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**Running Title: Effects of azadirachtins on Asian Long-horned Beetles**

# Inhibition of larval growth and adult fecundity in Asian Long-horned Beetle (*Anoplophora glabripennis*) exposed to azadirachtins under quarantine laboratory conditions.

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**Abstract**

BACKGROUND: The Asian Long-horned Beetle (*Anoplophora glabripennis* (Motschulsky)), is an invasive, wood-boring insect posing significant economic and ecological threats to the deciduous forests of North America. The risk is underscored by both a recent infestation in Ontario Canada and established populations in nearby U.S. states. As part of an integrated response strategy, development of efficacious and environmentally acceptable control techniques are necessary.

RESULTS: Results of our quarantine laboratory experiments demonstrate the multi-mechanistic effects of azadirachtins (TreeAzin™) on larval growth, adult ovipositon effort and fecundity of ALB. Growth inhibitory effects were greatest on early stage larvae, increased significantly with increasing concentration as well as with duration of exposure. ALB adults, exposed via maturation feeding on leaves and twigs imbibed with TreeAzin, showed significant, concentration-dependent inhibition of feeding, oviposition effort and fecundity.

CONCLUSIONS: Results suggest that appropriately timed stem injections of TreeAzin, could lead to acquisition of azadirachtin doses sufficient to reduce within-tree ALB populations as the combined inhibitory effects on adults reproduction and larval growth. Field efficacy studies are required to demonstrate this potential under simulated operational use scenarios.

**Keywords:** azadirachtins, Asian Long-horned Beetle, TreeAzin, inhibition, effects

## 1 INTRODUCTION

Alien invasive species are recognized as globally significant agents of change[1-3] with new introductions likely to increase in proportion to increasing international trade and transport of goods.[4,5] . Although relatively few introduced species become established in their new environments, those pests which do establish and become invasive tend to pose highly significant economic and ecological risks.[6,7]

Of the 450 non-native forest insects known to be established in the United States, wood-boring species are considered to pose the greatest risk of impacts.[8] In Canada, 546 known interceptions of wood-boring insect pests were reported during a single 3 year period (1997 to 2000).[9] Of these, members of the family Cerambycideae accounted for ~ 40% of the total; including the Asian Long-horned Beetle (ALB; *Anoplophora glabripennis* (Motschulsky)) which has been repeatedly intercepted in both Canada and the USA.

Excellent reviews detailing various aspects of ALB introductions to North America, basic biology and eradication efforts have recently been published.[10-12] A native of China and the Korean peninsula, this wood-boring insect pest is internationally recognized as one of the 100 worst invasive species,[13] and is known to attack multiple tree species in North America, including various members of maples (*Acer spp*).[10] ALB interceptions have been documented in more than 17 countries worldwide, typically at ports of entry. Infested solid wood packaging materials (e.g. crates, pallets) associated used in transport of goods have been identified as a key pathway by which ALB has been introduced to new environments and this vectoring mechanism continues despite implementation of improved phytosanitary measures requiring heat or fumigation treatment of solid wood packaging materials (e.g. ISPM-15 and revision).[10,14,15]

Since the original discovery of a breeding population of ALB in Brooklyn, New York in 1996,[16] a number of breeding populations have been documented in several northeastern states (New York, New Jersey, Illinois, Massachusetts) as well as in California.[17] In 2010, ALB was considered to be established in North America.[14] but prevention and eradication efforts continue to be identified as critical elements of a response strategy designed to mitigate risk and impacts.[15] Typically, attempts to eradicate ALB have involved cutting, chipping and burning of those trees known to be infested as well as those considered to be susceptible to attack within delineated management zones, often resulting in destructive removal of thousands of trees.

ALB was first discovered in Ontario Canada in 2003, in a commercial warehouse area bordering Vaughan and the city of Toronto.[17] This discovery triggered an extensive eradication effort lead by the Canadian Food Inspection Agency (CFIA), involving the establishment of a 152 $km^2$ regulated area and destructive removal of approximately 27,400 trees.[17] Although no new infested trees or ALB were observed within the regulated zone per se for several years, a subsequent discovery of an ALB adult in Mississauga ON, just to the east of the previously established regulated zone occurred in 2013. Genetic analyses of that specimen, together with physical evidence from a wood cut slice of a tree near the epicentre, provided evidence to conclude that this second site was a satellite of the original infestation, rather than a new introduction.[18] The Mississauga ON infestation site involved only approximately 50 trees, of which 25 were confirmed to contain live ALB, with at least 10 additional trees showing signs or symptoms characteristic of *A. glabirpennis* attack.[18] Approximately 8600 trees were destructively removed from this satellite area in 2014 (Turgeon personal

communication). To date, annual follow-up surveys since have reported no new infested trees in a broad area around the Mississauga ON site since 2014.

Carter et al. concluded that ALB infestations in northeastern North America can multiply to outbreak proportions in urban areas, even though genetic diversity of the these populations may be very low.[17] Recent evidence from the Worcester MA site demonstrates that ALB can disperse and develop under more natural forest scenarios as well[19] and raises the spectre of potential widespread establishment throughout the mixed wood and deciduous forest regions of eastern North America. In these regions, forest stands commonly contain or may even be dominated by tree species considered susceptible to ALB attack. Known host tree species in North America include members of the genera: *Acer* (maple), *Betula* (Birch), *Ulnus* (elm) and *Salix* (willow) *Aesculus* (Horse chestnut, Buckeye), *Populus* (Aspen), *Sorbus* (Whitebeam, mountain ash) and *Platnus* (Sycamore) among others.[14]

While evidence to date suggests that ALB disperse relatively slowly, should this invasive pest become broadly established throughout the contiguous range of deciduous forests in North America, economic and ecological impacts could be devastating. Estimates of potential economic costs associated with eradication, compensation and property value loss alone, range well into the billions of dollars.[8,20-22] In eastern Canada, maple syrup production which is broadly based on woodlots throughout the southern portions of Quebec and Ontario, comprises 80% of world production and in 2013 was valued at $408 million per annum.[23] The commercial hardwood forest industry in Canada produces more than $11 billion in wood products annually.[24]

Equally important, but more difficult to estimate, would be the loss of ecosystem services, aesthetic, recreational and ecotourism values provided by susceptible forest stands throughout this broad region. One particular example of an ecological value is the provision of habitat for a disproportionally high number of forest-dependent birds, terrestrial mammals, amphibians and reptiles in these regions which are listed as "at risk" in Canada (SARA, http://www.registrelep-sararegistry.gc.ca/).

Given the cumulative potential for economic and ecological impacts, hyper-vigilant prevention and aggressive eradication attempts must continue to be employed as the first lines of defense against ALB. However, research, development and deployment of effective and environmentally acceptable biological and/or chemical control tools are also essential components of a comprehensive, integrated pest management. Early intervention with such control tools could be used to slow the spread of this invasive alien pest per se, and also to reduce some of the costs and impacts of the destructive removal of susceptible trees in eradication attempts. Should the invasive pest actually become broadly established such techniques could also be used to protect particularly high value trees or stands including those in urban parks, conservation areas or in woodlots intensively managed for maple syrup production.

