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Lydia McCormick 04/09/2013

Honey Hydrogen Peroxide as a Form of Social Immunity in Honey bees (*Apis mellifera*)

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Abstract

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Social immunity involves cooperative mechanisms to defend against threats to colony health. For honey bees, this defense is critical and compensatory given their deficit in immunity genes. I propose that honey hydrogen peroxide (H_2O_2) is a biochemical social immune factor that bees regulate to promote colony health and defend against invasive pathogens. First, I tested in vitro whether experimentally adjusted concentrations of honey H_2O_2 affected the fitness of adult small hive beetle *Aethina tumida* (SHB). Results showed that pest survival was highest in the absence of honey H_2O_2 and was directly inhibited by higher concentrations. Second, I examined whether honey H_2O_2 content is spatially regulated in a healthy colony by measuring H_2O_2 content in capped samples from storage versus brood frames. I found that H_2O_2 concentrations were higher in brood frames suggesting that its defense role out weighs potential oxidative damage to brood. Third, I conducted a field study to examine whether supplemental feeding altered honey H_2O_2 content compared to nectar-fed controls. Results showed that high fructose corn syrup (HFCS) reduced honey H_2O_2 content while sucrose feeds increased honey H_2O_2 relative to control revealing that routine beekeeping feeds can modify the natural oxidant characteristics of colony honey. Fourth, I examined potential mechanisms by which supplemental feeding may alter honey H_2O_2, including feed concentration, induction of glucose oxidase (GO) activity by bee caste, and the statistical interaction between feed type and caste. I found that feed concentration did not alter honey H_2O_2 while bee caste GO induction was highest in receiver bees, and there was no statistical interaction between feed type and caste. Finally, I present two field studies in which colonies were infected with SHB and honey H_2O_2 content measured over the course of infestation. The results showed positive, but not statistically significant, trends between colony infestation and honey H_2O_2. When supplemental feeding was a covariate, the infection-driven increase in H_2O_2 was reduced in fed versus unfed bees. This work is consistent with the hypothesis that honey H_2O_2 is a form of social immunity, which may be negatively impacted by supplemental feeding, a common beekeeping practice.

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Introduction

Given ongoing honey bee declines, one of the foremost challenges confronting beekeepers today is managing their colonies to support honey bee social immunity. Social insects have evolved group strategies to defend their colony against infection by pathogens and pests. From an ecological perspective, group living itself poses greater threats to colony health by confining individuals in tight proximity, hence facilitating disease spread. Therefore, social immunity provides cooperative mechanisms that compensative for this increased transmission risk of group living. Evidence supports the role of diverse cooperative defense mechanisms in ants, wasps, termites, and bees (Cremer et al. 2007). Honey bees, however, display advanced colony-level immune responses that have parallel characteristics to the complex cellular immune systems active within organisms (Cremer and Sixt 2009, Parker et al. 2011). Not surprisingly, there is growing concern that customary beekeeping management techniques, which manipulate the colony habitat and potentially disrupt essential social immune mechanisms, may contribute to current honey bee disease susceptibility (Evans and Spivak 2010).

The potential importance of these group defense mechanisms is further magnified by data from the honey bee genome sequencing project showing honey bee specific deficiencies in the innate disease-resistance pathways and immune responses (Evans et al. 2006, Weinstock et al. 2006, Evans and Spivak 2010). Indeed, compared to non-social insects like *Drosophila*, honey bees possess a significantly reduced number of immunity-conferring genes (Evans et al. 2006). This genetic immune deficit not only supports the evolutionary pertinence of the already documented honey bee non-immunological responses, but also indicates that social immune responses may function as pivotal compensatory defense mechanisms for social insects (Cremer et al. 2007, Parker et al. 2011).

Honey bees display key social immune behaviors including mite grooming, necrophoresis (removing dead or diseased larvae to prevent pathogen transmission), and social fever (elevating colony temperature to kill pathogens) (Visscher 1983, Moore et al. 1995, Starks et al. 2000). Concerning chemically-mediated social defense mechanisms, honey bees have been shown to self-medicate by enhancing propolis production in parasitized versus healthy colonies (Simone-Finstrom and Spivak 2012). Moreover, the antimicrobial properties of propolis resin have been demonstrated to reduce infestation of *Paenibacillus larvae,* a causative agent of American Foulbrood (Antúnez et al. 2008).

Honey has long been recognized for its antimicrobial properties, yet, its potential role as a chemical mediator of social immunity against colony pathogens has not been explored. While several properties confer honey's antiseptic actions, including its viscosity, hyperosmolarity, and acidity, perhaps most intriguing is its hydrogen peroxide (H_2O_2) content. Hydrogen peroxide functions as a ubiquitous biological signal and a toxic metabolite. It generates oxygen free radicals that over time can induce oxidative stress, DNA damage, apoptosis and carcinogenesis (Song et al. 2007). Honey bees add H_2O_2 to honey via the hypopharyngeal gland enzyme, glucose oxidase (GO). During nectar transport, the enzyme irreversibly converts glucose to hydrogen peroxide and glutonic acid such that there is a proportional relationship between secreted enzyme and H_2O_2 honey content (White et al. 1963).

GO is present naturally in assorted fungi and a few insects (bees, larvae (*Helicoverpa zea* and *assulta, Spondoptera exigua*), and the grass hopper *Schistocerca americana*) (Candy 1979, Zong and Wang 2004, Wong et al. 2008). It is utilized widely in the industrial food market as a preservative because its enzyme activity maintains low levels of H_2O_2 that are both anti bacterial and anti fungal (Wong et al. 2008). This commercial application of GO underscores the

beneficial role that chronic low levels of H_2O_2 in honey may provide against pathogens, especially those that are in direct contact with honey or lack adequate biological antioxidant counter mechanisms.

The potential social immune role of GO in honey bees colonies was suggested in two studies wherein worker bee hypopharyngeal activity or GO gene expression were shown to increase with worker bee age (Huang and Otis 1989, Ohashi et al. 1999). In another study, GO activity was experimentally induced in caged young bees in response to dietary protein and, in particular, poly-floral compared to mono-floral pollens (Alaux et al. 2010). The investigators hypothesized that greater GO content in colony food stores may improve colony health (social immunity) by rendering food less infectious. While the use of added antibacterial agents for food preservation is rare for insects, this behavior is found in the burying beetle (*Nicrophorus vespilloides*), which uses antibacterial lysozymes to prepare and protect carcasses of small vertebrate prey for its larvae (Cotter and Kilner 2010). Moreover, as a possible honey bee defense, colony fitness has been suggested to diminish in response to decreased hypopharyngeal gland size resulting from *Varroa destructor* mite infection (Pinto et al. 2011).

While the role of H_2O_2 as a food preservative is recognized, the potential for this natural honey oxidant to have more widespread, even targeted social immune functions within colonies has not been explored. My thesis examines this novel concept. The foundation of this work evolved over six years and is based on original preliminary studies that were done while I was a high school student in Birmingham, AL between 2008-2010. These initial studies explored the potential importance of honey H_2O_2 as a colony defense mechanism via field studies on honey samples collected from state-wide apiaries and targeted in vitro experiments with small hive beetle (SHB). I selected SHB for this study because it decimates hives by damaging wax-comb

while consuming pollen, bee larvae, and most importantly, stored honey, during its reproductive cycle (Ellis et al. 2002). Moreover, other beetles have been shown to be susceptible to diet-related reactive oxidative species in natural plant defense mechanisms (Krishnan et al. 2007).

Through experimental studies at Emory and the University of Georgia (UGA) Bee Laboratory, I expanded the aforementioned preliminary work across all four of my undergraduate years at Emory (2010-2014), including three field seasons at the UGA bee lab. Given the broad scope of these experiments, I segregated this work in five chapters that develop the overarching theme of H_2O_2 in the context of social immunity and colony defense. Below is a short synopsis of each chapter, presented to aid in linking these chapters together.

Chapter 1: Small hive beetle (Aethina tumida) survival To directly address the defense potential of honey H_2O_2, I examined whether the experimental manipulation of honey H_2O_2 content affects small hive beetle *Aethina tumida* (SHB) survival. These experiments entailed replicated exposure of adult beetles to honey feed containing experimentally manipulated levels of H_2O_2, ranging between 0 (catalase control) and 4000 μg/ml added H_2O_2. I found that H_2O_2 negatively impacts SHB survival, with higher levels of H_2O_2 having a stronger negative effect. This result suggests that H_2O_2 plays a role in colony defense beyond acting solely as a food preservative.

Chapter 2: Variation of honey H_2O_2 at the colony level Based on the observations made in Chapter 1 that pest survival is adversely affected by higher H_2O_2, I aimed to determine whether honey H_2O_2 is spatially distributed within the hive as a mechanism of colony protection. In particular, there is a theoretical balance between the protective effects of honey H_2O_2 against colony pests, which contrasts with its potentially negative actions on developing bee larvae

(which also consume honey). Thus, H_2O_2 may be differentially expressed (positively or negatively) in frames of honey, which are general food stores for the entire colony, versus that found in honey cells on brood frames, from which developing bee brood and recently emerged workers are fed. To test this, I measured honey H_2O_2 collected from honey storage frames versus honey from brood (honey bee larvae) frames. I found significantly higher levels of H_2O_2 in brood frames as compared to honey frames, suggesting that the protective effects of H_2O_2 may outweigh the risks to developing bees.

Chapter 3: Supplemental feeding on honey H_2O_2 Serendipitous observations in 2012 suggested that supplemental feeding caused visually apparent differences in stored honey. Laboratory analysis showed that these more translucent honey samples had unusually low honey H_2O_2 content. I followed up on this finding with a replicated, controlled field study of feeding source types and H_2O_2 content, using high fructose corn syrup (HFCS) and cane sugar as carbohydrate sources. My pilot finding of reduced H_2O_2 in cane sugar-fed colonies relative to unfed controls was not supported, but I did find that HFCS feeding led to significantly reduced H_2O_2 relative to unfed controls and cane sugar fed bees.

