THE ASSOCIATION BETWEEN *LASIONYCTA WYATTI* (LEPIDOPTERA: NOCTUIDAE) AND THE TOXIC PLANT *AMBROSIA CHAMISSONIS* (ASTERACEAE)

by

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to the required standard

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March 1998

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Date 17 March 1998
ABSTRACT

A group of toxic sulfur-containing polyynes (thiarubrines) exhibit significant light-mediated antibiotic activity against microorganisms particularly against fungi. These phytochemicals have been isolated from several members of the Asteraceae including *Ambrosia chamissonis* Less. (Greene), a species found along sandy beaches in British Columbia. The larvae of a rare noctuid moth, *Lasionycta wyatti* (Barnes and Benjamin) (Lepidoptera: Noctuidae), were found to be associated with the stems and roots of this plant. High performance liquid chromatographic (HPLC) analyses of *L. wyatti* larvae and their frass found significant amounts of thiarubrines. Artificial diets containing thiarubrines were shown to be tolerated by these larvae. These same diets, however, were toxic to larvae of *Manduca sexta* (L.) (Lepidoptera: Sphingidae) and growth inhibitory to larvae of *Spodoptera litura* (Hubner) (Lepidoptera: Noctuidae). The combination of simulated sunlight and artificial diets containing thiarubrines, did not negatively affect the larvae of *L. wyatti*. However, the incorporation of UV-activated thiophenes did negatively affect the larvae of *L. wyatti*. Results of feeding assays suggest the escape mechanism used by *L. wyatti* is its ability to rapidly eliminate thiarubrines from its tissues.
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CHAPTER ONE
GENERAL INTRODUCTION

The existence of plants on Earth for hundreds of millions of years has not been without antagonism from herbivores such as insects. From the small home garden to large scale agriculture, insects have been a constant threat to the survival of plants. Insects not only feed on the stems, leaves and roots of a plant making it difficult to grow but they also transfer disease from one plant to another. Every year millions of dollars are spent on research, development and purchase of pesticides, but phytophagous (plant-feeding) insects continue to cause considerable damage. In spite of this constant antagonism, plants flourish today because herbivores do not have the ability to completely consume all the plants of the world.

Many plants have natural defenses against herbivory. Some of these defenses are physical such as, thorns, prickly hairs, spines, sticky gums and resins and/or a thick waxy cuticle. Plants with these defenses effectively ward off potential herbivores. Another plant strategy against herbivory is the production of toxic phytochemicals (plant chemicals) (Table 1.1). Toxic phytochemicals provide the plant with a formidable defense against even the largest of herbivores because they can interfere with growth and development, or could even be lethal to herbivores that consume them.

A unique group of phytochemicals that have an elaborate means of inducing toxicity in herbivores are called phototoxins. Phototoxins require light (including near Ultraviolet UV-A) to enhance their toxicity in the absence of light.
TABLE 1.1: Types of plant toxins and their animal targets (Harborne, 1988).

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>EXAMPLE(S)</th>
<th>TARGET(S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanogenic glycosides</td>
<td>Linamarin and lotaustral in <em>Lotus</em> sp.</td>
<td>Universal; fatal dose of HCN in humans ~ 50mg</td>
</tr>
<tr>
<td>Glucosinolates</td>
<td>Sinigrin in <em>Brassica</em> spp.</td>
<td>Cattle, insects</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>Atropine in <em>Atropa belladonna</em> berries</td>
<td>Mammals but not birds</td>
</tr>
<tr>
<td>Peptides</td>
<td>Amanitine in <em>Amanita phalloides</em></td>
<td>Mammals</td>
</tr>
<tr>
<td>Proteins</td>
<td>Abrin in <em>Abras precatorius</em></td>
<td>Mammals, Lethal dose in man 0.5mg</td>
</tr>
<tr>
<td>Iridoids</td>
<td>Aucubin in <em>Aucuba japonica</em></td>
<td>Insects, birds</td>
</tr>
<tr>
<td>Sesquiterpene lactones</td>
<td>Hymenovin in <em>Hymenoxys</em> sp.</td>
<td>Livestock, insects</td>
</tr>
<tr>
<td>Cardiac Glycosides</td>
<td>Ouabain in <em>Acokanthera ouabaio</em></td>
<td>Mammals, Insects</td>
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<tr>
<td>Saponins</td>
<td>Medicagenic acid in <em>Medicago sativa</em> leaves</td>
<td>Fish, insects</td>
</tr>
<tr>
<td>Furanocoumarins</td>
<td>Xanthotoxin from <em>Pastinaca sativa</em></td>
<td>Insects, microorganisms</td>
</tr>
<tr>
<td>Isoflavonoids</td>
<td>Rotenoids in <em>Derris elliptica</em></td>
<td>Insects, fish</td>
</tr>
<tr>
<td>Quinones</td>
<td>Hypericin in <em>Hypericum perforatum</em></td>
<td>Mammals, especially sheep</td>
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<tr>
<td>Thiophenes &amp; Polynyes</td>
<td>α-Terthienyl from <em>Asteraceae</em></td>
<td>Insects, microorganisms</td>
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The damage these phototoxic phytochemicals cause is termed “photosensitization” (sensitive to light) or “phototoxicity”. Photosensitization can result in severe lesions and ulcers on the skin of humans and large grazing vertebrates exposed to the sun (Ivie, 1982; Johnson 1982; Harborne, 1988). Insects suffering from photosensitization experience reduced growth, reduced feeding, and even death (Berenbaum, 1978; Wat et al., 1981; Arnason et al., 1981; Ivie et al., 1983; Downum et al., 1984; Champagne et al., 1984, 1986; Ellis et al., 1995; Aucoin et al., 1995; Guillet, et al., 1997).

Some of the more well known phototoxic chemicals include hypericin [1], a quinone produced by members of the Hypericaceae; 8-methoxypsoralen (xanthotoxin) [2], a linear furanocoumarin, produced by members of the Rutaceae, Apiaceae and Moraceae; and α-Terthienyl (2,2′:5′,2″-terthiophene) [3] a thiophene produced mainly by members of the Asteraceae.

The quinone hypericin [1] has been particularly well studied over the years because of the damage it causes to livestock. This compound is produced primarily by several species of Hypericum such as, H. perforatum. This European native plant species, commonly referred to as St. John’s-wort was accidentally introduced to North America and is now an invasive weed particularly in the Klammath area of Northern California, hence its other name, Klammath weed (Giese, 1980).

Hypericin is contained within resin glands distributed throughout the plant. It is virtually impossible for a herbivore to avoid these toxic glands. Sheep, cattle and horses are the main targets for hypericin photosensitization because they graze in the pastures where H. perforatum grows freely. Once the livestock consume H. perforatum, blindness, emaciation, erythema of the head, and nervous disorders results (Giese, 1980; Johnson 1982).
FIGURE 1.1: Structures of some phototoxic phytochemicals.

1. Hypericin
2. 8-Methoxypsoralen
   Xanthotoxin
3. alpha-Terthiényl
Linear furanocoumarins, such as xanthotoxin (8-methoxypsoralen) [2], and certain thiophenes including \( \alpha \)-Terthienyl [3], produce similar effects. Thiophenes are generally toxic to microorganisms and insects (Berenbaum, 1978; Wat et al., 1981; Arnason et al., 1981; Ivie et al., 1983; Downum et al., 1982; Champagne et al., 1984; Towers et al., 1985,1986; Aucoin et al., 1995). \( \alpha \)-Terthienyl, in particular, is extremely toxic to mosquito larvae (Arnason et al., 1981; Wat et al., 1981; Hasspieler et al., 1990) and shows great potential as a larvicide.

In full sunlight, \( \alpha \)-Terthienyl is more potent against mosquito larvae than the commercial pesticide DDT (Arnason et al., 1981). In simulated pond trials, a concentration of 100 parts per million (ppm) caused complete mortality in third and fourth instar larvae of the yellow fever mosquito, *Aedes aegypti* L. (Diptera: Culicidae), after only 15 minutes under natural sunlight. At 10 ppm, the same results were seen after 120 minutes in the same trial (Arnason et al., 1981). Similar results were seen in tests against *A. intrudens* Dyar (Philogene et al., 1986). Within two days at a concentration of 1 kilogram per hectare, 100% of the third- and fourth instar *A. intrudens* larvae were killed. The extreme potency of \( \alpha \)-Terthienyl against mosquito larvae, its biodegradability as well as the fact that it is not toxic to non-target aquatic organisms (Philogene et al., 1986) makes it an attractive alternative to synthetic pesticides like DDT.

**Phototoxic Modes of Action**

There are two modes of action that cause photosensitization in these and other organisms. The first, referred to as photodynamic photosensitization, requires oxygen in order for damage to take place. The second mode of action does not require oxygen and is termed non-photodynamic photosensitization (Arnason et al., 1983; McLachlan et al., 1984; Spikes, 1989).
Photodynamic photosensitization results from the generation of highly reactive free radicals from two separate pathways: Type I photosensitization and Type II photosensitization. The main difference between these two competing pathways is the resulting species formed. In the type I photosensitization, absorption of light causes a series of electron transfer steps that ultimately results in the formation of radicals including superanion oxide radicals ($\text{O}_2^-$). In the type II photosensitization, the sensitizer (phototoxin) absorbs light and is excited to a triplet state. This triplet state transfers this excited energy to oxygen and generates a highly reactive singlet oxygen species ($^1\text{O}_2$). These two species cause oxidative damage to lipids and proteins in cells and tissues of the organism. \(\alpha\)-Terthienyl and hypericin cause damage by this method.

The other phototoxic mode of action is non-photodynamic photosensitization. This mechanism does not require oxygen because the molecular target is the DNA of the organism. The compound 8-methoxypsoralen (xanthotoxin) is believed to cause this type of damage (Berenbaum, 1978; Arnason et al., 1983). The double bonds of the furan and lactone rings of xanthotoxin bind to DNA pyrimidine bases, such as thymine. Xanthotoxin intercalates itself between the DNA double helix and in a subsequent photochemical reaction forms mono- or dicyclobutane adducts (Figure 1.2). These adducts cause problems with cell transcription, mutations, or cell death (Arnason et al., 1983; Spikes, 1989). Despite the elaborate defense strategy of phototoxic plants, some insects have developed mechanisms that help them escape the phototoxicites of these compounds.