Ongoing efforts aimed at development of control techniques include assessments of various systemic insecticides such as imidacloprid, dinotefuran, emamectin benzoate and azadirachtins.[25-32] Research on potential biological controls is also ongoing and have included several investigations on parasitoid wasps from either the natural home range of ALB or parasitoids native to North America[33] Additionally, the use of fabric bands impregnated with entomopathogenic fungi (e.g. *Metarhizum anisopliae*) and possible use

of entomopathogenic nematodes have been investigated.[34-36] However, to date, there have been no biological controls deployed operationally against ALB in North America and only the neonicotinoid insecticide imidacloprid has been utilized in conjunction with eradication programs in the USA. Recent scientific and regulatory reviews indicate that widespread use of imidacloprid and other persistent neonicotinoid insecticides pose significant risk to the aquatic invertebrates, beneficial insects and their ecological functions.[37-39] As a result, the Canadian federal regulatory authority proposed a phase-out of most uses of imidacloprid in November 2016. [38]

As part of the ongoing effort to identify potential effective and more environmentally acceptable systemic insecticides for use against wood-boring insect pests in Canada, we have focused our attention on azadirachtins. Azadirachtins are a family of natural tetranortriterpenoid compounds extractable from the seeds of the neem tree, (*Azadirachta indica* Juss.). Neem seed extracts are particularly rich in two closely related compounds referred to as azadirachtin A and B (Aza-A and Aza-B) which are the putative active ingredients for observed effects on insects. Formulations prepared from these extracts have activity on a variety of wood-boring and foliar pests including growth inhibition effects on larvae of Emerald Ash Borer [40,41] and are rapidly taken up and translocated following stem injection into deciduous trees including *Fraxinus* (ash) and *Acer* (maple) species. [31,32,41] Azadirachtins are non-persistent both within the tree as well as in the environment generally, and also exhibit relatively low toxicity to mammals, birds, bees and other non-target invertebrates.[42,43] The azadirachtin-based systemic insecticide (TreeAzin™) is now being widely used for management of the Emerald Ash Borer in Canada. In this study, we report on a suite of experiments conducted under quarantine

laboratory conditions, to assess the effects of TreeAzin™ on larval and adult Asian Long-horned Beetle with a view to its potential use against this significant threat to managed woodlots, urban and natural forests of eastern Canada.

## 2 EXPERIMENTAL METHODS

### 2.1 Asian Long-horned Beetle Breeding Colony

All experiments were conducted within the Insect Production and Quarantine Laboratories at Natural Resources Canada, Great Lakes Forestry Centre in Sault Ste. Marie Ontario. These laboratories are approved by the Canadian Food and Inspection Agency as a level PPC-2 containment facility appropriate for studies on exotic invasive insect pest species such as Asian Long-horned Beetle. Test larvae and adults were third and fourth generation progeny from a pioneer colony originally obtained from the USDA-Forest Service quarantine facility in Ansonia, CT, USA in November 2009. The primary colony originated from adults that emerged from infested branch sections obtained from eradication zones in Chicago IL (1999) and Worcester MA (2009) in the United States. Keena has previously detailed the establishment and maintenance of the pioneer colony.[44,45]

Larvae derived from the breeding colony in Sault Ste. Marie were reared in individual plastic containers for two 5 week periods, with unvented and vented lids respectively, at 25°C, 60% RH and in complete darkness. Larvae were then chilled in a walk-in cold room at 4°C, for 10 weeks also in complete darkness, to induce diapause. Following diapause induction, larvae were placed back into original rearing conditions

(25 °C, 60% relative humidity; complete darkness) for 5-8 weeks and allowed to pupate. During the pre-pupation phase, cups were checked daily and pre-pupae were moved to a cavity excavated at the top of the diet within the same cup until they pupated. Pupae were transferred to a 50 mL centrifuge tube and held under the same rearing conditions until adults eclosed. As ALB adults are not sexually mature when they first emerge [16,46] they were maintained at 25 °C, 60% relative humidity and 16:8 L:D photoperiod cycle and provided with Striped Maple (*Acer pensylvanicum*) twigs to allow for prematurational feeding. Striped Maple has previously been shown to be preferred over sugar maple (*Acer saccharum*) as a feeding and oviposition substrate for ALB under laboratory conditions.[16]

**2.2 Selection of Experimental Larvae and Adults**

For experimental purposes, larvae at three different stages of development were selected from the breeding colony. Early-stage larvae (0.1-0.2 g) were 2 weeks old from hatch, whereas mid-stage larvae (0.5-0.7 g) were 5 weeks old, and late-stage larvae (1-2 g) were 13 weeks old. Experimental larvae were measured for length and weighed on a calibrated electronic balance [Mettler Toledo XP205, accurate to 0.015 mg] to determine body mass. A digital photo was taken of each uniquely identified larvae, before randomly placing it into diet of a particular treatment group. Adults which had recently emerged (3-10 days old) and begun to actively feed on Striped Maple twigs were selected for experimental use. A male and female pair were randomly placed into a bell jar for a given experimental treatment group as described below.

### 2.3 Azadirachtins in the Tested Systemic Insecticide Formulation

TreeAzin™ systemic insecticide is a proprietary formulation of azadirachtins owned by the Canadian Forest Service and licensed worldwide for distribution by BioForest Technologies Inc. TreeAzin contains 50 mg $mL^{-1}$ or 5% total azadirachtins A+B as the active ingredients. The formulation batch used for these experiments was manufactured May 2012 (Batch D0-94-1) and certified to contain total azadirachtins within 10% of the stated label claim. A formulation blank solution was prepared in the same manner as the commercial formulation, with the exception that the active ingredients were withheld. Comparative assessment among treatments involving the commercial formulation, distilled water controls, or formulation blanks allowed attribution of larval response either to the active ingredients or potentially to other formulants. The exact constitution of the commercial formulation is withheld to protect proprietary rights. Concentrations of the active ingredients in various experimental treatments were verified by mass spectrometric techniques at the Research Instiute for Pesticides and Water, University Jaume I, Castellon, Spain following the general methods which have been previously described.[31]

#### 2.3.1 Larval Growth Inhibition Experiments

The series of quarantine laboratory experiments focused on examining potential growth inhibition effects for ALB larvae at three different life stages (early, mid or late) following exposure to artificial diet treated with differential levels of TreeAzin.

Using a disposable pipet, appropriate volumes of the TreeAzin formulation were dispensed into the top of warm, unsolidified, 500 g batches of artificial diet contained in separate 500 ml glass beakers. Each batch was stirred vigorously using a clean metal stirring rod to achieve diet test concentrations ranging between 0.25 and 10 mg $kg^{-1}$ fresh weight (f.w.). Formulation blank and distilled water controls were similarly prepared by adding 10 mL volumes of the blank formulation (i.e. all ingredients excepting the active compounds) or of distilled water to the separate batches of artificial diet. Approximately 75 g of each treated diet batch was then dispensed into clean 4-oz or 8-oz plastic diet cups, to generate six replicates of each test concentration of the commercial formulation, as well as formulation and water blanks. Treated diet was allowed to cool and solidify for approximately 1 hour before use in larval feeding assays.

Larvae were transferred to the replicate diet cups containing the various treatments by carving out a pit in the diet with a clean stainless steel reagent digger. The larvae were placed head-first into this pit, and then covered again with the displaced diet. Diet cups were capped and placed in a covered crate, shielded from the light and held in a controlled environment chamber with set points of 25°C and 30-32% relative humidity.

ALB larvae were allowed to feed on artificial diet treatments in their individual cups for varying periods of exposure (i.e. 3, 4, 12, 20 or 35 days before being transferred to clean diet cups. Every 7 days until the end of the 70 day monitoring period, diet cups were opened, the solid diet was carefully broken apart by hand and the larva were gently extracted using clean forceps. Larval length and mass were measured and a digital photo taken to assess morphological deformities relative to pre-exposure photos before being placed back into the diet.

