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Lucy Anderson April 4, 2013

Distinguishing feral and managed honey bees (*Apis mellifera*) using stable carbon isotopes

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Abstract
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The honey bee (*Apis mellifera*) is integral to the human agricultural enterprise and is currently facing a variety of threats to its health, including disease. Efforts to protect the honey bee against the *Varroa* mite and other diseases are hindered by the inability to effectively distinguish feral from managed honey bees. Feral honey bees have experienced intense natural selection for resistance to colony pests and parasites and may provide a strong source of resistance genes. Genetic testing is typically unreliable given often-extensive interbreeding between feral and managed colonies. I evaluated a diagnostic test based on stable carbon isotopic ratios that holds promise for identifying feral versus managed honey bees. Carbon isotopes have long been used to determine honey adulteration with added sugars, and this method works on the same principle, based on the fact that managed colonies are nearly always fed some supplemental sugar. I set up four types of experimental colony types: feral, managed with no supplemental feed, managed with supplemental feed, and managed with ^{13}C labeled glucose added to supplemental feed. I analyzed carbon isotopic ratios in the tissues of individual bees using Isotope Ratio Mass Spectrometry. There was a significant difference between the isotopic signatures of colonies receiving supplemental feed and unfed feral colonies, demonstrating that there is a detectable fractionation of carbon isotopes in honey bee development. However, this difference only persisted for a few weeks after supplemental feeding was removed, suggesting that stable carbon isotopic ratios cannot be used to differentiate feral and managed bees on all timescales in all situations. This study highlights the potential for future ecological studies with a greater understanding of temporal turnover of carbon isotopic signals.

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Introduction

Honey bees are the primary pollinators of agricultural crops, pollinating crops that contribute to over one-third of the calories humans consume (Morse & Calderone 2000; Klein et al. 2007). Recently, much evidence has been found suggesting honey bee declines (e.g. Oldroyd 2007; Potts et al. 2010). Severe losses of honey bees pose a major threat to the American agricultural industry and food security worldwide. Honey bees face a range of threats including pesticides, a decline in forage pasture area due to urbanization, and pathogens, but disease is the most detrimental threat to honey bee health and survival. Specifically, the Varroa mite (*Varroa destructor*) is thought to be the single largest cause of overall honey bee colony death (Rosenkranz et al. 2010; Martin et al. 2012). Efforts to strengthen the honey bee against the *Varroa* mite and other diseases as well as develop more efficient pesticides have been hindered by the inability to effectively distinguish unmanaged or "feral" honey bees from managed honey bees (Oxley et al. 2010; Spivak et al. 2011).

Feral bees, unmanaged by people, have potentially experienced intense natural selection for resistance to colony pests and parasites, and may provide a strong source of resistance genes to honey bee breeders and scientists (e.g. Seeley 2007). Developing a consistent and inexpensive method for distinguishing the two types of honey bees could lead to improvement in resistance breeding programs as well as the ability to attain important information for ecological studies (e.g., if honey bees sampled in the course of diversity or pollination studies are feral or managed). Little is known about the relative population sizes of feral honey bees and managed honey bees or their respective habitat suitability and preference. Currently, the only method for identification of feral versus

managed honey bees is genetic testing (Delaney 2012), but this technique is unreliable in many or even most contexts given the often-extensive interbreeding between feral and managed colonies and is very expensive. Here, I propose and test an Isotope Ratio Mass Spectrometry (IRMS) diagnostic test to differentiate between feral and managed honey bees.

IRMS is used to detect the ratios of stable isotopes in a sample. Stable isotopes occur naturally in the environment, are not radioactive, and pose no direct threat to human health. Rarer isotopes chemically react in very similar ways to the most abundant isotope of the specific element. Isotopes of an element have the same atomic number but a different number of neutrons, which causes isotopes to have different atomic masses.

