January 2022

The Sweet Smell Of Mystery: The Scent Glands Of Oncopeltus Fasciatus

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THE SWEET SMELL OF MYSTERY: THE SCENT GLANDS OF *ONCOPELTUS FASCIATUS*

by

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THESIS

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

MAY 2022

MAJOR: BIOLOGICAL SCIENCES

Approved by:

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Advisor Date

ACKNOWLEDGEMENTS

I would first like to thank my PI and mentor, Dr. Aleksandar Popadić, for his enthusiasm and patience both in the classroom and in the laboratory. My time in the lab taught me how to think on my feet and the value of “thinking like an engineer” when conceptualizing ideas. Without a doubt, the skills that I have learned while in graduate school will help me in the future throughout medical school and eventually as a doctor.

I also thank my committee member, Dr. Joy Alcedo, for all her help throughout my time in the biology department. By including me in journal clubs and testing my conceptual thinking and reasoning, she overall helped me to become a confident presenter. In addition, I would like to thank my committee member Dr. Weilong Hao, for his insightful feedback of this project.

I would like to thank former lab member Lisa Hanna, who showed me the ropes in the lab and provided data that inspired this thesis project. The friends that I have made while in graduate school have made this time considerably more delightful, with special thanks going to Deniz Sifoglu.

Finally, I would like to thank my parents Mia and Tasos, my brother Alexi, and my dog Rambo for their unconditional love and support.

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CHAPTER I: INTRODUCTION

Insects: A Brief History

The Arthropod phylum includes crustaceans, myriapods, insects, and arachnids, and is the largest contributor to Earth's biodiversity (Dunlop, Scholtz, & Selden, 2013; Ortega-Hernández, 2016). The first appearance of arthropods in the fossil record begins in the ocean during the Cambrian period 640-665 million years ago (Mya) (Benton, 1995)), and the earliest evidence of terrestrial arthropods dates back to the Silurian Era (443 Mya- 419 Mya) (Kenrick, Wellman, Schneider, & Edgecombe, 2012). It has been speculated that the aquatic to terrestrial transition occurred presumably during the Ordovician-Silurian extinction 400-500 Mya, when oxygen deprivation in the aquatic environment prompted the transition to land (Hsia, Schmitz, Lambertz, Perry, & Maina, 2013). This environmental transition led to modification of the arthropod body plan, particularly in the respiratory system, the exoskeleton, and the communication system like glands and pheromones.

The significant differences in both density and viscosity between aquatic and terrestrial environments makes it metabolically more efficient to extract oxygen from air (Damen, Saridaki, & Averof, 2002; Hsia et al., 2013). The insect tracheal system, which shares the same genetic regulatory network with the gills of aquatic arthropods, adapted to utilize this increased oxygen availability through modification of respiratory morphology. The aquatic arthropod gills are positioned on the external surface, respiration occurs via gas exchange as oxygenated water makes contact with the hemolymph-filled gills, then a system of afferent and efferent veins to transport the oxygenated hemolymph throughout the body (Snodgrass, 2019). In contrast, the insect tracheal system uses a system of branching trachea that is similar to the bronchial system in mammals. The main tracheal branches make contact with the external environment through spiracles, openings in the body wall, which then terminate at tracheoles where gas exchange occurs (Hayashi & Kondo, 2018; Hsia et al., 2013). The shared basic design of using a system of

tubes as pathways for gas exchange, exemplifies a modification of an existing system that was required for survival in a new environment (Carroll, Grenier, & Weatherbee, 2013).

One major consequence of the terrestrial transition was the development of the modulatory, cuticular exoskeleton, as it was more advantageous in such an environment (Hopkins & Kramer, 1992). The hydrophobic cuticle protects against the lack of moisture in the terrestrial environment and is composed of multiple layers which impede the amount of water that is diffused from the body and can be selectively sclerotized depending on function (Krupp, Nayal, Wong, Millar, & Levine, 2020; K. Li et al., 2017). For example, insects that macerate, or chew their prey, require a hardened cuticle, whereas those with a sucking proboscis do not. Another example of cuticular modifications is the hard, shield-like forewings of beetles, which serve a dramatically different function than those of the membranous hindwings used for flight. Depending on the environment and body plan, certain features are more advantageous, and the modulatory potential of the exoskeleton contributed significantly to insect evolutionary success.

Finally, the ability to interact with and adapt to the environment is an essential part of the survival of a species. In general, there are several main forms of interaction: visual, physical, and chemical. Visual communication can involve coloration for sexual selection, aposematic pigmentation, or mimicry. Physical communication can include size or species-specific mating behaviors. Chemical communication can incorporate pheromones or other compounds, such as droplets or spray, which play a role in mating and defense. Chemical defense occurs through specialized exocrine glands, which are derived from the epidermis and store and secrete volatile, odoriferous substances that can be used to repel predators or disperse aggregated groups of the same species (Noirot & Quennedey, 1974; Staddon, 1979; Vane-Wright & Boppre, 1993). The earliest study of these specialized glands was done over a hundred years ago (Packard, 1895). These observational studies described the gland function as a direct adaptation to the needs and environment of insects and recorded the existence of scent glands in several insect orders such

as Lepidoptera, Blattodea, Orthoptera, Mantidae, Hymenoptera, Coleoptera, and Hemiptera (Packard, 1895).

Cell classes involved in gland development

Depending on the insect and the gland type, there are different gland cell classes that are utilized in the epidermis. There are three classes of gland cells that are categorized by general structure and method of secretion (Figure 1). Class 1 cells are gland cells that are covered by a cuticle, hence secretion occurs when the scent compound exits the body by crossing the cuticular barrier (Figure 1A and G1 in Figure 1C). Class 2 cells are composed of multiple cells functioning as a unit (Figure 1B and G2 in Figure 1C). In this cell class, the gland cells are surrounded by differentiated epidermal cells that are covered with a cuticle, similar to class 1 cells. However, the bottom half of the cell secretes the compound, which is then transferred into the above epidermal cell before being excreted through the cuticle. The oenocytes, lipid metabolizing cells, are associated with this class (Brückner & Parker, 2020; Noirot & Quennedey, 1974). Class 3 cells are structurally more complex (G3 in Figure 1C). This class of cells utilize a “bulb and duct” anatomical plan where the gland cell has a duct cell attachment that is continuous with the cuticle. The duct cell (D in Figure 1C) is connected to the gland cell that secretes the compound. Multiple cells of different classes can be involved in the scent gland (ScG) to produce the defensive secretion. The types of compounds produced are species-specific, whereby different proportions of gland cell classes result in the creation of a unique defensive secretion. (Figure 1D) (Brückner & Parker, 2020; Noirot & Quennedey, 1974).

Scent Gland Structural Variations in Different Species

In accordance with being the most diverse phylum, the body plans of arthropods are significantly modified to suit their habitats. Scent glands also followed this trend and developed different ways of delivering scent gland compounds. For example, chemical secretions can be

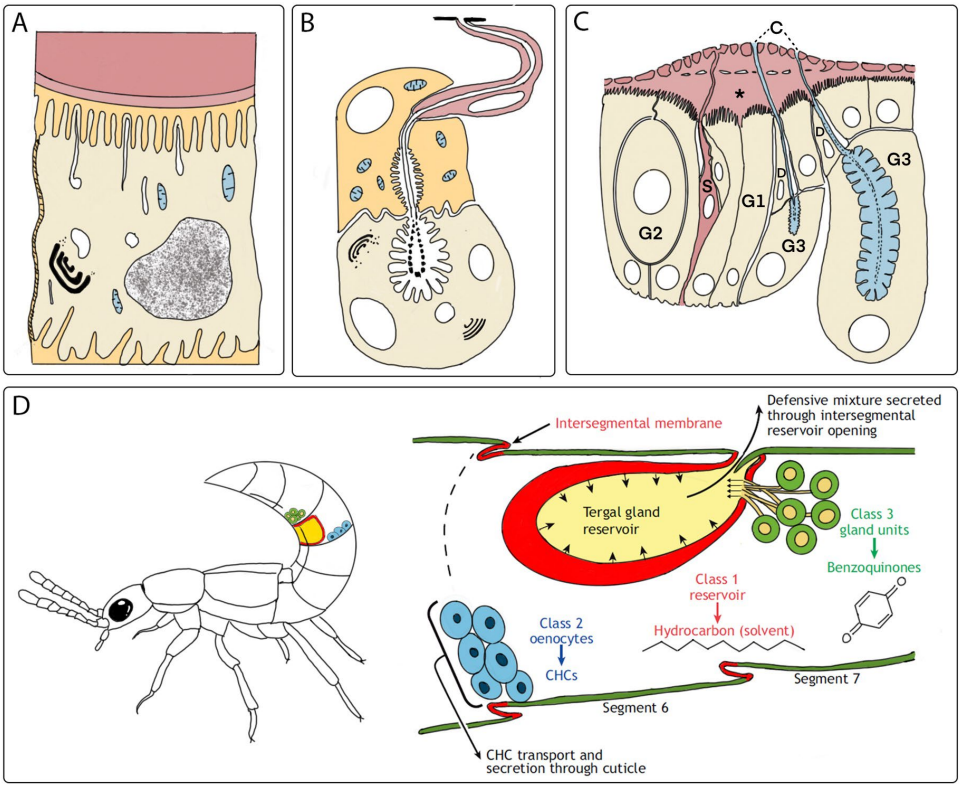

Figure 1. **Descriptions of epidermal gland cell types**. (A) A class 1, Gilson's gland from *Phryganea* larvae. (B) A class 2 cell type, from the dermal gland of *Tenebrio,* gland cell (tan) is on the bottom and modified epidermal cell (yellow) is on top. (C) The actual sternal gland in *Rhinotemitidae*, composed of all three classes of gland cells (G1, G2, G3, and G3') are shown in a complex that makes the gland. (D) Different gland cell classes used in the defense gland of the rove beetle. D; ductule, S; campaniform sensillae, C; perforated cuticle, (*) subcuticular space. Redrawn from (Brückner & Parker, 2020; Noirot & Quennedey, 1974)

sprayed out from the body to hit a "long range" target, or the secretion can be produced as a liquid droplet on the body and act in a smaller radius. Depending on the secretion method, scent gland openings, called ostioles, are structured differently and are divided into three types: Type 1 resembles a round hole in the body wall and the scent substance accumulates as a droplet directly over the ostiole (Staddon, 1979). Types 2 and 3 are known as divided ostioles and resemble rings that are pinched in the middle. The difference between the two is that type 2 is not truly divided because there is no adhesion between the touching sides, whereas type 3 is truly divided into two halves and the tissue is permanently fused together (Figure 2; Staddon, 1979). The scent substance in type 2 ostioles is slowly secreted and immediately spreads out over the surrounding cuticle in a film (Staddon, 1979). Insects with type 3 ostioles force the secretion out of the body

so quickly that it results in a spray (Staddon, 1979). By utilizing these different ostiole types, the scent glands facilitate survival of insects in different environments.

Figure 2. Types of abdominal ostioles. (A) Type 1 ostiole. (B) Type 2, pseudo divided, ostioles. (C) Type 3, divided ostioles. Adapted from Staddon 1979.

The majority of insight regarding scent gland development and organization comes from insects (Table 1), with only two reports from myriapods. As a matter of fact, the descriptions and distribution of these organs have been first reported in 1895 (Packard, 1895), with extensive coverage of Lepidoptera (moths and butterflies). Additionally, scent glands in several other insect groups have been described (Figure 2), including Coleoptera (beetles), Hemiptera (true bugs), Blattodea (cockroaches), and Phasmatodea (walking sticks).

Defensive glands first appeared in the form of ozadene glands in Myriapods (centipedes and millipedes), represented by Scolopendrellidae and Spirobolida orders in Table 1. These arthropods are older than insects on an evolutionary scale, with the oldest dated fossil being around 423 million years old (Engel & Grimaldi, 2004). Their body plan is composed of a head and metamerically segmented trunk covered by a tough exoskeleton (Qu et al., 2020). Consistent with the segmented trunk, the ozadene glands are arranged repeatedly on each segment, and are composed of integumental sacs that run along the lateral sides of the body wall and are released through openings called ozopores. The chemicals released include alkaloids, quinones, phenols, and cyanogenic compounds, which in turn, serve as paralytics, repellents, toxins, and as an adhesive compound that physically protects the animal (Qu et al., 2020). Given their phylogenetic position (Figure 3), myriapod ozadene glands are thought to have evolved separately from insect scent glands as a result of a separate, independent, terrestrialization event (Lozano-Fernandez et al., 2016).

