THE EFFECT OF SCOPOLAMINE ON LARVAL *MANDUCA SEXTA*

By

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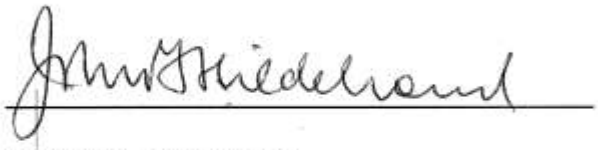

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Abstract

Scopolamine, a neurotoxic tropane alkaloid, is found in the natural diet of *Manduca sexta* in the Sonoran Desert. To establish the basic effects of scopolamine on larval *M. sexta*, two groups (control: 0% scopolamine diet; experimental: 0.2% dry weight scopolamine diet) were subjected to experimental studies of growth rate and diet preference. Leaves of the host plant *Datura wrightii* (Jimson weed) typically have 0.2% dry weight of scopolamine. There were no observed differences in growth rate (n = 100) or diet preference (n = 600) between the two groups. The apparent absence of effects suggests the existence of one or more innate defense mechanisms, protecting this species from intoxication with scopolamine. Based on previous studies of the effects of nicotine, another alkaloid present in one of the host plants (tobacco), on which *M. sexta* feeds, we predicted that mechanisms defending *M. sexta* against scopolamine might be present in both the gastrointestinal tract and the central nervous system (CNS). Direct injections of scopolamine into the hemolymph were administered to last (5th) instar larvae in order to bypass the suspected gastrointestinal defenses (n = 78). Mortality and pupal weight of control and experimental groups were not significantly different, which is consistent with the possible existence of a CNS defense mechanism. As a control, the unspecialized species *Galleria mellonella* underwent dietary and injected scopolamine treatments. *G. mellonella* experiments showed no significant difference in larval mass after treatment between the 0% and the 0.2% scopolamine groups. However, injected scopolamine was observed to incapacitate the larvae for an extended period of time. Thus, natural concentrations of scopolamine do not appear to be toxic even to unspecialized species, though injected scopolamine did cause transient effects.

Introduction

Scopolamine is a muscarinic receptor antagonist clinically used in humans as a sedative, antiemetic and amnesic drug in pharmacological doses. However, administered at greater concentrations, scopolamine can induce somnolence, coma, confusion, agitation, hallucination among other symptoms associated with anticholinergic intoxication (Renner et al. 2005). In insects, scopolamine has been implicated in interference with development of mushroom bodies in honeybees (Weinberger 2006) as well as in blocking flight stimulation in locusts (Buhl and Stevenson 2008).

Muscarinic receptors of multiple subtypes have been previously characterized in the central nervous system of the tobacco hornworm (*Manduca sexta*) (Torkkeli et al 2005, Qazi et al 1996). These receptors are not well classified because they do not correlate well with the subtypes known in vertebrates (Qazi 1992). Despite the absence of such classification, it has been shown that activation of postsynaptic nicotinic acetylcholine receptors is modulated by muscarinic receptors (Trimmer and Weeks 1993, Trimmer and Weeks 1989) and that the muscarinic agonist, pilocarpine, can induce crawling motor patterns when applied to desheathed larval nerve cords (Johnston and Levine 1996). These past studies indicate that muscarinic receptors play an active role in the central nervous system of *M. sexta* (Trimmer and Qazi 1996). Although some pharmacological studies have been performed with scopolamine on cultured *M. sexta* cells (Torkkeli 2005), the physiological and behavioral effects of scopolamine in *M. sexta* have not been previously investigated.

The effect of scopolamine on *M. sexta* is of particular interest because scopolamine is present in the natural diet of *M. sexta* native to Arizona. Specifically, local *M. sexta* feed on the

leaves and nectar of jimsonweed (*Datura wrightii*) (Hare and Walling 2006). Scopolamine belongs to a family of alkaloids known as tropane alkaloids, which contains the bicyclic tropane moiety. In nature, it is believed that alkaloids serve defensive purposes in plants (Zenk and Juenger 2007). In the case of nicotine, ingestion by unadapted herbivores showed a pronounced adverse affect in herbivore performance (Schmetz 1971). Yet, through evolution, specialized insects, such as *M. sexta*, have developed a resistance to physiologically toxic levels of nicotine (Steppuhn et al 2004). The mechanisms of the resistance of *M. sexta* to nicotine involve a multi-component defense system spanning the digestive, circulatory and nervous systems of larvae. The first form of defense against nicotine involves transport systems that remove nicotine from the hemolymph. For nicotine that successfully crosses the blood-brain barrier, the second form of defense involves the metabolic detoxification of nicotine in the neuropil of the insect CNS. Thirdly, if toxic nicotine remains in the neuropil, permeability glycoprotein (P-glycoprotein) putative pumps have been shown to remove nicotine from the neuropil (Murray et al 1994).

