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Impairing The Insect Immune System With Plant-Derived Substances

Katherine Haszcz

IMPAIRING THE INSECT IMMUNE SYSTEM WITH PLANT-DERIVED SUBSTANCES

A Masters Thesis

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Master of Science, Plant Science

By

Katherine Haszcz

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IMPAIRING THE INSECT IMMUNE SYSTEM WITH PLANT-DERIVED SUBSTANCES

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ABSTRACT

Worldwide resistance to current insecticides continues to grow at an alarming rate, with the danger of pesticide overuse reducing acceptable doses. Alternative strategies for insect pest management are therefore imperative. By compromising the insect immune system with plant-derived substances and studying the detailed processes involved in hemocyte-mediated responses, one can develop improved methods for insect control. Hemocyte numbers change in response to stress. This allows essential defense mechanisms such as phagocytosis and encapsulation to take place. In this thesis, plant-derived substances were used to challenge larval-stage *Galleria mellonella* (Family: Lepidoptera) due to their nontoxic nature and high value in pest management. Insects were subjected to chemicals of botanical origin including essential oils and various neem formulations. Azadirachtin, the presumed insecticidal compound of neem, was also tested in addition to a mycoinsecticide containing *Beauveria bassiana* fungi. Differential hemocyte counts were conducted, phagocytosis was assessed using Sumi ink, and nylon implants were inserted to measure encapsulation. Final results indicate a correlation between hemocyte release and insect cellular defenses. Interestingly, neem oil significantly disrupted pupation while simultaneously inhibiting plasmatocyte production. Azadirachtin similarly halted larval growth, but did not elicit a hemocytic response.

KEYWORDS: insect immunity, Lepidoptera, *Galleria mellonella*, bioinsecticides, essential oils, azadirachtin, hemocytes, phagocytosis, encapsulation

This abstract is approved as to form and content

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Dr. Maciej Pszczolkowski

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INTRODUCTION

Vertebrates have a well-developed immune system that protects them from various pathogenic organisms. This immune system is generally classified into two subsystems – innate and acquired immunity. Innate immunity involves a generic recognition and response to foreign invaders that is only temporary. Conversely, acquired immunity consists of specialized cells that identify specific agents and ultimately produce an immunological memory (Strand, 2008). Insects appear to lack the acquired immune response characteristic of vertebrates. Therefore, insects rely solely on their highly efficient innate immunity for defense against pathogens (Berger and Jurcova, 2012).

The innate immune system of insects is comprised of cellular and humoral responses, both of which are mediated through various signaling pathways (Lavine and Strand, 2002). Humoral defenses involve macromolecules with antimicrobial properties that control melanin formation and coagulation. Cellular responses refer to hemocyte-mediated processes such as phagocytosis, encapsulation, and nodulation (Strand, 2008). Due to difficulty and lack of research, little is known regarding the detailed mechanisms involved in insect cellular defenses (Ribeiro and Brehelin, 2006).

Hemocytes are insect blood cells found in the hemolymph, or insect blood. Insects generate a variety of hemocytes that are classified according to their morphological, histochemical, and functional characteristics (Strand, 2008). For the purpose of this thesis, the various lepidopteran hemocytes will be discussed. Different hemocyte classes found in lepidopterans include plasmatocytes, granulocytes, spherule

cells, oenocytoids, and prohemocytes (Lavine and Strand, 2002). Both plasmatocytes and granulocytes are involved in phagocytosis, encapsulation, and nodulation. Oenocytoids are non-adhesive hemocytes that contain phenoloxidase, an enzyme that appears to regulate melanization of hemolymph (Ribeiro and Brehelin, 2006). Spherule cells have been suggested to transport cuticular components, although their precise function remains unknown. Prohemocytes are hypothesized to be stem cells that differentiate into particular hemocyte types (Strand, 2008).

Plasmatocytes and granulocytes are regarded as the primary hemocytes involved in cellular responses due to their ability to adhere to foreign agents. Studies have shown that plasmatocytes and granulocytes collectively make up more than 50% of all circulating hemocytes in larval stage lepidopterans (Lavine and Strand, 2002). However, discrepancies exist with regards to which hemocyte class is directly involved in phagocytosis. In lepidopterans, some researchers have shown that granulocytes are the main hemocyte type that mediates phagocytosis, whereas others have found that plasmatocytes are the only hemocyte class to directly participate in this cellular defense (Tojo et al., 2000).

Granulocytes are morphologically distinguished by their larger size and ruffled, circular shape. Contrarily, plasmatocytes appear smaller and spindle-like due to rapid asymmetrical spreading on foreign surfaces. Hemocyte staining is used as an additional technique to improve cell counting. Common nonenzymatic stains include neutral red, toluidine blue, alcian blue, and biebrich scarlet (Chain and Anderson, 1983). Although light microscopy allows for total hemocyte counting, plasmatocytes are the only hemocytes that can be accurately differentiated. A phase-contrast microscope would be

necessary for further differentiation of other hemocytes. Therefore, studies conducted for this thesis categorized hemocytes into two main groups – plasmatocytes and hemocytes other than plasmatocytes.

All vertebrate blood cells originate from hematopoietic stem cells that have the ability to differentiate into a specific cell type (Lebestky et al., 2000). In insects, hemocytes are initially produced from mesoderm during embryogenesis. Lepidopteran hemocyte development continues throughout all larval stages via two ways: (1) differentiation of prohemocytes, or stem cells, from hematopoietic organs into plasmatocytes and (2) continued division of circulating hemocytes (Strand, 2008). The paired hematopoietic organs of lepidopterans are located in the meso- and meta-thorax (Lavine and Strand, 2002). Insects respond to stress, injury, or disease with a rapid increase or decrease in the number of total hemocytes. One can accordingly conclude that an insect's total hemocyte count is indicative of its immune response.

Plasmatocytes or granulocytes are the only hemocytes capable of phagocytosis, encapsulation, and nodulation (Strand, 2008). Therefore, insect cellular responses induce the transformation of circulating hemocytes from non-adhesive to adhesive cells (granulocytes or plasmatocytes) that are able to adhere to and get rid of foreign agents. Phagocytosis occurs when a target binds to the receptor of an immune cell, or hemocyte, and compels it to produce a phagosome (Strand, 2008). The hemocyte engulfs the target and the phagosome matures into a phagolysosome, which can be expelled from the hemolymph. Insect hemocytes phagocytize many different targets including yeast, bacteria, apoptotic bodies, and abiotic particles (Lavine and Strand, 2002). Research

indicates that hemocyte-mediated phagocytosis can be efficiently measured using abiotic particles such as latex beads or ink, which can also alter the hemocytic profile of insects.

Encapsulation occurs when multiple hemocytes adhere to a relatively large target that cannot be phagocytized (Strand, 2008). Previous research has shown that the proficiency of encapsulating a nylon monofilament by an individual insect is analogous to its ability of encapsulating an actual pathogen (Krams et al., 2012). Consequently, lepidopteran encapsulation is frequently measured by implanting a section of monofilament into the insect body cavity, or haemocoel. Established procedures use implants that contain a section of white nylon monofilament with one knotted end, 2mm long and 0.5mm in diameter (Grizanova et al., 2014). After the experiment is completed, the degree of encapsulation response can be evaluated using a computer program that indicates the extent of melanization in terms of gray-scale units and optical density.

Nodulation involves the specific binding of numerous hemocytes to clusters of bacteria (Lavine and Strand, 2002). Encapsulation and nodulation are essentially the same processes, with the latter primarily attacking bacteria. The relatively complex process of nodule formation begins when hemocytes aggregate around foreign bacterial cells. This confines the bacteria while the hemocyte microaggregation continues to expand. Eventually, the nodulation response is completed by activation of phenoloxidase and melanization of developed nodules (Shaurub, 2012). For the purpose of this thesis, nodulation was only studied with *Beauveria bassiana* fungi due to lack of necessary materials and equipment.

Agricultural crop production continuously relies on pesticides to protect crops from insect pests. A pesticide is defined as a synthetic substance used to prevent, kill,

repel, or eliminate insects and other organisms (Khater, 2012). Initially, synthetically-derived insecticides appeared very effective in terms of persistence and application procedures. Their carcinogenic, teratogenic, spermatotoxic, highly residual, and environmentally dangerous properties were unknown at the time (Khater, 2012). The long-term overuse of pesticides has subsequently produced many ecological consequences. Furthermore, insect resistance to current pesticides is forcing farmers to increase their application rates or switch to pesticides with different modes of action for effective pest management. With the global population continuously growing, crop production will have to increase considerably over the next decade to prevent food scarcity. An alternative pest management strategy is consequently imperative (Chandler et al., 2011).

