Effects of caffeine on the growth and post-embryonic development in Manduca Sexta

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Abstract:

This study evaluated the impact of caffeine on the growth, metamorphosis, and adult development of Manduca sexta larvae. A range of dosages were administered in an artificial diet according to insect weight. Corresponding growth and progress through development stages of the animals yielded a negative correlation between caffeine ingestion and maturation. Data indicated that caffeine caused significantly decreased growth rates and hindered development in Manduca sexta. Ingestion of caffeine retarded growth rates and reliably culminated in defective development and/or death of the insect. These results support the potential use of caffeine as a means for control of insect development.

Introduction

Over the past half century, the xanthine alkaloid compound, caffeine (1,3,7, trimethylxanthine) has been heavily ingested by humans.[1] Concerns have arisen as a result of observed negative effects on development and possible toxicity of the compound.[2,3] This knowledge, and the potential development for use as a pesticide, prompted an evaluation of how the continuous ingestion of caffeine, in varying dosages, impacts the growth, development, and metamorphosis of the tobacco hornworm, *Manduca sexta*.[4]

Caffeine is known as a stimulant drug that inhibits the enzyme cyclic AMP phosphodiesterase (E.C. 3.1.4.53). Cyclic AMP is released in the human body when it is in an excited state. In a typical adrenaline response, cyclic AMP is broken down by phosphodiesterase and the body returns to a resting state. The presence of caffeine causes a prolonged alerted response because cyclic AMP is not broken down. In humans, caffeine has also been found to cause the antagonization of adenosine receptors, increased cytosolic calcium levels, blocked stimulation of glucose transport, and inhibited intrinsic protein kinase activity.[5] These symptoms can have many negative implications for the human body, especially under long term exposure to caffeine.

The fact that caffeine has several mechanisms of action can help to explain its pleotropic effects and physiological properties. Studies of the silk moth, *Bombyx mori*, have shown that the enzyme cyclic AMP phosphodiesterase is active throughout the growth and development of the moth and that it is inhibited in the presence of caffeine.[6] In hornets, *Vespa orientalis*, and bees, *Apis mellifera*, significant sensory and physiological changes were observed when the insects were exposed to caffeine. These effects included but were not limited to alteration in motor abilities, sensory responses to light and noise, increased irritability, and changes in appetite, copulation, hibernation, and longevity. The insects did not development any observable tolerance or addiction to caffeine.[7] These studies give reason to consider that caffeine does impact insect behavior, growth, and development.

Several invertebrate studies have shown that growth and metamorphosis of the organism display a negative correlation with respect to caffeine ingestion through dietary means. For example, caffeine is known to be a potent inhibitor of adult development in the moth *Hyalophora cecropia* by disrupting the synthesis or release of a brain hormone among impacting other crucial events.[8] Caffeine arrested growth and severely affected pupae formation in *Musca domestica* (common house fly) larvae.[9] In the larvae of the moth fly, *Telmatoscopus albipunctatus*, caffeine is known to cause retardation of growth and development and along with a high mortality rate.[10] Additionally, caffeine was shown to prevent the initiation of adult development in various silk moth pupae, *Samia cynthia, Callosamia promethea*, and *Bombyx mori*.[6,11]

Caffeine has been shown to reliably inhibit growth and metamorphosis in a number of insects. However, the mechanism by which this effect manifests itself is unknown and may vary between species. Studies have produced data that suggests some reasonable possibilities. One potential explanation, suggested by the results of a study with moth flies, proposed that caffeine may affect larval growth by retarding DNA synthesis.[10] Administration of caffeine to moth larvae, *Hyalophora cecropia*, indicated that the synthesis or release of a brain hormone was disrupted and that the xanthine compound likely acts at other sites within the sequence of events leading to adult development.[8] In *Callosamia promethea*, giant silk moths, it was shown that caffeine caused extensive degenerative damage in the brain and segmental ganglia, and resulted in arrested adult development through augmentation of the central nervous system.[11] These studies illustrate the multiple mechanisms and sites of action that the caffeine compound has on invertebrate systems.