**Insect Escape Mechanisms**
FIGURE 1.2: Components of non-photodynamic photosensitization (a), mono- and/or dicyclobutane adducts formed between the thymine base of DNA and the furanocoumarin, xanthotoxin (b).
Insect escape mechanisms include behavioral adaptations, physical adaptations, physiological adaptations or a combination of these. For example, some species of Chrysolina, particularly the Klammath weed beetle, Chrysolina hyperici Forst. (Coleoptera: Chrysolmelidae), specializes on the toxic Hypericum perforatum and does not suffer any of the phototoxic effects mentioned above. The secret to its ability to escape hypericin toxicity lies partially in the behavior it displays during its early stages of development.

During the early stages of its development, the larvae feed on H. perforatum only at night and during the early morning hours before sunrise when light intensity is low. When the light intensity reaches a point where damage could occur, the larvae crawl down the plant and burrow into the surrounding soil until the same hours the following morning. When the larvae were prevented from burrowing into the soil during the intense sunlight hours they suffered increased mortality (Fields et al., 1990).

Other light avoidance behavior displayed by insects include leaf tying (Sandberg and Berenbaum, 1989) silk spinning and stem boring (Iyengar et al., 1987) provide similar protection against damaging light. Still other protective forms of insect behavior against phototoxicity include selective feeding of plant material around toxic glands. This behavior is displayed by Anaitis plagiata L. (Lepidoptera: Geometridae) which selectively feeds around the toxic hypericin glands found on H. perforatum (Fields et al., 1990; Fields et al., 1991).

Other insects have physical characteristics that protect against damaging rays of light. A good example of this is seen in adult C. hyperici. The behavior displayed during the early stages of development ceases as the larva matures to the adult stage. Adult C. hyperici beetle feed on H. perforatum in full sunlight but does not suffer any of the phototoxic effects discussed above.
Fields et al. (1990 and 1991) believe that these thick, opaque elytra act as a shield and prevents damaging rays of light from reacting with the toxin in cells and tissues (Fields et al., 1990).

Finally there are physiological mechanisms which protect insects from oxidative damage. Free radicals including superanion oxide radicals and singlet oxygen can be quenched (destroyed) with mixed function oxidases found within the tissues of the insect. These mixed function oxidases reduce the radicals making them more water soluble (Krieger et al., 1971; Bull et al., 1986; Larson, 1986). Dietary antioxidants, such as, α-tocopherol, β-carotene or asborbic acid (Larson, 1986) also help to reduce free radicals. The black swallowtail butterfly, *Papilio polyxenes* Stoll (Lepidoptera: Papilionidae), is an example of a species which has this ability.

*P. polyxenes* specializes on plants of the Apiaceae that produce linear furanocoumarins such as xanthotoxin. It has been well studied for its ability to rapidly detoxify xanthotoxin and escape the DNA damage the toxin causes (Ivie et al., 1983). Ivie et al. (1983) provided small twigs of parsley treated with ¹⁴C-labeled xanthotoxin to *P. polyxenes* larvae and the fall armyworm, *Spodoptera frugiperda* JE Smith (Lepidoptera: Noctuidae), larvae. When they compared the elimination rates of these two species and the xanthotoxin content of their frass, Ivie et al. (1990) found that both species have the ability to break down xanthotoxin to several less toxic metabolic products via mixed function oxidase enzymes found in their gut tissues. This detoxification ability alone, however, does not offer *S. frugiperda* enough protection against the toxic effects of xanthotoxin. *S. frugiperda* still suffered photosensitization and mortality while *P. polyxenes* did not.

Ivie et al. (1990) revealed that not only was *P. polyxenes* able to break down xanthotoxin into its less toxic metabolic products, it also had the ability to rapidly eliminate the toxin from its tissues. Within 90 minutes of oral administration of the treated diet laced with xanthotoxin, there
was 15 times more toxin in the gut tissues of *S. frugiperda* than there was in *P. polyxenes*. Further, only 1% of the toxin was recovered in the frass of *S. frugiperda* as compared to the 50% recovered in the frass of *P. polyxenes* (Ivie et al., 1983). The toxin was not eliminated by *S. frugiperda* quickly enough for it to escape the toxicity of the compound. *P. polyxenes*’ ability to escape furanocoumarin toxicity is due to its ability to detoxify the compound with endogenous enzymes as well as its ability to rapidly eliminate the toxin from its tissues.

Along with *P. polyxenes* and *C. hyperici*, the European cornborer, *Ostrinia nubilalis* Hubn. (Lepidoptera: Pyralidae), and the tobacco budworm, *Heliothis virescens* Fab. (Lepidoptera: Noctuidae) are two more examples of insects that employ more than one mechanism to escape phototoxicity (Iyengar et al., 1987). *O. nubilalis* displays both light avoidance behavior through its silk spinning behavior and the ability to rapidly eliminate toxins from its tissues. *H. virescens* bears a dark cuticle which protects it from harmful light rays (similar to *C. hyperici*) and also has the ability to rapidly eliminate toxins from its tissues.

**Case Study**

The research discussed in this thesis determines which method(s) is/are being used by a hitherto unidentified species of caterpillar found in close association to the toxic plant *Ambrosia chamissonis* (Asteraceae). The insect was later identified as the rare moth *Lasionycta wyatti* Barn. & Benj. (Lepidoptera: Noctuidae) (see Chapter Two). Until the discovery of *L. wyatti*, researchers believed *A. chamissonis* was extremely toxic to all microorganisms and insects because of its several toxic sulfur-containing polyynes and corresponding thiophenes (Figure 1.3).
FIGURE 1.3: Thiarubrine Compounds from *Ambrosia chamissonis*.
These toxic sulfur-containing polyynes, commonly referred to as thiarubrines, are produced exclusively by members of the Asteraceae (Ellis et al., 1995). *A. chamissonis* is one of several members of the Asteraceae which produces thiarubrines (Ellis, 1993). Of all the thiarubrine-producing plants, *A. chamissonis* produces the most diverse array of thiarubrines identified from the Asteraceae (Ellis, 1993). “Thia” refers to the sulfur component of the dithiacyclohexadiene ring and “rubrine” refers to the deep red color of the plant extract. Thirteen different thiarubrines have been identified thus far from 50 species of the Asteraceae (Page, 1997b), nine of which occur in *A. chamissonis* (Ellis et al., 1995; Page, 1997b).

*A. chamissonis* is commonly referred to as silverburweed; “silver” from the silvery appearance of the plant cast by the hairs on the stems and leaves and “bur” from the spiny bracts that encapsulate the fruit. *A. chamissonis* is distributed along sandy beaches on the west coast of North America ranging as far north as Queen Charlotte Island off the coast of central British Columbia to Baja California (Figure 1.4) and the west coast of Chile (Ellis et al., 1993). *A. chamissonis* is a succulent, perennial herb which usually grows in large clumps roughly 20-100 centimeters tall (Figure 1.5) (Pojar and MacKinnon, 1994).

The toxic thiarubrines are contained within resin canals in the cortex and periderm of stems, leaves and roots which run longitudinally along the plant (Figure 1.6) (Ellis, 1993; Ellis et al., 1995). The orientation of these resin canals within the stems, leaves, and roots of *A. chamissonis* are situated in such a way that suggest that thiarubrines are produced as a defense against attack from subterranean herbivores. It would be difficult for any potential herbivore to avoid these glands (Figure 1.6).

The production of thiarubrines by the plants are believed to confer defense against attack from nematodes, microorganisms or herbivorous insects because of the extreme toxicity
FIGURE 1.4: Distribution of *Ambrosia chamissonis* (shaded region) and the range of known locations of four *Lasionycta* spp. *Lasionycta* spp. specimens are archived at the Canadian National Insect Collection (CNC) in Ottawa, Canada.
FIGURE 1.5: *Ambrosia chamissonis* early growth in the Spring (a) and a mature plant in the Fall (b).
FIGURE 1.6: Resin canals (rc) found in the cross section and longitudinal section of roots. Shown here *Chaenactis douglasii* (Asteraceae).
thiarurubrines exhibited against these types of organisms (Towers et al., 1985). The activities of 3-(1-propynyl)-6-(5-hexen-3-yn-1-ynyl)-1,2-dithiacyclohexa-3,5-diene, or thiarubrine A (Figure 13) and its corresponding 2-(1-propynyl)-5-(5-hexen-3-yn-1-ynyl) thiophene, or thiophene A, were tested against various representatives of bacteria, viruses and fungi both in light and in dark. Thiarubrine A displayed the characteristic difference in toxicity in light versus dark.

Thiarubrine A activity was tested against several bacteria including *Bacillus subtilis* and *Staphylococcus aureus*. In the absence of light, only slight activity was shown against these bacteria. However, in the presence of UV-A irradiation a concentration of 0.1 microgram per disk (μg/disk) generated significant activity (Towers et al., 1985; Ellis, 1993). Tests were also conducted against viruses, both membrane bound and non-membrane bound viruses (Hudson et al., 1986a,b). In antiviral assays, thiarubrine A showed activity only against membrane containing viruses (Hudson et al., 1986a,b). From this result, it was suggested that thiarubrine A affects viruses and possibly other organisms by damaging their cell membranes (Hudson et al., 1986a,b). In darkness, there was no antiviral activity. However, in the presence of UV-A irradiation, an extremely low concentration of 0.01 μg/disk generated significant activity. From these data, thiarubrines were considered phototoxic because of the characteristic differences in activity in light versus dark. Tests against fungi, however, showed thiarubrines were not just toxic in light, but overtly toxic.

Fungi, including the human pathogenic yeast *Candida albicans*, were tested for susceptibility to thiarubrine A. *C. albicans* is the agent responsible for candidiasis, vaginal yeast infections as well as other fungal infections (Bodey & Feinstein, 1985). For the first time, significant activity of thiarubrine A against the yeast occurred in complete darkness. In darkness, antifungal activity against *C. albicans* was detected at a low concentration of 0.1μg/disk (Towers
et al., 1985; Ellis, 1993). In light, thiarubrine A was active against *C. albicans* at an even lower concentration of 0.01 μg/disk (Towers et al., 1985). These findings led to the placement of US and Canadian patents on the use of thiarubrines as antifungal agents (Towers, pers comm.). The therapeutic use of these thiarubrines against fungi, unfortunately, is non-existent today because it was also discovered during these tests that thiarubrines are extremely cytotoxic to mammalian cells (Hudson et al., 1986a; Ellis 1993).

Nematocidal and insecticidal activity was also examined. Thiarubrine A (Towers et al., 1985) and thiarubrine C [3C] (Wat et al., 1981) proved fatal to the soil nematode, *Coenorhabditis elegans*. Mortality resulted when *C. elegans* was exposed to 5.0 micrograms per milliliter (μg/ml) thiarubrine A in dim light (Towers et al. 1985). In light, a low concentration of 0.03 μg/ml produced the same effect. These results suggested a possible defensive role for the thiarubrines against soil nematodes.