### 2.3.2 Adult Feeding, Oviposition and Fecundity Effects Experiments

In addition to larval exposure studies noted above, a series of laboratory experiments on adult ALB were used to examine potential effects on feeding activity, oviposition effort and fecundity. In the adult experiments, ALB were exposed via feeding on Striped Maple twigs which had been imbibed with water (controls), a dilute aqueous solution of the formulation blank (1:16) or various dilute solutions of the TreeAzin formulation (1:8, 1:16, 1:24 or 1:32 TreeAzin:water) for a period of four days. Individual adult ALB were allowed to feed on these systemically loaded twigs for a period of 7 days.

Systemically loading the treatments involved selecting 6-7 twigs of approximately 0.5 cm diameter and 15 cm length, freshly cutting the lower end of each twig and allowing the twigs in each treatment group to uptake the appropriate aqueous test solutions from separate glass beakers. Beakers were covered tightly in parafilm to prevent evaporation and each contained a total of 40 mL of the treatment solution. Following the 4 day uptake period, twigs were air-dried, weighed and supplied as a feeding substrate to male and female ALB adults approximately 7 days following emergence from their pupal cases. ). Adults were allowed to feed on the imbibed twigs for 7 days in bell jars housed in a controlled environment chamber set at 25°C, 30-32% humidity and with a 15h light / 9h dark photoperiod. Twigs were analyzed to determine azadirachtin concentrations in the feeding substrates using methods as previously described.[31] Average concentrations were determined for each treatment level as: 64 mg $kg^{-1}$ (1:8 dilution), 38 mg $kg^{-1}$ (1:16 dilution), 24 mg $kg^{-1}$ (1:24 dilution) and 8 mg $kg^{-1}$ (1:32 dilution). Beetles were checked daily for health and feeding activity. After 7 days, twigs were removed and weighed to

quantify the mass loss of each twig as a measure of feeding activity. Following the 7 day exposure period, males and females were paired and placed into new bell jars containing 3-4 fresh, uncontaminated Striped Maple twigs and an uncontaminated larger diameter bolt of Striped Maple (18 cm long x 3-5 cm diameter) serving as feeding and potential oviposition substrates respectively. The larger oviposition bolt had ends sealed with wax to preserve moisture content. Bell jars containing adult ALB pairs were retained under the controlled environment chamber conditions for a period of 5 weeks and checked daily for health, mating activity and survivorship. Each week, the pairs of adults were transferred to new mating jars containing fresh twigs and oviposition bolts. The number of oviposition pits on each bolt was recorded weekly as a measure of oviposition effort and the bark was subsequently peeled to allow collection and counting of the total number of viable and non-viable eggs as a measure of fecundity. Egg viability was determined by visual inspection. Viable eggs were characteristically white and plump in appearance, while non-viable eggs were characterized by darker colour and often having a flattened surface.

To examine whether ALB adults show a preference to feed on untreated or treated plant material, a feeding preference test was conducted. In this test, individual males and females were allowed a choice to feed on single treated or untreated twigs contained within the same bell jar. Again differential replication was used, with 9 replicates of water and formulation blank controls as well as 1:16 dilution (38 mg $kg^{-1}$) of the commercial TreeAzin formulation and 3 replicates of a 1:32 dilution treatment (8 mg $kg^{-1}$).

To determine whether oviposition and fecundity effects occur following more realistic exposure scenarios, ALB adult pairs were similarly exposed to branchlets (twigs and foliage) freshly excised from one of six experimental sugar maple trees (*Acer saccharum*). In this experiment, 3 trees were stem injected with TreeAzin under typical operational protocols and 3 were left untreated. Experimental trees averaged 19.8 ± 1.2 cm in diameter with no significant difference between control and treated diameter means (t-test $P = 0.163$). Treated trees were injected with TreeAzin formulation at a rate of 0.25 g/cm of tree diameter at breast height using the Ecoject injection system, 4 injection ports per tree and a total of either 5 or 6 20 mL canisters, depending upon individual tree diameter, as required to deliver appropriate total formulation volumes per tree. Branchlets to which ALB adults were exposed were excised with pole pruners from the mid-canopy of treated trees 4 days following injections.

**2.4 Statistical Analysis**

All statistical analysis including calculation of means and standard errors (SE), t-tests, standard Analysis of Variance (ANOVA) and Repeated Measures Analysis of Variance (RM-ANOVA) were completed using SigmaPlot v.12.3 (Systat Software Inc., 2008). A probability value of $P = 0.05$ was used to declare statistically significant effects in all tests.

For the larval growth inhibition experiments, raw larval weight (g) data for each individual were converted to % weight gain for any given observation. Percent weight gain for each individual insect was calculated as: % gain = [observed mass – initial mass)/initial mass]*100). This approach normalized for slight differences in initial size

of larvae randomly assigned to treatment groups. Means and standard errors (SE) were calculated for each treatment group and compared using RM-ANOVA. For adult experiments, feeding activity was determined based on percent mass loss of twigs (Initial mass – final mass/initial mass * 100) during the 7 day exposure period. Treatment means were compared by ANOVA.

Effects on female oviposition effort were assessed based on ANOVA comparisons of weekly average number of oviposition pits excavated by females in each treatment group. The cumulative total number of oviposition pits per female over the 4 week observation period were also calculated and compared. Fecundity effects were assessed by comparing the mean cumulative number of viable eggs laid by females in each treatment group over the observation period.

## 3 RESULTS AND DISCUSSION

### 3.1 Larval Growth Inhibition

TreeAzin exposure induced significant, concentration-dependent growth inhibition on early-stage ALB larvae for all tested exposure periods. Growth inhibition increased with both TreeAzin concentration and with increasing periods of exposure ranging from 3 to 21 days (Fig. 1). Figure 1 shows a threshold of activity approximating 1 mg $kg^{-1}$ and statistically significant ($P = 0.05$) reduced growth at 5 mg $kg^{-1}$ thru 42 days of observation.

In contrast, no significant differences in cumulative mean % growth were observed for mid-stage larvae exposed for either 3 days (observation period = 70 days; ANOVA $P =$ 0.637) or for 12 days (observation period = 77 days; ANOVA $P =$0.359). However,

under a 12 day exposure regime (Fig. 2), both 1 mg $kg^{-1}$ and 5 mg $kg^{-1}$ test levels showed consistently lower mean % growth relative to controls on day 56, 63 and 70 of the observation period. Although there was evidence of concentration-dependence, comparative mean responses within any specific day typically failed to show statistically significant differences among the various concentrations tested. These findings were interpreted as indicating a weak inhibitory effect on mid-stage larvae at diet concentrations exceeding 1 mg $kg^{-1}$ of TreeAzin. Very low exposure concentrations approximating 0.25 mg $kg^{-1}$, appeared to show a weak stimulatory effect.

Results for the 35 day exposure period (Fig. 3) showed a strong growth inhibitory effect for mid-stage larvae exposed to the 5 mg $kg^{-1}$ test concentration. The inhibitory effect was sustained throughout 70 days of observation. A weaker inhibitory effect was observed for the 1 mg $kg^{-1}$ concentration, with depressed growth relative to controls sustained between day 7 through day 49. Linear regression of larval weights (g) versus test concentration (mg $kg^{-1}$) for the day of maximal effect (42) and for the last day of observation (70) were both significant ($P$ <0.001), with slopes indicating that each unit increase in concentration resulted in reduction of larval weight by 0.18 g.