In this project, we attempted to demonstrate that the resistance to the effects of scopolamine may be caused by mechanisms similar to those for nicotine, involving a gut-blood barrier as well as a blood-brain barrier. To determine if a gut-blood barrier exists, various physiological and behavioral effects of dietary scopolamine in *M. sexta* were investigated. Based on the findings on nicotine, we hypothesized that dietary scopolamine will not affect the growth rate, development time or mortality of *M. sexta*. To provide a comparison between insects of the same order (Lepidotera), the effect of dietary scopolamine was tested on *Galleria mellonella*, whose natural diet does not contain scopolamine. We also hypothesized that *M. sexta* larvae will

not have a gustatory preference for scopolamine-laced diet. To determine if a blood-brain barrier exists, injection experiments to bypass any gut-blood barrier were performed on both species. We expected no significant growth or behavioral effects on *M. sexta*, but significant effects on *G. mellonell*a.

Methods

Larval care

M. sexta larvae were reared from eggs on a wheat-germ-based artificial diet (31g agar, 360g wheat germ, 24g salt mix, 6g sucrose, 5g cholesterol, 12g ascorbic acid, 6g sorbic acid, 5g methyl paraben, 30mg nicotinic acid, 15mg riboflavin, 7mg thiamine, 7mg pyridoxine, 7mg folic acid, 0.6mg biotin, 9mL linseed oil, 60mL formalin, 1830mL ddH_2O) at 26°C in a growth chamber (Environmental Growth Chambers) under a 15h L: 9h D photocycle. For experiments requiring dietary scopolamine, the scopolamine concentration of the diet was determined to be 0.2% scopolamine of the dry ingredients, based on the natural concentration of scopolamine in the host plant, *Datura wrightii* (9). To create the scopolamine diet, scopolamine hydrobromide trihydrate (1.11g, Sigma Aldrich) was dissolved into the distilled water of the regular diet recipe.

All *G. mellonella* larvae were reared from eggs on artificial diet prepared by combining 15mL honey, 1mL distilled water, 20mL glycerol, 10g milk powder and 40g wheat germ. The larvae were raised under constant darkness at 26°C. To create the scopolamine diet, scopolamine hydrobromide trihydrate (0.191g, Sigma Aldrich) was dissolved into the distilled water of the regular diet recipe.

Growth Curve

The dietary effect of scopolamine on growth rates of control (0% scopolamine diet, n = 50) and experimental larvae (0.2% scopolamine diet, n = 50) was tested. All larvae used in this part of the experiment were reared from eggs. In order to ensure that all larvae hatched within

one hour of each other, the larvae were placed into individually-labeled (29.6) soufflé cups with an excess of the appropriate diet as the larvae hatched. The average time between which the first and last larvae selected had hatched was determined to be t = 0h. Mass measurements were taken at t = 18h, 42h, 66h, 90h, 114h, 186h, 258h, 330h, and 402h. At these times points, the developmental stage and mortality were noted as well. To maintain hygiene and food quality, the cups and diet (in excess) were replaced every 3 days. Upon reaching the 3rd instar (t = 90h), the larvae were placed into larger individually-labeled (414mL) cups to allow sufficient space for growth.

Late in-star *G. melonella*, weighing 35mg (±5mg) were selected from the colony and placed into individual 2mL plastic cups with either normal diet or 0.2% dry-weight scopolamine-laced diet. To maintain hygiene and food quality, the cups and diet (in excess) were replaced every 3 days. To prevent over handling, the larvae were weighed only after pupation. Pupal mass and development time were recorded.

Injections

To test the effect of injected scopolamine, the growth and developmental differences between control (70μL injection of saline, n = 38) and experimental (70μL injection of 0.114M scopolamine hydrobromide trihydrate in saline, n = 45). The physiological saline consists of 149.9mM NaCl, 3.0mM KCl, 3.0mM anhydrous $CaCl_2$ and 10mM TES, titrated with 1N NaOH to obtain a pH of 6.9. The concentration of scopolamine injection was determined based on, firstly, the predicted scopolamine consumption derived from larval growth models and, secondly, on a previous titration experiment in which 70μL injections of 0.011M (0.5X daily scopolamine consumption), 0.023M (1X), 0.080M (3.5X) and 0.114M (5X) scopolamine hydrobromide

trihydrate were performed (data not shown). Because of an apparent lack of scopolamine effect for lower concentrations, 0.114M scopolamine hydrobromide trihydrate was determined to be the most suitable concentration for the experiment. This concentration of scopolamine is equivalent to 1100mg scopolamine per kg of larval mass, which, for the sake of comparison, is lower than the LD_{50} in rats (3800 mg/kg).