Several studies were conducted against insects using various thiophenes and non-phototoxic polyynes from the Asteraceae. There have only been a handful of reports which focus specifically on the effects of thiarubrines on insects (Wat et al., 1981; Ellis et al., 1995; Aucoin et al., 1995; and Guillet et al., 1997). Ellis et al. (1995) described the deterrent nature of thiarubrine A against the Oriental cockroach, *Blatta orientalis* (L.) (Dictyoptera: Blattidae). The cockroaches avoided all contact with dog food laced with 10 μg/g thiarubrine A. Aucoin et al. (1995) subjected *O. nubilalis* to choice test assays. The average larval consumption rate of an artificial diet treated with thiarubrine A decreased as its concentration in the diet increased above 100 μg/g (Aucoin et al., 1995).

Guillet et al. (1997) examined the effects of thiarubrine A on neonate larvae of the tobacco hornworm *Manduca sexta* L. (Lepidoptera: Sphingidae) and second instar larvae of *Aedes*
**atropalpus** Say (Diptera: Culicidae). Thiarubrine A significantly inhibited the growth of neonate *M. sexta* with a concentration of 50 micrograms of active ingredient per gram (μg a.i./g) of diet resulting in 62% mortality as compared to the 5% mortality in the group of larvae fed an artificial diet lacking thiarubrine A (Guillet et al., 1997). The lethal concentration against 50% of the larvae tested (LC₅₀) of thiarubrine A for *A. astropalpus* larvae was 0.09 micrograms of active ingredient per milliliter (μg a.i./ml) in the dark and 1.8 μg a.i./ml in the presence of near UV/visible light.

Constabel and Towers (1989) examined the role light plays in the toxicity of these toxic sulfur-containing polyynes. Exposure to near UV light converts thiarubrines to their corresponding thiophenes (Constabel, 1983). In the antifungal research, thiarubrines displayed both light dependent and light independent activity. Exposure of thiarubrines to light results in the extrusion of a sulfur atom from the 1,2-dithiin ring (Figure 1.7). Constabel and Towers (1989) believe it is this light mediated conversion from the thiarubrine to the thiophene that causes the toxic effects seen in microorganisms. Comparisons of the antibiotic activity of thiarubrines to that of the resulting thiophenes showed that the latter are far more active against micro-organisms than thiarubrines are alone when activated with UV light (Constabel & Towers, 1989). The cumulative results of these investigations have led to a better understanding of the light dependent nature of thiarubrines as well as the effects they have on microorganisms and insects.

The discovery of *L. wyatti* in close association to roots and subterranean stems of *A. chamissonis* added an interesting dimension to what is known about this plant. The phenomenon of an insect that is able to withstand the toxicity of these thiarubrines generated interest and excitement. This thesis investigates the possibility that *L. wyatti* is specially adapted to feeding on
Toxicity:

Antibacterial
Antifungal
Nematocidal
Antifeedant
Insecticidal

FIGURE 1.7: Conversion of thiarubrines to their corresponding thiophenes.
A. chamissonis in nature and determines which mechanism(s) is/are used by it in dealing with the toxic thiarubrines.

Chapter Two describes the primary investigation into possible alternatives to ingestion through which L. wyatti could be exposed to thiarubrines in the field. This study includes analyses of randomly collected samples of sand surrounding A. chamissonis plants at varying distances from the roots, wild caught L. wyatti larvae, frass from wild caught larvae, and young emerging A. chamissonis stems. It was determined from the sand analyses that exposure to thiarubrines by contact is eliminated as a possibility as no thiarubrines were detected in the sand. Analyses of wild larvae and their frass confirm the ingestion of plant materials rich in thiarubrines by L. wyatti larvae in the wild.

Chapter Two also describes analyses done on laboratory reared larvae raised on an artificial diet spiked with thiarubrines. Analyses of dissected parts of L. wyatti larvae were conducted to determine the fate of the thiarubrines in larval tissues. Frass was also examined. The highest concentration of thiarubrines was recovered from the frass demonstrating L. wyatti's ability to rapidly eliminate thiarubrines from its tissues. This ability to rapidly eliminate the toxins may be a physiological means of escaping thiarubrine toxicity.

Chapter Three describes the collection and identification of L. wyatti larvae as well as the rearing techniques used in maintaining a laboratory colony. No previous literature on the laboratory rearing of these insects exists.

Chapter Four describes an experiment designed to determine the effect of thiarubrines in an artificial diet on the larval weight gain of neonate larvae of L. wyatti, M. sexta and Spodoptera litura Hubn. (Lepidoptera: Noctuidae). Experiments were conducted both in the presence and absence of UV/visible light. Experiments show, in complete darkness, thiarubrines in an artificial
diet do not affect larval weight gain in *L. wyatti* but do inhibit the growth of *S. litura* larvae and cause significant mortality in *M. sexta*. In the presence of UV/visible light, there was no significant difference in the weight gained by *L. wyatti* larvae compared to cohorts fed an untreated diet regardless of the presence of light. However, with the incorporation of UV activated thiophenes from *A. chamissonis* into an artificial diet, the weight gain of *L. wyatti* larvae was significantly affected.
CHAPTER TWO

ANALYSES OF THIARUBRINE CONTENT IN LARVAE, FRASS, AND STEMS OF AMBROSIA CHAMISSONIS PLANTS

INTRODUCTION

Prior to carrying out any experimental analyses, the insect found in close association to *A. chamissonis* was properly identified by Jim Troubridge, an insect taxonomist with Agriculture and Agri-Food Canada (AAFC). He identified it as the rare noctuid moth *Lasionycta wyatti* Barnes & Benjamin (Lepidoptera: Noctuidae). There is some ambiguity with this insect in terms of its proper identification. This insect is one of four species (*L. wyatti* B. & Benj., *L. arietis* Grt., *L. insolens* Grt., *L. ochracea* Riley) that does not fit the normal habitat distribution of the genus *Lasionycta* (Troubridge pers. comm.). Normally the members of the genus *Lasionycta* are found in alpine meadows of British Columbia. These four species, however, have been collected from sandy dune habitats along the west coast of North America spanning as far south as central California (La Fontaine pers. comm.) (Figure 1.4). Because their distribution is different from that of other members of *Lasionycta*, these four species may be reassigned to a separate genus. Further, the striking similarities in morphology of these four species may reveal that they are really two morphologically variable species (La Fontaine pers comm.).

Following the species identification, a literature search was conducted but no information on the natural history of *L. wyatti* was found. *L. wyatti* and the other four sandy dune moths
species have only been collected from relatively few beaches on the west coast of North America. These specimens are currently archived in the Canadian National Insect Collections (CNC) in the Agriculture Canada Research Branch in Ottawa (La Fontaine pers. comm.).

Preliminary HPLC analyses (data not included) on methanolic extracts of *L. wyatti* larvae collected from the bases of *A. chamissonis* plants in 1995, indicated the presence of thiarubrines. Based on these findings, further investigation into the relationship between this insect and plant began. The first test in this chapter investigates the sand surrounding bases of *A. chamissonis* plants for the presence of thiarubrines and their related thiophenes. A presence of thiarubrines in the sand could be the means by which *L. wyatti* acquired thiarubrines in its tissues.

Plant leachates, including phototoxins, have been detected in the sand and soil surrounding the bases of several plants. For example, the pappus of achenes of *Tagetes* spp. (Asteraceae) display defensive properties. When the pappi are spread on an agar petri plate inoculated with bacteria or fungi and irradiated with near UV-light, α-Terthienyl is released into the medium. Similarly, furanocoumarins are exuded by the fruits of some apiaceous plants (Towers, 1989). Lam et al. (1989) reported evidence of leachates from *Santolina chamaecyparissus* (Anthemideae). In sand surrounding *S. chamaecyparissus* plants, thiophene-furan acetylenes were detected and α-Terthienyl was detected in the soil surrounding *Echinops shaerocephalus* plants (Lam et al., 1989). Further, Berenbaum and Larson (1988) detected the release of singlet oxygen from *Zanthoxylum americanum* (prickly ash) and *Pastinaca sativa* (wild parsnip) at levels which may cause detrimental effects in insects without actually causing any damage to the plants.

In keeping with these findings, tests were conducted to investigate the effects of a topical application of polyynes on insects (Downum et al., 1984; Iyengar et al., 1987). Contact with such leachates have been shown to be readily absorbed by the tissues of some insects. Downum et al.
(1984) showed that topical application of α-Terthienyl on *Manduca sexta* resulted in tissue necrosis in fifth instar larvae and severe problems with pupal case development.

In a similar study with three species of caterpillar, Iyengar et al. (1987) showed the greatest penetration of α-Terthienyl was in the cuticle where damage could severely affect the health of the insect. The presence of these chemicals in the soil and the detrimental effects they cause through contact could be a means of defense by plants against subterranean, herbivorous insects such as *L. wyatti*. My HPLC analyses of the sand samples, however, precludes the possibility that *L. wyatti* larvae acquire the thiarubrines through contact with subterranean chemicals as no thiarubrines were detected.

Investigations into the elimination processes of insects exposed to toxic phytochemicals provide valuable information on the kinetics of the compounds *in vivo* as well as determining the degree to which the insect becomes poisoned. Insects that have the ability to rapidly eliminate toxic compounds from their tissues avoid most of the negative effects. It was determined through analyses that wild *L. wyatti* contained thiarubrines in its tissues through the ingestion of *A. chamissonis* plant material. From feeding trials it was determined that *L. wyatti* avoided the negative effects experienced by other insects (Chapter Four). It was important to determine whether *L. wyatti* was able to escape thiarubrine toxicity by virtue of an ability to rapidly eliminate the toxin from its tissues.

An investigation into the distribution of administered thiarubrines in *L. wyatti* larval tissue is also included in this chapter. The information these analyses provide lends some insight into the possible mechanism by which *L. wyatti* larvae escape thiarubrine toxicity.
MATERIALS AND METHODS

Field Specimens

Beginning in late March through May of 1996 (with subsequent trips in April and May of 1997), field trips were made to Centennial Beach in Boundary Bay Regional Park in Tsawwassen, BC for the collection of *L. wyatti* larvae (site of the first collection), stems from *A. chamissonis* plants, and beach sand. Permits to collect plant materials and insects were obtained from the Greater Vancouver Regional District (GVRD) Parks Department.

After first locating and identifying *A. chamissonis* plants, scrupulous digging was done around the base of randomly selected plants. Sand to a depth of 5 to 10 cm was brushed aside using either a hand held trowel, shovel or simply by hand. Once larvae were uncovered, they were immediately placed in a plastic container and covered with sand.

