ABSTRACT

Title of Thesis: IN VITRO MEASURES OF MDR-TRANSPORTER FUNCTION AND WHOLE-HIVE EXPOSURE DYNAMICS USING FLUORESCENT DYES

Grace Regina Kunkel, Master of Science, 2014

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We need to study *Apis mellifera* both in vivo and ex vivo to better understand honey bee biology. In vivo synergism of chemicals can occur when xenobiotic transporters are inhibited by one chemical, allowing a second chemical to accumulate and become toxic. I have conducted assays between 2010 and 2013 that demonstrated RhB dye- a xenobiotic transporter substrate, is fed in the presence of the xenobiotic inhibitor verapamil, it is found in higher levels in the hemolymph of the *Apis mellifera* Two types of bee food combined with two dyes were tested in 2012 for the impact of food type, and the impact of dye type on the fate of the dye in a *Apis mellifera* hive. Slightly hydrophobic RhB and slightly hydrophilic UrO were used. Dyed syrup persisted longer in hives than dyed pollen patties, and dyes did not spread uniformly throughout the hive.

IN VITRO MEASURES OF MDR-TRANSPORTER FUNCTION AND WHOLE-HIVE EXPOSURE DYNAMICS USING FLUORESCENT DYES

By

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Thesis submitted to the Faculty of the Graduate School of the
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Advisory Committee:
Professor David Hawthorne, Chair
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Dennis vanEngelsdorp

Dedication

This work is dedicated to my family who has always been incredibly supportive of me. I feel so lucky to have the love and support of so many people, and it is thinking of them that kept me going to finish this project. My Mother has always provided me with encouragement and enthusiasm. She was always a phone call away with a pep-talk ready to go. When I was stressed out she would always make me feel better. She is responsible for getting me interested in science and encouraging me to pursue research. To my Father, who has always inspired me to try harder, achieve more, and most importantly put my best effort forward. He has been a huge resource, and always gives me great advice and guidance. To my sisters, Helen, Katie, and Claire who shared their experiences and difficulties in pursuing their own degrees to encourage me to keep going. They have been incredible role models and I strive to be more like them all of the time.

To my friends who never turned away when I needed a respite from work. And to my peers who provided me with measureless resources throughout our time at Maryland together. I never expected to grow so close to my fellow students, and yet I find I have made an entomology family, Particularly Ryan Gott and Ashley Jones. Finally this work is dedicated to my partner, Ryan Matis who has only known me for the last part of this thesis but inspires me to be better, and finish what I started.

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I am especially grateful to the wonderful people at the USDA Bee Research Laboratory for letting me experiment on their hives, and giving me tons of help along the way. Jeff Pettis for the advice, and guidance in designed my hive experiment. Nathan Rice, and Andy Ulsamer for their help setting up my experiment. I also had a lot of help from many undergraduates, Sarah Davies, Alex Guseman, Julius Goldberg, Erin Norcross, Andrew Garavito, Kaliah Miller. Thank you.

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Chapter 1: *In Vivo* Assessment of Xenobiotic Transporter Function in *Apis mellifera*

Abstract

Honey bees are an important and vulnerable agricultural resource utilized worldwide. They are susceptible to sub-lethal effects from many chemicals that in combination may have a lethal synergistic effects. One mechanism through which sub-lethal effects may arise is inhibition of ABC transporters, a key part of the xenobiotic processing system in most, if not all eukaryotes. An ABC transporter is a trans-membrane protein found in many tissues of the honey bee that actively efflux toxins away from sensitive tissues and cells towards excretion. Changes in these proteins have been shown to be important in some cases of pesticide resistance. Given the large number of potential inhibitors, standard feeding assays to measure dose-mortality relationships of inhibition of actual individual chemicals is very time consuming. Development of an assay to quickly and reliably test a chemical's inhibition of these transporter proteins is necessary to identify compounds that may be increasing bees' sensitivity to pesticides. An assay was developed using Rhodamine B (RhB), a fluorescent dye that is a known substrate and verapamil, a known inhibitor of the main xenobiotic trafficking transporters. Following a pretreatment with verapamil, bees were fed RhB + Verapamil—laced sucrose syrup. To determine if transporter inhibition increased the rate of dye transit into the hemolymph and slowed its excretion, the amount of RhB remaining in the hemolymph was measured. Bees treated with verapamil had consistently higher levels of RhB in their hemolymph than bees fed inhibitor-free sucrose syrup. Results provided proof-of-concept that the fluorescent dye RhB could be used as a surrogate representing a chemical substrate of these key transporters.

Introduction

Honey bees are an essential element of our agricultural industry, contributing an estimated $14.6 billion in value (Morse & Calderone, 2000). The almond industry is the largest user of honey bees for pollination in the U. S., employing 60 percent of the nearly 2.5 million managed honey bee colonies nation-wide to ensure a full crop (vanEngelsdorp *et al*., 2008). Several large and unexplained losses of honey bee colonies, across much of North America, were reported following the winter of 2006/2007 (vanEngelsdorp *et al*., 2009). These losses were associated with an unusual collection of symptoms including: 1) *rapid loss of adult worker bees from affected colonies as evidenced by weak or dead colonies with excess brood populations relative to adult bee populations, 2) lack of dead worker bees both within and surrounding the affected hives, and 3) the delayed invasion of afflicted colonies by hive pests* (vanEngelsdorp *et al*., 2009). These symptoms were labelled "Colony Collapse Disorder" (CCD). There have been many hypotheses proposed for the cause of CCD including pathogens such as Israeli Acute Paralysis Virus (Cox-Foster *et al*. 2007) vectored by the parasitic mite *Varroa destructor*, exposure of bees to pesticides such as the widespread neonicotinoid insecticides (Chensheng *et al.* 2012), and broader threats like poor nutrition resulting from monocrop farming systems (Aluax *et al.* 2012). All of these candidate causes for CCD are supported by intriguing but inconclusive evidentiary threads; none are clearly and individually implicated.

High overwintering losses have continued since 2006, but because the symptoms associated with CCD do not always accompany the losses, CCD may not be the sole cause of those losses. Historically, acceptable overwintering losses are considered to be below 15%, but since 2006 they have averaged 30.5%, peaking in 2012-2013 at 31.1% (vanEngelsdorp *et al*.

2013). The cause of these losses, as with CCD itself, are unknown and may be due to a combination of the factors listed above.

Pesticides in particular have been singled out as a possible cause of CCD and high overwintering mortality of bee colonies (Mullin *et al*., 2010, Hawthorne & Dively, 2011, Johnson *et al*., 2010). This is because pesticides are routinely encountered by honey bees while foraging, and are also placed within their hive for pest control. The effects of many pesticides on honey bees have been tested individually as a requirement for commercial registration in the United States and Canada and in Europe. Only a few have been tested in combination with other pesticides or in-hive medications. Given the very large number of pesticides, environmental toxins and plant chemicals to which honey bees are exposed, it is becoming increasingly clear that exposure to multiple compounds (each at individually harmless concentrations) should be examined as a possible cause for colony losses (Hawthorne & Dively, 2011, Johnson *et al*., 2009).

One of the ways in which adverse consequences of a combination of pesticides could occur is through inhibition of a key detoxification or excretion mechanism by one or more of the chemicals—thus rendering the bees more susceptible to another. Inhibition of a family of membrane-bound proteins, often called xenobiotic transporters, is a candidate mechanism for adverse effects of multiple pesticide exposures. A xenobiotic transporter is a cellular pump that effluxes harmful molecules out of cells for removal from the organism (Karnaky *et al*., 2003). These transporters were first examined in detail in the realm of chemotherapy resistance in tumors, which were deemed multi-drug resistant (MDR) cells that are especially good at removing chemotherapy drugs (Klaassen, 2002). One class of these transporters or pumps are the ATP binding cassette (ABC) transporters (Leonard *et al.*, 2003). These transporters utilize

ATP to push materials into or outside of the cell in an active process (Bosch & Croop, 1998, Lage, 2003). Members of the ABC-B family of these transporters, also called p-glycoprotein (p-gp) play an important role in xenobiotic metabolism and excretion (Leonard *et al*, 2003).

Honey bees, like virtually all living organisms have p-gp's that are responsible for transporting an unusually broad range of substrate compounds. These transportable compounds include a very wide range of environmental toxins, including secondary plant compounds and insecticides. These transporters are known to contribute to insecticide resistance in several pest species, including mosquitos (Porretta *et al*., 2008) and body lice (Yoon *et al*., 2011), and are likely important to many more cases of resistance (Buss & Callaghan, 2008). The widespread foraging and subsequent concentration of floral nectar and pollen within the hive may lead to bees being exposed to extraordinarily diverse collections of chemicals. One landmark study found over 121 different pesticides in samples taken from beehives across North America, with some hives having residues from as many as 39 different pesticides (Mullin *et al*., 2010).

This comingling of chemicals within the hives, and individual bees is alarming because we don't know the effects of the interactions that most of these chemicals have with each other inside of an organism. If honey bees are utilizing xenobiotic transporters to prevent poisoning, then it is important to know if, for example, oxytetracycline, a common antibiotic applied to beehives is also an inhibitor for p-gp. Coumaphos, a once widely used in hive treatment against varroa mites, has also been shown to inhibit this family of transporters in human multi-drug-resistant (MDR) cell lines, which suggests it could inhibit honey bee transporters as well (Bain *et al*., 1997). That type of knowledge would allow for better honey bee management decisions, and maybe even more precise and thoughtful insecticide applications to crops where bees are actively foraging. In order to get this knowledge we need to be able to identify the undesirable

combinations, preferably quickly and cheaply, because there are so many combinations to be tested.

The role of p-gp in metabolism and excretion of a toxin is to establish a concentration gradient of potentially toxic compounds, continuously shunting the toxin towards less-sensitive tissues and towards excretion. By reducing the concentration of a toxin within cells, the efficiency of metabolic processes is increased and the most sensitive tissues and targets are protected from reaching critical toxin concentrations (Klassen, 2013). The location of these transporters can also be an indication of their role in detoxification and excretion. They are found at the blood-brain barrier of fruit flies in order to protect the brain (Mayer *et al*. 2009). They are also found in the midgut lining, the malpighian tubules, and the cuticle, all tissues involved in protection of insects from exterior or ingested toxins (Labbe *et al*., 2011., Lanning *et al*., 1996). These transporters have also been observed increasing in number following exposure to toxic substrates (Yoon *et al*., 2011).

The role of these transporters can be tested by co-exposing an insect to a p-gp inhibitor and a toxin that is a substrate of the transporter. Inhibition of the transporters will, under these conditions, increase the organism's sensitivity to the toxin, increasing mortality. This assay can be performed with insects; the effects of an inhibitor measured by mortality at a single dose or over a range of toxin dosages to estimate the LD50. But this increased sensitivity to toxins could also be due to inhibition of other detoxification mechanisms, such as cytochrome p450 metabolic enzymes. To complement inhibitor mortality assays, a functional assay is needed to measure the rate of chemical transport in the presence of a p-gp specific inhibitor.

We can somewhat narrow down the chemicals that specifically are a target for p-pg based on some broad chemical properties. Typically, the molecular weight is greater than 399 (Bain *et*

al. 1997, Didziapetris *et al*., 2003), the compound is moderately hydrophobic (usually 1 < log K_{ow} < 2 to 3) (Bain *et al*., 1997, Didziapetris *et al*., 2003), it has at least one six- or more-membered ring structure (Bain *et al*., 1997), and is has the ability to be a hydrogen bond donor and acceptor (Bain *et al*., 1997, Osterberg & Norinder, 2000, Penzotti *et al*., 2002). It is important to note that these characteristics, while useful as guide, do not include all known substrates of p-gp. Exceptions to these properties, include substrate compounds with a lower molecular weight, and a higher log K_{ow}, so a chemical cannot be ruled out based only on these rules (Bain, McLachlan and Leblanc, 1997). Interestingly, in the same survey of chemicals this group also found that coumaphos, an acaridae and long-lasting hive contaminant, strongly inhibits pg-p.

Rhodamine B (RhoB) is a fluorescent dye that is non-toxic to honey bees at the concentrations I used and is also a substrate of p-gp based on its chemical properties and observed transport across the blood brain barrier (Meyer et al., 2009, Bain et al., 1997). This makes it a useful tool for measuring p-gp activity by looking at the relative amounts in different honey bee tissues. Given a model of RhB excretion that includes retention of the dye in the gut lumen (aided by p-gp in the midgut epithelium), and rapid removal of the dye that has entered the hemolymph by the malpighian tubules (also aided by p-gp in the tubules), I expected to see the concentration of RhoB in the hemolymph of bees fed that dye to be low and to diminish rapidly following exposure.

