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Indole sensitivity in various insect herbivores.

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Indole Sensitivity in Various Insect Herbivores

By

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Background

Plants use a variety of mechanisms to defend themselves against herbivores [1]; [2], and chemical defenses are especially well developed in plants. The defensive chemicals used by plants can be volatiles or dissolved structural compounds. [1]; [3]. The goal of this study was to understand how one volatile compound commonly produced by plants, indole, affects the growth of herbivores.

A volatile is a substance with a high vapor pressure, meaning it will easily evaporate at room temperature (23C). In nature, plants release certain volatiles when they are damaged by insects and other herbivores. These volatiles, called Herbivore-Induced Plant Volatiles (HIPVs), help plants defend themselves by acting as a deterrent and serve as a signal to neighboring plants that an herbivore attack is happening nearby. [4]. The release of HIPVs is thought to be an energetically expensive process for a plant to undergo, thus it tends to be highly regulated. Some plants release varying concentrations of volatiles depending on the identity of the herbivore or the magnitude of the attack. [5].

One common HIPV released in nature is the compound indole (C_8H_7N). [6]. Indole is an aromatic, bicyclic organic compound that has toxic effects of many herbivores. [7]. Indole is an intermediate in the biosynthesis of the amino acid Tryptophan. In this process, indole is part of the Tryptophan Synthase Complex. There are two gene duplications that have occurred from the original Tryptophan Synthase Pathway, both creating pathways with new functions. The first duplication occurred through the gene that encodes the alpha-subunit of the Tryptophan Synthase Complex, creating the gene BX1. This pathway is responsible for the biosynthesis of DIMBOA, a defense compound that is present in the developing tissues of young seedlings. The second duplication that occurred encoded the gene indole-3-glycerol phosphate lyase (IGL). This gene is responsible for the emission of the volatile form of free indole, which is used specifically in response to herbivore attack. [8].

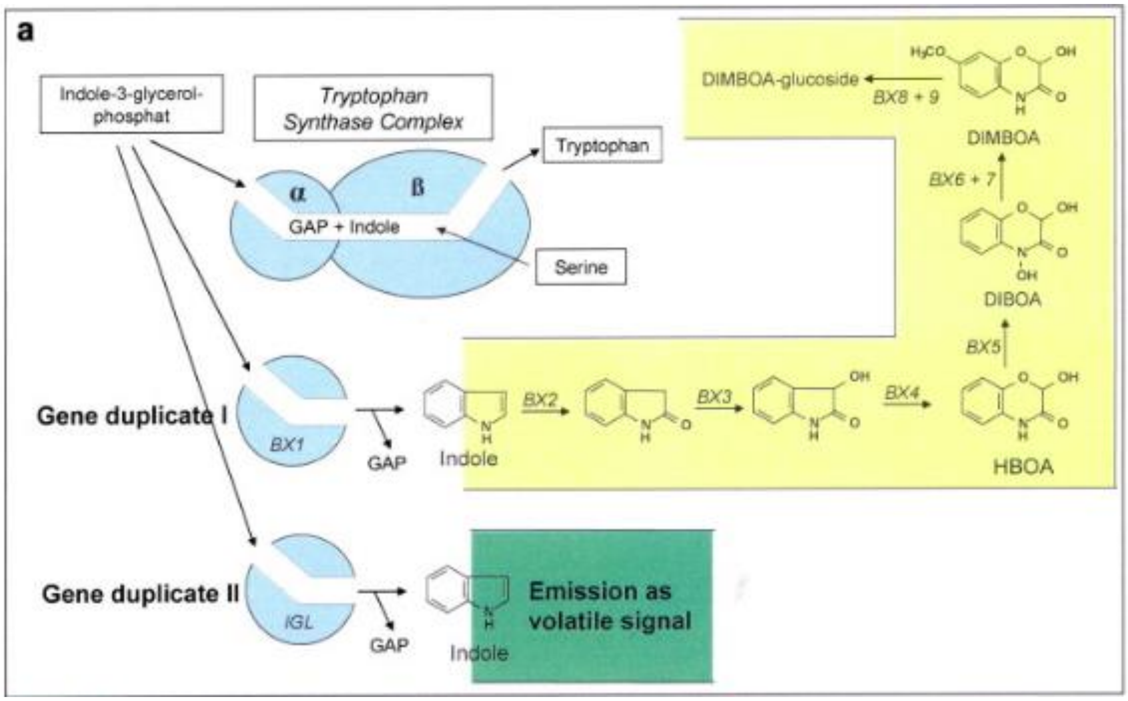

Figure 1: Illustration of Indole Gene Duplications. The original tryptophan synthase pathway and the pathways created by gene duplications I (BX1) and II (IGL). The pathway from gene duplication I, as shown, leads into the synthesis of DIMBOA, while gene duplication II releases indole in its volatile form. [8].

