Toxicity of Moist Snuff and Impact on Various Stages of Darkling Beetles (*Tenebrio molitor*)

Lisa Collins
with **Latisha Edwards**
Fayetteville State University
Faculty Mentor: Shirley Chao
Fayetteville State University

ABSTRACT

Moist snuff is a manufactured form of smokeless tobacco that, when consumed chronically, contributes to the leading cause of premature deaths in the world. Snuff contains several known toxins, such as nicotine, that have been proven to adversely affect organisms exposed to it through tobacco products. The objective of this study was to test whether moist snuff adversely affects darkling beetles by increasing mortality and hindering normal development of Tenebrio larvae. Results indicate that moist snuff did increase mortality, but also increased the developmental rate of survivors. In addition, a positive trend was observed in which moist snuff increased the amount of protein per insect without increasing average weight. Increases in protein amounts suggest possible compensatory responses to the toxic insult- grounds for further research.

According to the Centers for Disease Control and Prevention (2011), tobacco use is the leading cause of preventable death in the United States and a major cause of premature death worldwide. Tobacco is produced and manufactured into several products, which may be divided into the categories of smoking tobacco and smokeless tobacco. Smokeless tobacco comes in two forms: chew and snuff (Connelly, 2006), which contain more than 7,000 chemicals, 60 of which are known carcinogens (American Cancer Society, 2011). One of the most toxic ingredients found in tobacco products is nicotine. Nicotine is a powerful neurotoxic, psychoactive compound that occurs naturally in the leaves of tobacco plants, *Nicotiana tabacum* and *Nicotiana rustica* (Higa de Landoni, 1999). Chemically, nicotine is an alkaloid, or nitrogen containing substance (Naff, 2007), and functions as a natural insecticide. Variable amounts of nicotine are found in tobacco products, and depend on brand and product type. Studies show that nicotine in cigarettes account for approximately 1.21 mg to 2.16 mg per cigarette (Keithly, Cullen, & Land, 2004). For the purposes of this study, the focus is on moist snuff. Among the amalgam of chemicals, nicotine has been found in concentrations ranging from 0.59 percent up to 3.35 percent of snuff products per container (Tilashalski, Rodu, & Mayfield, 1994). Once nicotine is consumed in the form of moist snuff, it quickly enters the bloodstream through mucus membranes lining the mouth (LeVert, 2007), and acts on the brain as well as other organs within seconds (Naff, 2007). Tobacco products have been studied in great depths and are known to have adverse health effects on infants when consumed by expecting mothers. These effects include, but are not limited to, low birth weight and spontaneous abortion. In

addition, neurological effects are reported to be caused by tobacco use during pregnancy (Ernst et al., 2001). Though little research has been conducted on the direct effect of snuff use during pregnancy, there is evidence that its use may lead to premature delivery and preeclampsia (England et al., 2003). Several studies have determined the toxic effects of tobacco and nicotine on vertebrates such as humans, rats, and mice (Abreu-Villaca, Seidlera, Tatea, & Theodore, 2003); however, few experiments have been conducted to determine toxicity of tobacco products in insects such as mealworms.

The mealworm (*Tenebrio molitor*) has been a major pest of grain worldwide, costing high losses of stored grains, specifically in mills. As a result, pest management strategies have been studied to understand reproduction and development of this pest species (Taibi et al., 2003). In addition, the mealworm is an ideal animal model to understand animal development. Humans and mealworms may seem to have nothing in common, yet they share similar factors that control the nervous system and development in both (Brown, 2006).