The tissues responsible for the majority of the excretion of toxins in insects are the malpighian tubules (Labbe *et al*., 2011, Gaertner *et al*., 1998, Wang *et al*., 2004). The blood-brain barrier also contains ABCs to protect the brain from toxins (Mayer *et al*., 2009). Fluorescent dyes that are substrates of these transporters can be used in place of toxins and can

measure functionality of transporters through spectrophotometry without killing the insect. If the p-gp's in our system are keeping the substrate dye within the lumen of the gut, and the malphigian tubules to be excreted out of the animal, then we would expect to see higher levels of dye in the hemolymph of the inhibited animals. The transporters mop up the dye in order to limit exposure and get rid of it as they would a toxin. This has been seen in fruit fly blood-brain barriers, where dye is constantly pumped away from the brain (Mayer *et al*., 2010). The goal of this assay was to demonstrate transporter mediated activity in honey bees. Here, I developed an assay to assess the function of xenobiotic transporters *in vivo* following exposure to inhibitors by measuring the concentration of RhB in the hemolymph of the honey bee.

RhB was used as the fluorescent transporter substrate to quantify the amount of transporter activity happening inside the bee in the presence and absence of an inhibitor. This dye has previously been used as a ABC transporter substrate in experiments measuring transport across the blood brain barrier (Mayer *et al*., 2010). It has also been used in MDR function assays devleoped in mussells, measuring the transport of RhB across the gills in the presence of various chemicals to determine their potential as transporter inhibitors (Cornwall *et al*., 1994). In preliminary assays, I found that RhB in the range of concentrations I used, was non-toxic to bees, and did not appear to be metabolized over 24-48 hr periods within the bee. RhB is also used in biological risk assessment by measuring efflux by transporters in animals commonly used for ecosystem monitoring like mollusks. Animals with more, or more functioning transporters efflux RhB more rapidly (Smital *et al*., 2000).

Uranine O (UrO), a dye typically used as water tracing dye in environmental assessments, was used as a negative control. UrO, a water soluble form of fluorescein dye, is not a known substrate of p-gp, perhaps because it does not meet the slightly lipophilic requirement

(L. Bain *et al.* 1997). Its excitation and emission spectra also do not overlap with RhB, facilitating simultaneous measurement of both dyes. UrO was also non-toxic and did not degrade over time in honey bees in preliminary lab assays. Verapamil was selected as the inhibitor for our assay because it is a well characterized inhibitor of p-gp (Gatouiliat *et al*., 2008). It has been used previously in transporter assays in insects (Mayer *et al*, 2010). Verapamil was not toxic to the honey bees in lab testing.

Methods

Source of Bees

Honey bees were obtained from established field colonies located at the Central Maryland Research and Education Center, Beltsville facility at Beltsville, MD. Brood frames with signs of emerging bees were removed from colonies and brought to the laboratory where they were reared in a dark incubator maintained at 33±2°C and (70–80%) RH. Emerging bees were collected daily and maintained in groups of 15-20 in 7 oz wax paper cups with a muslin covering. Bees were fed sucrose solution (30%; w:w) *ad libitum* from holes in the bottom of 2.0 ml microfuge tubes, until they were used for assays.

Treatments

RhB was used as a transporter substrate that would serve as a surrogate for a pesticides and other environmental chemicals that are substrates of p-gp. I determined in preliminary assays that RhB was non-toxic to honey bees, even at concentrations exceeding 2mM, and did not break down over time within the bee. Uranine O (UrO), a water soluble form of fluorescein dye, typically used as a water tracing dye in environmental assessments, was also used as a negative control when co-fed with RhB, because it was a non-substrate of our target transporter. Its

excitation and emission spectra also do not overlap with RhB, making it a good candidate for a dye to use alongside RhB. UrO was also non-toxic and did not degrade over time in honey bees in preliminary lab assays. Verapamil was selected as the main inhibitor because it is a well characterized inhibitor of p-gp transporter function (Gatouiliat *et al*., 2008), and used previously in transporter assays in insects (Mayer *et al*,. 2010). Verapamil at dietary doses of 1mM was non-toxic to honey bees in previous studies (Hawthorne & Dively, 2011). Ketoconazole was tested as another inhibitor, known to have strong effects in mammalian cells and because it is in the same chemical family as some fungicides used in field crop sprays.

Both dyes, verapamil and ketoconazole were obtained from Sigma Aldrich. Spectrophotometers used were Molecular Devices SpectramaxPlus (Method A), and FilterMax F5 (Method B). All assays were performed using 96-well assay plates (Costar).

Experimental Design

Frames with emerging bees were taken from research hives and placed into a dark incubator maintained at 33±2°C and (70–80%) RH. Emerging bees were collected daily and maintained in cohorts of 15-20 in 7oz wax paper cups with a muslin covering. After 3-7 days bees were fed either a control sucrose solution or a sucrose + inhibitor solution for 24 hours (Figure 1). Two different protocols were used in the following experiments, for feeding bees labelled sucrose solutions and obtaining hemolymph, differing in the method of honey bee restraint and in the spectrophotometer and excitation/emission settings used to measure fluorescence. For the first method, (method A), bees were anesthetized with CO2, and the legs and wings and body were restrained to a support surface with tape such that their head, mandibles and antennae could move freely (Figure 2). Bees were then fed 10ul of experimental solutions containing the fluorescent dyes via pipettor. The process of restraining bees resulted in

approximately one hour without food before dye treatment. After feeding, bees, still restrained, were kept in a dark and humid chamber at room temperature for 24 hr. Hemolymph (1ul) was collected from bees through a minute slit between the terminal sternites of the abdomen using a micro-capillary tube, mixed with 50ul of 0.01% SDS, and transferred to a 96 well plate. Fluorescence of hemolymph samples was measured using a Molecular Devices SpectramaxPlus spectrophotometer (ex/em RhB 540:625, ex/em UrO 490/625). For the second method, (Method B), cohorts of 10-15 bees in cups were shallowly anesthetized through chilling. During recovery, bees were held by the wings and thorax and fed 10ul of experimental solutions from a pipettor. Bees were then placed individually into a 15 mL centrifuge tube for two hours to ensure that each bee fully consumed the solution and then returned to their original cups along with similarly treated cohort members and allowed to continue feeding on their assigned inhibitor treatment *ad libitum*. To sample hemolymph, bees were anesthetized on ice, a meso-thoracic leg was removed and 1ul of hemolymph collected in a micro-capillary. The hemolymph was prepared and fluorescence measured as in method A, however a FilterMax F5 spectrophotometer was used (ex/em RhB 540:625, ex/em UrO 490/625). Because fluorescence readings of the two spectrophotometers differed, data cannot be compared between assays using the two methods.

Experiment 1. Does consumption of verapamil or ketoconazole alter hemolymph concentrations of RhB?

Cups of four-day old bees were assigned to control or inhibitor treatments and continued on 30% sucrose solution, or switched to a 30% sucrose solution containing the p-gp inhibitor verapamil (1mM) or the fungicide ketoconazole (1mM). After 24 hr, bees were fed (Method A) 10ul of a

30% sucrose syrup containing 0.125mg/ml RhB. After 24 hours 1ul of hemolymph was collected and fluorescence measured. Plates also contained SDS-only control samples and hemolymph samples from bees fed sucrose syrup without any added dyes. Preliminary standard curves were estimated in the linear range of the standard curve for each dye.

Experiment 2. Does consumption of verapamil alter hemolymph concentrations of RhB and UrO?

Using Method A, bees were similarly fed sucrose or sucrose + verapamil (1mM) solutions, and after 24 hour fed a 30% sucrose syrup containing 0.125mg/ml RhB and 0.125mg/ml UrO. Hemolymph was collected and fluorescence measured as described above, with the additional reading of UrO fluorescence using 490/625 nm.

Experiment 3. Is there a dose-response of verapamil and hemolymph concentrations of RhB?

Using Method B, cups of 3-7 day old bees were pretreated for 24 hour with 30% sucrose solutions containing one of a range of verapamil concentrations (0, 0.05, 0.1, 0.33, 0.66, 1.0 mM), then fed 0.125 mg/ml solutions of RhB in 30% sucrose syrup. Hemolymph was sampled from bees after 24 or 48 hour, and fluorescence measured.

Statistical Analysis

Differences in the mean hemolymph concentrations of RhB between bees fed inhibitors and controls were tested using a t-test (SAS Institute 2002-2008).

Results

RhB concentrations in hemolymph (24 hour) were significantly greater in bees fed either verapamil or ketoconazole than those fed only sucrose syrup ($t = -2.37$, $df = 10$, $P < 0.04$) (Figure4). RhB concentrations in hemolymph similarly increased in verapamil treated bees in experiment 2 ($t = -2.06$, $df = 14$, $P < 0.06$), whereas UrO concentrations were not significantly different in the verapamil and control bees ($t = 1.66$, $df = 9$, $P = 0.13$) (Figure 5, 6). These results show that verapamil increases the concentration of RhB in hemolymph, supporting our expectation of p-gp inhibition causing increased hemolymph concentrations of a p-gp substrate. I also show, by the lack of an effect on the non-substrate UrO, that the effect was not generalized to all fluorescent compounds.

There was a significant increase in RhB concentrations in hemolymph with increasing dosages of verapamil after both 24 and 48 hour post dye feeding (Figure 7). Levels of RhB, as inferred from fluorescence, were lower in all hemolymph samples from bees treated with verapamil 48 hours after treatment compared to 24 hours after treatment.

After 24 hours, bees that were treated with 0.1, 0.33, 0.66, or 1.0 mM of verapamil all had significantly more RhB in their hemolymph than bees that received no inhibitor and bees that received only 0.05 mM of inhibitor (Figure 5). Hemolymph fluorescence after 24 hours following the 0.33mM verapamil treatment was not different from the fluorescence in hemolymph in bees treated with 0.66mM verapamil. Hemplymph fluorescence in bees treated with 1.0 mM did not differ from the fluorescence in bees treated with 0.66mM verapamil; however, hemolymph fluorescence in bees treated with 0.33mM of verapamil was lower than the fluoresces observed in the hemolymph of bees treated with 1.0mM verapamil (Figure 7).

Although levels of dye in the hemolymph of bees after 48 hours were less than 24 hour bees, there were still differences among verapamil concentration treatments. Hemolymph dye

levels of 0.33, 0.66, and 1.0mM verapamil treatments are all significantly higher than the control. The level of fluorescence in hemolymph resulting from two lowest concentrations of verapamil (0.05 and .1mM), are not statistically different from each other. The level of fluorescence resulting from the two highest concentrations of verapamil are also not statistically significant from each other (1.0 and 0.66mM), but levels of fluorescence resulting from the 0.66 and 1mM treatments are both significantly higher than the 0.33mM (Figure 7).

Discussion

These data provide evidence supporting the use of verapamil as an inhibitor of at least one class of the xenobiotic handling ABC transporters in honey bees, and that RhB was a useful substrate and indicator of the function of those transporters. I also show that the hemolymph concentration, and possibly the dynamics of metabolism and excretion of a non-substrate dye, UrO, are unaffected by exposure of bees to verapamil (Figure 5, Figure 6). This difference in transport of dyes supports the conclusion that higher levels of RhB dye in bees that are inhibited with verapamil was because p-gp was inhibited and not because verapamil was acting in some other way allowing the dye to more rapidly enter or to remain in the hemolymph. It supports that I was actually characterizing functionality of p-gp, and not a non-target mechanism.

This assay was designed to determine if a fluorescent substrate of the ABC-B transporters in honey bees could be used to measure a reduction in transporter function following exposure to a candidate inhibitor. Previous work (Hawthorne & Dively, 2011) has demonstrated that orally dosed verapamil increases honey bee sensitivity to insecticides, and the results reported here suggest that the mechanism of that increased sensitivity was indeed reduced functionality of the

xenobiotic transporters. This combination of assay strategies provide a complementary set of approaches that could be used to identify inhibitors of these transporters in honey bees.