Stems were collected from plants where *L. wyatti* larvae were found. Stems collections were made from five different *A. chamissonis* plants. Each stem included both above and below ground portions. Care was taken to collect stems of similar girth as well as similarity in foliage. Stems were immediately placed in separate brown paper bags which were then placed in a black bag to prevent light exposure.

Four separate samples of sand were collected from the bases of *A. chamissonis* plants. Four Simport plastic specimen containers were wrapped with aluminum foil to prevent exposure of the contents to sunlight. Once larvae were collected from the base of a plant (Chapter Three), the lid was removed from the plastic container and sand was scooped into the container at the precise location where larvae were found. The container was filled to the top, immediately
capped and placed into a black bag. Light exposure was carefully minimized by working quickly and physically blocking the sunlight.

After each collection, the larvae, stems and sand were brought to the laboratory. Individual larvae were identified by comparing them with voucher specimens caught at the same location on previous trips that had been positively identified. Voucher specimens are archived at the Canadian National Insect Collection (CNC) in Ottawa and the Spencer Entomological Museum at the University of British Columbia. A few larvae randomly selected for analyses of thiarubrine content were weighed and immediately frozen to -80°C until extracted. The remaining larvae formed the laboratory colony (Chapter Three). Stem and sand samples were also weighed and immediately frozen to -80°C until extracted.

**Laboratory Specimens**

In a separate set of analyses, four randomly selected fourth instar larvae and their frass were taken from the laboratory colony. Four fifth instar larvae were fed a thiarubrine-laced diet for 48 hours then immediately frozen to -80°C for 24 hours. Frass and the uneaten diet were also frozen to -80°C for 24 hours.

After 24 hours frozen larvae were thawed and dissected. Hemolymph was drawn out by carefully cutting the last proleg with small scissors until no more liquid exuded. A scalpel was used to carefully make a shallow longitudinal incision along the ventral side of the larva. Once the incision was made, the gut contents (excluding mouth parts) were carefully removed with forceps and immediately placed in 3.0 milliliters (ml) CH₂Cl₂. The remaining carcass was placed in 7.0 ml of CH₂Cl₂. All specimens were weighed and stored at 2°C until extracted.
Preparation of All Specimens for Analyses

All specimens, with the exception of dissected larvae, were prepared using the following methods. In complete darkness, specimens were thawed, placed in liquid nitrogen then crushed in a mortar and pestle to a fine powder. A household type coffee grinder (Braun model KMS2) was used in place of a mortar and pestle to grind the stem samples. The powders of frass, larvae and diet were each suspended in 15.0 ml CH$_2$Cl$_2$. The finely ground stems were suspended in 50 ml CH$_2$Cl$_2$ and sand samples were suspended in 100 ml of CH$_2$Cl$_2$. All suspended samples were then stored for 48 hours at 2°C.

After 48 hours, the solids from all specimens, including the dissected larvae, were removed through vacuum filtration and discarded. The remaining extracts were dried with MgSO$_4$ then concentrated by evaporating the solvent in vacuo. The oily resin was then resuspended in 1.0 ml of High Performance Liquid Chromatographic (HPLC) grade CH$_3$CN. Prior to HPLC analysis, the extracts were filtered through a 0.45 micrometers (µm) Gelman filter to remove any minute particles.

HPLC and Spectrophotometric Analyses

Extracts were analyzed by HPLC methods adapted from Page et al. (1997). Water used for HPLC analyses was Milli-Q plus (Millipore) and all other reagents were HPLC grade (Fisher). The HPLC system controlled by Millennium 2.1 software designed for Microsoft Windows application consisted of a Waters 600 pump controller, 996 photodiode array detector and 717 plus autosampler. The solid phase consisted of a C-18 Waters Novapak 3.9 x 150 mm (4 µm) HPLC with a Novapak precolumn. The liquid phase consisted of the following step-gradient
water-CH$_3$CN elution system: 55:45 (pre-injection); 30:70 (0 minutes); 10:90 (10 minutes); 0:100 (15 minutes); 55:45 (32 minutes). Injection volume was 20 microliters (µl) with a flow rate of 1 milliliter per minute (ml/min). UV detection range was set at 200-600 nanometers (nm) with specific detection set at 340 nm.

Spectrophotometric analyses were conducted using a Philips PU 8720 UV/Visible Scanning Spectrophotometer with wavelength set at 490 nm. All samples were blanked with HPLC grade (Fisher) CH$_3$CN. The limit of detection of thiarubrines by the UV spectrophotometer was determined with serial dilutions of a known concentration of crude plant extracts. UV spectrophotometric analyses were run on these dilutions until a zero absorbance reading at 490 nm was achieved.

RESULTS AND DISCUSSION

The results of the analyses on sand samples collected from the bases of A. chamissonis plants precludes the possibility of that L. wyatti larvae acquire thiarubrines through contact. UV spectrophotometric analyses of sand samples show an average absorbance of 6.8 x 10$^{-3}$. The limit of detection of crude thiarubrine extracts by the UV spectrophotometer is 1.0 x 10$^{-2}$, which was a result of a crude plant extract that was diluted 4000 times. The next serial dilution (8000 times) did not generate a different absorbance reading. The reading remained 1.0 x 10$^{-2}$. The spectrophotometer becomes unreliable at these low concentrations.

If, however, this absorbance is due entirely to thiarubrines and no other compound, the concentration of thiarubrines found in the sand would be 3.52 x 10$^{-3}$µg/g based on the Beer-Lambert equation (Appendix, Table A). At such a low absorbance, one can not be absolutely sure
thiarubrines are the only compounds present. The probability that no other compounds occur in
the sand surrounding bases of *A. chamissonis* plants is extremely low. Further, the idea that
thiarubrines are the only compounds in the sand that absorb at 490 nm is also highly unlikely. I
feel that this insignificant concentration can be ruled out with a high degree of certainty as
thiarubrines. Randomly collected samples of sand from beaches where *A. chamissonis* does not
exist would probably generate a similar reading.

HPLC analyses of wild *L. wyatti* larvae collected from the same locations around the bases
of the plants contained an average of 4.24 µg/g thiarubrines and frass from these larvae contained
an average of 36.8 µg/g of thiarubrines (Table 2.1). These concentrations are higher than what
could potentially be found in the surrounding sand. The only means by which wild larvae could
have acquired these amounts is by the ingestion of plant materials. Further, significant
subterranean feeding damage was observed from various *A. chamissonis* plants where *L. wyatti*
were collected (Figure 2.1).

Above and below surface stem samples of *A. chamissonis* were analyzed for thiarubrine
content via HPLC. These parts of the plant were analyzed because of evidence of feeding damage
as well as the fact that larvae were discovered more often among subterranean stems than among
woody root material (Figure 2.1). The concentration of thiarubrines found in the stems was 147.6
± 110.2 µg/g dry weight (Table 2.1). These analyses provided the thiarubrine concentration to
which *L. wyatti* larvae were exposed in their natural habitat. This thiarubrine concentration
formed the basis for the concentration incorporated in artificial diets (Appendix, Table B). The
presence of other endogenous plant compounds in the crude extracts may contribute to the
variation seen here. Crude extracts were used instead of pure thiarubrines to mimic as closely as
possible the true conditions *L. wyatti* larvae are exposed to in nature.
TABLE 2.1: UV spectrophotometric and HPLC analyses of Sand, *A. chamissonis* Stems, Wild *L. wyatti* Larvae and their Frass

Concentration (μg/g dry wt.)

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Spectrophotometric Analyses(^1)</th>
<th>HPLC Analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sand</td>
<td>(3.52 \times 10^{-3} \pm 1.7 \times 10^{-3})</td>
<td>nt</td>
</tr>
<tr>
<td>Stems</td>
<td>nt</td>
<td>147.6 ± 110.2</td>
</tr>
<tr>
<td>Larvae(^2)</td>
<td>nt</td>
<td>4.24</td>
</tr>
<tr>
<td>Frass</td>
<td>nt</td>
<td>36.8 ± 15.5</td>
</tr>
</tbody>
</table>

Means ± Standard Deviation. nt = not tested
\(^1\)Concentration determined using the Beer-Lambert equation (Appendix, Table B)
\(^2\)Samples combined (n=7)
FIGURE 2.1: Feeding damage on *Ambrosia chamissonis* by subterranean *Lasionycta wyatti* larvae.
The concentration of total thiarubrines incorporated into the diet was higher than the concentrations used in previous tests with other insects (Ellis et al., 1995; Guillet et al., 1997). Also, these previous tests with thiarubrines and insects only used pure thiarubrine A (Ellis et al., 1995; Aucoin et al., 1995; Guillet et al., 1997). The use of crude extracts takes into consideration the diversity of thiarubrines found in the *A. chamissonis* plants. It seemed only fitting to use crude extracts in this study as HPLC analyses of wild larvae and their frass indicated a complex pattern of thiarubrines and thiophenes.

*Tissue Distribution of Thiarubrines in Lasionycta wyatti*

A 1.0-2.0 gram artificial diet laced with thiarubrines was given to larvae for 48 hours to determine the fate of the toxins. The highest concentration of recovered thiarubrines appeared in the frass and relatively little was recovered in the gut contents and integument (Table 2.2). This result is similar to that reported by Iyengar et al. (1987) who investigated the toxicokinetics of α-Terthienyl in three Lepidoptera, *Manduca sexta, Heliothis virescens,* and *Ostrinia nubilalis*. From all three insects, the highest amount of administered toxin was recovered from the frass. Of the three species tested, the highest amount of α-Terthienyl was recovered from the frass of *O. nubilalis*, the most tolerant species (Iyengar et al., 1987). The authors proposed that the ability of *O. nubilalis* to better withstand the effects of α-Terthienyl than *M. sexta* can be attributed to its ability to rapidly eliminate the toxin.

