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HIDE AND SEEK: AN EXPLORATION OF ANTIPREDATOR AND PREDATOR AVOIDANCE MECHANISMS IN ORTHOPODOMYIA SIGNIFERA IN RESPONSE TO PREDATION FROM TOXORHYNCHITES RUTILUS

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HIDE AND SEEK: AN EXPLORATION OF ANTIPREDATOR AND PREDATOR AVOIDANCE MECHANISMS IN ORTHOPODOMYIA SIGNIFERA IN RESPONSE TO PREDATION FROM TOXORHYNCHITES RUTILUS

by

Nathaniel James-Lasser Dahlberg

A Thesis
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ABSTRACT

There have been many observations of larval *Orthopodomyia signifera* coexisting with the predator *Toxorhynchites rutilus.* There are three hypotheses that could explain how *Or. signifera* resists predation from *Tx. rutilus*. The first hypothesis states that larvae adapt behavioral changes that limit predation. The second hypothesis states thoracic setae serve as a physical defense that prevents *Tx. rutilus* from grasping *Or. signifera*. The third hypothesis states *Or. signifera* possess a chemical defense indicated by aposematic coloration. To test the first hypothesis larval *Or. signifera* were exposed to conspecific and heterospecific predation cues and their behavior was observed. Both cues caused *Or. signifera* to change their behavior, with conspecific cues causing the most stringent defensive behavior and heterospecific cues causing moderate defensive behavior. This led to the confirmation of the first hypothesis. To test the second hypothesis the rate of successful strikes of *Tx. rutilus* on *Or. signifera* and *Aedes albopictus* were compared. There was no significant difference in the successful strike rate of *Tx. rutilus* on *Or. signifera* and *Ae. albopictus* and the second hypothesis was rejected. For the third hypothesis the functional response of *Tx. rutilus* on *Ae. albopictu*s and *Or. signifera* when exposed to a natural and artificial diet was compared. Diet was not a factor, however, *Or. signifera* had significantly longer handling time and fewer 2nd strikes in comparison with *Ae. albopictus*. The third hypothesis was inconclusive as the results could indicate a physical or chemical defense.

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DEDICATION

I would like to dedicate this thesis to my loving family. This would not have been possible without my incredible wife Kristen whose unconditional love and support kept me motivated. I am extremely grateful to my parents, John and Victoria, who always encouraged my interest in entomology and whose support was invaluable. Without the encouragement of my sister and brother, Carolyn and Griffin, and their willingness to let me sneak insects into the house, I would never have developed my love of insects. I would also like to dedicate this to my two wonderful nieces, Lydia and Adeline whose artwork and motivational speeches kept me focused.

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1.1 Orthopodomyia signifera Ecology

Orthopodomyia signifera is a mosquito (Family: Culicidae) widely distributed throughout the continental United States (Darsie & Ward 1981). Larvae are found primarily in phytotelmata, which are plants that form structures that retain water (Kitchling 2000). Phytotelmata preferred by *Or. signifera* include large permanent tree holes with a high pH and a high tannin-lignin content (Bradshaw & Holzapfel 1988). *Orthopodomyia signifera* larvae are considered benthic filter feeders or browsers and consume detritus and microorganisms (Bradshaw & Holzapfel 1984). Other mosquito larvae commonly associated with *Or. signifera* in the United States include: *Aedes albopictus*, *Ae. triseriatus*, *Ae. sierrensis*, *Anopheles barberi*, *Culex pipiens*, and *Toxorhynchites rutilus* (Lewis & Tucker 1978, Bradshaw & Holzapfel 1984, Qualls & Mullens 2006, Farajollahi et al. 2009). *Orthopodomyia signifera* can be encountered in treeholes and tires in the southern U.S. They are found co-inhabiting with *Ae. albopictus* and *Ae. triseriatus* (Farajollahi et al. 2009, Qualls & Mullens 2006). In southern Mississippi *Or. signifera* showed a preference for treeholes over tires and were found in 48.9% of treeholes (n = 47) sampled at a density of 21.8 larvae/L (Yee et al. 2012). In comparison, *Or. signifera* were found in only 18.8% of tires (n = 48) at a density of 12.6 larvae/L (Yee et al. 2012). Competition between larval *Or. signifera* and other mosquitoes has not been widely studied however, it has been found to be outcompeted by *Aedes triseriatus* (Livdahl 1984).

The ecology of adult *Or. signifera* has been examined primarily through oviposition and its ability to transmit arboviruses. *Orthopodomyia signifera* are thought to select oviposition locations mainly through the cues associated with the permanence of the site (Woodward et al. 1998). Oviposition sites that are long lasting are preferential and thought to help reduce interspecific competition (Woodward et al. 1998). This species has been found to be a competent vector of West Nile Virus (Granwehr et al. 2004). Surveys on vectors of arboviruses often do not capture enough adult *Or. signifera* to attempt to extract viral RNA to determine if this species is an important arbovirus vector (Cupp et al. 2003, Gilland et al. 2005, Young 2005). In addition, *Or. signifera* has been thought to be a competent vector of Eastern and Western Equine Encephalitis due to their ability to take blood meals from both humans and avian hosts (Hanson et al. 2005, Chamberlain et al. 1954).

1.1.1 Toxorhynchites rutilus Ecology

Mosquitoes in the genus *Toxorhynchites* are found throughout the tropics with a single species, *Tx. rutilus,* found in the continental U.S. (Focks 2007). There are two subspecies, *Toxorhynchites rutilus rutilus* and *Toxorhynchites r. septentrionalis*, and these can be distinguished only via the morphology of the males (King 1960). *Toxorhynchites rutilus* larvae inhabit a variety of phytotelmata and artificial containers but prefer larger volume habitats because they provide a larger density of prey items (Farajollahi et al. 2009, Yee et al. 2012). In southern Mississippi *Tx. rutilus* was shown to prefer tree holes (46.8% of tree holes (n = 47) at a density of 2.92 larvae/L) over tires (6.25% of tires sampled (n = 48) at a density of 1.14 larvae/L) (Yee et al. 2012). *Toxorhynchites rutilus* are predatory as larvae and can consume a variety of different aquatic insects including other mosquitoes (Focks 2007). *Toxorhynchites rutilus* are opportunistic hunters and do not actively hunt prey; instead, they use their sensilla to detect disturbances in the water column (Donald et al. 2020). The mouthparts of *Tx. rutilus* are modified for predation and the mouth brushes have developed into comb like structures to grasp prey (Donald et al. 2020). Common prey mosquitoes in the United States include: *Ae. aegypti, Ae. albopictus*, *Ae. triseriatus*, *Culex quinquefasciatus*, *Cx. pipiens*, *Or. signifera*, and other *Toxorhynchites rutilus* (Focks et al. 1982, Lounibos et al. 1996, Bradshaw & Holzapfel 1988, Farajollahi et al. 2009). A single *Tx. rutilus* can consume over 6000 mosquito larvae during their development (Focks et al. 1980). Due to this and that the adults do not take blood meals, they have been examined as a possible method of biocontrol for medically important mosquitoes such as *Ae. aegypti* and *Ae. albopictus* (Collins & Blackwell 2000, Focks 2007).