The nervous system of an insect such as the mealworm is similar to that of a human because they share common neurotransmitters such as acetylcholine (Brown, May 2006). Acetylcholine plays a major role in the healthy functioning of the peripheral nervous system as well as muscle functioning in both humans and insects. The nicotine in tobacco products has the ability to mimic acetylcholine once it enters the body, which affects major systems of the body (LeVert, 2007). Due to similarities between the nervous systems of humans and insects, mealworms are excellent model organisms for toxicology research. In addition, mealworms undergo predictable patterns of development; therefore, they are ideal candidates for investigating factors that control animal development. Contrary to popular belief, the mealworm is not a worm, but the larval stage of the mealworm beetle, also called the darkling beetle of *Tenebrio molitor* species. The mealworm goes through four stages of development: (1) egg, (2) larva, (3) pupa, and (4) adult. The female darkling beetle lays hundreds of tiny, white, oval eggs which hatch into tiny mealworms (the larval stage). Each mealworm eats a tremendous amount and sheds its exoskeleton as it grows. It then enters its pupa stage. After pupating, a white adult darkling beetle emerges from the pupa; it soon turns brown, then almost black, completing the life cycle (Lawrence Hall of Science, July 2009). Since mealworms undergo complete metamorphosis during its lifecycle, the effects of a tobacco product such as snuff could be easily observed and analyzed (Gosselin & Fernandez, May 2011).

The purpose of our study is to test the effects of snuff exposure on darkling beetles, beginning in the larval stage, and to observe any adverse effects during development. Our study tested the following hypothesis: moist snuff adversely affects darkling beetles by increasing mortality and hindering normal development of *Tenebrio* larvae.

MATERIALS AND METHODS

The chemical used in the experiment was moist snuff, which is a finely ground form of smokeless tobacco. The brand was Longhorn Fine Cut Natural, manufactured at Pinkerton Tobacco Company LP, located in Owensboro, KY USA. This product contains at least 50% domestic tobacco. Kretschmer Original Toasted Wheat Germ processed in Manhattan, KS USA was used as the food source for the mealworms. A total of 30 mealworms (larval stage of darkling beetles) were obtained from PetSmart (Fayetteville, NC). Weighing of all materials was done

using a triple beam balance and organisms were tested and stored in 100mL plastic containers purchased from Thompson and Little Supply Store (Fayetteville, NC). All animals were stored at room temperature ranging from 20-25°C.

Procedure

The study was conducted over a five week period with treatments done one day a week on three occasions. We weighed each group of five mealworms, whose average weight was 0.5g, and 0.5g of moist snuff for a 1:1 ratio. For each snuff treatment, we added 0.5g snuff to 50-mL of water and incubated for 10 minutes. For each control, only 50mL of water was added to a cup. We then placed a group of mealworms in designated treatment cups and exposed for one minute, noting observations. The water was then drained, larvae dried on a paper towel, and transferred into their habitat which included wheat germ, a moist paper towel, and air holes in the lid. Mealworms were weighed each week before they were exposed to water or the snuff treatment for a total of three weeks. Once a larva entered the pupa stage, they were weighed, but not exposed to water or the snuff treatments. Upon entering the adult beetle stage, they were weighed and once again exposed. Development and body weight through various stages of the mealworm's lifecycle were documented each week (Figures 2 and 3). Mortality was noted and only survivors were maintained (Figure 1).

Protein Assay

Homogenization. Three adult beetles were obtained from the control group and the experimental group and placed into separate test tubes containing 1.0mL of sodium phosphate buffer (0.01M, pH 7.0). Each beetle was homogenized separately and centrifuged at 12000g for 3 minutes. The supernatant was removed from each sample and transferred into labeled tubes to be frozen until assayed for total protein using the Bradford method (Bradford, 1976).

Bradford Protein Assay. Bovine Serum Albumin (BSA) stock of 1mg/mL was prepared and used as the standard. Three milliliters of Bradford reagent (Thermo Fisher Scientific Inc., Waltham, MA) was added to each BSA standard (20, 40, 60, 80, 100µg). Absorbance was read for each BSA standard at 590nm, the peak wavelength determined. Each 15 µL sample of the darkling beetle supernatant with Bradford reagent was transferred to a cuvette and absorbance was read at 590nm.