The significant positive dose response of RhB concentration in the hemolymph in response to increasing verapamil dosage further supports that the differential transport of RhB was due to p-gp. Verapamil is a well-known p-gp inhibitor, and so it makes sense that increasing the concentration would cause reduced p-gp function. Given our logic model for the consequences of reduced transporter function in honey bees, we expected verapamil to create a situation where more RhB would remain in the hemolymph of the bee (Figure 4, Figure 5, and Figure 7).

The observation that hemolymph collected 48 hours after RhB feeding still showed the effects of verapamil exposure suggests two things. First, it shows that the verapamil was not immediately metabolized or excreted and remains partially effective at inhibiting the transporters 48 hr after dye exposure, but secondly, that the levels of verapamil are either reduced over time allowing recovery of transporter function and removal of the RhB from the hemolymph, or that transporter function was not fully inhibited, especially in the malpighian tubules and the bees were able to eventually remove the majority of RhB from the hemolymph.

It is important to realize however that even shutting down only a small fraction of the available p-gp's could still increase accumulation of a substrate. If that substrate was toxic to the insect the partial inhibition of p-gp would decrease the LD50 of the insect to that substrate, rendering it more sensitive to the toxin. It is also of value to note that honey bees would not naturally encounter verapamil, an artificially synthesized pharmaceutical. We used verapamil here to study the function of xenobiotic transporters. Ketoconazole is a fungicide that has inhibited p-gp function in a wide array of cell-based assays, and here behaved similarly to

verapamil. Interestingly, fungicides with similar chemistry to that of ketoconazole, such as propiconazole, and tebuconazole are used in agriculture to protect crops, suggesting one of the means by which a honey bee would encounter powerful transporter inhibitors while foraging (Chowdhary *et al*., 2013).

In addition to expanding the assay to the realm of chemicals encountered by bees, it would also be interesting to pair experiments where an inhibitor and RhB are fed to one group of bees, and the same inhibitor and a toxin is fed to another group of bees. It would be interesting to relate a reduction of the LD50 of a toxin via inhibition of p-gp to decreased function of p-gp in moving RhB due to the same inhibitor. This would provide further evidence that the mechanism of synergism of toxicity is due to p-gp inhibition and not another pathway.

More evidence of the inhibition of p-gp could potentially be obtained through direct observation of transport of dye using isolated malpighian tubules. Tubules could be dissected out of the bee, and suspended in a bath of just dye, or dye and inhibitor mixture using the Ramsay assay (O'Donnell, 2009). Observing the tubule removing the dye from solution and concentrating it in the tubule lumen would confirm the larger picture that is believed to be happening in the honey bee. The dye is getting absorbed by the malpighian tubules for excretion, thereby being removed from the hemolymph.

Developing this line of research further is important as we shift from considering the effect of a single chemical, compound, or condition on honey bees to considering the interplay between several factors. Honey bees interact with so many plant compounds, chemical treatments, pesticides etc. that they are a prime candidate for this type of research. This is underscored by their key role in food production in the United States.

Figure 1: Rearing cages for the newly emerged bees.

Figure 2: Feeding 30% sucrose-fluorescent syrup to individual honey bees.

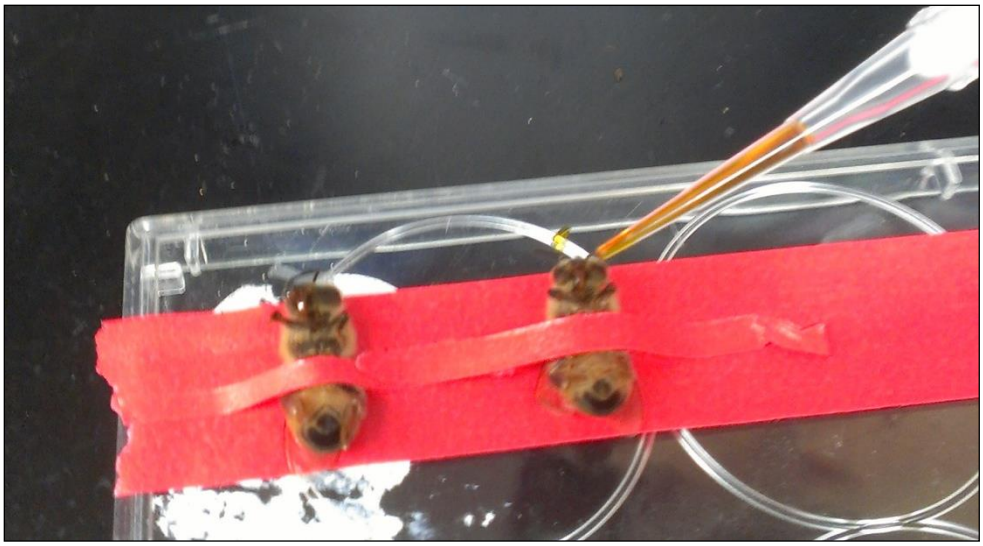


Figure 3: Removing hemolymph from the honey bees using a 1uL micro capillary tube.

Figure 4: Mean levels of RhB in bees fed 30% sucrose containing verapamil (1mM), ketoconazole (1mM), and a sucrose only control.

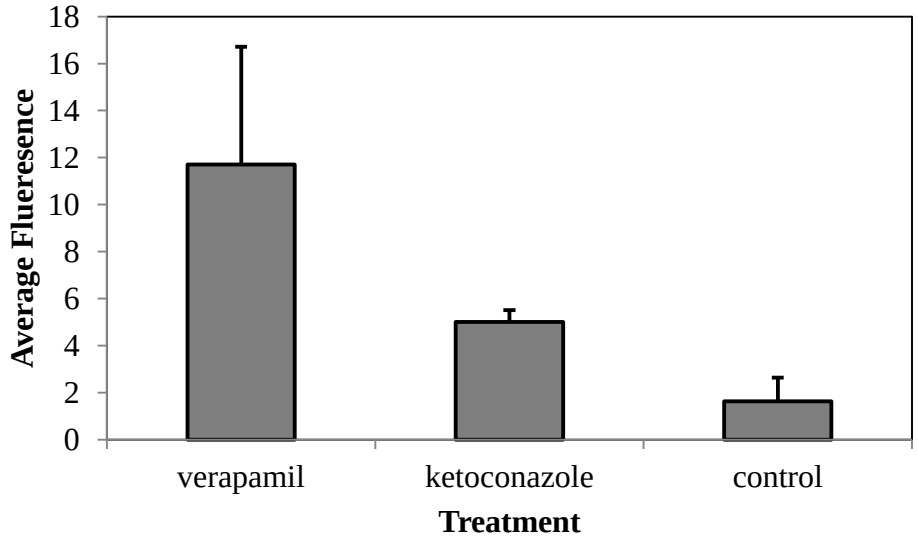


Figure 5: Mean levels of RhB in bees fed 30% sucrose containing verapamil (1mM), and a sucrose only control. There was a significantly higher level of RhB in the hemolymph of verapamil treated bees than control bees. (P=0.0002)

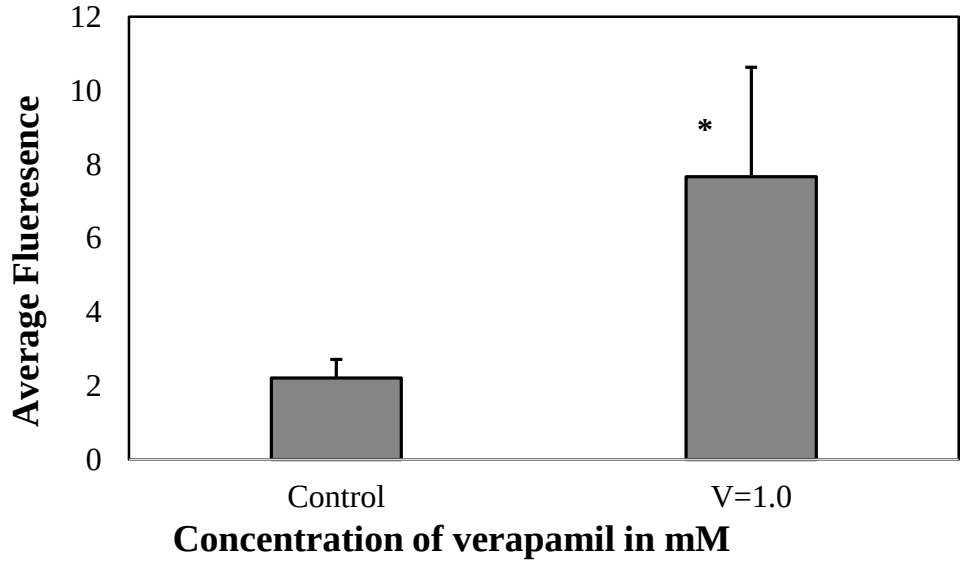


Figure 6: Mean level of UrO fluorescence in untreated and treated bees. The mean levels of UrO in hemolymph did not differ among verapamil treated versus untreated bees (P=0.3055).

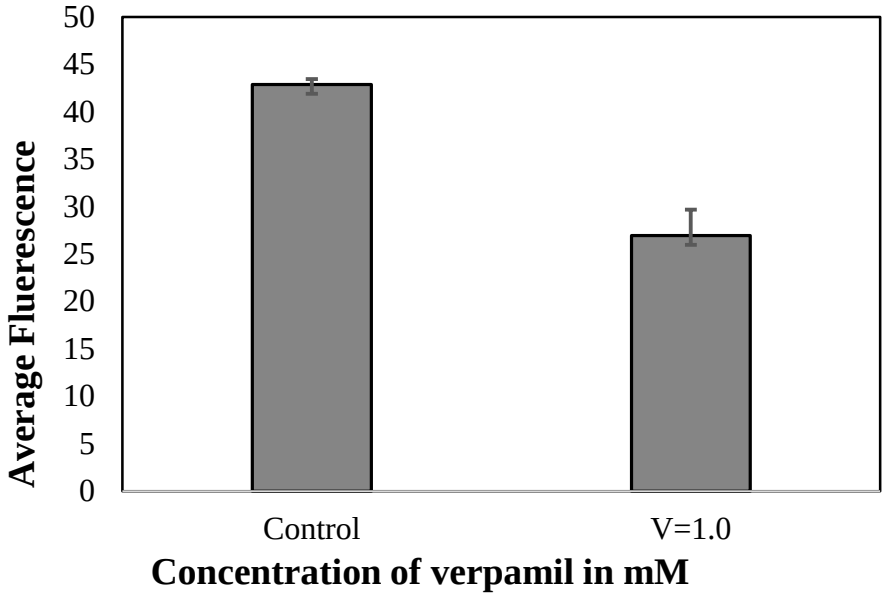
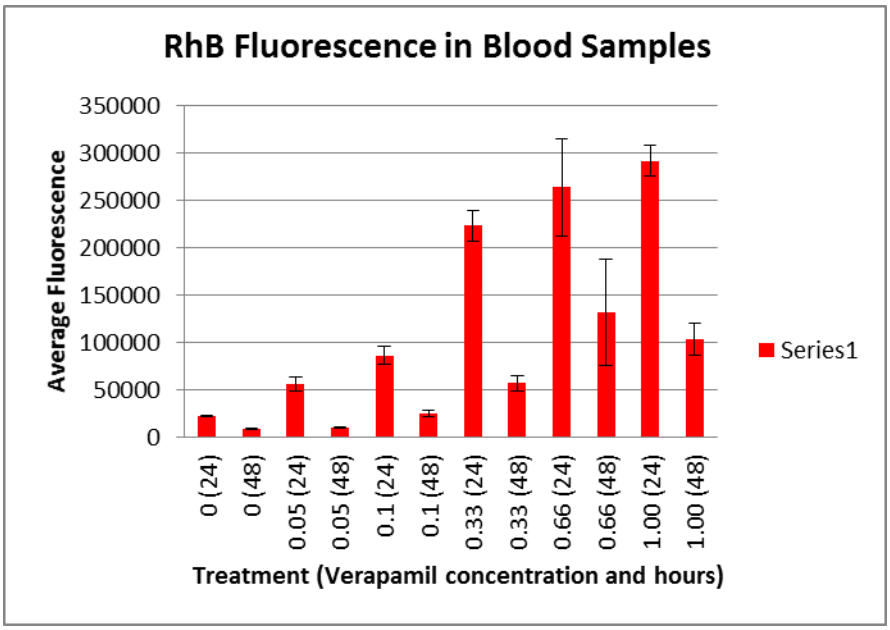


Figure7: Mean fluorescence of hemolymph RhB from bees treated with a series of verapamil dosages (mM) at 24 and 48 hours after dye treatment.