Similar results to those noted above were observed for larvae exposed for a period of 20 days (Fig. 4). The greatest inhibitory effect was observed at the 5 mg $kg^{-1}$ test level, which resulted in sustained growth inhibition from day 14 through day 42, followed by a period of recovery from day 42 to day 70. Linear regression analysis applied to the data on day 42, showed a significant reduction in % weight gain with increasing level of TreeAzin in the diet ($P < 0.001$; $df$ =1,25; $a$ = -23.13; $r^2$= 0.82) . Despite evidence of slow recovery when larvae were moved to clean diet and fed ad libitum, those individuals

exposed to the 5 mg $kg^{-1}$ concentration for 20 days, never attained total weights equivalent to those of the control group. Thus, such levels of exposure would likely inhibit growth of ALB throughout a typical Canadian summer and we postulate that resultant reduced larval mass may impair over-winter survivorship and emergence in the following year.

### 3.2 Inhibition of Feeding, Oviposition and Fecundity of Adult ALB

#### 3.2.1. Feeding Inhibition

Two separate experiments were used to examine potential feeding inhibition for adult ALB exposed to TreeAzin under simulated maturation feeding conditions. Adults were allowed to feed on Striped Maple twigs imbibed with dilute solutions of TreeAzin, the formulation blank or distilled water as an untreated control. The first experiment was limited to only 3 males and 4 females in each treatment group owing to constraints on adult availability from our newly developing ALB colony. For the group exposed to TreeAzin (38 mg $kg^{-1}$), loss of twig mass resulting from ALB feeding, was 7-11% lower than that observed for the control or formulation blank treatment groups, with means of twig mass (± SE) of 24.1 ± 4.7, 31.6 ± 4.3 and 34.8 ± 2.6, respectively. Two-way RM-ANOVA analysis of the experimental data showed no significant differences in feeding activity between sexes ($P = 0.41$), among control, formulation blank or TreeAzin (38 mg $kg^{-1}$) treatments ($P = 0.19$), and no significant interaction between sex and treatment ($P = 0.75$). The lack of statistical significance in this case was considered to be attributable to the low level of replication and marginal inhibitory response.

In a similar follow-up experiment, involving greater numbers of males and females as well as additional dilution levels for the TreeAzin treatments, a significant interaction between sex and treatment ($P < 0.001$) was observed. A subsequent t-test confirmed that females consumed a significantly greater mass of twigs ($n = 8$; 40.9 ± 3.4%) than males ($n = 9$; 18.9 ± 1.9%), possibly reflecting their generally greater body size and needs for energy acquisition to support egg development. As such, data for male and female adult ALB were analyzed separately via one-way ANOVA. For males, no significant differences in mean % mass loss of twigs were observed among treatment groups ($P = 0.79$) (Fig 5). However, a significant feeding inhibition effect ($P < 0.001$) was observed for females exposed to the 38 mg $kg^{-1}$. Mean mass loss of twigs (20.4 ± 1.5% g) in the TreeAzin treatment group was far less than that observed in controls (40.7 ± 3.0 g) or formulation blank treatments (42.3 ± 4.0), indicating an approximate 50% reduction in feeding. For females exposed to the different levels of TreeAzin formulation, mean mass loss differed significantly ($P < 0.05$) from that observed for formulation blank and control groups, indicating that the azadirachtin active ingredients rather than other formulation ingredients were responsible for the observed inhibition of feeding activity.

As shown in Fig 5, the mean % mass loss for the 64 mg $kg^{-1}$ TreeAzin treatment as observed for females (26.6 ± 4.3%) was also substantially lower than that observed for control and formulation blank treatments for females, but the difference was not statistically significant. This was considered an artefact of the unequal replication ($n = 3$) for this treatment as compared to the larger number of replicates that were assigned to the 38 mg $kg^{-1}$ TreeAzin dilution, controls and formulation blank treatments. This postulate was supported by post-hoc t-tests which showed significant differences ($P = 0.05$) for

both the 64 mg $kg^{-1}$ and 38 mg $kg^{-1}$ TreeAzin treatments relative to the control, but no such differences for lower exposure levels 24 mg $kg^{-1}$ or 8 mg $kg^{-1}$ TreeAzin treatments. Overall, under quarantine laboratory study conditions, ALB adults exposed to twigs systemically loaded with TreeAzin showed concentration dependent feeding inhibition that differed between males and females. In North America, ALB are known to feed extensively on both twigs and foliage of at least 18 different tree species, but show a particular preference for maples.[16,46] We have previously demonstrated that azadirachtins are taken up and rapidly translocated throughout the canopy of deciduous trees, resulting in foliage and twig residues ranging from approximately 1-6 mg $kg^{-1}$ in various ash and maple species.[31,41] In 3 of 4 tree species (Norway Maple, Red/Freemani Maple and London Planetree) that are known hosts for ALB in North America, maximal foliage levels ranged from 2-6 mg $kg^{-1}$ and declined with time. Foliar residues were typically greater than those in whole twigs, reflecting the transpiration-driven translocation process where foliage acts as a temporary sink for azadirachtin residues. As such, typical foliar and twig residue levels in systemically injected host tree species may be anticipated to be below those required to inhibit female ALB feeding. Therefore, we consider that ALB adults are likely to feed normally on host trees systemically injected with TreeAzin and acquire dose levels of azadirachtin through that maturation feeding process. However, since the relative ingestion rates of twigs and leaf material, as well as the duration of active feeding in systemically injected trees has not been examined under natural conditions, field studies designed to quantify real world exposure duration and magnitude in relation to possible effects on feeding inhibition, oviposition and fecundity as discussed below are required as key next steps.

### 3.2.2 Effects on Oviposition Effort

The mean number of oviposition pits excavated by females exposed to twigs imbibed with TreeAzin (38 mg $kg^{-1}$) was consistently lower than that observed for females exposed to control or blank formulation treatments in each of the five weeks of observation post-treatment (Fig 6).

Peak oviposition effort occurred within the first two weeks in all treatment groups and generally declined thereafter through the remainder of the observation period. The temporal pattern in oviposition effort is similar to that reported by Keena under similar laboratory conditions.[45] The greatest mean (± SE) number of oviposition pits observed for controls in weeks 1 and 2, were 26.1 ± 4.4 and 27.0 ± 3.9 and were somewhat lower than the average of 30-37 pits observed in week 2 as reported by Keena.[46] Over the 5 week observation period, the cumulative total number of oviposition pits excavated by females exposed to TreeAzin (38 mg $kg^{-1}$) was approximately 61% of that observed in controls (Table 1). Median number of oviposition pits excavated by females over the 5 week period of observation following a 7 day exposure to the TreeAzin 38 mg $kg^{-1}$ treatment was also significantly lower ($P = 0.05$) than that observed for controls or blank formulations.

The results indicate that female ALB feeding on twigs systemically loaded with a dilute solution of TreeAzin expend significantly less effort in excavating oviposition pits as compared to females exposed to untreated twigs or those treated with a formulation blank. The reduced oviposition effort effect can be directly attributed to the active ingredients in the TreeAzin formulation, rather than to other formulants. As only the

highest test concentration was examined in this study, the relative effects of lower exposure concentrations are unknown.

### 3.2.3. Effects on Fecundity

Following 7 days of exposure of both males and females via feeding on Striped Maple twigs, the number of viable eggs produced was consistently lower for mated pairs exposed to the TreeAzin (38 mg $kg^{-1}$) treatment as compared to controls or formulation blanks. The effect was sustained through 4 weeks of the post-treatment observation period. In contrast, exposures to lower concentrations of TreeAzin (8 mg $kg^{-1}$) treatment resulted in mean viable egg production similar to those of controls and blanks. Due to insufficient availability of ALB adults, replication for the more dilute TreeAzin treatment was constrained (N=3) and hence this data was excluded from statistical analysis. RM-ANOVA for the higher (38 mg $kg^{-1}$) TreeAzin treatment data which involved 7 replicates, showed a significant effect of time (i.e. week of observation; $P = 0.02$) and for TreeAzin treatment ($P = 0.01$), with no significant interaction between time and treatment variables ($P = 0.09$). Post-hoc multiple comparison tests showed that the significant treatment effect was restricted to comparison with the controls ($P = 0.01$) while the comparison to formulation blanks was marginally insignificant ($P = 0.07$). The significant temporal effect was associated with the comparison between weeks 1 and 2 only ($P = 0.01$).