The HIPV indole is toxic to herbivores. [7]. When plants are under attack or receive signals from neighboring plants that are under attack, they produce indole. Not only is indole released into the air, but it is also consumed by the herbivores that are attacking the plant because indole is present in the leaf tissue being consumed. Indole has many negative effects in nature, including attracting natural enemies of the herbivores that are attacking the plant, signaling to other herbivores that the plant is already under attack and has begun producing deterrent compounds, signaling to other plants that it is under attack, and serving as a precursor for metabolites that are toxic to herbivores. [9]. Many plants in nature produce the compound indole, including soybean, sunflower, rice, millet, kidney bean, buckwheat, wheat, corn, oats, seeds of oats, and peas. [10]. Though the exact concentration of indole released by these plants is unknown, we do know that certain plants such as corn, rice, and oats produce more indole in relation to others. [10]. This project focuses on the effects of indole on insect herbivores – specifically Lepidoptera larvae (hereafter, caterpillars).

Caterpillars can be affected by HIPVs such as indole throughout all stages in their life cycle. Many such insects exhibit complete metamorphosis, with egg, larva, pupa, and adult stages. The larval (caterpillar) stage is when most feeding and growth occurs. As caterpillars develop, they progress through different instars, or incremental periods of growth. [11]. With each instar, the herbivores grow larger and closer to their adult form. The first instar of an herbivore is the stage immediately following hatching and is thought to be the stage in which the caterpillar in most vulnerable to its surroundings. [11].

Many different species of plant-feeding Lepidoptera exist in nature, and many are affected by the toxic effects of indole. Six such herbivore species include the fall armyworm (*Spodoptera frugiperda*), the cabbage looper (*Trichoplusia ni*), the beet armyworm (*Spodoptera exigua*), the velvetbean caterpillar (*Anticarsia gemmatalis*), the tobacco budworm (*Heliothis virescens*), and the corn earworm (*Helicoverpa zea*). These species are common agricultural pests and are often used in laboratory studies. [11].

One important difference among these species is their host range. Host range refers to the range of plants that a certain species is able to consume. [12]. Herbivores with a broader host range are ones that are able to thrive in a wide variety of conditions, whereas herbivores with a narrower host range have a limited range of environmental conditions in which they can survive. [13]. Species with narrower host ranges are not able consume as many types of plants as those species with a broader and more generalized host range. [12]. It is predicted that species with a wider host range are better equipped to handle different chemicals and metabolites that they may encounter, whereas species with a narrower host range are tolerant of chemicals pertaining more closely to that particular host range. [14].

Indole is toxic and lethal to some caterpillars, but the exact concentration of indole that causes caterpillar mortality is unknown. In this study, I explored the effects of different concentrations of indole on various species of caterpillars that differ in host range. I hypothesized that the lethal concentration of indole would vary consistently among species based on their host range. Specifically, I predicted that herbivore species with a wider host range would be more tolerant of higher concentrations of indole (i.e., have higher LC_{50}) than herbivore species with a narrower host range.