Similarly, in their study with *Papilio polyxenes* and *Spodoptera frugiperda*, Ivie et al. (1983) showed that rapid elimination of xanthotoxin was recovered from the specialist *P. polyxenes*. Within 90 minutes of ingestion *P. polyxenes* eliminated 50% of the toxin while *S. frugiperda* only eliminated 1%. Ivie et al. (1983) believe it is this ability to rapidly eliminate the
TABLE 2.2: Recovery of thiarubrines in micrograms per gram (μg/g) from Frass, Gut Contents and Carcass of dissected *Lasionycta wyatti* larvae 48 hours after oral administration (n=4 for each treatment)

<table>
<thead>
<tr>
<th>Specimen</th>
<th>μg/g</th>
<th>% Recovery</th>
<th>% Uneaten</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frass</td>
<td>14.6 ± 4.7</td>
<td>69.7</td>
<td></td>
</tr>
<tr>
<td>Gut</td>
<td>6.1 ± 3.7</td>
<td>28.1</td>
<td></td>
</tr>
<tr>
<td>Carcass</td>
<td>0.5 ± 0.2</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>Uneaten Diet</td>
<td>36.1 ± 11.2</td>
<td>52.2</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Recovery based on pure thiarubrines consumed from diet.
toxin from its tissues that has allowed *P. polyxenes* to escape its toxic effects. *S. frugiperda* was not able to excrete as much of this toxin and therefore suffered from the exposure (Berenbaum, 1978). This finding coupled with the discovery that *P. polyxenes* contains high levels of mixed function oxidases, detoxification enzymes, in its gut (Bull et al., 1986), helps to elucidate the mechanism by which *P. polyxenes* is able to tolerate the toxins. Based on my results from similar feeding assays, I strongly suggest that *L. wyatti* larvae escape thiarubrine toxicity by rapid elimination.

The relatively low levels of thiarubrines found in the gut contents of laboratory reared larvae (Table 2.2) and whole wild larvae (Table 2.1) suggest thiarubrine levels in the insect is kept at very low levels despite the high levels found in plant tissues. Ivie et al. (1983) reported that *P. polyxenes* contained only 0.7% of the administered amount in its body (Ivie et al., 1983). Similarly, wild caught *L. wyatti* larvae contained only 2.9% of the amount of thiarubrines found in stems (Table 2.1).

The analysis of wild larvae (Figure 2.2) uncovered an unidentified peak that was not seen in analyses of stem or root extracts. An investigation of the UV spectrum of this peak reveals it is neither a thiarubrine nor a corresponding thiophene (data not shown). This peak may represent a metabolic break down product of a thiarubrine. Isolation and identification of this compound to determine what role, if any, this compound plays in *L. wyatti* metabolism should be carried out in future studies.

Finally, it is not known at this time if the conversion of the thiarubrines to their corresponding thiophenes can be achieved enzymatically or if the conversion of thiarubrines can result in products other than their corresponding thiophenes. Identification of this unknown compound may help to answer these and other questions about thiarubrines.
FIGURE 2.2: HPLC Analyses of thiarubrines standards (a) *A. chamissonis* root extract (b) and Wild caught *Lasionycta wyatti* larvae (c). The arrow reveals the presence of an unidentified peak in the larvae which is absent from the root extract. Upper case letters denote thiarubrines; lower case letter denote thiophenes.
CHAPTER THREE

COLLECTION AND LABORATORY REARING OF Lasiomycta wyatti
(LEPIDOPTERA: NOCTUIDAE)

INTRODUCTION

The discovery of L. wyatti larvae in the sand surrounding the roots of A. chamissonis plants sparked my curiosity because of the presence of toxic thiarubrines in the plant (Ellis, 1993). As was shown in Chapter Two, L. wyatti contained toxic thiarubrines in its tissues. Prior to any experimental analyses, a laboratory colony of L. wyatti needed to be established.

The techniques outlined in this chapter resulted in the successful rearing of three generations. An attempt at simulating overwintering conditions of the local habitat, resulted in complete mortality of eggs from the third generation of moths. Given the success of the first two generations, it is believed that had simulation of these overwintering conditions not been attempted, further generations would have been successfully reared.

METHODS and MATERIALS

Laboratory Rearing of Larvae

After identification of wild larvae by comparison against voucher specimens, (Chapter Two), each individual was placed into a 15 millimeters (mm) x 60 mm round petri dish and given 1.0 gram (g) of artificial diet (Table 3.1), covered then placed into a dark environment at 25°C.
<table>
<thead>
<tr>
<th>Table 3.1: Ingredients used in the 100 g artificial diets</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Diet ingredients:</td>
</tr>
<tr>
<td>17.5 g dry diet mix$^1$ per g total weight</td>
</tr>
<tr>
<td>b) Wet ingredients:</td>
</tr>
<tr>
<td>2.50 g agar per g total weight</td>
</tr>
<tr>
<td>80.0 ml distilled water per g total weight</td>
</tr>
</tbody>
</table>

$^1$Ingredients in Appendix, Table C
FIGURE 3.1: *Lasionycta wyatti* larvae in insect assay trays with artificial diets.
Moisture was provided by the food cubes. Food cubes and petri dishes were changed once a week during the first through third instars and then once every 48 hours during the last stages prior to pre-pupation.

**Pre-pupae and Pupae**

Upon pre-pupation, individual larvae were placed in clean 15 mm x 60 mm covered petri dishes without food cubes and maintained in a dark environment at 25°C until pupation. Larvae which had pupated were placed into a large, round 14.5 cm uncovered petri dish lined with sand collected from Centennial Beach, Boundary Bay Regional Park in Tsawwassen. This petri dish was then placed into a 24 cm³ Plexiglas flight cage which had a nylon mesh top and front secured with silicone for ventilation and easy access. A 25 ml Erlenmeyer flask filled with a 10% aqueous sucrose solution and a cotton wick was placed in the flight cage to provide emerging adults with a food source. This flight cage was then placed into a 25°C room with a 14:10 hour light regime adapted from Jacobson (1970). Once adults began emerging, they were captured and placed into another 24 cm³ flight cage for egg laying.

**Eggs and Neonate Larvae**

Eggs, roughly 1.0 mm in diameter (Figure 3.2), were laid individually against the walls, roof and floor of the flight cage. Eggs were carefully collected using a fine, sable, artist’s paint brush dipped into a weak bleach solution (two to three drops of hyperchlorite bleach in 250 ml of distilled water). The excess liquid was tapped off the brush. The brush was used to gently get between the egg and the surface to which it was attached (e.g. mesh, Plexiglas). Once the eggs
FIGURE 3.2: *Lasionycta wyatti* eggs at various stages of development, increasing in development from right to left. Measurement in millimeters.
adhered to the bristles of the brush, they were carefully deposited into a sterile 15 mm x 60 mm petri dish. The petri dish was covered and stored in a dark environment at 25°C.

Once neonate larvae began to emerge, they were placed in a cell of an insect bioassay tray with approximately 1.0 g of food and maintained in the dark at 25° Celsius. Food cubes and bioassay trays were replaced once a week. Larvae were kept in insect bioassay trays until prepupation at which time they were placed in petri dishes as described above.

RESULTS AND DISCUSSION

Collection of Larvae

In spring, when collections of insects began, the larvae were of the second or third instar. This is merely an approximation since no literature on the life cycle of this species exists. When larvae were uncovered in the sand, they were quite conspicuous as their cream color did not provide any camouflage and they were large enough to distinguish from the surrounding flora.

Capture of live moths is usually accomplished with black light traps or pheromone traps at night to insure the collection of the intended species. However, because the interaction of interest is between the plant and the larvae, it was more appropriate to collect the insect at an earlier stage of development with identification taking place afterward.

The distribution of larvae found at the bases of randomly selected *A. chamissonis* plants was not even. Some plants had no larvae in the sand surrounding their bases while at others, anywhere from 2-20 larvae were found. Further, larvae were almost always found around the bases of *A. chamissonis* plants that showed subterranean feeding damage. This finding provides further evidence that suggests *L. wyatti* larvae were feeding on the subterranean stems as opposed to the woody roots as was first assumed. Further evidence of this is shown by the depth at which
most of the larvae were found. Most of the larvae were collected from the top 5-10 cm of sand surrounding the subterranean stems and not 20-30 cm below the surface where the woody roots begin.

No larvae were ever found in the sand surrounding the bases of other species of randomly selected plants in close proximity to *A. chamissonis*. On only one occasion was a larva found in the sand between two plants, but one of the two plants was *A. chamissonis*.

The most larvae collected at one time was 43, collected from over 70 randomly selected *A. chamissonis* plants. A total of 91 larvae were collected in the spring of 1996 and a total of 65 larvae were collected in the spring of 1997 to start laboratory colonies. Of the 91 larvae collected in 1996, 50 pupated and emerged as adults. In 1997, only 65 larvae were collected of which 60 pupated and emerged as adults (Table 3.2).

**Laboratory Rearing of Insects**

In the laboratory, fungal contamination proved to be the main obstacle to rearing the fourth and fifth instar larvae as moisture built up in the bioassay trays from respiration and a rapid accumulation of frass. Similar problems were found when fourth and fifth instar stages were placed in 15 mm x 60 mm petri dishes. While no mortality occurred in the early stages of development (first through third), some mortality occurred in the later stages as a results of the moisture and fungal contamination.

The pre-pupal stage was brief (Table 3.3) lasting only 3 to 5 days. Larvae entering the pre-pupal stage ceased to feed 1 to 2 days prior to this. The pre-pupal larvae decreased in size but slightly increased in girth and took on a more oval shape.
TABLE 3.2: Survivorship of Wild Caught *Lasionycta wyatti* Larvae collected in 1996 and 1997

<table>
<thead>
<tr>
<th></th>
<th>1996</th>
<th>1997</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Number of Larvae Collected</td>
<td>91</td>
<td>65</td>
</tr>
<tr>
<td>Number of Larvae to Pupae Stage</td>
<td>57</td>
<td>64</td>
</tr>
<tr>
<td>Number of Pupae to Adult Stage</td>
<td>50</td>
<td>60</td>
</tr>
</tbody>
</table>
The posterior end of the larva became more tapered and the anterior end more rounded characteristic of other noctuids.

The amber colored pupa darkened as the insect advanced through the metamorphosis, eventually becoming a coffee color. This transformation lasted between 15 and 19 days (Table 3.3). At the end of this stage, when the pupae had darkened in color, the adult emerged as a silver colored moth with black and white speckles on the forewings mimicking the sand grains found at Centennial Beach. The adult measured between 2.0 cm and 2.75 cm from head to wing tip (Figure 3.3).

Adults lived between 14 and 21 days. During this time, they displayed a nocturnal behavior pattern. During the 14 hours of light, the adults remained perfectly still within the flight cage and did not move even when prodded. However, during the 10 hours of night, the adults became active. They flew around the flight cage, fed on the sucrose solution, mated, and the females laid eggs.