All larvae for the injection experiments were raised on 0% scopolamine diet. Larvae during the 4^{th} molt that weighed 1.500g (±0.100g) were carefully screened and selected in order to reduce variation in results due to mass. The selected larvae were placed into individually-labeled cups with an excess of diet. 48h after selection, the larvae were starved for 90min. The larvae were then placed on ice for 30min. 30.5 gauge needles with 1mL syringe were used to deliver the 70μL injections to the hindmost left proleg in the A6 region. After injection, the injection site was sealed with VetBond (3M). The larvae were replaced into their respective cups and given an excess of fresh diet. Upon pupation, the mass and sex of the pupae was recorded. The reported results include only those individuals that survived to pupation.

Larvae from the *G. mellonella* colony fed on 0% diet weighing 170mg (±20mg) were selected to be used in the injection experiments. The selected larvae were starved for 30min. The larvae were anesthetized by placement on ice for 10min. The concentration and amount injected were based on the injection experiments with *M. sexta*, which determined the appropriate injection amount relative to body mass. The injections were performed on the third proleg (numbered from the anterior end) on the left side of the larvae. After injection, the injection site was sealed with VetBond. The larvae were placed in individual wells in a 24-well plate with excess food of the appropriate scopolamine concentration based on experimental group. To

prevent over-handling, the larvae were weighed only after pupation. Pupal mass and development time were recorded.

Food Preference

Larvae were raised from eggs on 0% scopolamine and 0.2% scopolamine diet. Larvae (n = 50 from each diet group, total n = 100) in the early 3rd instar (one day after the 2nd molt) were selected. Two 1.25cm x 1.25cm x 0.63cm cubes of each diet type were placed into clear circular dishes with gridlines as shown in Figure 1. The selected larvae were then placed into the dishes such that the larvae were parallel with the center gridline (see Figure 1). After 24h, the position of the larvae was noted.

The above procedure was repeated for early 4th instar (total n = 100) and early 5th instar (total n = 100) larvae, increasing the diet cube size to 1.25cm x 1.25cm x 1.25cm and 1.25cm x 1.25cm x 2.50cm, respectively.

Results

Dietary scopolamine has no effect on larval growth rate, development or mortality for both M. sexta *and* G. mellonella.

Dietary scopolamine experiments showed that 0.2% scopolamine did not have a significant effect on larval growth rate, development or mortality for both *M. sexta* (Fig. 1) and *G. mellonella* (Fig. 2). *M. sexta* experiments showed no significant different in larval mass after treatment between the 0% (13.22 ± 1.09g) and the 0.2% (12.70 ± 2.06g) experimental groups. All of the M. sexta reached developmental maturity at approximately the same rate (data not shown). The mortality rate was not significantly different between 0% (14% of n = 50) and 0.2% (20% of n = 50) experimental groups. *G. mellonella* experiments showed no significant difference in larval mass after treatment between the 0% (255.71 ± 11.64mg) and the 0.2% (269.17 ± 12.00mg) experimental groups. There was no mortality in the 0% and the 0.2% experimental groups and no significant difference in developmental rate (data not shown).

Injected scopolamine has no effect on pupae development time and mass for both M. sexta *and* G. mellonella.

Injected scopolamine experiments showed that 0.2% scopolamine did not have a significant effect on larval growth rate or development for both *M. sexta* (Fig. 3, 4) and *G. mellonella* (Fig. 5). In Figure 4, *M. sexta* experiments showed no significant difference in larval mass after treatment between the 0x daily projected intake injections (5.274 ± 0.417g) and the 5x (5.276 ± 0.560g) experimental groups. *G. mellonella* experiments showed no significant different in larval

mass after treatment between the 0x daily projected intake injections (190 ± 3.8mg) and the 5X (186 ± 5.1mg) experimental groups. Both *M. sexta* and *G. mellonella* larvae in both experimental groups reached the same developmental stage as the controls (data not shown).