Growing concerns regarding the harmful effects of pesticides have driven agriculturalists to search for biological methods. Plants, bacteria, and fungi are the sources of bioinsecticides, synthesizing numerous compounds for defense against herbivory and pests (Khater, 2012). Such substances are generally regarded as safe and nontoxic, thus providing an alternative strategy for pest control. Furthermore, the registration process for biopesticides is significantly quicker than that of conventional insecticides. Bioinsecticides are usually registered in less than a year whereas regular pesticides require a minimum of three years (Dutta, 2015). Research involving effective botanicals, as well as their exact mode(s) of action, is becoming increasingly popular. Three biologicals investigated in this thesis include essential oils, neem, and entomopathogenic fungi.

Essential oils are composed of different volatile organic compounds that many plants produce as secondary metabolites for protection (Khater, 2012). Throughout history, steam distillation of aromatic plants has been used to generate essential oils for perfume fragrances and food flavoring. Recent studies have indicated that most plant extracts have insecticidal properties. For instance, various essential oils demonstrate direct toxicity, feeding deterrence, and/or repellence of several insect orders including Lepidoptera. Essential oils exhibit these effects because they interfere with basic functions of the insect nervous system (Copping and Menn, 2010). The current trend strongly advocates use of insecticidal essential oils in agriculture because they are generally regarded as nontoxic to mammals. However, their highly volatile nature and short half-life remains an issue to be solved before they can become effective commercial bioinsecticides (Copping and Menn, 2010).

The genus *Artemisia* belongs to Asteracea, a large plant family that contains approximately 300 different plant species (Zibaee and Bandani, 2010). Most species belonging to *Artemisia* produce essential oils with renowned medicinal and insecticidal properties (Kohan and Sendi, 2013). One particular species, *Artemisia annua* or sweet wormwood, has been widely researched for its presumed anti-insect effects. Research suggests that several isolated compounds from this species demonstrate anti-malarial, antibacterial, anti-inflammatory, and plant growth regulatory activities. For instance, deterrent effects of *A. annua* extracts on codling moth larvae were investigated and substantiated by Dr. Maciej Pszczolkowski in the current laboratory (Durden et al., 2009). *Artemisia annua* essential oil is consequently a highly investigated plant extract.

The neem tree, *Azadirachta indica*, is a legendary plant that is native to Burma and India (Schmutterer, 1995). Villagers throughout India have used neem trees to protect agricultural crops from insects during growth and in storage for more than 2,000 years. In 1959, Dr. Heinrich Schmutterer observed neem trees resisting attack by locust swarms in Sudan. All other plants were completely destroyed during this locust invasion, while neem trees remained fully green and healthy (Schmutterer, 1995). After many years of research, scientists now distinguish *Azadirachta indica* as a highly insecticidal plant. Raw neem extracts have been shown to affect insect reproduction and development, particularly disrupting their feeding ability (Azambuja et al., 1991). Consequently, the global market for commercial neem formulations is expanding quickly as farmers become increasingly aware of organic pesticides as alternatives for pest management.

The effective insecticidal properties of neem trees are primarily and directly associated with a nortriterpenoid limonoid known as azadirachtin (Nisbet, 2000). Butterworth and Morgan (1968) discovered the processes required to isolate azadirachtin from neem seeds. The complete structure of azadirachtin (Figure 1) was determined about 20 years later with the combined efforts of several laboratories. Azadirachtin is a potent antifeedant that interferes with insect development by preventing molting and producing malformed adults. These developmental effects classify azadirachtin as an Insect Growth Regulator (IGR), and thus explain how azadirachtin leads to insect mortality (Khater, 2012). Although azadirachtin is regarded as generally nontoxic to mammals, its exceptionally short half-life and rapid degradation creates issues for long-term efficacy as a bioinsecticide (Azambuja et al., 1991).

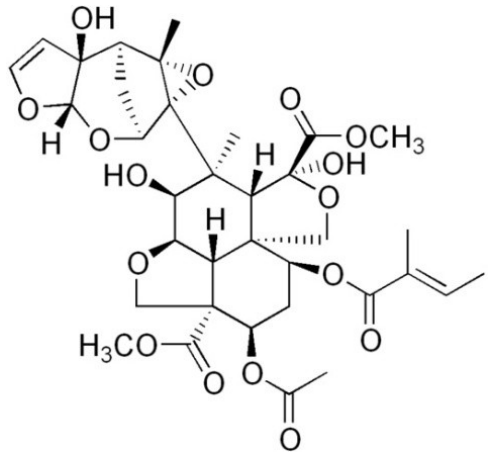

Figure 1. Structural formula of azadirachtin, $C_{35}H_{44}O_{16}$ (Wikipedia, 2016).

The endocrine pathways involved in insect development are relatively understood. Ecdysteroids and juvenile hormone (JH) are the two primary metamorphic hormones found in insects. Molting occurs only in response to a specific cascade of events. In Lepidoptera, neurosecretory cells in the brain produce a peptide hormone called prothoracicotropic hormone (PTTH), which is transported to the corpora cardiaca (CC) for storage. The CC are paired endocrine glands that release PTTH when signaled by the brain. PTTH then stimulates the prothoracic glands (PG) to secrete ecdysone (Muszynska-Pytel et al., 1993). The corpora allata (CA) are brain-controlled endocrine structures that produce and release JH upon stimulation by allatotropins. Ecdysone initiates molting, while the presence or absence of JH regulates what stage the insect develops into (Nijhout, 1998).

Azadirachtin disrupts insect development at a cellular level by targeting specific endocrine events. However, studies involving the precise mode of action involved in azadirachtin-controlled insect development are scarce. Some research has shown that levels of ecdysone are decreased in the presence of azadirachtin (Khater, 2012).

According to Khater, release of PTTH from the brain is inhibited in the presence of azadirachtin and therefore signaling for ecdysone production from the prothoracic glands is absent. Barnby and Klocke (1990) found that oral injections of 1µg azadirachtin reduced PTTH and ecdysone levels in the hemolymph of *Heliothis virescens*, the tobacco budworm. Nevertheless, additional studies are needed in order to obtain a complete understanding of the endocrinological mode of action involved in azadirachtin-controlled growth disruption.

Entomopathogenic fungi produce substances that attack and/or kill insects, which could potentially contribute to pest management. Insect pathogenic fungi provide an alternative to synthetic insecticides because they are environmentally safe and biologically persistent (Gul et al., 2014). *Beauveria bassiana* is a commercially used entomopathogen that combats insect attack using a specific mode of action (Zibaee and Bandani, 2010). First, the fungus releases spores that germinate and grow through the cuticle of susceptible insect species upon contact. Next, *B. bassiana* proliferates in the hemolymph while producing harmful toxins and depleting the insect of essential nutrients (Groden, 1999). This eventually results in insect mortality. There are several commercial *B. bassiana* formulations currently available including Mycotrol, Mycotrol O, BotaniGardES, and NaturalisL (Groden, 1999). Although *B. bassiana* is generally regarded as non-toxic to humans and safe for most beneficial insects, additional research needs to be conducted involving its effects on insect immunity to increase the efficacy of current and future formulations.

Greater wax moth larvae (*Galleria mellonella*) are exemplary model organisms for studying the immune response of challenged insects. The greater wax moth is of the

order Lepidoptera and family Pyralidae (Krams et al., 2015). Their entire life cycle from egg to adult lasts approximately 5 weeks at 35°C and 80% relative humidity. During this time, the caterpillars undergo seven larval stages until they pupate and eventually metamorphose into adult moths. These larvae primarily rely on honeycomb inside bee hives for nourishment as they develop, and are therefore recognized as pests of apiculture (Krams et al., 2015). Advantages of using *G. mellonella* as a model system to study insect immunity include inexpensive rearing costs, simple injection achievability, and recognition as an ethically suitable animal model (Mukherjee et al., 2010).

Numerous botanical extracts can stimulate insecticidal activity, repel a wide variety of insect pests, and cause major disturbances in insect growth. However, little is known regarding the specific immunological response that is involved in these effects. It was mentioned previously that insects rely heavily on different hemocyte-mediated processes to combat foreign substances. Hemolymph extracted from a model insect (i.e. *Galleria mellonella*) that was formerly treated with a botanical extract could potentially contain hemocytes undergoing specific immune responses. It can therefore be hypothesized that bioinsecticides administered to *G. mellonella* larvae orally or by injection will modify the hemocytic profile of the insect, indicating how plant-derived substances affect the insect immune system. Such defenses can be observed with an ink injection to visualize changes in phagocytosis, differentiated hemocyte counts to show which hemocyte type(s) are involved, and implant insertions to describe alterations in the hemocyte-mediated encapsulation response.

