.1
Glutathione S-transferase	25703* , 26064*	2076, 2281	3.1e-160, 3.6e-108	AdoMet_MTases (D, L)	*C. floridanus* (D, L)	EFN73893.1
NADH dehydrogenase	26390* , 20200**	1836, 1846	2.2e-125, 0.0	PRK06074 (D, L) Complex1_51K (L); NADH_4Fe-4S (L); SLBB (L)	*A. gambiae* (D) *T. castaneum* (L)	XP_316497.2 NP_001164305.1
Superoxide dismutase	30579* , 3402*	1237, 1132	5.2e-103, 8.3e-100	PLN02957 (D); HMA (D); Cu-Zn_Superoxide_Dismutase (D); Sod_Fe_C (L); Sod_Fe_N (L); SodA (L)	*S. salar* (D) *A. gambiae* (L)	NP_001133786.1 XP_314490.4

* Indicates multiple contigs with homology with the same protein, the contig shown has the lowest e-value.
** Indicates multiple contigs with homology with the same protein that have the same e-value, the contig shown has the greatest length.

Table 3.6 The total number of sequences from *F. fusca* with BLAST hits from the GenBank nr (non-redundant) protein database that are associated with xenobiotic metabolism.

Gene name	No. adult sequences	No. larval sequences
Carboxylesterase	39	26
Catalase	14	45
Cytochrome P450	211	204
Glutathione s-transferase	40	36
NADH dehydrogenase	27	31
Superoxide dismutase	17	18

Table 3.7 Contigs selected by the lowest e-value that were sequenced from adult (D) and larval (L) *F. fusca* with homology (E-value ≤ E-3) to proteins from the GenBank nr (non-redundant) protein database that are involved in insect growth and development.

Protein Name	Contig (D, L)	Length (bps) (D, L)	E-value (D, L)	Conserved domain(s)	Best match to NR protein database	
					Organism	Accession no.
Allatostatin precursor	2474* , 27452*	880, 9762	4e-30, 1.2e-29	none	*A. mellifera* (D, L)	P85798.1
Allatostatin receptor	77189 , 13843	1417, 1035	5.6e-126, 2.8e-128	7tm_4 super family (D, L); 7tm_1 (D, L)	*P. american* (D, L)	AAK52473.1
Coenzyme A reductase	18781 , 23549	568, 2049	1.5e-12, 0.0	HMG-CoA_reductase_classI (D, L)	*B. germanica* (D) *I. paraconfusus* (L)	P54960.1 AAD20975.2
Diphosphomevalonate decarboxylase	26356* , 29773*	1432, 1481	8.8e-124, 2.5e-154	GHMP_kinases_N super family (D, L); Diphosphomevalonate_ decarboxylase (D, L)	*B. mori* (D, L)	NP_001093300.1
Eclosion hormone	-- , 32699*	-- , 684	-- , 4.4e-13	Eclosion super family	*D. willistoni*	XP_002069
Farnesoic acid methyltransferase	26966* , 29573	1368, 1362	5.5e-133, 5.1e-133	Methyltransf_FA (D, L); DM9 (D,L); DUF3421 (L)	*S. gregaria* (D, L)	ADV17352.1
Farnesyl pyrophosphate synthase	20429* , 31785*	1227, 1630	6.3e-75, 1.3e-37	Trans_IPPS_HT (D) Isoprenoid_Biosyn_C1 super family (L); Draxin super family (L)	*B. terrestris* (D) *A. pisum* (L)	AFI55104
Farnesyl pyrophosphate synthetase (FPP synthetase)	-- , 911583	-- , 136	-- , 9.1e-5	none	*C. militaris*	EGX94931.1
Juvenile hormone acid methyltransferase	11138 , 834	992, 781	1.7e-63, 3.3e-37	AdoMet_MTases (D, L)	*T. castaneum* (D, L)	BAG30999.1
Juvenile hormone epoxide hydrolase	29499 , --	2207, --	4.3e-166, --	EHN (D); MhpC (D)	*T. castaneum*	NP_001161927.1
Juvenile hormone esterase	30118* , 32940	2344, 2197	4.8e-119, 9.0e-78	Esterase_lipase super family (D, L)	*R. flavipes* (D, L)	ACT53736.1
Mevalonate kinase	31377* , 29161*	3408, 2891	2.1e-68, 1.1e-78	GHMP_kinases_N (D, L); GHMP_kinases_C (D, L); PLN02677 (D, L)	*C. floridanus* (D) *P. xuthus* (L)	EFN64406.1 BAM18406.1
Phosphomevalonate kinase	27323* , 27847*	637, 1016	6.8e-48, 6.2e-29	P-mevalo_kinase super family(D, L)	*B. mori* (D, L)	BAF62110.1
Vitellogenin receptor	31924* , 24285*	5767, 1912	0.0, 0.0	LDLa (D, L); EGF_CA (D, L); LY (D, L); Ldl_recept_b (D, L); Ldl_recept_b super family (D, L); FXa_inhibition (D, L)	*P. americana* (D, L)	BAC02725.2
Vitellogenin	32814**, 339075*	3265, 801	0.0, 6.0e-59	Vitellogenin_N (D, L)	*L. deyrollei* (D) *P. stali* (L)	BAG12118.1 BAA88076.1

-- Indicates a homolog was not found in the corresponding library.
* Indicates multiple contigs with homology with the same protein, the contig shown has the lowest e-value.
** Indicates multiple contigs with homology with the same protein that have the same e-value, the contig shown has the greatest length.

Table 3.8 The total number of sequences from *F. fusca* with homology (E-value ≤ E-3) to proteins from bacterial genera.