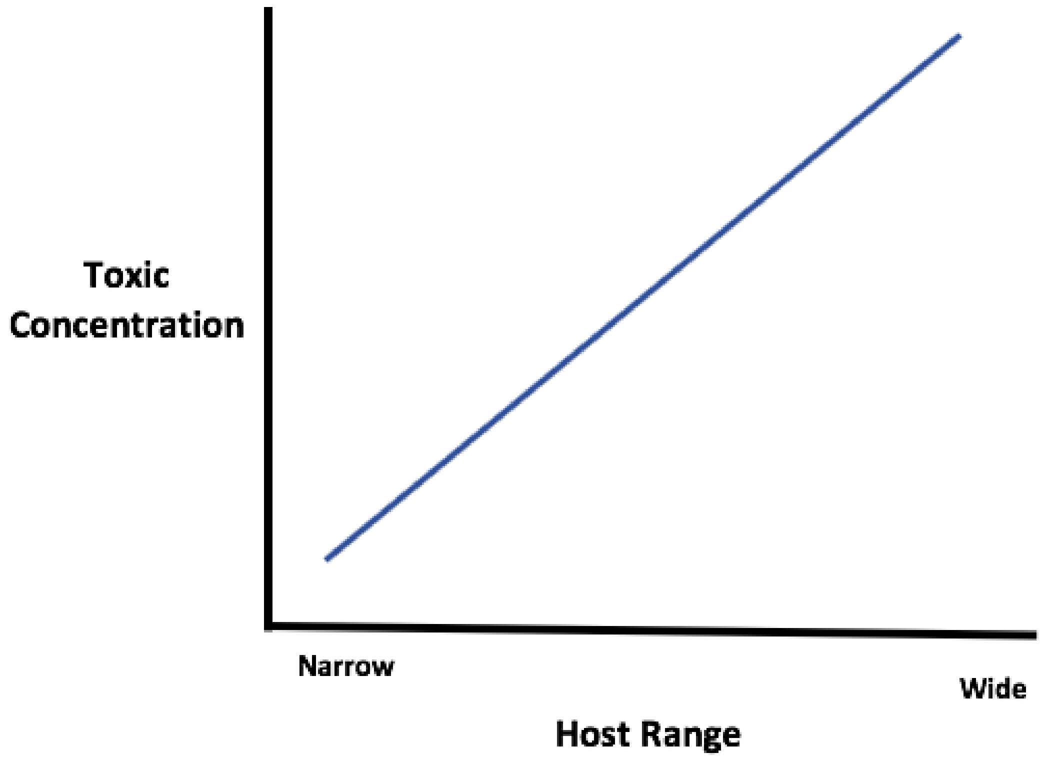

Figure 2: Prediction of Host Range and Toxic Concentration Relationship. The prediction that the concentration of indole needed to become toxic will increase as the host range of the caterpillar increases.

Experimental Approach – Methodology

I tested the effects of indole consumption on six different caterpillar species (Table 1) by using indole as a diet additive. I also tested the volatile form of indole for one of the species. The herbivore species used in this study were the fall armyworm (*Spodoptera frugiperda*), the cabbage looper (*Trichoplusia ni*), the beet armyworm (*Spodoptera exigua*), the velvetbean caterpillar (*Anticarsia gemmatalis*), the tobacco budworm (*Heliothis virescens*), and the corn earworm (*Helicoverpa zea*). Each week, the egg masses of one of the species of herbivore listed above were ordered from

Benzon Research Inc. USA. Upon arrival, the egg masses were immediately placed into clear plastic cups and left at room temperature for approximatly 72 hours. Once the eggs hatched, ten first-instar herbivores were transferred to each experimental cups using a fine-tipped paint brush. The cups were arranged on an open benchtop in an array based on the concentrations of indole added. The cups were in a room that did not have windows. Temperatures in the laboratory space ranged between 20-25°C.

The herbivore diet was prepared on the same day that the herbivores were transferred to their experimental cups. Approximately 32 g of diet powder (from Southland Products Inc., Arkansas, USA) was added to 100 mL of boiling water to prepare the diet. The diet was then transferred into designated 50 mL plastic centrifuge tubes, each containing specific amounts of indole (see below). Once the diet was added to the tube, the tube was placed on a VWR Analog Vortex mixer for one minute to ensure that the indole was evenly distributed within the diet. For the experiments in which indole was tested directly as a diet additive, one tube contained no indole and served as the control diet for the experiment, while the rest of the tubes contained indole crystals to generate experimental concentrations ranging between 0.01-5.0 mg/mL. Experimental concentrations of indole were chosen based on results from previous experiments and were intended to mimic the concentrations that could be found in nature. Specifically, the concentrations tested included values that caused a decline of survivorship in pilot studies. The increments at which indole concentrations were tested was chosen to help calculate an accurate LC_{50}. The diet solidified in the centrifuge tubes for approximately one hour before being cut into 10mm x 5 mm cylindrical pieces and placed in their respective cups. Each indole concentration and the control diet had 6-8 replicates.

The effects of volatile indole in the herbivore's headspace were also tested for the cabbage looper (*Trichoplusia ni*). The experimental cups and diet were prepared in a similar manner as the diet experiments described above, with the exception that no indole was added to the diet. Varying concentrations of indole were instead added to 2.0 mL amber glass vials from Agilent Technologies which all contained 1 mg of glass wool. The control vials in this experiment contained only the glass wool and no indole. The vials were sealed using a rubber septum and the septum was pierced with a hollow 18-gauge needle. The sharp end of the needle was pointing away from the interior of the vial and was used to pierce into the lid of the experimental cup, thereby attaching the vial to the cup. The flat end of the needle was inside the amber vial while the sharp end was inside of the experimental cup with the tip remaining approximately 2.5 cm above the bottom of the cup. This apparatus allowed for the volatile to diffuse into the experimental cups without escaping into the outside air. The amount of indole left in each vial after the experiment was completed was not measured, so the exact amount of volatile released into the headspace is unknown. This experimental protocol assumed that higher amounts of indole inside the amber vials would generate higher amounts of volatile indole in the headspace.

For each experiment, the number of live herbivore larvae was counted at 24 hours and 72 hours after being placed in the experimental cups. The experimental cups were frozen at 72 hours to complete the experiment. In the experiments in which indole was tested in headspace, the average weight of the surviving caterpillars was calculated by weighing all of the individuals of each experimental cup as a pool, and then dividing by the number present. Survivorship plots were generated for each species and the lethal concentration (LC_{50}) value was calculated.