METHODS

Materials

Galleria mellonella larvae were acquired from Knutson's Live Bait (Brooklyn, MI) and kept in glass mason jars with breathable lids. Insect diet was prepared by combining 37ml glycerin, 25ml purified water, and 25g cane sugar in a plastic dish. This mixture was then heated for ~20 seconds to dissolve the sugar, stirred, and added to 400ml of Gerber multigrain cereal (Fremont, MI) in a plastic Ziploc bag. Gerber multigrain cereal contains several key vitamins (A, B1, B2, B3, B6, B12, C, E) and minerals (calcium, iron, phosphorus, zinc). In order to ensure an even distribution of the liquid throughout the cereal, the Ziploc bag was hand-mixed for ~2 minutes. The diet was cooled to room temperature before use and excess diet was stored at 4°C for up to 2 months. Larvae were maintained in a dark incubator at 35°C and 80% relative humidity to provide the conditions necessary for a normalized life cycle. Larvae that reached day 3 of their 7^{th} instar stage were used for all experiments.

Anticoagulant buffer and neutral red stain were used for nearly all studies with *Galleria mellonella*. Therefore, stock solutions of anticoagulant buffer (98mM NaOH, 145mM NaCl, 17ml Na_2EDTA, and 41mM citric acid, pH 4.5) and neutral red stain (2mg bacto-neutral red in 1ml anticoagulant buffer) were prepared prior to experimentation. NaCl and NaOH were purchased from Sigma-Aldrich, St. Louis, MO; Na_2EDTA and citric acid were acquired from Thermo Fisher Scientific, Pittsburgh, PA; bacto-neutral red was obtained from BD, Franklin Lakes, NJ. Additional chemicals used are described in the proceeding methods.

Experiment One: Toxicity of essential oils and effects on development

The toxicity of essential oils from sweet wormwood, *Artemisia annua*; neem tree, *Azadirachta indica*; and narrow-leaved peppermint, *Eucalyptus radiata* on *Galleria mellonella* larvae was determined. *Artemisia annua* and *Eucalyptus radiata* were acquired from Rocky Mountain Oils, Orem, UT while 100% pure neem oil was purchased from NOW Foods, Bloomingdale, IL. All essential oil solutions were stored in a refrigerator at 4°C until further use. Concentrations of 10%, 20%, 40%, and 80% *A. annua* or *E. radiata* (v/v) and neem essential oil (w/v) were prepared in cholesterol-free soybean oil (Cal Western Packaging Corporation, Memphis, TN). Neem essential oil was carefully weighed and dissolved in soybean oil to achieve the desired concentrations due to its semi-solid nature. One untreated group and one soybean oil-treated group were additionally maintained as controls.

Galleria mellonella larvae of the 7th instar, day 3 weighing 100 ± 10mg were used for each toxicological analysis. Prior to treatment, insects were anesthetized by submersion in water for 10-15 minutes. The larvae were then dried and injected through a proleg with 5µl of essential oil solution using a Hamilton microliter syringe, 24G/2"/30° (Reno, NV). This procedure was applied to 30 larvae per concentration of essential oil. Treated insects were stored in a dark incubator at 35°C and 80% relative humidity. Mortality was recorded 24, 48, and 72 hours post-injection. Pupation was additionally assessed for up to 20 days after initial treatment. Appropriate essential oil concentrations for future experiments were determined by visually inspecting the resulting toxicity curves.

Experiment Two: Impact of Sumi ink on hemocyte mobilization

Galleria mellonella larvae were subjected to KY2 Black Sumi ink (Yasutomo, San Francisco, CA) to observe its effects on the insect immune system. Before each experiment, large ink particles were eliminated by centrifuging 1ml of Sumi ink at 2,000g for 10 minutes. The supernatant was then collected and diluted in double-distilled water to the desired concentration(s). Ink was additionally vortexed for 5 seconds upon initiation of injections. Insects were challenged with Sumi ink at various concentrations and time intervals in order to accurately establish their response.

As previously, *G. mellonella* larvae of the 7th instar, day 3 weighing 100 ± 10mg were used. Prior to treatment, insects were anesthetized by submersion in water for 10-15 minutes. The larvae were then dried and injected through a proleg with 5µl of ink solution using a Hamilton microliter syringe, 26G/2"/30° (Reno, NV). Double-distilled water (5µl per larva) was injected into control insects. Preliminary experiments suggested that the response to ink should be assessed after 4 hours. Treated insects were stored in a dark incubator at 35°C and 80% relative humidity during this time. All future studies employed the same procedure.

Dose-dependence. Four different concentrations of Sumi ink (v/v) were diluted with double-distilled water to obtain 10%, 20%, 40%, and 80%. Microcentrifuge tubes containing 40µl anticoagulant buffer and 4µl neutral red stain were prepared (1 per larva) before hemolymph was collected 4 hours post-injection. In order to extract the hemolymph, a proleg was carefully cut off with micro iris scissors and the larva was bled onto a piece of parafilm. 5µl of hemolymph was aspired from the parafilm and dispensed into a prepared microcentrifuge tube using a 1-5µl capillary micropipet (Drummond

Scientific Company, Broomall, PA). The hemolymph solution was vortexed and incubated (with neutral red) for 5-10 minutes before counting to stain the hemocytes. This procedure was applied to 12 larvae per concentration of Sumi ink.

An improved Neubauer hemocytometer (Hausser Scientific, Horsham, PA) facilitated the observation of hemocytes using a specific procedure. First, cover glass with thickness 22 x 22mm (Corning Inc., Corning, NY) was placed over both chambers of the hemocytometer. Each hemolymph sample was additionally vortexed for a minimum of 5 seconds immediately before use. Next, 10µl of hemolymph solution from the 1st larva was slowly pipetted under the cover glass into the upper chamber. The pipette tip was discarded and replaced to eliminate cross-contamination of hemolymph samples. Lastly, hemolymph solution (10µl) from the 2nd larva was pipetted under the cover glass into the lower chamber.

Hemocytes were classified into one of two groups – plasmatocytes and hemocytes other than plasmatocytes (referred to as "other hemocytes") – based on the classification of Altuntaş et al. (2012). A hemocytometer containing two hemolymph samples was placed under a light microscope (Olympus BX40, Tokyo, Japan) to observe, differentiate, and count hemocytes based on their morphological differences. Plasmatocytes exhibit rapid cytoplasmic spreading whereas other hemocytes retain a circular shape. Phagocytosis of ink particles was assessed by visually examining each hemocyte. Non-phagocytizing hemocytes remained clear-pink and phagocytizing hemocytes turned black in color. The numbers of black plasmatocytes and other hemocytes versus normal plasmatocytes and other hemocytes was recorded. All future studies employed the same methods for hemocyte profiling and phagocytosis assessment.

Time-dependence. Preceding results from the dose-dependent mobilization of hemocytes study indicated that KY2 Black Sumi ink produces the greatest immune response in *Galleria mellonella* larvae at a concentration of 40%. Accordingly, the time-dependent effects of 40% Sumi ink on *G. mellonella* hemocytes were examined for up to 6 hours after injection. Hemolymph was extracted for hemocyte counting at 30, 120, 180, 240, and 360 minutes post-injection. This procedure was applied to 10 larvae per time interval. Hemocytes were subsequently counted and phagocytosis was assessed at each time period. The numbers of black, phagocytizing plasmatocytes and other hemocytes versus clear, non-phagocytizing plasmatocytes and other hemocytes was recorded to measure the percentage of phagocytosis.

Experiment Three: Effects of two essential oils on hemocytes

Differentiated hemocyte counts and hemocyte-mediated phagocytosis were determined for ink-challenged *Galleria mellonella* larvae responding to essential oils from sweet wormwood, *Artemisia annua* and narrow-leaved peppermint, *Eucalyptus radiata*. Based on preceding toxicity results from experiment one, *Artemisia annua* and *Eucalyptus radiata* (v/v) were diluted in cholesterol-free soybean oil to concentrations of 2%, 5%, 10%, and 30%. Larvae were injected with 5µl of 40% Sumi ink followed by a second injection (5µl) of essential oil solution. Control insects were also injected with 5µl of 40% Sumi ink, with a subsequent injection (5µl) of pure soybean oil. These procedures were applied to 10-14 larvae per concentration of essential oil. Hemocytes were counted and phagocytosis was assessed after 4 hours.

Experiment Four: Hemocytic response to different forms of neem

The immune response of ink-challenged *Galleria mellonella* larvae to pure neem essential oil from the neem tree, *Azadirachta indica*, was studied. Neem essential oil was carefully weighed and dissolved to achieve the desired concentrations due to its semi-solid nature. Based on preceding toxicity results from experiment one, neem essential oil (w/v) was prepared in cholesterol-free soybean oil at concentrations of 0.1mg, 0.25mg, 0.5mg, and 1.5mg per larva. Larvae were injected with 5µl of 40% Sumi ink followed by a second injection (5µl) of neem oil solution. Control insects also received 5µl of 40% ink, with a subsequent injection (5µl) of pure soybean oil. These procedures were applied to 10-14 larvae per concentration of essential oil. Hemocytes were counted and phagocytosis was assessed after 4 hours.