1.1.2 *Or. signifera* & *Tx. rutilus* Coexistence

There have been reports of significant numbers *Or. signifera* being found alongside *Tx. rutilus* (Bradshaw & Hozapfel 1983, Farajollahi et al. 2009). In southern Mississippi both were found to coexist in 29.79% of tree holes (n = 47) at a density of 25.22 and 3.354 larvae/L for *Or. signifera* and *Tx. rutilus* respectively (Yee et al. 2012). This coexistence is unusual because *Tx. rutilus* usually suppress the abundance of other mosquito larvae in shared habitats (Collins & Blackwell 2000). One hypothesis is that *O. signifera* can evade predation from *Tx. rutilus*, although the mechanism for this remains unclear (Bradshaw & Hozapfel 1983, Farajollahi et al. 2009).

1.1.3 Predator Avoidance & Antipredator Mechanisms

There are two ways in which prey can avoid a predator: predator avoidance and antipredator mechanisms (Brodie et al. 1991). Predator avoidance is when prey increases its survivorship by decreasing the probability that they will inhabit the same foraging habitat as the predator (Brodie et al. 1991). Antipredator mechanisms are when the prey is able to reduces the probability of the predator consuming them during an encounter (Brodie et al. 1991). Insects have evolved both predator avoidance and antipredator mechanisms to avoid predation.

Aedes triseriatus possess predator avoidance mechanisms. For example, when in the presence of water-borne chemical cues from *Tx. rutilus, Ae. triseriatus* will change their behavior to reduce the probability of predation (Kesavaraju & Juliano 2007). There are 4 main types of behavior that mosquito larvae can exhibit: 1) browsing, when the larva propels itself along a feeding surface with its mouthparts, 2) filtering when the larva propels itself through the water column with its' mouthparts 3) resting, when the larva is drifting through the water column or remaining still and 4) thrashing, when the larva moves through the water by flexing its body in a sigmoidal motion (Yee et al. 2004). To avoid predation *Ae. triseriatus* spent less time thrashing and browsing and more time resting in the presence of cues from *Tx. rutilus* (Kesavaraju & Juliano 2007). These behaviors are less attractive to *Tx. rutilus* and therefore, *Ae. triseriatus* are less likely to be consumed.

Aside from changing their behavior, *Ae. triseriatus* will also change their location in the water column in the presence of water-borne cues from *Tx. rutilus* (Kesavaraju & Juliano 2007). *Toxorhynchites rutilus* spend most of their time on the bottom of the habitat (Steffens & Evenhuis 1981). To avoid predation, *Ae. triseriatus* will spend the majority of its time at the surface of the water column (Kesavaraju & Juliano 2007). This change in behavior decreases the probability of interspecific encounters between *Ae.*

triseriatus and *Tx. rutilus* and therefore increases the formers survivability (Kesavaraju & Juliano 2007).

Another antipredator mechanism in insects is chemical defense. A variety of herbivorous insects can sequester plant-derived secondary metabolites that decrease predation (Hartman 2004). The secondary metabolites can cause the insects to be distasteful to predators and this will decrease the probability that the predator will feed on them (Hartman 2004, Poinar et al. 2007). In some instances, the chemical defense can prove fatal for the predator (Poinar et al. 2007). Another example of antipredator mechanisms in insects is a physical defense. These physical defenses are a physical structure that decreases the probability of predation. For example, larvae of the midge, *Cricotopus sylvestris* (Diptera: Chironomidae) possess long setae that discourage predation by Hydra (Hershey & Dobson 1987). A further example can be found in larval tortoise beetles, *Charidotella bicolor* and *Deloyala guttata* (Coleoptera: Chrysomelidae). These larvae form a shield of feces and exuviae that prevent predation from carabid predators with short mandibles (Olmstead & Denno 1993).

1.1.4 Hypothesized Antipredator & Predator Avoidance Mechanisms

There have been two proposed antipredator mechanisms in the literature that explain how *Or. signifera* may evade predation from *Tx. rutilus*. The first hypothesis is that *Or. signifera* has long, stout setae emerging from its thoracic, abdominal, and lateral tufts that reduce the likelihood of a successful strike by *Tx. rutilus* (Bradshaw &Holzapfel 1983). The second hypothesis suggests that *Or. signifera* unusual orange coloring could be a form of aposematism, perhaps linked to chemical defense which discourages *Tx. rutilus* consumption (Farajollahi et al. 2009).

Besides the hypothesis mentioned above another way that *Or. signifera* could avoid predation from *Tx. rutilus* is through behavioral modification. Other container dwelling mosquitoes such as *Ae. triseriatus* change their normal behavior to a lower risk behavior in the presence of *Tx. rutilus* or the predatory midge *Corethrella appendiculata* (Diptera: Corethrellidae) (Juliano & Gravel 2002, Kesavaraju & Juliano 2007). Specifically, they decrease browsing for food and this likely reduces interspecific encounters between *Ae. triseriatus* and either predator (Juliano & Gravel 2002, Kesavaraju & Juliano 2007). It is possible that *Or. signifera* modifies its behavior in a similar way to avoid predation.

I tested the hypothesis that *Or. signifera* changes their behavior in the presence of *Tx. rutilus*. Testing was done by placing *Or. signifera* in water that contained chemical cues from *Tx. rutilus* preying on *Or. signifera* and *Ae. albopictus*, and I then video recorded their responses to those cues. I hypothesized that *Or. signifera* would modify their behavior equally in the presence of predation cues of *Tx. rutilus* created in the presences of *Or. signifera* or *Ae. albopictus*. The reason for this is because of a similar behavioral modification observed in *Ae. triseriatus* (Juliano & Gravel 2002, Kesavaraju & Juliano 2007). To test the above hypothesis, I proposed the following null hypotheses: *Or. signifera* do not modify their behavior in the presence of cues predation cues from *Tx. rutilus* on *Or. signifera*. The second null hypothesis was: *O. signifera* do not modify their behavior in the presence of predation cues from *Tx. rutilus* on *Ae. albopictus*. I predicted that *Or. signifera* modify their behavior in the presence of *Tx. rutilus* in the same manner regardless of the prey species. This is based on the fact there is no literature demonstrating that any mosquito larvae responded differently to heterospecific predation cues.

1.2 Materials and Methods

1.2.1 Collection & Husbandry

Orthopodomyia signifera and *Tx. rutilus* larvae were collected from two locations in southern Mississippi in the August of 2021: Ragland Hills (31.20405786031081 N, -89.17505734393647 W) and the POW Camp Recreation Area of the Desoto National Forest (30.648425794628672 N, -89.01974934394521W). These habitats consisted of large stretches of conifers intermixed with small stands of mixed deciduous trees. Both species were collected as 1st or 2nd instar larvae and reared in captivity. *Toxorhynchites rutilus* were kept individually in clear, 100 ml urine cups with 50 ml of deionized water and feed *Ae. albopictus* larvae *ad libitum* that were of corresponding instar. The environmental chamber was set at a photoperiod of 14:10 (Light: Dark) and 26-29°C during the day and 21-24°C at night (Schiller et al. 2019). The *Or. signifera* from both locations were randomly sorted 50 at a time in clear 900 ml mosquito breeders (BioQuip.com #1425) with 250 ml of deionized water and 250 ml of filtered tree hole water. Breeders were provided with 20 g of detritus, dry weight, found in the tree-holes and 0.05 g of pulverized dog food (Purina Puppy Chow Complete) every 48 hrs. The detritus from the tree holes was created by drying, homogenizing, and combining all the detritus from all the different tree holes where *Or. signifera* larvae were collected. The environmental chamber was set at a photoperiod of 14:10 (L:D) and 28-31°C during the day and 26-29°C at night (Chambers 1985). Both larvae were raised to the 4th instar stage and then randomly selected for the trials.