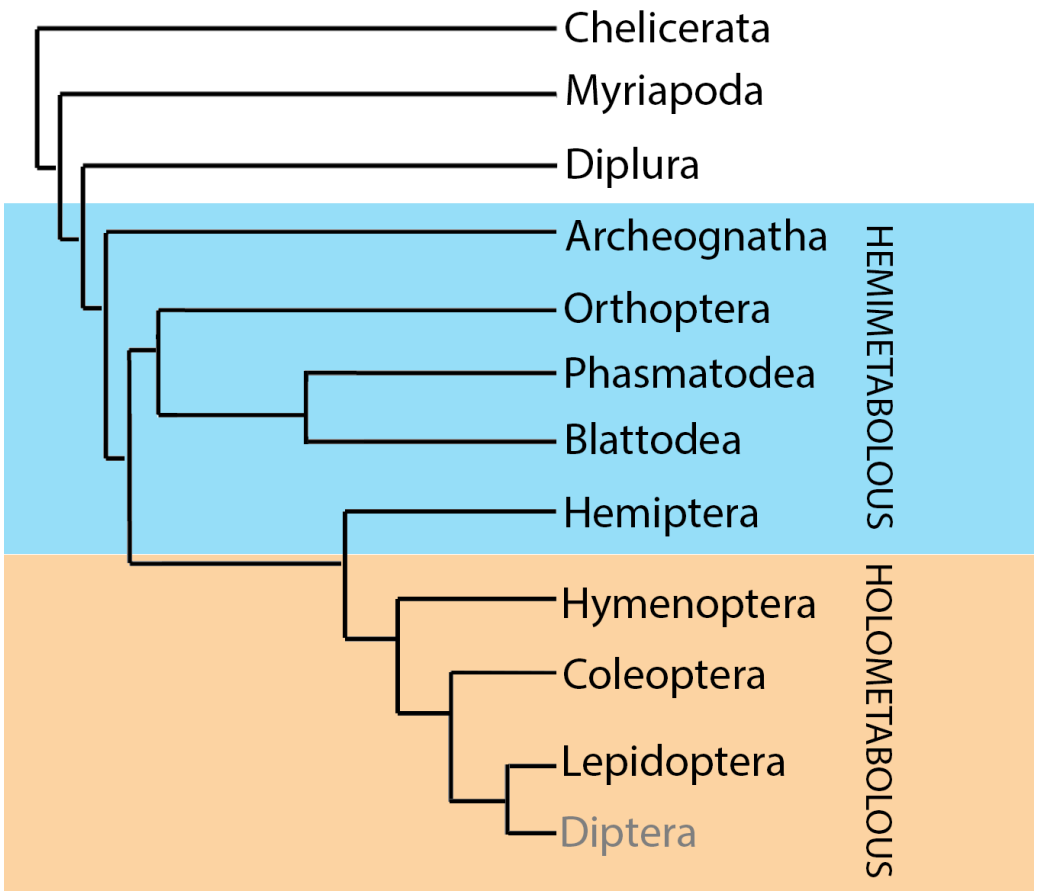

Figure 3. Phylogeny of insect orders processing defensive scent glands. Diptera (grey) is the only order without ScGs. Phylogeny is adapted from Misof et al. (2014).

Lepidopterans have the best descriptively studied scent glands of the insect groups. The larvae of Lepidoptera (caterpillars) do not have eversible scent glands in the same body regions as adults due to the stark difference in body morphology between the two developmental stages. Caterpillars possess a gland on their head called the osmeterium, which secretes a strong repellent that is applied to the caterpillar's surroundings and can also serve a secondary function: defensive mimicry. When the glands are stimulated and evert, they display a snake tongue-like set of horns on top of the head which, in addition to the eyespots on the thorax,

Table 1. Insect and myriapod species with documented ScG.

ORDER	FAMILY	SPECIES
Scolopendrellidae	Scolopendrella	Scolopendrella immaculata
Spirobolida	Trigoniulidae	Trigoniulus corallinus
Diplura	Campodeidae	Campodea staphylinus
		Campodea cookei
		Campodea mexicana
Archaeognatha	Machilidae	Machilis
Orthoptera	Rhaphidophoridae	Ceuthophilus maculatus
Phasmatodea	Diapheromeridae	Diapheromera femorata
	Heteropterygidae	Heteropteryx dilatata
	Phasmatidae	Phasma putidum
	Pseudophasmatidae	Anisomorpha buprestoides
Blattodea	Blattidae	Eurycotis floridana
		Periplaneta americana
		Periplaneta orientalis
	Corydiidae	Corydia carunculigera
	Ectobiidae	Blattella germanica
		Ectobius lapponicus
Hemiptera	Aphididae	Lachnus strobi
	Cimicidae	Cimex lectularius
	Coreoidae	Coreus marginatus
	Lygaeidae	Oncopeltus fasciatus
		Lygaeus saxatilis
	Pentatomidae	Halyomorpha halys
	Pyrrhocoridae	Pyrrhocoris apterus
		Dysdercus intermedius
Hymenoptera	Cimbicidae	Cimbex americana
	Tenthredinidae	Craesus septentrionalis
		Croesus varus
Coleoptera	Carabidae	Pheropsophus verticalis
	Melyridae	Malachius bipustulatus
		Anthocomus equestris
	Tenebrionidae	Tribolium castaneum
		Luprops tristis
	Staphylinidae	Ocypus fulvopilosus
		Lomechusa pubicollis
Lepidoptera	Erebidae	Catocala
		Orgyia antiqua
		Orgyia ericae
		Orgyia gonostigma
		Orgyia gulosa
		Orgyia vetusta
		Parorgyia clintonii
		Dasychira fascelina
		Leucoma salicis
		Laria rossii
		Psilura monacha
		Ocneria dispar
		Parorgyia leucophaea
		Parorgyia paralella
		Dasychira pudibunda
		Lymantra concolor
		Choerotricha plana
		Charmidas exclamationis
		Artaxa vitellina
		Liparis detrita
		Liparis rubea
		Artaxa scintillaris
		Artaxa guttata
		Daschira dalbergiae

ORDER	FAMILY	SPECIES
Lepidoptera	Noctuidae	Bryophila
		Cucullia formosa
		Cucullia scrophulariae
		Habrostola
		Cleophana linearie
		Leucania staminea
		Leucania hispanica
		Leucania nonagrioides
		Plusia gamma
	Nolidae	Nola strigula
		Nola ovilla
		Nola cucullatella
	Notodontidae	Pheosia rimosa
		Schizura concinna
		Danima banksii
		Macurocampa marthesia
		Heterocampa pulverea
		Cerura vinula
		Cerura furcula
		Cerura borealis
		Cerura multiscripta
	Nymphalidae	Limenitis disippus
		Astyanax archippus
		Vanessa io
		Melitaea artemis
		Melitara
		Vanessa
		Argynnis
		Dryas iulia
		Heliconius apsuedes
	Papilionidae	All the species
	Pieridae	Aporia crataegi
		Aporia nebulosa
	Megalopyge	Megalopyge crispata
	Yponomeutidae	Hyponomeuta evonymella
	Hemileucidae	Hyperchiria io
		Hemileuca yavapai
		Hemileuca maia
		Pseudohazis eglanterina
	Lycaenidae	Lycaena damon
		Lycaena baetica
		Lycaena pseudargiolus
		Lycaena icarus
		Lycaena scudderi
	Mimallonidae	Lacomosa chiridota
		Perophora melsheimerii

The table is based on species studied by Packard (1895) and supplemented with representative insects and arthropods from more recent studies. Listed species are organized by orders and families, respectively. (Abhitha, Vinod, & Sabu, 2010; Burns, Gutzwiller, Tomoyasu, & Gebelein, 2012; Packard, 1895; Silberglied, 1977; Staddon, 1979)

mimics a snake's head (Cong, Borek, Otwinowski, & Grishin, 2015). In contrast, adult Lepidopterans can have glands on the wings, legs, or body. These organs can take the form of androconia (scent scales or hairs) or coremata (inflatable sacs). Furthermore, unlike other insect groups, which solely use the scent glands to release aversive substances, Lepidopteran adults also utilize the scent glands to emit sex pheromones. The pheromones were shown to be secreted by class 1 gland cells (Brückner & Parker, 2020). Studies of Lepidopteran scent substances have been limited to chemical analysis of the active compounds in pheromones and found them to be identical to the compounds in defensive secretions (Silberglied, 1977). Although pheromones are utilized in all insects, including those without scent glands, further studies are needed to determine if the similarity between pheromones and scent secretions are shared in other scent gland possessing insects.

While not all Coleoptera have scent glands, some members of this insect order display extraordinary examples in this regard. Bombardier beetles are also one of the most distinguished, as this beetle stores a reactant solution of hydrogen peroxide and *p*-hydroquinones. When stimulated, the solution flows into a reaction chamber where different peroxidases and enzymes catalyze the formation of *p*-benzoquinones that react explosively and propel the noxious substance from the pygidial (abdominal) gland (Arndt, Moore, Lee, & Ortiz, 2015). Another "sprayer" is the darkling beetle (*Luprops tristis*), which uses a spraying mechanism that release an acerbic compound from the adult abdominal pygidial glands and lateral depressions in the second and third tergite in larvae. This spray can cause blisters on human skin (Abhitha et al., 2010). Rove beetles, in contrast, only release a vesicating secretion when crushed, causing the skin condition *dermatitis linearis* (blistering) (Cressey, Paniz-Mondolfi, Rodríguez-Morales, Ayala, & Da Silva, 2013). Based on molecular analysis, this species provided an excellent model for understanding how multiple gland class types contribute to organ function (Figure 1D). Class 1 cells differentiate to create the lumen of the reservoir and secrete fatty acid derivatives. Class 3 cells are situated posterior to the reservoir and release benzoquinones into the solvent of fatty

acids, creating the final secretion mix (Figure 1D) (Brückner & Parker, 2020). In contrast to the caustic secretions of the aforementioned species, *Tribolium* adult beetles utilize glands that function to create quinone-based irritants and repellents. *Tribolium* possess two sets of scent glands (known as stink glands); one on the prothorax (the prothoracic or the anterior gland) and the other on the abdomen (the pygidial glands). As a widely utilized genetic model system, RNA sequencing, transcriptomics, and RNAi were performed to determine the genetic mechanisms of quinone production, derivatives of which compose *Tribolium* secretion (J. Li et al., 2013). Gene ontogeny annotations of the volatile secretions from the stink glands show high expression of genes involved in metabolism, catalytic activity, and binding activity (J. Li et al., 2013). While it can be intuitively stated that the function of all scent glands would require the involvement of genes responsible for metabolism, this can only be confirmed with further transcriptomic studies of the glands in other insect orders.

Hemiptera are true bugs that undergo hemimetabolous development, in which there are multiple nymphal stages before achieving the adult stage, in contrast to the complete metamorphosis utilized in Lepidoptera and Coleoptera species. In fact, the scent gland system is a distinct morphological feature in the sub-order Heteroptera (Staddon, 1979). The insects of this sub-order have scent glands in both nymphs and adults that secrete droplets or spread out as a film over the cuticle. The dorsal abdominal scent glands are nymphal glands that become obsolete upon the adult molt, whereas the metathoracic gland is exclusively an adult organ. The substances that are secreted from the glands are mainly short to medium unbranched hydrocarbons with the exception of rare odd branched compounds in some pentatomids and pyrrhocorid species (Staddon, 1979). Secretion compounds can be acquired from the diet in some species. For example, in the cotton stainer *Dysdercus intermedius,* linalool oil, which is found in the cotton plant was also isolated from the secretions. Additionally, the large milkweed bug *Oncopeltus fasciatus* sequesters cardiac glycosides from the large milkweed plant in its body and adds it to the defense secretions. Both scent gland systems are composed of class 3 gland cells

with 3-cell (2 duct cell/secretory cell) and 2-cell (1 duct cell/secretory cell) secretory units in the abdominal and metathoracic gland, respectively. While hemimetabolous development generally implies that the structures present in nymphs are also maintained in the adults, the scent glands seems to be an exception to this rule, as the metathoracic scent gland is present only in the adult insect when the abdominal scent glands become obsolete. Similar “translocation” is also observed in phylogenetically distant insects such as Lepidopterans (Figure 2) indicating that the process of “translocation” is independently acquired between these two orders. It is reasonable to believe that the morphological differences between nymphs/larvae and adults, as seen in both Lepidopteran and Hemipteran orders, ushered in this event in order to preserve functionality of the scent glands as a response to these changes.