Plant extract from neem bark in aqueous propylene glycol (Sigma-Aldrich, St. Louis, MO) was tested on larvae of *Galleria mellonella* to compare its activity to that of neem essential oil. Neem bark extract was diluted in double-distilled water to concentrations of 10%, 20%, 30%, and 100%. Anesthetized insects were injected with 5µl of 40% Sumi ink followed by a second injection (5µl) of neem bark extract solution. Control larvae were injected with 5µl of 40% ink and received a subsequent injection (5µl) of pure double-distilled water. This procedure was applied to 16-20 larvae per concentration of neem bark extract. Hemocytes were counted and phagocytosis was assessed after 4 hours.

Larval stage *Galleria mellonella* were injected with Ferti-Lome Triple Action Plus RTU (Voluntary Purchasing Goods Inc., Bonham, TX) and hemocytic responses were observed. This particular neem insecticide contains the following active

ingredients: 0.90% neem oil extract, 0.20% piperonyl butoxide technical, and 0.02% pyrethins. The insecticide (v/v) was prepared in cholesterol-free soybean oil at concentrations of 10% and 30%. Larvae were injected with 5µl of 40% Sumi ink followed by a second injection (5µl) with neem insecticide solution. Control insects received only one 5µl injection of 40% ink. This procedure was applied to 15-20 larvae per concentration of Ferti-Lome Triple Action Plus RTU insecticide. Hemocytes were counted and phagocytosis was assessed after 4 hours.

Experiment Five: Effects of azadirachtin on hemocytes and pupation

Ink-challenged larvae of *Galleria mellonella* were injected with azadirachtin (Sigma-Aldrich, St. Louis, MO), the presumed compound in neem trees responsible for impairing the insect immune system. The injected dose of Sumi ink was lowered from 40% to 20% due to high mortality. Azadirachtin was prepared in 35% ethanol and administered at a single dose of 2.5µg per larva. Control insects were injected with 35% ethanol alone. This procedure was applied to 20 larvae per treatment. Hemocytes were counted and phagocytosis was assessed after 4 hours. The percentage of larvae that pupated was additionally evaluated for up to 20 days post-injection. Azadirachtin was stored at -18°C until further use to prevent degradation.

Experiment Six: Toxicity of *Beauveria bassiana* and effects on development

Galleria mellonella larvae were subjected to various concentrations of mycoinsecticide in order to observe the nodulation response of hemocytes. Mycotrol O (LAM International, Butte, MT) was obtained from BioWorks, Inc. for this experiment.

This particular biological insecticide contains 10.9% *Beauveria bassiana* Strain GHA, or 2 x 10^{10} viable spores per gram of active ingredient. Previous research indicates that *B. bassiana* is active at a concentration of 1 x 10^{7} spores per ml (Zibaee and Bandani, 2010). Consequently, Mycotrol O was diluted in double-distilled water to concentrations of 10^{7}, 10^{8}, 10^{9}, and 10^{10} spores/ml.

A toxicological study was conducted to test and observe the activity of *Beauveria bassiana* spores. Experimental larvae were anesthetized and injected with 5µl of spore solution. Control insects were injected with 5µl of double-distilled water. This procedure was applied to 20 larvae per treatment. Mortality was recorded throughout 5 days after injection and hemolymph was observed using phase contrast microscopy.

Experiment Seven: Encapsulation response to neem essential oil

Findings from preceding experiments involving the response of *Galleria mellonella* larvae to three different essential oils (*A. annua, E. radiata,* and neem) strongly suggested that neem essential oil particularly impacted the insect immune system. In order to further investigate this immune response, an additional study involving the encapsulation of nylon implants by hemocytes was conducted. Neem essential oil (w/v) was prepared in cholesterol-free soybean oil at concentrations of 0.5mg and 1.5 mg per larva based on prior results.

Sections of nylon monofilament (Spiderwire, Spirit Lake, IA) 5mm long and 0.2mm in diameter were implanted into *G. mellonella* larvae. Each larva received one implant and was subsequently injected with 5µl of 0.5mg or 1.5mg neem essential oil. Two different control groups were maintained: insects implanted with one nylon implant

followed by an injection of cholesterol-free soybean oil alone and insects that only received one nylon implant. These procedures were applied to 40 larvae per treatment. The implants were dissected and photographed under a stereomicroscope after 24 hours. To determine the degree of encapsulation, resulting images were evaluated for optical density using ImageJ (National Institutes of Health, Bethesda, Maryland). This software processed images by converting the image to binary and then comparing the numbers of black versus white 'grey' scale units (Figure 2).

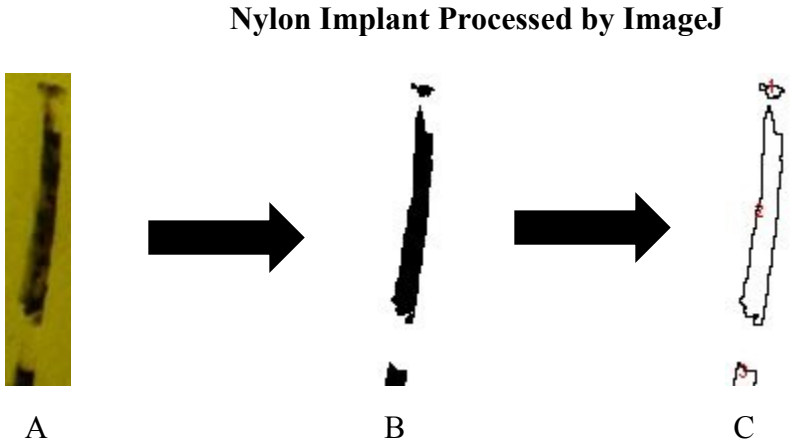

Figure 2. Example of a nylon implant processed by ImajeJ that was extracted from an untreated *Galleria mellonella* larvae. A. Original photograph. B. Conversion of image into binary. C. Final image with analyzed particles.

RESULTS

Experiment One

Galleria mellonella larvae were subjected to three different essential oils including *Artemisia annua*, *Eucalyptus radiata*, and neem essential oil at concentrations of 10%, 20%, 40%, and 80%. Mortality was assessed 48 hours after injection for each treatment (Figure 3). Both *A. annua* and *E. radiata* produced nearly 80% mortality, with the former exhibiting a higher mortality at lower concentrations. Contrarily, larvae treated with neem essential oil exhibited an exceptionally low percent mortality of 25%.

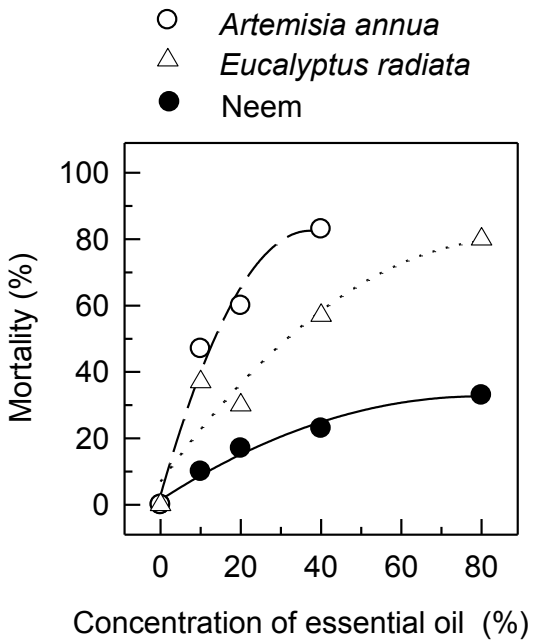

Figure 3. Toxicity curves of three different essential oils on *Galleria mellonella* larvae. Solid points and solid line represent neem essential oil; open triangles and dotted line represent *Eucalyptus radiata*; open points and dashed line represent *Artemisia annua*. N=30 larvae per mean. Mortality was assessed 48 hours after injection.

Percent mortality and pupation were also measured cumulatively 12 days after injection with essential oil (Table 1). Neem doses (w/v) were converted into mg/larva for accurate conclusions. *A. annua* and *E. radiata* produced nearly 100% mortality at all concentrations, with the former exhibiting a slightly higher mortality. Larvae treated with neem essential oil demonstrated a percent mortality of only 40-60%. However, no pupation was observed in neem-treated insects at each individual concentration. Nearly all remaining larvae treated with *E. radiata* produced pupae. Since *A. annua* was highly toxic, pupation only occurred at the lowest concentrations where a few insects subsisted.

Table 1. Effects of selected essential oils on pupation and mortality of *Galleria mellonella* larvae 12 days after injection. N=30 larvae per mean.

Oil concentration	Pupae (%)	Mortality (%)
0 (control)	56	16
Artemisia annua (%)		
10	8	92
20	0	100
40	0	100
80	0	100
Eucalyptus radiata (%)		
10	20	80
20	16	84
40	0	100
80	0	100
Neem oil (mg/larva)		
0.5	0	56
1.0	0	40
2.0	0	36
4.0	0	64

The effects of neem essential oil on mortality and pupation were observed for up to 20 days after treatment (Table 2). Mortality varied considerably between concentrations, with 84% at 4 mg/larva and 80% at 0.5 mg/larva. Untreated insects exhibited 24% mortality with 76% pupation. No pupation was observed at 1, 2, and 4 mg/larva of neem essential oil, and only 8% pupation occurred at 0.5 mg/larva.