Larval M. sexta do not have a dietary preference for scopolamine-laced diet

According to Figure 6, of the third, fourth and fifth instar larvae reared on control diet without scopolamine, 45.3%, 46.4% and 53.6% of each respective instar group selected 0.2% scopolamine diet after 24h. Of the third, fourth and fifth instar larvae reared on 0.2% scopolamine diet, 50.5%, 47.4% and 59.8% of each respective instar group selected 0.2% scopolamine diet after 24h. There is no signficiant difference between the experimental dietary choice and dietary choice predicted by random selection. It should also be noted that for fifth instar larvae, both types of diet were consumed. Thus, larval *M. sexta* does not have an apparent dietary preference for scopolamine, regardless of developmental stage or original diet.

Discussion

In this study, we explored possible defense mechanisms of *M. sexta* that protect it from the neurotoxic effects of scopolamine. We found that scopolamine does not have a significant growth or behavioral effect on *M. sexta*. The larvae do not appear to have a gustatory preference for or against scopolamine-laced diet. Contrary to our hypothesis, the same concentrations of scopolamine when used on G. mellonella did not have a significant growth effect but did have possible behavioral effects. Although these behavioral effects for *G. mellonella* were not quantified in this project, there is observational evidence that injected scopolamine incapacitated the larvae for an extended period of time when compared to the control injection group.

The dietary scopolamine experiments on *M. sexta* and *G. mellonella* strongly suggest a gut defense. In both organisms, but especially in *G. mellonella*, scopolamine is most likely not absorbed in significant amounts by the digestive tract to cause neurotoxicity. Also, it is possible that significant amounts of scopolamine are absorbed into the hemolymph, but transport systems, similar to those involved in the removal of nicotine in the gut of larval *M. sexta*, may actively remove it from the hemolymph to be excreted. The injection data suggest that *M. sexta*, but not *G. mellonella*, larvae may also possess a blood-brain barrier that protects the CNS. This barrier can be physical, physiological or even metabolic. It is possible that the CNS membrane is selectively impermeable to scopolamine. Another possibility, similar to the case of the larval response to nicotine, is the presence of putative pumps that actively remove scopolamine from the CNS. Thirdly, it is possible that scopolamine is metabolized either in the blood or in the hemolymph into non-toxic substances. Without further biochemical and histological studies, it is unclear whether the protection from the effects of scopolamine results from one or a combination of the aforementioned mechanisms.

The findings also have implications on the understanding of the role of scopolamine in plant defense against herbivory. The results for dietary scopolamine contrast with experiments on *M. sexta* with dietary nicotine. Nicotine at any concentration reduces consumption of diet (Wink and Theile 2002). However, scopolamine at natural concentrations found in *D. wrightii* does not significantly affect larval mass. Unlike nicotine, which plays an important role in determining larval feeding sites and nutrient consumption (Steppuhn et al 2004, Thompson and Redak 2007), the lack of dietary preference for scopolamine suggests that these specialized insects do not utilize or perceive scopolamine and nicotine in the same manner. Thus, the role of these alkaloids in the evolution of *M. sexta*'s defensive mechanisms most likely differs as well. Though the relative toxicity of scopolamine versus nicotine is not well documented in insects, nicotine is much more toxic in rats where the LD_{50} for nicotine is 50mg/kg and for scopolamine is 3800mg/kg. Thus, whereas nicotine at natural concentrations found in tobacco plants is highly toxic to unspecialized insects, dietary scopolamine at natural concentrations does not appear to cause the same fatal consequence as was seen in this study with the lack of an adverse developmental or growth effect on *G. mellonella*. Assuming that higher toxicity of the diet leads to higher evolutionary pressure for the consumer, dietary scopolamine is not as strong a selective agent as nicotine since scopolamine consumption had no effect on specialized or unspecialized species. This suggests a lesser role for scopolamine in plant defense against herbivory in comparison to more potent alkaloid compounds, such as nicotine.

From this study, the specific defense mechanisms against scopolamine are still unclear. To further confirm the excretion hypothesis, GC analysis of larval fecal matter should be performed. This may also give us insight into scopolamine metabolism in M. sexta. We know that muscarinic receptors are present in the CNS of the larvae, and there is evidence that

scopolamine induces inconsistent biochemical changes when acting on these receptors (Trimmer and Qazi 1996). It is interesting to note that of the muscarinic receptor agonists and antagonists used in Trimmer and Qazi 1996, only scopolamine produced inconsistent effects on the concentration of secondary messengers. Given this interesting result, electrophysiological studies still need to be performed in order to see if such biochemical changes impact the physiological response of the CNS.