Chapter 2: Whole hive dynamics of fluorescent dyes delivered in sugar syrup and pollen patties.

Abstract

Many laboratory studies have reported sublethal effects on individual honey bees (*Apis mellifera*) by exposing them to single doses of chemicals such as insecticides. Some argue that these effects cannot be extrapolated to the overall health of a functional colony which can compensate as a super organism for many stress factors. Hence, sublethal effects of pesticide exposure are now being assessed by feeding colonies treated sucrose syrup and/or treated pollen supplements. Because the pharmacokinetics of a pesticide within a honey bee colony could change the potential impacts of a toxin, it is important to assess the fate of pesticides within colonies and the exposure doses to bees, brood, queen, and other hive matrices. However, it is very expensive to analyze hive components for tracking the fate of chemical residues, which limits the number of samples and replicate colonies. This study evaluated the utility of using two chemically different fluorescent dyes, RhB (slightly hydrophobic) and UrO (highly hydrophilic), as surrogate agents to track the movement of a simulated pesticide within colonies. Honey bees were exposed to dye-labelled pollen supplement patties and sucrose syrup. To measure movement of the dyes, adult workers, larvae, pupae, wax, pollen, and honey were sampled at 3 weekly intervals, and royal jelly was sampled once at the end of the study. The concentration of dye present in samples was measured using a spectrophotometer. Significant differences in dye levels in bees and hive matrices were found and varied according to the exposure method. Dye from pollen patties did not persist in the hive as long as dye from sucrose syrup. The movement and decay patterns between the dyes, particularly evident in wax, suggested that there was some partitioning of the dye based on lipophilicity. Overall results indicated that fluorescent dyes can

be used to mimic the movement over time of pesticides within colonies with greatly reduced cost and without harm to honey bees.

Introduction

A comprehensive assessment of risks to honey bees (*Apis mellifera*) from environmental toxins should include three tiers of experiments: an analysis of the acute effects to individual bees, usually performed in laboratory studies; an evaluation of the sublethal effects on cohorts of bees over a longer period; and a field colony study examining chronic lethal and sublethal effects of dietary exposure to toxins over multiple brood cycles (EPA, 2012). Many laboratory studies have reported sublethal effects on individual honey bees (*Apis mellifera*) by exposing them to single doses of chemicals such as pesticides (Blacquière *et al*. 2012). Some argue that whole colony analysis of bee responses to toxic exposure is essential because a functional colony as a superorganism exhibits many social interactions and feedback mechanisms to compensate for stress factors, and these are unpredictable from extrapolation of responses of individual bees (Cresswell, 2010). For example, trophallaxis between honey bees can spread and dilute compounds across a hive population of bees, and thus can buffer bees and brood from exposure to toxins (Crailshem, 1990). Alternatively, dilute toxins might become concentrated in honey increasing the exposure dosages to bees later feeding on that honey.

Few field studies have been conducted using honey bee colonies to assess sublethal effects of dietary exposure to pesticides. Of those published reports, researchers have either placed colonies in isolated treated or untreated bee-friendly crops or exposed bees directly to known residues in sucrose syrup or pollen supplements and then measured various parameters of colony performance, foraging activity, and overwintering (Unpublished work- Galen Dively,

Cutler & Scott-Dupree, 2007, Henry *et al*., 2012, Lu *et al.*, 2012, Nguyen *et al*., 2009**,** Faucon *et al.* 2005, Tremolada et al., 2004). The pharmacokinetics of a pesticide within a honey bee colony can change the potential impacts of a toxin, so it is important to measure the fate of a toxin moving through the colony and its accumulation in different hive matrices such as honey, wax, beebread, pollen, and the bees themselves. However, it is costly to quantitatively analyze hive matrices for the presence of residues, thus the high cost of chemical analyses has limited the number of samples and replicate colonies that are usually tested in field studies.

Another potential limitation with colony studies that use treated sucrose syrup or pollen supplements as exposure routes is whether the bees actually process these foods the same as they would naturally foraged nectar or pollen. For instance, beekeepers and researchers generally agree that pollen supplements are consumed immediately by honey bees and not stored in the hive in the same way that foraged pollen is processed. Pollen supplements (commonly fed as MegaBee diet patties) are readily consumed by bees and closely resemble the nutritional value of pollen (DeGrandi-Hoffman, 2008). However, while they provide supplemental protein for brood production, the rapid consumption of pesticide-treated pollen patties may expose bees over a shorter time to higher doses of pesticides and thus not accurately represent the fate of a toxin entering the colony in foraged pollen. Hive bees mix foraged pollen with a little honey and enzymes from their saliva to form a fermented blend called "bee bread" which is stored for future use (Herbert & Shimanuki, 1978). During this process, degradation of a toxin due to microbial activity and abiotic conditions can result in a different exposure dose, especially after the bee bread is further processed as brood food and honey jelly (Winston, 1987). In contrast, sucrose syrup is thought to be treated by hive bees similarly to that of foraged nectar. Sugar syrup is commonly fed to colonies as a supplemental feed during the early spring and late

summer when natural nectar sources are scarce; however, much smaller volumes are usually used to expose bees to a potential toxin in field experiments. Nevertheless, pesticide-treated pollen and sucrose supplements as routes of exposure could have very different fates and resultant effects on a honey bee colony than those of contaminated bee-collected pollen or nectar. Therefore, it is important to determine the relative levels and distribution of pesticide residues within colonies fed treated food supplements before drawing conclusions that their effects are representative of foraging exposures.

The physical properties of pesticide residues present in pollen or nectar (either foraged or supplemented) may also influence their fate, distribution and exposure routes within a honey bee colony. For instance, the highly lipophilic coumaphos, an organophosphate acaricide, is known to accumulate in fatty substances, such as wax, which acts as a sink for the chemical and allow it to persist in the hive for long periods (Tremolada *et al*., 2004). Conversely, a more hydrophilic compound, such as imidacloprid, is likely to accumulate in honey which could have a greater direct impact on bees, but may not persist and accumulate in hives. Thus, it would be useful to know how the physical properties of toxins affect their exposure dynamics within the hive.

A more efficient way to track the fate and distribution of potential toxins within honey bee colonies are fluorescent tracing dyes, which have been used by environmental scientists for years to study the flow of one matrix over time in fresh water and soil systems (Smart & Laidlaw, 1977). Dyes have also been used to track the flow of pesticides in the environment after an application (Pang & Close, 2001). Fluorescent dyes allow the tracing of a material by direct visualization or by the use of a fluorometer if quantitative detection of lower concentrations is required. This method of detection could serve as a proxy for chemicals to study the movement and fate of pesticides within colonies, at less cost than a residue analysis of a sample and without

harm to honey bees. A similar approach was performed by Crailsheim (1990, 1992) who used proteins tagged with radioactive isotopes to trace royal jelly through the hive and from bee to bee.

To explore the possibility of using fluorescent tracing dyes in honey bee risk assessment, I evaluated the utility of using two chemically different dyes, RhB (slightly hydrophobic) and UrO (highly hydrophilic), as surrogate agents to track the movement of a simulated pesticide within colonies. First, I completed laboratory assays with caged cohorts of bees to determine if dye consumption had any direct effect on survival and whether dyes would accumulate and persist in bees. Field colonies were then exposed to dye-labelled pollen supplement patties and sucrose syrup, and then samples of bees and hive matrices were taken over time and quantitatively analyzed using a spectrophotometer to measure dye concentrations. I predicted that UrO would accumulate more in aqueous matrices such as honey, while RhB would be found at higher levels in the wax and larvae.

Methods

Source of bees

Honey bees used in laboratory studies were obtained from established field colonies located at the Central Maryland Research and Education Center, Beltsville facility at Beltsville, MD. Brood frames with signs of emerging bees were removed from colonies and brought to the laboratory where they were reared in a dark incubator maintained at 33±2°C and (70–80%) RH. Emerging bees were collected daily and maintained in groups of 20-27 in 7 oz wax paper cups with a muslin covering. Bees were fed sucrose solution (30%; w:w) *ad libitum* from holes in the bottom of 2.0 ml microfuge tube, until they were the right age for assays. Colonies used for the

hive study were located at the USDA-ARS Bee Research Laboratory in Beltsville, Maryland. All colonies consisted of a single-deep Langstroth hive box, each with 10 fully drawn frames. Prior to the field study, colonies were all queen right and equalized to contain 8-10 frames of bees, 6 frames of brood, and similar amounts of pollen and honey.

Dyes and food supplements

Rhodamine B, [9-(2-carboxyphenyl)-6-diethylamino-3-xanthenylidene]-diethylammonium chloride, (RhB) (MW = 479.01, Kow = 190) was used as a surrogate of a pesticide with lipophilic properties. Uranine O, the disodium salt of fluorescein, (UrO) (MW = 376.15, Kow = 0.047) (Kasnavia *et al.* 1999, typically used as a water tracing dye in environmental assessments, was also used as a as a surrogate of a more hydrophilic pesticide. RhB and UrO were selected based on their differing physical and chemical properties, stability over several weeks, and different excitation and emission frequencies. Both dyes were obtained from Sigma Aldrich. The sucrose syrup was prepared by mixing 2 parts of granulated sugar to 1 part of water (w/w). The pollen supplement was prepared by adding MegaBee powder (Dadant & Sons, Inc., Hamilton, IL) in a 1.7:1 diet to sucrose solution. This produced soft, moist dough which was formed into 227 g patties. Dyes were either added to the sucrose syrup or to the MegaBee powder and thoroughly blended in the mixing process to produce the specific concentration of dye for each test.

Laboratory assays

Feeding assays were conducted to determine if the survival of bees fed dyes was affected and how quickly dyes accumulated and persisted in bees. To address survival, 12 cages (8 X 10cm) with 20-25 bees (four days old) in each cage were kept in a 34 degree C. incubator in the dark. The cages were randomized into three groups assigned to three treatments: 1) four cages

were fed 30% sucrose containing RhB and UrO, each at a concentration of 1g/L for 24 hours, followed by untreated syrup for the remainder of the 7 day period; 2) four cages were fed the same sucrose syrup with both dyes for the entire 7days; and the third group of four cages served as a control and were fed only untreated sucrose solution. The number of live bees in each cage were recorded daily.

To address the persistence of dyes in bees, four cages each with 10 bees were assigned to two groups: one fed 1g/ L of RhB and the other fed 1g/l of UrO in sucrose syrup for 24 hours. After the dye exposure period, the food was replaced with untreated sucrose solution. One bee was removed daily from each cage and prepared for fluorescence analysis.

Colony study treatments

Twenty colonies, equalized for bee and brood strength, were spaced 3 m apart in two parallel rows of ten, separated by a 6 m open area. Colonies were randomly assigned to five treatment groups (each with 4 replicates). Each hive in control group #1 was fed 1 liter of sucrose syrup and one 277 g pollen patty on day 0 and again on day 7. The syrup was provisioned in a 2 liter in-hive feeder by removing one frame. The patty was placed on the top bars of frames inside each hive to allow bees *ad libitum* access to the syrup and pollen supplements. Treatment group #2 was fed the same quantities of both food supplements on both days but the pollen patty on day 0 contained 1g each of RhB and UrO dyes. Treatment group #3 was exposed to the same food supplements as in treatment #2 but fed twice, on days 0 and 7. Colonies in treatment groups #4 and #5 were provisioned with the same food supplements and timings of feeding as in treatments #2 and #3, respectively, but the sucrose syrup contained 1g/liter each of RhB and UrO dye. The dyed sucrose syrup and pollen patties delivered to hives

contained the same amount of each dye (1g), so all treated colonies fed either once or twice were exposed to the same level of fluorescent dye by both types of food supplements.

To measure the stability of the two dyes within colonies over time, 50 ml tubes with small amounts of dyed syrup were placed inside the hives and then removed at regular intervals to determine if any degradation of the dye occurred due to abiotic factors within colonies.