The apparent trend of increasing numbers of viable eggs through time for the TreeAzin 38 mg $kg^{-1}$ treatment group, as evident in Fig. 7, was determined to be non-significant via linear regression ($P = 0.4$; $r^2$=0.01), suggesting that the strength of the

reproductive impairment effect did not diminish with time. The cumulative total number of viable eggs observed over the 4 week period for ALB mated pairs exposed to the two TreeAzin treatments was markedly lower than that observed for controls or formulation blank treatment groups (Table 2). The effect was concentration-dependent, with the 8 mg $kg^{-1}$ treatment showing a 58% reduction in total fecundity, whereas the more concentrated 38 mg $kg^{-1}$ TreeAzin treatment induced a 70% reduction in total viable eggs relative to the control group. Moreover, a comparison of results for the blank formulation and the TreeAzin treatments shows that the effect is generated by exposure to the azadirachtin active ingredients rather than by other formulants. The 4 week cumulative mean number of viable eggs/female for the TreeAzin 38 mg $kg^{-1}$ treatment was significantly lower than observed for the control or formulation blank treatment groups. However the more dilute TreeAzin (8 mg $kg^{-1}$) treatment, showed no such significant effect, perhaps as a result of the reduced replication for this treatment (n=3 pairs versus n=7 pairs for other treatments) or a threshold level for fecundity inhibition effects.

The mean number of eggs produced per female per day in controls (Table 2) was quite similar to the range observed for 1st and 2nd generation females (0.63-0.92 eggs/female/day) from two different populations[45] and resulted in weekly averages very similar to that reported by Hajek and Kalb for ALB reared on *A. pensylvanicum*.[16] Overall, we observed significant concentration-dependent inhibition of adult fecundity that was sustained through 4 weeks post-exposure, even where exposure periods were artificially constrained to 7 days. Results suggest that exposure concentrations approximating exceeding 8 mg $kg^{-1}$ may be required to induce the fecundity inhibition effect. However, the exposure magnitude and duration used in these lab studies may or

may not simulate exposure dynamics under real world conditions and thus require further investigative field study under scenarios where ALB populations are established in North America and which are representative of the most likely potential uses for protection of high value trees in urban forests or managed woodlots. In our previous field study, stem injections of TreeAzin resulted in rapid uptake and translocation of the azadirachtin active ingredients into foliage and twig matrices in a variety of different ALB host tree species.[31] Although sugar maple showed exceptionally low residue levels in twigs and foliage in that urban scenario study, our ongoing field study demonstrates more typical rapid uptake, translocation and expression of residues in foliage and twigs of sugar maple under managed woodlot conditions with residue levels in foliage and twigs approaching the exposure thresholds required to impair ALB fecundity as determined in this laboratory study. We note that in this study, females were provided with high-quality feeding substrate, uncontaminated oviposition logs, and were maintained under optimal environmental conditions throughout the post-exposure period. All of these conditions would be anticipated to minimize the inhibitory effects on fecundity. In contrast, under sub-optimal real-world scenarios adults may have to expend greater energy to reach sexual maturity and produce eggs and when thus be comparatively more susceptible to fecundity impairment induced by exposure to TreeAzin.

## 4 CONCLUSIONS

The suite of quarantine laboratory experiments described herein, demonstrate statistically significant, concentration-dependent inhibition of larval growth and adult feeding activity, oviposition effort and fecundity in ALB concentrations of azadirachtins

as the active ingredients in the TreeAzin formulation. We estimate that thresholds of exposure magnitude (> 0.25 mg $kg^{-1}$ of azadirachtins) and exposure period (> 3 days) would be required to induce significant growth inhibition effects in larvae and that early stage larvae are likely to be most sensitive. ALB adults exposed to TreeAzin via simulated systemic uptake in twigs, also showed significant, concentration-dependent, impairment of female feeding activity, oviposition effort and fecundity. All response variables showed sustained impacts throughout a 4-5 week observation periods, but required exposure concentrations (> 8 mg $kg^{-1}$ of azadirachtins) slightly greater than those typically observed in host trees systemically injected with TreeAzin. Overall, results of this laboratory study demonstrate significant deleterious effects of azadirachtins (TreeAzin) on both larval and adult life stages of Asian Longhorned Beetle via a variety of activity mechanisms. Further research is required to verify the dual mechanisms of effect are in fact realized under real-world exposure regimes and result in localized population reductions and protection of treated trees. Since a host of environmental variables are known to influence uptake, translocation and loading of the active ingredients in critical feeding matrices, as well feeding behavior, body mass and energy reserves required for successful reproduction of the insects field studies designed to more directly quantify and demonstrate efficacy under environmentally realistic conditions are an essential next step to further development of TreeAzin as a potential component of a comprehensive and integrative response strategy as required to address the massive threat that the Asian Long-horned Beetle poses to the southern deciduous forests of Canada. Such utilization strategies could include stem injections of TreeAzin

as a chemical buffer around destructive removal zones during eradication attempts or as part of a "slow-the-spread" strategy where ALB actually becomes established.