Eggs were laid individually, not in clusters or plaques. There was no one particular location where eggs were laid (e.g. walls of flight cage as opposed to the floor of the flight cage). Fortunately, the cream colored eggs were 1.0 mm in diameter (Figure 3.2) and easily located. In 1996, approximately 170 eggs were laid by the first generation of wild adults, 120 of which hatched, and approximately 150 eggs were laid by the second and third generations of (laboratory reared) adults. Approximately 100 eggs hatched from the second batch of eggs but no eggs hatched from the third batch as they were the subject of a failed attempt to simulate overwintering conditions in the laboratory. All the eggs desiccated as a result of this attempt. In the 1997 collection, approximately 500 eggs were laid by adults caught that year of which 400 hatched.
TABLE 3.3: Time (Days) required for development of Eggs, Larvae, Pre-pupae, and Pupae of Laboratory Reared *Lasionycta wyatti*

<table>
<thead>
<tr>
<th>Life Stage</th>
<th>Number of Days</th>
<th>Head Capsule Width (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg</td>
<td>21-28</td>
<td>N/A</td>
</tr>
<tr>
<td>Instar 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.8 ± 1.1</td>
<td>0.5 - 0.8</td>
</tr>
<tr>
<td>&quot; 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.2 ± 1.2</td>
<td>0.8 - 1.2</td>
</tr>
<tr>
<td>&quot; 3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.6 ± 1.8</td>
<td>1.2 - 1.5</td>
</tr>
<tr>
<td>&quot; 4</td>
<td>7 - 14</td>
<td>1.5 - 2.5</td>
</tr>
<tr>
<td>&quot; 5</td>
<td>11 - 14</td>
<td>2.5 - 3.0</td>
</tr>
<tr>
<td>Pre-Pupae&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.4 ± 1.4</td>
<td>N/A</td>
</tr>
<tr>
<td>Pupae&lt;sup&gt;e&lt;/sup&gt;</td>
<td>17.1 ± 2.7</td>
<td>N/A</td>
</tr>
<tr>
<td>Adult</td>
<td>14 - 21</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Means ± Standard Deviations. No error was given for instar 4, 5 and adults due to complications with identification and no error was given for egg stage due to large numbers involved.

<sup>a</sup>(n=20)
<sup>b</sup>(n=19)
<sup>c</sup>(n=18)
<sup>d</sup>(n=57)
<sup>e</sup>(n=47)
FIGURE 3.3: Adult *Lasionycta wyatti* moth. Measurement in centimeters.
The eggs that did develop showed the same color change exhibited by the pupae during the course of development. Newly laid cream colored eggs darkened to a peach color and finally became opalescent. The last few days before hatching, the translucent egg took on the color of the gray-cream larvae encased within the egg which gave the mature egg an illusion of opalescence.

The eggs developed over 21 to 28 days (Table 3.3). Beginning with the seventh to tenth day, the tiny larvae encased within the egg could be seen with the aid of a hand lens or microscope. The tiny larvae did not show any circadian hatching rhythm as eggs hatched during all hours.

The proportion of eggs that hatched increased in 1997 over those in 1996. Higher success rates in both pupation and hatching rates can be attributed to better overall care in the handling of the larvae during the incubation period. Specifically, in 1997 the incubation temperature was increased from 20-22°C to 25°C. A higher temperature may better simulate field conditions. A summary of the life cycle of *L. wyatti* is shown in Figure 3.4.
FIGURE 3.4: A summary of the life cycle of *L. wyatti*
CHAPTER FOUR

COMPARISON OF THE EFFECTS OF THIARUBRINES ON THREE INSECTS:

LASIONYCTA WYATTI, SPODOPTERA LITURA, AND MANDUCA SEXTA

INTRODUCTION

Some insects evolved over time the ability to escape the phototoxic effects of some phytochemicals through behavioral, physical, and physiological mechanisms or a combination of these (Chapter One). After tests showed the presence of thiarubrines in the tissues and frass of wild *L. wyatti* larvae (Chapter Two), the first objective, as outlined in this chapter, was to determine (in complete darkness) if *L. wyatti* experienced any of the negative effects experienced by non-specialized herbivores. The mere presence of thiarubrines in the tissues and frass of *L. wyatti* larvae did not preclude the possibility of toxic effects on the growth and development of the insect. *L. wyatti* larvae were fed a thiarubrine-laced diet for the entire lives of one generation of insects. Simple observations were made on their growth and development which were compared to cohorts fed an untreated diet. No observable difference was noted in the two groups of larvae.

Separately, another group of neonate *L. wyatti* larvae were exposed to an artificial diet treated with *A. chamissonis* plant extracts over a shorter time frame of four days to determine what immediate effect(s), if any, the thiarubrines had on the weight gain of neonate larvae. Along with testing *L. wyatti*, the same feeding assay was conducted using *S. litura* and *M. sexta* to provide a basis for comparison. *S. litura* belongs to the same family of moths as *L. wyatti* i.e., Noctuidae. Comparison of two related species could possibly demonstrate whether there is an
inherent ability to withstand toxic compounds in related species. *M. sexta* was reported in several papers (Downum et al., 1984; Champagne et al., 1986; Iyengar et al., 1987; Aucoin et al., 1995; Guillet et al., 1997) as being extremely sensitive to thiarubrines and other phototoxins. Including it in these feeding assays served as a positive control.

The results of these feeding assays showed a significant difference in the larval weight gain of *S. litura* larvae fed the treated diet as compared to cohorts fed an untreated diet. More dramatic results were seen with *M. sexta* larvae. Larvae fed the treated diet suffered mortality while the larvae fed the untreated diet suffered none. Tests on *L. wyatti*, however, revealed no mortality or significant difference in the weight gain in larvae fed the treated diet compared to cohorts fed an untreated artificial diet.

Feeding assays were also conducted in UV/visible light to determine if the activity of thiarubrines would be enhanced with the presence of light. Previous tests on bacteria and viruses showed no activity in the dark, but light in addition to exposure to thiarubrines showed significant activity (Towers et al., 1985; Hudson et al., 1986a,b; Ellis, 1993).

This feeding assay experiment would also determine whether a behavioral mechanism was being used by *L. wyatti* larvae as a means of escaping the toxic effects of thiarubrines. As was reported with *Chrysolina hyperici*, tests which prevented larvae from burrowing into the surrounding soil (effectively forcing them to sunlight exposure) showed the larvae suffered photosensitization and mortality. Similarly, this feeding assay looked at the effect of UV/visible light in combination with a thiarubrine-laced diet on the weight gain of *L. wyatti* larvae. The results suggest that *L. wyatti* larvae are resistant to the toxic effects of the thiarubrine compounds in the presence of the UV/visible light regime. However, when the larvae were exposed to UV
activated thiophenes, the conversion product of thiarubrines in UV, they suffered significant weight loss as compared to their cohorts fed an untreated diet.

MATERIALS AND METHODS

A. Preparative Methods

Plant Materials

*A. chamissonis* roots were collected from Centennial Beach in Boundary Bay Regional Park in Tsawwassen, British Columbia during the months of April through May 1996 and 1997. Individual plants were identified and then roots were harvested by digging sand away from the roots to a depth of two to three feet. Sections of root were then cut away with gardening shears and immediately placed into brown paper bags then in large, black garbage bags to eliminate any exposure to sunlight. Root collections brought to the Department of Botany at the University of British Columbia were first weighed (fresh weight) then placed in -80°C freezer until extracted.

Drying Root Material

The following procedures were conducted in dim light to avoid any photoconversion of endogenous plant compounds. Frozen roots were thawed and allowed to dry on paper towels or in cardboard boxes within a laboratory fume hood for approximately five days. Roots were considered dry when they were no longer pliable and easily broken in half.

Roots were weighed again (dry weight) and placed in an industrial size Thomas Wiley-Mill grinder model #4. Roots were ground to a fine powder then suspended in 2.0 liter (L) CH$_2$Cl$_2$ to
completely submerge the fine powder within a 4L Pyrex Erlenmeyer flask. This mixture was allowed to sit for 48 hours at room temperature in complete darkness.

**Preparation of Crude Extract from Roots**

After 48 hours, the contents of the 4L Erlenmeyer flask were vacuum filtered through a Whatman 1 filter paper. The desired plant compounds were isolated by evaporating the CH$_2$Cl$_2$ solvent *in vacuo* at 30°C from the liquid filtrate. The residue was then resuspended in 850 ml of CH$_3$OH and stored in a Pyrex Erlenmeyer flask at 2°C.

**Conversion of Thiarubrines to Thiophenes**

Four hundred ml of the stock solution were placed in a round bottom flask, stoppered then exposed to UV light 6.0 W/m$^2$, provided by four Sylvania black-light bulbs, for 24 hours at 22°C. After 24 hours, any solids which formed at the bottom of the flask were removed by vacuum filtration using a Buchner funnel and Whatman 1 filter paper.

**Verification of Thiarubrine concentration of Crude Extract Stock Solution**

Five 1.0 ml samples of the prepared crude extract were analyzed using a Philips PU 8720 UV/Visible scanning spectrophotometer to determine the total thiarubrine concentration of the crude extract. Individual absorbance readings (spectrophotometer wavelength set at 490 nm) were averaged and included in a mathematical computation utilizing the molecular weight and molar extinction coefficient of thiarubrine A (Appendix, Table A). Concentration is reported as milligrams per milliliter (mg/ml).
Verification of Thiophenes converted from Thiarubrine Stock Solution

Three to five 1.0 ml samples of thiophene solution were analyzed using a UV spectrophotometer (wavelength set at 490 nm). Zero absorbance at the set wavelength confirmed the complete conversion of thiarubrines to thiophenes.

Preparation of Artificial Diet Containing Crude Thiarubrine Extract

One hundred-fifty micrograms (μg) of crude thiarubrines per gram fresh weight of diet (150 μg/g or 150 ppm) was used in the artificial diets administered to the insects. The volume of crude extract solution needed to achieve the 150 μg/g artificial diet concentration was determined using the concentration of the “stock” (crude extract) solution and the Beer-Lambert equation (Appendix, Table A).

Once the amount of stock solution was determined, the solvent was evaporated from the solution in vacuo until only the resin remained in the flask. The dry diet mixture (Table 3.1) was then added to the flask and mixed with the resin until the mixture was homogeneous.

Ingredients for the wet mixture (Table 3.1) were combined in a 100 ml beaker and heated to 90°C. The solution was stirred constantly as it was heated to avoid burning the agar. Once this solution reached 90°C, the solution was removed from the heat source and cooled to 55°C. At 55°C, the dry diet mixture was added to the wet ingredients and stirred until the dry and wet ingredients were evenly distributed.

This final liquid mixture was then poured into a square Integrid 100 mm x 15 mm plastic petri dish and allowed to solidify in a refrigerator set at 2°C. Artificial diets not containing
thiarubrines (untreated diets) were prepared in the same manner excluding the addition of thiarubrines to the dry diet mixture.