Origins of Scent Glands

Novel organs, such as exocrine glands, require the creation of new cell types that need to synthesize compounds (Brückner & Parker, 2020). However, there is limited data that describes the developmental regulation that would give rise to the cells that form the scent glands. Such data is limited to functional testing of *cephalothorax*, an ortholog of *sex combs reduced*, in *Tribolium* prothoracic stink glands, where the glands were unaffected by *Cx* RNAi (Clark-Hachtel, Moe, & Tomoyasu, 2018). Additionally, functional testing of Hox genes in *Oncopeltus fasciatus*, revealed that a simultaneous knockdown of *abdominal A* and *Ubx* everts the scent glands on the dorsal abdomen, though either of the genes knocked down individually do not influence gland formation (Angelini, Liu, Hughes, & Kaufman, 2005). Furthermore, a*bdominal B* RNAi resulted in ectopic formation of abdominal scent glands on segment A7 (Angelini et al., 2005). This data revealed that while Hox genes are involved in scent gland regulation and development are a part of overall segmental identity, we don’t know the actual genetic mechanisms that specifically regulate scent gland formation.

Previous studies have given insight to the effect of pheromones in insect communication, as Lepidopteran defense secretions and pheromones share identical compounds and changes in

pheromone producing organs can affect *Drosophila* sexual behavior (Boppré, Petty, Schneider, & Meinwald, 1978; Fernández et al., 2010). These pheromone-producing organs are called oenocytes and are present ubiquitously in insects (Makki, Cinnamon, & Gould, 2014). These cells resemble hepatic cells in mammals, and are responsible for lipid metabolism and detoxification, (Gould, Elstob, & Brodu, 2001; Makki et al., 2014). These cells have abundant smooth endoplasmic reticulum, express lipid synthesizing enzymes, catabolizing enzymes, fatty acid synthase, fatty acid desaturase, and beta-oxidation enzymes, which show their lipid processing capability (Makki et al., 2014). Additionally, these cells are involved in producing cuticular hydrocarbons and pheromones (Figure 1D) as shown by their function in tracheal waterproofing and modulation of mating behavior (Fernández et al., 2010; Makki et al., 2014). The function of the oenocytes in lipid metabolism is consistent with results from *Tribolium* stink gland transcriptome data, which shows over 70% of genes were involved of catalytic and metabolic genes (J. Li et al., 2013). The ubiquity of oenocytes and their functional resemblance with scent glands make these cells a good candidate as a precursor to scent gland origin.

***Oncopeltus fasciatus*: The Scent Gland Model**

As stated previously, ScGs are a common feature of Hemipteran insects making these species excellent models for studying ScG development. In particular, the large milkweed bug, *Oncopeltus fasciatus*, is easy to rear, has a high fecundity, and is sensitive to RNA mediated knockdowns. *Oncopeltus* undergoes 5 nymphal stages after hatching before reaching the sexually mature adult, taking roughly 4-5 weeks before final adult ecdysis (Feir, 1974). Throughout nymphal development, the general body plan remains the same except for the development of the wing pads, which begins at 3rd nymphs and become most prominent at 5th (Figure 2 B-C). Traditional model systems like *Drosophila melanogaster* are not suitable for ScG studies because *Drosophila* do not possess any scent glands. Similarly, *Tribolium* ScGs are rather small and inconspicuous making these organs less accessible.

Various Functions of the *Oncopeltus* Scent Glands

As with all ScGs, the main purpose of the *Oncopeltus* ScG is to defend the animal. When disturbed, the brown, pungent, secretion is discharged from the metathoracic scent gland (Mtx ScG) in adults and abdominal scent glands (Abd ScG) in nymphs, and mixes with a discharge from the rectum, forming a droplet at the tip. After ejection, a nymph may use its legs to brush the liquid onto the surface that it is standing on, however there have been no reports of other deliberate transfers of the liquid as a defensive action (Games & Staddon, 1973b). Interestingly, the glands have been shown to have an additional role in communication between sexes, similar to previous studies in Lepidoptera (Boppré et al., 1978; Silberglied, 1977). In fact, *Oncopeltus* adults have sexually dimorphic ratios of acetate in their secretion. When males are reared separately from females the amount of esters that are produced increases as compensation, demonstrating that the scents released from the ScGs are involved in sexual selection as well. (Staddon, 1979).

Genetic Regulatory Networks of Endocrine, Exocrine, and Tracheal Systems

The genetic mechanisms responsible for tracheal control are best studied in *Drosophila*, and show that the primordia originate from serially homologous organs (Sánchez-Higueras, Sotillos, & Hombría, 2014). Specifically, it was discovered that tracheal development genes, *ventral-vein less* (*vvl*) and *trachealess* (*trh*), were found in the endocrine glands of *Drosophila* (Sánchez-Higueras et al., 2014). Complementary studies in *Oncopeltus* not only confirmed these findings, but also documented mRNA localization of *vvl* and *trh* in the ScGs (Hanna & Popadić, 2020). Both *Drosophila and Oncopeltus studies* uncovered that the precursor cells of trachea and glands are subjected to Hox gene regulation for cell determination (Hanna & Popadić, 2020; Sánchez-Higueras et al., 2014). One gene in particular is found downstream of the Hox gene pathway in all three organ systems: a zinc finger transcriptional repressor called *spalt* (*sal*). This pleiotropic gene has been found to differentiate tracheal cell migration, localize in endocrine glands, and act as a developmental switch between oenocytes and chordonotal cells in both *Drosophila* and *Tribolium* (Hanna & Popadić, 2020; Rusten et al., 2001; Sánchez-Higueras et al.,

2014). In the *Drosophila* tracheal system, *sal* inhibits the function of trunk Hox gene in the head and restrict *vvl* and *trh* to the thorax and abdomen and is responsible for the migration and fusion of the dorsal tracheal trunk. It is also localized in the prothoracic gland and induces the formation of oenocytes in the abdomen (Rusten et al., 2001; Sánchez-Higueras et al., 2014). Since the oenocytes are a possible candidate for the origin of ScGs, understanding the genetic regulatory network involved in oenocyte development can give insight for candidate genes involved in ScG development.

The molecular mechanism of oenocyte formation is well documented in *Drosophila* larvae. In the abdomen, Engrailed determines the posterior half of the abdominal segment while Spalt is expressed on the dorsal side. Within the posterior half of the abdominal segment, chordotonal organ precursors (COPs) can either become chordotonal organs, involved in mechanoreception, or induce oenocyte formation from the dorsal ectoderm. The COPs are linearly spread out from the dorsal to ventral side, with only the most dorsal, the primary COP, located within the dorsal Spalt domain. In this case, Spalt acts as switch that induces oenocyte cell fate in the primary COP. Complementary studies in both *Drosophila* and *Tribolium* have revealed that genes involved in oenocyte differentiation include *sal, vvl,* and *hepatocyte nuclear factor-4 (hnf4)*. Data from *Oncopeltus* has shown that *vvl* is expressed in the abdominal ScGs and are under Hox gene regulation (Hanna & Popadić, 2020). This provides valuable information as it confirms shared genetic regulation of the scent glands with that of the endocrine and tracheal systems. Therefore, the genes within this network can be considered candidates for scent gland development genes.

Current Knowledge of Genetic Mechanisms of ScG Evolution

The present understanding of molecular evolution of exocrine glands is based on the duplication model in which a duplication of ancestral enzyme-coding loci, in combination with functional divergence of their paralogues, allow insects to metabolize different compounds (Brückner & Parker, 2020). In terms of developmental mechanisms, it is likely that transcription factors physically interact in new ways to form novel functions, and *cis*-regulatory modification

can lead to biosynthetic modifications that change the chemical profile of the gland secretions (Brückner & Parker, 2020). Although only limited genomic and transcriptomic analysis of ScGs have been performed, the genetic profile of genes expressed in *Tribolium* and millipede ScGs revealed significant expression of genes involved in organic substance metabolism and enzymatic activity (J. Li et al., 2013; Qu et al., 2020). At this moment, knowledge of the shared genetic regulatory network of the tracheal, endocrine, and exocrine systems is the best starting point for gaining insight into scent gland development.

This work examines the role *spalt* and its role in the development of adult and nymphal scent glands through functional testing and *in situ* hybridization. In addition, I examined the role of *hnf4*, an oenocyte marker, to explore the genetic relationship between ScG and oenocytes. This work documents the gross anatomy of the abdominal and metathoracic scent glands, reveals that *spalt* promotes the formation of both abdominal and metathoracic scent glands, and identifies several embryonic regions that express *hnf4*.

CHAPTER II: MATERIALS AND METHODS

Cloning and amplification of sequences

RNA extraction and cDNA amplification was performed according to the protocol from Li and Popadić (H. Li & Popadić, 2004). Eggs ranging from day 1-5 were used and any embryos showing cuticle development were not selected. PCR amplification was performed using nested primers (see table below), including previously designed primers for *sal* and *vvl* (Hanna & Popadić, 2020; Simmons, 2017). Nested fragments of expected lengths were transformed using pCR4-TOPO vector cells following manufactures' protocols. For every gene, 6 clones were prepared and the plasmid DNA was extracted using the PureYield Mini Prep Kit (Promega) and sent for sequencing. Sequences were confirmed using EMBOSS Needle software.

Primers

Gene	Sequence	Length
sal	1F/1R 5' TACCATCCCCCTCTTCTG 3' 5' TCAGAACAGACTTCATCTCC 3' 2F/2R 5' GATAGGTGCTCACCAAAGTC 3' 5' GTTCTGACGACTTACTCAAA 3'	200
vvl	1F/1R 5' GGTTTGAAGCTTTGCAACTG 3' 5' TAGTGAGCGGTAAGCGAGGT 3' 2F/2R 5' GAGGACCAGTATCGAAGTGT 3' 5' TAGTGAGCGGTAAGCGAGGT 3'	501
HNF4	1F/1R 5' GACAAACGAAACCAGTGCAGA 3' 5' GCAACTCCAAATAGTCGAGCAA 3' 2F/2R 5' GAACGGATTGGGTGCTTCCT 3' 5' CATCTGTCAATGGGCGGACT 3'	245

In situ hybridization

Riboprobes were synthesized following the protocols from Li and Popadić (2004) and Mahfooz et al (2004). Collected embryos ranged from day 3.5 to 5. Probes for *vvl an HNF4* were conjugated to Digoxigenin-UTP and the *sal* probe was conjugated to Biotin-UTP, all probes were visualized with NBT+BCIP.

RNA interference

Double stranded RNA was synthesized using T3/T7 RNA Polymerase kits following company protocols (Ambion). Maternal RNAi was performed by injecting *sal* dsRNAi in concentrations of 1.5, 2, 3, and 4µg/µL into the abdomen of gravid females. Nymphal RNAi was performed by injecting *sal* dsRNA in concentrations ranging from 0.5µg/µL to 2µg/µL, the maximum injection volume was 2µL. The injection sites were all located ventrolaterally between the 2nd and 3rd abdominal segments. All injections were performed using a Hamilton syringe with a microinjector holder attachment (WPI) and glass capillary needles pulled to the dimensions following Chesebro et al (2009).

Injection Type	Concentration	Concentration	Concentration	Concentration
Maternal 20190816	2µg (Set I)	4µg (Set II)		
Maternal 20191005	1.5µg (Set I)	3µg (Set II)		
Nymphal 20191005	1µg (10)	2µg (10)		
Nymphal 20191005	1µg (10) 4ths	2µg (10) 4ths	1µg (8) 5ths	2µg (8) 5ths
Nymphal 20200206 (Other fragment)	1µg (10)	2µg (10)		
Nymphal 20200224 (Other fragment)	1µg (10)	2µg (1/10)		
Nymphal 20210528	0.5µg (10)	0.75µg (10)	1µg (10)	1.5µg (10)
Nymphal 20210722	2µg (1/6)			

Table 2. Injection summary organized by type and date. Concentration and stages are recorded. Sets represented by 2 females per 1 male. Number of injected nymphs are represented in parentheses. All injected nymphs were at 5th nymph stage unless otherwise indicated.

Dissections

Abdominal dissections of insects required the removal of the forewings and hindwings, followed by scoring of the abdomen around the perimeter of the ScG (Figure 5). After scoring, sharp forceps were used to gently lift and tear the scored perimeter. After the perimeter epidermis was removed from the body wall, the flap of the epidermis was slowly lifted from the body starting from the posterior end. As the resection progressed, the ScG (Figure 5 C") or the intima (Figure 5 C) would "pop out" of the body wall. Resection of the body cavity was performed using forceps.

The Mtx ScG was accessed from the dorsal side of the insect. The wing structures (either pads or mature wings) were removed before the scutellum is split down the middle with dissecting scissors. The head and the scutellum were also removed with the scissors (Figure 6). The lateral flaps of the T1 segment were lifted as the tissue is resected until the glands are visible underneath. The flaps and any excess tissue were cut away with the scissors after which the legs and their muscles are removed.