The brain and associated neural hormones are crucial to normal insect development. For example, caffeine induces changes in gene expression in the honey bee, *Apis mellifera*, brain that exert both cognitive and developmental effects.[12] The *Hyalophora cecropia* silkmoth exhibits a change in the levels of cyclic AMP prior to the initiation of normal adult development, potentially providing a significant opportunity for caffeine to impact subsequent development.[13] Additionally, caffeine has been shown to specifically attack the central nervous system in the giant silk moth, *H. cecropia*, resulting in changes that successfully inhibit growth and metamorphosis.[11]

Manduca sexta (tobacco hornworm) serve as an important model for the study of the

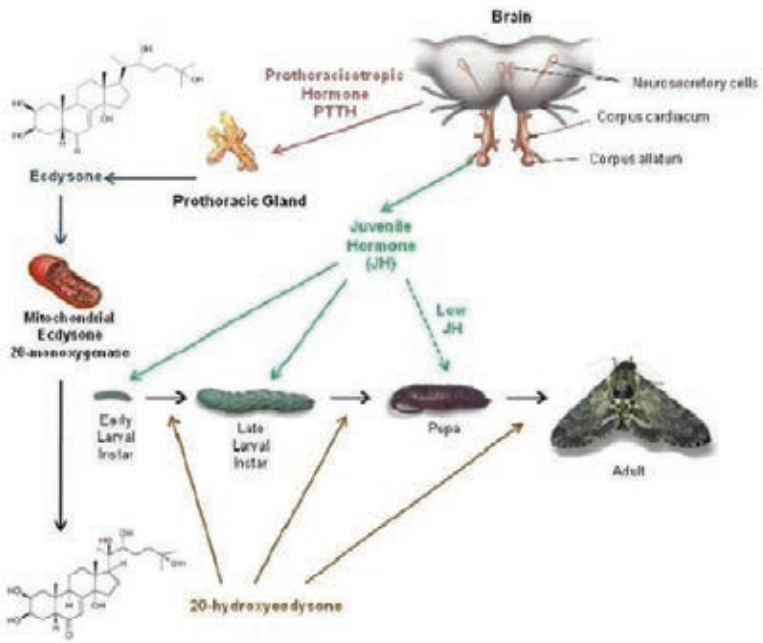

Figure 1: Regulation of metamorphosis in Manduca sexta by key developmental hormones.[24] The insect brain secretes PTTH (prothoracicotropic hormone) and JH (juvenile hormone). PTTH acts on the prothoracic gland to promote the synthesis of ecdysteroids such as ecdysone. Ecdysone is converted to the active form of the molting hormone by the mitochondrial ecdysone 20-monooxygenase. Together 20-HE (20-hydroxyecdysone) and JH work synergistically to regulate larval molts as the insect grows through each instar. Ultimately, the presence of ecdysone relative to the absence of JH results in the formation of a pupa and eventually the emergence of an adult.

growth, development, and metamorphosis of insects. They are an ideal candidate due to their pest reputation, rapid growth, large size, and ease of laboratory rearing.[14]*Manduca sexta* have a complete life cycle lasting 30-50 days with several larval stages.[14] When the larvae have reached the fifth instar, the process of metamorphosis begins. A variety of hormones are responsible for the steps involved in this process. An endocrine cascade begins once the larvae have reached a critical weight.[15] Once the process has been initiated metamorphosis is continually regulated by ecdysteroid hormones which ensure that each step occurs in a coordinated fashion.[16] The brain controls the secretion of the hormones controlling development based on the action of its tropic hormone (prothoracicotropic hormone) on the prothoracic glands.[17] The secretion of the brain peptide hormone prothoracicotropic hormone (PTTH) positively regulates the production of ecdysteroids by activation of ecdysone synthesis.[18,19] Subsequent conversion of the molting hormone ecdysone to the active form 20-hydroxyecdysone (20-HE) by the NADPH-utilizing ecdysone 20-monooxygenase (E20-M; E.C.1.14.99.22) is essential to the development from larvae to pupae.[20] Lower concentrations of JH result in an ecdysone-induced molt that produces a pupa.[21] Following the final larval-pupal molt, the larvae develop into opaque green pupae that gradually tan.[22] Juvenile hormone (JH) levels are responsible for the induction of larval molting and regulate the cuticular melanization of the new endocuticle formed when the pupa is generated.[23] Eventually there is no active JH present and the pupa gives rise to an adult.[24] The roles of these hormones in the process of insect development are displayed in Figure 1.