Table 2. Effects of neem essential oil on pupation and mortality of *Galleria mellonella* larvae 20 days after injection. N=30 larvae per datum point.

Neem concentration (mg/larva)	Pupae (%)	Mortality (%)
0	76	24
0.5	8	80
1.0	0	72
2.0	0	76
4.0	0	84

Experiment Two

Dose-dependence. *Galleria mellonella* larvae were injected with KY2 Black Sumi ink at concentrations of 10%, 20%, 40%, and 80%. The differential hemocyte count showed an initial drop in plasmatocytes at 10% ink (Figure 4A). This drop was followed by a dramatic rise and eventual peak in plasmatocyte numbers at 40% ink. Contrarily, other hemocytes did not exhibit this dose-response. Plasmatocyte numbers changed from 100,000 cells/ml at 10% ink to nearly 350,000 cells/ml at 80% ink. On the

other hand, numbers of other hemocytes remained at approximately 300,000 cells/ml despite increasing doses of ink.

Both plasmatocytes and other hemocytes exhibited a significant rise in phagocytosis with increasing concentrations of ink, achieving maximum phagocytosis at 20% (Figure 4B). However, the percentage of hemocytes that underwent phagocytosis differed between the two hemocyte groups. Nearly 100% of plasmatocytes subjected to 20% ink were involved in phagocytosis while other hemocytes reached only 60% phagocytosis at this same ink concentration. Phagocytosis of both hemocyte groups remained consistent at doses greater than 10% ink.

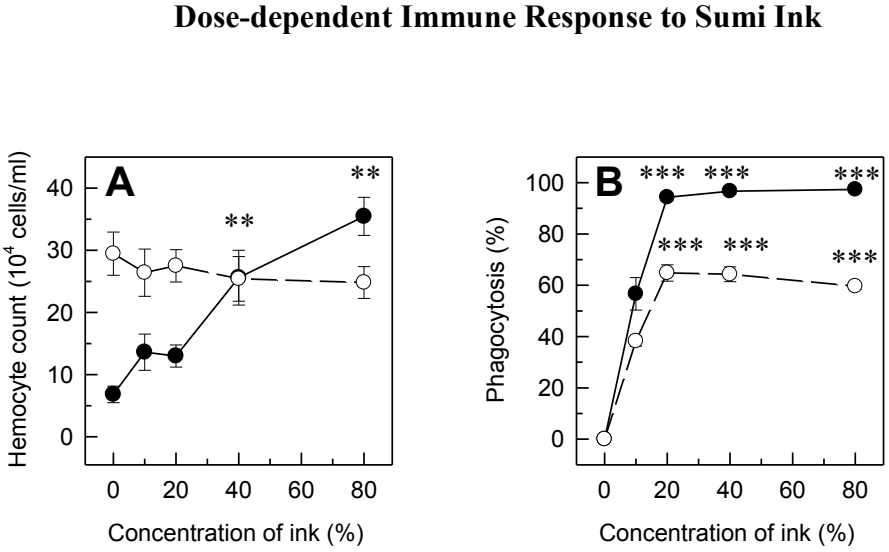

Figure 4. Dose-dependent immune response of *Galleria mellonella* larvae to different concentrations of Sumi ink. A. Changes in total hemocyte numbers. B. Dynamics of phagocytosis. Solid points and solid line represent plasmatocytes; open points and dashed line represent other hemocytes. Data are expressed as means ± SEM. N=10-11 insects per mean. ** $P<0.01$, *** $P<0.001$ in ANOVA followed by Bonferroni comparison of means.

Time-dependence. Hemolymph of larvae challenged with 40% Sumi ink was observed for up to 6 hours after injection. Hemocytes were differentiated and phagocytosis was assessed at time intervals of 30, 120, 180, 240, and 360 minutes post-injection. Both plasmatocytes and other hemocytes demonstrated a slow increase in hemocyte count until 240 minutes after injection (Figure 5A). At this time, the hemocyte counts of both groups were identical and maximum hemocyte numbers were achieved.

Time-dependent Immune Response to Sumi Ink

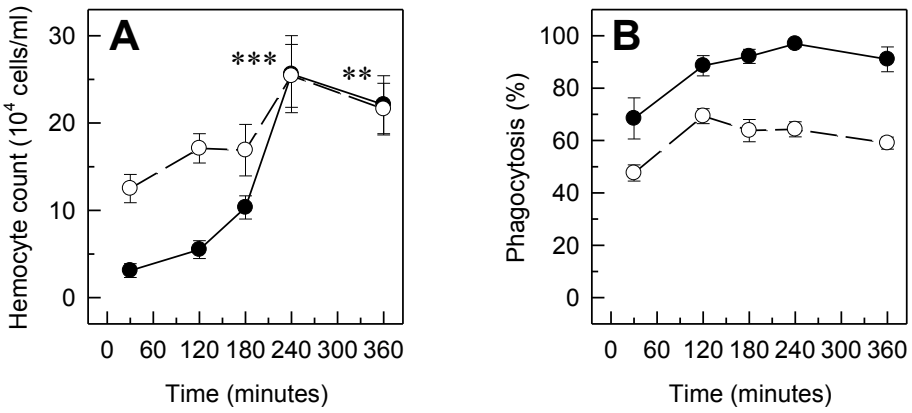

Figure 5. Time-dependent immune response of *Galleria mellonella* larvae to 40% Sumi ink. Hemocytes were observed 30, 120, 180, 240, and 360 minutes after injection. A. Changes in total hemocyte numbers. B. Dynamics of phagocytosis. Solid points and solid line represent plasmatocytes; open points and dashed line represent other hemocytes. Data are expressed as means ± SEM. N=10 insects per mean. ** $P<0.01$, *** $P<0.001$ in ANOVA followed by Bonferroni comparison of means.

The percentage of phagocytosis increased significantly for both plasmatocytes and other hemocytes between 30 and 120 minutes post-injection. However, the maximum phagocytosis by these two hemocyte groups differed in both percentage and

time interval (Figure 5B). Plasmatocytes exhibited a maximum phagocytosis of nearly 100% at 240 minutes after injection. Other hemocytes reached their maximum 120 minutes post-injection, with only about 70% of cells phagocytizing.

Experiment Three

Galleria mellonella larvae were challenged with 40% Sumi ink and subsequently exposed to *Artemisia annua* and *Eucalyptus radiata* at concentrations of 2%, 5%, 10%, and 30%. No significant changes were observed for hemocyte counts of both plasmatocytes and other hemocytes responding to different concentrations of *Artemisia annua* (Figure 6A). Overall, there were less plasmatocytes than other hemocytes, with

Dose-dependent Immune Response to *Artemisia annua*

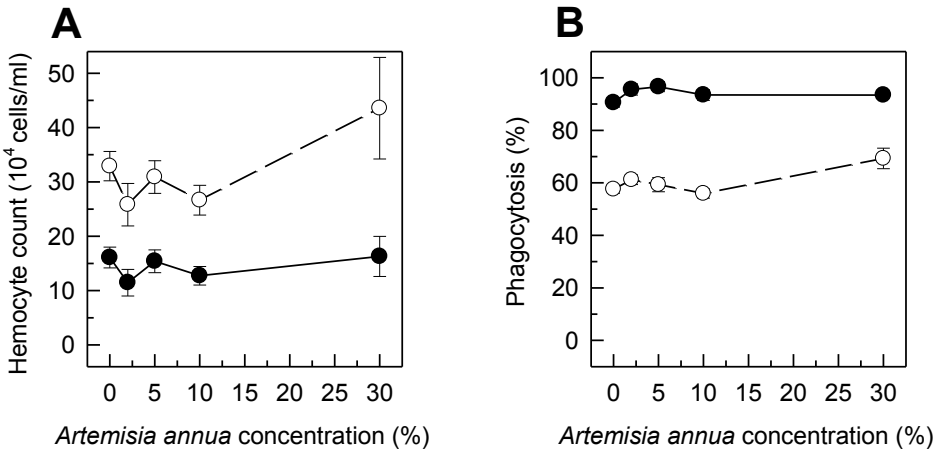

Figure 6. Dose-dependent immune response of *Galleria mellonella* larvae to sweet wormwood (*Artemisia annua*) essential oil. A. Changes in total hemocyte numbers. B. Dynamics of phagocytosis. Solid points and solid line represent plasmatocytes; open points and dashed line represent other hemocytes. Data are expressed as means ± SEM. N=8-11 insects per mean. There were no significant differences between means as determined by ANOVA.

about 400,000 plasmatocytes and 100,000 other hemocytes per ml of hemolymph at 30% *A. annua*. The percentage of phagocytosis for both hemocyte groups also remained essentially the same between all four doses (Figure 6B). Nevertheless, phagocytosis in plasmatocytes was higher (100%) when compared to that of other hemocytes (60-65%).