Imaging

After the first couple clutches of eggs were laid (to account for eggs already fertilized in a WT environment), eggs were separated for observation until hatching. Nymphs and adults were dissected (except for first nymphs, which were directly imbedded in Aqua-Poly/Mount) in 1xPBT using forceps and needles before being transferred to new dissecting dishes with fresh PBT. Images were taken on SZX16 Olympus microscope. To capture the magnified ScG, microscope slides were prepared by first making a dime-sized ring of petroleum jelly then placing a large drop of Aqua-Poly/Mount in the center. Insects were euthanized in PBT, and the wings were removed, before being embedded in the center of the drop. After the slide was slightly dried the position of the nymph was adjusted as necessary, and a small drop of Aqua-Poly/Mount was added before placing the slide cover. ScG images were taken using the Zeiss Axoiscope upright microscope.

CHAPTER III: RESULTS

The *Oncopeltus* Scent Glands

Hemimetabolous Development and the Scent Glands

Throughout development, the abdominal scent glands (Abd ScGs) become proportionally larger in respect to the increase in body size (Figure 4). These insects have type 2 ostioles (Figure 4A”-C”, A”’-C”’) and shed the scent gland intima (inner content of the scent gland) during ecdysis (Staddon, 1979). In *Oncopeltus* nymphs, the ScGs are located on the dorsal abdomen where the anterior ScG (ScG A) is found on segment A5, and the posterior ScG (ScG P) is found on A6. The ostioles are located within the characteristic black abdominal spots (Figure 4A-C). At the 1st nymph stage, the dorsal black pigmentation on the abdomen, marking the ScG localization resemble small circular dots (Figure 4A-A’). Structurally, the Abd ScGs ostioles have yet to show the characteristic “pinching” of the center of type 2 ostioles and remain completely open (Figure 4A”-A”’). The wing pads become discernable at the 3rd nymph stage as small triangular shaped pouches extending from the dorsal thoracic segment (Figure 4B). As for the Abd ScGs, the ostioles now take a characteristic type 2 shape with the pseudo-closure in the middle (Figure 4B”-B”’). The pigmentation around the surrounding openings have expanded proximally, and the region appears more oblong (Figure 3B’). At the 5th nymphal stage, the wing pads are at their largest size and the forewings and hindwings are dissectible (Figure 4C). The pigmentation of the Abd ScGs change throughout development as well. In 1st nymphs, the ostiole bisects the black dot (Figure 4A’), whereas in 5th nymphs, the ostioles take up only a fraction of the black area (Figure 4C’). There is little change in the ostiole itself, with the exception of the pinching being more centralized in 5th nymphs (Figure 4C”-C”’) in comparison to the 3rd nymph ostioles which show the pinching being offset to the left (Figure 4B”-B”’). Coinciding with the appearance of fully developed wings in adults, the abdominal scent glands become obsolete and seal shut (Figure 4D’-D”’). Additionally, there is a loss of pigmentation on the dorsal A5-A6 segments (Figure 4D’)

that had black spots in previous stages. The “new” metathoracic scent glands (Mtx ScG) appear laterally between the second and third thoracic segment (Figure 4E-E’).

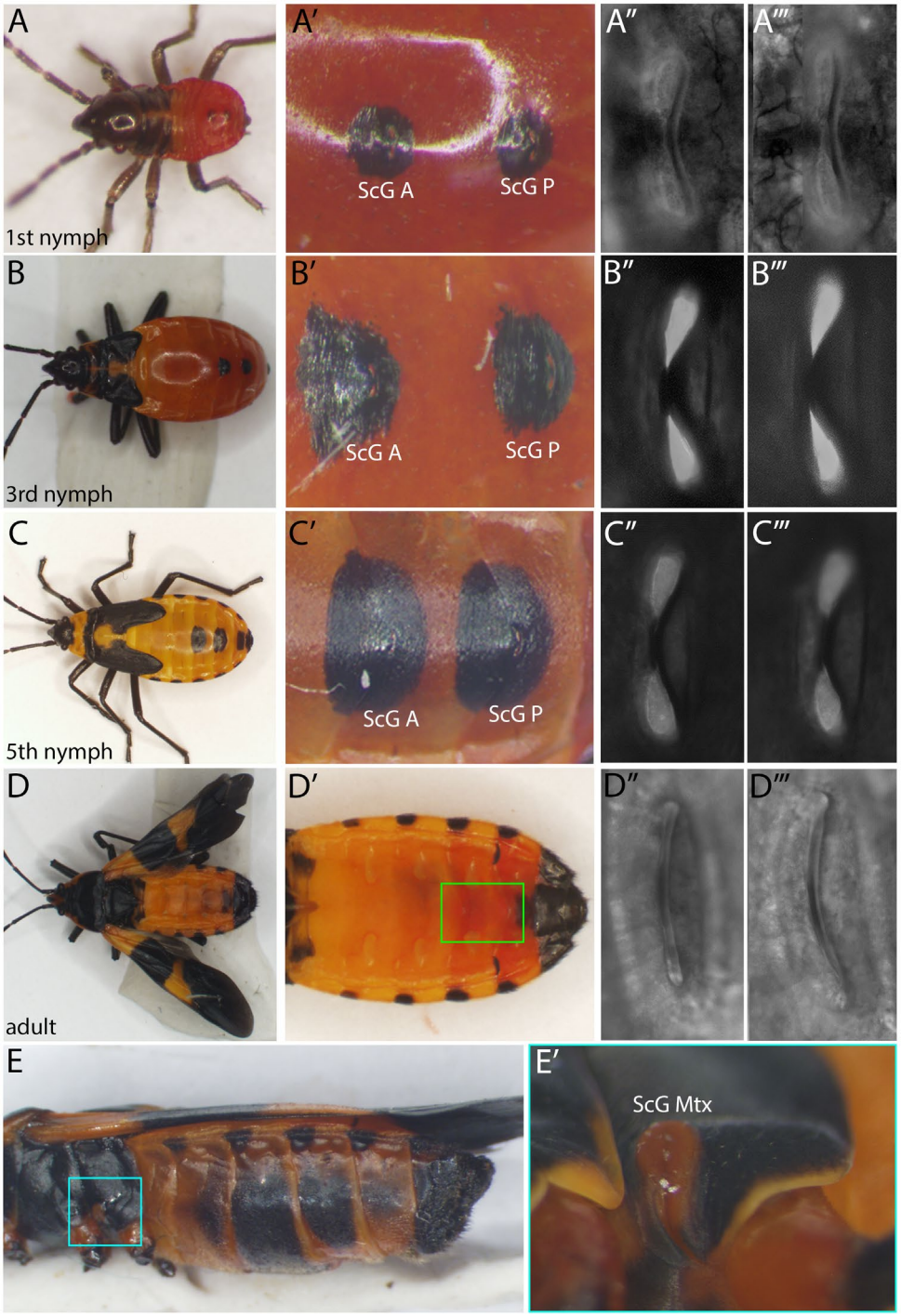

Figure 4. Development of *Oncopeltus* from 1st nymph to adult. (A’-D’) shows magnified A5-A6 abdominal region. (A”-D”) DIC of ScG A. (A”’-D”’) DIC of ScG P. (D’) Green box indicates ScG region. (E) Lateral view of adult Mtx ScG. (E’) Magnified view of Mtx ScG.

Gross Anatomy of the Scent Glands

Abdominal Scent Glands in 5th nymphs

The nymphal ScGs appear as orange sacs imbedded in the body wall and resemble peach-shaped pouches (Figure 5A). The glands are filled with a gel-like intima and attach to the ostioles on the underside of the dermis (Figure 5A). In adults, the ScGs remain attached to the ventral dermis (Figure 5B), but the intima doesn't regenerate in adults, rendering the sacs completely empty (Figure 5B).

Metathoracic Scent Glands

The adult metathoracic scent glands (Mtx ScG) are found on the ventrolateral side of the thorax between the T2 and T3 segments and resemble an orange drop embedded in the body wall (Figure 4E-E'). The primordia appear as an underdeveloped Mtx ScG structure in 5th nymphs (pre-ecdysis stage), where the median reservoir appeared as orange tissue bridging the two sides of the thoracic segment (Figure 5C). The fully developed adult gland is symmetrical and composed of two parts: a yellow gourd-shaped median reservoir (mr), and a white lateral reservoir (lr) attached on the superior side of each bulb (Figure 5D). The two reservoirs play different roles in making the scent substance. The lateral reservoir is surrounded by secretory tubules that utilize 2-cell secretory to which synthesize the scent precursors, whereas the median reservoir has regions of aqueous and liquid phases which combine with the precursors that are made in the lateral reservoir. Enzymes in the medial reservoir and accessory gland (located within the median reservoir) catalytically make the final scent substance. This is then stored and released into the external gland orifice through a duct on the lateral side of the median reservoir (Staddon, 1979).

Embryonic *sal* expression

As previous work by Hanna and Popadić (unpublished) suggested *sal* localization in the ScG primordia, we wanted to further document and confirm these observations. Based on their positioning on the dorsal abdomen in nymphs, the ScG primordia would likely be found in a late-stage embryo during dorsal closure. However, we wanted to detail if there was ScG development

prior to dorsal expansion, therefore day 3.5 embryos (when the embryo is still “flat” with well-developed leg structures) were used for the youngest stage for the *sal in situ*. We found that indeed there was a signal in the distal abdomen (Figure 6A-A’) where the ScG primordia appear as dots (Figure 6A’). From this point until dorsal closure, the signal gets stronger, and the primordia begin to invaginate and form a crescent pattern (Figure 6C’). This signal that correspond to the strong signal on the dorsal abdomen of day 5 embryos (Figure 6D-E’). Once dorsal closure begins (day 5) the ends of the crescents pinch together before meeting in the dorsal midline. This forms a pattern similar to the 4-dots on the side of a die (Figure 6E).

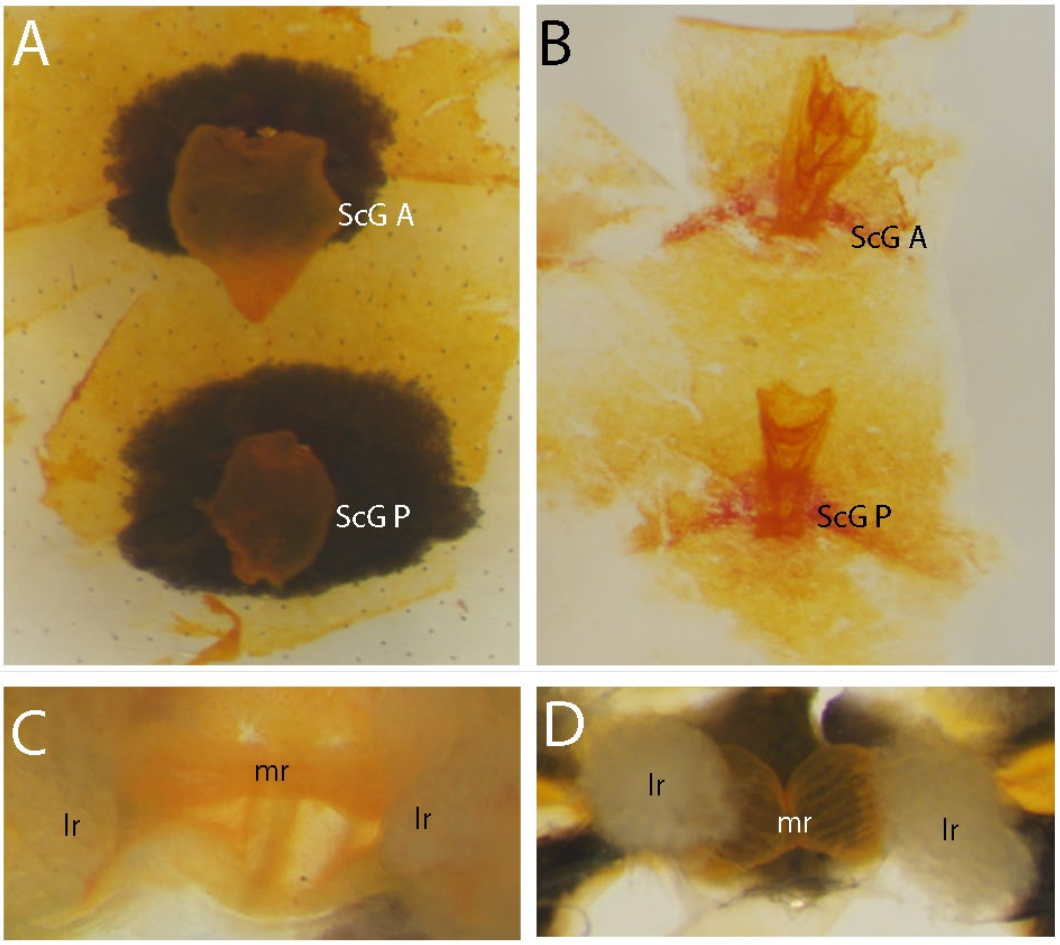

Figure 5. The internal anatomy of the ScGs in 5th nymphs and adults. (A) The ventral side of the 5th nymph abdominal region A5 (ScG A) and A6 (ScG B) showing the orange ScG sacs attached to the ostioles. (B) Ventral side of adult abdominal region A5 (ScG A) and A6 (ScG P). (C) Internal structure of 5th nymph Mtx ScG. (D) Internal structure of adult Mtx ScG when development is complete. *lr*- lateral reservoir, *mr*- medial reservoir.