Chapter 4: Mechanistic drivers of the relationship between supplemental feeding and H_2O_2. Given the findings from Chapter 3, I explored potential mechanisms that could produce variation in honey H_2O_2 driven by supplemental feeding. I assessed three mechanisms: 1) the effect of feed concentration on honey H_2O_2, 2) regulation of worker bee caste glucose oxidase (GO) activity, and 3) the effect of feed type on worker bee caste glucose oxidase activity. Examining whether feed concentration alters honey H_2O_2 was considered because the nectar dehydration

process involves repeated association with the mouthparts near the hypopharyngeal gland such that dilute supplemental feeds may involve longer exposure to enzyme secretion (hence have greater H_2O_2). Secondly, I investigated three worker caste's GO activity (content at Vmax) because of previous knowledge of age determined GO expression (Ohashi et al. 1999). Therefore, this mechanism analyzed whether key feed-processors have markedly reduced GO activity, which could result in lower H_2O_2 honey. Thirdly, I examined the interaction between feed type and GO caste activity to determine if the composition of feeds may affect GO secretion/expression. I found no evidence for the concentration effect, the worker-development effect, or an interaction between the feed type and caste-associated GO activity in driving variation in honey H_2O_2 shown in my previous studies. Still, my results produced an interesting result in that they contrasted with published work which suggested that young honey bees do not produce GO (Ohashi et al. 1999). In addition, the results of this study contrasted with the results from Chapter 3, in that HFCS did not lead to reduced honey H_2O_2 relative to cane sugar feed. This discrepancy was likely driven by two factors. First, this study took place during the spring nectar flow, and thus both cane sugar fed bees and corn syrup fed bees were able to acquire substantial amounts of nectar. This is in contrast to the studies described in Chapter 3, which took place during a nectar dearth in the early autumn, which greatly limited nectar inputs into the colony. Second, I fed bees at a lower rate in this study relative to the study in Chapter 3, so again the signal of supplemental feed versus nectar was lower.

Chapter 5: Small hive beetle experimental infection To ultimately confirm honey H_2O_2 as a novel social immune defense, a pathogen infection study is essential. SHB was selected as an experimental model for colony infection because of its rapid reproduction involving a voracious

larval stage (high consumption of honey and pollen) and relative ease for in vitro testing. The latter in vitro results supported adverse effects of higher honey H_2O_2 on SHB fitness and, notably, improved SHB survival with the lowest H_2O_2. In addition, random field collection studies in 2008 suggested that SHB infested hives had 50% higher honey H_2O_2 relative to healthy hives. . To causally link SHB infection with a social, colony-mediated honey H_2O_2 response, longitudinal measurements of honey H_2O_2 before and during SHB infestation (versus control colonies) are required. Of added importance, observations from my supplemental feeding studies (Chapters 3 and 4) indicated that the field study design needed to incorporate feeding as a covariant influence on honey H_2O_2. Two field studies aims were, therefore, developed. To test aim 1, I measured H_2O_2 content in colonies that were experimentally infected vs. uninfected with SHB. To test aim 2, I conducted a full-factorial experiment with all four combinations of fed/unfed and infected/uninfected bees. In Aim 1, I found that H_2O_2 increases in experimentally infected colonies relative to uninfected colonies, supporting the idea that H_2O_2 could be induced. In Aim 2, my data were consistent with the presence of an interaction between supplemental feeding and infection status in honey H_2O_2, but this pattern was not statistically significant. Power analyses suggest that repeating the experiment with approximately 15 colonies per group would provide the statistical power necessary to document this interaction.

Chapter 1: Small hive beetle (Aethina tumida) survival in relation to varying H_2O_2 concentrations

Introduction:

While numerous studies have proven the effectiveness of the antimicrobial qualities of honey H_2O_2, it has rarely been explored as a potential defense (Molan 1992, Taormina et al. 2001a, Wilkinson and Cavanagh 2005). Moreover, at the molecular level, H_2O_2 is recognized for its production of tissue-damaging oxidative free radicals (Song et al. 2007). Here I propose that honey H_2O_2 is a colony-wide defense that can cause oxidative harm and decrease the survival of honey bee pathogens. In order to test this aim experimentally, I selected the ubiquitous ideal target pest, the small hive beetle (*Aethina tumida*) (SHB).

SHB is an invasive species from South Africa which has become particularly virulent in the United States, Australia, and Europe consequent to commercial colony transport (Neumann and Ellis 2008). These beetles have a rapid reproductive cycle including a larval and pupation stage that occurs over a period of about 48 days (Murrle and Neumann 2004). As a main diet, SHB adults and larvae rigorously consume honey and pollen stored within a honey bee colony. These high levels of scavenging can lead to hive absconding within weeks (Neumann and Elzen 2004). Thus, given that SHB ingests constant amounts of honey and undergoes rapid metamorphosis and cell divisions during it's life cycle, the beetle may be particularly susceptible to altered levels of honey H_2O_2.

In this study, I monitored the effect of experimentally altered honey H_2O_2 levels on the survival of adult SHB for 7 weeks. Given knowledge of the damaging oxidative properties of H_2O_2, I predicted that the highest beetle survival would be in groups fed lower concentrations of honey H_2O_2. Furthermore, in regard to social immunity, honey bees have already been recognized to display characteristics of social defense against the small hive beetle. For example,

guard bees at the hive entrance aggressively block the intrusion SHB and, within the colony, specialized workers corral the beetles into crevices and then encapsulate them with propolis (Neumann and Elzen 2004). These behaviors further indicate the possibility of other forms of non-immunological social defense against SHB. Honey hydrogen peroxide could provide additional defense against these pests.

Methods: *Small hive beetle survival*

In order to study the effects of honey hydrogen peroxide on hive pests, I assayed the survival of small hive beetle (SHB) on honey substrates with differential H_2O_2 contents. In June 2010, I collected SHB from one apiary within the University of Georgia honey bee farms. These beetles were then reared according to standard protocols (Ellis et al. 2002, Murrle and Neumann 2004). I incubated adult SHB in 4oz Ziploc containers with screen mesh lids, prepared with corrugated cardboard squares, cut dry beeswax comb, 6 grams granulated pollen, and 4 grams experimental honey. Two positive control honey samples were used; one from Athens, GA (5μg/ml H_2O_2) and the other from Birmingham, AL (40μg/ml H_2O_2). Honey (Birmingham, AL) was adjusted to the following H_2O_2 concentrations; 0ug/ml (catalase negative control), 250 μg/ml, 750 μg/ml, 1500 μg/ml, 4000 μg/ml H_2O_2. The catalase negative control was prepared according to previous methods in order to remove the endogenous honey H_2O_2 (Osato et al. 1999). There were three replicates of each experimental honey concentration and ten adult SHB were added to each incubation container. I measured their survival once a week for 7 weeks. In order to maintain humidity, I misted all the containers with water twice a week.

Results:

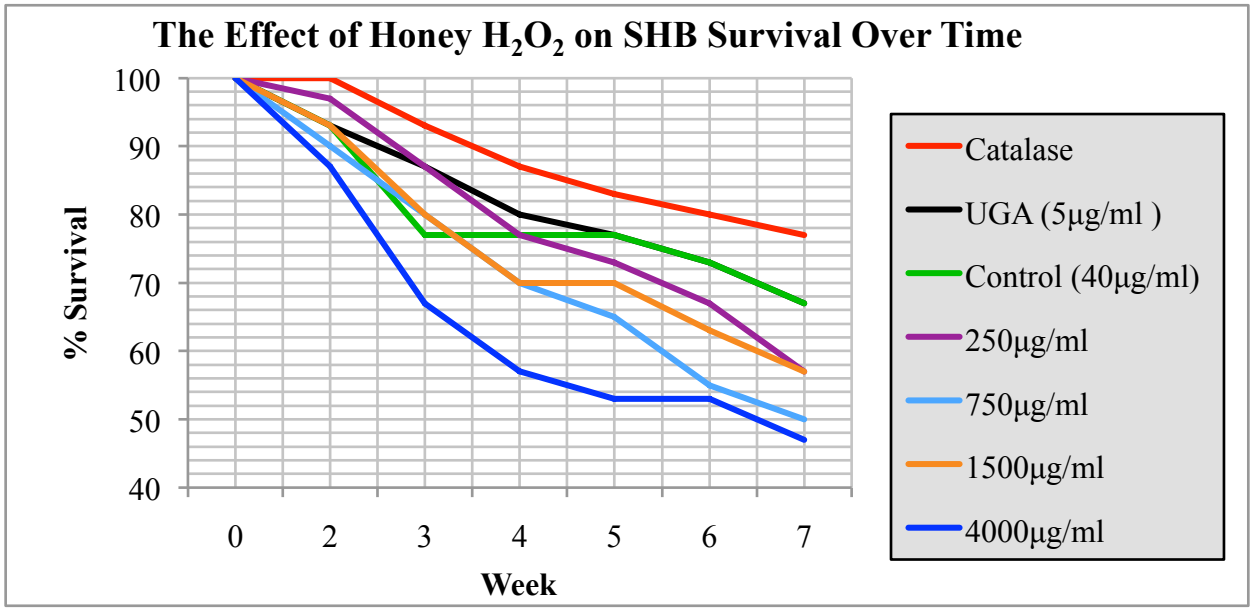


Figure 1: The Effect of Honey H_2O_2 on SHB Survival: Groups of 10 adult SHB were incubated in triplicate with various concentrations of honey H_2O_2. Percent survival was calculated over 7 weeks. The catalase group was the negative control (0µg/ml H_2O_2). Two unadjusted natural honey samples were included; UGA (5µg/ml H_2O_2) and Control (40µg/ml H_2O_2) General linear model analysis revealed a marginally significant (p=.055) result between catalase and unaltered 5µg/ml H_2O_2 control.