Stable isotopes have been used widely in ecology and nutrient cycling studies, including among others looking at food webs, nutrient exchange, and migration patterns (Hobson 1999; Kelly 2000; Kennedy et al. 1997; O'Brien & Martinez 2000; Ostrom et al. 1997; Phillips 2001). One early use of stable isotopes was to detect the addition of supplemental sugars (cane sugar and/or corn syrup) to honey, using carbon isotopic signatures (Doner & White 2007; Elflein & Raezke 2008). In addition, it has been shown that Royal Jelly, a secretion of protein, sugars, and amino acids fed to honey bee larvae, produced by honey bees with supplemental feed can be distinguished from Royal Jelly produced by honey bees not receiving this feed (Daniele et al. 2011).

Stable carbon isotopes include ^{13}C and ^{12}C, and different ratios of ^{13}C to ^{12}C are produced in different plants as they undergo photosynthesis. The relative ratio of ^{13}C to ^{12}C can be detected using IRMS. The standard for expressing the ratios is a $\delta^{13}C$ value signifying parts per thousand (‰). These ratios are reported relative to the ratio in Pee

Dee Belemnite (PDB), a cretaceous fossil from North Carolina, which has been chosen as the arbitrary standard, i.e. PDB is defined to have a $\delta^{13}C$ value of zero (Craig 1957). As it contains a relatively high concentration of ^{13}C, most compounds found in nature have a negative $\delta^{13}C$ signature in comparison.

In particular, stable isotopes of carbon have been heavily used to distinguish between plants with different photosynthesis pathways. Photosynthesizing plants have developed three distinct metabolic pathways of carbon fixation: C3, C4, and CAM. The C4 carbon fixation process produces higher ratios of ^{13}C to ^{12}C in the plant relative to C3 fixation, yielding a distinct isotopic $\delta^{13}C$ signature in IRMS (Farquhar et al. 1989). In the United States, honey bees typically forage on nectar-producing wildflowers and broad-leaved plants, both of which undergo C3 carbon fixation. Many monocots, including the grass family (*Poaceae*)—of which corn, sugarcane, and wheat are members—undergo C4 carbon fixation (Koziet et al. 1993). As beekeepers supplement their colonies with sugar solutions generally made from corn syrup or sugarcane, both of which are C4 grass species, these solutions tend to be higher in ^{13}C. The standard $\delta^{13}C$ range for plants undergoing C3 photosynthesis is between -33 and -24 ‰, and is between-16 and -10 ‰ for C4 photosynthesizing plants (O'Leary 1988). One study found that cane sugar in particular has a $\delta^{13}C$ value of -11.2 ± 0.23 ‰ (Koziet et al. 1993).

Because of the well-documented ability of carbon isotopes to detect adulteration of honey and royal jelly with supplemental sugars, I predicted that carbon isotopes will also allow for distinguishing feral from managed honey bees, given that most managed bees are given supplemental C4 sugars. Specifically, in this study I had two distinct objectives. Objective 1: Determine if there is a difference in isotopic signatures between honey bee

tissue from managed and feral bees. In order for this test to be completely reliable in all situations, there would be no overlap between signatures of bees from managed colonies and signatures of bees from feral colonies. Ideally, the heaviest signature from any feral bee will still be lighter (i.e. more negative) than the lightest signature from a managed bee. Objective 2: Determine how long a distinct signal will persist between the two types of bees after supplemental feeding is removed. Understanding the temporal trends in signal persistence is key, because supplemental feeding typically occurs periodically rather than year-round. Though little is known about the temporal turnover of carbon in bee tissues, I hypothesized that distinct ^{13}C signals will persist in bees from colonies receiving supplemental feed for several months, assuming that ^{13}C from supplemental feed is incorporated into the durable chitin in the honey bee exoskeleton.

Methods

Study Site

This study took place in Winter 2011 through Fall 2012 in Athens, Georgia, in and around the University of Georgia Bee Lab (located at the UGA Durham Horticulture Farm, Watkinsville, GA). I utilized nine pre-existing honey bee colonies from the UGA bee lab stocks, each composed of a brood box and a shallow honey super (except for one of the nine colonies, FM 2, due to limited resources). Three each were randomly designated fed labeled (FL), fed managed (FM), and unfed managed (UM).