Plasmatocytes and other hemocytes of larvae injected with *Eucalyptus radiata* followed identical patterns to that of *Artemisia annua* in total hemocyte counts at all four concentrations. Minor variations were seen between control insects and larvae injected with 10% *E. radiata* (Figure 7A). However, the overall number of plasmatocytes

Dose-dependent Immune Response to *Eucalyptus radiata*

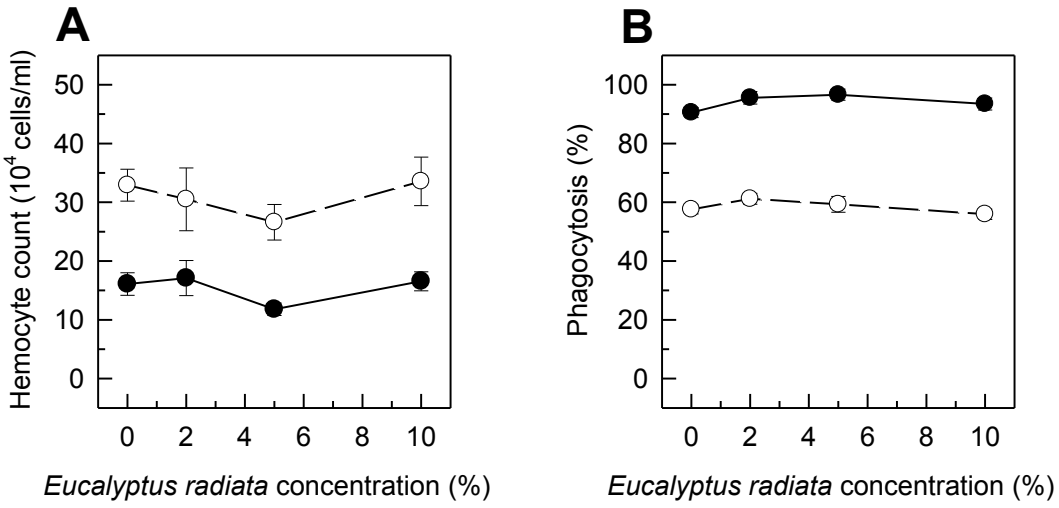

Figure 7. Dose-dependent immune response of *Galleria mellonella* larvae to narrow-leaved peppermint (*Eucalyptus radiata*) essential oil. A. Changes in total hemocyte numbers. B. Dynamics of phagocytosis. Solid points and solid line represent plasmatocytes; open points and dashed line represent other hemocytes. Data are expressed as means ± SEM. N=9-11 insects per mean. There were no significant differences between means as determined by ANOVA.

(150,000 cells) was lower than that of other hemocytes (350,000 cells). The percentage of phagocytosis by both hemocyte groups followed a similar trend, with no apparent dose-response to *E. radiata* (Figure 7B). As with previous findings, 100% of plasmatocytes and 65% of other hemocytes underwent phagocytosis in response to ink.

Experiment Four

Galleria mellonella larvae were challenged with 40% Sumi ink and subsequently exposed to four different concentrations of neem essential oil at 0.1mg, 0.25mg, 0.5mg, or 1.5mg per larva. There were significant changes in the plasmatocyte count of larvae treated with neem essential oil (Figure 8A). Control insects exhibited approximately

Dose-dependent Effect of Neem Essential Oil on Hemocyte Count

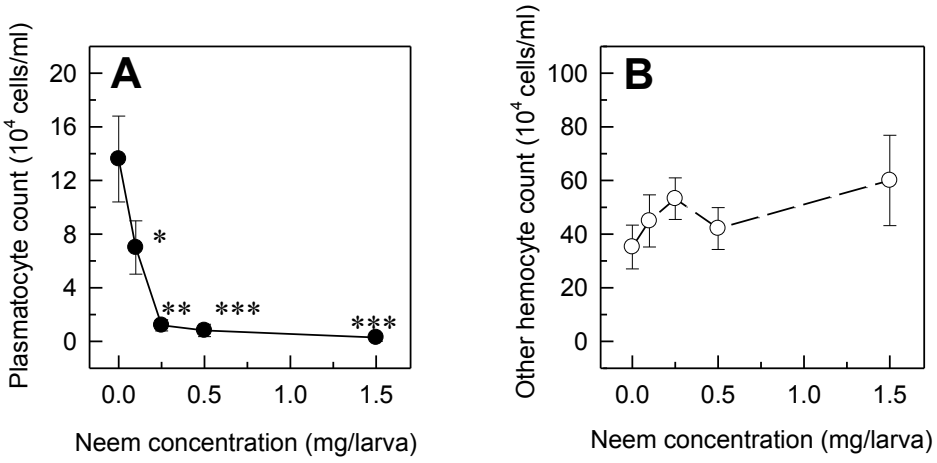

Figure 8. Dose-dependent effect of neem essential oil on hemocyte counts of ink-challenged *Galleria mellonella* larvae. A. Changes in plasmatocyte numbers. B. Variations in numbers of other hemocytes. Solid points and solid line represent plasmatocytes; open points and dashed line represent other hemocytes. Data are expressed as means ± SEM and N=5-11 insects per mean. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ in ANOVA followed by Bonferroni comparison of means.

130,000 plasmatocytes per ml of hemolymph. This number decreased to nearly zero at 0.25 mg/larva of neem oil and stayed the same for the remaining doses. Other hemocytes did not exhibit this pattern, with similar levels at all four doses of neem (Figure 8B).

Phagocytosis by both plasmatocytes and other hemocytes was unchanged between different neem concentrations (Table 3). In most hemolymph samples, plasmatocytes underwent about 100% phagocytosis whereas only 40-60% of other hemocytes phagocytized. However, the percentage of phagocytosis by plasmatocytes was not accurately calculated because as much as 87.5% of larvae injected with neem doses higher than 5% exhibited an absence of plasmatocytes.

Table 3. Effects of neem essential oil on phagocytosis by hemocytes in ink-challenged larvae of *Galleria mellonella*. Data are expressed as means ± SEM. N=5-11 larvae per mean. Means followed by the same letter do not differ significantly (P>0.05, ANOVA followed by Bonferroni comparison of means).

Neem Concentration (mg/larva)	Phagocytosis (%)	
	Plasmatocytes*	Others
0	92.60 ± 3.43 a	42.40 ± 2.16 a
0.1	96.00 ± 2.05 a	49.40 ± 2.02 a
0.25	100.0 ± 0.00 a	54.40 ± 2.65 a
0.5	100.0 ± 0.00 a	51.45 ± 3.06 a
1.5	100.0 ± 0.00 a	53.14 ± 3.20 a

* As much as 87.5% of larvae injected with neem concentrations higher than 5% exhibited an absence of plasmatocytes and phagocytosis could not be calculated.

Ink-challenged *Galleria mellonella* larvae were injected with neem bark extract at concentrations of 0%, 10%, 20%, 30%, and 100%. Full-strength neem extract produced

100% mortality and results could not be collected. The numbers of both plasmatocytes and other hemocytes remained the same for all doses of neem bark extract (Figure 9A). Similarly, phagocytosis by hemocytes was maintained by both hemocyte groups, regardless of increasing neem bark concentrations (Figure 9B).

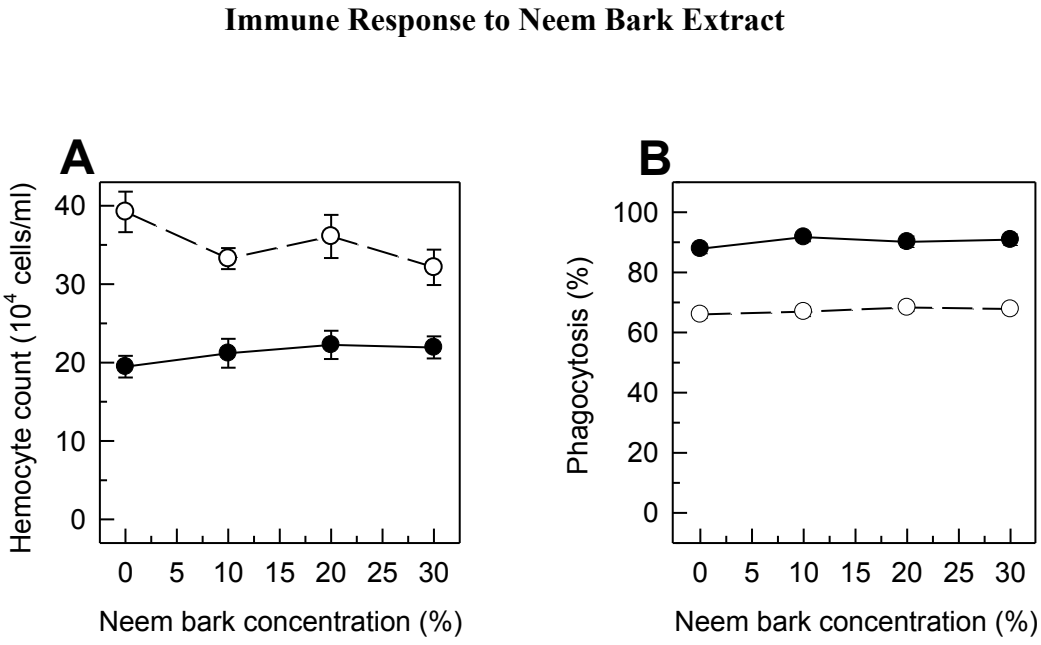

Figure 9. Dose-dependent immune response of *Galleria mellonella* larvae to neem bark extract. A. Changes in total hemocyte numbers. B. Dynamics of phagocytosis. Solid points and solid line represent plasmatocytes; open points and dashed line represent other hemocytes. Data are expressed as means ± SEM. N=12-18 insects per mean. There were no significant differences between means as determined by ANOVA.