Overall, I found that SHB adult survival decreased when fed increasing concentrations of experimentally adjusted honey H_2O_2. The reduction in beetle survival ranged from 20-50% for each concentration of honey H_2O_2. Most notably, the catalase group (endogenous H_2O_2 removed) promoted the best SHB survival with only a 23% reduction in survival by 7 weeks compared to the 33% reduction in the unadjusted controls. In order to analyze the natural peroxide range compared to the catalase group, I used general linear model (GLM) binomial analysis based on the final survival at week 7. This analysis was conducted using R Statistical Programming language (R Development Core Team 2012). I found that there was barely insignificant result between catalase (0µg/ml) and control 5µg/ml (p= .055). In addition, although currently in progress, I plan to conduct a mixed effects, interval-censored survival analysis.

Discussion:

The results conformed to my prediction that small hive beetle would be adversely effected when consuming increased levels of honey H_2O_2. Most importantly, the survival of this hive pest was optimal in the study group that had removed natural H_2O_2. Although I did not find a statistically significant difference in the natural range (catalase vs. control), the marginally significant difference was very close to p=.05. Therefore, this indicates there is a trend here, and with additional samples it is likely that I would have found a significant difference. Thus, my results suggest that this chemical in honey may function as a protective response for the honey bee besides its recognized role of food preservation.

While honey bees will never be able to produce honey with H_2O_2 concentrations as high as even 250μg/ml, I wanted to visualize a spectrum of free radical stress for this beetle. In particular, the diet of SHB also includes pollen (although not as a main intake), which sometimes can contain catalase, an enzyme that breaks down H_2O_2 (Murrle and Neumann 2004). Speculatively, this consumption of pollen could play an important antioxidant coping response for the beetle's high consumption of honey. Thus, the wide range of H_2O_2 concentrations in this study account for this idea (note the study beetles are also fed pollen). Yet, the marginally significant result for catalase, suggests that the hive pest thrived best when endogenous H_2O_2 was removed. Therefore, this has implications to suggest that colonies that are not able to produce higher levels of honey H_2O_2 may be especially vulnerable to this pest.

Given that I have demonstrated an adverse effect of honey H_2O_2 on the SHB adults (presumably due to oxidative free radical stress), it would be intriguing to conduct similar experiments using SHB larvae. The rapid growth rate and cell division of larvae would make them more susceptible to oxidant stress and, perhaps, cause an effect at much lower honey H_2O_2

concentrations. In addition, to test the broader implications of honey H_2O_2 as a social immune defense, I would also like to test its potential oxidant toxicity on other honey bee pests. Of interest, varroa mite (*V. destructor*) and other pathogens, have been shown to specifically attack the hypopharyngeal gland (affecting development). This directed action could possibly be a defensive response to the gland's peroxide forming capabilities (Pinto et al. 2011). Finally, it may be that honey H_2O_2 provides a low level, background anti-microbial and anti-fungal function within the colony infrastructure that enhances its overall tolerance to more invasive pests (such as SHB and varroa) by reducing the colony baseline pathogen load.

Chapter 2: Variation of honey H_2O_2 within the colony level

Introduction:

As demonstrated in the previous chapter, honey H_2O_2 can have negative effects on pest fitness and therefore may serve as a social immune defense. These findings suggest that it may be regulated within the colony in order to best promote colony protection. While pathogens such as SHB may plague an entire colony, it may be energetically favorable for workers to produce the highest peroxide honey where it could serve as most beneficial to colony health. In order to test this question, I measured honey H_2O_2 collected from honey storage frames versus honey from brood (honey bee larvae) frames. I proposed that higher levels of peroxide surrounding honey bee offspring could provide optimal protection against pathogens (especially from microorganisms like fungi or bacteria) Of note, honey bees have already been shown to display spatial patterns in nest architecture in order to optimize brood protection (Pie et al. 2004). Therefore, I expected to find higher levels of honey H_2O_2 on brood frames versus honey only frames. Yet, a tension may evolve between pest suppression and damage to brood because bee larvae also feed from this honey and could be vulnerable to elevated H_2O_2 concentrations. This possible adverse effect may result in only slightly elevated levels to be suitable for brood consumption.

Methods:

I collected honey samples from 47 different colonies from four different apiaries throughout Athens, Georgia in June and July 2011. Capped adjacent honeycomb cells were obtained in order to fill 1.5ml centrifuge tubes. Honey was selected from both brood frames (honey bee larvae present) as well as honey (only) frames within each colony. Although slightly varying in colony amount present, I collected about 15-20 honey samples total from each apiary.

These samples were stored at -20°C. I measured honey hydrogen peroxide content (µg/ml honey) using a colorimetric microplate detection kit (Enzo Life Sciences, Plymouth Meeting, PA). This spectrophotometer assay was completed in duplicates for each sample. The honey was diluted accordingly between 1:10 to 1:50 by volume with phosphate buffer in order to fit the standard curve.

Results:

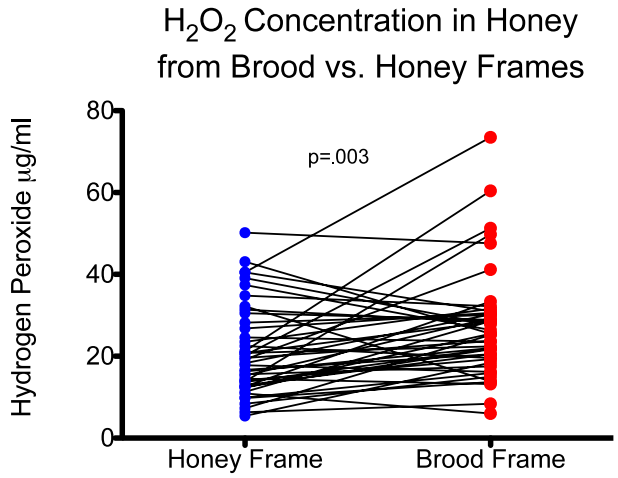


Figure 2: Honey H_2O_2 Concentrations from Brood vs. Honey Frames: Honey samples were taken from 47 hives in various apiaries in Athens, GA during the summer 2011. Capped samples were from brood frames (n=43) and honey frames (n=43). Honey was measured for H_2O_2. Statistical differences are p=.003 by *paired* t test and by *unpaired* t test p=.02

After two months of sampling, I found that honey H_2O_2 concentration for a given colony was higher from the brood frames (n=43) than from honey storage-only frames (n=43). These results were statistically significant by both paired t-test (p=.003) and by unpaired t-test, (p=.02). Of the 43 paired samples, 10 had a doubling or more of the honey H_2O_2 concentration.

Discussion:

The aim of this study was to determine if honey bees regulate their honey H_2O_2 within the colony with the implication that it may serve as a potential defense to pathogens. I found that honey from brood frames is significantly higher than from honey frames. Moreover, I have shown the potential for oxidative free radical stress to hive pests from honey consumption (chapter 1). This result conforms to my prediction that honey bees may control their production

of peroxide at a spatial level within the colony to best protect their offspring. Honey bees have adapted a well organized hive building pattern that consists of a compact brood sphere within the center of the colony (Camazine 1991). Thus, it is possible that adding honey with higher H_2O_2 to the perimeter cells of the brood nest could serve as a defense barrier to scavenging pests and microorganisms.

Conversely, one must consider the potential adverse effect of the high peroxide diet on developing bee larvae and newly emerged workers who also consume brood frame honey (Winston 1991). This warrants further investigation, yet it is plausible that the nurse bee and/or larvae develop protective antioxidant responses. Indeed, adaptive antioxidant responses have been observed in other insects. For example, the Colorado potato beetle (*Leptinotarsas decemlineata*) develops protective mid-gut antioxidant enzymes to tolerate the oxidative stress from the ingestion of their main diet, potato leaves (Krishnan et al. 2007). Furthermore, brood defense responses have also been studied in insects. In particular, the burying beetle (*Nicrophorus vespilloides*) utilizes antibacterial exudates to defend their offspring's food source from competitors (Cotter and Kilner 2010). These antibacterial secretions from the adults harm microorganisms and pathogens that attempt to ingest their offspring's resource. The close resemblance of this unique response in honey bees supports the function of honey H_2O_2 as a brood protection against microorganisms, fungi, and hive pests like SHB.

Chapter 3: Effects of Supplemental feeding on honey H_2O_2

Introduction:

During sampling from studies in the previous chapter, I casually noted during field collections that the honey stores in hives being supplementally fed sucrose syrup were often translucent in color. Analysis of six of these samples (from random hives) revealed that H_2O_2 was significantly lower in supplementally fed colony samples than both honey and brood frame honeys from naturally nectar foraging bees. My past SHB survival data suggested that lower levels of honey H_2O_2 promote SHB longevity (chapter 1). Taken together, these observations were concerning given the universal beekeeping practice of supplemental feeding because this seemingly innocent practice might be adversely lowering immunoprotective levels of honey H_2O_2. To thoroughly examine this possibility, I examined the effect of high fructose corn syrup feeding HFCS (commercially used) and cane sugar water (most commonly used) on the honey H_2O_2 content in 15 colonies. Understanding how this widespread hive management method can manipulate H_2O_2 levels could be beneficial in maintaining social immunity in colonies.