Additionally, 17 feral colonies were collected in swarm traps in the Chattahoochee-Oconee National Forest from December 2011 through May 2012. These colonies were

larger and were sampled at various times and intervals throughout this time. These were designated unfed feral (UF) colonies. Each of the 26 colonies were given a unique colony ID.

There were two colony types that received supplemental feed (FM and FL) and two colony types that did not (UF and UM). I assumed that Unfed Feral colonies were unfed, as they were captured from swarms. Unfed Managed colonies were not given any supplemental feed and were assumed to only forage on flowers. This was to simulate the fact that supplemental feeding is often irregular and seasonal, and thus some managed colonies can go sometimes months at a time without supplemental feeding. Fed Managed and Fed Labeled colonies were given supplemental feed once per week for three weeks and were assumed to forage on flowers as well as the sugar-water solutions provided. Fed Labeled colonies were fed a solution that included "labeled" glucose, which was enriched with ^{13}C, to allow me to track the turnover of carbon in honey bee workers over time.

Colonies were kept in different apiaries managed by the UGA Bee Lab, separated by type, and apiaries were located at least 2 miles away from each other. Separate apiaries were used to reduce the possibility of honey or sugar solution robbing and therefore unknown contamination of samples between the types of colonies.

Supplemental Feeding

Supplemental feed for Fed Managed (FM) colonies was prepared by mixing 1840.36 g of pure sugarcane sucrose with 1840.36 mL of deionized-distilled water, creating 3 L of 50:50 water sucrose solution by weight, with molarity 2.92144 M. Supplemental feed for Fed Labeled (FL) colonies was prepared by mixing 1.0 g of 99 atom % ^{13}C-glucose (Omicron Biochemicals, Inc.) with 1838.53 g pure sugarcane sucrose and 1840.36 mL of

deionized-distilled water, creating 3L of solution with the same molarity as the Fed Managed colonies, and nearly a 50:50 water sugar solution by weight. This solution had 0.54 mg of 99 atom % ^{13}C-glucose per gram sucrose. Mixtures were homogenized in 3L Erlenmeyer flasks with a magnetic stirrer bar and a hotplate-stirrer and refrigerated until use.

In mid June of 2012, one 3L batch of supplemental feed was mixed for the Fed Manged (FM) colonies and one 3L batch of supplemental feed was mixed for the Fed Labeled (FL) colonies. One liter of the appropriate solution was put into a sterile glass jar with a metal lid containing several small (~3mm diameter) punched holes, and placed upside down on the top of each of the six colonies as a top-feeder. Feed was replaced twice following the initial feeding, once per week. Three weeks after the initial feeding, all feeding was removed from the six supplemental-feeding colonies.

Sampling

Honey bee individuals were collected from each of the nine colonies starting at the time of the first supplemental feed. For the first three weeks, individuals from each colony were collected. Individuals from the three FL colonies continued to be collected weekly for 12 more weeks, for a total of 15 weeks, in order to follow comprehensive tracking of the fractionation of the ^{13}C in the honey bee tissue. In the other three colony types, UF, UM, and FM, samples were collected biweekly after the first three weeks, for a total of nine sampling dates over 15 weeks from June-September 2012.

Ten individuals were collected from each of the colonies by scraping bees from a frame into a labeled Ball jar filled ¼ of the way with 70% ethanol. Each colony was

collected into a different Ball jar to prevent contamination. Once taken back to the lab, bees were placed into small vials filled with 90% ethanol until they could be pinned and labeled. Bees were pinned on separate clean paper towels to prevent cross-contamination.

Pinned bees were dried at 50°C for 3 hours. One hind leg was removed, put into a tin envelope, and weighed using a microbalance measuring to the accuracy of .01 mg. Bees without wing damage or pollen present on their hind leg were chosen for analysis. Weights ranged from .50 mg to 1.05 mg. To avoid cross-contamination, tools and workspace were thoroughly cleaned with ethyl alcohol between samples.