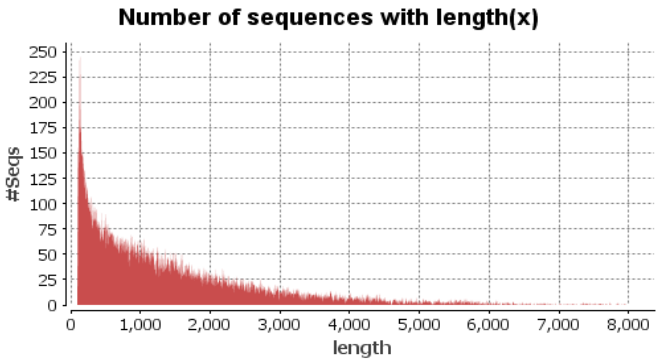
Genera	No. adult sequences	No. larval sequences
Candidatus	1	16
Curvibacter	1	0
Escherichia	5	8
Rickettsia	1	1
Streptococcus	1	7
Wolbachia	8	16

Table 3.9 Contigs that were sequenced from larval (L) and adult (D) *F. fusca* with homology (E-value ≤ E-3) to viral RNA polymerases.

					Best match to NR protein database	
Protein Name	Contig (D, L)	Length (bps)	E-value	Conserved domain(s)	Organism	Accession no.
RNA-dependent RNA polymerase	12959L	378	3.8e-7	RT_ZFREV_like	*R. delemar*	EIE78134.1
RNA-dependent RNA polymerase	30026L	3820	6.1e-174	Methyltrans_Mon super family; Mononeg_mRNAcap	Pike fry rhabdovirus	ACP28002.1
RNA-directed RNA polymerase	149740D	388	2.0e-15	none	*Homalodisca vitripennis* reovirus	YP_002790884.1
RNA-directed RNA polymerase	2131D	544	2.1e-13	none	Rice dwarf virus	NP_620544.1
RNA-dependent RNA polymerase	30023D	1977	2.66e-11	Phytoreo_S7	Wound tumor virus	CAA32438.1
RNA-directed RNA polymerase	159082D	337	8.1e-9	none	Rice gall virus	ADF59186.1
RNA-directed RNA polymerase	206420D	253	6.0e-9	none	Rice dwarf virus	BAA14222.1
RNA-dependent RNA polymerase	289349D	272	5.36e-7	none	Rice dwarf virus	BAA01074.1

Figures

A)



B)

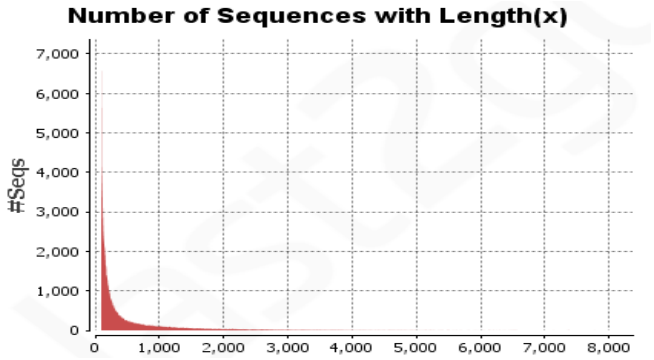


Figure 3.1 Contig length of assembled reads from Illumina® sequencing of the transcriptome from whole bodies of larval (A) and adult (B) *F. fusca*.

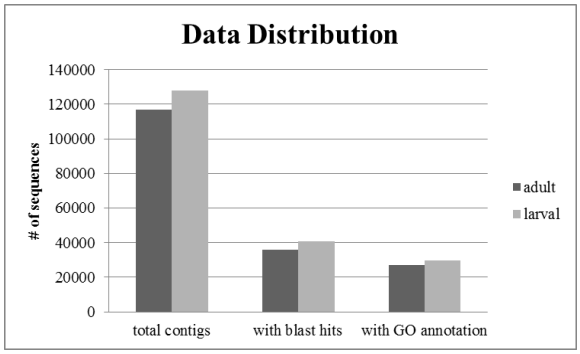


Figure 3.2 The number of contigs assembled from whole body sequencing of *F. fusca* (total contigs), the number of sequences with matches in the Basic Local Alignment Search tool at a cutoff e-value of 10^{-3} (with blast hits) and the number of sequences annotated with gene ontology terms (with GO annotation).

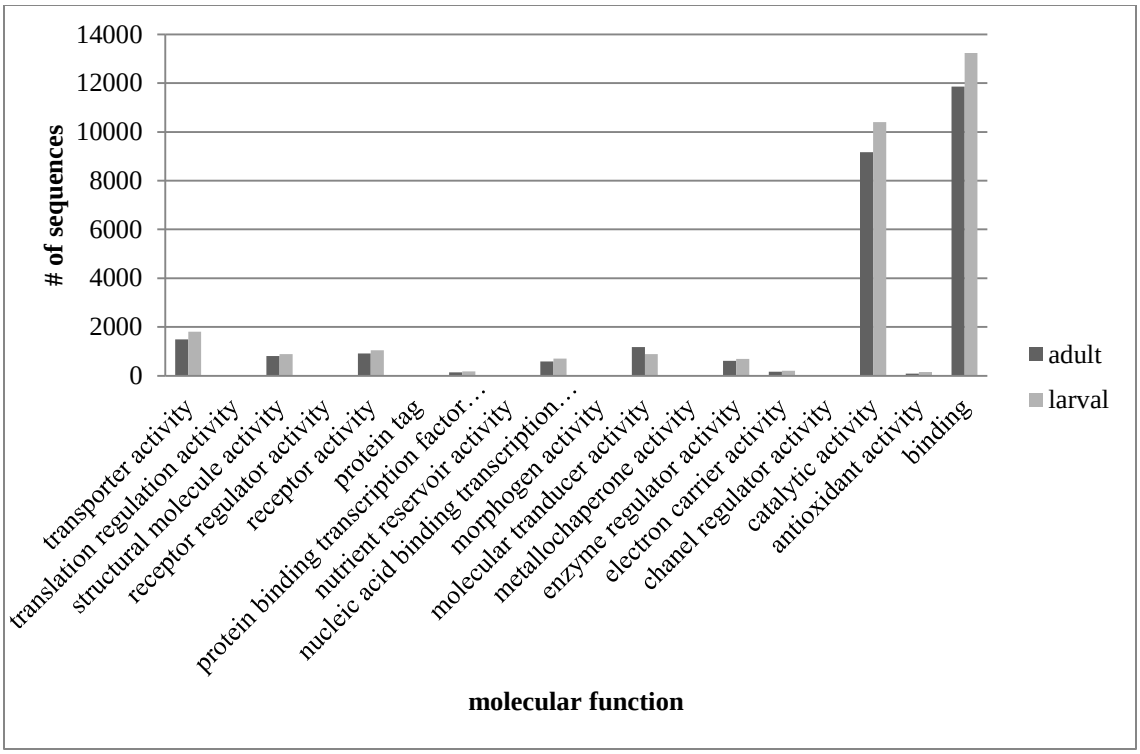


Figure 3.3 Sequences assigned by GO analysis to molecular function (x) for whole body adult and larval *F. fusca*.