Species	**Geographic Range**	**Host Plants**	**Image of Herbivore**
Spodoptera frugiperda (fall armyworm)	Native to the tropical regions of the Western Hemisphere. [15].	Wide host range. Consumes various types of corn and grass. [15].	 Photograph by James Castner
Trichoplusia ni (cabbage looper)	Found throughout most of the US in summer months. [16].	Wide host range with preference for the Brassicaceae family. Prefers cruciferous vegetables such as broccoli, cauliflower, cabbage, etc. [16].	 Photograph by James Castner
Spodoptera exigua (beet armyworm)	Originated in southeast Asia but now occur throughout the southern half of the US. [17].	Wide host range. Consumes many vegetables and flower crops (asparagus, broccoli, corn, chicpea, spinach, tomato, potato, etc.). [17].	 Photograph by John Capinera

Anticarsia gemmatalis (velvetbean caterpillar)	Found throughout the southeastern US. [18].	Narrow host range primarily consisting of soybean. [18].	 Photograph by Lyle J. Buss
Heliothis virescens (tobacco budworm)	Found in the eastern and southwestern US. [11].	Narrower host range. Consumes field crops such as alfalfa, clover, cotton, and tobacco. [11].	 Photograph by John Capinera
Helicoverpa zea (Corn earworm)	Found throughout most of North America. [19].	Wide host range. Includes many vegetable crops such as corn, tomatoes, cucumbers, cabbage, lettuce, and potatoes. [19].	 Photograph by John Capinera

Table 1: The six different caterpillar species used in this study, including the geographic range, host plants, and a photo of each species.

<u>Results</u>

Results were obtained for all six herbivore species with indole in the diet and, for *Trichoplusia ni*, with indole in headspace. The headspace experiment with *T. ni* showed that indole had no significant effect on their survivorship across the range of concentrations tested (Fig. 3). As the concentration of indole in headspace was increased, the survivorship of the herbivores stayed fairly consistent with that of the herbivores in the control experimental cups (Fig. 3). Likewise, the weight of surviving caterpillars was consistent across all tested concentrations (Fig. 4). Even at extremely high concentrations, indole in heasdspace did not have a lethal effect on the *T. ni* larvae. Though *T. ni* is a species with a narrow host range, the caterpillars survived at all concentrations of indole in headspace, even concentrations as high as 5 mg. At all of the concentrations tested, the weight of the caterpillars also stayed fairly constant, meaning that the indole in the headspace of these herbivores may not have had a large effect on their health.