*Preparation of Artificial Diets containing Thiophenes*

The concentration of thiophenes in an artificial diet was determined using the same mathematical computation used for a thiarubrine-treated diet (Appendix, Table B). The amount determined from the equation was then decanted into a round bottom flask and the solvent was evaporated *in vacuo* in a rotovap. When only the thiophene resins remained, the dry ingredients (Table 3.1) were added to the flask and combined with the resins until homogeneous. The remaining steps of this process (heating of liquid media and the total combination of all ingredients) are identical to those mentioned above. Similar cooling and storing methods were used here as stated above.

*Insects*

*L. wyatti* larvae were collected from the field and raised in the laboratory at the Department of Botany at the University of British Columbia (Chapter Three). First generation of eggs collected from laboratory reared, wild *L. wyatti* were used in the bioassays outlined in this chapter. The other species used in the bioassay for comparison was the Asian armyworm *Spodoptera litura* Hubn. (Lepidoptera: Noctuidae) obtained from a continuous culture in the Department of Plant Science at the University of British Columbia, and the tobacco hornworm *Manduca sexta* L. (Lepidoptera: Sphingidae) purchased from Carolina Biological Supply Company in South Carolina, USA.
B. Bioassays

Dark Treatment

Sixty two-day-old larvae were collected after hatching and individually weighed (initial weight) using a micro-balance. Each larva was then placed into a cell of an insect bioassay tray with a 1.0 g food cube. Half of the larvae (30 individuals) were given food cubes treated with thiarubrines and the other half were given untreated food cubes. Food cubes and insect assay trays were replaced with fresh food cubes and clean trays after 48 hours (Figure 3.1). The larvae remained in these cells with their food cubes for a total of four days in total darkness at 25°C. After four days, each larvae was weighed again for a final weight. In a separate experiment, the same methods were used replacing the thiarubrine treated diets with thiophene treated diets.

UV and Visible Light Treatment

Four separate groups of two-day-old larvae each consisting of 20 individuals per group were used in this two factor bioassay. Each individual was weighed (initial weight) prior to being placed into a cell in an insect bioassay tray with a 1.0 g food cube. Two groups of 20 larvae were given food cubes treated with thiarubrines and the other two groups of 20 larvae were given untreated food cubes. These groups were then placed in total darkness for 48 hours at 25°C. After 48 hours, all the larvae were removed from their individual cells and placed into 15 mm x 60 mm petri plates without any food for four hours. During the four hours, one group of 20 larvae fed treated diets and one group of larvae fed untreated diets were exposed to UV and visible light at an intensity of 6.3 W/m² supplied by two Sylvania cool white bulbs and two General Electric black-light bulbs. In all 40 individual larvae were exposed to this light treatment. The remaining
two groups of 20 larvae were also removed from their cells and placed into 15 mm x 60 mm petri dishes without food for four hours in complete darkness. All four groups were kept at 25°C.

At the end of the four hours, all larvae were returned to fresh trays and given fresh food cubes. Groups given treated diets were again given treated food cubes at this time. All larvae were then placed in total darkness at 25°C for four days further. Fresh food cubes and clean trays were given to the larvae every 48 hours. On the fourth day after the light exposure treatment, all 80 individuals are weighed again.

The identical methods were used in a separate experiment where the treated diets were made with the thiophene extract instead of the thiarubrines extracts.

RESULTS

*Comparative biological effects of thiarubrines on larval weight gain of* L. wyatti, S. litura and M. sexta larvae

The incorporation of crude thiarubrine extracts from *A. chamissonis* plant extracts into an artificial diet significantly reduced weight (*p*<0.01) of 4-day-old *S. litura* and *M. sexta* larvae (Table 4.1) in complete darkness, compared to cohorts fed untreated diets. Larval growth of *S. litura* fed the thiarubrine-laced diet was inhibited by 25% with no mortality. *M. sexta*, however, experienced 50% mortality within four days of the exposure (Table 4.1). In contrast, thiarubrine-laced artificial diet did not cause any mortality nor significant reduction in larval weight gain *L. wyatti* (*p*>>0.05) (Table 4.1).
TABLE 4.1: Average weight gained (mg) by neonate larvae exposed to an artificial diet treated with and without thiarubrines over 4 days in complete darkness.

<table>
<thead>
<tr>
<th>Species</th>
<th>Treated Diet [150µg/g]</th>
<th>Untreated Diet</th>
<th>P</th>
<th>% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <em>L. wyatti</em></td>
<td>11.6 (± 0.8)</td>
<td>10.3 (± 0.2)</td>
<td>p&gt;0.05</td>
<td>0.0</td>
</tr>
<tr>
<td>2. <em>S. litura</em></td>
<td>19.9 (± 0.4)</td>
<td>26.3 (± 0.3)</td>
<td>p&lt;0.01</td>
<td>0.0</td>
</tr>
<tr>
<td>3. <em>M. sexta</em></td>
<td>0.8 (± 0.0)</td>
<td>24.0 (± 0.1)</td>
<td>p&lt;0.01</td>
<td>57.5</td>
</tr>
</tbody>
</table>

Means (± Standard Deviation)

\(^1\)Standard deviation = 7.3E-0.7
The light dependent effects of thiarubrines on *L. wyatti* larval weight gain was tested to determine if light enhances the effects of thiarubrines in an artificial diet. Results showed that exposure to UV/visible light had no significant effect (p>0.05) on larval weight gain of *L. wyatti* larvae (Table 4.2). At the onset of feeding trials, larvae readily ate artificial diets. There was no visible difference in the amounts of diet ingested by larvae fed untreated diet from those fed the treated diet. A two-way analysis of variance (ANOVA) showed no interaction between the presence of light and thiarubrines. Therefore, *L. wyatti* larvae were not effected by the combination of dietary thiarubrines and the presence of near UV/visible light (Table 4.2). The weight gained by *L. wyatti* larvae in the feeding trials (days), however, was not the same in both replicates.

In the first feeding trial, the larvae fed the treated diet fared much better than their cohorts fed an untreated diet. These larvae gained more weight over four days than those fed the untreated diet regardless of the presence of UV/visible light. These data were not replicated in the second feeding trial. There was no significant difference in the weight gain of larvae fed the treated diet compared to those fed the untreated diet. No significant difference was detected in the second trial. However, I personally noted visible differences in groups fed the treated diet over those fed an untreated diet during their entire life cycle. Those larvae fed the treated diets fared better overall in that their molting patterns were more uniform and the days between each molt was shorter.

*Effect of Thiophenes in an artificial diet on the larval weight gain of Lasionycta wyatti larvae*

A crude extract of thiarubrines, photoconverted to their corresponding thiophenes with the use of UV light, was incorporated into an artificial diet and fed to 4-day-old *L. wyatti* larvae.
TABLE 4.2: Two-way analysis of variance of the weight gained by 4 day old *Lasionycta wyatti* larvae exposed to an artificial diet with and without thiarubrines in both darkness and UV/visible light.

Two-way ANOVA

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet</td>
<td>1</td>
<td>13.880</td>
<td>3.786</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Light</td>
<td>1</td>
<td>1.836</td>
<td>0.501</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Days¹</td>
<td>1</td>
<td>250.627</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>D x L</td>
<td>1</td>
<td>0.000</td>
<td>0.000</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Error</td>
<td>3</td>
<td>3.666</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹Replicates conducted on different days.

NT = not testable. Treatments are fixed.
Results showed that the thiophenes significantly affected the larval weight gain of *L. wyatti* larvae. Over two replicates, the average weight gained by 4-day-old *L. wyatti* larvae exposed to a 150 μg/g thiophene-laced diet reared in dim light was 8.6 ± 0.2 milligrams (mg) while the average weight gained by cohorts fed an untreated diet was 15.0 ± 1.2 mg (0.0005<p<0.001).

**DISCUSSION**

*L. wyatti* fed an artificial diet laced with thiarubrines over their entire lives did not experience any problems in their development. There was some evidence that larvae fed an artificial diet with thiarubrines may have actually incurred some benefit over their cohorts fed untreated diet (data not included). Larvae fed the treated diet molted more consistently than those fed an untreated diet and the days between each molt was shorter than those fed the untreated diets. This observation lends further support towards the hypothesis that *L. wyatti* larvae specialize on *A. chamissonis* and are tolerant of thiarubrines.

Further, tests conducted in dim light showed that the larval weight gain of neonate *L. wyatti* larvae was not significantly affected while the larval weight gain of *S. litura* and *M. sexta* were (Table 4.1). Tests against bacteria and viruses reported the need for UV-A irradiation to generate significant results (Towers et al., 1985; Hudson et al., 1986a,b; Ellis, 1993). The results from tests against *S. litura* and *M. sexta*, as well as other insects (Ellis et al., 1995; Aucoin et al., 1995; Guillet et al., 1997) suggest that thiarubrines are not just phototoxic to insects, they are overtly toxic as no light was necessary to produce any negative effects.

*S. litura* larvae exposed to the treated diets did not seem deterred by the presence of toxins in the diet as there was no major difference in the accumulation of frass between larvae fed control diets and larvae fed the treated diets. However, the growth of neonate *S. litura* larvae
was inhibited (Table 4.1). This suggests that the effects of thiarubrines in *S. litura* are physiological and not behavioral, as they were with *B. orientalis* (Ellis et al., 1995) and *O. nubilalis* (Aucoin et al., 1995). *B. orientalis* simply avoided all contact with commercial dog food laced with 10 μg/g thiarubrines. *O. nubilalis* decreased its consumption of an artificial diet laced with thiarubrines as the concentration of thiarubrine A increased in the diet.

*M. sexta*, however, was not deterred by the thiarubrines. *M. sexta* is highly susceptible to α-Terthienyl and other phototoxins (Downum et al., 1984; Champagne et al., 1986; Iyengar et al., 1987; Aucoin et al., 1995; Guillet et al., 1997). Yet, as shown in this study as well as a study conducted by Aucoin et al. (1995), it is not able to detect the toxins and feeds on treated diets. Aucoin et al. (1995) looked at the index of consumption of thiarubrine A by *M. sexta* larvae. They found that as the thiarubrine concentration increased in an artificial diet, the larvae did not change their consumption rate and became intoxicated. Over 50% of the *M. sexta* larvae given an artificial diet treated with 150 μg/g thiarubrine extracts suffered mortality within 2 to 3 days of the administration. Yet, despite efficacy of thiarubrines against other insects, in complete darkness, *L. wyatti* was able to tolerate the thiarubrines compounds.