1.2.2 Experimental Design

These experiments consisted of three treatment levels: *Ae. albopictus* predation water (hereafter AA water), *Or. Signifera* water (hereafter OR water), and DI water. The first treatment level consisted of one, 4th instar *Or. Signifera* placed into a 100 ml vial with 60 ml of AA. The AA water was created with 3 individual trays each containing 1200 ml of DI water, 1 4th instar *Tx. rutilus,* and 30 4th instar *Ae. albopictus*. The predator then spent 72 hrs feeding on *Ae. albopictus* and every 24 hrs *Ae. albopictus* were replenished to maintain the density at 30 larvae. After 72 hrs the 3 trays were filtered through 106 um mesh to remove any detritus and the water was put in one large container. The OR water was created in the same manner as the AA, except the 30 *Ae. albopictus* larvae were swapped for 30 *Or. signifera*. The third treatment was a no prey control.

For the experiment each replicate consisted of a single tripour beaker divided on the exterior into 3 equal vertical zones: low, medium, and high, using a black marker. The low zone consisted of the lowest 20 ml of water, the medium zone consisted of middle 20 ml of water, and the high zone consisted of the highest 20 ml of water. A camera was placed above two of the tripours in an isolated room and the larvae were recorded for a total of 41 minutes. The first 10 minutes were an acclimation period for the larvae and the remaining 31 minutes were later analyzed for behavior. Every minute during those 31 minutes instantaneous scan census occurred, and I recorded the vertical location (top, middle, bottom) and behavior (thrashing, filtering/resting, or browsing). The filtering and resting behavior had to be combined as they could not be visually distinguished during the recording. The aforementioned, behaviors have been determined to be important behaviors exhibited by other mosquitoes in behavioral studies, and the location have also been indicated to be an important behavior of mosquitoes in the presence of predators (Kesavaraju, & Juliano 2004, 2007). There were 20 replicates of each of the three treatments levels.

1.2.3 Analysis

Raw data for both species found in the middle of the environment violated normality so it was arcsine transformed where normality was achieved. Because some behaviors are likely correlated (e.g., resting at the top) data were processed first using Principal Components Analysis (PCA). The independent PCs scores from this analysis were then analyzed using a Multivariate Analysis of Variance (MANOVA), with the three levels of one treatments (AA, OR, and DI) as independent variables. Standardized Canonical Coefficients (SCCs) were used to determine the important dependent variables that explained the multivariate effects (Scheiner & Gurevitch 2001).

1.3 Results

The PCA reduced the three activities and three positions down to three axis that summarized 92.87% of the variation in these measures (Table 1.1). On PC1 individuals with positive PC scores were thrashing at the bottom and those with a negative score were resting/filtering at the top (Table 1.1). For PC2, individuals with positive PC scores spent time browsing and those with negative scores resting/filtering (Table 1.1). For PC3 individuals with positive PC scores were found in the middle and those with negative scores were at the bottom (Table 1.1).

Table 1.1 *Principal Component Analysis for larval behavior and location. Values ≥ 40 are listed with an * and indicate strong loadings on each principal.*

Response Variables	PC1	PC2	PC3
Thrashing	92*	-11	20
Resting/Filtering	-82*	-50*	-9
Browsing	-10	98*	-2
Bottom	95*	-13	-11
Middle	11	0	99*
Top	-81*	80*	-44*
Interpretation	Thrashing, Bottom vs Resting/Filtering, Top	Browsing, Top vs Resting/Filtering	Middle vs Bottom

There were significant differences based on the MANOVA (Pillai's Trace=$0.402_{6.37}$, P<0.0001). PC1 (SCC=1.122) and PC2 (SCC=0.371) contributed most to these differences compared to PC3 (SCC=0.089). For PC1 *Or. signifera* reacted to OR water and AA water differently in comparison to the control. In the presence of either prey type (OR, AA) *Or. signifera* would rest/filter at the top and in the presence of the control (DI) larvae would thrash at the bottom. On PC2 *Or. signifera* responded more strongly to OR water by resting/filtering, in comparison to browsing when exposed to AA water.

1.4 Discussion

1.4.1 Interspecific & Intraspecific Behavioral Responses

When exposed to a cue from a predator consuming *Or. signifera* or *Ae. albopictus*, *Or. signifera* larvae adapted less risky behavior by staying motionless at the top of their container. This lack of movement has been demonstrated to allow other mosquito species such as *Ae. triseriatus, Ae. siensis,* and *Or. anopheloides* to avoid predation from *Toxorhynchites* mosquitoes (Yasuda & Takashi 1992, Juliano & Gravel 2002, Kesavaraju & Juliano 2007, Zuharah et al. 2015). In Japan *Or. anopheloides* adopts a similar strategy of becoming motionless to avoid predation from *Tx. towadensis,* and in comparison, with another native species, *Ae. albopictus,* are much less likely to be preyed upon (Yasuda & Takashi 1992). Field surveys have demonstrated that *Or. anopheloides* and *Tx. towadensis* can coexist in discarded tires and bamboo nodes but that *Ae. albopictus* cannot coexist with *Tx. towadensis* (Yasuda 1996). In addition, *Or. signifera* and *Tx. rutilus* coexist in tires, suggesting that perhaps the genus *Orthopodimya* as a whole could be specialized in avoiding predation from *Toxorhynchites*. This specialization would allow them to persist in habitats that other more susceptible mosquitoes cannot. Further research on the behavior of other *Orthopodi*mya and *Toxorhynchites* could further develop this hypothesis.

When exposed to AA water however, *Or. signifera* showed a less stringent response to predation pressure then when conspecifics were being consumed. *Orthopodomyia signifera* stopped the riskiest behavior (thrashing) and adapted a less risky behavior (browsing). Because *Tx. rutilus* do not actively hunt and are ambush predators, browsing could still lead to mortality as when an *Or. signifera* accidentally enters their strike range

(Donald et al. 2020). In contrast to AA cues, the presence of predation cues on conspecifics caused *Or. signifera* to rest/filter and during these behaviors the larvae remained relatively motionless. The differences in response to the predation cues could be the result of *Or. signifera* recognizing a specific chemical released by the consumption of conspecifics.

1.4.2 Effect of Invasive Specie

A possible explanation for *Or. signifera* no responding equally to predation cues could be that of *Ae. albopictus* is an invasive species in the study area. *Aedes albopictus* is native to southeast Asia, and was first detected in Harris County, Texas in 1985 and spread into Mississippi within five years (Moore & Mitchell 1997). *Aedes albopictus* is highly adapted to both urban and rural areas, whereas *Or. signifera* is more of a rural species that is occasionally found in urban areas (Ramasamy et al. 2011). Both species can be found in the same larval habitat in south Mississippi, but cooccurrence is not common (Yee et al. 2012, Pers. Obs.). Therefore, perhaps *Or. signifera* cannot detect the same cues found in the environment when *Ae. albopictus* is consumed because it has not co-occurred with this species long enough to develop an ability to sense when it is being consumed. To attempt to address this hypothesis further studies could test how *Or. signifera* responds to the predation cues of other mosquitoes native to south Mississippi such as *Ae. triseriatus* and *Culex territans* (Yee et al. 2011). Because *Or. signifera* has spent significantly more time coexisting with the native species it maybe more adept at detecting predation cues from native species compared to a relatively new invasive.