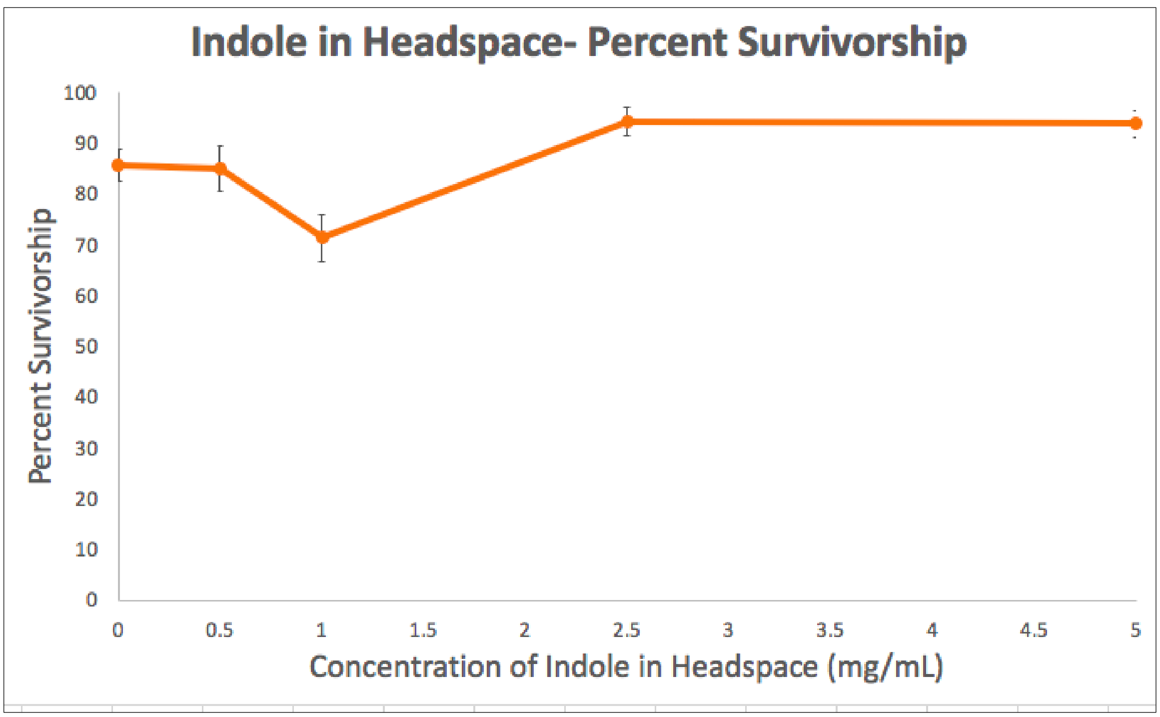

Figure 3: Indole Toxicity in *T. ni* Headspace. A graph of the survivorship of first-instar *T. ni* caterpillars at varying concentration of the volatile form of indole in headspace.

Concentration of Indole	Sample Size	Mean of % Survivorship	Standard Error
0.00	48	91.96	1.84
0.01	24	98.24	1.02
0.025	8	93.75	2.63
0.05	32	63.99	5.57
0.075	24	39.88	8.63
0.10	24	23.69	7.59
0.50	8	0	0
1.00	8	0	0

Table 2: The raw data for *Trichoplusia ni* indole in headspace.

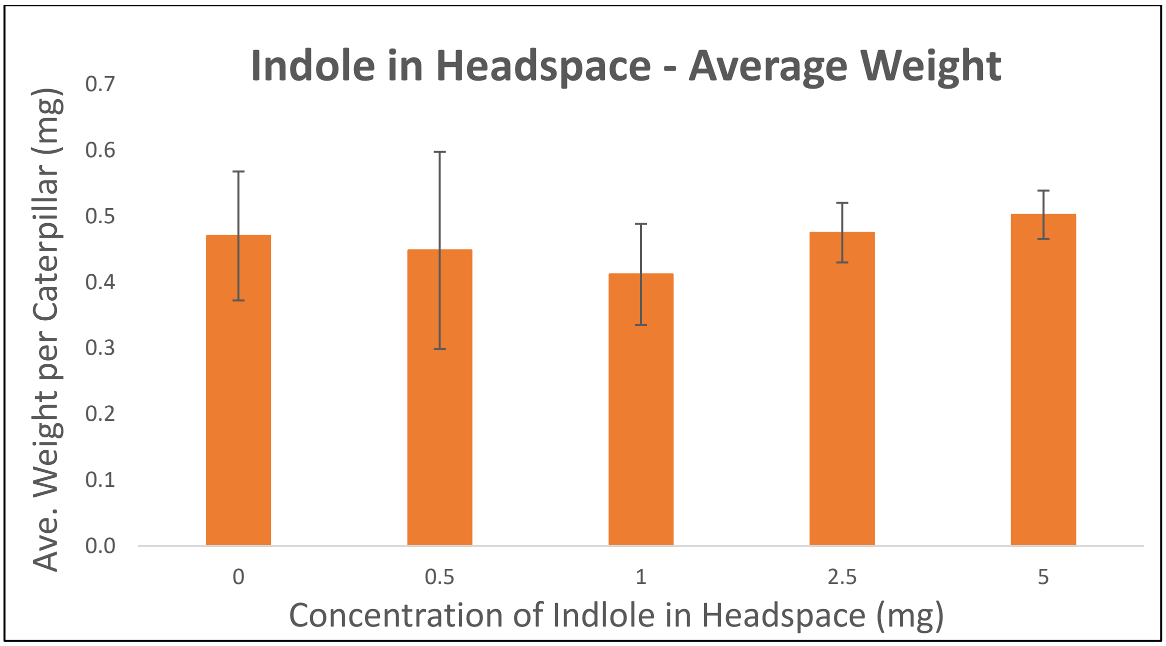

Figure 4: *T. ni* Wet Mass for Indole in Headspace. A graph of the average weights of *T. ni* caterpillars at the different tested concentrations of the volatile form of indole in headspace.

Experimental addition of indole to the caterpillar diet resulted in a significant difference in survivorship for all of the species tested across the concentration gradient. For each species tested, survivorship decreased as the concentration of indole increased (Fig. 5). However, the concentrations at which survivorship began to decrease differed among species (Fig. 5). The species *Anticarsia gemmatalis* and

Trichoplusia ni were the most sensitive to indole concentration (LC_{50} = 0.05 mg/mL), followed by *Heliothis virescens* (LC_{50} = 0.18 mg/mL), *Helicoverpa zea* (LC_{50} = 0.27 mg/mL), *Spodoptera frugiperda* (LC_{50} = 0.29 mg/mL), and *Spodoptera exigua* (LC_{50} = 0.35 mg/mL) (Fig. 3). The herbivore species with more specialized host ranges such as *A. gemmatalis* and *T. ni* were significantly more sensitive to the toxic effects of indole in their diet than were the herbivores with more generalized host ranges. Species such as *S. exigua*, *S. frugiperda,* and *H. zea*, which have wider host ranges, were not as sensitive to the toxicity of indole. These species survived at much higher concentrations of indole in diet than did the species with narrower host ranges.