Colony sampling

Four subsamples of larvae, pupae, bees, stored pollen, capped honey, uncapped honey, and wax were collected at random from the interior six frames of each hive on days 3, 7 and 14 of the study. Once an individual frame was selected, a 10 cm x10 cm cardboard square was tossed onto a frame to outline an area to remove samples. The square was tossed repeatedly until each sample type was removed from the frame. If not all subsamples were collected from the first frame, another frame were removed and sampled. Bees were removed by gently skimming the bees off of the top bars into 50 ml centrifuge tubes when hive was first opened. Pupae and larvae were removed from cells using tweezers and placed in Eppendorf tubes. Wax from these same cells was then collected into two 50 ml centrifuge tubes. Capped honey, uncapped honey, and pollen samples were removed by pressing a 15 ml centrifuge tube into the cells and pulling the tube away with the section of the comb containing the matrix, which was later removed from the wax cells in the laboratory. Royal jelly production was stimulated by removing the queens from all hives on day 7. After 5 days of being queen-less, each colony was examined for queen cups. Royal Jelly was collected from all queen cells present using a 1 ml syringe and stored in a 1.5 ml Eppendorf tube. All samples were kept cold and dark until they were returned to the lab, where they were stored at -20^{O} C until analyzed.

Sample processing

Samples were removed from cold storage and weighed in portions of approximately 0.1 g prior to processing. Each sample was prepared as a homogenized solution to allow for spectrophotometric measurements of fluorescent. A 0.01% stock solution of sodium dodecyl sulfate (SDS), a detergent that helps to breakdown tissue cells, was added at a rate of 10 ul per mg of sample to create uniform concentrations of samples. Because royal jelly was collected in limited amounts, less SDS was added to the royal jelly to avoid over diluting it. Samples were transferred to small centrifuge tubes and either pulverized by hand grinding, blended using a vortex for 30 seconds, or homogenized in a Hammer Genie shaker with steel beads, depending on the particular matrix. The mixture was then centrifuged at 2000 rpm for 2 minutes, and 100 ul of the supernatant was transferred to individual wells of a 96-well plate for measurement by the spectrophotometer. The excitation/emission of 540 nm/625 nm for RhB and 490 nm/525 nm for UrO was used to measure the fluorescence level of each dye.

Statistical analysis

All data sets were evaluated before analysis for normality and homogeneity of variances by examining residual plots and Shapiro-Wilk statistic. For data not meeting the assumptions of ANOVA, an appropriate transformation was used. A mixed model procedure (SAS Institute, version 9.1.3) was used to test for dye effects on bee survival. Each caged cohort of bees represented a single experimental unit and the endpoints of mortality recorded over the 7 day period were treated as repeated measures and thus corrected for autocorrelation. For the colony study, separate analyses were performed on different subsets of the data. A three-way ANOVA tested each fluorescent dye for main and interaction effects of the food supplements (sucrose syrup, pollen patty), hive matrices (larvae, pupae, bees, stored pollen, capped honey, uncapped

honey), and time after exposure (3, 7 and 14 days). Fluorescence was the response variable, food supplements and matrices were fixed factors, and time was treated as repeated measures. This analysis only included data from colonies that were exposed once at day 0. A second two-way ANOVA tested each fluorescent dye for main and interaction effects of the food supplements and matrices as fixed factor but only used data for day 14 from colonies that were exposed twice at days 0 and 7. Mean differences in all analyses were separated following a significant *F* test by using Tukey's multiple comparison adjustment ($P < 0.05$). Arithmetic means and standard errors were computed and summarized in all graphs. For day 7 data, ratios of the amounts of dye in each hive matrix were determined by dividing the fluorescence units of UrO by the units of RhB. These ratios were then compared to the ratio of the dyes in sucrose syrup or pollen patties fed to the bees on day 0 (baseline). A higher ratio in a matrix relative to the baseline ratio suggested that UrO accumulated in the matrix more than RhB, while a ratio lower than baseline suggested the opposite effect.

Results

Laboratory assays

When bees were fed dyed sugar syrup there were no obvious negative effects reflected in the number of dead bees when compared to bees that were just fed sugar syrup. (Table.1)

Preliminary Studies: Persistence of Dyes

Both RhB and UrO appear in the bees after day one. Neither RhB nor UrO appeared to break down within the honey bee, even four days after the dyed sugar syrup was completely removed from the cage. Levels of dye per bee were variable which was expected because the bees fed ad libitum and so likely ingested differing amounts of dye. The average amount of dye

at day four was equal to or greater than the amount at day one for both RhB and UrO. (Figure 10, Figure 11)

In-Hive Dye Dynamics Field Study

In 2012 a combined 1,919 samples of adult bees, larvae, pupae, pollen, capped honey, and uncapped honey were collected from 20 hives and analyzed. 36 samples of royal jelly were collected and analyzed. Control vials of dyed sugar syrup were placed in the hives and removed at regular intervals to test for degradation of the dye within the hives. The RhB remained highly fluorescent throughout the two weeks of sampling (Figure 12).

The main effects of treatment, were significant for RhB concentration, ($t=10.35$ $df=8.92$ $P<0.05$) Mean fluorescence measures for the no-dye control treatment pooled across substrates was: 4.53+/- 0.56, the mean for the once-fed patty treatment was 15.94+/- 3.77 and the mean for the once- fed syrup treatment was 41.83+/- 12.24. The main effect for sample time was also significant for RhB, ($t=3.44$, $df=168$, $P<0.03$), the mean for sample day 3 was 23.15+/- 10.78. The mean for sample day 7 was 15.94+/- 3.77, and the mean for sample day 14 was 19.65+/- 5.8. The main effect of matrix was also significant ($t= 33.94$, $df=168$, $P<0.0001$). The pooled mean for pollen was 9.96+/- 1.53, the pooled mean for uncapped honey was 40.91+/-22.54, the pooled mean for capped honey was 5.43+/- 2.34, the pooled mean for larvae was 10.69+/-2.19, the pooled mean for pupae 9.90 +/- 2.34, and the pooled mean for adult bees was 39.086+/-8.46.

The main effects of treatment, sample time and matrix were also significant for UrO. For treatment ($t= 10.19$, $df= 8.94$, $P<0.01$) and the pooled mean for the control treatment was 77.61+/- 5.89, the mean for the once fed pollen patty treatment was 144.98+/-26.02, and the pooled mean for the sugar syrup treatment was 267.03+/- 41.15. For sample time ($t=1.64$, $df=168$, $P<0.20$) the pooled mean for sample day 3 was 178.43+/- 40.44, the pooled mean for

sample day 7 was 151.07+/- 20.15, and the sample mean for day 14 was 161.49+/-23.13. For matrix (t=111.05, df=168, P<.0001). The pooled mean for pollen was 91.20+/- 6.32, the pooled mean for uncapped honey was 78.68+/-25.61, the pooled mean for capped honey was 28.06+/- 5.21, the pooled mean for larvae was 249.43+/-39.34, the pooled mean for pupae 200.50+/- 32.78, and the pooled mean for adult bees was 333.78+/-70.04.

There was a significant interaction between feeding method and sample matrix in samples taken at day 14 for both RhB and UrO ($F_{(10,51)} = 4.83$, $P < 0.001$), ($F_{(10,51)} = 3.24$, $P < 0.003$) (Figure 12, Figure 13). Overwhelmingly RhB and UrO accumulated at higher levels from dyed sucrose syrup than dyed pollen patties by sample day 1. Within the sucrose syrup treatment dye accumulated significantly higher in all matrices for RhB, and most matrices for UrO. (Figure 12, Figure 13).

There was a significant interaction between the treatment the colonies were fed and the time the samples were taken for both RhB and UrO concentrations ($F_{(10,168)} = 10.63$, $P < 0.001$), ($F_{(10,168)} = 9.24$, $P < 0.001$) (Figure 14 & Figure 15). Sugar syrup was always the highest at each day. By day 14 sugar syrup remained at high levels while pollen patties approached control levels.

There was a significant interaction of treatment by matrix interaction for both RhB and UrO concentrations ($F_{(10,168)} = 4.83$, $P < 0.001$), ($F_{(10,168)} = 2.23$, $P < 0.02$). RhB accumulated the most from the sucrose treatment in the uncapped honey matrix, whereas UrO accumulated the most from sucrose in the adult bees, pupae and larvae (Figure 16 & Figure 17). Interestingly, for the interaction between matrix and sample date pooled across feeding treatments, only UrO concentration was significant ($F(10,168) = 2.12$, $P < 0.001$)(Figure 18). The interaction when looking at RhB was not significant.

Both dyes were found in royal jelly. The level of RhB was significantly higher in royal jelly when bees were fed the sugar syrup treatment then the pollen patty treatment ($p=0.007$) The twice fed pollen patty hives also had significantly higher levels of RhB than the hives only fed one dyed pollen patty ($p=<0.0001$). The once fed pollen patty treatment was almost identical to the control and had virtually no dye present. The once fed syrup treatment and twice fed pollen patty treatments were not different from each other ($p=0.16$) (Figure 19).

There was significantly more UrO in the royal jelly from the sugar syrup treated hives than from hives fed one dyed pollen patty ($p=0.0004$). There was also significantly more UrO in royal jelly from hives fed two dyed pollen patties than hives fed one ($p= <0.0001$). The royal jelly form the sugar syrup hives, and the twice-fed pollen patty hives are not statistically different from each other ($p=0.73$) (Figure 20).

The ratio of UrO to RhB concentrations was lower in the wax than in the dyed syrup samples removed from the hive boxes, but it was not statistically significant. The ratio of UrO to RhB was higher in honey, adult bees, and similar in royal jelly to the ratio in wax (Figure 21).

Discussion

Pesticide analysis via high performance liquid chromatography (HPLC) is a costly endeavor because you have to send samples to a lab with the proper equipment and staff. However pesticides are being detected in honey bee colonies at unprecedented levels and combinations (Mullin *et al.*, 2010). Being able to approximate the movement of those pesticides through a hive by using a fluorescent dye as a surrogate is a valuable tool. Here, I examined the in-hive dynamics of two chemically different fluorescent dyes, to determine if feeding method impacted where the dye ended up, and to see if the dyes were found in royal jelly. I found that

both dyes distributed throughout the hive in similar ways with some subtle difference. Feeding method had a large impact on the concentrations of both dyes, and the sampled hive matrix also had a large impact on the level of dye found. And I did find dye in royal jelly samples.

Laboratory assays showed that the dye gets into the honey bees quickly, it does not kill, and it does not break down over time, but exposure and readings are variable from bee to bee. This variation was important to keep in mind when using these dyes because a very small difference in the amount consumed can have a large impact on the fluorescence reading from a bee or matrix.

The higher pooled mean, over all matrices, from the syrup treatment show that both dyes from syrup accumulated within the hives to higher levels, than when delivered to the hives in pollen patties. Based on high levels of dye from sucrose treatments in the uncapped honey, but very little dye from the pollen patty's in the stored pollen, this data shows pollen patties were consumed by the adult bees, but apparently not stored in the hive. The bees appeared to be using the patties as a source of immediate fuel whereas they were storing the sugar syrup within the hive for consumption later.

The overall level of dye in matrices from the pollen patty was lower than dye from sugar syrup. The level in adult bees was not high enough to account for all of the dye fed in the pollen patties, so where was it going? Because the adult bees are eating it right away, the pollen patty was likely also being excreted in frass when bees left the hive. This would reduce the exposure of the hive to the dye in the pollen patty treatment.

Based on these data I can recommend that pollen patties are good to deliver a "pulse-like" exposure to a hive; they were not stored so the exposure was not long-lasting. The sugar

syrup would be better for an extended exposure, because it persisted in the hive throughout the two weeks of the study.

Some matrices also had higher overall means than others. It would make sense for adults, larvae, and uncapped honey to have higher levels of dye; particularly from the syrup treated hives because the adults consumed the syrup, exposing themselves first, then they either stored the syrup or fed the larvae. Some dye remained in the larvae and at the next sample date has transformed into dyed pupae. The dyed uncapped honey became capped by the next sample date with the dye inside. A small amount of dye may have gotten in with fresh pollen to make bee bread. This movement meant dyes were diluted and spread throughout the hives, but not in a uniform way.

The higher mean level of dye at sample day 14 indicates that dye was accumulating in the hives over time. This suggests that the "metabolism" of the hives were not fast enough to eliminate these materials and so they caused prolonged exposure.

I observed an interaction between sample day and matrix for UrO but there was no significant interaction for RhB (Figure 20). The significant interaction meant that depending on sample date, UrO accumulated differently in the hive matrices, whereas RhB did not accumulate differently from day to day. This difference could be apparent with UrO because it fluoresces much higher than RhB and so the differences were larger with UrO leading to a significant interaction that was not observed with RhB.