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Figure Legends

Figure 1: Dietary scopolamine does not affect growth rate of *M. sexta*. A plot of the mass of the larvae under control (0% scopolamine ●; n = 50) and experimental (0.2% scopolamine ○; n = 50) conditions after hatching until pre-pupation. Error bars are shown at each data point. There is no significant difference in mass or mortality (not shown) between the two groups.

Figure 2: Dietary scopolamine does not affect growth rate of *G. mellonella*. A graphical comparison of larval mass before treatment (dark grey; n = 24) and after 480h of scopolamine diet consumption (light grey; n = 24). Dietary scopolamine treatment does not appear to have a significant effect on larval mass.

Figure 3: Injected scopolamine does not affect *M. sexta* pupal mass and development time. Dose-response experiment (n = 25) of larvae injected with different concentrations of scopolamine, 0X, 0.5X, 1.0X, 3.5X and 5.0X daily projected intake. Mass was taken at the pre-injection 5^{th} instar (yellow) and post-injection pupal (blue) stages. Although there appeared to be no significant difference in mass or mortality (not shown), 5.0X demonstrated the greatest potential difference in mass.

Figure 4: Injected scopolamine does not affect *M. sexta* pupal mass and development time. Repeat trials with 5.0x daily projected intake injections were performed (n = 73). Mass was taken at the pre-injection 5^{th} instar (dark grey) and post-injection pupal (light grey) stages. There was no apparent difference in mass or mortality (not shown) between control and 5.0X daily projected intake injection groups.

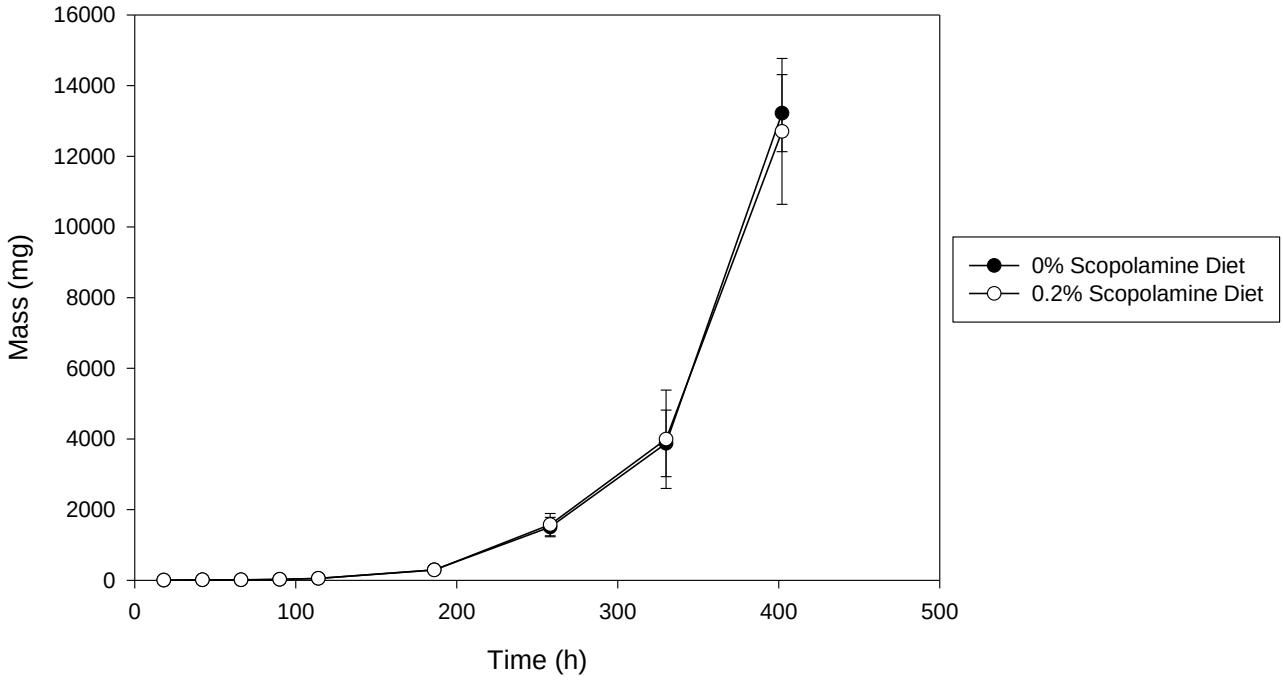
Figure 5: Injected scopolamine does not affect *G. mellonella* pupal mass and development time. Injected scopolamine treatment does not appear to have a significant effect on larval mass

(n = 24). Mass was taken at the pre-injection 5th instar (dark grey) and post-injection 240h (light grey) stages. There was no apparent difference in mass or mortality (not shown) between control and 5.0X daily projected intake injection groups.