RESULTS AND DISCUSSION

Our results were drawn by averaging data from three control groups and comparing the average of three experimental groups. We report observations on the following data: mortality after moist snuff exposure (Figure 1), total number of survivors at various stages of development (Figure 2), average weight of control and test groups (Figure 3), and average protein per beetle following treatments of moist snuff (Figure 4).

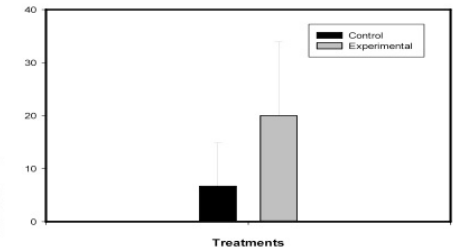

Figure 1. Mortality of Insects Following Treatments. Average percent mortality (±SE) after three weeks of treatments is shown. Control groups (n=15) were treated with water and Experimental groups (n=15) were treated with moist snuff.

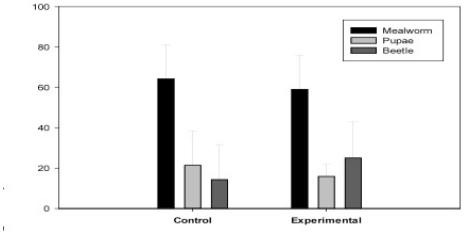

Figure 2. Effects of Moist Snuff on Rate of Development. The development of larva through its life cycle was observed and recorded following treatments of snuff (Experimental n=15) and water (Control n=15). The average percentage of each developmental stage is shown.

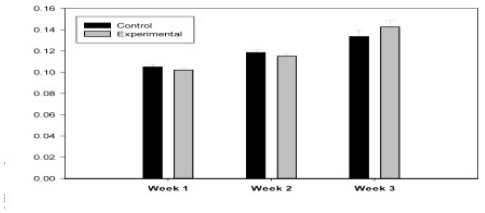

Figure 3. Average Weight of Insects Following Treatments. The weight of each group of insects was taken using an analytical scale before every treatment. The average weight (±SE) per insect was calculated for each treatment group each week for a total of three weeks. Week 1 Control: n=15; Week 1 Exp: n=15. Week 2 Control: n=14; Week 2 Exp: n =12. Week 3 Control: n=14; Week 3 Exp: n=12.

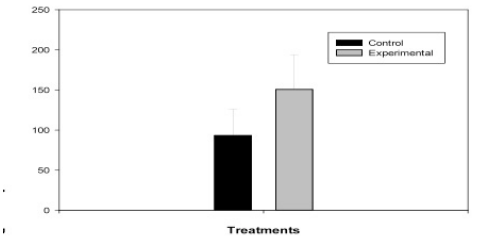

Figure 4. Average Protein per Adult Beetle (µg/15µL) Following Treatments with Snuff. The amount of protein of control beetles exposed to water (n=3) and experimental beetles exposed to moist snuff (n=3) was determined using the Bradford protein assay. The average amount of protein per insect was determined for the control and experimental group.

Mortality Following Moist Snuff Treatments

After the first week, one of fifteen insects in the control group and one of fifteen insects in the experimental group died. No other deaths were observed in control groups. However, more deaths were noted among the experimental group than the control group although significance was not reached (based on T-test analysis with significance set at *alpha* = .05).

We observed an overall 93% survival among the control group, and an 80% survival among the treatment group. The life stages appear to have progressed more quickly among the experimental group, although resulting in an equal number of adult beetles between the two groups at the end of the three weeks. All of the surviving darkling beetles in the experimental group were black, indicating that they had reached a later stage of development than the control group, which had one white beetle (newly emerged) and one brown beetle. In summary, after three weeks of exposure to moist snuff, 20% of experimental insects died. Control groups exposed to water alone only exhibited 7% mortality as shown in Figure 1.