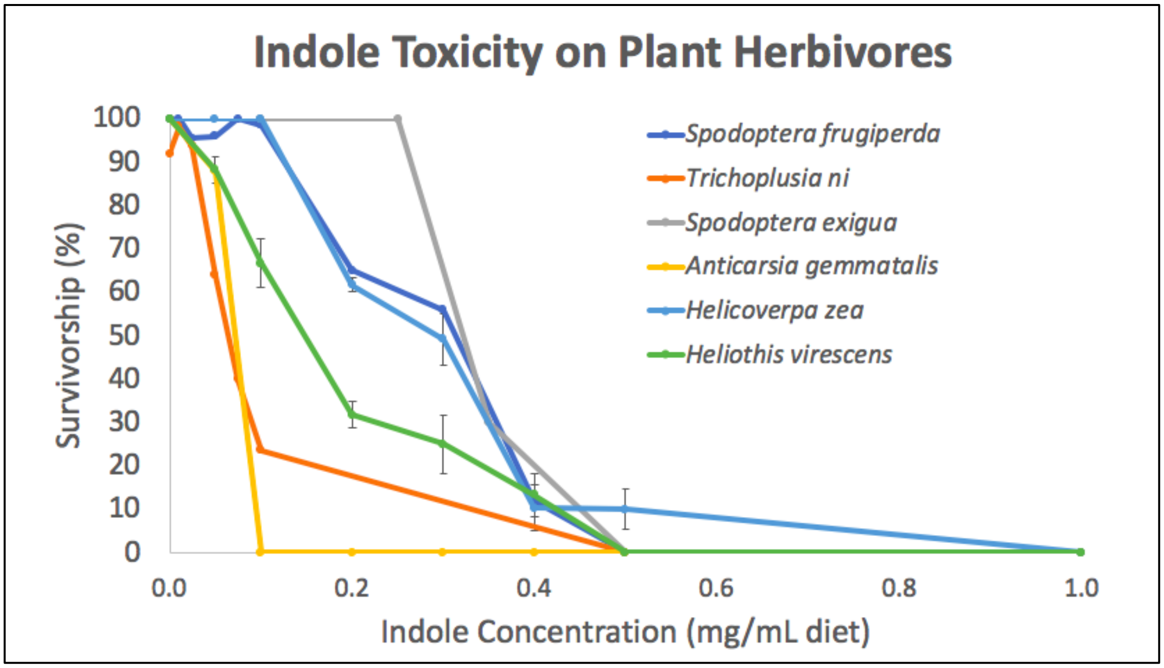

Figure 5: Toxicity of Indole in Diet. A graph of the average survivorship of six different caterpillar species against a concentration gradient of indole as a diet additive. Bars indicate ± 1 SE and are not shown for all means.