Considering the reviewed examples and the paucity of information linking caffeine to specific developmental pathways, the synthesis and activity of these brain-associated hormones could potentially be affected by caffeine ingestion. The results of this study seek to clarify and characterize the impact of caffeine on insect larval development.

Materials and Methods

Manduca sexta Rearing

Manduca sexta eggs and artificial diet were purchased from Carolina Biological (Burlington, NC). *Manduca sexta* were grown on an artificial diet in a Percival Intellus Environmental Controller (Perry, IN) at a constant temperature of 27°C at 63% humidity with a 12-hr light/dark cycle. Caffeine powder was purchased from Sigma-Aldrich (CAS# 58-08-2). Caffeine was carefully mixed with the artificial diet and administered through dietary ingestion. Each insect was raised in an individual specimen cup. Evaluation and maintenance of insects was performed on a daily basis as per Hatakoshi, Nakayama, and Riddiford.[22] All food prepared with caffeine was retained with that particular larva until completely consumed. Caffeine allotments were recalculated periodically as animals increased in size in order to maintain correct proportions to respective lethal dose values.

Experimental Design

Three experimental trials were conducted. The first trial was designed to establish the lethal dosage (LD) of caffeine in *Manduca sexta* and consisted of five experimental groups: control, LD_{25}, LD_{50}, LD_{75}, and LD_{100}. Insects used in this trial ranged from early third instar to mid-fourth instar. Dosages for each experimental group were developed with the goal of simulating mammalian toxicity equivalents. Calculations were performed in accordance to the lethal dosage recorded in humans where LD_{50} in humans is approximately 150-200 mg/kg.3 Caffeine doses were calculated for each experimental group based on an average of 175 mg/kg for LD_{50}. Values are listed in Table 1.

Dosages were calculated based on the initial weight of the animal. The amount of caffeine was increased by one order of magnitude in order to encourage more pronounced results as it became apparent in initial trials that *Manduca sexta* are not as sensitive to caffeine as humans. Insects naturally have higher levels of P450 enzymes that allow them to metabolize harmful compounds at a faster rate than humans.[25] As a result, increased LD values were established as a baseline for *in vivo* assessments. Doses were measured and incorporated into two grams of the artificial diet.

The second trial was designed based upon the results of the first trial with the goal of obtaining a higher degree of fatality. The dosage was increased by another order of magnitude in order to account for the high P450 metabolism of toxic compounds noted in insects and with hopes of obtaining consistent lethal results. Three experimental groups were established: control, LD_{50x100}, and $LD_{100x100}$. Insects used for this experiment were in late second to early third instar. In this age range insects were large enough to receive a measurable caffeine dosage, but also young enough to provide sufficient time to observe long-term

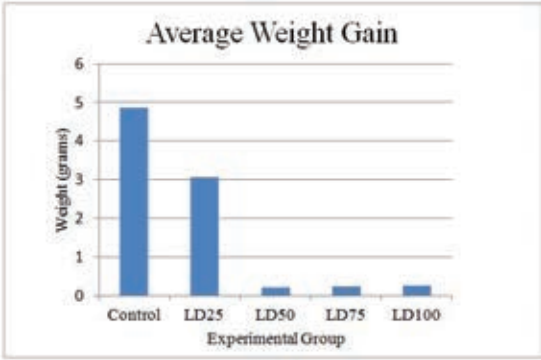

Figure 2: Average weight gain in Manduca sexta upon exposure to caffeine in trial 1. Average weight gain values were determined as per the protocol described in the Materials and Methods section under Evaluation and Analysis. The experimental groups LD_{50}, LD_{75}, and LD_{100} receiving higher dosages of caffeine experienced significantly lower levels of growth than the Control and LD_{25} groups.