Development of Larva

Differences in development were first observed two weeks after initial exposure. During week 1 of observations, of the initial 15 organisms, there were 14 survivors in each group. Of the 14 in the control group, 13 remained larval, and 1 had pupated. Of the 14 in the experimental group, 12 remained larval, and 2 had pupated. The following week (week 2), we observed 14 living organisms in the control group (7 larvae, and 7 pupae). There were only 12 survivors in the experimental group (6 larvae, 3 pupae, and 3 darkling beetles).

Week 2 marked the first observation of beetle development. The following week (week 3), our third and final observations were made. Our control group still included 14 living organisms (7 larvae, 1 pupa, and 6 beetles: 2 beige, 1 brown, and 3 black. These beetles were the first for the control group). The experimental group continued to have 12 living organisms (5 larvae, 1 pupa, and 6 darkling beetles: 1 dark brown, 5 black). It was noted that all of the beetles from the experimental group were very active. By the end of three weeks of treatment, the control group consisted of 64% larvae (mealworms), 21% pupae, and 14% adult beetles (Figure 2). The experimental group consisted of 59% larvae, 16% pupae, and 25% adult beetles. The beetles in the experimental group were darker than those in the control groups, indicating that the adults in the experimental group were older and had emerged before the control beetles. It was noted that the beige and brown beetles in the experimental group were also more active than the control beetles. While significance was not reached based on T-test analysis ($p>0.05$) of treatment groups by developmental stage, experiments should be repeated with more animals to confirm the trend observed.

Average Weight per Model Organism

Average weights of the control group in week one was 0.11g, and 0.10g for the experimental group (Figure 3). By week 2 (deaths taken into consideration) average weights were 0.12g for the control group, and 0.12g for the experimental group. By the third week, the control group weighed 0.13g, and the experimental group weighted 0.14g. No significant differences were observed for weights between the treatment groups based on T-test analysis ($p>0.05$).

Protein Assay

The Bradford protein assay was used to determine the concentration of protein in 6 adult darkling beetles; 3 protein samples from the control group and 3 protein samples from the experimental group. The control protein samples had an average of approximately 94µg of protein per 15µL of sample (Figure 4). The experimental samples had an average 151ug of protein per 15µL of sample. These results indicate that snuff may increase the amount of protein present in an organism. However, T-test analysis revealed that significant differences were not observed between control groups ($p>0.05$).

Because animals were exposed to water that contained moist snuff, it is unclear which chemical component of snuff was responsible for the results that we observed. Specific components should be isolated to determine which tobacco plant chemical could affect mortality and alter development as seen in this study.

Based on the results of our study, we must reject our original hypothesis. Although mortality was higher with insects exposed to the moist snuff, mortality was also observed with the control group. Further studies examining several concentrations of snuff (or nicotine) should be explored to determine LC50 (lethal concentration to produce 50% mortality), a more statistically consistent number and useful for comparisons.

While we cannot conclude that snuff had a significant impact on overall developmental rate of beetle larvae, specific developmental stages should be explored further, especially the development from pupae to adults. Our findings appear to indicate that snuff may increase the rate of development to the adult stage. More animals need to be tested with a more relevant exposure concentration however to reach statistical significance.

Our results may also indicate that snuff caused the organisms to be more active. It is unclear if nicotine was the direct cause

of any of these effects. However, our protein data suggest that moist snuff might have induced protein synthesis in insects as a possible defense mechanism as shown in many animals exposed to a particular toxin. While we cannot conclude that snuff significantly impacted overall weight and protein in these animals, the overall weight was measured for all developmental stages rather than each developmental stage. Because the protein data reflect only adult protein levels, it remains to be seen whether snuff actually increased the overall weight of the adults. Further studies should be conducted to clarify the discrepancies in weight and overall protein. In addition, further research should be conducted to understand the impact of nicotine (at low concentrations) on the growth rate of insects. Such research would benefit the application of pest management strategies, particularly those that involve insecticides that inhibit acetylcholinesterases (enzymes that degrade the neurotransmitter, acetylcholine). Because nicotine mimics the actions of acetylcholine, such research would prove to be useful in developing pest management strategies.

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