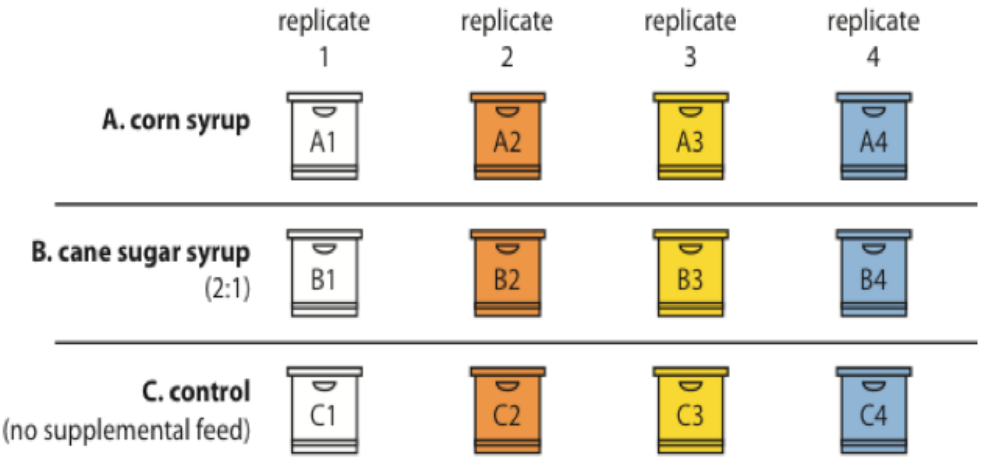
Given the reduction of honey H_2O_2 in pilot colonies fed supplemental sucrose water, it seemed plausible that there could be a disturbance in the regurgitative production of honey from the feed. Indeed, my result was intriguing because biochemical literature suggests that H_2O_2 formation by honey bees is possible with a sucrose substrate (Huang 2010). While glucose oxidase is highly specific to glucose, invertase, and hypophyarngeal enzyme secreted by foragers, cleaves the sucrose disaccharides into fructose and glucose monosaccharides (Huang 2010). Thus, rather than substrate limitation, I hypothesize that a simpler mechanism could be responsible due to the length of the regurgitation process during honey production. In this study, I expected to find lower honey H_2O_2 in cane feeds than unfed controls. The corn syrup fed

colonies could have lower H_2O_2 because of its higher concentration of fructose, which is not a substrate for glucose oxidase.

Methods: *Honey hydrogen peroxide concentrations*

I examined the impact of supplemental feeding on honey hydrogen peroxide concentrations. Three experimental groups were set up at the UGA bee lab in September 2013 1) 55% high fructose corn syrup (55% fructose, 42% glucose) -this is the standard "heavy" syrup that is used by commercial beekeepers 2) 2:1 (by weight) cane sugar solution group (100% sucrose) - this is most commonly used by beekeepers, and 3) control unfed group, floral nectar was the only source of nutrition (see diagram). I isolated the control unfed colonies in Tiger, GA to prevent robbing of the supplementally fed hives and to secure that nectar collection only came from floral sources. Honey bees sometimes steal honey and nectar stores from nearby colonies, therefore, it was particularly important to separate control so these colonies would not have frames contaminated with supplemental honeys (Winston 1991). The experimentally fed colonies were maintained on the UGA bee lab Horticulture Farm in Watkinsville, GA, which separated them from the control hives by 113 kilometers. All colonies in each study group were placed at the edge of field and woodland landscapes. I used nucleus colonies for each group that were created in the week before the study. These colonies were produced with 3 lb honey bee packages using 5 frame double deep hive boxes. To ensure that sampling only occurred with honey stores added during the time of the study, marked honey-absent comb frames were added at the beginning of the experiment. While movement of older nectar honey stores to the marked frames may occur, it is generally rare and any such movement that appears in samples was averaged over the many samples I collected. The colonies were fed half-gallon jars, twice a week for 8 weeks and 8 weeks of sampling began after the feeding period ended in October 2013.

Sampling began after feeding due to the unavailability of field help at UGA to collect samples during prior weeks. Two random capped honey samples were taken from each colony each week in addition to one observer selected "sugar water" sample (very clear honey), and one "nectar" sample (uncapped and viscous golden honey). To monitor collection, the spatial area of selection in the hive was recorded such as "top-far," "top-near," "center," "bottom-far," and "bottom-near" The honey samples were stored at -20°C. Hydrogen peroxide was measured using a colometric assay (Enzo Life Sciences). This spectrophotometer assay was completed in duplicates for each sample. The honey was diluted accordingly from 1:5 to 1:50 by volume with phosphate buffer in order to fit the standard curve.



Honey H_2O_2 statistical analysis:

I tested the statistical significance of honey hydrogen peroxide values between the different feeding groups (corn syrup, cane sugar syrup, and control/unfed) by using linear mixed effects models ("LMMs') with the colony ID as a random effect, with the "lme4" package (Bates et al. 2011) in the R Statistical Programming Language (R Development Core Team 2012). Because the different honey samples from the same colony cannot be considered independent samples, linear mixed effects models adjust for this non-independence of colony groups while

also utilizing all collected data points. I used qq-plots to assess whether the honey H_2O_2 data was normally distributed and met the assumptions of my random-intercepts model. Given that the result did not appear to be normally distributed, I conducted a power transform ("Box-Cox transform), after which the data conformed to the assumptions of normality. The "lmer" function from the "lme4" package does not estimate p-values, thus, I used ANOVA analysis to compare the model including feeding type to a null model with respect to the random effect of colony number. This approach was applied to both the original and transformed data. In order to test the effect of corn syrup and sugar syrup feeding compared to the control unfed colonies, I applied the "lmerTest" package in R Statistical Programming Language to my untransformed mixed effects model. This function calculates p-values.

Results:

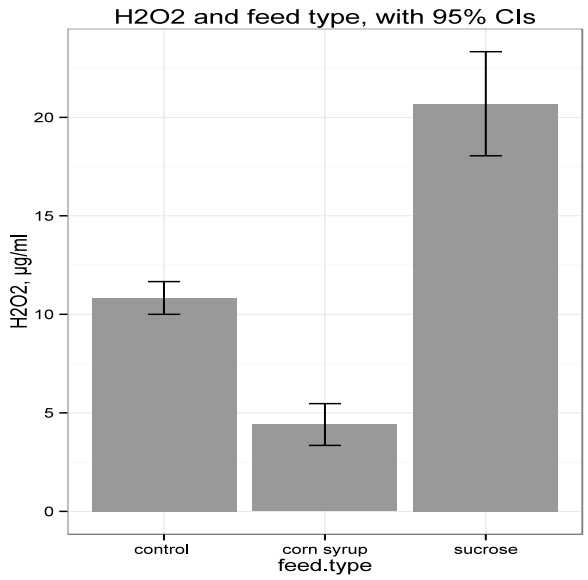


Figure 3:The Effect of Supplemental Feeding Type on Honey H_2O_2: Honey samples were collected from three experimental groups each with 4 colonies; cane (sucrose fed), corn (corn syrup fed) and control (unfed). Colonies were fed twice a week 8 weeks prior to another 8 weeks of sampling. Statistical analysis was by linear mixed-effects models (see methods) with n=73 to 76. Corn vs control p=.0001 and cane vs. control p=.006.

Compared to controls (n=73) there was an approximately 60% reduction in honey H_2O_2 in the corn fed (n=76) colonies (p=.0001) and a two fold increase in the sucrose fed (n=76) colonies (p=.006). However, when the corn versus control cohorts were compared using power transformation statistics, the p-value (.000006) was significantly more robust. Also, there was no

significant difference in H_2O_2 levels attributed to selection bias between observer selected "sugar water" (clear and nonviscous) and "nectar" (viscous and golden) samples. This result supports that visual sampling did not confound my past data. Finally, there was no difference in honey H_2O_2 based on sampling location on the marked frames.

Discussion:

This chapter analyzes the effect of supplemental feeding type on honey H_2O_2 concentrations. I found that *control* nectar foraging colonies had statistically *higher* honey H_2O_2 than *corn* syrup fed colonies and *lower* H_2O_2 than *cane* sucrose-fed colonies. These data did not conform to my predictions from my pilot data, suggesting that the cane-fed colonies would have less honey H_2O_2. However, the pilot study contained only a small sample size (n=6) compared to the follow-up study presented herein.

I considered whether differences were due to the unequal glucose content of the feeds tested. Quantitatively, there is greater glucose substrate in cane sugar water versus corn syrup (42% glucose) yet, this mechanism would not account for the entire 75% reduction of honey H_2O_2 seen in the corn-fed colonies. Another potential explanation for the reduced H_2O_2 content with corn syrup could be the presence of inhibitors of GO activity in this feed type. Along these lines, it is interesting to speculate that byproducts from the factory processing of corn syrup could be interfering with GO enzyme function. Indeed, the toxic impact of hydroxymethylfurfural (HMF) or mercury contamination in corn syrup has been described (Parker et al. 2010). Given that HMF causes gastrointestinal distress in the bee gut, it is conceivable that it might have similar detrimental effects on the hypopharyngeal GO mechanism. Also of possible relevance, mercury has been shown to inhibit GO activity directly (Wilson and

Turner 1992). Future studies could explore this *in vitro* by examining the effect of HFCS and its toxic byproducts on purified bee GO.

It is important to mention that the control colonies may have had reduced honey H_2O_2 because their floral source was 113 kilometers away from the experimental groups. The control colonies foraged within an area of sourwood tree bloom (*Oxydendrum arboreum*) which is known to have one of the lowest glucose contents of honey sources (White and Doner 1980). In addition, sourwood honey has been shown to have high free radical scavenging ability which maybe could dampen endogenous peroxide levels (Moniruzzaman et al. 2013). It would be intriguing to assay the H_2O_2 content of sourwood honey compared to other floral honeys to determine the extent to which low glucose substrate has an effect on H_2O_2 concentration.