1.4.3 Cue Source

Besides understanding how *Or. signifera* can detect different cues, it could also be asked the source of the cue. No other mosquito larvae have been demonstrated to possess glands that produce defensive substances and thus it is unlikely that *Or. signifera* possess these structures. Instead, *Or. signifera* could shed these chemical cues passively through its hemolymph. The OR water was filtered through a 106 um filter that removed particulate matter created by *Tx. rutilus* feeding on prey. However, as a liquid, the hemolymph is small enough to fit through this filter and thus could be detected by *Or. signifera*.

Other organisms have demonstrated the ability to detect hemolymph and change their behavior accordingly. In aquatic systems the Caribbean spiny lobster, *Panulirus argus*, will adapt more stringent defensive behavior when it is exposed to conspecific hemolymph then when exposed to the hemolymph of other crustaceans (Shabani et al. 2008). When exposed to hemolymph from other crustaceans *Pa. argus* will not always adapt defensive behavior, and if it does, the behavior is less extreme then when exposed to conspecific hemolymph (Shabani et al. 2008). In terrestrial insects, the common eastern bumble bee, *Bombus impatiens*, anti-predator behavior occurs when in the presence of hemolymph from other bees (Goodale & Nieh 2012). *Bombus impatiens* actively avoid foraging in areas that researchers had covered in hemolymph from the western honeybee, *Apis mellifera* (Goodale & Nieh 2012). Conversely, *Ap. mellifera* showed no such response to cues from the hemolymph of *Bo. impatiens* (Goodale & Nieh 2012). The behavior of both the *Pa. argus* and *Bo. impatiens* share similarities with *Or. signifera*. If hemolymph is a predation cue, then both species do not need specialized structures to store and release the chemical. Instead, the chemical cue would be released passively when the individual is damaged via a predator.

Besides, behavioral defenses it has been hypothesized that *Or. signifera* can avoid predation through a chemical defense, largely based on apparent aposematic coloration (Farajollahi et al. 2009). Many insects, such as the aphid *Myzus. persicae* (Hemiptera: Aphididae) may acquire a chemical defense through the consumption of plants (Verheggen et al 2010). Certain compounds in plants can be used by the insect as chemical base for which the insect then develops their defense (Verheggen et al 2010). As *Or. signiferas'* diet can consist of the plant matter found in the water column it may gain a chemical defense from its diet in treeholes. Chapter 2 will seek to unravel if *Or. signifera* gains any chemical defense from nutrients found in the water column.

CHAPTER 2 – PHYSICAL & CHEMICAL DEFENSE

2.1 Chemical Defense

Diet has been shown to be an important factor in how some insects develop their chemical defense (Verheggen et al. 2010). For example, the caterpillar of the monarch butterfly, *Danaus plexippus* (Lepidoptera: Nymphalidae) feed on milkweed in the genus *Asclepias* (Malcolm & Brower 1989). These plants contain steroids, known as cardenolides, that caterpillars will sequester within their body (Malcolm & Brower 1989). Cardenolides are toxic to ants and a deterrent for birds and provide late instar caterpillars and adult *Da. plexippus* with a chemical defense that prevents them from becoming prey (Malcolm & Brower 1989). Like *Da. plexippus* it has been hypothesized that *Or. signifera* contain a chemical defense that help them avoid predation (Farajollahi et al. 2009). The source of the chemical defense is not known however the larvae's diet may play a role in the development of this anti-predator defense.

2.1.1 Tannins

The environment in which tree-hole mosquitoes develop contain a variety of edible plant material including leaves, bark, and heartwood that the larvae feed on (Mercer 1992, Rey et al. 2009, Pers. Obs,). The breakdown of these materials can release allelochemicals from the plant tissue including tannins. Tannins are type of polyphenol that plants synthesize to serve as a chemical defense against phytophagous insects (Khanbabaee & Van Ree 2001). For example, when fed artificial diets high in tannins caterpillars of the common cutworm, *Spodoptera litura* (Lepidoptera: Noctuidae) experienced slower development times and lower body weights (Nomura & Itioka 2002). The authors of the aforementioned study hypothesized that this has an indirect effect on the population of *Sp. litura,* as lower body weight and long development times negatively affect fecundity and increase predation pressure (Nomura & Itioka 2002). Therefore, the tannins defend the plant by decreasing the population of *Sp. litura* over time (Nomura & Itioka 2002).

Further work on tannins effects on phytophagous insects have determined a likely mechanism by which tannin's inhibit insect growth. In the fall webworm *Hyphantria cunea* (Lepidoptera: Erebidae) high concentrations of tannic acid, a derivative of tannins, significantly affects the detoxification enzyme acetylcholinesterase, AChE, and this coincided with a decrease in the ability of the larvae to effectively digest their food (Yuan et al. 2020). The decrease in digestion results in more time spent feeding and slower development periods (Yuan et al. 2020).

Larvae of culicids are negatively affected by the presence of tannins in treeholes. The larvae of *Aedes sierrensis* exhibit delayed growth and smaller body size when raised in high tannins (Mercer 1992). The effect of tannins on mosquito larvae can differ between species and some are more susceptible to tannin's negative effects. For example, in the Alps tree-hole communities can be shaped by the levels of tannins in the water (Rey et al. 1999). *Culex pipiens* is very sensitive to tannin levels in their environment and if it reaches a certain threshold, it can be fatal (Rey et al. 1999). Studies have further demonstrated that certain *Aedes* mosquitoes have different sensitivities to tannins with *Ae. aegypti* being more sensitive than *Ae. albopictus,* which itself is more sensitive than *Aedes rusticus* (Rey et al 1999). These differences in sensitivities allow different species to inhabit different larval habitats based on the concentration of tannins (Rey et al. 2000).

Rey and other (2000) hypothesize that these sensitivities are based on individual species abilities to successfully clear tannins from their system before any negative effects occur.

It is not currently known how tannins affect larval *Or. signifera* development. However, it is known that their preferred habit are large volume, permanent tree-holes with high tannin content (Bradshaw & Holzapfel 1988, Pers. obs.). I have observed that *Tx. rutilus* spends significantly more time-consuming individual *Or. signifera* in comparison with *Ae. albopictus;* and *Tx. rutilus* will eat fewer *Or. signifera* in comparison with *Ae. albopictus* over a 24 hr period. These observations mirror those of *Hu. cunea* spending more time feeding when given a diet high in tannins (Yuan et al. 2020). I hypothesize that *Or. signifera* sequester tannins from their larval environment and when consumed by *Tx. rutilus* these sequestered tannins serve to decrease the predator's appetite. This decrease in appetite allows other *Or. signifera* larvae to avoid predation.

Aside from chemical defense it has been hypothesized that *Or. signifera* possess a physical defense to evade predation. The hypothesis was put forth by Bradshaw & Hozapfel (1983), who suggest that the long, stout setae emerging from *Or. signifera's* thoracic, abdominal, and lateral tufts reduce the likelihood of a successful strike by *Tx. rutilus.* It has not been demonstrated that culicids possess such a defense however some other dipterans do. For example, larvae of the midge, *Cricotopus sylvestris* (Diptera: Chironomidae) possess long setae that discourage predation by Hydra (Hershey & Dobson 1987). These hydra cannot grasp the midge due to the long setae and therefore they can escape predation.