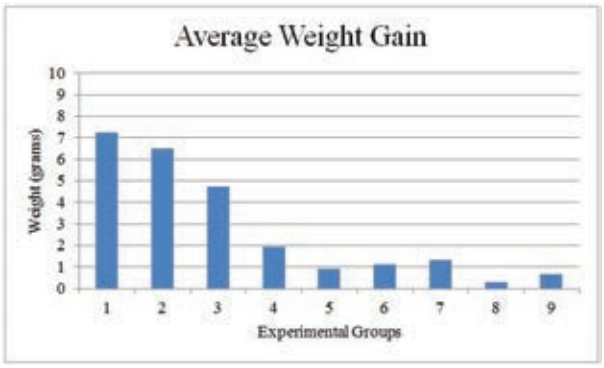

Figure 3: Average weight gain in Manduca sexta upon exposure to caffeine in trial 3. Average weight gain values were determined as per the protocol described in the Materials and Methods section under Evaluation and Analysis. Experimental groups are designated as control (1), LD_{25} (2), LD_{50} (3), LD_{75} (4), LD_{100} (5), LD_{25x100} (6), LD_{50x100} (7), LD_{75x100} (8), and $LD_{100x100}$ (9). The following experimental groups receiving higher dosages of caffeine, LD_{75}, LD_{100}, and LD_{25x100}, LD_{50x100}, LD_{75x100}, $LD_{100x100}$, experienced significantly lower levels of growth than the control, LD_{25} and LD_{50} groups.

effects.

The purpose of the third trial was to further test the effects of various caffeine dosages on *Manduca sexta* larvae in terms of lethal dosage and developmental impacts. Nine experimental groups were established in the third trial: control, LD_{25}, LD_{50}, LD_{75}, LD_{100}, LD_{25x100}, LD_{50x100}, LD_{75x100}, and $LD_{100x100}$. Insects chosen for this trial were also in the late second to early third instar. Dosages were calculated as previously described using increases of one and two orders of magnitude to account for increased P450 metabolism.

Evaluation and Analysis

Animals were maintained and evaluated through pupation. All resulting pupae were stored in the incubator following the experiment for comparison between experimental groups. Weight, behavioral observations, and physical observations were recorded daily. Insects were weighed on a Fisher Scientific scale (Model S-300D). Behavioral and physical observations consisted of an overall assessment established by determination of the level of relative activity (based on reaction to touch and amount of movement on the scale), the presence/quantity of feces, whether or not food was consumed, and the condition of the animal in terms of size and coloring as per Hatakoshi, Nakayama, and Riddiford.[22]

Table 1: Established caffeine dosage for each experimental group. This table shows the relative dosages used to calculate measured amounts of caffeine. Each amount was used to determine the amount to feed each individual and corresponds to a certain experimental group.

Experimental Group	Dosage (mg/kg)
Control	0.0
LD_{25}	87.5
LD_{50}	175.0
LD_{75}	262.5
LD_{100}	350.0

Results from each trial included a quantitative analysis of overall weight gain and growth rate. Experiments were replicated in order to obtain a compilation of accurate and consistent results. Behavioral and physical observations were also noted. For all trials the change in total weight for each insect was calculated and then averaged to determine an average change in total weight for a particular experimental group. This number is referred to as average weight gain. For an individual insect, the change in weight was calculated by subtracting the initial weight from the final weight at the end of the trial. Chi-squared analysis was performed for the first and the third trial on the average weight gain data to determine significance of the values.

For each experimental group in the third trial, the maximum change in growth was taken for each animal and averaged. The maximum change in growth for an individual was designated as the day in which the insect gained the most weight.

Results

The results of the first trial indicated that increased dosages of caffeine corresponded to decreased overall weight gain. This was especially notable in the LD_{50}, LD_{75}, and LD_{100} groups as shown in Figure 2. In terms of general post-embryonic development, *Manduca sexta* in the LD_{50}, LD_{75}, and LD_{100} groups exhibited difficulty accomplishing normal developmental molts between instars in the final molt prior to pupation. If the insects do not reach a critical weight, they cannot mature correctly.[21] A number of insects tanned without attempting the final larval-pupal molt or forming a pupa. The majority of the animals that were fed caffeine died before reaching maturity and did not proceed through development in a successful manner.