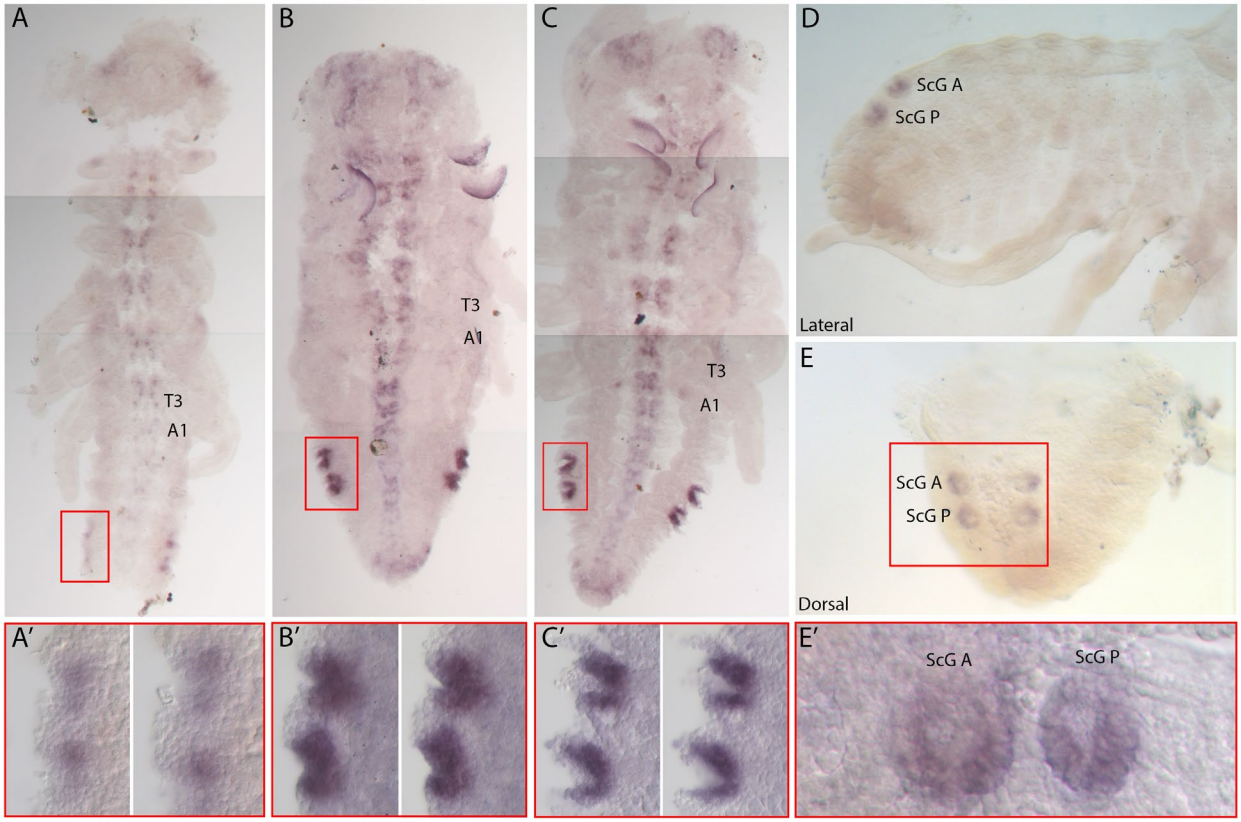

Figure 6. *sal* mRNA localization during mid to late embryogenesis. (A-C) Ventral view of day 3.5, 4, 4.5 embryos, respectively. (A'-C') abdominal segments A5 (top) and A6 (bottom) showing *sal* localization. (D) Lateral view of day 5 embryo, showing crescent-shaped expression of ScGs on the dorsal surface. (E) Dorsal view of day 5 embryo abdomen, ScG are showing symmetric expression of ScG pairs. (E') Magnified view of ostiole precursors in day 5 embryos
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In situ* Hybridization of *HNF4

Previous *in situ* hybridization of *vvl* in *Oncopeltus* reveled localization in ring-like structures along the lateral abdomen (Figure 7). Based on data found in other insect models, these cells are likely associated with the *Oncopeltus* oenocytes (Burns et al., 2012; Makki et al., 2014). To determine further if there was a connection between the oenocytes and ScGs, we wanted to visualize the embryonic expression of the lipid metabolizing marker, *HNF4*. We found that *HNF4* was localized in several regions of the embryo including the ScGs, presumed oenocytes, gut, and trachea (Figure 8-9). In the abdominal ScG, *HNF4* is localized in both the secretory cells and duct cells however the signal strength is not as pronounced as that of *vvl* (compare panel A' vs C', Figure 8).

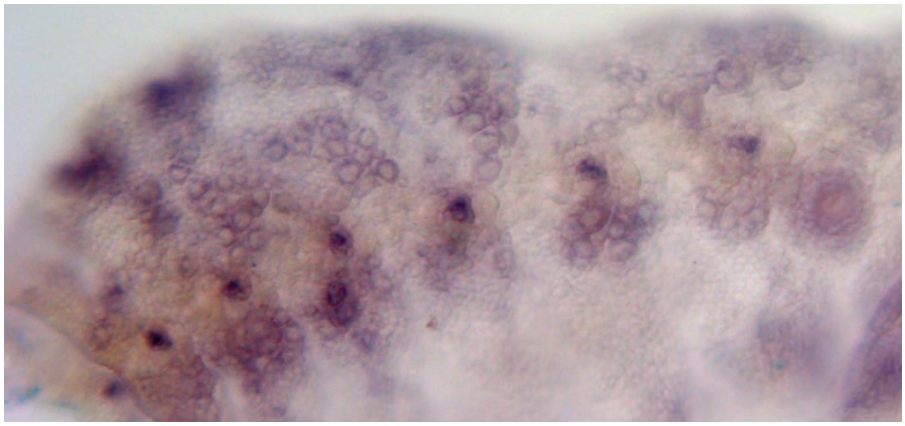

Figure 7. *vvl* staining of presumptive *Oncopeltus* oenocytes. The lateral rings located dorsal and ventral to the spiracles (dots) are likely oenocytes, or oenocyte-associated structures.

As the oenocyte marker, *HNF4* was expected to be found in the oenocytes, as was described for *Drosophila* and *Tribolium* (Burns et al., 2012). Although there were clusters of cells in the lateral abdomen that were expressing *HNF4* (Figure 9C), the lack of signal intensity compared to background precludes us from any conclusive inference regarding the identity of these cells.

The dorsal trunk and dorsal branches of the abdominal trachea also exhibit weak *HNF4* localization (Figure 9B arrows). While there are no current reports of *HNF4* being actually expressed in the trachea directly, oenocytes have been shown to be responsible for waterproofing of the trachea (Burns et al., 2012; Gutierrez, Wiggins, Fielding, & Gould, 2007; Makki et al., 2014; Palanker, Tennessen, Lam, & Thummel, 2009). Hence, our results in *Oncopeltus* provide a putative link between tracheal cells that express *HNF4* and waterproofing.

The strongest expression of *HNF4* is in the gut, spanning the thoracic and abdominal regions (Figure 9A arrows and inset). This expression corresponds well with Oncopeltus diet, as both laboratory and wild populations feed on seeds, in which most of the calories come from unsaturated fats. Hence, *Oncopeltus* has likely developed a digestive system that is built to efficiently metabolize fat because of this fat-rich diet, as the primary function of *HNF4* is to break down lipids.

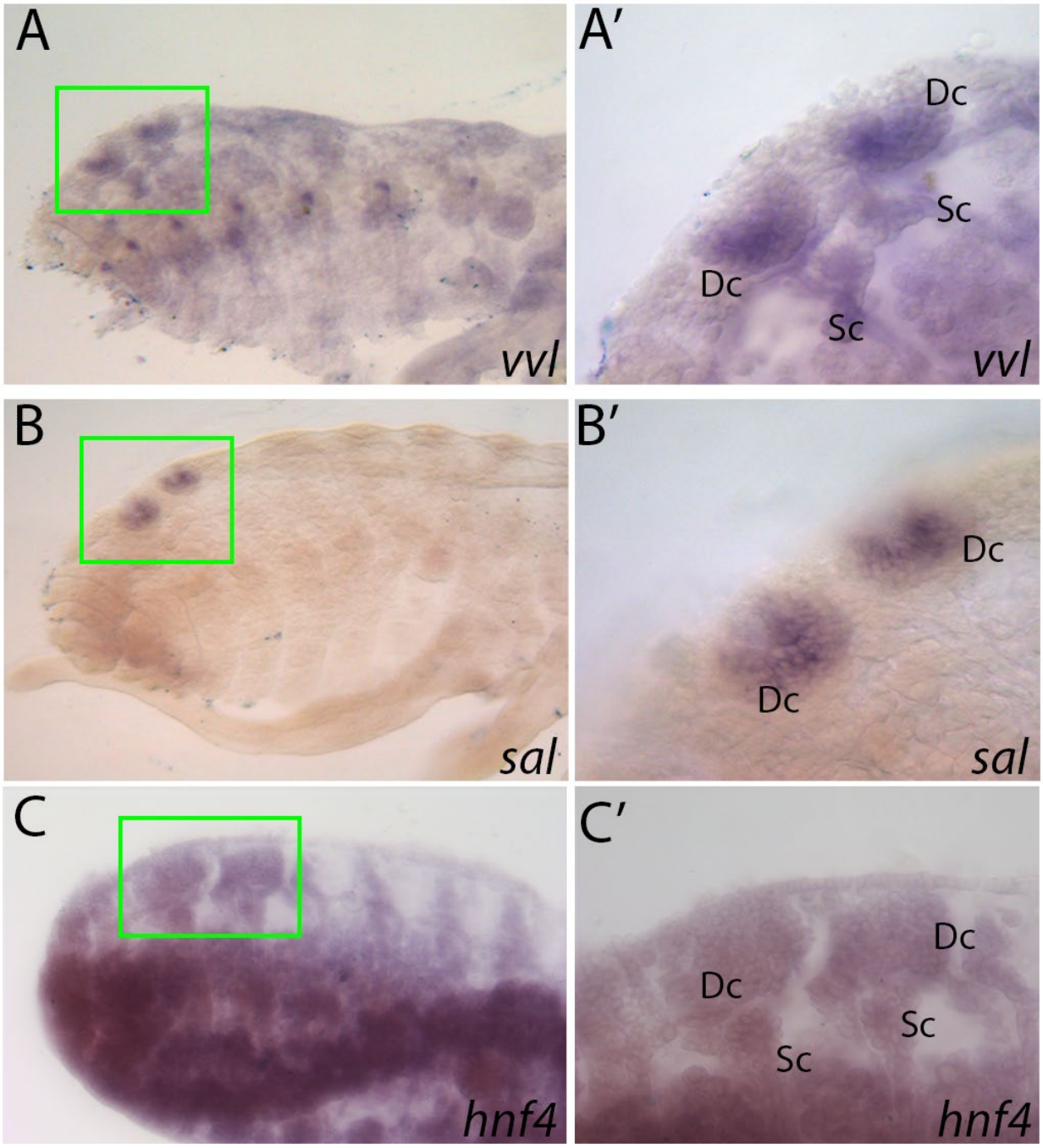

Figure 8. mRNA localization of *vvl*, *sal*, and *hnf4* in day 5 embryonic scent glands. (A'-C') magnified view of the green boxes of (A-C). (A) *vvl* expression in the ScGs and spiracles. (A') *vvl* is localized in both the duct cell and secretory cell regions of the ScG. (B) *sal* is localized solely in the Abd ScGs of the embryo. (B') *sal* is localized in the duct cell region of the ScG only. (C) *hnf4* is shows strong signal in the gut of the embryo with localization in the ScG (green box). (C') *hnf4* is weakly localized in the both the duct cells and the secretory cells of the ScG, as well as the peripheral cells. Dc- duct cells, Sc- secretory cells.