2.2 Hypotheses

The hypotheses for this chapter seek to examine if diet or the long setae play a role in how *Or. signifera* develops its hypothesized defense from *Tx. rutilus* predation. For hypothesis 1 I exposed *Or. signifera* to two different diets, a natural diet and an artificial diet, and observed any changes in the handling time of *Tx. rutilus*. I hypothesize that *Or. signifera* will have a higher handling time and there will be more unsuccessful strikes when given a natural diet containing tannins and other chemicals when opposed to an artificial diet. To compare how diet affects handling time rates for *Or. signifera* will be compared to rates of *Ae. albopictus*. *Toxorhynchites rutilus* preferentially feed on *Ae. albopictus* that seem to lack antipredator capabilities (Griswold & Lounibos 2005). To address the above hypothesis, I have developed the null hypotheses that handling time for *Or. signifera* and *Ae. albopictus* will not vary when consumed by *Tx. rutilus*. The second null hypothesis is that the proportion of successful strikes by *Tx. rutilus* on *Ae. albopictus* and *Or. signifera* will not vary. I predict that the handling time for *Tx. rutilus* on *Or. signifera* will be higher than that of *Ae. albopictus*.

For the second hypothesis I predict that *Or. signifera* with artificially shortened setae will have a higher mortality from *Tx. rutilus* compared to *Or. signifera* with normal setae. I propose that the setae are a physical defense that decrease *Tx. rutilus* from successfully grasping *Or. signifera* during predatory encounters. My null hypothesis is that: *Or. signifera* with artificially shortened setae will have the same mortality when exposed to *Tx. rutilus* as unaltered *Or. signifera*. I predict that *Or. signifera* with artificially shortened setae will have higher mortality from *Tx. rutilus* compared to *Or. signifera* with normal setae.

2.3 Methods

2.3.1 Experimental Design For Chemical Defense

The larvae used in this experiment were collected in September 2021 at the same locations in Chapter 1. For these experiments there were 2 treatments with 2 levels each. Treatment 1 consisted of either 1, 4th instar *T. rutilus* and 5, 4th instar *O. signifera* or 5, 4th instar *Ae. albopictus* raised under sterile conditions. Sterile conditions consisted of a tray containing 1200 ml of DI water and a diet of dried liver powder. Every 2 days 50 ml of water was added along with 0.25 g of liver extract. Each tray contained 50, 1st instar larvae randomly selected from either collection location.

Once the larvae reached the 4th instar, they were randomly combined into 125 ml urine cups with 120 ml of DI water three separate times to remove any excess debris. Then the larvae were placed in a replicate with each replicate containing 5, 4th instar *Or. signifera* and 1, 4th instar *Tx. rutilus*. Two replicates at a time were placed in an empty room for 4 hours and their behavior was recorded. Every 5 mins larval density was recorded, and any larvae consumed by the predator were replaced to ensure a density of 5 larvae. There were four replicates of each treatment level. Each recording was analyzed, and the total number or larvae consumed was recorded for each replicate. Handling times were also calculated by recording the time between consuming an individual larva and the predator consuming another larva. Also, the number of successful and unsuccessful strike by *Tx. rutilus* was recorded to discern how successful *Tx. rutilus* was in capturing prey.

Treatment 2 modeled the natural environment for *Or. signifera*. The natural conditions consisted of tree-hole water that was gathered at the same locations the larvae were collected. The water from all locations was filtered with 106um mesh and the debris set aside, and all the water was combined into a single container. The detritus was sorted between plant and animal matter and all the animal matter was discarded. Animal matter discarded due to wide variety of species, and they could not be consistently represented in each tray. Plant debris (25 g) was added to each tray contained 1200 ml of tree-hole water and two leaves, one from *Liquidambar styraciflua* (Saxifragales: Altingiaceae) and the other *Liriodendron tulipifera* (Magnoliales: Magnoliaceae) that were recovered from tree-holes. These leaves were selected because they were the most commonly encountered in the tree-hole environments, and all the tree-holes the larvae were collected from were in either of the two aforementioned trees. Each tray contained 50 randomly assorted 1st instar larvae. Once the larvae reached the 4th instar, they were randomly combined into a 125 ml urine cup with 120 ml of DI water three separate times to remove any trace amounts of tree-hole water and debris. Larvae were then randomly selected from this cup to be in each replicate. The larvae were recorded and the results analyzed in an identical manner to those in the first treatment. Level 2 consisted of a similar setup however, *Or. signifera* larvae were replaced with *Ae. albopictus*. For each treatment level there were 4 replicates.

2.3.2 Analysis of Chemical Defense

Results were analyzed through Multivariate Analysis of Variance (MANOVA) with four dependent variables (time between consuming prey, time consuming prey, handling time, and proportion of successful strikes) and two independent variables (species and treatment) (SAS 2014). Raw data did not meet the assumptions of the MANOVA, however after transforming the handling time and strike accuracy using a square root and lnx+1 respectively, the variance were equal and normality improved.

2.3.3 Experimental Design of Physical Defense

Larval *Or. signifera* used in the physical defense experiments had their setae mechanically shortened. These larvae were collected from the same locations in August 2021 as mentioned in Chapter 1. Once collected, 200 random 4th instar larvae were cooled in a refrigerator for 15 minutes to reduce their activity. The setae were trimmed to half their original length using a razor blade. To ensure the trimming did not affect mortality the shortened setae larvae morality was compared to normal larvae. This was done by having five replicates of one treatment with two levels, Treatment level 1 contained 20 shortened setae larvae and treatment level 2 contained 20 unaltered larvae. There were 5 replicates of each treatment levels. Larvae were placed in clear, 900 ml mosquito breeders (BioQuip.com #1425) with 250 ml of deionized water and 250 ml of tree hole water. Larvae were fed every 48 hrs using a 0.05 g of pulverized dog food (Purina Puppy Chow Complete). Both levels were placed in an environmental chamber using the same conditions as larval rearing.

2.3.4 Analysis of Physical Defense

The number of living larvae was counted every 24 hrs and the survival of each treatment was analyzed using PROC PHREG in SAS. Differences between means were identified using Tukey tests, which control for comparison-wise error rates. To determine if the number of *Tx. rutilus* strikes varied before consuming the first and second prey a repeated measures ANOVA tested for significant difference between species and the species by time interaction, with time (strikes before consuming the first larvae vs. strikes before consuming the second larvae) as the repeated factor.

2.4 Results

2.4.1 Chemical Defense Results

When fed an artificial and natural diet *Tx. rutilus* consumed an average 4.5 and 7 *Ae. albopictus* respectively and for *Or. signifera* these values were 1 and 1.5. The results of the MANOVA indicated there were no significant differences due to treatments (Pillai's $\text{Trace}_{4,48} = 0.155$, $P = 0.084$) or by the treatment between species interaction (Pillai's $\text{Trace}_{4,48} = 0.079$, $P = 0.401$). However, there was a significant difference by species (Pillai's $\text{Trace}_{4,48} = 0.561$, $P = 0.001$). Specifically, the handling time for *Ae. albopictus* between *Tx. rutilus* was significantly shorter than that of *Or. signifera* (Table 2.1) Also, *Tx. rutilus* time spent less time consuming *Ae. albopictus* in comparison with consuming *Or. signifera* (Table 2.1). There was no significant difference between the accuracy of *Tx. rutilus* strikes on *Ae. albopictus* and *Or. signifera* (Table 2.1).