To determine if the activity of thiarubrines against *L. wyatti* was dependent on light as it was in the cases against bacteria (Towers et al., 1985; Ellis, 1993) and viruses (Hudson et al., 1986a,b), neonate *L. wyatti* larvae were subjected to feeding assays which looked at the effects of UV/visible light in combination with a thiarubrine-laced diet on their weight gain over four days. A two factorial experiment was designed and analyzed. The two-way analysis of variance (ANOVA) results showed that the individual factors “diet” (presence or absence of thiarubrines) or “light” (presence or absence of UV/visible light) did not affect the weight gain of *L. wyatti* larvae (Table 4.2). The results also showed that the combination of these two factors did not
affect *L. wyatti* growth (Table 4.2). It appears from these data that *L. wyatti* are immune to the toxic effects of the thiarubrines. The ability to escape the thiarubrine toxicity may be a function of its ability to rapidly eliminate the toxins from its tissues (Chapter Two). An interesting note to point out, however, is that there was a difference in the two days which the feeding trials were conducted. Although the $F$ statistic is not testable because the variables are fixed, the data from the first feeding trial suggested, the larvae fed the treated diet gained more weight over four days than their cohorts fed the untreated diet, regardless of the presence of light. These experimental group larvae fared better than the control group. In the second trial, this results was not seen, however. There was no significant difference in the weight gain of larvae fed the treated diets compared to those fed the untreated diet. This result is an interesting one and should be investigated further with more replicate trials. Since visual observations also suggest that larvae on the treated diets had a more uniform molting pattern than cohorts fed the untreated diet, further replication could show that *L. wyatti* do in fact do much better and possibly even require a thiarubrine rich diet.

The enhancement of thiarubrine activity that was reported in past experiments (Towers et al., 1985; Hudson et al., 1986a,b; Ellis, 1993; Guillet et al., 1997) could have been a consequence of the unavoidable conversion of thiarubrines to their corresponding thiophenes in the presence of light, both near UV and visible light. Investigators are not sure if the enhanced toxic effects of the thiarubrines against microorganisms in the presence of light is attributed to the activated oxygen species formed during the actual photoconversion process or to the resulting photoconversion products, thiophenes (Constabel, 1988).

Owing to this ambiguity, separate feeding assays were conducted with thiophenes, the resulting compounds after the conversion of thiarubrines in UV light. UV activated thiophenes
from *A. chamissonis* were placed into an artificial diet and given to 4-day-old *L. wyatti* larvae. This feeding assay better simulated the effects these thiarubrines have on the health and development of *L. wyatti* larvae. *L. wyatti*’s ability to rapidly eliminate the thiarubrines from its tissues prevents the accumulation of the thiarubrines in its tissues which ultimately prevents the eventual conversion of these thiarubrines to their corresponding thiophenes. In their natural habitat, *L. wyatti* larvae are found below the surface of the sand therefore, exposure to thiophenes (activated by UV light) is not likely to occur. Regardless, UV activated thiophenes were incorporated in an artificial diet.

The incorporation of UV activated thiophenes into an artificial diet revealed that *L. wyatti* larvae were susceptible to them (Figure 4.1). *L. wyatti* exposed to a thiophene-laced diet suffered significant weight reduction (8.6 ± 0.2 mg) compared to their cohorts fed an untreated diet (15.0 ±1.2 mg) (p<0.001). This result suggests that should *L. wyatti* consume thiarubrines and then be exposed to UV rays, they would be photosensitized. The reason why the light treatment in the two factorial feeding assay did not reveal any effects of the larval weight gain of *L. wyatti* may be due to its ability to rapidly eliminate the thiarubrines from its tissues before the conversion of the thiarubrines could take place. The complete conversion of the thiarubrines to their corresponding thiophenes required 24 hour exposure to UV light (6.0 W/m^2). The duration of the light exposure treatment in the two factorial feeding assay only lasted four hours at 6.3 W/m^2 offering *L. wyatti* the chance to eliminate the toxin from its tissues and avoid any phototoxic effects.

An alternative hypothesis could be that *L. wyatti* is not susceptible to the activated oxygen species formed during the actual photoconversion process but to the resulting photoconversion products, thiophenes. Realistically, noctuids like *L. wyatti* would not be exposed to UV light in
FIGURE 4.1: Weight gained (mg) by *Lasionycta wyatti* larvae exposed to a thiophene-laced diet.
their natural habitats for more than a few seconds at most, since they display light avoidance behavior. So, photosensitization by activated thiophenes is highly unlikely.

The results of the thiarubrine feeding assays conducted in darkness and in UV/visible light strongly support the notion that *L. wyatti* are tolerant of *A. chamissonis* plant extracts rich in thiarubrines as long as the exposure to UV is minimized. Further, wild *L. wyatti* larvae and their frass contained thiarubrines which suggests that they are feeding on *A. chamissonis* plants in their natural habitat. Moreover, the sites along the west coast of North America where *L. wyatti* have been collected closely resemble the distribution pattern of *A. chamissonis* (Figure 1.1). An depth ecological field study on the interaction between *L. wyatti* and *A. chamissonis* may reveal a host plant/specialist relationship and continued work in the laboratory would reemphasize the fact that *L. wyatti* are able to escape toxic effects of the thiarubrines and incurs some benefit from the ingestion of them.
GENERAL SUMMARY AND CONCLUSIONS

The research described in this thesis concerns the relationship between the larvae of the rare noctuid moth, *Lasionycta wyatti* (Barnes & Benjamin) (Lepidoptera), and the toxic plant *Ambrosia chamissonis* (Less.) Greene (Asteraceae). The findings in this thesis are summarized as follows: 1) *L. wyatti* larvae discovered in the sand surrounding the bases of *A. chamissonis* plants contained thiarubrines in their tissues. The thiarubrines were not acquired as a result of contact through leachates as no thiarubrines were detected in the sand. Acquisition of thiarubrines by *L. wyatti* larvae therefore resulted from ingestion of the plant. 2) In darkness, *L. wyatti* larvae were not affected by dietary thiarubrines while *Spodoptera litura* and *Manduca sexta* larvae were significantly affected. Growth of 4-day-old *S. litura* larvae was inhibited by 25% and *M. sexta* larvae experienced 50% mortality. 3) *L. wyatti* larvae were not affected by the combination of UV/visible light and dietary thiarubrine. 4) The highest proportion (33%) of administered thiarubrines were recovered in the frass. This finding coupled with the finding that very little thiarubrines were found in the gut contents, suggested that a possible mechanism of detoxification is rapid elimination. 5) UV-activated thiophenes incorporated into an artificial diet negatively affect *L. wyatti* larvae.

In addition to the insight this study gives into the relationship between *L. wyatti* and *A. chamissonis*, laboratory rearing techniques for *L. wyatti* are outlined in Chapter Three. These techniques resulted in the successful rearing of three generations. Suggestions for future research include isolation and identification of the unidentified metabolite shown in Figure 2.2 to determine its role, if any, in the metabolic detoxification of thiarubrines by *L. wyatti* larvae. Further research
into the ecological distribution of *L. wyatti* in its natural habitat needs to be made to better understand the relationship it has with *A. chamissonis*. Although the data included in this thesis strongly suggests *L. wyatti* is a specialist on *A. chamissonis*, more information on the interaction between the two species needs to be conducted before any absolute statement could be made.

A personal note: there is very little information on the natural history of this rare insect. Conservation efforts of *L. wyatti*'s natural habitat(s) need to be made in order to insure the survival of this rare moth. Recently, plans have been implemented that incorporate horse riding trails along some portion of Centennial Beach in Tsawwassen, BC (G. Bradfield pers. comm.), the beach where I personally collected larvae. The sandy beach locations where *A. chamissonis* and *L. wyatti* are found are often considered for prime locations for development. Plants in these locations, such as *A. chamissonis*, are considered unimportant weeds. It would be a tragedy, should *A. chamissonis* and *L. wyatti* become victims of overdevelopment. The information that has been drawn from research on this toxic plant and its interaction with this rare moth so far is little compared to what can be drawn from further research.

There are relatively few interactions between phototoxic plants and specialist insects that have been studied over the past twenty years. The phototoxic thiophene, α-Terthienyl, is currently the most studied. It would be interesting to see if *L. wyatti* would tolerate α-Terthienyl as well as it tolerated thiarubrines. More research needs to be conducted on the various toxic polyyynes and thiophenes from the Asteraceae as well as their relationships to insects like *L. wyatti*. 
REFERENCES


Bradfield, G (1997) The University of British Columbia, Vancouver. Personal communication


Ellis, SM (1993) MSc Thesis. Thiarubrine production in roots and root cultures of *Ambrosia chamissonis*. The University of British Columbia, Vancouver, pp. 1, 4


Towers, GHN (1997) The University of British Columbia, Personal communication


Towers, GHN, PA Spencer and E Rodriguez (1989) Recent topics in phytochemical ecology research In: *Phytochemical Ecology: Allelochemicals, Myotoxins and Insect Pheromones and Allomones* CH Chou and GR Waller (eds.) Institute of Botany, Academia Sinica Monograph Series No.9, Taipei, ROC

Troubridge, J. (1996) Agriculture Canada and Agri-Food Canada, Agassiz. Personal communication

APPENDIX

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TABLE A: Determination of Thiarubrine Concentration of a Crude Extract using the Beer-Lambert Equation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average UV spectrophotometer absorbency</td>
<td>$4.824 \pm 0.3074$</td>
</tr>
<tr>
<td>Molar absorptivity of thiarubrine A at 490 nm ($\varepsilon$)</td>
<td>3000</td>
</tr>
<tr>
<td>Molecular weight of thiarubrine A</td>
<td>228 g/mol</td>
</tr>
</tbody>
</table>

Concentration = $\frac{4.824 \times 228}{3000} = 0.3666 \pm 0.02336$ mg/ml

\(^1\text{(Ellis, 1993)}\)
TABLE B: Calculation used to determine the amount of stock solution needed to achieve 150 μg/g treated artificial diet

Thiarubrine concentration of artificial diet = 150 μg/g or 0.150 mg/g
Total weight of artificial diet = 100 g
Concentration of stock solution = 0.3700 mg/ml

\[
0.150 \text{ mg/g} \times 100 \text{g artificial diet} = 15.0 \text{ mg of thiarubrines needed}
\]

Volume of stock solution needed = \( \frac{15.0 \text{ mg of thiarubrines needed}}{0.3700 \text{ mg/ml}} \) = 40.5 ml

\(^1\text{from Table A}\)
**TABLE C: Ingredients Contained in the Commercial Dry Diet Mix**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Velvetbean Caterpillar Diet</td>
<td>85.7%</td>
</tr>
<tr>
<td>Alfalfa Meal</td>
<td>5.7%</td>
</tr>
<tr>
<td>Vanderzant’s Vitamins</td>
<td>8.6%</td>
</tr>
</tbody>
</table>

1 purchased from Bio-Serv, Frenchtown, NJ USA