Figure 3.4 Multiple sequence alignment (ClustalW) of the deduced amino acid sequences of putative *F. fusca* argonaute-2 proteins (larval contig 26183 and adult contig 31592) with the top hit from *Tribolium castaneum* (NP_001107828.1). Start and end of conserved domains PLNO3202 (A), DUF1785 (B), PAZ_argonaute_like (C) and Piwi_ago-like (D) are indicated. An asterisk (*) denotes identical sequences, a colon (:) indicates conservation between groups of strongly similar properties and a period (.) denotes conservation between groups of weakly similar properties.

```
                                                                A
F.fusca_L       MPPPVAQAPIQAPPPSWGQPTPQQSQAPRQPSSAPGGQSTPHQGQQPRQMAPAKAAATPS      60
F.fusca_A       MPPPVAQAPIQAPPPSWGQPTPQQSQAPRQPSSAPGGQSTPHQGQQPRQMAPAKAAATPS      60
T.castaneum     MGAPQQQQPQRGPLPQQQ-----QQQQ----------------QRRPQQQQPPVKSPVPH      39
                *  *  * * :.* *.       *.*                 ::*:*  *   : .*

F.fusca_L       GDAQSTPRSVQPRGAQA-GK-SSQAVVLPRRVDCPNEPKSGRQIVLEVNHLLLKIADPGV      118
F.fusca_A       GDAQSTPRSVQPRGAQA-GK-SSQAVVLPRRVDCPNEPKSGRQIVLEVNHLLLKIADPGV      118
T.castaneum     PEPSSPPRQEPAPPLSGGGDCLSGALV-------VTPGTKGRRIQIESNHLSLNLGK-LT      91
                 : .* **.      .. *.  * *:*        .  ..**:* :* *** *::..  .

F.fusca_L       KIYHYDVSIDPDKPKRFLRLVMDEMQRKCYPQNFPGFDGSKNLYSKGPLPFKDEITEEVN      178
F.fusca_A       KIYHYDVSIDPDKPKRFLRLVMDEMQRKCYPQNFPGFDGSKNLYSKGPLPFKDEITEEVN      178
T.castaneum     EAYHYDVAITPDTPKCLLRDVMNLFGRKHYPQNHPAFDGRKNLYSPKKLPFPNDTKSDTI      151
                : *****:* **.** :** **: : ** ****.*.*** *****   *** :: ..:.

F.fusca_L       VPDSERQDKKLKTFKVTIKLARNNISLQELFAYQQKGKSTSIPQDVVQAVDVILRSASAQ      238
F.fusca_A       VPDSERQDKKLKTFKVTIKLARNNISLQELFAYQQKGKSTSIPQDVVQAVDVILRSASAQ      238
T.castaneum     EVEGE---NRKKEFKVEVKLARTV----DLTPLHDIMRTTQSPQDALQCLDIVLRNAPSN      204
                  :.*   :: * *** :****.     :*   ::  ::*. ***.:*.:*::**.* ::
                 B                                                  B,C
F.fusca_L       KFVQVGRSFFCPPMTP--RDLGNGMELWYGFFQSFVMGSKPYLNIDVAHKGFPKSQPLLS      296
F.fusca_A       KFVQVGRSFFCPPMTP--RDLGNGMELWYGFFQSFVMGSKPYLNIDVAHKGFPKSQPLLS      296
T.castaneum     ACIIAGRCFFTPPRDGQIIPLGDGMELYYGFYQSAIRGWKALLNVDVAHKAFPKASNVLD      264
                  : .**.** **       **:****:***:** : * *  **:*****.***:. :*.

F.fusca_L       LLKAMDPR-------AEKPSDALRYKRNEFERFIKGLKVEYQLPNVETSKRTYRVNGLVR      349
F.fusca_A       LLKAMDPR-------AEKPSDALRYKRNEFERFIKGLKVEYQLPNVETSKRTYRVNGLVR      349
T.castaneum     IVCEIGSDFRTTMTRANLSQPLREFVQRDFEKFIKQLKVKYEIPNQSSSKRIHRVNGLGE      324
                ::  :          *:  .   .: :.:**:*** ***:*::** .:*** :*****  .
                                                                          C
F.fusca_L       NAFDERFQLDDSGRTMSIGEYFEKEKKYTLRYPDLPCVHVGHRD--KTIYVPMELCVLSP      407
F.fusca_A       NAFDERFQLDDSGRTMSIGEYFEKEKKYTLRYPDLPCVHVGHRD--KTIYVPMELCVLSP      407
T.castaneum     PPSQAKFKLDD-GRMTTVERYYQEVKRCKLQYPHLPTLWVGSRERESKILLPLEFCTVVG      383
                   : :*:*** **  :: .*::: *: .*:**.** : ** *:  ..* :*:*:*.:
                                                        D
F.fusca_L       GQATMKKLDETQTAAMVRIAATNVQDRKNKIKEAMQRANLNSSPHVKELGLSVATEFAKI      467
F.fusca_A       GQATMKKLDETQTAAMVRIAATNVQDRKNKIKEAMQRANLNSSPHVKELGLSVATEFAKI      467
T.castaneum     GQAINRKMNENQTSAMIRKAATSTDVRKDKIMQTLRTANYNNDPCIREFGFSVSNNFEKL      443
                ***  :*::*.**:**:* ***..: **:** :::: ** *..* ::*:*:**:.:* *:

F.fusca_L       NGRILPAPKLAYYQDRTVQPARGVWRMDGMQFRETRELDKWICLVINSRVRPDDIRSFTE      527
F.fusca_A       NGRILPAPKLAYYQDRTVQPARGVWRMDGMQFRETRELDKWICLVINSRVRPDDIRSFTE      527
T.castaneum     DARVLNPPSLLYADNAQIKPSKGVWRADRNRFLVGATINKWTIASGTR-YPSRDADKLAD      502
                :.*:*  *.* * ::  ::*::**** *  :*     ::**     .      *  .:::

F.fusca_L       NLRSEAKRVNMVMAAPAPPV-IMDVRDQRKIMGELSNRLGKFRQEGHKLVLVVISGYNKQ      586
F.fusca_A       NLRSEAKRVNMVMAAPAPPV-IMDVRDQRKIMGELSNRLGKFRQEGHKLVLVVISGYNKQ      586
T.castaneum     MIFRMASSNGMQITSKATPSTHIGGR------QGLRDFIDYFKGKQDYDLIIVVVPNSGP      556
                 :   *.   * ::: * *   :  *        * : :  *: : .  :::*:   .

F.fusca_L       IYGQVKQVAELQVGVLTQCVKDETVTRRNNQATVHNIMLKINAKFNGVNQVIADQSKVKC      646
F.fusca_A       IYGQVKQVAELQVGVLTQCVKDETVTRRNNQATVHNIMLKINAKFNGVNQVIADQSKVKC      646
T.castaneum     QYSFVKQAAELNVGCLTQCIKERTIGRLN-PQTVGNILLKINSKMNGTNHRLSPNSRPLI      615
                 *. ***.***:** ****:*:.*: * *   ** **:****:*:**.*: :: :*:

F.fusca_L       FNSKVLVFGADVTHPSPDSKDIPSVAAVTASHDIKGFKYNMRVRLQAPRQEMIEDLAAIV      706
F.fusca_A       FNSKVLVFGADVTHPSPDSKDIPSVAAVTASHDIKGFKYNMRVRLQAPRQEMIEDLAAIV      706
T.castaneum     MKRPCMIMGADVTHPSPDARDIPSVAAVTASHDPNAFQYNICWRLQPPKVEIIEDLCNIT      675
                ::   :::**********::************* :.*:**:  *** *: *:****. *.

F.fusca_L       YEQLAIYKKETGFLPEHIVFYRDGVSEGQFHEVISKEVTAIFRACEQLR---TKPKLTFL      763
F.fusca_A       YEQLAIYKKETGFLPEHIVFYRDGVSEGQFHEVISKEVTAIFRACEQLR---TKPKLTFL      763
T.castaneum     VEQLKFFYQKTGFKPESIVFFRDGVSEGQFKQVQRAEIAAIQKACKMLQKDDYEPKITFL      735
                 *** :: ::*** ** ***:*********::*   *::** :**: *:    :**:***
```

```
F.fusca_L        VVQKRHHTRFFPTDNRDSQDKNGNVPAGTIVDTEITHASEIDFYLVSHASIQGVARPTKY      823
F.fusca_A        VVQKRHHTRFFPTDNRDSQDKNGNVPAGTIVDTEITHASEIDFYLVSHASIQGVARPTKY      823
T.castaneum      VVQKRHHTRLFPTNPRDSEDKNNNVPAGTCVDTHITNPRMQDFYLVSHASIQGVAKPTKY      795
                 *********:***: ***:*** ****** ***.**.    **************:****
                                                                      D
F.fusca_L        HVLWDDAHMSENDIQLMTYNLCHLFTRCDRAVSYPAPTYYAHLAASRGRVYLEGKNVRIE      883
F.fusca_A        HVLWDDAHMSENDIQLMTYNLCHLFTRCDRAVSYPAPTYYAHLAASRGRVYLEGKNVRIE      883
T.castaneum      CTLWDDNNMNNDDIEELTYHLCHMFTRCNRSVSYPAPTYYAHLAAARAKVYIENDKLDMS      855
                  .**** .*.::**: :**.***:****:*:**************:*.:**:* .:: :.
                 A
F.fusca_L        DKKRLGERKQHNTNRDEFYKYLTNVFCVDMF     914
F.fusca_A        DKKRLGERKQHNTNRDEFYKYLTNVFCVDMF     914
T.castaneum      QLKRHQEKCQIQEK---IVKG-KPMFFV---     879
                 : **  *: * : :   : *  . :* *
```