Table 2.1 *LS Means for dependent variables. Means are back transformed data*

	Or. signifera	*Ae. albopictus*	P value
Mean Handling Time	11536.3 (+795.6, -858.1)	4772.8 (+743, -1013.1)	<0.001
Mean Time Consuming Prey	4505.5 (+1048.7, -939)	355.8 (+126, -106)	<0.001
Mean Time Between Prey	6391.2	3749.4	0.0586

	(+928.389, -1089)	(+666.3, -813.8)	
Mean Percent Strike Accuracy	37.6% (+0.04, -0.05)	42.2% (+0.02, -0.02)	0.386

To measure if consuming *Or. signifera* led to reduced *Tx. rutilus* predation rates the number of strikes before the first larvae was consumed and the number of strikes before the second larvae was consumed was recorded for both species and treatment levels. The repeated measures ANOVA showed that there was a significant difference between the species and the species time interaction (Table 2.2). Specifically, *Tx. rutilus* decreased the number of strikes after consuming an *Or. signifera* (Table 2.2). On average, *Tx. rutilus* struck at *Or. signifera* 3.75 and 3.25 times before consuming a single *Or. signifera* in the natural and artificial diets, respectively. After consuming a single *Or. signifera* the average number of strikes decreased to 0 and 0.5 in the same diets (Fig.2.1).

Table 2.2 *Results of three-way (time between strikes, species, and treatment) repeated measure ANOVA.*

	DF	P Value
Time	1, 24	0.007
Treatment	1, 24	0.763
Species	1, 24	0.616
Treatment x Species	1, 24	0.763
Species x Time	1, 24	0.028
Treatment x Species x Time	2, 24	0.512

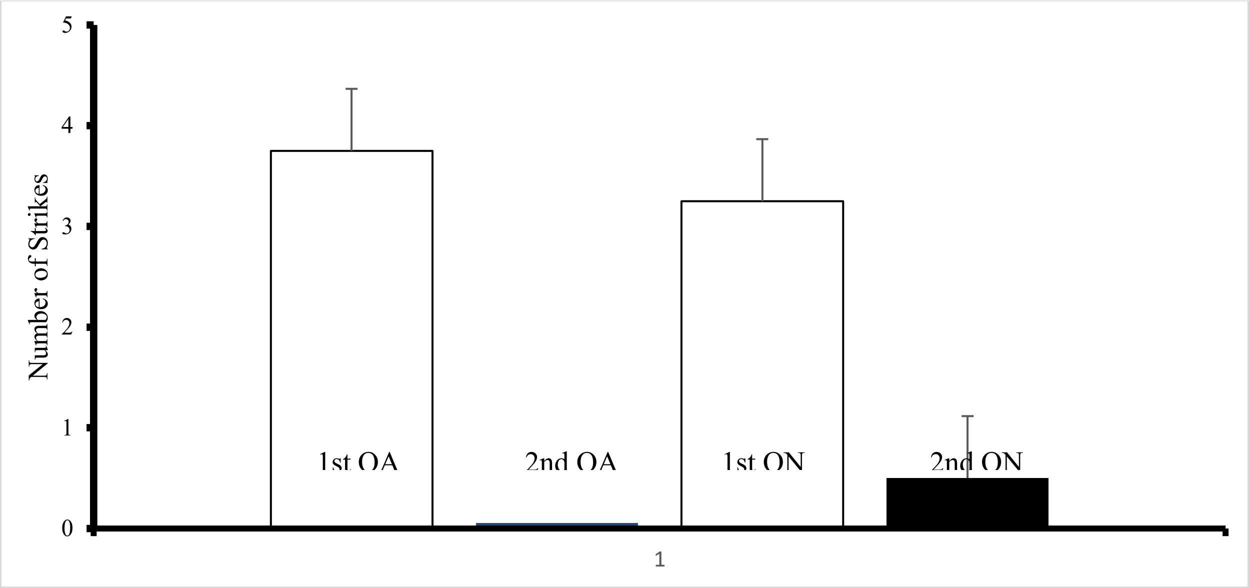

Figure 2.1 *Average number of strikes for Tx. rutilus before and after consuming one larvae. OA is for Or. signifera fed an artificial diet and ON is for Or. signifera fed an artificial diet. White columns are for the number of strikes before consuming the first larvae and the black columns are for the number of strikes after consuming the 1st larvae.*

2.4.2 Physical Defense Results

Manually trimming the setae of *O. signifera* resulted in significant mortality. Within 24 hrs 86.5% of setae shortened *O. signifera* died. Due to mortality the hypothesis that setae serve as a physical defense was abandoned. It was apparent from observations that trimming setae in this fashion caused the larvae to be unable to access the surface for breathing, and thus they drowned.

2.5 Discussion

2.5.1 Lack of Evidence for Physical Defense

Bradshaw & Holzapfel (1983) proposed that the long thoracic setae of *Or. signifera* serve as a physical defense against predation from *Tx. rutilus*. Because of the similar successful strike rate of *Tx. rutilus* on *Ae. albopictu*s and *Or. signifera* it is unlikely the thoracic setae serve as a physical defense for *Or. signifera*. Coupled with the observation that trimming the setae causes disorientation in larvae, the hypothesis that long setae prevent *Tx. rutilus* from grasping *Or. signifera* is rejected. It has been hypothesized that other insects, such as larval lepidopterans, have developed spines and long hair to avoid predation from other arthropods (Greeney et al. 2012). However, possessing long hairs to reduce predator grasping success is not a generalized predator defense in lepidopterans (Greeney et al. 2012). The effectiveness of these hairs is based largely on the guild of predator that fed on the caterpillars and it is more likely that these long hairs have evolved as a mechanism for physically detecting the presence of a predator (Greeney et al. 2012). Perhaps the long setae of *Or. signifera* have evolved a similar role in dark tree holes.

Larval culicid *Tx. brevipalpis* also possess long thoracic setae which are attached to neurons in the body (Mciver & Beech 1986). It is hypothesized that due to their internal structures that these setae serve as sensory structures that respond to mechanical sensations that assist the *Tx. brevipalpis* in detecting prey movement in the water column (Mciver & Beech 1986). Because *Or. signifera* are not predators but still possess these setae, it is probable that they also serve to detect mechanical sensations.

2.5.2 Diet & Chemical Defense Rejection

The substantially longer handling time for *Or. signifera* in comparison to *Ae. albopictus* gives evidence of *Or. signifera* possibly possessing a predator defense that *Ae. albopictus* lacks. Because there was no significant difference between natural and artificial environments for individual species, I reject the hypothesis that the sequestration of tannins (or other chemicals) leads to the development of chemical defense for *Or. signifera*. However, this does not rule out chemical defense overall as some insects can develop their chemical defenses regardless of diet (Verheggen et al. 2010).

2.5.3 Possible Post Ingestion Physical and Chemical Defense

Toxorhynchites rutilus spent significantly longer time consuming *Or. signifera* compared to *Ae. albopictus* and this could be evidence of a physical or chemical defense post ingestion. Some insects such as *Cacopsylla chinensis* (Hemiptera: Psyllidae) can have different predation rates based on the amount of chitin in their exoskeleton (Ge et al. 2019). The wintertime morph has more chitin in its exoskeleton, compared to the summertime morph, and this caused a decrease in the handling time for the predator *Harmonia axyridis* (Coleoptera: Coccinellidae) (Ge et al. 2019). One of the reasons for this decrease in handling times was the high chitin content being harder to consume and this increased the amount of time the predator spent consuming the prey (Ge et al. 2019). Based on the evidence provided perhaps larval *Or. signifera* also possess some type of hardened structure in their body and this is responsible for the increase in time spent consuming prey, and therefore a higher handling time. However, at this time I do not have a way to assess such differences.