Queens are the sole reproductive unit of the hive and are expected to live between 1 and 3 years and can produce 2000 eggs a day at their peak (Corona *et al.*, 2007). Royal jelly is produced in the hypopharyingeal gland of workers and fed to honey bee larvae as they develop. Royal jelly makes up a much higher percentage of the food for queen larvae than ti does for

worker larvae, therefore contaminants present in the royal jelly would impact all larvae but could potentially have a larger impact on queen bees (Winston, 1987). Dye in the royal jelly likely originated from labelled food consumed by workers while they are producing royal jelly. Dye from both the patty and syrup were present in royal jelly, although not for the once fed patty treatment, just the twice fed patty treatment was present. This tells us that it was not the delivery method per se but the time since feeding that influences the level of contamination of royal jelly. The syrup from both syrup treatments was taken into the hive and stored so when the bees were making the royal jelly they were still being exposed to dye that was within the hive. Dyes originating from the single patty treatment were gone when royal jelly production was stimulated because it was not stored in the hive. In contrast, hives that received two dyed patties were still actively consuming the second patty at the time that royal jelly was produced, and the dyes were then found in the royal jelly.

Hive components are complex materials. Honey contains over 181 different components including several sugar and enzymes, and royal jelly is made up of water, sugars, proteins and fats (Viuda-Martos *et al.,* 2008). This can make it hard to determine where a more lipophilic dye might accumulate versus a hydrophilic dye. Wax was the only compound in this study that is mainly a lipid, and has been shown to accumulate lipophilic pesticide residues like coumaphos (Chauzat & Faucon, 2007). Here, I saw that that the ratio of UrO (hydrophilic) to RhB (lipophilic) dye is less than the starting ratio of those dyes in the sucrose syrup, indicating that there is a relatively higher amount of RhB, the more lipophilic dye in the wax. If I had chosen dyes that are even more different in their polarity this partitioning may have been more substantial.

Even though this data does not demonstrate clear partitioning of dyes in matrices besides in the wax, this does not rule out the use of a dye as surrogate for an insecticide exposure by matching chemical properties. Levels of dye in this study were very concentrated to make sure we would recover dye in the sampling. This high initial level may have masked any subtle differences in accumulation across different materials.

Using these data, dyes should be used to target the best sampling method for a pesticide trial by helping establish the optimal timing and location of sampling. This would avoid wasting money on analyzing more samples than necessary. Particularly if a dye is co-fed with a pesticide of interest, spot sampling to establish presence of the pesticide in dyed areas could be coupled with a much more extensive sampling where only dye concentration is measured. By relating dye concentration to pesticide concentration, this could provide a detailed picture of where the pesticide is reaching in the hive. In addition no material taken into a beehive will be spread throughout the hive uniformly. The spread may be impacted by the chemical properties of the material, and dyes are one tool to help these differences.

Table 1: Cumulative numbers of dead bees over seven days (n=four cages) for three dye feeding treatments +/- standard error.

Day	Mean bee deaths: No dye fed	Mean bee deaths: RhB/UrO mixture fed for 1 day	Mean bee deaths: RhB/UrO mixture fed for 7 days
1	0 ± 0	0.5 ± 1.44	0 ± 0
2	0 ± 0	1 ± 3.53	0 ± 0
3	0.25 ± 1.25	1.5 ± 5.95	0 ± 0
4	0.5 ± 1.44	5.5 ± 15.24	0 ± 0
5	4.25 ± 16.6	6.5 ± 12.99	7.5 ± 27.5
6	4.5 ± 16.13	7.5 ± 13.61	7.5 ± 27.5
7	4.9 ± 13.39	10.5 ± 9.68	7.5 ± 27.5

Figure 8: Mean RhB fluorescence in bees following feeding on day 1.

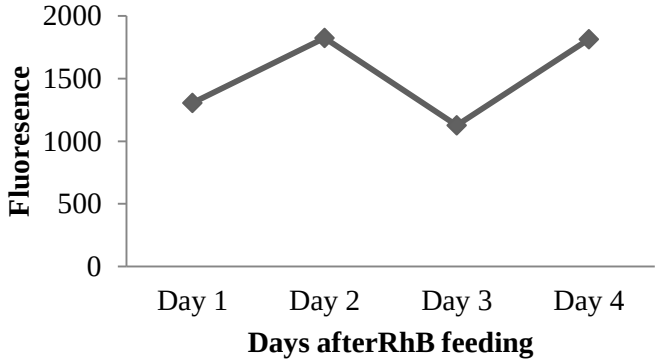


Figure 9: Mean level of UrO fluorescence in bees following feeding on day 1.

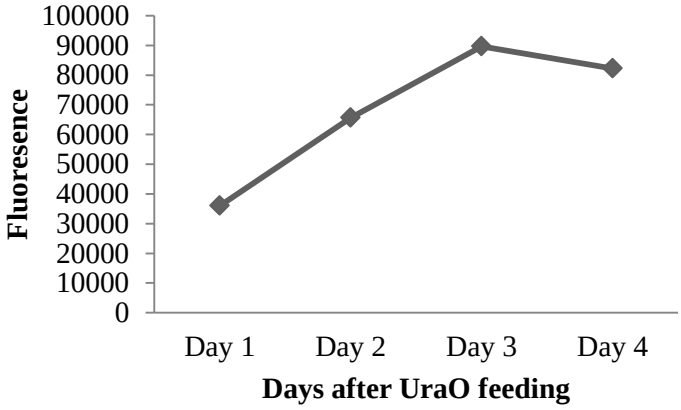


Figure 10: RhB fluorescence in dyed sucrose syrup vials placed inside of hive boxes, but inaccessible to bees, and removed throughout the study.

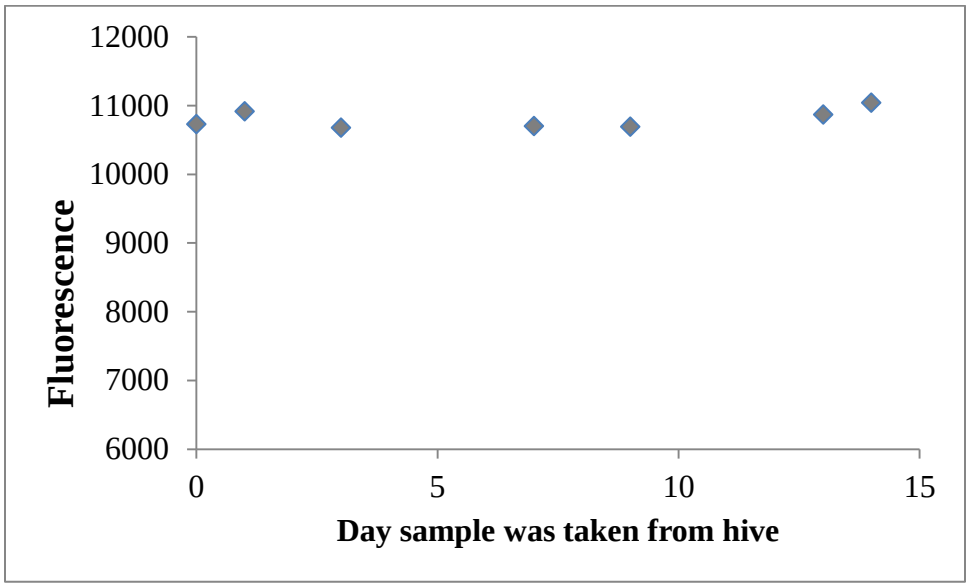


Figure 11: Mean (±SE) fluorescence of RhB in different hive matrices from colonies fed undyed food supplements, dyed pollen patties and dyed sucrose syrup. All colonies were exposed to the food treatments on days 0 and 7 and samples collected on day 14. The matrix by treatment interaction was highly significant ($F_{(10,51)} = 4.83$, $P < 0.001$).

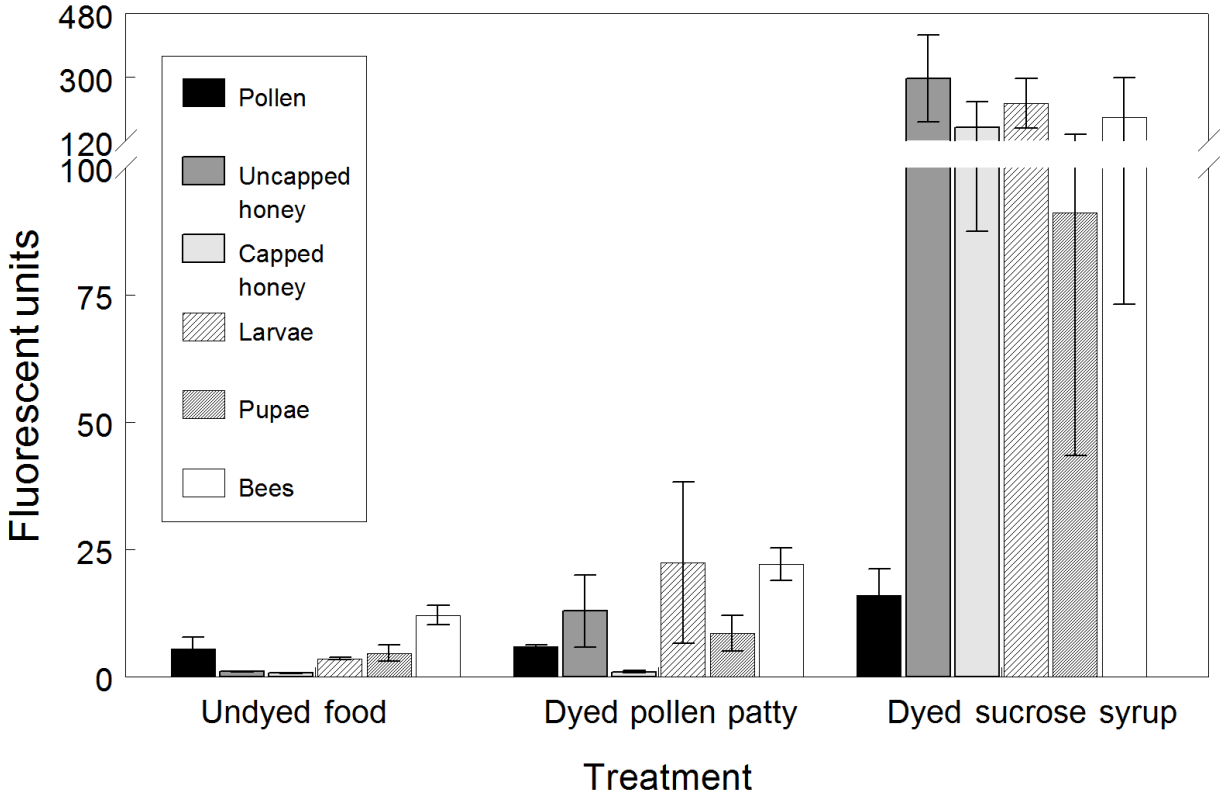


Figure 12: Mean (±SE) fluorescence of UrO in different hive matrices from colonies fed undyed food supplements, dyed pollen patties and dyed sucrose syrup. All colonies were exposed to the food treatments on day 0 and day 7 of the study and samples of matrices were collected on day 14. The matrix by treatment interaction was highly significant ($F_{(10,51)} = 3.24$, $P = 0.003$).