Figure 3.5 Pairwise sequence alignment (ClustalW) of the deduced amino acid sequence of a putative *F. fusca* nicotinic acetylcholine receptor protein (30107) with the top hit from *Ctenocephalides felis* (CBX19382.1). The sequences are 74.4% identical. Conserved extracellular loops (Loop A, Cys loop, Loop B and Loop C) are indicated in light grey with their tyroseine and tryptophane residues denoted by triangles (▼), and their half-cystines and ligand binding sites denoted by circles (°); three predicted transmembrane helices (TM1, TM2 and TM3) are indicated in dark grey (Dederer et al. 2011). An asterisk (*) denotes identical sequences, a colon (:) indicates conservation between groups of strongly similar properties and a period (.) denotes conservation between groups of weakly similar properties.

```
F.fusca      --MAKKGD-MTRGLLLVAVLGAVLLGCARPADANPDAKRLYDDLLSTYNRLIRPVSNNTH      57
C.felis      MYLTKSARSAIQTLLV------FLVLDLREVICNPDAKRLYDDLLSTYNRLIRPVSNNTD      54
               ::*..    : **:      .*:   * . .******* ******************

F.fusca      TVLVKLGLRLSQLIELNLKDQILTTNVWLEHEWQDHKFKWDPSEYGGVTELYVPSEHIWL     117
C.felis      TVLVKLGLRLSQLIDLNLKDQILTTNVWLEHEWQDHKFQWDPTEYGGVTELYVPSEHIWL     114
             **************:************************:***:*****************
               Loop A                                  Cys loop     Loop B
F.fusca      PDIVLYNNADGEYVVTTMTKAVLHYTGKVLWTPPAIFKSSCEIDVRYFPFDQQTCFMKFG     177
C.felis      PDIVLYNNADGEYVVTTMTKAVLHHTGKVVWTPPAIFKSSCEIDVRYFPFDQQTCFMKFG     174
             *************************:****:******************************
                                                                  Loop C
F.fusca      SWTYDGFQIDLQHINQKVGENMVEVGIDLKEYYPSVEWDILGVPAERHERYYPCCNEPYP     237
C.felis      SWTYDGDQIDLKHINQKLGDNKVEVGIDLREYYPSVEWDILGVPAERHEKYYPCCAEPYP     234
             ****** ****:*****:*:* *******:********************:***** ****
                                TM1                                TM2
F.fusca      DIFFNITLRRKTLFYTVNLIVPCVGISYLTVLVFYLPADSGEKIALCINILLSQTMFFLL     297
C.felis      DIFFNITLRRKTLFYTVNLIVPCVGISYLSVLVFYLPADSGEKIALCISILLSQTMFFLL     294
             ******************************:*****************.***********
                                          TM3
F.fusca      ISEIIPSTSLALPLLGKYLLFTMFLVGVSVVITIIVLNVHYRKPSTHKMASWVRKLFIRW     357
C.felis      ISEIIPSTSLALPLLGKYLLFTMLLVGLSVVITIIILNVHYRKPSTHKMAPWVRKFFIKR     354
             ************************:***:*******:************ ****:**:

F.fusca      LPKVLLMRVPDTMLADLVGNRRLFRLSTKKARQMQTSASQVVASSSTASSPDSFRLPPGR     417
C.felis      LPKLLLMRVPKDLLKDLAMNKIAGRGK--KSKFSAALAAQQAHAASGGSSPDSIRHMQGR     412
             ***:******. :* **. *:   * .  *::   : *:* . ::* .*****:*   **

F.fusca      PGGCNGLHSSGATNRFAGL-----------------------SSVLAGLDDSLSDVAIRK     454
C.felis      PSGCNGLHTTTATNRFSGLVGALGGGIGGMGGLGIGGGYNGLPSIMSGLDESLSDVVPRK     472
             *.******:: *****:**                        *:::***:*****. **

F.fusca      KYPFELEKAIHNVLFIKHHMQRQDEFDAEDQDWRFVAMVMDRLFLWIFTVVSLAGTFMIL     514
C.felis      KYPFELEKAIHNVMFIQHHMLRQDEFNAEDQDWGFVAMVLDRLFLWIFTIASIVGTFAIL     532
             *************:**:*** *****:****** *****:*********:.*:.*** **

F.fusca      CEAPSLWDETKAIDTELSNVAQQQYLPDFASMSHAAGVE553
C.felis      CEAPALYDDTKPIDMELSSVAQQQFLPDV----------561
              ****:*:*:** ** ***.*****:***.
```