In the first trial, control animals developed normally and gained an average of 4.85 grams. LD_{25} animals exhibited an average weight gain of 3.05 grams. In the LD_{50} experimental group, the average growth was 0.22 grams. In the LD_{75} group, the average growth was 0.24 grams. In the LD_{100} group, average growth was 0.26 grams. Physical effects of caffeine ingestion manifested themselves primarily in the final stages of adult development and varied between individuals. Symptoms included failure to complete the final molt prior to pupation and premature tanning with no final larval-pupal molt and no pupa formation. In general, insects either produced malformed pupae or failed to complete the last larval-pupal molt prior to pupation hindering any further development. Some successful pupation was noted, but resulting pupae appeared squished or were malformed. *Manduca sexta* were observed repeatedly to have difficulty molting at each instar. This difficulty resulted in constrictions of molted skin that could have contributed to premature death. Several individuals in the LD_{75} and LD_{100} groups died with no significant growth and no sign of any progress toward adult development. Those insects in the higher dosage experimental groups that did accomplish some weight gain tanned with no final molt and did not

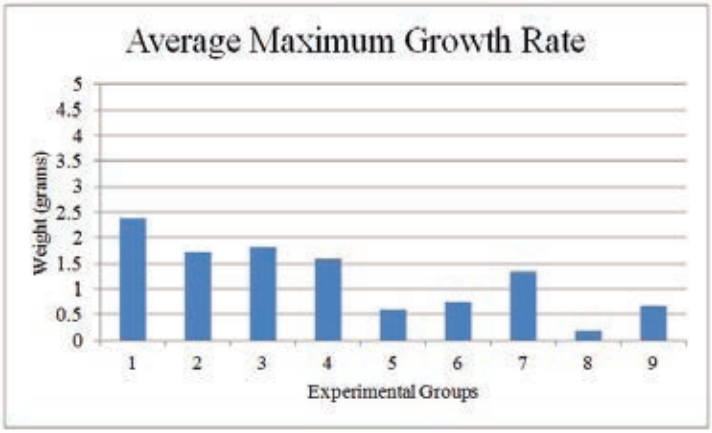

Figure 4. Average maximum growth rate of Manduca sexta upon exposure to caffeine in trial 3. Average maximum growth rate values were determined as per the protocol described in the Materials and Methods section under Evaluation and Analysis. Experimental groups are designated as control (1), LD_{25} (2), LD_{50} (3), LD_{75} (4), LD_{100} (5), LD_{25x100} (6), LD_{50x100} (7), LD_{75x100} (8), and $LD_{100x100}$ (9). Control and lower caffeine dosage groups had higher maximum growth rates overall.

survive. Chi-squared analysis showed a significant decrease in average weight gain for the LD_{50}, LD_{75} and LD_{100} groups.

Results of the second trial are replicated in the third trial and are therefore not shown. Results from the third trial were obtained in much the same way as the first trial. It is apparent from these data that the animals receiving a diet with caffeine did not achieve the same degree of weight gain as the control. Increasing caffeine dosage corresponded to decreasing overall weight gain. Excluding a few animals from LD_{25x100} and LD_{50x100} groups, respectively, all of the animals in the LD_{100}, LD_{25x100}, LD_{50x100}, LD_{75x100}, and $LD_{100x100}$ groups showed no significant growth and died prior to reaching the final stages of development before they could even have a chance to attempt pupation. Control, LD_{25}, and LD_{50} experimental groups experienced significant overall weight gain throughout development as seen in Figure 3.

General observations regarding how the final stages of development for the insects in the third trial progressed revealed a low level of successful pupation and a high level of mortality. Many of the animals that received caffeine did not achieve successful adult development and could not form a normal pupa. Results were similar to observations made in the first trial. Control insects developed normally. In the LD_{25} group, animals tanned without pupating and some failed to complete the final larval-pupal molt as indicated by white stripes on the side.[21] In the LD_{50} group, all the insects in the group formed an unhealthy pupa. Pupae typically had a clear, fragile membrane area on the underside or had misshapen hooks. In the LD_{75} group, all animals failed to complete the final larval-pupal molt and died. Insects in the groups above LD_{75} died prior to achieving any successful development. Any individuals that did manage to accomplish some weight gain reached the same fate as those in the LD_{75} group. Chi-squared analysis found the decrease in average weight gain values to be significant for LD_{75}, LD_{100}, $LD_{25 x 100}$, $LD_{50 x100}$, $LD_{75 x 100}$, and $LD_{100 x 100}$.