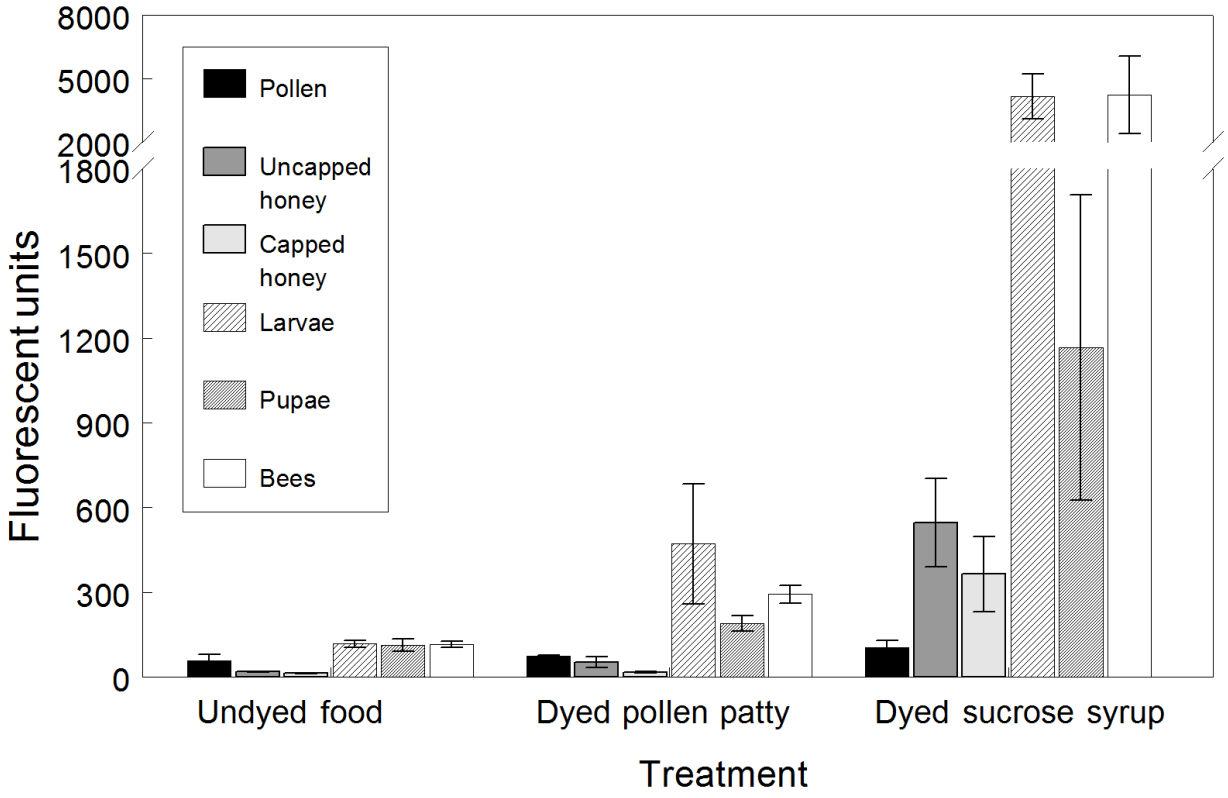


Figure 13: Mean (±SE) fluorescence of RhB at 3, 7 and 14 days in all hive matrices from colonies exposed to undyed supplemental food, dyed pollen patties, and dyed sucrose syrup. Colonies were fed treated food on day 0 only. The treatment by time interaction was highly significant ($F_{(10,168)} = 10.63$, $P < 0.001$).

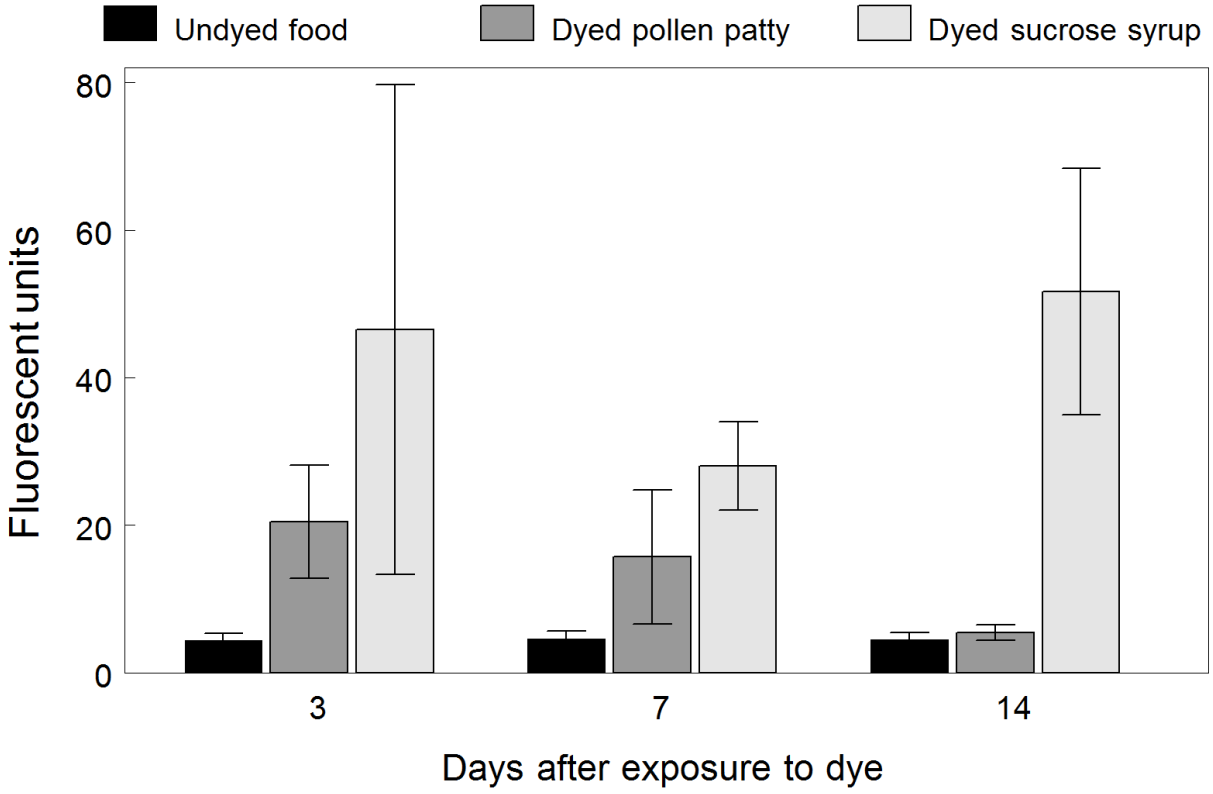


Figure 14: Mean (±SE) fluorescence of UrO at 3, 7 and 14 days in all hive matrices from colonies exposed to undyed supplemental food, dyed pollen patties, and dyed sucrose syrup. Colonies were fed treated food on day 0. The treatment by time interaction was highly significant ($F_{(10,168)} = 9.24$, $P < 0.001$).

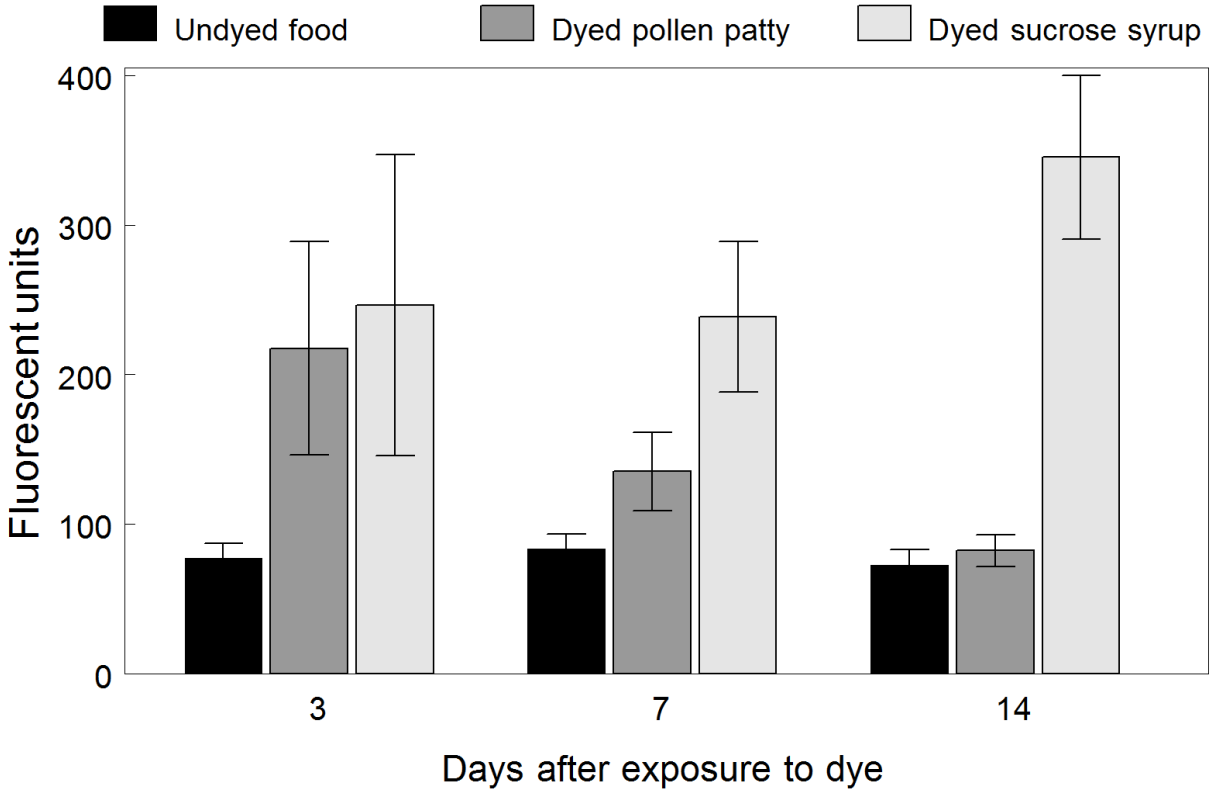


Figure 15: Mean (±SE) fluorescence of RhB in different hive matrices from colonies exposed to undyed supplemental food, dyed pollen patties, and dyed sucrose syrup. Colonies were fed treated food on day 0 only. The treatment by matrix interaction highly pooled across all sample days was significant ($F_{(10,168)} = 4.17$, $P < 0.001$).

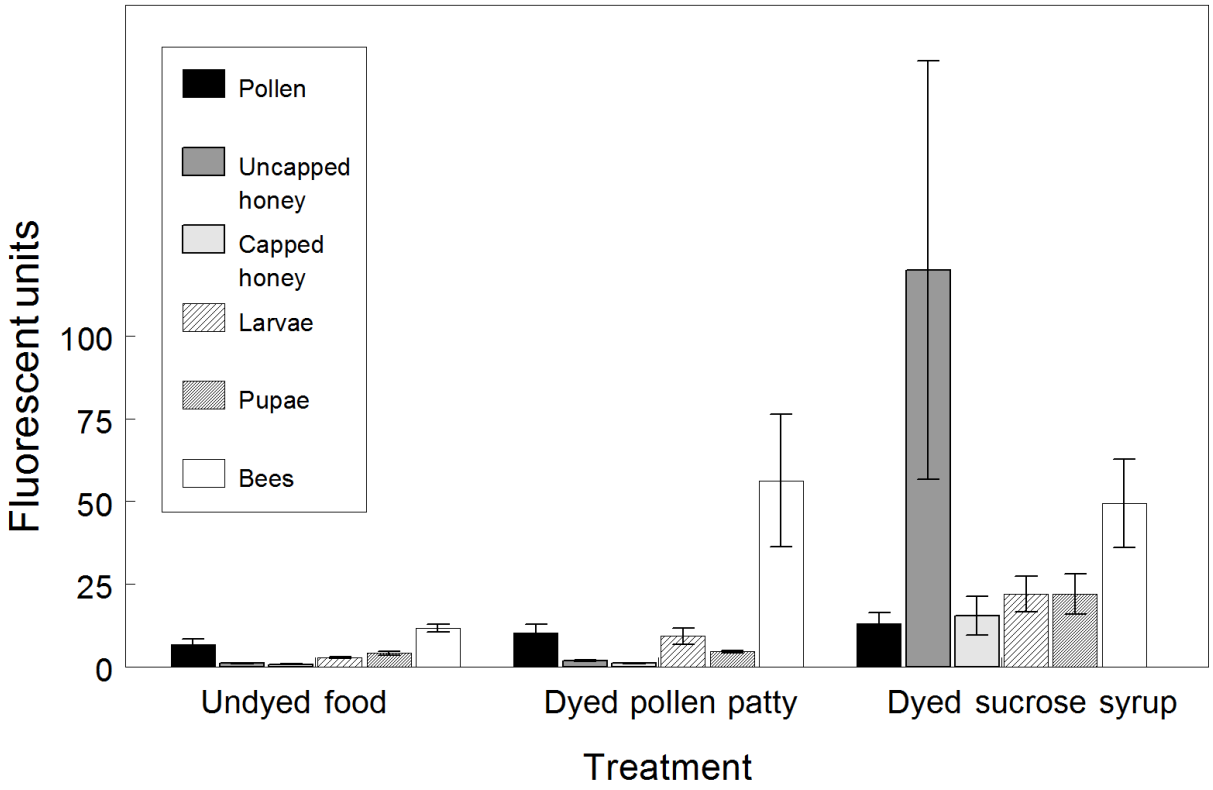


Figure 16: Mean (±SE) fluorescence of UrO in different hive matrices from colonies exposed to undyed supplemental food, dyed pollen patties, and dyed sucrose syrup. Colonies were fed treated food on day 0 only. The treatment by matrix interaction pooled across all three sample days was significant ($F_{(10,168)} = 2.23$, $P = 0.018$).