The results presented in this chapter suggest that corn syrup feeding may have an unfavorable influence on colony health by lowering honey H_2O_2 concentrations. This deduction would be particularly warranted if there were further statistical strength from the work in chapter 1 indicating an inverse relationship between pest fitness and honey H_2O_2 (SHB experiments in chapter 1). Yet, honey H_2O_2 has indeed been shown to adversely effect microorganisms such as fungi and bacteria that could decrease colony health (Osato et al. 1999, Taormina et al. 2001b, Brudzynski 2006). It is possible that even a small, chronic deficit in the beneficial antibacterial function of honey H_2O_2 could have major implications for beekeeping management practices. This is especially relevant because commercial beekeepers primarily use corn syrup feeding given its convenience and cost-savings. This presumption is even more concerning because of increased pressure of horizontal disease movement given the cross country transportation of bee yards in order to meet pollination demands (Ellis 2012).

Chapter 4: Mechanistic drivers for the relationship between supplemental feeding and H_2O_2

Introduction:

In the previous chapter, I revealed that honey H_2O_2 levels are manipulated by supplemental feeding. As a potential social immune response, it is critical to determine the regulatory mechanisms that may produce variation in H_2O_2 from supplemental feeding. Beekeeping techniques can then be catered to enhance higher honey peroxide concentrations. Here, I explore three potential mechanisms 1) the effect of feed *concentration* on honey H_2O_2, 2) regulation of worker bee caste glucose oxidase activity, and 3) the effect of feed type on worker bee caste glucose oxidase activity.

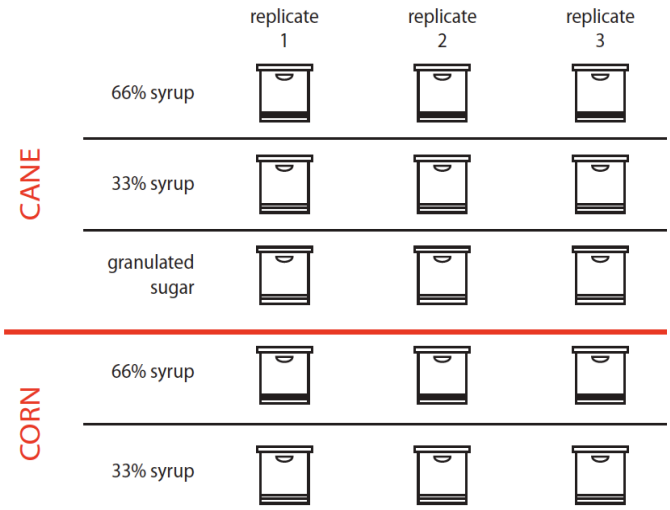
In the first aim, I examined the effect of two feeding concentrations (66% and 33% by weight of corn syrup and sucrose water) on honey hydrogen peroxide levels. I have suggested in the previous chapter that there could be an effect of the worker bee regurgitation process on H_2O_2 formation. Given that glucose oxidase (GO) is secreted from the hypopharyngeal gland near the mouthparts, it is conceivable that repeated passage of nectar over the mouthparts would increase GO, and H_2O_2, additions to honey (Nicolson and Human 2008). By contrast, more concentrated and viscous syrup might have less physical opportunity to interact with the mouthparts during processing. According to this "residence time" hypothesis, I predicted that worker bees processing more dilute concentrations would undergo more regurgitation and, hence, have higher H_2O_2 levels then if fed concentrated feeds.

The second focus of this chapter was to examine honey bee age-determined polytheism and biochemical development. Of particular interest, there is genetic evidence of undeveloped hypopharyngeal gland GO expression until the elder forager stage responsible for nectar collecting (Ohashi et al. 1999). If sugar syrup is processed primarily by within hive workers as

opposed to foragers, there may be insufficient GO addition. For this study, I measured GO activity in three worker bee castes. In addition to sampling foragers, the youngest caste of nurse bees (no GO expression) was collected as well as "receiver" bees (intermediate age and unmeasured GO expression by Ohashi et al.), which were taken directly from feeding source (Ohashi et al. 1999, Huang 2010). If there is an effect of age-related biochemical development, nurse and receivers might have lower GO activity than forager bees. Furthermore, I assessed the correlation between honey H_2O_2 and GO production to examine if other factors could be affecting this pathway.

Finally, I considered the effect of supplemental feeding type on the GO activity per bee caste. The purpose of this approach was to examine if disparate feeds (sucrose or corn syrup) could potentially alter GO activity of a given caste. I predicted that there should be no interaction unless the feed type could induce hypopharyngeal development or GO expression. Thus, these three study aims help shed light on the mechanistic background for the differences I have previously shown for honey H_2O_2 in fed managed bees.

Methods:



I explored the mechanistic regulation of honey H_2O_2 during supplemental feeding by investigating worker bee castes and supplemental feed concentration. At the University of Georgia bee lab (located at the UGA Durham Horticulture Farm, Watkinsville, GA) I fed fifteen colonies two concentrations (33% and 66% wt/wt) of 55% high fructose corn

syrup (corn) and sucrose (cane) as well as solid granulated sugar. Each of these groups was represented by three replicate colonies (see diagram). During sampling in May to July 2013, I fed each colony a 2-quart jar every other week and caloric content was equalized among all feeds. To test the first aim (effect of feed concentration on honey H_2O_2), samples of capped honey were collected from marked frames that were added at the beginning of the study. I measured honey H_2O_2 using a colorimetric assay (Enzo Life Sciences).

To explore the second aim (regulation of worker bee caste GO), I also measured hypopharyngeal glucose oxidase activity from several age-dependent worker honey bee castes. Three honey bees were collected from each colony every other week over a period of 41 days and frozen at -20°C until analysis. The oldest worker bee castes, forager bees, were sampled returning to the colony after collecting pollen and nectar. The youngest bees, or nurse bees, were collected from brood frames (Ohashi et al. 1999). Finally, receiver bees (age unknown or intermediate) were sampled directly while feeding on the supplemental food source. These receiver bees are likely analogous to "nectar receiver" bees, which are characterized by their role in the transfer, processing, and storage of nectar collected from returning forager bees (Anderson and Ratnieks 1999) (Huang 2010).

In order to measure glucose oxidase activity, I isolated extracts of the hypopharyngeal gland, which is located near the bee mandible. Bee heads were rinsed three times (to remove adherent sugar), homogenized with phosphate buffer, centrifuged (2 minutes), and the supernatant was used to measure glucose oxidase activity. The enzyme activity per bee head was measured using the Amplex Red Glucose/Glucose Oxidase Assay kit (Invitrogen Molecular Probes). I incubated the assay micro plates at room temperature for 30 minutes (with excess glucose substrate) in the dark after the reagent was added.

As the third focus of this study, I examined the relationship between worker bee caste GO activity and the experimental feeding groups (corn, cane, and granulated). The sampling and feeding methods are presented above.

Statistical analysis:

To test the statistical power of the hypopharyngeal glucose oxidase concentrations, I used a linear mixed effects model ("LMMs") with colony ID as the random effect. The "lme4" package (Bates et al. 2011) was utilized with R Statistical Programming Language (R Development Core Team 2012). The mixed effects model was the appropriate approach for this analysis because the individual bee samples collected from the same colony were not independent and can be accounted for at all data points in this model. I used qq-plots of my data to reveal that it was normally distributed and to determine that a power transform was unnecessary. The mixed effects model with the random effect of colony ID was used to analyze the fixed effects of worker caste, time, and feeding type (cane sugar or corn syrup). I also conducted basic statistical analysis to determine if there was a correlation between GO activity and honey H_2O_2 concentration per colony.

Results:

1) Supplemental feed concentration on honey H_2O_2:

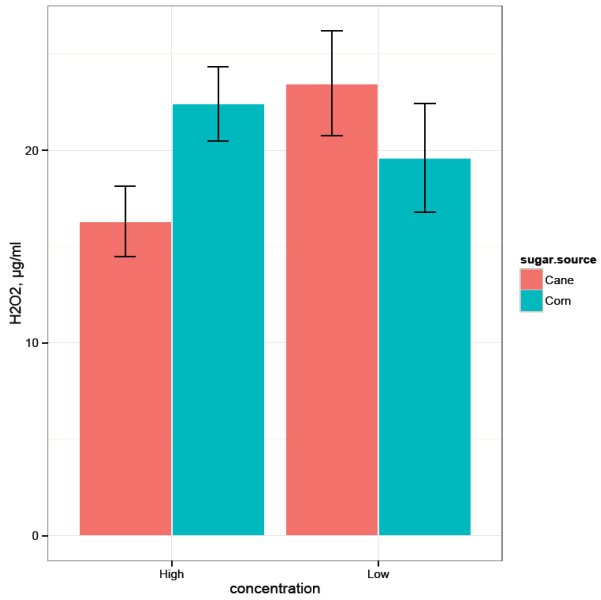


Figure 4: The Effect of Concentration in Cane and Corn Feeds on Honey H_2O_2: A total of twelve colonies at UGA were fed two different types of supplement feeds (corn syrup and sucrose water), each at two different concentrations (33% and 66% by wt.). There were no statistical differences between high vs. low or cane vs. corn. Results are expressed mean ± SEM.