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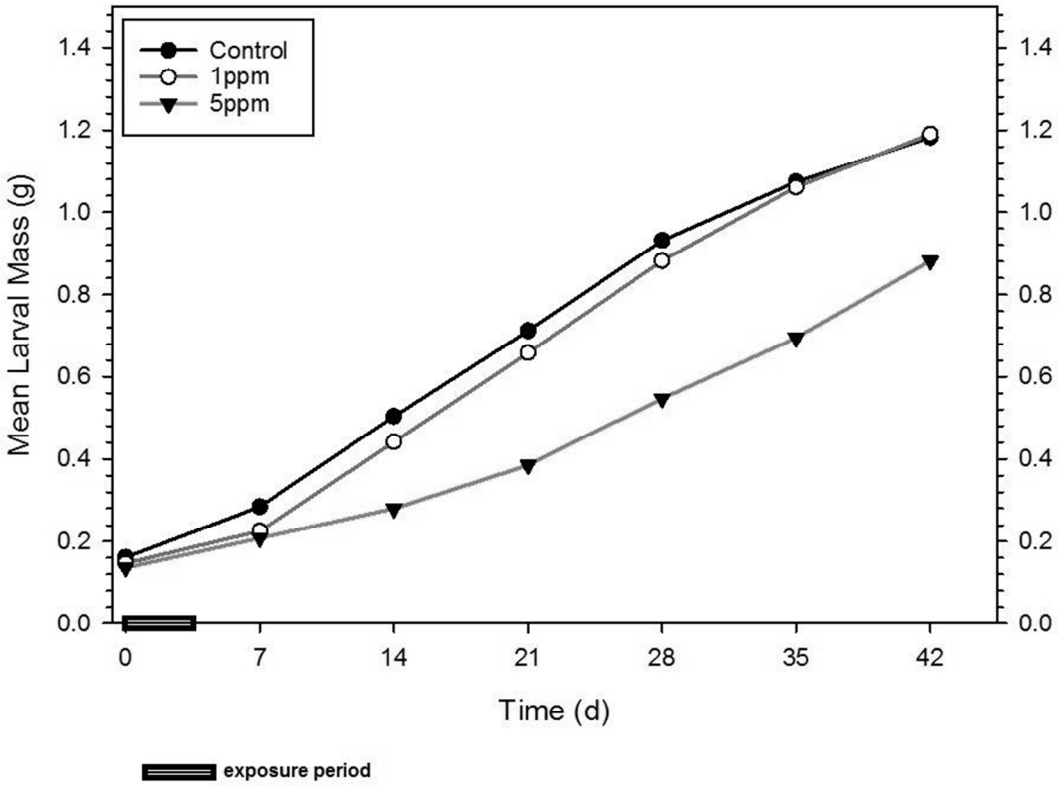
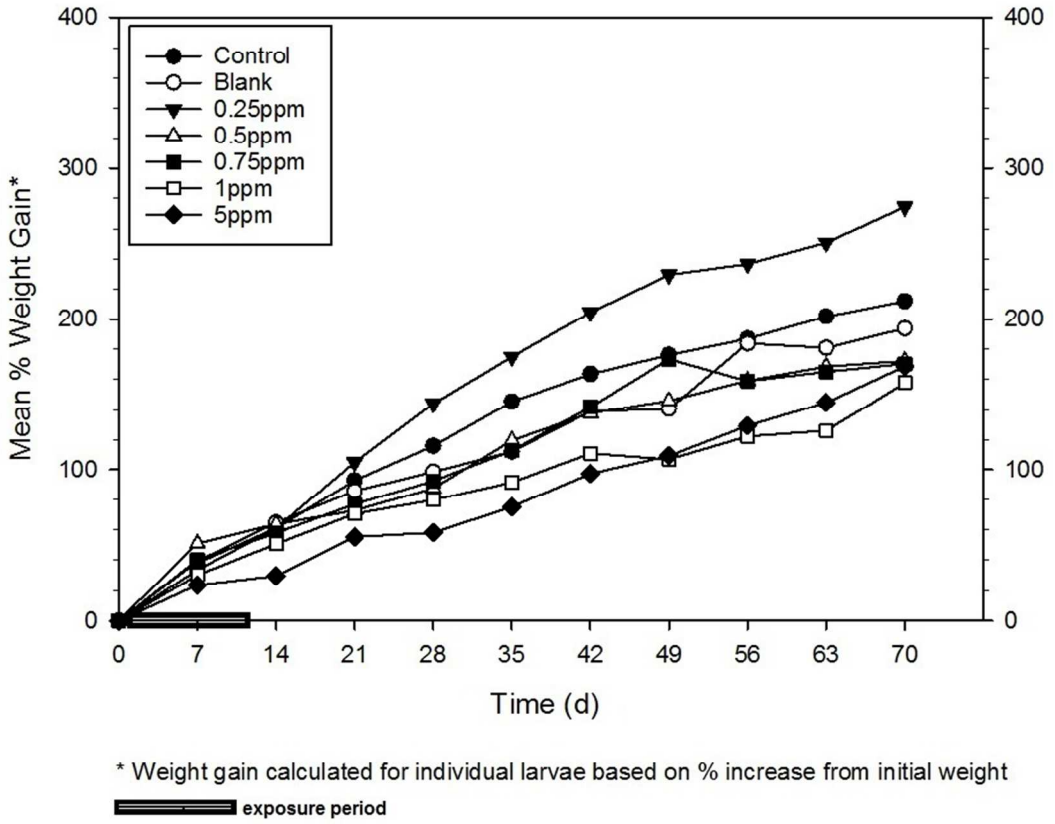
**Figure 1. Growth Inhibitory Effect of TreeAzin on Early Life Stage ALB Larvae Exposed to TreeAzin in Artificial Diet for 3 days.**



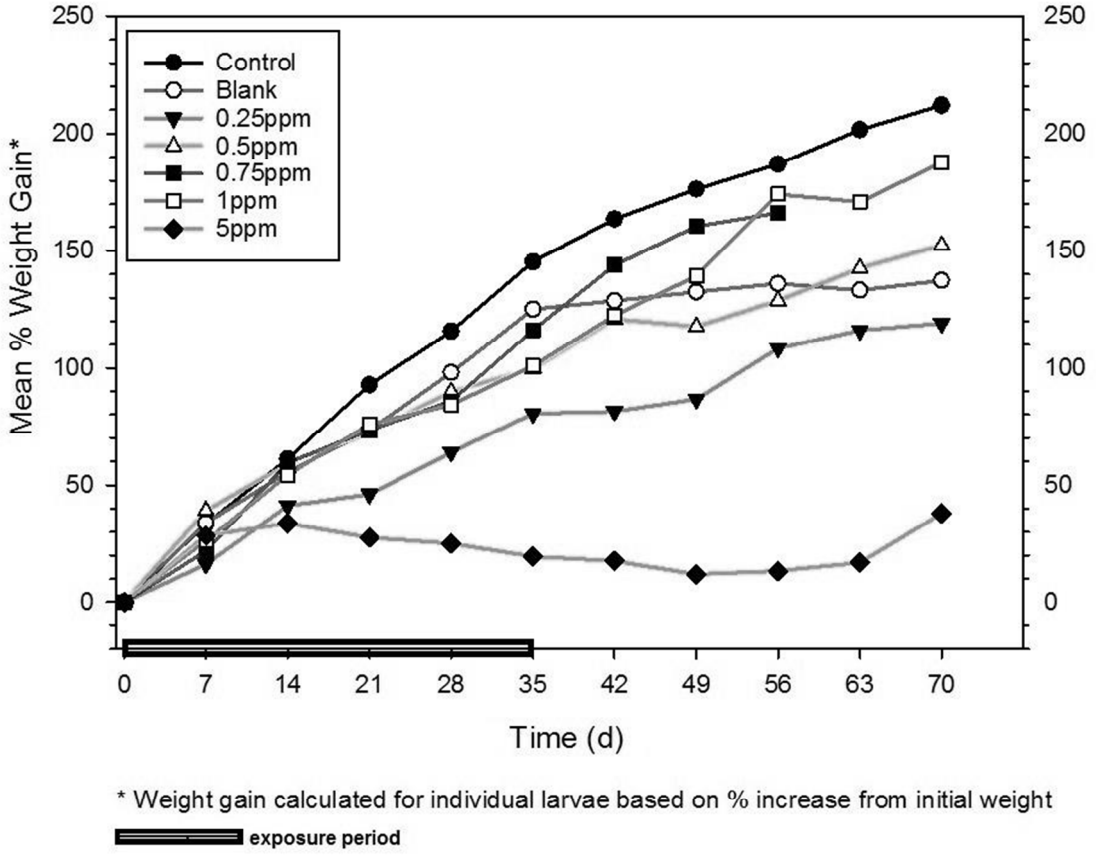

Inhibition of larval growth was statistically significant ($P = 0.05$) only for the 5 mg $kg^{-1}$ thru 42 days of observation.