Figure 3.6 Multiple sequence alignment (ClustalW) of the deduced amino acid sequence of putative *F. fusca* cytochrome p450 proteins (larval contig (L) 16756 and adult contig (A) 28270) with the top hit from *Cimex lectularius* (AFD50507.1). Conserved features are marked; ▼ denote NADP binding pockets, ♦ denote FAD binding pockets, boxes denote catalytic residues, light gray boxes denote phosphate binding motifs and dark gray boxes denote beta-alpha-beta structure motifs. Start and end of conserved domains CYPOR (A), SDR super family (B) and CysJ (C) are indicated. An asterisk (*) denotes identical sequences, a colon (:) indicates conservation between groups of strongly similar properties and a period (.) denotes conservation between groups of weakly similar properties.

```
                                                                C
F.fusca_L          MSDPKETLVEAE-PEAEAWFGLLDVVILVALAAFGVWWVLSNKRKQAEQAAAHKSYSLQP     59
F.fusca_A          MSDPKETLVEAE-PEAEAWFGLLDVVILVALAAFGVWWVLSNKRKQAEQAAAHKSYSLQP     59
C.lectularius      MGDATEMLKEVGVESEEPLFGTLDIALFLALAGVGFWWLMKDRQSK--SSLKDKSYTIQP     58
                   *.* .* * *.   . *  ** **:.:::***..*.**::.::::.:  .:  .***::**

F.fusca_L          TSL--ATQTTDNSFLKKLKTSGRSLVAFYGSQTGTAEEFAGRLAKEGIRYQLKGMVADPE     117
F.fusca_A          TSL--ATQTTDNSFLKKLKTSGRSLVAFYGSQTGTAEEFAGRLAKEGIRYQLKGMVADPE     117
C.lectularius      MAMSSMPQNTDNSFIKKLKASGRSLIVFYGSQTGTGEEFAGRIAKEGIRYKMKGMVADPE     118
                    ::    *.*****:****:*****:.********.******:*******::********

F.fusca_L          EYDMEELVNLKDIPNSLAVFCLATYGEGDPTDNAMEFYEWLQNGDANLTGLNYAVFGLGN     177
F.fusca_A          EYDMEELVNLKDIPNSLAVFCLATYGEGDPTDNAMEFYEWLQNGDANLTGLNYAVFGLGN     177
C.lectularius      EYDMEDLMQMKNIPNSMAVFCLATYGEGDPTDNAMDFYEWLQNGEGDLTGLNYAVFGLGN     178
                   *****:*:::*:****:******************:********:.:*************
                                                   B                   B
F.fusca_L          KTYEYYNEVAKYVDKRLEELGATRVYELGMGDDDANIEDDFITWKEGFWPAVCEYFGIET     237
F.fusca_A          KTYEYYNEVAKYVDKRLEELGATRVYELGMGDDDANIEDDFITWKEGFWPAVCEYFGIET     237
C.lectularius      KTYERYNEVAIYVDKRLQELGGTRVYQLGLGDDDANIEDDFITWKDKFWPTVCEYFGIEG     238
                   **** ***** ******:***.****:**:****************: ***:********
                                                        A
F.fusca_L          MGEDVSVRQYKLTEHSDIDPSRLYNGEVARLHSFVNQRPPFDTKNPFLANIKVNRELHKT     297
F.fusca_A          MGEDVSVRQYKLTEHSDIDPSRLYNGEVARLHSFVNQRPPFDTKNPFLANIKVNRELHKT     297
C.lectularius      AGEDVSVRQYKLTEHIDNIPERVYTGEVARLHSLINQRPPFDVKNPYLAPVKVNRELHKG     298
                    ************** *  *.*:*.********::*******.***:** :********

F.fusca_L          GDRQCMHLEFDIEGSKMRYDAGDHLALYPVNHTELVNKLGKLLDADLDTAITLTNTDEES     357
F.fusca_A          GDRQCMHLEFDIEGSKMRYDAGDHLALYPVNHTELVNKLGKLLDADLDTAITLTNTDEES     357
C.lectularius      GDRSCMHIEFDIEGSKMRYDTGDHLAVYPMNNLDLVDKLGKLLNVNLDTIFTLTNTDEDS     358
                   ***.***:************:*****:**:*. :**:******:.:*** :*******:*

F.fusca_L          SKKHPFPCPTTYRTALTYYLDITSNPRTHVLKELAEYTKNNKEQEMLRLMASTSPEGKAL     417
F.fusca_A          SKKHPFPCPTTYRTALTYYLDITSNPRTHVLKELAEYTKNNKEQEMLRLMASTSPEGKAL     417
C.lectularius      SKKHPFPCPCSYKTALTYYIDITSNPRTHILKELAEYASDPKEAEKLKLMASTSIEGKQM     418
                   ********* :*:******:******** *:*******:.: ** * *:****** *** :

F.fusca_L          YQKWINQDNRNIIHILEDLPSCKPEIDHICELLPRLQCRYYSISSSPKLYPTTVHVTAVV     477
F.fusca_A          YQKWINQDNRNIIHILEDLPSCKPEIDHICELLPRLQCRYYSISSSPKLYPTTVHVTAVV     477
C.lectularius      YNQWINQDNRNIVHILEDMPSVKPKLDHICELLPRLQCRYYSISSSSKVYPTCVHITAVK     478
                   *::*********:*****:** **::****************** **** *:*** **:***

F.fusca_L          VEYETPTKRINKGVATTWLREMKPVDPEKKHLVPVFIRKSQFRLPTRTQTPIIMIGPGTG     537
F.fusca_A          VEYETPTKRINKGVATTWLREMKPVDPEKKHLVPVFIRKSQFRLPTRTQTPIIMIGPGTG     537
C.lectularius      VEYDTPTGRRNEGVATSWLGKKIPNDSNSLPLVPIFIRKSQFKLPTRTNTPIIMIGPGTG     538
                   ***:*** * *:****:** :  * * :.  ***:*******:*****:*********

F.fusca_L          LAPFRGFIQERALAVEEGKPVGETILYFGCRKRSEDFLYEEELTEYEKKGVLKLHLAFSR     597
F.fusca_A          LAPFRGFIQERALAVEEGKPVGETILYFGCRKRSEDFLYEEELTEYEKKGVLKLHLAFSR     597
C.lectularius      LAPFRGFIQERALAKQEGKPVGDTILYFGCRKKAEDFIYQEELEAFVENGNLKMHVAFSR     598
                   **** ********** :******:*********::***:*:***  : ::* **:*:****

F.fusca_L          DQAKKVYVTHKLQENAEEIWRVLGENNGHVYVCGDARSMAKDVHNIILKVVQEKGKMSEQ     657
F.fusca_A          DQAKKVYVTHKLQENAEEIWRVLGENNGHVYVCGDARSMAKDVHNIILKVVQEKGKMSEQ     657
C.lectularius      DQKEKVYVTHLLAQNADQIWNVLGENNGHVYVCGDARTMAHDVHGILLKVFQEQGKMTEA     658
                   ** :****** * :**::**.************:****:**:*** *:***.**:***:*

                                  A,C
F.fusca_L          EAIAYVKKMEAQKRYSADVWS      678
F.fusca_A          EAIAYVKKMEAQKRYSADVWS      678
C.lectularius      EAQAYLKKMEVQKRYSADVWS      679
                   ** **:****.**********
```