As expected, the control group had the largest maximum growth rate. These data did not show as much of a change in rate as might have been expected, but it was clear that insects fed caffeine grew significantly less each day and reached an overall smaller weight as shown in Figure 4. Average maximum growth rate for control animals was 2.38 grams. The average maximum growth rate for animals received caffeine ranged from 0.2 to 1.59 grams.

Throughout the experiment, it was observed that animals that received caffeine often had an aggressive or irritable response to touch. This was exhibited with flailing and biting motions. Some individuals showed a high level of activity during handling and when placed on the scale. A few insects sat on the scale with their heads up in the air swaying back and forth. In comparison, the animals in the control group were relatively docile. They did not significantly react to touch and were not overly active.

Discussion

As reported, caffeine retarded the growth of *Manduca sexta*. Overall, feeding caffeine via artificial diet to *Manduca sexta* appeared to lower the rate of growth, decrease the extent of weight gain, and hindered the ability to complete healthy development. Insects receiving caffeine administered through their diet did not match the growth rate or size of control insects. Indeed, total growth was significantly decreased in all experimental trials along in correlation with increased caffeine ingestion and exposure. As caffeine administration was increased, weight gain was substantially lessened and eventually caused mortality. In addition to decreased weight gain, it was also apparent that caffeine slowed down the growth rate of the larvae. Decreased observed weight gain and growth rate could be the result of diminished appetite in treated animals; the cause for this could be two-fold. The first possibility is the detection of caffeine, resulting in rejection of the artificial diet and depreciated nutrition. The second is that their ingestion of the caffeine brought about a decreased appetite response that also resulted in lowering overall food consumption. These reasons are reviewed by John Glendinning.[26]

Behavioral modifications were also noted for animals treated with caffeine. *Manduca sexta* that were fed and exposed to the intermediate amount of caffeine on a regular basis had more extreme reactions to stimulation via touch or noise and were more active overall during observation periods. Consequently, these results suggest that caffeine impacts the animals in a similar way to how it impacts humans as described by Isaac et al., and causes to some degree a heightened state of alert or hyperactivity.[27]

It was also observed that the animals receiving caffeine had difficulty completing the final stages of larval development. *Manduca sexta* from the highest dosage groups perished before reaching the fourth and fifth instars and therefore never pupated. However, animals from the lower dosage groups typically did survive until this stage and several different observations were made. Typically, one of two things happened: mortality was observed prior to the final larval-pupal molt or they exhibited premature tanning with no indication of complete pupation. Animals that showed such mortality developed pronounced lateral white striping. The animals that tanned prematurely during the fifth larval instar did so without pupating and did not display these white strips. These results suggest that the overall impact of caffeine administration lead to the inhibition of *Manduca sexta* growth and post-embryonic development. It is possible that caffeine contributes to the activity or inactivity of important developmental hormones, for instance

different ecdysteroids like ecdysone.[16] Another potential is that the inhibited growth of the organism overall negatively impacts the stages of its development. If the animal is moving through stages of development too quickly or too slowly, an early or delayed response by hormones, such as PTTH (prothoracicotropic hormone), ecdysone, and JH (juvenile hormone), could be causing these kinds of results. If the animal is not at a critical weight, then the release of the developmental hormones is delayed as described by Nijhout and Williams.[28]

It is known that various flavonoids, other plant allelochemicals, and compounds such as caffeine have an effect on insect growth, development, and reproduction. However, the mechanisms for these effects require more study. The inhibition of post-embryonic development in insects by allelochemicals and other compounds such as caffeine is considered. It is recognized that these compounds could potentially function as biopesticides that act to control insect development.[24] By preventing the insects from reaching sexual maturity, the pest can be effectively controlled. The mechanism of how ingested caffeine affects the insect, as well as how it impacts humans, mammals, and other insects, must first be understood in order to determine the impact of the compound on subsequent development. These evaluations would yield information that should be taken into consideration when considering caffeine and any other compound as a potential biopesticide. Further investigations are required to determine the feasibility of this application due to the high concentrations of caffeine required to achieve a negative effect.

The results of this *in vivo* study demonstrate that caffeine causes significantly decreased growth rates and hinders development in *Manduca sexta*. While the exact biochemical mechanisms of this inhibition have yet to be elucidated, a correlation between caffeine and invertebrate development is evident. Certainly further study of this model system may lead to a better understanding of the effects of caffeine on insect development.

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