Lastly, we found localization of *HNF4* in paired structures on the lateral A1 segment, just posterior to the T3 leg (inset, Fig. 9A). These organs are likely the pleuropodia, which has been also found to express *HNF4* in *Tribolium* (Burns et al., 2012). The function of the pleuropodia is to assist the embryo in emerging from the egg upon hatching using enzymatic digestion (Konopová, Buchberger, & Crisp, 2020). Previous transcriptomic studies in grasshoppers reported that genes involved in lipid metabolism are upregulated in the pleuropodia (Konopová, Buchberger, & Crisp, 2020). Our observations are consistent with this report, and suggestive of HNF*4* being involved in breaking down the chorion so that the nymph can hatch.

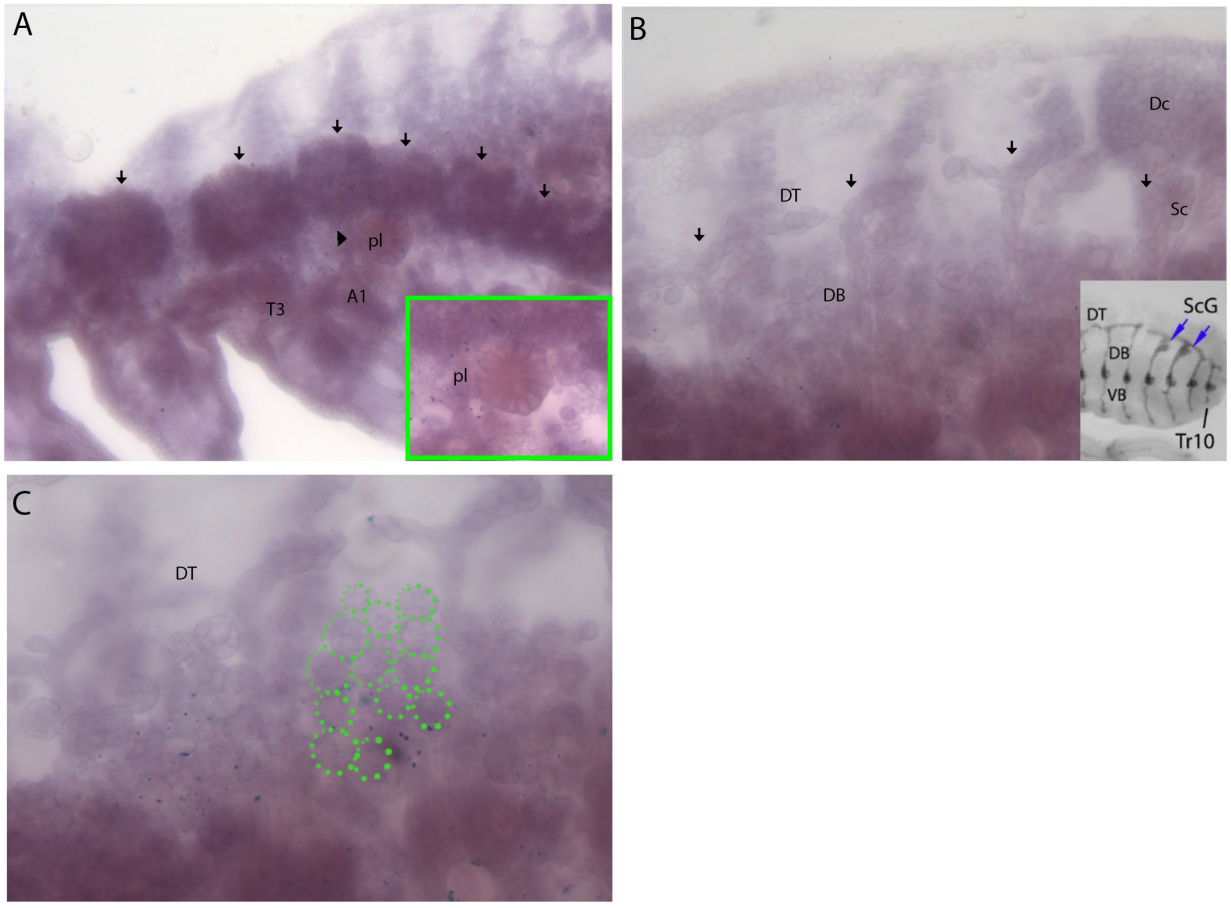

Figure 9. *HNF4* mRNA localization in other body regions. (A) Strongest signal is localized in the gut (arrows) of the embryo. The pleuropodia (arrowhead and green inset) is also identifiable with *HNF4* localization. (B) *HNF4* is localized in the peripheral tracheal branches (arrows) of the dorsal trunk (DT) and dorsal branches (DB). *Inset*- expression of *trh* in the trachea of the WT embryo showing the dorsal trunk, dorsal branches, and ventral branches (VT) (Hanna & Popadić, 2020). (C) *HNF4* localization in the center cluster of putative oenocytes (green outline). Additional adjacent clusters can be seen to the left and right of the group outlined in green.

sal RNAi causes ScG reduction and alterations in pigmentation

Maternal sal RNAi

Given that the signal in late-stage embryos is localized to the ScGs only, we wanted to examine the functions in first nymphs. Maternal injections were performed in 4 sets (Table 2). After the first couple of clutches were laid (to account for eggs already fertilized in a WT environment), eggs were separated for observation. We found that the phenotypes of the hatched 1st nymphs were lethal regardless of dsRNA concentration. All nymphs were unable to move their legs, and after emerging from the egg they were "inching their body like a worm". The nymphs were also unable to melanize or sclerotize, therefore the texture of the body was soft and sticky, and the nymphs remained their bright reddish-orange color. Structurally, the ScG openings were either entirely absent or significantly reduced and lost their characteristic "figure-eight" structure (Figure 10B).

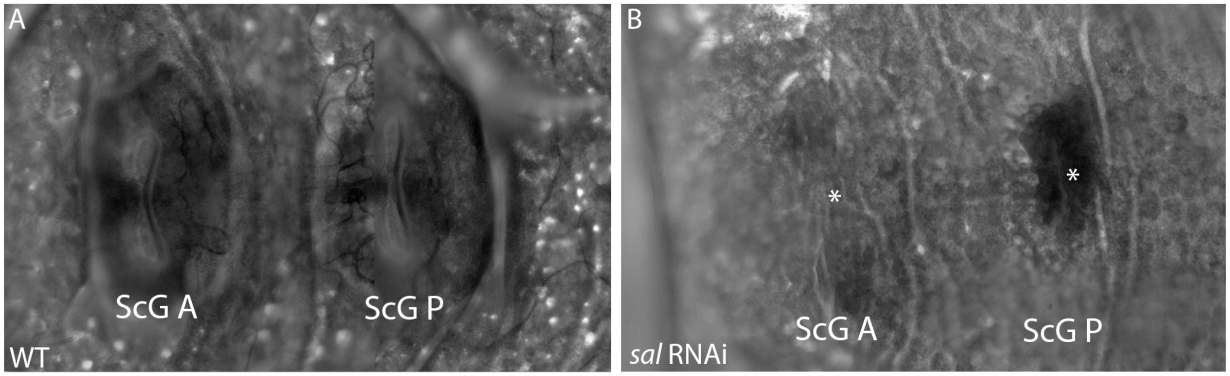

Figure 10. WT and _sal_ RNAi nymphal abdominal scent glands. DIC images of the anterior (ScG A) and posterior (ScG P) scent glands corresponding to the 5th and 6th abdominal segments, respectively. (A) WT Abd ScGs. (B) *sal* RNAi Abd ScGs.

Nymphal RNAi

Finally, we wanted to determine the function of *sal* in late nymph and adult stages. Nymphs were injected at either the 4th or 5th instar stage, however all injected nymphs died during the final adult molt with the exception of 2 insects (Table 2). The same fragment was used in a previous study and resulted in replication of the viable adult phenotypes, indicating that the high lethality rate was unrelated to the fragment itself (Simmons, 2017). The two viable phenotypes showed

alterations in wing and thoracic pigmentation as well as the dorsal abdomen (Figure 11). In the forewings, the proximal black stripe loses definition and expands into the neighboring anterior orange region. Additionally, there is a loss of pigmentation in the center of the wing and the texture of the distal tip changes and appears much flimsier than wild-type (Figure 11B-B'). Similarly, the hindwing loses the well-defined proximal border and there is an increased pigmentation gradient (Figure 11C'). There is also a change in pigmentation in the thorax, where the lateral orange stripe on the scutellum is encroached by the black pigmentation (Figure 11D'). The external structure of the Mtx ScG does not show any morphological change (Figure 11E'), although color variation is a common occurrence in WT insects. Finally, on the dorsal abdomen the indents lateral to the stripe between the Abd ScG change from a concave chape to a sideways teardrop shape (Figure 11F'). These findings are consistent with previously reported adult *sal* RNAi wing phenotypes in *Oncopeltus* (Simmons, 2017).

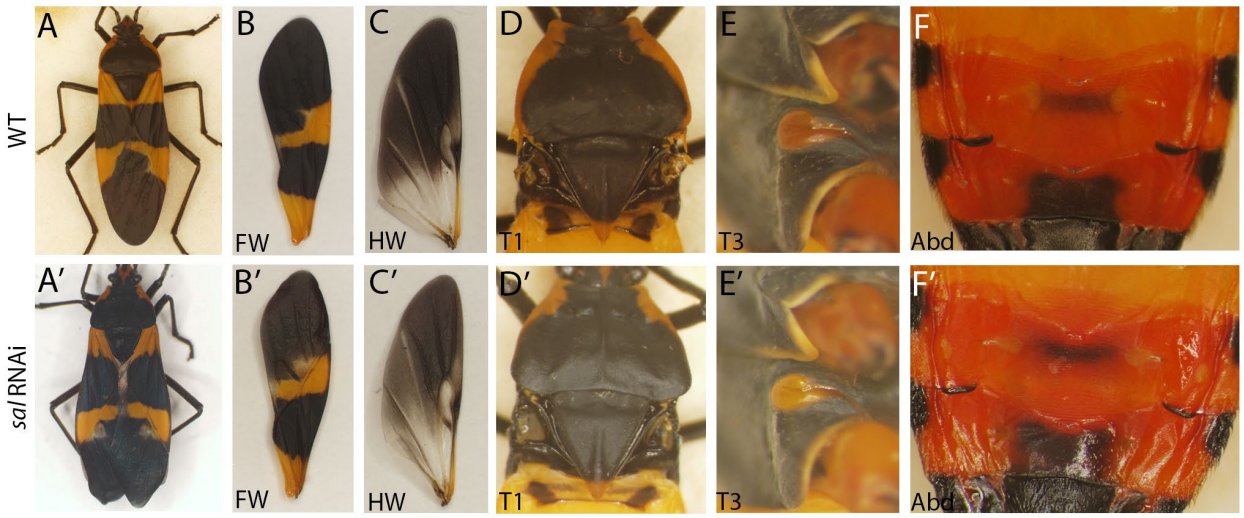

Figure 11. Comparison of WT and *sal* RNAi adult phenotypes. (A-A') Dorsal view of adult WT and *sal* RNAi *Oncopeltus*, respectively. (B-B') Dorsal view of forewings. (C-C') Dorsal view of hindwings. (D-D') Dorsal view of the thoracic body region. (E-E') Ventrolateral view of the right side of the thoracic region. (F-F') Dorsal view of the distal abdominal segments A5 and A6.

The internal structures of the scent glands were also affected by *sal* RNAi in both nymphs and adults (Figure 12 and Figure 13). The nymphs were dissected pre-ecdysis to ensure that the WT and *sal* RNAi individuals were at a similar developmental stage to compare their internal

structures. At this stage, new cuticle is being deposited on the epidermis and does not fully develop until after molting is complete. Therefore the epidermis is relatively delicate, and the force of resecting the dorsal epidermis is enough to tear the tissue connecting the epidermis to the ScG. (Zhang, Lu, Kong, Zhang, & Ling, 2014). When 5th nymphs undergo ecdysis the exuviate is shed as well as both scent gland intima, which have a smooth texture that conformed to the shape of the ScG pouch (Figure 12C). In *sal* RNAi nymphs, the texture of the body wall (Figure 12D'), intima, and the ScG pouches (Figure 12D') are all distorted. The intima of the ScG become wrinkled and thin (Figure 12C'), while the body wall of the abdomen as well as the ScG pouches becomes more translucent (Figure 12D') than that of the WT nymphs (insert, Figure 12D).