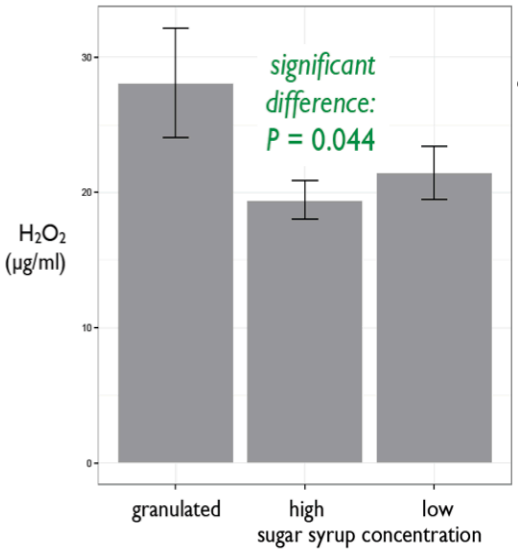


Figure 5: Aggregated Corn and Cane Syrup Vs. Granulated: This data set is from the same experimental group of Figure 4 but includes the granulated sucrose fed colonies. Data from the liquid feed types were pooled by concentration. Statistical differences were by ANOVA between high (n=33) and low (n=33) syrup vs. granulated (n=17) with p=0.044. Results are expressed mean ± SEM.

As illustrated in Figure 4, there was no statistical difference between high versus low or corn versus cane feeds. However, as shown in Figure 5, there was a significant difference (by ANOVA) between granulated (solid) versus both the high and low concentrations of corn and cane syrups ($p < .05$). While this was a non-ideal experiment lacking a nectar-fed control, I compared my current results to mixed nectar fed controls from previous studies completed in June of 2011 and 2012. The average concentration of H_2O_2 (µg/ml) from 2011 was 24.9±9.1 µg/ml H_2O_2 (n=19) and the average from 2012 was 21.8±12.9 (n=47). According to this assessment, the historical nectar average is similar to the high and low liquid feeds but, again, lower than granulated.

2) Honey bee worker castes:

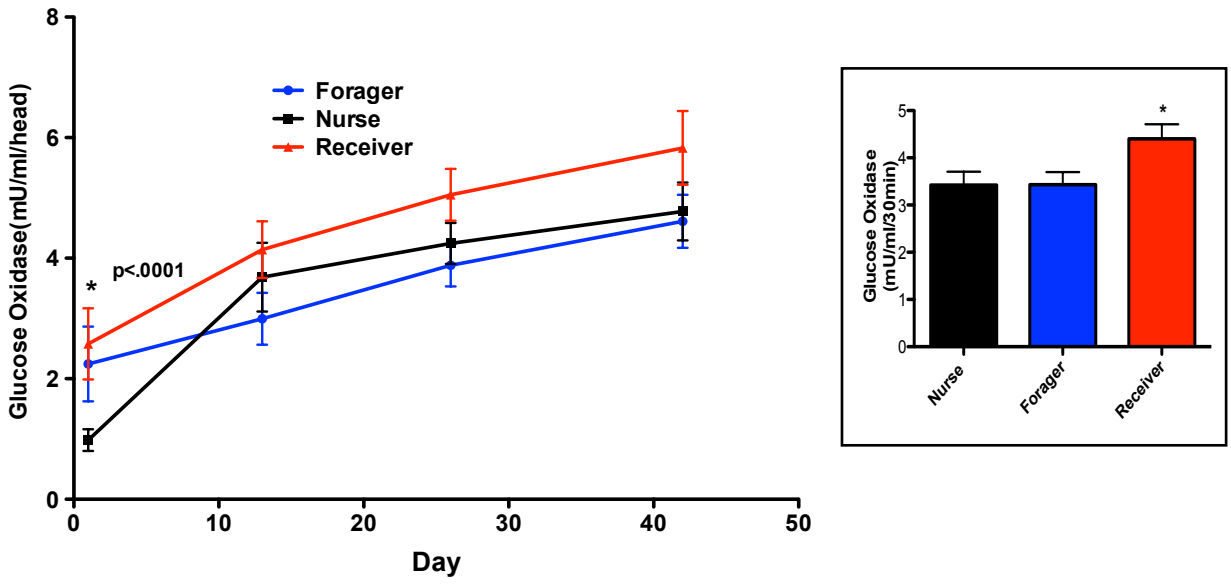


Figure 6: Glucose Oxidase Activity in Honey bee Castes Over Time (with INSERT GRAPH): Supplemental feeding began after day 0. Forager, nurse, and receiver bees were collected from the 15 colonies during the 41 day study period. Glucose oxidase was measured in bee heads. Results are given as mean ± SEM (n=15). Statistics were by unpaired t-test (★p<.0001, receiver versus nurse bee). The inserted graph shows average oxidase activity per bee caste at all time samplings. Results are given as mean± SEM (n=60 bees per group). The receiver group was statistically different by linear mixed effects-model analysis *p=.0055

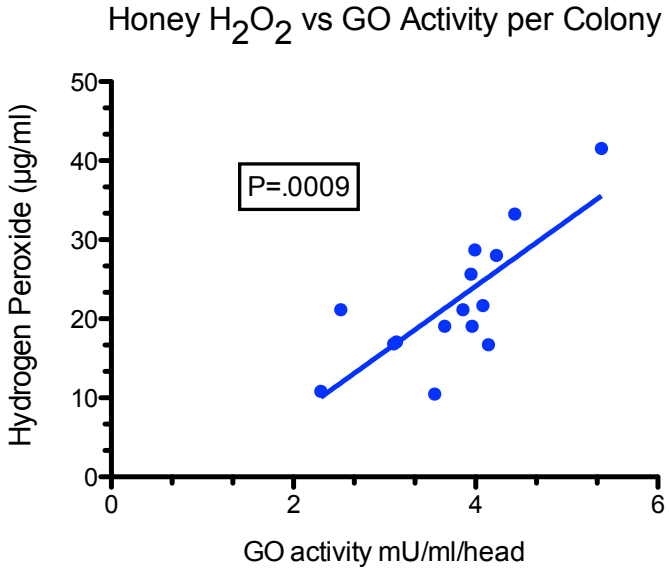


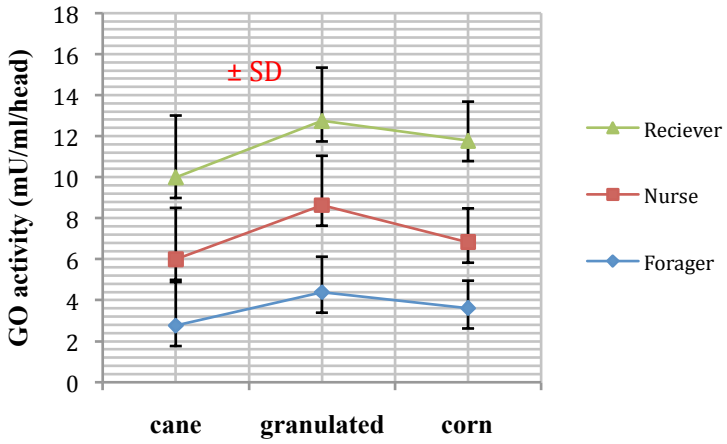
Figure 7: Honey H_2O_2 versus Glucose Oxidase Activity per Colony: The honey samples that were collected from the 15 colonies in Figure 6 were correlated with GO activity (see methods). For each colony, the GO measurement was calculated by averaging the GO activity from nurse, receiver, and forager bees (n= 12 bees per colony).The correlation of these data sets was statistically significant (p=.0009).

As demonstrated by Figure 6, there was a significant (p=.039) temporal increase in glucose oxidase activity in all bee castes over the 41 day study period. Moreover, at the study's commencement, prior to supplemental feeding, I also found a significant difference between receiver and nurse bees (p<.0001). This was a particularly intriguing result because nurse bees reportedly have negligible GO activity, yet I found that feeding appears to have induced hypopharyngeal gland development after only 10 days. This trend continued throughout the study (Ohashi et al. 1999). Furthermore, the insert graph of Figure 6 displays the average GO activity of each worker bee caste from all colonies represented in the study. Receiver bees had statistically higher GO activity than nurse bees by linear mixed-effects analysis (p=.0055). However, there was no difference in GO between forager and receiver bees.

Figure 7 illustrates the strong correlation (p=.0009) between honey H_2O_2 (n=6 honey samples per colony) and average glucose oxidase activity (n=12 bees per colony) in 15 study colonies. Therefore, I can conclude that an increase in honey bee hypopharyngeal gland GO activity can be equated to an increase in honey H_2O_2.

3) Feed type on worker bee caste glucose oxidase activity:

Figure 8: Effect of Feed Composition on Glucose Oxidase Activity in 3 Bee Castes



In Figure 8, I analyzed my formerly discussed data from the 15 supplementally fed colonies to determine if there was an effect of feed composition on worker caste GO expression or excretion (n=20 bees per caste per group). The data show no interaction between feed type and GO activity between the castes. This finding was consistent with my prediction, which suggested that feed type would not induce or reduce hypopharyngeal gland production of GO differently among the castes.

Discussion:

The focus of this study was to access three mechanistic explanations for the variation of honey H_2O_2 acknowledged in chapter 3 in colonies managed with supplemental carbohydrate feeds. The three mechanisms investigated were the effect of feed *concentration* on honey H_2O_2, GO activity in age-determined worker bee castes, and the interaction of feed composition on GO activity of these castes. Furthermore, I wanted to confirm that there was a correlation between measured honey H_2O_2 and GO activity.

Regarding the first mechanism, I found no statistical effect of feed concentration but there appeared to be opposite trends between high and low concentrations of corn and cane liquid feeds (Figure 4). The dilute concentration of cane sugar water had slightly more honey H_2O_2 than the more concentrated group, which could comply with my "residence time" theory given the possibility of increased duration of mouth interaction to achieve dehydration. The reverse trend for high vs. low in corn-fed colonies is less evident and is currently unexplained. Likewise, qualitative field observations denote that many corn-fed samples were "clear" and appeared "watery" which may suggest that worker bees were not effectively devoting time to dehydrate this honey. By contrast, the majority of the samples from the granulated (cane) fed colonies were described as "golden" and "viscous." The thicknesses of these honey samples and

the solid property of granulated sugar could suggest a rehydration might be involved in order to produce honey. This rehydrating process could require even more time and hypopharyngeal contact than dehydration of liquid feeds allowing for increased GO discharge throughout the procedure (Figure 5).