Figure 3.7 Multiple sequence alignment (ClustalW) of the deduced amino acid sequence of putative *F. fusca* vitellogenin receptors (larval contig (L) 24285 and adult contig (A) 31924) with the top hit from *Periplaneta americana* (BAC02725.2). Start and end of conserved domains Low Density Lipoprotein Receptor Class A domain (A-K), Calcium-binding EGF-like domain (M), Low-density lipoprotein-receptor YWTD domain (N-P), Low-density lipoprotein receptor repeat class B domain (Q-R) are indicated; gray boxes denote cysteine residues. An asterisk (*) denotes identical sequences, a colon (:) indicates conservation between groups of strongly similar properties and a period (.) denotes conservation between groups of weakly similar properties.

```
                                                A
F.fusca_L       ------------------------------------------------------------------------------  0
F.fusca_A       -MPRSSNMWAALCLAVLVNTCQSSIAFESLSHCDDGFFACLDGECLRSSRHCDGVRDCRD                   59
P.americana     MLLYAEIMWIIIQL----------HTVQGESSCPSGFFTCHNGECINDDKHCDGTSDCKD                   50

                      A     B                                       B       C
F.fusca_L       ------CEFIKCEPPMFFRCQNDKCVSTSFVCDEENDCGDWSDEHDCHNYTPPSVSNGCS                   54
F.fusca_A       GNDEWDCEFIKCEPPMFFRCQNDKCVSTSFVCDEENDCGDWSDEHDCHNYTPPSVSNGCS                   119
P.americana     GSDEFDCDMVLCKEPHWFRCHNGRCTSKSFHCDGVDDCGGWSDEEDCYEME--SKPANCT                   108
                      *::: *: * :***:* :*.*.** **  :*** ****.**::    *    *:

                                                C
F.fusca_L       SKEFQCLDKLCIPNDMVCDGEPDCLDGSDETLGCSTKISCDDGFMCKNHHCIMSEFRCDG                   114
F.fusca_A       SKEFQCLDKLCIPNDMVCDGEPDCLDGSDETLGCSTKISCDDGFMCKNHHCIMSEFRCDG                   179
P.americana     ADEWRCVDNNCIFMDWVCDGKQDCMDGSDELQGCSHKMSCEDGFVCGNYHCIPNSFLCDG                   168
                :.*::*:*: **  * ****: **:*****  *** *:**:***:* *:** ..* ***

                                                                D
F.fusca_L       SKDCTDGSDEEDCHMITHNVPIEECVPEKLLFACKDHVGCLDIRQVCDNTSHCLDDSDEH                   174
F.fusca_A       SKDCTDGSDEEDCHMITHNVPIEECVPEKLLFACKDHVGCLDIRQVCDNTSHCLDDSDEH                   239
P.americana     FDDCGDNSDEKLCPSV-RNVPPEDCKLEKNLFLCADRQECVEVRELCDGTPHCYDGSDEG                   227
                 .** * ***: *  : :*** *:*  ** ** * *:  *:::*::** * ** * ***

                                D
F.fusca_L       GRCSE---SCESSNCTHQCYRTPDGPVCICPKGTK-MENGACVDFDECTSYGICDQKCNK                   230
F.fusca_A       GRCSE---SCESSNCTHQCYRTPDGPVCICPKGTK-MENGACVDFDECTSYGICDQKCNK                   295
P.americana     PACNASRAACPTVGCSHQCIPSPQGPLCVCQVGYKTVDNKTCVDVDECMEYGICDQRCRN                   287
                  *.    :* :  *:***  :*:**:*:*  * * ::* :***.*** .******:*.:

F.fusca_L       KPGGYQCYCDLGYVLQKDGHSCKADGGEALMVFSAGSQIRGYYMTSQTLFRVASKLGQVA                   290
F.fusca_A       KPGGYQCYCDLGYVLQKDGHSCKADGGEALMVFSAGSQIRGYYMTSQTLFRVASKLGQVA                   355
P.americana     LQGSYSCYCDEGYEVGSDKRSCKATGPDALMLFSSTKEIRGLYVHKDFYYVVAQSLERAV                   347
                  *.*.****** ** : .* :**** * :***:**: .:*** *: .:  : **..* :..

                  Q                     N                     Q
F.fusca_L       GIGYDGFHIYWTNVMQGEESIMRSNEDGSDQEQLVSAGLGLPEDLAVDWITQNVYFTDSQ                   350
F.fusca_A       GIGYDGFHIYWTNVMQGEESIMRSNEDGSDQEQLVSAGLGLPEDLAVDWITQNVYFTDSQ                   415
P.americana     GISYDGNHVYWTELMLGEEAIVRSKDDGSHIEAIVTAGVYQPEDLAVDWITGNIYFTDME                   407
                **.*** *:***::* ***:*:**::***. * :*:**:  ********** *:**** :

                N                                 R                       O
F.fusca_L       KKHIGVCSSDGSSCTVVVNEDIDQPRSLALLPSQRMMYWSDWGEKPMIASSGMDGSKPKV                   410
F.fusca_A       KKHIGVCSSDGSSCTVVVNEDIDQPRSLALLPSQRMMYWSDWGEKPMIASSGMDGSKPKV                   475
P.americana     AQHIGVCVNNGSSCAVLVNEDIDKPRAIALMPLEGLMFWSDWGERPLIARAGMDGSQPEV                   467
                 :****** .:****:*:******:**::**:* : :*:******:*:** :*****:*:*

                                R                       O
F.fusca_L       LVSSDIRWPNGLALDYHNDRLYWVDAKRQKIETIKL-----------------------                   446
F.fusca_A       LVSSDIRWPNGLALDYHNDRLYWVDAKRQKIETIKLDGSDRRVILEKVLKHPFDIAVFEG                   535
P.americana     FVSTDLRFPNGLTIDYHNSRLYWVDAKLLVIESIKLDGSDRRVVLKDVVKHPYAIAVFED                   527
                :**:*:*:****::****.********   **:***

F.fusca_L       ------------------------------------------------------------                   446
F.fusca_A       SLYWSDWEGRQIQACDKFTGKNHRVLIRERK--NKVYGVHIFHSAMQHRNITNPCVDAGC                   593
P.americana     TLYWSDWHGRDIQACNKFTGKDHRIIIREKSKGDFIYGVHIYHPSMM-KLVTNPCHNNWC                   586

F.fusca_L       ------------------------------------------------------------                   446
F.fusca_A       SDLCVLSPN-TLGYSCACPEDRQLAADKHNCHEVEKRQVVIVGAGHNLYLVETRVLGKLA                   652
P.americana     SDICLLAPNKTYTYTCACPENKQLGADKHTCHEIRKQELVVVAAGHKLTAVGHQFLGRQT

F.fusca_L       ------------------------------------------------------------                   446
F.fusca_A       VTTIPLRAVHKMGALAYNSLTGSLFISDYVQKKIYSYNPKAARIDLLVGGAIGVVSGMDF                   712
P.americana     LYDMTLKNVHTIGAVTYNSLTDHIIIFDSEQQKLFTLGLKTMKLSLLLS-HVGKIDAMDF                   705