**Figure. 2. Growth Inhibition for Mid-stage ALB Larvae Exposed for 12 Days to Various Concentrations of TreeAzin Incorporated into Artificial Diet**



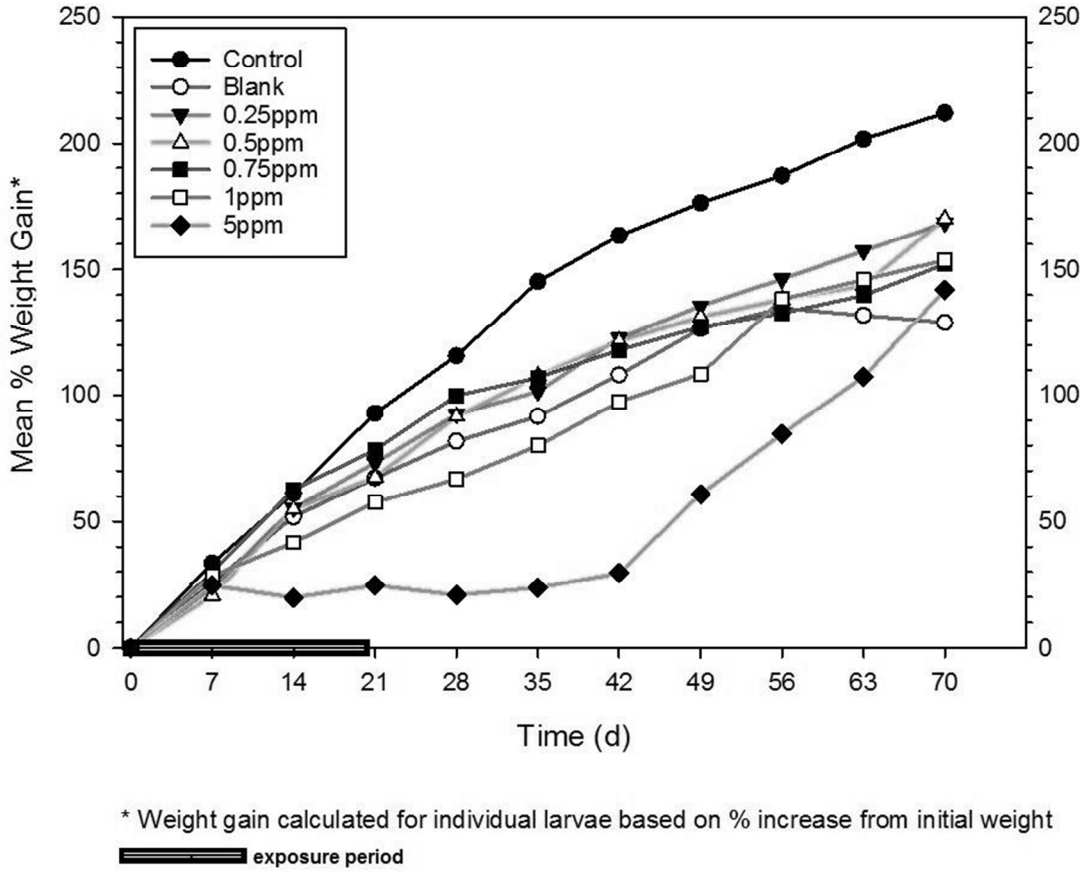

Although both 1 mg $kg^{-1}$ and 5 mg $kg^{-1}$ test levels showed consistently lower mean % growth relative to controls, results were not statistically significant ($P > 0.05$).

**Figure 3. Growth Inhibition for Mid-stage ALB Larvae Exposed for 35 Days To TreeAzin Incorporated Into the Artificial Diet at Various Concentrations**



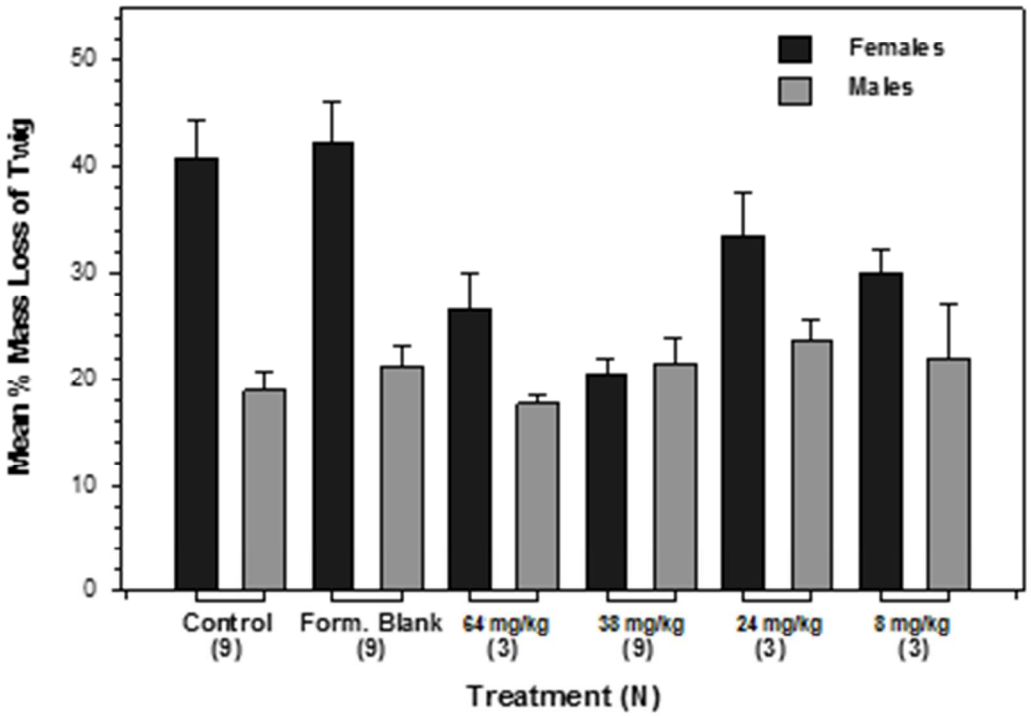

Linear regression of larval weights (g) versus test concentration (mg $kg^{-1}$) for the day of maximal effect (42) and for the last day of observation (70) were both significant ($P < 0.001$), with slopes indicating that each unit increase in concentration resulted in reduction of larval weight by 0.18 g.

**Figure 4. Growth Inhibition for Midstage ALB Larvae Exposed for 20 Days to Various Concentrations of TreeAzin Incorporated into Artificial Diet**




Linear regression analysis applied to the day 42 data showed a significant reduction in % weight gain with increasing level of TreeAzin in the diet ($P < 0.001$; $df$ =1,25; $a$ = -23.13; $r^2$= 0.82)

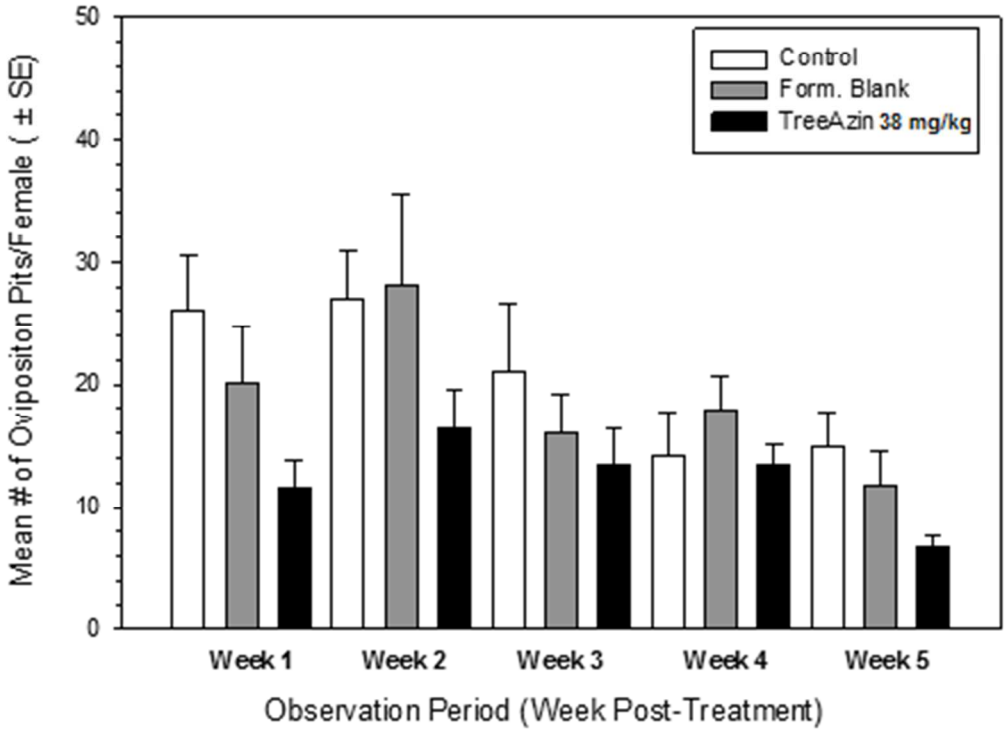
**Figure 5. Comparative Mass Loss in Striped Maple Twigs Resulting From Adult ALB Feeding Over a Period of 7 Days**




Note that since there is a significant interaction between sex and treatment effects, means should only be compared within sexes. Ratios indicate TreeAzin to water dilutions in solutions used to imbibe twigs and numbers in brackets indicate replicates for each treatment.