Larvae of *G. mellonella* were challenged with ink and subjected to Ferti-Lome Triple Action Plus RTU, an insecticide that contains neem essential oil, at concentrations of 0%, 10%, and 30%. Control injection caused only 6% mortality. Complete mortality was reached at only 30% neem insecticide and thus results were only acquired for one other dose of 10%. At this concentration, 65% morality of larvae was achieved. No

significant differences in hemocyte counts of both plasmatocytes and others were seen between control and treated larvae (Table 4). Furthermore, no changes in phagocytosis by these two hemocyte types were also observed at 10% neem insecticide.

Table 4. Effects of Ferti-Lome neem insecticide on hemocytes of *Galleria mellonella* larvae. Data are expressed as means ± SEM. N=7-15 larvae per mean. Means followed by the same letter do not differ significantly (P>0.05, ANOVA followed by Bonferroni comparison of means).

Treatment	Hemocyte count (10^4 cells/ml)		Phagocytosis (%)	
	Plasmatocytes	Others	Plasmatocytes	Others
Ink only	24.53 ± 1.41 a	41.13 ± 1.80 a	91.80 ± 1.74 a	65.80 ± 0.93 a
Ink + 10% Insecticide	21.43 ± 2.64 a	32.14 ± 3.64 a	87.14 ± 2.58 a	63.14 ± 2.99 a

Experiment Five

Larvae of *Galleria mellonella* were challenged with 20% Sumi ink and subsequently injected with azadirachtin (Figure 1, page 8) at a single dose of 2.5µg per larva. Control insects were injected with 35% ethanol solvent alone. Hemocyte counts for both plasmatocytes and others did not differ between control and treated larvae (Table 5). These counts (10^4 cells/ml) remained at 14.66 ± 1.21 plasmatocytes and 31.87 ± 2.72 other hemocytes. Pupation of larvae 30 days after injection with 2.5µg/larva azadirachtin decreased dramatically. The number of days required for larvae to pupate was only 7.22 ± 0.62 for untreated insects versus 28.63 ± 0.24 for azadirachtin-treated larvae. It is important to note that the majority of insects treated with 2.5µg/larva azadirachtin did not

undergo pupation at the termination of the study. These larvae eventually died off in larval stage.

Table 5. Effects of azadirachtin on hemocyte counts of ink-challenged *Galleria mellonella* larvae. Data are expressed as means ± SEM. N=26 larvae per mean. Means followed by the same letter do not differ significantly (P>0.05, ANOVA followed by Bonferroni comparison of means).

Treatment	Hemocyte count (10^4 cells/ml)	
	Plasmatocytes	Other hemocytes
Solvent †	14.66 ± 1.21 a	31.87 ± 2.72 a
2.5µg/larva azadirachtin	13.00 ± 1.25 a	34.04 ± 2.24 a

† 35% ethanol

Experiment Six

Galleria mellonella larvae were treated with Mycotrol O (2 x 10^{10} *Beauveria bassiana* spores per gram of active ingredient) at concentrations of 10^7, 10^8, 10^9, and 10^{10} spores/ml. Toxicological results showed that all concentrations of *B. bassiana* caused 100% mortality by the 5th day (Table 6). Mortality was only 25% in control, untreated insects. Spores were visually observed in hemolymph samples of treated larvae, with spore numbers increasing daily. However, nodulation by hemocytes was not seen despite the fact that spores were actively germinating. In other words, hemocytes did not aggregate around *B. bassiana* spores or form nodules. Nodulation by hemocytes is typically seen when foreign spores invade insect hosts.

Table 6. Toxicity of Mycotrol O (*Beauveria bassiana*) on *Galleria mellonella* larvae. N=20 larvae per mean. Mortality (%) was assessed for up to 5 days after injection.

Concentration (spores/ml)	24 hours	48 hours	72 hours	96 hours	120 hours
0	0	10	20	20	25
10^6	0	10	20	65	100
10^7	10	30	75	95	100
10^8	5	30	90	95	100
10^9	0	40	100	100	100
10^{10}	100	100	100	100	100

Experiment Seven

The encapsulation of nylon implants by hemocytes of *Galleria mellonella* larvae was studied in association with neem essential oil. Insects treated with neem oil (w/v) received doses of 0.5mg or 1.5 mg per larva. Four different treatments were administered: implant only, implant + solvent, implant + 0.5 mg neem/larva, and implant + 1.5 mg neem/larva. Cholesterol-free soybean oil was used as the solvent for all neem preparations. Neem essential oil did not change the encapsulation response at 0.5mg/larva (Table 7). The average encapsulation for untreated insects and larvae treated with solvent was 806.82 ± 71.95 gray scale units. However, neem at a concentration of 1.5 mg/larva significantly decreased encapsulation to 552.52 ± 57.02 units ($P<0.05$, ANOVA followed by Bonferroni comparison of means).

Table 7. Effects of neem essential oil on encapsulation of nylon implants by *Galleria mellonella* larvae. Data are expressed as means ± SEM. N=30-46 larvae per mean. Means followed by the same letter do not differ significantly (P>0.05, ANOVA followed by Bonferroni comparison of means).

Treatment	Encapsulation (gray scale units)
Implant only	865.13 ± 71.95 a
Implant + solvent †	748.50 ± 71.95 a
Implant + 0.5 mg/larva neem oil	697.00 ± 89.57 a
Implant + 1.5 mg/larva neem oil	552.52 ± 57.02 b

† Cholesterol-free soybean oil

DISCUSSION

All seven experiments were essential for fully understanding the various immune defense mechanisms of larval-stage *Galleria mellonella*. Copping and Menn (2010) found that many essential oils cause direct toxicity to lepidopteran insects. Experiment one verified that essential oils *Artemisia annua*, *Eucalyptus radiata*, and neem were toxic to larvae at certain concentrations. Findings from Azambuja et al. (1991) indicated that raw neem extracts affect insect development. When *G. mellonella* larvae were injected with neem essential oil, pupation was entirely disrupted and larvae were unable to molt into adult moths. Both *A. annua* and *E. radiata* did not cause any changes in larval growth since surviving insects molted normally.

Insect hemocytes are capable of phagocytizing abiotic particles such as India ink (Lavine and Strand, 2008). Hemocytes of *G. mellonella* larvae injected with KY2 Black Sumi ink visually underwent phagocytosis. Small black particles were observed in phagocytizing hemocytes whereas non-phagocytizing cells remained naturally clear. Dose- and time-dependent studies were additionally conducted to obtain optimum hemocyte-mediated phagocytosis. Results indicated that 40% ink produced the highest peak of hemocytes, with optimal hemocyte mobilization four hours post-injection. This suggests that the signal received by the retrocerebral complex must be consolidated to initiate the cascade of processes leading to hemocyte production.

As mentioned previously, phagocytosis is an important hemocyte-mediated defense mechanism in lepidopterans. According to Strand (2008), plasmatocytes and granulocytes are the only hemocytes capable of phagocytosis. Ink-challenged *Galleria*

mellonella larvae exhibited a plasmatocyte phagocytosis of nearly 100% whereas phagocytosis by other hemocytes averaged 60-65%. Granulocyte phagocytosis could not be accurately measured due to lack of necessary equipment. However, these results clearly contradict findings from Ratcliffe (1993), which showed that plasmatocytes were the only hemocytes capable of phagocytosis in *G. mellonella*. Since 60% of other hemocytes underwent phagocytosis of Sumi ink, plasmatocytes cannot be the only hemocytes that are involved in this defense response. Sumi ink also stimulated plasmatocyte mobilization, with plasmatocyte numbers increasing dramatically 4 hours after injection.