Another possible mechanism behind this increased handling time and hardened anti-predator structure can be explained by an increase in digestion time and nutrient absorption, known as specific dynamic action (SDA) (McCue 2008). It is hypothesized that prey with a hardened features result in a meal that decreases SDA; and that predators would avoid this prey item as the nutrient levels gained from them are decreased (McCue 2008). For example, larval *Tenebrio molitor* (Coleoptera: Tenebrionidae) have smaller quantities of exoskeleton compared to adult beetles (Barnes et al. 2018). When fed to *Latrodectus mactans* (Araneae: Theridiidae) the spiders were not able to consume and assimilate as many nutrients from high density exoskeleton adults compared to the low density exoskeleton larvae (Barnes et al. 2018). The authors of the aforementioned study concluded that the *La. mactans* would favor the larval prey because their soft exoskeleton allowed them to digest and assimilate nutrients easier, despite the fact that adult *Te. molitor* have a higher nutrient content (Barnes et al. 2018).

Orthopodomyia signifera's increased handling time maybe the result of a hardened exoskeleton, or other hardened structures, that decreases *Tx. rutilus's* SDA. The decrease in number of strikes after consuming an *Or. signifera* could be indicative that *Tx. rutilus* decreases its predation rate as it needs more time to digest *Or. signifera* compared to *Ae. albopictus.* This hypothesis could be explored by comparing the nutrient content, nutrient quality, digestion times, and nutrient assimilation of *Or. signifera* and *Ae. albopictus* when fed to *Tx. rutilus*.

Besides a physical defense, a chemical defense could also explain the differences in handling time and time spent consuming prey. Some anti-predator chemical defenses completely prevent predation of an individual insect. For example, when consumed by the toad *Bufo japonicus* (Anura: Bufonidae) the bombardier beetle *Pheropsophus jessoensis* (Coleoptera: Carabidae) produce a noxious chemical from a gland in their abdomen that caused 47% of toads to vomit up a still living and active beetle (Sugiura & Sato 2018). It is unlikely that if *Or. signifera* larvae contain a chemical defense that allows some to escape alive, as this was not observed in any of the experiments. The hypothetical chemical defense would likely be more similar to that of adult *Danaus plexippus* (Lepidoptera: Nymphalidae). Adult *Da. plexippus* are chemically defended from cardenolides that they sequester as larvae from feeding on plants belonging to the genus *Asclepias* (Malcolm & Brower 1989). These cardenolides can reach toxic levels in avian predators and to avoid this *Icterus galbula* (Passeriformes: Icteridae) and *Pheucticus melanocephalus* (Passeriformes: Cardinalidae) will adapt a cyclic feeding cycle (Brower & Calvert 1985). These birds will feed on adult *Da. plexippus* until they feel the effects of the cardenolide buildup and then switch to other insect prey for an average of 7.85 days (Brower & Calvert 1985). This break in feeding allows the birds to clear the cardenolides from their body so they can resume feeding on the plentiful and nutrient rich *D. plexippus* (Brower & Calvert 1985). This phenomenon mirrors *Tx. rutilus's* longer handling times for *Or. signifera* and perhaps the predator needs a "cooling off period" to remove the toxic effects of the hypothesized chemical defense, and this results in an increased handling time.

In many cases *Tx. rutilus* can reduce the population of culicid*s* in a treehole to zero (Donald et al. 2020). To prevent starvation *Tx. rutilus* can enter a state of torpor and 4th instar larvae have been recorded to survive as long as 60 days without food (Focks & Hall 1977, Donald et al. 2020). This adaption of torpor would allow for *Tx. rutilus* to clear the buildup of toxins from consuming *Or. signifera* and perhaps this time period is long enough for the remaining *Or. signifera* to pupate and eclose. This would allow them to escape predation by becoming terrestrial adults. I hypothesize that if *Or. signifera* possess a chemical defense that it causes *Tx. rutilus* to adapt a cyclic feeding cycle and that this cyclic cycle is long enough to allow for the remaining larval *Or. signifera* to eclose and escape predation. However, more research will need to be done to explore this hypothesis.

2.5.4 Primary and Secondary Defenses

Because *Or. signifera* demonstrated a behavioral defense, as demonstrated by Chapter 1, and its ability to increase handling time after consumption it likely reduces predation through a multifaceted approach. Prey that contain multiple anti-predator mechanisms can divide their defenses into primary and secondary defenses (Ruxton et al. 2004) The function of a primary defense is to avoid detection from a predator through visual cues (aposematism and crypsis) or behavioral changes (Ruxton et al. 2004). The decreased movement and foraging that *Or. signifera* adapt in the presence of predation cues from *Tx. rutilus* would be classified as a primary defense. The two functions of secondary defenses are: to increase the odds of a prey avoiding predation once it is detected by the predator (deflection or predator startle); or for the encounter to be unprofitable for the predator (spines, stings, and toxic effects) (Ruxton et al. 2004) The unknown mechanism that increases *Or. signiferas'* handling time and time spent consuming prey would be classified as a secondary defense.

An example of an insect that contains a primary and secondary defense are weevils in the genus *Pachyrhynchus* (Coleoptera: Cuculionidae). These weevils' abdomen and thorax contain blue metallic spots and a hardened exoskeleton (Wang et al. 2018). This hardened exoskeleton can allow beetles to survive predation from the lizard *Japalura swinhonis* (Squamata: Agamidae) (Wang et al. 2018). The hardened exoskeleton allowed 100% of large beetles to escape predation from the lizard in laboratory trials (Wang et al 2018). After the failed predation attempt the lizards learned that the distinctive blue spots are a unique identifying feature for the beetle and would not attempt to consume more for up to 23 days (Wang et al. 2018). Therefore, the hardened exoskeleton was classified as a secondary defense because it was not profitable for the lizard to attempt to consume the beetle. The blue coloration was determined to be aposematic, and this defense is classified as a primary defense because it caused the lizard to avoid consuming the beetle.

2.5.5 Aposematic Coloration

It has been hypothesized that the unique orange to purple coloration of larval *Or. signifera* is a form of aposematism to prevent predation from *Tx. rutilus* (Farajollahi et al. 2009). Predators have been demonstrated to avoid prey that have aposematism as it likely indicates a longer handling time (Cyriac & Kodandaramaiah 2019, Wang et al. 2018). Two members of the genus *Orthopodomyia* have demonstrated the ability to avoid predation from *Toxorhynchite*s mosquitoes and also possess orange coloration, *Or. signifera* and *Or. anopheloides* (Yasuda & Mitsui 1992). However, the aforementioned study only examined *Or. anopheloides* behavioral response to predation cues and the predators handling time of the larvae was not examined. Therefore, it is unknown if *Or. anopheloides* also contains a secondary defense like *Or. signifera*. Very little is known about the larval traits of species in the genus *Orthopodomyia*, but the orange to purple variation in coloration is present in most described species (Table 2.3). However, it should be noted that we lack information for all 38 described species of *Orthopodomyia* and some species' larvae are not known to science (Zavortink 1968, Mosquito Taxonomic Inventory 2022).