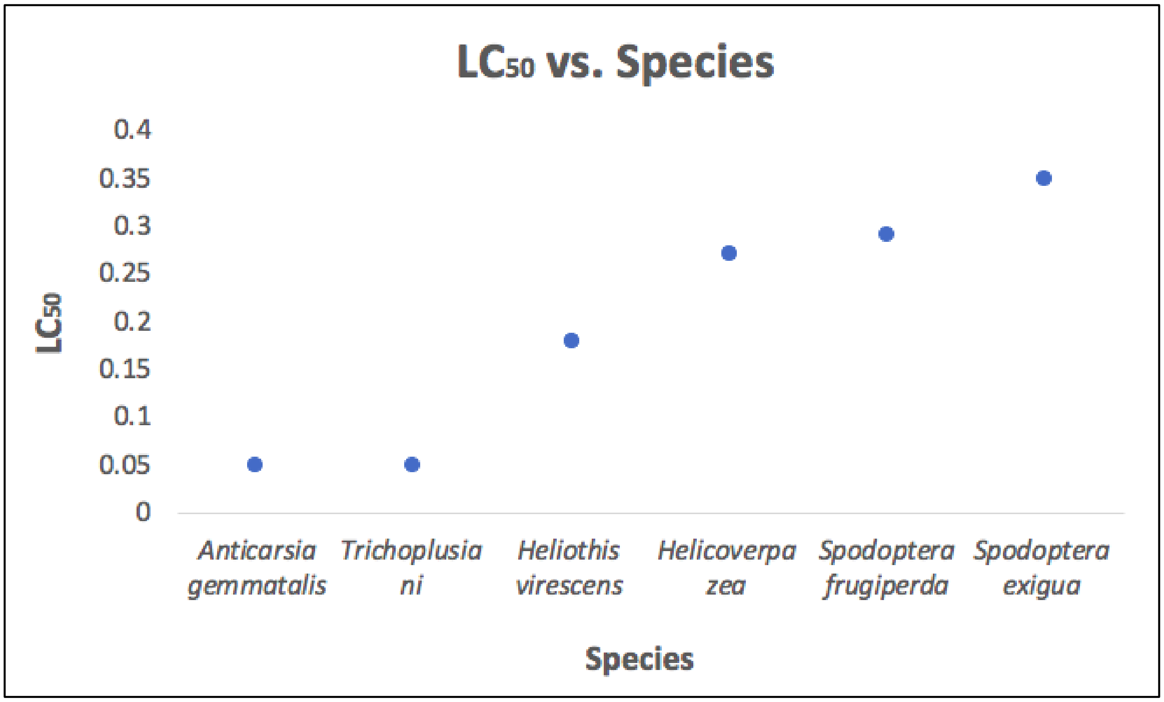

Figure 6: LC_{50} Data of Indole in Diet. A graph of the relationship between the lethal concentrations of indole found and the caterpillar species tested. The species are listed in order from narrow host range to wide host range.

Species	LC_{50}
Anticarsia gemmatalis	0.05
Trichoplusia ni	0.05
Heliothis virescens	0.18
Helicoverpa zea	0.27
Spodoptera frugiperda	0.29
Spodoptera exigua	0.35

Table 3: The LC_{50} values for indole as a diet additive.

Concentration of Indole	Sample Size	Mean of % Survivorship	Standard Error
0.00	6	100	0
0.05	6	88.33	4.77
0.10	6	0	0
0.20	6	0	0
0.30	6	0	0
0.40	6	0	0
0.50	6	0	0
1.00	6	0	0

Table 4: The raw data for *Anticarsia gemmatalis* indole in diet.

Concentration of Indole	Sample Size	Mean of % Survivorship	Standard Error
0.00	48	91.96	1.84
0.01	24	98.24	1.02
0.025	8	93.75	2.63
0.05	32	63.99	5.57
0.075	24	39.88	8.63
0.10	24	23.69	7.59
0.50	8	0	0
1.00	8	0	0

Table 5: The raw data for *Trichoplusia ni* indole in diet.

Concentration of Indole	Sample Size	Mean of % Survivorship	Standard Error
0.0	6	100	0
0.05	6	88.33	3.07
0.10	6	66.67	5.58
0.20	6	31.67	3.07
0.30	6	25.00	6.71
0.40	6	13.33	4.94
0.50	6	0	0
1.00	6	0	0

Table 6: The raw data for *Heliothis virescens* indole in diet.

Concentration of Indole	Sample Size	Mean of % Survivorship	Standard Error
0.0	6	100	0
0.05	6	100	0
0.10	6	100	0
0.20	6	61.7	1.67
0.30	6	49.2	5.85
0.40	6	10.3	5.30
0.50	6	10.0	4.77
1.00	6	0	0

Table 7: The raw data for *Helicoverpa zea* indole in diet.

Concentration of Indole	Sample Size	Mean of % Survivorship	Standard Error
0.00	26	100	0
0.01	14	100	0
0.025	14	95.5	2.31
0.05	14	96.0	2.04
0.075	14	100	0
0.10	12	98.5	1.12
0.20	6	65.0	4.28
0.30	6	56	4.48
0.40	6	12	4.01
0.50	6	0	0
1.00	6	0	0

Table 8: The raw data for *Spodoptera frugiperda* indole in diet.

Concentration of Indole	Sample Size	Mean of % Survivorship	Standard Error
0.00	12	100	0
0.25	10	99	1.00
0.50	7	0	0
1.00	5	0	0

Table 9: The raw data for *Spodoptera exigua* indole in diet.

Discussion

Plants use a variety of compounds to deter herbivores [2], many of which are volatile organic compounds. [20]. Some of these volatile compounds include green leaf volatiles such as cis-3-hexenol, cis-3-hexenyl acetate, and trans-2-hexenal, terpenes such as β-caryophyllene and linalool, and aromatic compounds such as indole. Knowledge about the toxicity of compounds such as these is very limited. A previous study examined the toxic effects of various monoterpenoids as insecticides on the European corn borer moth [21], but there is little information on the direct toxicity of volatile compounds on various caterpillar species.