Figure 6: *M. sexta* does not have a dietary preference for scopolamine. To account for possible imprinting effects, two sets of larvae were each raised on either 0% (A, n = 300) or 0.2% (B, n = 300) scopolamine diet prior to experimentation. The data includes 24h preference data collected from the 3rd, 4th and 5th instar. There is no significant preference difference. It should be noted that most of the 5th instar larvae consumed both types of food. The dark grey section represents the proportion selecting the 0.2% scopolamine diet, and the light grey section represents the proportion selecting 0% scopolamine diet.

Tables and Figures

Figure 1:



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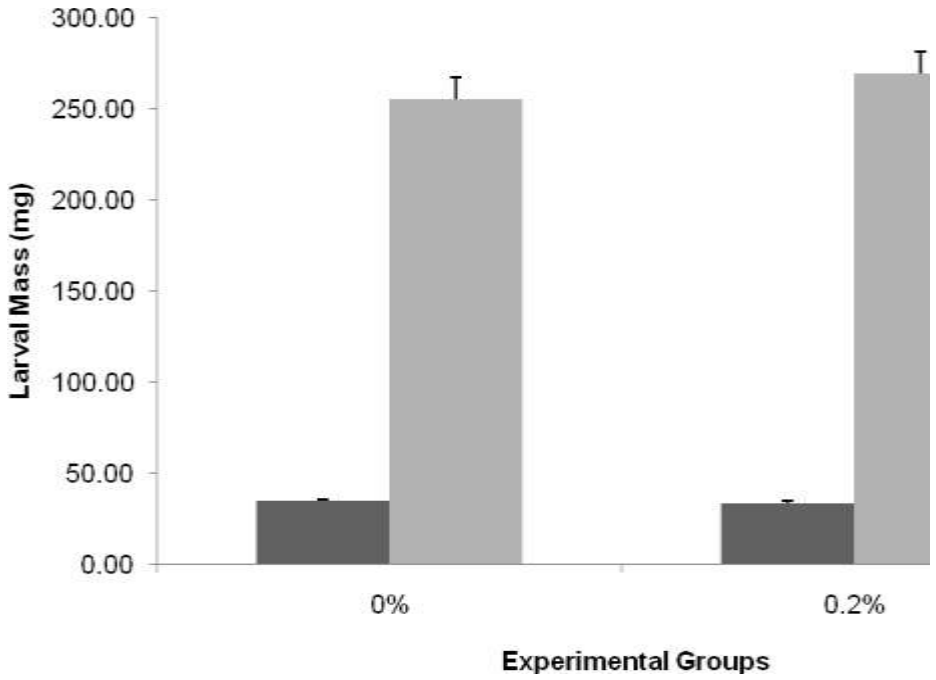
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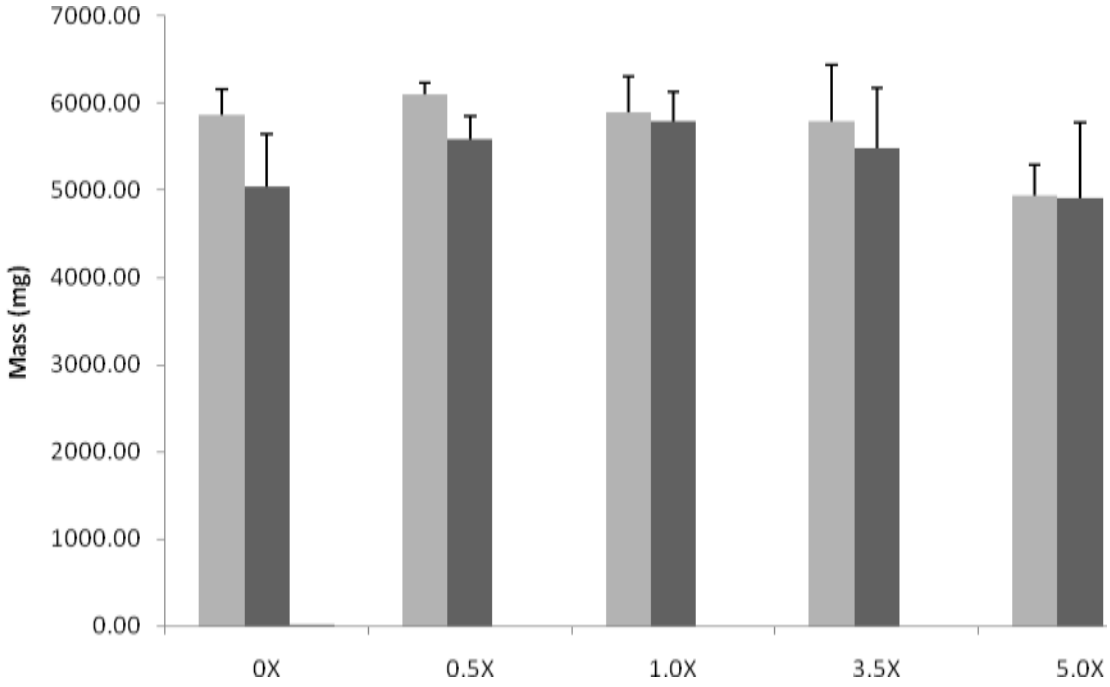
Figure 3: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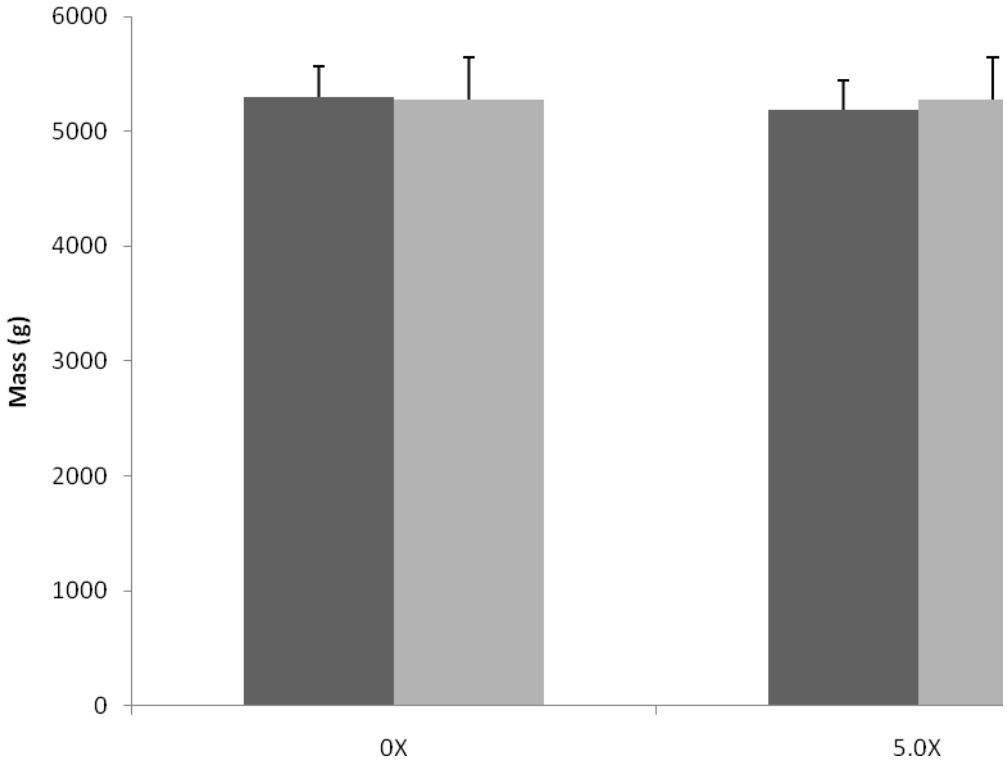