Secondly, I analyzed the glucose oxidase activity from three worker bee castes as a mechanism for altered honey H_2O_2 (Figure 6). Specifically, I was interested in a study by Ohashi et al. 1999 that revealed age-determined GO expression among worker bee castes. Contrary to these findings, I actually showed elevated GO activity in nurse bees comparable to that of foragers. Likewise, receiver bees have significantly the highest enzyme activity and they are in direct contact with the supplemental source. This response suggests an inductive effect of feeding on GO activity and, possibly hypopharyngeal gland maturation by nurse bees causing them to initiate enzyme expression. Moreover, initial data taken prior to feeding revealed that nurse bees had significantly lower GO activity compared to receivers, further supporting an induced response. With regard to honey processing, others have identified receiver bees to be the sole processors of nectar because they obtain it from returning foragers (Anderson and Ratnieks 1999). Accordingly, it makes intuitive sense for my sampled receivers to have increased GO activity; especially given that they were sampled while directly feeding from supplemental jars.

As a sub-analysis of worker caste GO activity, I also examined the interaction between enzyme and product. Indeed, I found a significant correlation between measured GO activity and honey H_2O_2 per colony. This result was expected given the understanding of the GO enzymatic reaction to produce H_2O_2 in honey. This finding supports either measurement as an interchangeable investigative tool in honey bee research. However, it is important to mention

that this result could be colony specific and may not be an accurate response variable across studies given genetic, seasonal, and floral variations.

Finally, the third potential mechanism that was proposed found no interaction between feed composition type and GO activity among the different worker bee castes. Thus, managing bees with different carbohydrate compositions does not effect hypopharyngal gland GO production individually between the castes. However, while feed type may not effect enzyme secretion, it may have biochemical implications on GO's production of H_2O_2 once added to the honey.

In retrospect, there were some unanticipated design flaws in this study but two shortcomings are worth discussion. First, the timeline of this experiment occurred in May-July 2012, which is the peak "nectar flow" season for the UGA vicinity. During these several weeks, honey bees are extremely active collecting nectar, attending to brood, and building wax comb to increase colony size (Winston 1991). This high-energy expenditure by worker bees likely results in the immediate consumption of supplemental feed rather than storing it in honey frames (Huang 2010). Secondly, I fed these highly active colonies one two-quart jar of feed every other week. This amount may not have been enough to promote honey storage after consumption needs were fulfilled. Moreover, field notes often noted empty feeding jars in between the feeding schedule indicating rapid feed consumption and intermittent nectar dependence. Hence, a sub optimal feeding schedule occurring during a peak nectar flow may have altered the exclusive impact of the experimental feeds in this study.

I would also like to address the discrepancy between my supplemental study results in chapter 3 and 4. It is important to note that despite the chapter sequencing, the study from chapter 3 (Sept 2013) was a follow-up experiment to adjust for the feeding frequency and honey

flow shortcomings of the study completed in May-July 2012 (chapter 4). Given that the feeding study presented in this occurred during a nectar flow, and the knowledge that nectar collection is not influenced by the amount of internal honey stores (Fewell and Winston 1996), there was an increased probability of sampling naturally nectar-foraged honeys. In other words, the external floral factors regulating nectar collection and storage were much more prominent in the May-July 2012 study than the September 2013 study. This allowed for a much larger sampling pool of natural nectar honeys over experimental feed honey in the summer study. The September 2013 study (chapter 3) was improved by increasing feeding frequency to biweekly. In addition, fall is a seasonal period of reduced hive activity, which would increase the likelihood that supplemental feed is stored rather than consumed. Thus, the effect of supplemental feeding on honey H_2O_2 was more accurately accessed in September 2013 (chapter 3) than May-July 2012 study (chapter 4).

Another incongruity between the two feeding studies is the lower honey H_2O_2 content in the corn-fed colonies in Fig. 3, chapter 3 compared to Fig. 4, chapter 4. This difference cannot be attributed to GO activities based on its lack of regulation relative to feed type shown in Figure 8, chapter 4. While I have shown a correlation between honey H_2O_2 and GO activity in Figure 7, these results may be colony-specific and thus I cannot assume that the colonies from the September 2013 study were identical in their GO production/expression to those in Chapter 4 (May-July 2012). Yet, the 75% reduction in corn syrup fed honey H_2O_2 observed between 2012 and 2013, may have additional etiologies. It is possible that by quadrupling the feeding frequency for the September 2013 study previously unrecognized inhibitory factors were magnified (see discussion on HFCS byproducts in chapter 3). As a final note, the lower control group H_2O_2 levels noted in 2013 (Fig. 3) compared Fig. 4 and historical controls (see discussion of Fig 4) might be attributed to the sourwood nectar flow (lower glucose content) where these

colonies were located (White and Doner 1980).The GO enzyme would therefore have less glucose substrate to produce H_2O_2. Undeniably, further studies are warranted to clarify whether GO has intrinsic colony regulation that is seasonal or possibly related to colony age or founder queen type (genetics).

All in all, more work is required to fully understand the mechanisms underlying how supplemental feeding alters honey H_2O_2. Future studies will need to account for the influences of nectar sources in addition to seasonal issues. It would be intriguing to test whether purified honey bee glucose oxidase enzyme activity is directly altered by supplemental feed types independent of their glucose content. Simple laboratory bee studies could also be designed to test whether individual honey bee GO is induced or inhibited by selected experimental feeds. Understanding these mechanisms could contribute to the improvement of beekeeping management strategies to enhance honey H_2O_2 and its potential as a regulated social immune response. Conversely, one might avoid basic husbandry methods that diminish honeybee GO if this is shown to be detrimental to colony health. On the other hand, targeted breeding or inducers of GO, especially in forager and receiver bees who process honey, might be beneficial.

Chapter 5: Small hive beetle experimental infection

Introduction:

In order to determine whether honey H_2O_2 functions as a social immune defense, an experimental infection with small hive beetle was imperative. Two infection studies were conducted; 1) one with mostly natural nectar feeding, and, 2) another with supplemental feeding as a covariate. After each infection, I measured honey hydrogen peroxide levels. If honey H_2O_2 is a social defense response by bees then under pathogen stress, bees may up-regulate the production of the chemical to retaliate. Moreover, I have already shown that increased H_2O_2 is detrimental to small hive beetle fitness while removal of H_2O_2 (with catalase) promotes survival (chapter 1). For the first study, I expected to find higher levels of H_2O_2 in the SHB infested colonies. The second study aimed to sort out how supplemental feeding might independently effect the outcome.

Methods: (5A) *Preliminary SHB infection study 2011*

I prepared 10 nucleus colonies (5 frame colonies with mated queens) at the University of Georgia Bee Lab in June 2011. Five colonies were infected with adult and larval small hive beetles on two occasions. The SHB were lab reared according the procedure by Murrel and Neumann 2004. The first infestation was on day one with 100 adult SHB and 50mL of larvae per colony. The second treatment was on day fifteen with 200 adult SHB and 50mL larvae per colony. Pollen patties mixed with Brood Builder (protein supplement) were added the second week to promote beetle growth within the colonies. The five control colonies received no treatment and were located ten miles away to prevent cross-infestation. Once a week, I collected honey samples from brood and honey frames from each of the 10 colonies. Samples were stored

at -20ºC and honey hydrogen peroxide concentrations were measured with a colorimetric assay (Enzo Life Sciences).

Methods: (5B) *Secondary SHB infection study 2012 (supplemental feeding covariate)*

A second small hive beetle infection study was conducted in June 2012 at UGA except with supplemental feeding as a co-variable. 12 colonies were prepared with a brood box and medium deep box (20 frames total); 6 colonies were infected with SHB and 6 were uninfected controls. I fed cane sugar water (1:1 by wt. standard) to 3 colonies from each of these two groups. Each fed colony received one 2-quart jar of sugar water every other week for 32 days.

New SHB infection approaches were attempted given results from previous infection in 2011. Instead of only adding SHB individuals, I promoted an infection environment within the colonies. This method included reducing bee space between frames to hinder worker bee ability to clear frames of SHB eggs and larvae. I also added intraframe beetle traps (1 per colony) filled with Brood Builder (protein supplement) and pollen patties to promote beetle reproduction and hiding. By contrast, in the control colonies, I added these beetle traps, but filled them with the appropriate apple vinegar and oil treatment to eliminate endogenous beetles. To begin colony infection, I created an incubation container with hung honey frames and allowed adult SHB to infest these frames with larvae and eggs. I graciously received 500 lab-reared adult SHB from Dr. William Meikle from the Carl Hayden Bee Research Center in Arizona. A week after adding SHB to the rearing container, one of these infected frames was added to each of the 6 infected colonies. In order to accurately sample newly added honey, I also added a marked empty frame to both control and SHB colonies. Honey samples were collected every other week from the marked frame and stored at -20ºC until measured for honey H_2O_2 using a colorimetric assay (Enzo Life Sciences). Upon analyzing the results of this study, I conducted a power analysis to

determine the necessary sample size in order to produce significant results. The power analysis was calculated using R Statistical Programming Language (R Development Core Team 2012).