Additionally, there are changes that occur in the Mtx ScG internal structures of adults (Figure 13). Normally, the gland is separated into two parts: the white, cluster-like lateral reservoir that is surrounded by secretory tubules (Figure 13B and C), and the yellow median reservoir that resembles a "pair of balls" (Figure 13B). When *sal* is knocked down, the constitution of the lateral reservoirs become significantly reduced (Figure 13B' and C') and the median reservoir loses its WT shape, and instead resembles the median reservoir of the pre-ecdysis 5th nymphal Mtx ScG (Figure 13D and D').

Overall, the data generated here document the progression of ScG development during the *Oncopeltus* life cycle, starting from the primordia of mid-stage embryos to the adults (Figure 6 and Figure 13). Furthermore, the gross anatomy of the internal structures of the ScGs have been described for the first time using light microscopy (Figure 5). *In situ* hybridization of *HNF4* documented localization in the ScGs, presumed oenocytes, gut, pleuropodia, and trachea. However, these preliminary results do not show a discriminating signal, indicating that further investigation needs to be performed. Finally, RNAi results reveal that *sal* is responsible for development of ostioles in the 1st nymph (Figure 10) and is necessary for the complete development of the internal structures of both the abdominal and metathoracic ScGs in 5th nymphs and adults (Figure 12-13).

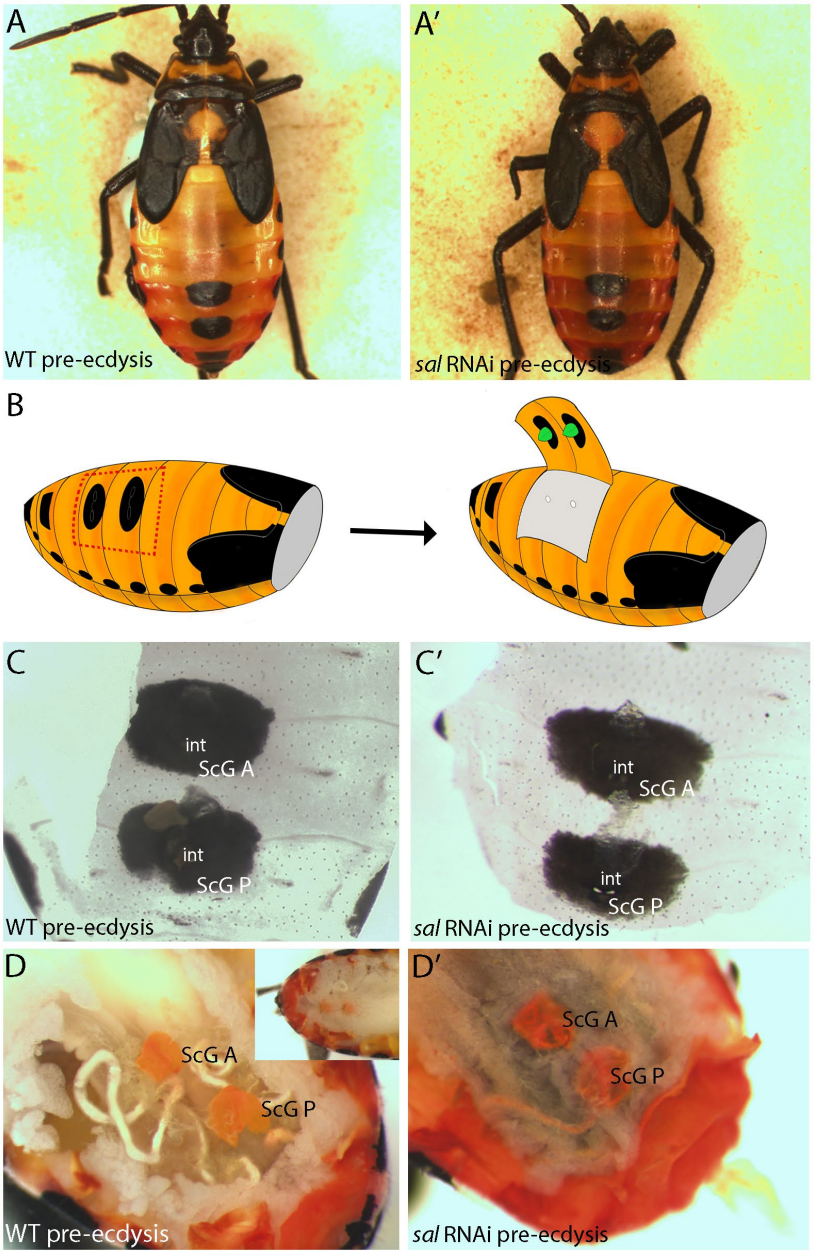

Figure 12. *sal* RNAi affects the abdominal nymphal scent gland. (A-A') 5th nymphs days before the final adult molt. (B) Schematic of how the dorsal Abd ScGs were dissected from the body. (C-C') Ventral view of the exuviate covering the Abd ScG, *ScG A*- anterior (A5) ScG, *ScG P*- posterior (A6) ScG, *int*- ScG intima. (C") Ventral view of the dermis that covers the adult Abd ScG. (D) WT Abd ScG pouches. *Inset*- how the pouches appear before resection, after the epidermis is dissected. (D') *sal* RNAi Abd Pouches.

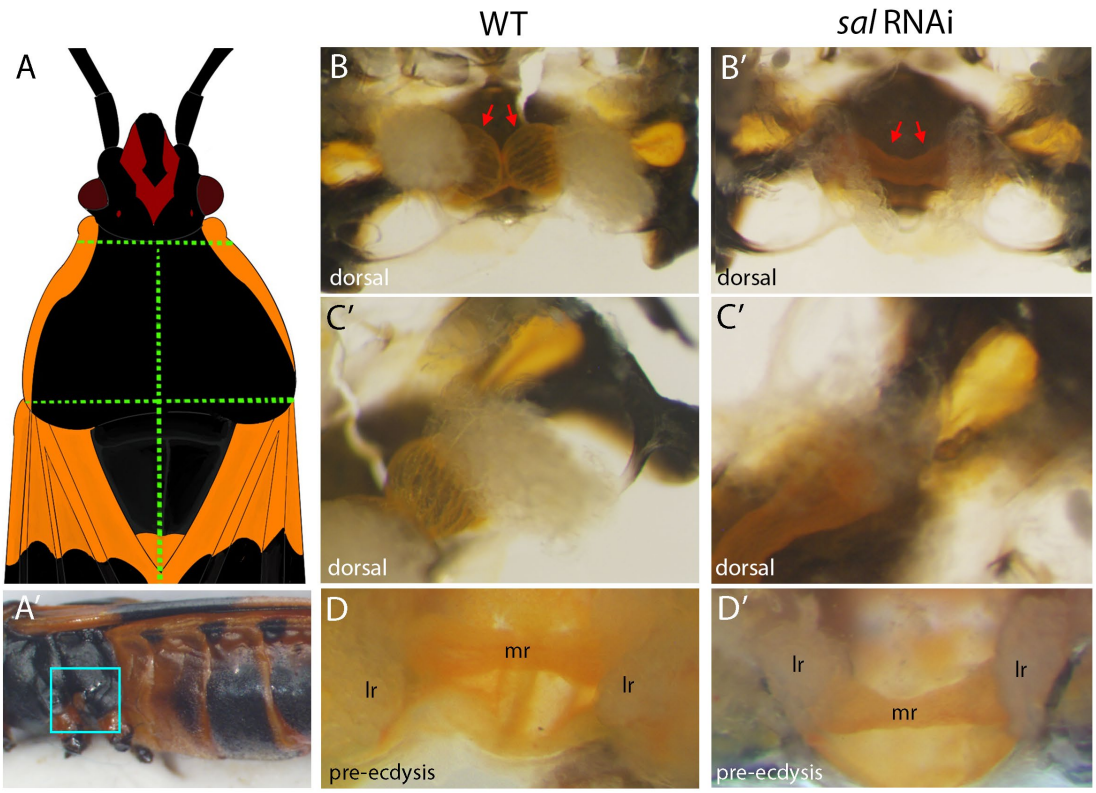

Figure 13. *sal* RNAi affects internal structures of the adult metathoracic scent gland. (A) A schematic showing the lines of dissection. The Mtx ScG were exposed by resecting from the dorsal to the ventral side of the body. (A') Lateral view of a WT adult, Mtx ScG in blue box. (B-B') A dorsal view of the ScG openings, the lateral reservoirs, and the median reservoir in WT and *sal* RNAi nymphs, respectively. (C-C') An increased magnification view of the right side of the Mtx ScG system, detailing the textures of the lateral and median reservoirs. (D-D') Pre-ecdysis dissection of the developing Mtx ScG in WT and *sal* RNAi nymphs, respectively. *lr*-lateral reservoir, *mr*-median reservoir

CHAPTER IV: DISCUSSION AND FUTURE DIRECTIONS

The translocation of *Oncopeltus* ScGs

Although ScGs have been observed and studied since the turn of the 20th century, their internal structures have only previously been described with either diagrams or focused on the microstructure using SEM (Packard, 1895; Staddon, 1979). This is the first work that documents the gross anatomy of the scent glands in *Oncopeltus* with light microscopy (Figure 5 and Figures 12-13). While the loss of function in the adult abdominal scent glands were previously reported in Staddon (1979), this work details the changes that occur in the internal structure of the abdominal ScGs, leading to its eventual obsolescence in adults. For example, intima does not regenerate even after cuticle development is completed (Figure 5B), which is why the orange outer layer (of the scent gland) appears “deflated” while still being attached to the epidermis. Observing the failure of intima regeneration in the adult stage contributes new information to the mechanism of Abd ScG obsolescence. In parallel, dissection of the thorax of pre-ecdysis 5th nymphs revealed that the presence of a Mtx ScG precursor in 5th nymphs, which completes development either during or after ecdysis.

The mechanism of “translocation” (from Abd ScG to Mtx ScG) remains an open question. However, it is possible to deduce the developmental “logic” behind the necessity of the “translocation”. As the adult *Oncopeltus* is the only developmental stage with fully developed wings, having the abdominal glands covered by the wings would be impractical. Interestingly, other evolutionary adaptations such as aposematism have also been correlated with changes in the scent gland morphology (Staddon, 1979). This question can be addressed in the future by using CRISPR/Cas9 approach. This technique has proven to be an efficient system for genome editing, and has recently been used to knock out the gene *white* in *Oncopeltus* (Reding & Pick, 2020). Additionally, CRISPR has been used to create transgenic lines in other insects, in particular GFP knock-ins (Kimura, Hisano, Kawahara, & Higashijima, 2014; Kina, Yoshitani,

Hanyu-Nakamura, & Nakamura, 2019). By inserting a GFP reporter construct next *sal*, the progression of the ScG cells can be tracked throughout development.

HNF4 is found in lipid metabolizing areas

Previous studies in *Drosophila, Tribolium,* and *C. elegans*, have shown that *hnf4* is required for lipid catabolism that is involved in β-oxidation and pheromone (Burns et al., 2012; Fernández et al., 2010; Gutierrez et al., 2007; Palanker et al., 2009). Functionally, the ScGs should perform a similar function, as these glands synthesize and secrete different compounds comparable to pheromones (Brückner & Parker, 2020). Furthermore, previous studies of endocrine glands documented expression of genes that were also expressed in the *Oncopeltus* ScGs (Burns et al., 2012; Hanna & Popadić, 2020; Makki et al., 2014; Sánchez-Higueras et al., 2014). Therefore, it was inferred that the ScGs either directly employ oenocytes or utilize oenocyte-like cells that undergo similar regulation to produce the necessary compounds (Brückner & Parker, 2020; Games & Staddon, 1973a, 1973b; Staddon, 1979).

My results of the *HNF4 in situ* did not clarify the role of *HNF4* or oenocytes in the development of ScGs as the localization in the ScGs were indistinct from the surrounding signal (Figure 8C). In a similar fashion, the oenocytes failed to be clearly distinguished in the abdominal region (Figure 9C), although presumptive clusters were found in regions correlating to the regions where *HNF4* was localized in other insects (Burns et al., 2012). Additional experiments, such as functional testing with RNAi, need to be performed in order to clarify the oenocyte-ScG connection.