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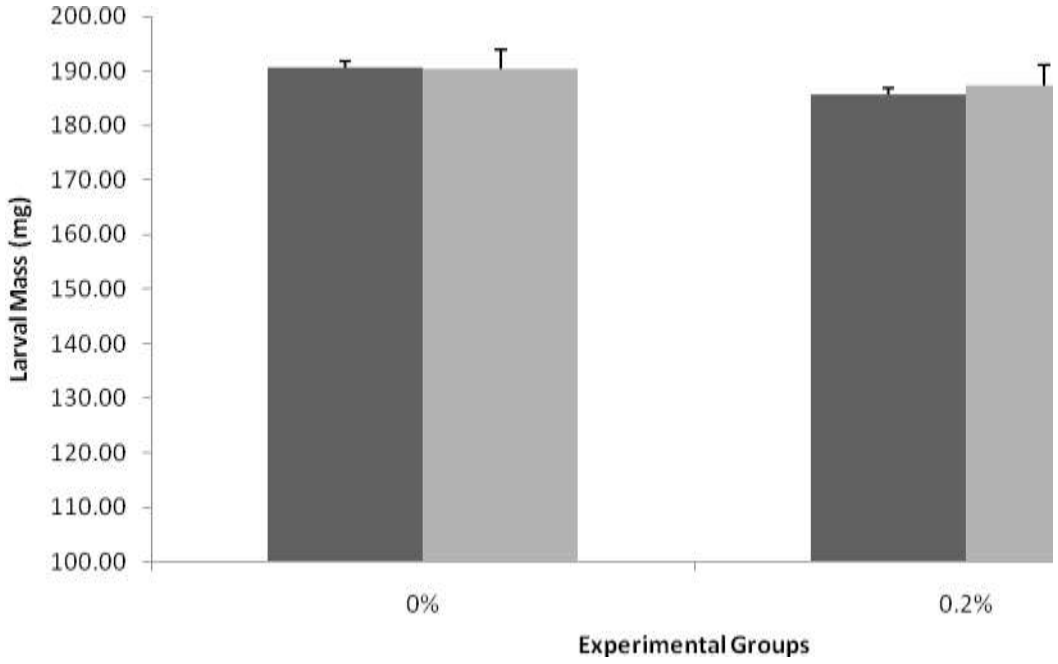
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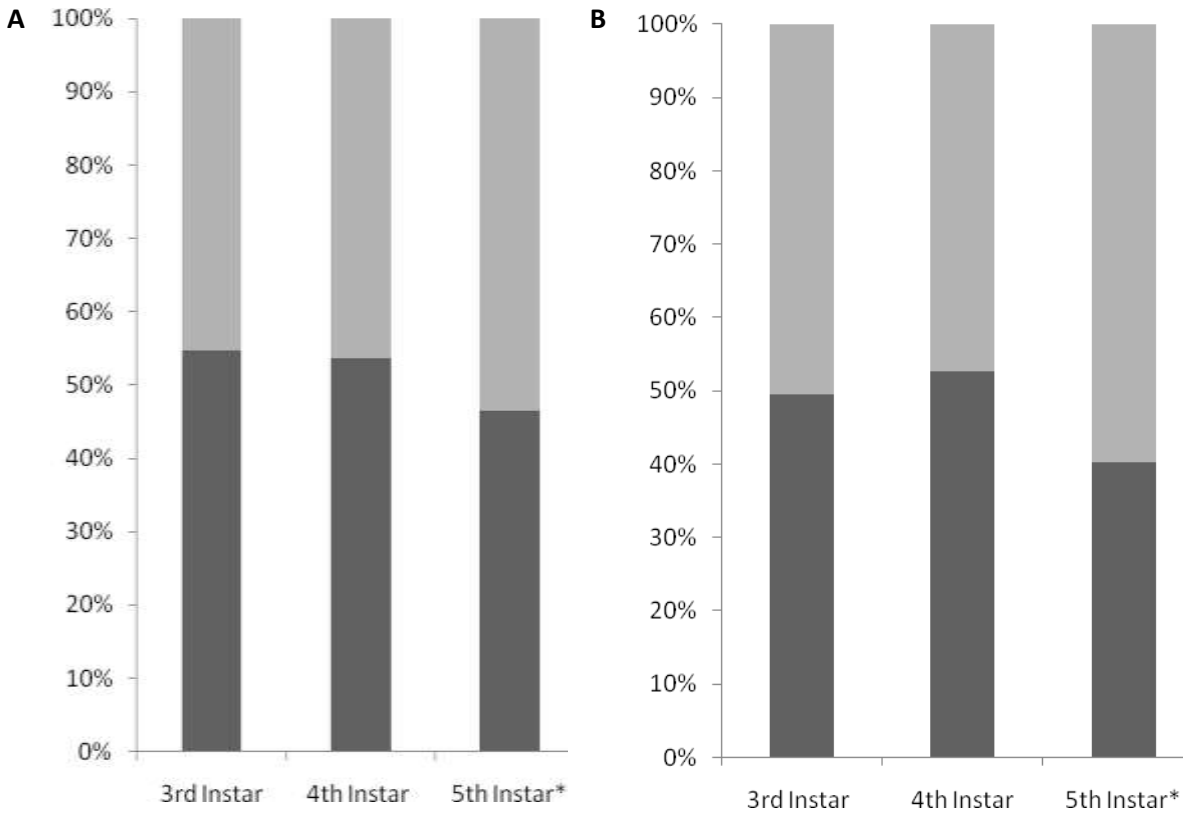
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