Results:
(5A) *SHB infection study 2011:*

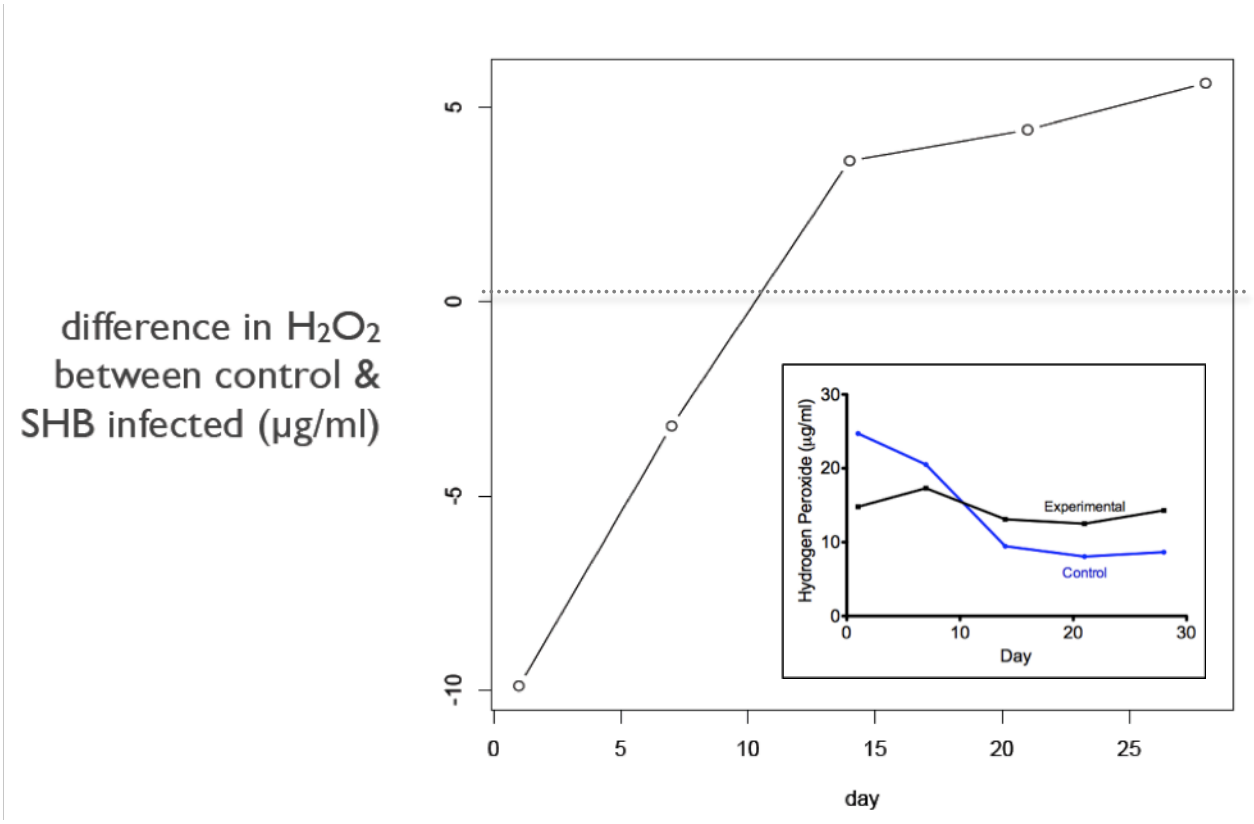


Figure 9: Differences Between SHB Infested Colonies vs. Control Colonies (same data presented two ways): 10 nucleus colonies at UGA were prepared; 5 served as a control group and 5 were infected with SHB adults and larve on day 1. Honey samples were collected weekly for measurement of H_2O_2. The main graph shows the difference in H_2O_2 in the experimentally infected versus the control colonies. *Insert graph*: honey H_2O_2 over time in SHB infected (**black**) vs. control (**blue**)

Figure 9 shows the difference of honey H_2O_2 over 28 days in control versus SHB infected nucs. The main graph shows that there was a positive difference in the infected colonies after day 10 relative to the control group. The figure insert shows the weekly trends of honey H_2O_2. Linear model analysis of the slopes of this insert graph revealed that the confidence intervals do not overlap (control, -0.065±0.35; experimental, -0.087±0.17). Thus, there is a statistically significant difference between control vs. infected responses of H_2O_2 over time. At onset, the

control group H_2O_2 concentration was 10µg/ml higher than the infected group, which may be explained by the arbitrary selection of stored honey frames in the nucs set up by the UGA lab. Given this, and considering the overall trends, the control H_2O_2 level declined over time to a minimum plateau of 10µg/ml /ml by study completion. By contrast, the experimental group maintained its honey H_2O_2 level throughout the study such that there was a maximum difference of about 7µg/ml honey H_2O_2 over control.

(5B) *SHB infection study 2012 (supplemental feeding covariate):*

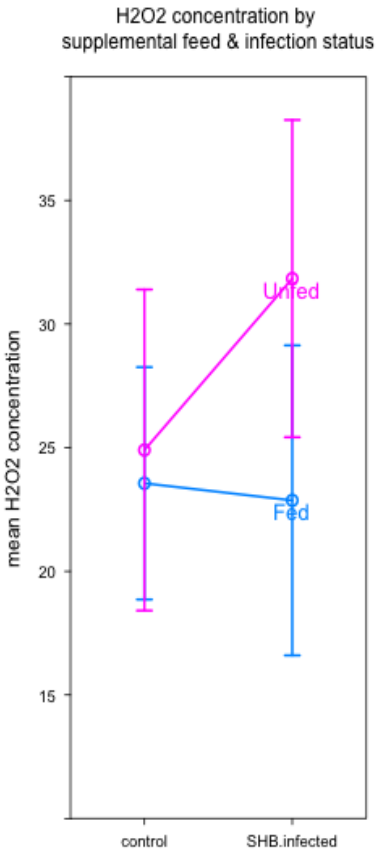


Figure 10: H_2O_2 Concentration (µg/ml) by Supplemental Feed and Infection Status 12 colonies at UGA were prepared; 6 served as a control group and 6 were infected with SHB adults and larvae infested frames on day 1. In each group, 3 of the colonies were fed sucrose water every other week. Honey samples were collected every other week for measurement of H_2O_2. Here, the honey H_2O_2 is averaged by SHB treatment and feed (n≈20 samples per group). Results are expressed with 95% CI.

In 2012, a second SHB infection study was conducted except with the addition of sucrose water fed colonies. Figure 10 plots the average honey H_2O_2 collected throughout the 32 day study for the four groups. While there was no significant between groups, there was a promising trend of honey H_2O_2 increasing in nectar foraging colonies between control and infection. This trend was not apparent in the fed colonies, which seemed to maintain steady H_2O_2 concentrations despite infection compared to control. Given these trends, I conducted a power analysis to determine the ideal number of colony replicates to produce statistically significant divergence. The power analysis revealed that 60 replicates (15 per control/infected and fed/unfed/ combinations) are required.

Discussion:

I conducted two separate small hive beetle infection studies in two consecutive years and measured honey H_2O_2. Both of these field studies were especially challenging given that there have been no documented methods on how to properly inoculate honey bee colonies with SHB. However, despite this obstacle, I was still able to demonstrate some promising trends in regards to honey H_2O_2 as potential social immune response.

In the first infection study in 2011, I found that there was an increase in honey H_2O_2 in relation to control colonies even though over time the experimental group seemed to maintain its honey H_2O_2 concentration. Because these were newly packaged nucleus colonies (5 frame nucs), they were fed sucrose water per UGA protocol. At this early experimental point, I was unaware of any confounding effect of supplemental feeding on honey H_2O_2 because that observation was not identified until the following year. Given this, it is possible that sucrose feeding altered the initial downward trend in honey H_2O_2 in both control and infected. Once stabilized, after day 10, the infected colonies maintained higher H_2O_2 levels than control. Moreover, it is also important to note that the nucs were inoculated with SHB day 1, and I expected that the impact of infection would take time to manifest. Therefore, this data was consistent with the prediction that honey bees may increase honey H_2O_2 as a social defense mechanism against small hive beetle. However, due to potential manipulation by supplemental feeding, I repeated the experiment the following year with improved SHB infection strategies and with the addition of supplemental feeding as a covariate.

Also in 2011, in an attempt to examine the effect of native SHB infection that year, separate experiments quantified the SHB load in stable colonies with Neumann diagnosis traps (Neumann and Hoffmann 2007). These studies revealed a non-significant linear relationship

between trapped number of adult SHB and honey H_2O_2 levels, yet results suggested slight positive trends (data not included). It should be mentioned that some investigators question whether bottom board Neumann traps accurately measure total colony SHB load. In the future, it would be intriguing to examine the honey H_2O_2 concentration of visually severe SHB colony infection (spontaneous occurrences) versus healthy co-localized colonies.

The second infection study in 2012 resulted in promising, although not significant, trends showing an increase in honey H_2O_2 in infected versus control colonies. Conversely, this increase was dampened between control and infected during supplemental feeding. This study provided a new set of frustrating obstacles that could have possibly contributed to the reduction of statistical power. Firstly, the supplementally managed cohort was only fed once every other week during a nectar flow (May-July), which may have reduced the effect of feeding (this pitfall is explained in chapter 4). Secondly, while this SHB infection was much more successful than first study in 2011, UGA was concerned with colony absconding and recommended beetle death traps to slow seemingly rapid progression of infection. Regrettably, this intervention was so effective that infection rate stalled. This may have attenuated the magnitude of the final results.

In conclusion, it is clear that another infection study is warranted. Therefore, I conducted a power analysis and determined that 60 colony replicates are needed to show statistical divergence given the data set. Unfortunately, however, when I contacted UGA about conducting a third study in 2013, they did not have the resources, given other studies underway at the time. To sum, both studies show a trend of a honey H_2O_2 difference during SHB infection stress. Particularly, there is an increasing trend during infection in the second SHB study (Figure 10). This observation is consistent with the proposed social immune defense response in honey bees.

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