The results of this study show that indole, a common plant defensive compound, affects the survivorship of many different species of caterpillars. Of the species tested, each has specific plants that can be consumed, and certain species have a wider range of consumable plants than others. It was hypothesized that herbivore species with narrower host ranges would be more sensitive to the toxic effects of indole, while species that have a broader host range could withstand indole at higher concentrations. This could be because species with a narrower host range are more specialized to handle defense compounds released by the specific plants that they can consume. These species could have traits to defend themselves against defensive compounds specific to their host range plants but may not have developed a way to defend themselves against indole as well as those species with wider host ranges. Species with a wider host range, however, could be more equipped to deal with a broader variety of defense compounds, including indole, because they come in contact with many different types of plants that release a variety of defense compounds. Indole is a very common compound released in nature by many plants [7], and caterpillars are bound to be exposed to indole as they attack various plants within their host ranges. If a species has a narrower host range, however, it is not as likely to be exposed to the same amounts of indole as a species with a wider host range.

The hypothesis that there is a positive correlation between indole toxicity and herbivore host range was supported. As the host ranges of species got wider, the sensitivity to indole decreased. This could mean that the species with wider host ranges can tolerate various different defense compounds more effectively than species with narrower host ranges. The species that have wider host ranges could be more tolerant to indole because they are able to tolerate a wider range of toxic compounds in general. Species with wider host ranges consume a wide range of plants, meaning they are exposed to a wide variety of defensive compounds. In order to survive, these species must be able to tolerate these various compounds. This could be the reason why species with wider host ranges are more tolerant of the defensive compound indole.

Species with narrower host ranges were shown to be more sensitive to indole. These species could be more sensitive to indole for a similar reason to why wider host range caterpillars are less sensitive. Species that have narrower host ranges can only feed on specific plants, so these caterpillars may only effectively tolerate the defensive compounds of those specific plants. Since these species do not feed on a large variety of plants, they have no need to develop mechanisms to tolerate large quantities of a wide range of defensive compounds that are not produced by the few plants that they attack. These caterpillars could be very well equipped to tolerate the defensive compounds released by the plant that they consume but may not be able to tolerate large amounts of indole because they had no need to.

Many plants produce indole as a defense compound, including soybean, sunflower, rice, millet, kidney bean, buckwheat, wheat, corn, oats, seeds of oats, and peas. [10]. However, these plants produce indole at different concentrations. Thought the exact amount produced is unknown, it is known that certain plants such as corn produce higher amounts of indole than plants such as soybean. [10]. Of the caterpillar species tested, the three with the widest host ranges (*S. exigua, S. frugiperda, and H. zea*) all consume corn. [17]; [15]; [19]. These species were also the ones that were most tolerant to indole at higher concentrations as a diet additive. This supports the claim that species with wider host ranges are more tolerant to indole because they are exposed to more plants that produce the compound in higher amounts. Soybean, a plant that is crucial to the diet of the narrow host range species *A. gemmatalis* [18] does not produce as much indole as corn. [10]. As a result, the species *A. gemmatalis* was one of the most sensitive to indole, further supporting the hypothesis.

The data for indole in the headspace of *T.ni* showed that there was no significant change in survivorship of the caterpillars as indole concentration was increased. This could mean that indole is not as toxic if just presented in the air as a volatile. Indole may need to be consumed within plant tissue to be toxic to these herbivores. The indole in this experiment was tested at very high quantities (up to 5 mg) and still did not prove to be lethal to the caterpillars, meaning that indole in this form is not toxic to *T.ni* caterpillars at the quantities tested.

The data showing the average weight of the *T. ni* species when indole was presented in headspace also showed no significant difference among varying concentrations of indole. If the indole was beginning to have a lethal effect on the caterpillars, a decrease in weight would have been shown as the concentration of indole was increased. This lack of change in weight as concentrations were increased could mean that the indole was not having a significant effect on the caterpillars in headspace, regardless of the amount of indole presented. It could also be possible that the concentrations tested may not have been high enough to be toxic. Indole in volatile form may become toxic to *T.ni* caterpillars when it is presented at higher amounts, but concentrations higher than the ones tested are unlikely to be found in nature.

This study could have been improved by also testing indole as a volatile in headspace for the remaining 5 caterpillar species. This would have allowed comparison between the toxicity of indole in headspace for multiple species of caterpillars. The study also could have been improved by testing more species of caterpillars with indole as a diet additive. This would have solidified the data and helped further support the hypothesis.

The results of this project raised a number of questions regarding the effects of indole on herbivore development. Specifically, does indole have similar effects on these herbivore species if they were tested at different instars? Would a different HIPV have similar effects on the first instar species tested? Do all HIPVs have a correlation with the host range of different herbivore species? These questions would serve as great starting points for future studies as an extension of the work done in this experiment. Future studies such as these may have broader implications of understanding the chemical ecology of plants.

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