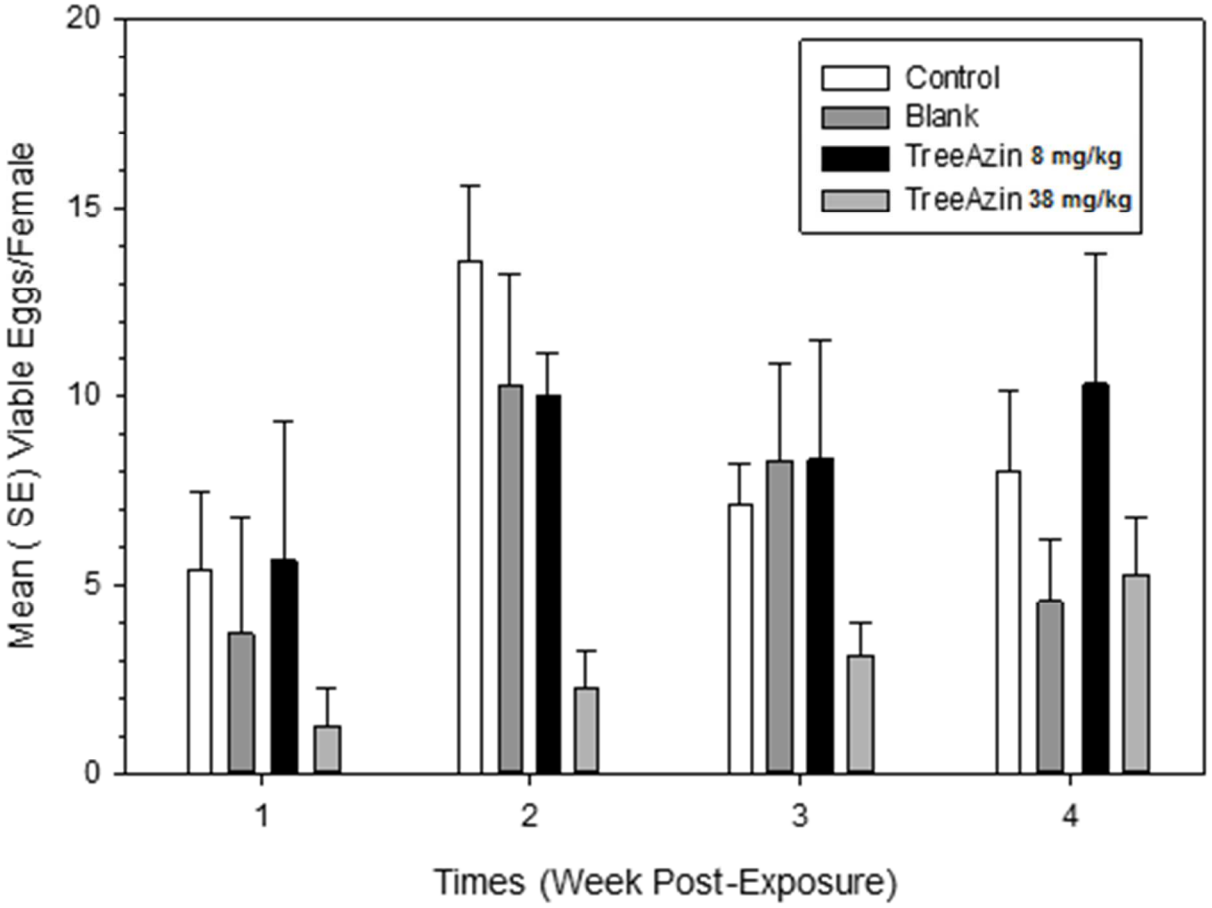
For females exposed to the different levels of TreeAzin formulation, mean mass loss differed significantly ($P < 0.05$) from that observed for formulation blank and control groups. Similar comparisons showed no significant differences for males ($P > 0.05$).

**Figure 6. Female ALB Oviposition Effort Following Exposure to TreeAzin Residues In Systemically Loaded Striped Maple Twigs**




The median number of oviposition pits excavated by females over the 5 week period of observation following a 7 day exposure to the TreeAzin 38 mg $kg^{-1}$ treatment was significantly lower ($P$ = 0.05) than that observed for controls or blank formulations.

**Figure 7. Weekly Mean Production of Viable Eggs by ALB Mated Pairs Following 7 Day Exposures to Various Treatments in Systemically Loaded Striped Maple Twigs.**




The 4 week cumulative mean number of viable eggs/female for the TreeAzin 38 mg $kg^{-1}$ treatment was significantly lower ($P = 0.05$) than observed for the control or formulation blank treatment groups. However the more dilute TreeAzin (8 mg $kg^{-1}$) treatment, showed no such significant effect, perhaps as a result of the reduced replication for this treatment (n=3 pairs versus n=7 pairs for other treatments) or a threshold level for fecundity inhibition effects

**Table 1. Comparative Oviposition Effort for Female ALB Exposed to Striped Maple Twigs Imbibed with Various Treatment Solutions.**

| | Control | Formulation Blank | TreeAzin (38 mg $kg^{-1}$) |
|---|---|---|---|
| **N** | 31 | 26 | 31 |
| **Mean/week/female (± SE)** | 21.0 ± 2.1 | 19.5 ± 2.3 | 12.8 ± 1.2 |
| **Mean/Day/female** | 3.0 | 2.8 | 1.8 |
| **Median/week/female** | 20.0[a] | 17.5[a] | 11.0[b] |
| **5 Week Total** | 651 | 506 | 398 |

[a] Median values followed by different letters are significantly different based on Kruskal-Wallis One Way ANOVA on ranks ($P = 0.003$) and Dunn's multiple comparison ($P < 0.05$).

**Table2. Mean Cumulative Viable Egg Production for Mated ALB Adults Exposed to Striped Maple Twigs Imbibed with Various Treatment Solutions.**

| | Control | Formulation Blank | TreeAzin ($8$ mg $kg^{-1}$) | TreeAzin ($38$ mg $kg^{-1}$) |
|---|---|---|---|---|
| **N** | 28 | 28 | 12 | 28 |
| **Mean/week/female (± SE)** | 8.75±0.97[a] | 7.43±1.33[a] | 8.58±1.42 [a] | 2.64±0.46[b] |
| **Mean/day/female** | 1.25 | 1.06 | 1.22 | 0.38 |
| **Median/week/female** | 8.50 | 6.00 | 10.5 | 2.5 |
| **4 Week Total** | 245 | 208 | 103 | 74 |

[a] Mean values followed by different letters are significantly different based on One Way ANOVA with Holm Sidak multiple comparison ($P < 0.05$)