Of the 17 *Orthopodomyia* species with reliable data on larval color, 15 have a range and habitat that overlap with at least one known *Toxorhynchites* species (Table 2.3). Of these 15 species, 11 contain similar coloration to *Or. signifera* and therefore if the orange coloration is aposematic, one could hypothesize that the hypothesized physical/chemical defense and resulting aposematic coloring could be an ancestral trait for the genus. Of the two species that do not overlap with *Toxorhynchites* only one, *Or. pulchripalpis,* possess the aposematic orange to purple coloration. *Orthopodomyia arboricollis*, which lacks the proposed aposematic coloration, is native to the island Mauritius and as there are no *Toxorhynchites* native to Mauritius. Therefore, one could propose *Or. arboricollis* lacks this hypothetical aposematic coloration is due a lack of predation pressure to maintain it. On the other hand, *Or. pulchripalpis,* which is native to Northern Europe and Northern Asia, has maintained its coloration and it is unclear as to why it has been maintained.

It is unclear why the four species whose habitat preference and range that overlap with *Toxorhynchites* lack this hypothetical aposematic coloration. There are anecdotal reports that *Or. alba* prefer treeholes in *Tilia, Aesculus*, and *Ulmus* and *T. rutilus* were not recorded in association with *Or. alba* in these habitats; even though their range and habitat preference overlap in North America (Darsie & Ward 1981). This could lead one to hypothesize that *Or. alba* lacks both the aposematic coloration and the hypothesized physical/chemical defense of *Or. signifera* as it does not face predation pressure from *Tx. rutilus*. However, there is no known research conducted on larval *Or. alba* and how it interacts with predators. The remaining three species that lack the orange-purple pigmentation, *Or. flavicosta*, *Or. flavithorax*, and *Or. waverleyi,* are also found in phytotelmata that local *Toxorhynchites* inhabit (Zavortinik 1968, Donald et al. 2020). Like *Or. alba* there is no known research examining how these three species interact with *Toxorhynchites* predators. Therefore, it is not possible to speculate if they have lost the hypothesized aposematic coloration due to lack of predation pressure or if they never obtained it to begin with. Further research should be done on the genus *Orthopodomyia* to determine if the orange to purple coloration is consistent across species and whether or not its presence is dependent on predation pressure.

Table 2.3 *List of all described larval Orthopodomyia and their species identification, color, distribution, presences of Toxorhynchites in the same habitat, and the species identification of that Toxorhynchites*

Orthopodomyia Species	Color	Range	Presence of *Toxorhynchites* in the same habitat	*Toxorhynchites* Species	Source
Signifera	orange-purple	North America	Yes	*rutilus*	Farajollahi et al. 2009
Anopheloides	Orange	Southeast Asia	Yes	*towadensis*	Yasuda & Mitsui 1992
Waverleyi	Brown	Jamacia	Yes	*moctezuma*	Zavortink 1968, Rawlins & Ragoonansingh 1990
Alba	straw colored	North America	Yes	*rutilus*	Zavortink 1968, Donald et al. 2020
Pulchripalpis	pink-red-purple	Northern Europe and Asia	No	na	Zavortink 1968
Kummi	pink-red-purple	Central America	Yes	*moctezuma*	Zavortink 1968, Donald et al. 2020
Albicosta	Red	South America	Yes	*violaceus*	Zavortink 1968, Albany et al. 2011
Fascipes	Red	Central and South America	Yes	*moctezuma*	Zavortink 1968, Donald et al. 2020

Table 2.3 (Continued)

Sampioi	Red	Brazil	Yes	*violaceus*	Zavortink 1968, Albany et al. 2011
Phyllozoa	Purple	Central and South America	Yes	*moctezuma* and *violaceus*	Zavortink 1968, Albany et al. 2011
Milloti	Purple	Madagascar	Yes	*brevipalpis*	Zavortink 1968, Donald et al. 2020
arboricollis	pigment absent	Maurautius	No	na	Zavortink 1968
flavicosta	pigment absent	Southeast Asia	Yes	*splendens*	Zavortink 1968, Donald et al. 2020
flavithorax	pigment absent	Southeast Asia	Yes	*splendens*	Zavortink 1968, Donald et al. 2020
madrensis	Purple	Philippines	Yes	*splendens*	Zavortink 1968, Donald et al. 2020
siamensis	Purple	Southeast Asia	Yes	*splendens*	Zavortink 1968, Donald et al. 2020
albipes	Purple	Southeast Asia	Yes	*splendens*	Zavortink 1968, Donald et al. 2020

There are two major pieces of information that suggest the orange to purple coloration of *Or. signifera* is not aposematic. First, *Toxorhynchite*s mosquito larvae are not visual predators and they detect their prey based on vibrations in the water column (McIver & Beech 1986, Donald et al. 2020). Thus, aposematic coloration makes little sense as the predator does not use visual cues to select prey. In fact, *Tx. rutilus* has trouble distinguishing inanimate objects floating in the water column from living prey and will strike at random debris (Pers. Obs.). Second, the water of treehole habitats that *Or. signifera* prefer are often highly turbid and often shaded from sunlight. This lack of light and dark water makes the visual cue associated with aposematism likely unable to be detected by *Tx. rutilus*. Therefore, it seems like this orange-purple coloration associated with *Or. signifera* is unrelated to aposematism and possibly a byproduct of an unknown biochemical or structural process.

2.6 Conclusions

My research has determined that *Or. signifera* possess primary and possible unidentified secondary defenses that significantly reduce predation from *Tx. rutilus*. The primary defense consists of a behavioral change in the presence of predation cues created by the predator feeding. In the presences of interspecific predation cues from *Ae. albopictus*, *Or. signifer*a browsed or remained motionless at the top of the water column. In the presence of conspecific predation cues *Or. signifera* remained motionless at the top of the water column. Behavior adapted by both cues would reduce predation by reducing the odds of a predatory encounter. I hypothesize that the difference in responses to interspecific and conspecific cues is the result of *Or. signifera* adapting the most optimal behavior to decrease the odds of a predatory encounter. If *Tx. rutilus* is feeding on a different species of mosquito it is not as urgent for *Or. signifera* to abandon feeding as compared to if *Tx. rutilus* is feeding on conspecifics. If *Or. signifera* detect *Tx. rutilus* feeding on conspecifics, browsing is not an efficient method of avoiding predation and more stringent measures, becoming motionless, must be adapted. The differences in the response to interspecific and conspecific consumption could allow for *Or. signifera* to be adapt the optimal behavior that would still allow it to ultimately escape predation by eclosing into an adult.

In addition to behavioral responses *Or. signifera* possess an unknown mechanism that increases *Tx. rutilus* handling time and time spent consuming prey. In some instances, insects can develop a chemical defense by consuming allelochemicals (e.g. tannins) in plants and then adapting them into their own chemical defense. However, *Tx. rutilus* did not show any difference in handling time in *Or. signifera* reared on a natural or artificial diet. This strongly suggests that diet, and perhaps plant secondary compounds like tannins, do not play a role in the development of a chemical defense for *Or. signifera*. However, it must be noted that some insects can develop chemical defenses independent of diet (Verheggen et al. 2010). Another possible mechanism for the longer handling time for *Or. signifera* is a hardened structure that slows the predator's SDA. It has been demonstrated that other insects that possess hardened exoskeletons can increase a predators SDA and thus are not selected for predation. Based on the experiments conducted in this thesis, I cannot conclude whether the secondary defense possessed by *Or. signifera* is a chemical or physical defense and further experiments are needed to elucidate its true mechanism.

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