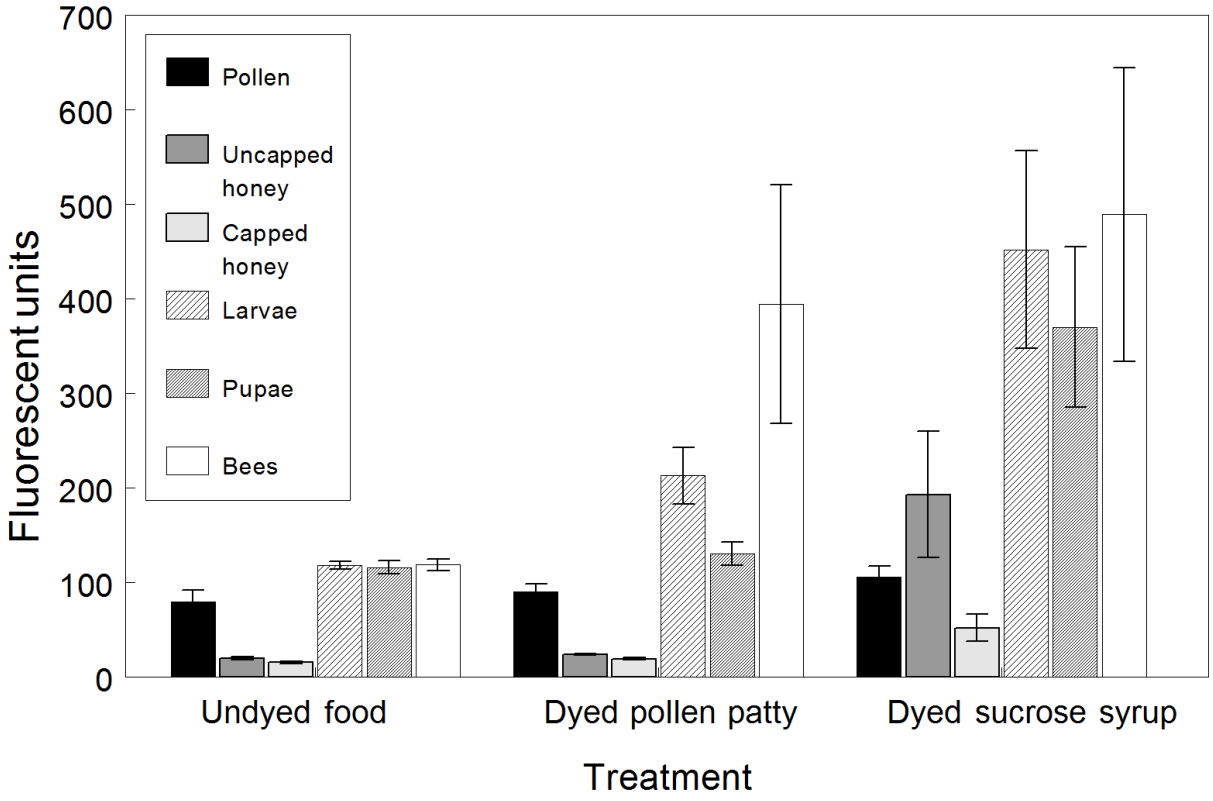


Figure 17: Mean (±SE) fluorescence of UrO at 3, 7 and 14 days in different hive matrices from colonies exposed to undyed supplemental food, dyed pollen patties, and dyed sucrose syrup. Colonies were fed treated food on day 0. Data are mean units pooled over all three food treatments. The matrix by time interaction was significant ($F_{(10,168)} = 2.12$, $P < 0.001$).

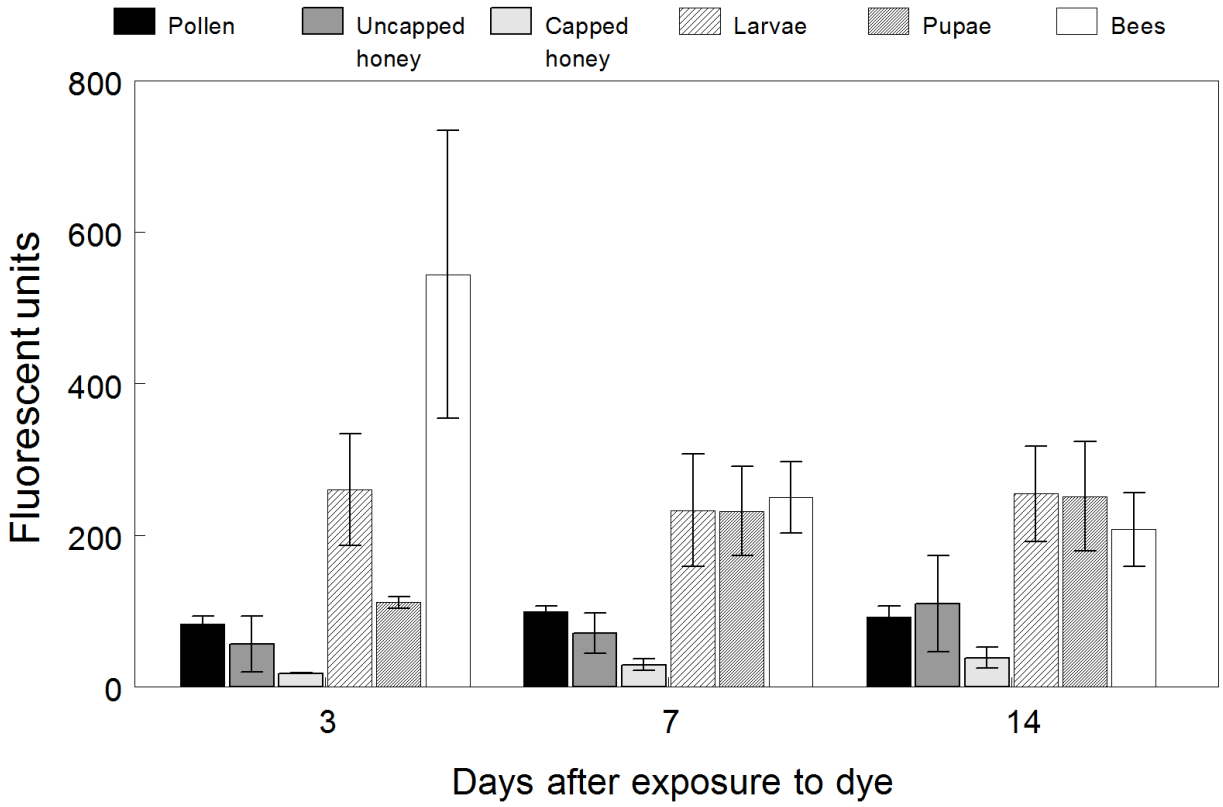


Figure 18: The mean fluorescence of RhB in royal jelly across three feeding treatments; Once dyed sucrose syrup (1xs), once fed pollen patty (1xp), and twice fed pollen patty (2xp). Bars are means, lines are standard error. * indicates p<0.05, ** indicates p<0.001

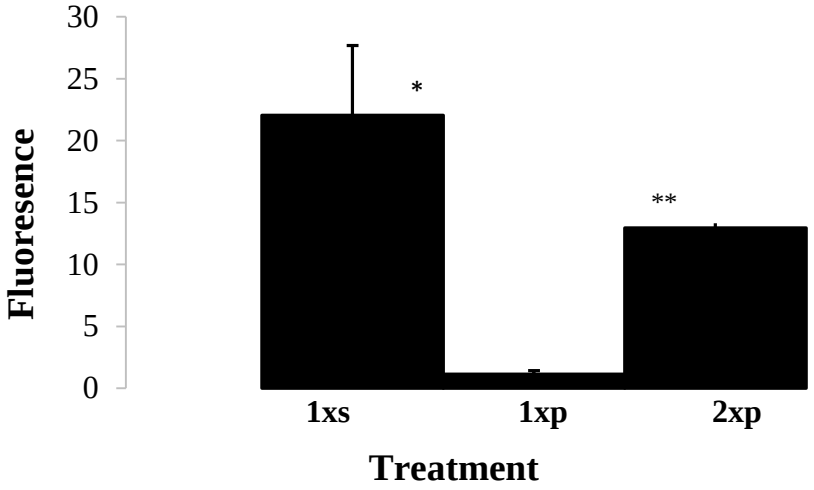


Figure 19: The mean fluorescence of UrO in royal jelly across three treatments. Once dyed sucrose syrup (1xs), once fed pollen patty (1xp), and twice fed pollen patty (2xp). Bars are means, lines are standard error. * indicates p<0.05, ** indicates p<0.001.

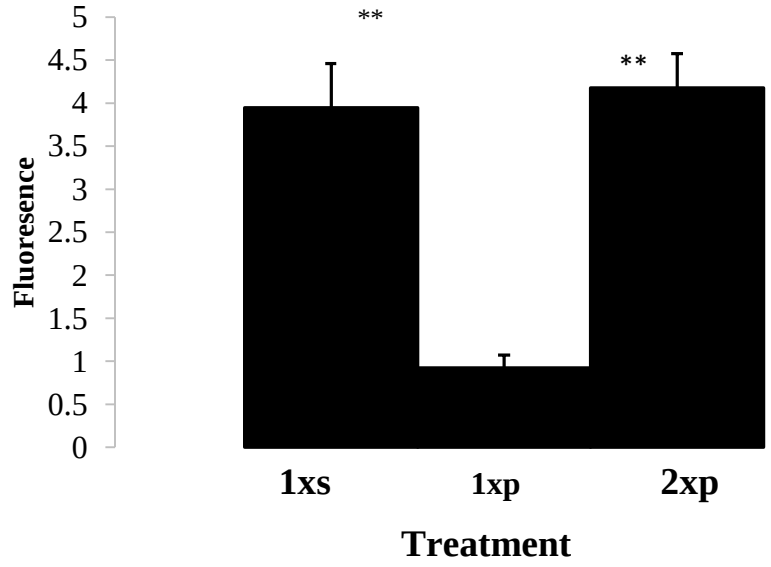
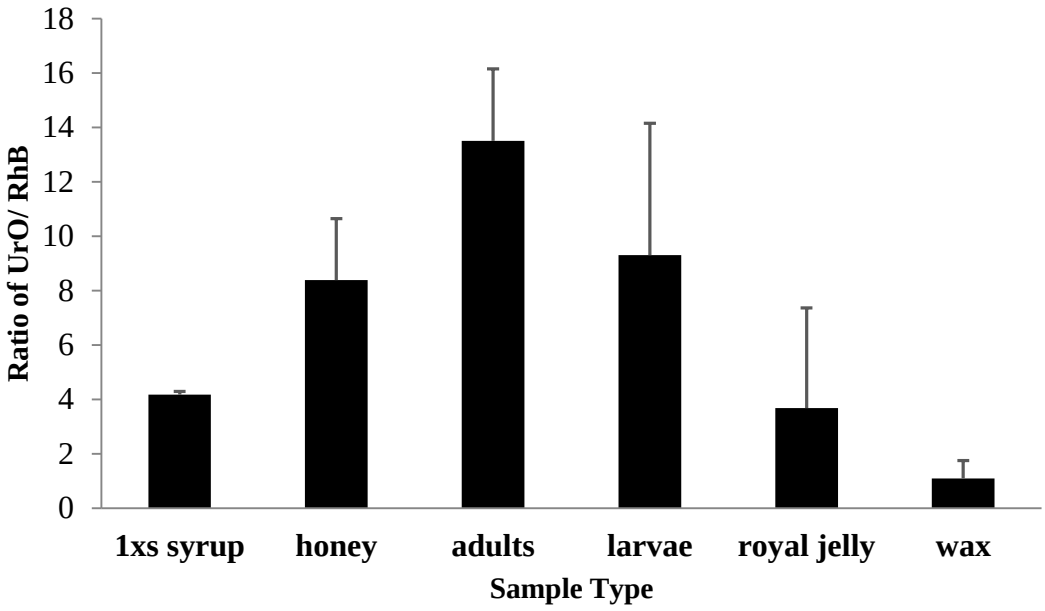


Figure 20: The ratios of UrO to RhB (average of fluorescence units) in matrices sampled at day 7 from syrup treated hives. Bars are means, lines are standard error.



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