F.fusca_L       ------------------------------------------------------------                   446
F.fusca_A       DNLANNLYWCDVEKNTVEIISLNTNERAVILRDLGNEVPLDLALVPSSGVMYIAFSS---                   769
P.americana     DYMGNNLYWCDGERATVEILSLNTMERAILIHTLEGEIPLDVAVIPEEGVMFVAFSRHVI                   765
```

```
F.fusca_L       ------------------------------------------------------------   446
F.fusca_A       DHGVHIDRFNMDGSG-RTHVIETGLEGPEVSLWYDQELDRVFWTDNGLGLIDSTSVEGTD   828
P.americana     GDGPHIDRMNMDGRGAHTHVIETSLDGPIISLFYDSDLHRVFWTDPNNEEIGSAAADGMD   825

F.fusca_L       ------------------------------------------------------------   446
F.fusca_A       RHAFRA-LSTSVIDVTTISSDVFWVDRHSSLLHWADKYDGYAHTKKLDLGINDGLDSVHL   887
P.americana     QHVFRSDVEGSPIDIASVGRDMFWTMWAHPYLYWASKFNSQSRMKRLLLDVED-SDKLPL   884

                                                                     E
F.fusca_L       ------------------------------------------------------------   446
F.fusca_A       VGVRGILSSPNHPCQDNNGGCSHLCLLLGKNHVCACPEGMILKHDNHTCVAPLHCTSSQF   947
P.americana     VAVRGVRAQPDHPCHKNNGGCSHICALALKHTVCLCPVGMVLNRDNKTCTTPVHCSGEMF   944

                                          E   F
F.fusca_L       ------------------------------------------------------------   446
F.fusca_A       KCKSDDICIPKSQRCNGRRDCPSGEDEE-G-CKPTCHYGQFACNNGQCIDESLKCDTNYD   1005
P.americana     KCKTDNFCIPGRMRCDGKIDCPNGGEDELNCHKVNCRDDQFVCHNGQCISITKKCDGDSD   1004

                        F   G                                   G   H
F.fusca_L       ------------------------------------------------------------   446
F.fusca_A       CTDRSDETNCHMVSCDPDTQFTCESGQCISLQWRCNFAEDCHDGSDEANCAESTCEPGKM   1065
P.americana     CRDGSDEYYCFEEECNEDLQFKCRTGDCIVKSWYCDGSKDCEDGSDEENCEEVTCEPS-A   1063

                                        H       I
F.fusca_L       ------------------------------------------------------------   446
F.fusca_A       FRCKSGACIPSTWECDHEIDCADGSDEHEKCAPPPCESPRFQCTNKVCIDERLKCDGHDN   1125
P.americana     FKCALGQCIPEEWVCDGQSDCVDDTDE-QNCAPPTCGPGAFSCGNGRCIDQTLLCNNVDD   1122

                    I                           J
F.fusca_L       ------------------------------------------------------------   446
F.fusca_A       CGDGSDESGCVYAPAIMTSKPDTEKIDKELVCAEGDFKCHIE----PTCLPAGARCNGTA   1181
P.americana     CGDRSDEDFCRKPAN------EEEERLSVILCKEGEYTCHPHGKNVTICLPSSGRCNGTA   1176

                        J K                                 K
F.fusca_L       ------------------------------------------------------------   446
F.fusca_A       ECVDGEDEMDCSGCAGHEFQCHNGHCIPTAWLCDKYNDCGDNSDEELKECKSSRAPSRIF   1241
P.americana     ECPLGDDERGC-GCQDFQFTCYNGKCIPSEWVCDGINDCGDGSDENNARCQLPSS----V   1231

                  L                         L
F.fusca_L       ------------------------------------------------------------   446
F.fusca_A       EATKKCNTFQCPTGECLPWSMVCNVHEDCPDAADEGGRCQTACSQGHPCLGICVRTPHGP   1301
P.americana     GTPGPCTDYACNDGQCISLSLACNNKRNCEDGSDEGGQCDIACNAKSPCDQICQPTLAAQ   1291

                                M                               M
F.fusca_L       ------------------------------------------------------------   446
F.fusca_A       HCSCNAGYELQGDGRTCQDINECED-DPCAQLCENTEGSFQCSCAPEFVLRADKVSCKAT   1360
P.americana     DATVHKGYVLSSDGAKCGDIDECEIGGACAQVCHNTRGSFSCSCHPGFQLRSDHVSCKAL   1351

F.fusca_L       ------------------------------------------------------------   446
F.fusca_A       GSPLEYLFSTGEQIRKASASLTAFHIIAENSGLDISGLDIDSRAQHIYWTTEANGILHRM   1420
P.americana     GEPMQFIFSAGNQIRKVSHELRFTDVVYPEAELKVTGLDVDSASNEVYWSTDVTSTIYRL   1411

F.fusca_L       ------------------------------------------------------------   446
F.fusca_A       SLGTGVLSHVRGLGVPTKLAVDWLSKNVYYTNMAERGSVNVCNLEKQKCAKLLHGEDGTY   1480
P.americana     SLRGGEKAYATGIGTPGDIAVDWISRNVYYVDKSTPQAIRACNLDEHRCAKVLVIEHGFS   1471
```

```
                                                          P
F.fusca_L       ------------------------------------------------------------   446
F.fusca_A       TGAIAVDPKSSLLFWSRIYPGDGGAPTSQLMVSDLSGQNGSSIANGN--LISGIALDLIK   1538
P.americana     VPKIAVDPIAGFIFWPEVTKWVFGEPSTDLFRSELTGRHKTMIETNNLMLVNGLTLDIVR   1531

                                P
F.fusca_L       ------------------------------------------------------------   446
F.fusca_A       RQVYWADQHVGTIARVDYSGENRIM-IRQDDVYHPLGLNLFEDTLFWINGGTGTLTRCRM   1597
P.americana     QRIYFADQHKRTIECMDYNGEDRHIIVHNEHVQNPIDMALFEGTLYWLTAGTGQLTSYKL   1591

F.fusca_L       ------------------------------------------------------------   446
F.fusca_A       YGKLQN-CEKLTLSHTDVKNFAVMQATRQTLGEDQCGAINCTHMCVLSLKG-PKCVCEDG   1655
P.americana     YGPHERRIGKLQLYIYSSDQFTILQQAIQPAAVNPCANHSCSELCVMNPGGTPSCLCSGG   1651

F.fusca_L       ------------------------------------------------------------   446
F.fusca_A       LIVKPGKSCSEARNAEDVPEEKKPSFHMVVETAEAYGK----SHSSSVVGPILA---VIM   1708
P.americana     QVVEMGELCPTSEVGEG------PWFEKVTPRGKQGGKSEEMQHSSNVGGIIIAILVIAL   1705

F.fusca_L       ------------------------------------------------------------   446
F.fusca_A       VIGAGLAFLAYKRRASGNGMNWPNLPLGFNIGKFGGGGTNVTFDNQGFGMASSRPDPFEP   1768
P.americana     VVGGVAAVYYYKR-FG---YKGPKLN------------FSLHFKNPTFGIKESDV-AVPQ   1748

F.fusca_L       ------------------------------------------------------------   446
F.fusca_A       TMKPGSHEYENPTMKDKTDGKLSMSTSVQSWSAPKKIPVDKMLLEDEDSDAEYMDGENDN   1828
P.americana     VLVPGQHQYTNPFDNAEALKQLEGS--VIQESRL-KKLADHIQLEDEDAED-YAPDGSDK   1804
```