Research regarding effects of essential oils on phagocytosis in *Galleria mellonella* is scarce, and thus no comparisons were made to pre-existing literature. Both *Artemisia annua* and *Eucalyptus radiata* essential oils did not change the hemocytic profile in treated larvae. Furthermore, insects treated with either essential oil maintained hemocyte-mediated phagocytosis of Sumi ink at normal rates. It can therefore be concluded that although *A. annua* and *E. radiata* are highly toxic to *G. mellonella* larvae, they do not affect immune responses involving hemocyte mobilization or phagocytosis of insects responding to Sumi ink.

The fourth experiment focused on further investigation of various neem preparations given the abnormal results that were observed in previous studies with neem essential oil. Interestingly, there were statistically significant decreases in plasmatocyte counts of larvae injected with neem essential oil. All four doses of neem oil inhibited plasmatocyte mobilization to almost zero. Contrarily, numbers of other hemocytes remained constant and phagocytosis by both hemocyte groups was maintained at normal

rates. There is currently no literature that shows or explains this phenomenon, although azadirachtin could presumably be responsible for inhibited plasmatocyte production and/or secretion.

Neem bark extract appeared relatively toxic but did not elicit an immune response in ink-challenged larvae of *Galleria mellonella*. Ferti-Lome Triple Action Plus RTU neem insecticide caused high mortality in treated insects and thus was difficult to study on hemocytes of *G. mellonella* larvae. This insecticide proved to be highly effective against caterpillars, as was indicated on the product label. Hemocytes were only observed for a single dose of 10% neem insecticide. However, no impact on hemocyte mobilization or phagocytosis was evident at this dose.

Butterworth and Morgan (1968) verified that azadirachtin is primarily responsible for the insecticidal activity observed in neem trees. The insect endocrine system involves a series of specific organs and hormones that regulate insect development (Figure 10). Azadirachtin disrupts insect growth by directly or indirectly altering the endocrine pathways necessary for a normalized development. Findings from Khater (2012) suggest that azadirachtin inhibits release of prothoracicotropic hormone (PTTH) from the brain. Since PTTH stimulates the prothoracic glands to produce ecdysone, levels of ecdysone in the hemolymph would ultimately decrease. Unfortunately, research regarding the effects of azadirachtin on the hemocytic profile of lepidopteran insects is limited.

In contrast to neem essential oil, plasmatocytes of ink-challenged *Galleria mellonella* larvae injected with azadirachtin were unaffected. This suggests that PTTH is not involved in the immune response of plasmatocytes to Sumi ink and inhibition of plasmatocytes in neem-treated larvae is not caused by azadirachtin. Consequently, an

additional chemical in neem essential oil is presumably responsible for lowering plasmatocyte numbers. Both plasmatocytes and other hemocytes exhibited phagocytosis at normal levels while other hemocyte numbers were unchanged, further proving that azadirachtin does not affect hemocytes directly.

Schematic Drawing of the Insect Endocrine System

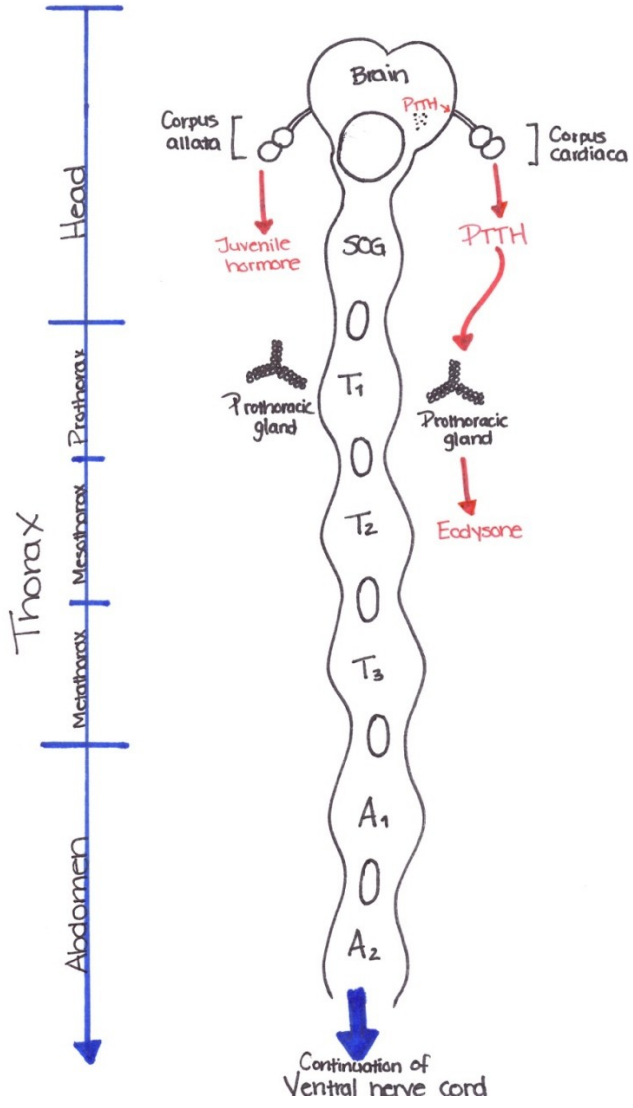

Figure 10. Schematic drawing of endocrine system involved in the development of *Galleria mellonella* larvae. SOG: suboesophageal ganglion; T_1: first thoracic ganglion; A_1: first abdominal ganglion.

Entomopathogenic fungi such as *Beauveria bassiana* have widely been studied for control of various insects (Zibaee and Bandani, 2010). Mycotrol O, a mycoinsecticide containing spores of *B. bassiana*, was highly toxic to *Galleria mellonella* larvae since all concentrations caused 100% mortality within 5 days after injection. Hemolymph extracted from dead larvae indicated that mortality was most likely caused by spore germination. Visual observation of hemolymph showed spore numbers increasing daily and pre-existing spores elongating. Results suggest that Mycotrol O can be an effective, biological method for insect control of larval stage lepidopterans. However, the manner by which Mycotrol O killed the larvae remains unknown. It is theoretically possible that the insects were unable to identify *B. bassiana* spores as foreign invaders. Another hypothesis involves the presence of an additional substance(s) in the formulation of Mycotrol O that is responsible for the observed mortality.

Findings from Bidochka and Khachatourians (1987) showed that *B. bassiana*-infected *Melanoplus sanguinipes*, the migratory grasshopper, underwent hemocyte-mediated nodulation. Contrarily, no nodulation was evident in larval hemolymph of *Galleria mellonella* injected with Mycotrol O despite the active germination of *B. bassiana* spores. Studies with a melanic morph of *G. mellonella* from Dubovskiy et al. (2013) indicated that the species is becoming increasingly resistant to *B. bassiana* strains. This could explain why no hemocyte-mediated nodulation response was observed in hemolymph of *G. mellonella* larvae injected with Mycotrol O. Furthermore, Vilcinkas et al. (1997) demonstrated the suppression of phagocytosis and encapsulation in fungi-

infected insects. *B. bassiana* could be affecting the cellular defense responses of infected *G. mellonella* larvae, resulting in absence of nodulation.

As mentioned previously, encapsulation is another primary hemocyte-mediated defense response in insects (Strand, 2008). Krams et al. (2012) measured hemocyte-mediated encapsulation through the use of nylon implants. Thus, sections of nylon monofilament were implanted (one per larva) into *Galleria mellonella* treated with neem essential oil in order to observe effects on encapsulation response. There are no comparisons to literature because research in this area is scarce. Results showed that high doses of neem oil (1.5 mg/larva) significantly decreased the degree of encapsulation in nylon implants. It can therefore be concluded that neem essential oil impairs the immune system of *G. mellonella* larvae by inhibiting both plasmatocyte mobilization and encapsulation by hemocytes.

In conclusion, simultaneous injections of plant-derived substances and Sumi ink effectively portray the hemocyte-mediated responses involved in *Galleria mellonella* larvae. Additional methods, including entomopathogenic fungi and nylon implants, allowed further understanding of specific mechanisms involved in the insect immune system. Results showed that Sumi ink injections considerably stimulated plasmatocyte production, thus suggesting that ink elicits an immune response in *G. mellonella* larvae. Plasmatocytes appeared to be the primary hemocytes responsible for phagocytosis and encapsulation of foreign agents, although differentiation of granulocytes was inaccurate due to material limitations.

Interestingly, findings indicated that neem essential oil strongly impacts insect growth and plasmatocyte production. Both neem essential oil and azadirachtin disrupted

Galleria mellonella pupation, with only the former inhibiting plasmatocyte production. It is therefore presumed that neem essential oil contains a chemical, in addition to azadirachtin, that produces the observed plasmatocyte increase. Insects responding to azadirachtin do not exhibit changes in hemocyte levels. This suggests that azadirachtin does not affect hemocytes indirectly through hematopoietic organs, or directly via mitosis of pre-existing hemocytes.

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