The role of *spalt* in ScG development

Most of the molecular insight of scent gland development stems from *Tribolium* studies of quinone synthesis in the stink glands (J. Li et al., 2013). Our *in situ* hybridization of *sal* validated the localization of *sal* mRNA in the abdominal ScGs (Figure 6). However, given its function in cell determination in other models, and its role in oenocyte and tracheal formation, the localization of *sal* in only the ScGs and CNS was rather surprising (Barrio, de Celis, Bolshakov, & Kafatos, 1999;

Kühnlein, Brönner, Taubert, & Schuh, 1997; Ott, Parrish, Bond, Schwaeger-Nickolenko, & Monaghan, 2001; Rusten et al., 2001). Similarly, there was no localization of *sal* in the thoracic region of the embryo marking the metathoracic scent glands (Figure 6D). One disadvantage to the *Oncopeltus* model is that we lack an ability to visualize mRNA localization post-embryonically, therefore the expression of *sal* in nymphs remains ambiguous. Thus, the remaining information regarding *sal* function was collected via *sal* maternal and nymphal RNAi.

Injection of *sal* dsRNA into gravid females resulted in the loss of ostioles, consistent with the mRNA localization in the apical duct cells (Figure 6B-B'). Hatched nymphs also exhibited paralysis, correlating with the localization of *sal* in the CNS embryonically (Figure 6A-C) and desiccated within hours after molting. Nymphal RNAi resulted in an overwhelming majority of lethal phenotypes where the nymphs died during the final molt, despite previous studies showing a 47% survival rate (Simmons, 2017). Only 2/13 injection sets yielded a viable phenotype, with 1/10 and 1/6 of viable insects within the two sets, respectively (Table 2).

The 5th nymph knockdown phenotype documented the compromised structure of the abdominal ScGs and the intima (Figure 12C'-D'). Although we have shown that *sal* is expressed in duct cells of the ScGs, further histological studies need to be performed to determine exactly how the secretory cell and duct cell primordia in the embryo (Figure 8A') correlate to the formation of the orange sac of the nymphal abdominal ScGs. The Mtx ScG precursor in pre-ecdysis 5^{th} nymphs provided insightful information in that regard, as the internal structure (medial and lateral reservoirs) showed no observable difference between WT and *sal* RNAi glands (Figure 12D-D'). In the adult, however, the morphology of the lateral and median reservoirs seemed to be affected differently by *sal* RNAi (Figure 12B'-C'). There was a visible reduction of the lateral reservoir from the opaque and condensed structures in WT adults to transparent and wispy ones in *sal* knockdowns (Figure 12B'-C'). In contrast, the median reservoir seemed to simply stop developing from the 5^{th} nymph precursor (Figure 12B'-D'). Previous descriptions of the Mtx ScGs revealed that the lateral reservoir consisted of three-cell secretory units similar like the abdominal glands,

likely meaning that *sal* is only involved in the development of the lateral reservoirs (Staddon, 1979). These results demonstrate that *sal* is necessary but not solely sufficient for the development of the Mtx ScGs, indicating similar regulation between nymphs and adults. By performing transcriptome analysis on both WT and *sal* RNAi Mtx ScGs, gene networks responsible can be identified. When combined with a transgenic *sal* GFP line, transcriptomics of both nymphal and adult stages will generate comprehensive data regarding the genetic mechanisms involved in the development of these specialized organs.

APPENDIX 1

Appendix 1. Amplification and cloning of *Oncopeltus fasciatus spalt* (*sal*)

Primers:

Forward: 5’ GATAGGTGCTCACCAAAGTC 3’

Reverse: 5’ GTTCTGACGACTTACTCAAA 3’

Amplicon length: 200bp

Apollo Gene ID: OFAS014214

Length of clone: 200bp

Length of gene (CDS): 2,946bp

Clone Sequence:

5’
GATAGGTGCTCACCAAAGTCTCCCGCCGGGGCCACCACCCTTTCCTCCCCAGTTTTCACC
CTTGTACCGCCCGCCCCACGACCTCGTCCTGCCCCCTCAAAGGTCTCATGATCCCGGCAA
CAAGCCCATCTTACCCGTCACGGTTTTTAGTCCAAGGATAGAGCAAGATATGCCGGCAGAT
TTGAGTAAGTCGTCAGAAC 3’

APPENDIX 2

Appendix 2. Amplification and cloning of *Oncopeltus fasciatus ventral veinless* (*vvl*)

Primer set 1:

Forward 5’ GGTTTGAAGCTTTGCAACTG 3’

Reverse 5’ TAGTGAGCGGTAAGCGAGGT 3’

Amplicon length: 501bp

Primer set 2:

Forward 5’ GAGGACCAGTATCGAAGTGT 3’

Reverse 5’ TAGTGAGCGGTAAGCGAGGT 3’

Amplicon length: 360bp

Apollo gene ID: OFAS011077

Length of clone (CDS): 1,275bp

Length of gene: 501bp

Clone sequence:

5’
GGTTTGAAGCTTTGCAACTGAGTTTTAAAATATGTGCAAATTGAAGCCCCTCCTTCAAAAGT
GGTTGGAAGAAGCTGATTCGACTACCGGCTCGCCGACAAGCATCGACAAGATTGCTGCCC
AGGGAGGGAAAAGGAAAAAGAGGACCAGTATCGAAGTGTCTGTCAAAGGAGCCCTCGAAC
AACATTTTCATAAACAACCCAAGCCTTCGGCACAAGAAATCACTTCTCTGGCCGATACTCTT
CAGCTAGAGAAGGAGGTCGTCCGAGTCTGGTTCTGTAACAGGCGACAGAAGGAAAAGAGG
ATGACACCCCCCAACACCCTCGGACCACAGGAAGGAATGATGGAAGGGCAGATGGGCCA
GACACCTCACGGCCTGCACGGATACCATCACCAAGATCACCTGCACGGCTCACCGATGGC
ACCGCACTCCCACAGCCACAGTCCGCCGATGCTGTCGCCCCAGAACATGCCCACCCATAC
CTCGCTTACCGCTCACTA 3’

APPENDIX 3

Appendix 3. Amplification and cloning of *Oncopeltus fasciatus*

***cuticular protein 1* (*cpr1*)**

Primer set 1:

Forward: 5' TCATAGTCGCTTGTGCTGTC 3'

Reverse: 5' AGAGAAGTCGCTGGATGAGTA 3'

Amplicon length: 359bp

Primer set 2:

Forward: 5' GAGGACAAGAACACCACAAAGA 3'

Reverse: 5' ATGAGTACGCACTCTCTCCT 3'

Amplicon length: 306bp

Apollo gene ID: OFAS015699

Length of clone: 306

Length of gene (CDS): 399

Clone sequence:

5'
GAGGACAAGAACACCACAAAGAAGTGGACTACTATGCGCCTCCACACTACAGATATGAGTA
CTCTGTTCATGACTCTCATACCCACGACATCAAGAAACAAGAAGAGGAACGCAAAGACGAC
CACGTCAAGGGCTCGTACTCCCTCCACGAGGCTGACGGTACAATACGAGAGGTACACTAC
AGCTCGGACAAGAAGAACGGCTTCAACGCGGTGGTACACAGGTCGGGGAAGGCCCAGCA
CCCGCAGAAGTACGGCAGCTCAGGGGAGTCTTACCATGGTCACGGAGGAGAGAGTGCGT
ACTCAT 3'

APPENDIX 4

Appendix 4. Amplification and cloning of *Oncopeltus fasciatus*

***cuticular protein 2* (*cpr2*)**

Primer set:

Forward: 5’ CTTCCAGTTACGATGCCTCAA 3’

Reverse: 5’ AGCACCAGGAGTACGTTG 3’

Amplicon length: 900bp

Primer set 2:

Forward: 5’ CCTCTTCTGCTTCCTCTTCTTAC 3’

Reverse: 5’ AGCACCAGGAGTACGTTG 3’

Amplicon length: 399bp

Apollo gene ID: OFAS010387

Length of clone: 399bp

Length of gene (CDS): 1,305bp

Clone sequence:

5’
CCTCTTCTGCTTCCTCTTCTTACTCGGCTCAATCCTCCTACGCTGCCGGCGCTTCTGGTTC
CGCCTACTCCTCGTACTCCGCTCCGGTCGTGTCGAGCTATAAGTCCGCTCCCATTGTCGTC
GCCCCTGCCCAATCGCCTGCTCCGGTATACAGCCCGGCTCCTATCGTCTCTTCCTACAGC
TCGGCTCCCATCGTCTCTTCCTACAGCTCGGCTCCTATCGTCGCTTCCTACAGTCCTGCTC
CTGCAATTTCTTCATACAATGCGGCTCCTATCGTCGCTGTCAAACCTGCCCCAATAAAGTAC
GCCAGTGAATCCCCTGCTGTTTCGGGATACAGTGCCTCTGCTGTGAAACTTGTTGGAGCTT
CTGCTGCTAAGAATAAGCACCAGGAGTACGTTG 3’

APPENDIX 5

Appendix 5. Amplification and cloning of *Oncopeltus fasciatus cuticular protein 3* (*cpr3*)

Primer set 1:

Forward: 5' AGGTACATCAGAGACCAC 3'

Reverse: 5' GATTCAGCTCCTTCAGCTAC 3'

Amplicon length: 278bp

Primer set 2:

Forward: 5' CAGAGACCACTACAGCAAGTTC 3'

Reverse: 5' GATTCAGCTCCTTCAGCTAC 3'

Amplicon length: 268bp

Primer set 3:

Forward: 5' GCACCTAGCCAATCCTATCTTC 3'

Reverse: 5' GTATCCCAAGCCAGTCATGTAG 3'

Amplicon length: 222bp

Apollo gene ID: OFAS004861

Length of clone: 268bp

Length of gene (CDS): 1,011bp

Clone sequence:

5'
CAGAGACCACTACAGCAAGTTCAGAAGAGCTTGCTTCAGACATTTCACCAGTAAGTGAAAA
CACCAATACGGAATCTGAAAAAGACAATAGTGCATCAGATGAAGAATCTGTACACAACTTG
GAAAGTAGTCCAGCTCCTGAGCAAAAAGAAAACGACTCTCAGCAGGTCACAGATTCAAAAA
GTAAAGAATCCGACTCAAGAGAAGGCACAAGTGACTCCAATGAAGAAAATAGCAATGGAAA
CGAAGTAGCTGAAGGAGCTGAATC 3'

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ABSTRACT

THE SWEET SMELL OF MYSTERY: THE SCENT GLANDS OF *ONCOPELTUS FASCIATUS*

by

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May 2022

Advisor: Dr. Aleksandar Popadić

Major: Biological Sciences

Degree: Masters of Science

Scent glands (ScGs) are defensive glands that are found among a wide variety of insect orders. These glands represent a specialized function of the exocrine system; however, the developmental mechanism remains unclear. Previous functional studies of the endocrine glands revealed that the endocrine and tracheal systems utilize similar genetic regulatory networks which indicate that these systems have common primordia. In *Drosophila*, *sal* is localized in the prothoracic gland while in *Oncopeltus* the gene is localized in the duct cells of the abdominal scent glands (Hanna & Popadić, 2020; Sánchez-Higueras et al., 2014). RNAi mediated knockdown of *sal* resulted in significant reduction of the abdominal ScG in nymphs and the metathoracic (Mtx) ScG in adults. Interestingly, there is no localization of *sal* in the thoracic segments, indicating that the activation of *sal* on the Mtx ScG is temporally restricted to later developmental stages. Linked by their lipid metabolism function, as ScGs secretions are largely composed of hydrocarbons, oenocytes served as a possible ancestral precursor to ScG. Studies on oenocytes show that genes involved in ScG and endocrine gland development are involved in their differentiation (Burns et al., 2012; Gould et al., 2001; Makki et al., 2014). *HNF4*, an oenocyte marker, was found to localize in the embryonic abdominal ScG in addition to the gut and pleuropodia. These results provide further clarification on the genetic regulatory network of ScG development.

AUTOBIOGRAPHICAL STATEMENT

DESPINA TSITLAKIDOU

I completed my undergraduate studies at Wayne State University and received a BS, majoring in Biological Sciences with a minor in modern Greek languages. During that time, I was an NCAA athlete and lettered all four years on the Wayne State Fencing team. I was also a supplemental instructor for CHM 1040 with the Wayne State Academic Success Center. In my junior year, I took Developmental Biology with Dr. Alcedo, which sparked my interest in research and developmental genetics. Later in my senior year, I took evolution with Dr. Popadić and eventually joined his lab as an undergraduate student. It was during this time I was accepted into the MS program where I studied the development of the scent glands of *Oncopeltus*. In addition, I received the Heberlein Award for Excellence in Teaching in 2020.

During the first year that I was in the Master's program, I was considering switching to get a PhD, however after realizing how much I enjoyed teaching physiology and desired to do dexterous work, I chose to go to medical school and will begin my studies in January 2022. I am confident that the skills I acquired both in genetics and teaching, will benefit my medical school studies and eventually my future patients.