Table 1. Quantities of managed bees per colony selected for analysis. Each "Sample Date" corresponds to a unique date where each Sample Date is one week apart. Sample Dates run from 21 June 2012 (Sample Date 1) through 27 September 2012 (Sample Date 15).

			Sample Date														
			1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Colony Type	UM	**1**	2	2	2		2		2		2		2		2		2
		2	2	2	2		2		2		2		2		2		2
		3	1	1	1		1		1		1		1		1		2
	FM	**1**	2	2	2		2		2		2		2		2		2
		2	2	2	2		2		2		2		2		2		2
		3	1	1	1		1		1		1		1		1		1
	FL	**1**	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
		2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Table 2. Quantities of feral bees per colony selected for analysis.

Colony	Number of Sample dates	Number of individuals per sample date
1.2	1	3
1.3	1	3
1.4	1	3
1.7	1	3
1.8	3	5/4*
2.1	2	3
2.2	1	3
2.3	1	3
2.4	2	5
2.5	2	5
2.11	1	3
3.1	1	3
3.3	1	3
3.5	3	5
3.6	1	3
3.9	1	3
3.11	1	3

* One of the three sample dates had only four samples due to mass restrictions; the remainder had five samples.

Once weighed, samples were placed in the tin envelopes, crimped, and then placed into a microwell plate. Samples were sent to the Boston University Stable Isotope Facility (Department of Biology, 5 Cummington Street, Boston, MA) and analyzed using an elemental analyzer interfaced to a GV Instruments Isoprime isotope ratio mass spectrometer (GV Instruments Ltd, Manchester, UK). Mass corrections were made to ensure mass did not bear an effect on the value of the isotopic signature.

Sample Analysis

Ratio measurements of naturally abundant isotopes of an element most often compare the less abundant to the more abundant isotope. For carbon, ^{13}C is the less abundant isotope compared to ^{12}C. This ratio is usually referenced against an international standard and the result, symbolized as lowercase delta (δ) is measured in parts per thousand ‰ relative to the international standard. The formula for $\delta^{13}C$ is as follows:

$$\delta^{13}C = ((R_{sample} / R_{standard}) - 1)*1000$$

where R_{sample} is the ratio of $^{13}C/^{12}C$ of the sample and $R_{standard}$ is the ratio of $^{13}C/^{12}C$ of the international reference standard, Pee Dee Belemnite (PDB).

To test for statistical differences in $\delta^{13}C$ values between different management (feral vs. managed) and feeding groups, I used linear mixed-effects models ("LMMs") with colony ID as a random effect, with the "lme4" package in R. I took this approach because different bee individuals from the same colony cannot be considered independent samples, and mixed-effects models allow for the use of all data points while taking into account the non-independence of colony groups (e.g. Bolker et al. 2009). I used Gaussian errors because the response variable ($\delta^{13}C$) is continuous and errors were approximately normal. To test for the significance of colony type (UF vs. UM; UF vs. FM; UF vs. UM+UF), for each test I compared a model including colony type (as the only fixed effect) to a null model that included only the random effect (colony ID), with ANOVA in a model-comparison context (e.g. Bolker et al. 2009), using the "anova" command in R.

Results

I analyzed the carbon isotopic signature of 257 honey bee individuals. Of these, 91 individuals were from UF colonies (*N* = 17 colonies), 45 were from FM colonies (*N* = 3 colonies), 46 were from UM colonies (*N* = 3 colonies) and 75 were from FL colonies (*N* = 3 colonies). Distributions of isotopic signatures within each colony type are shown in Figure 1. The $\delta^{13}C$ values of the samples ranged from -26.76 ‰ to -13.80 ‰. Values on both ends of the spectrum reside within the standard ranges for C3 and C4 plants, but not within the average range of isotopic signatures for pure cane sugar (-11.65‰ to -10.75‰).

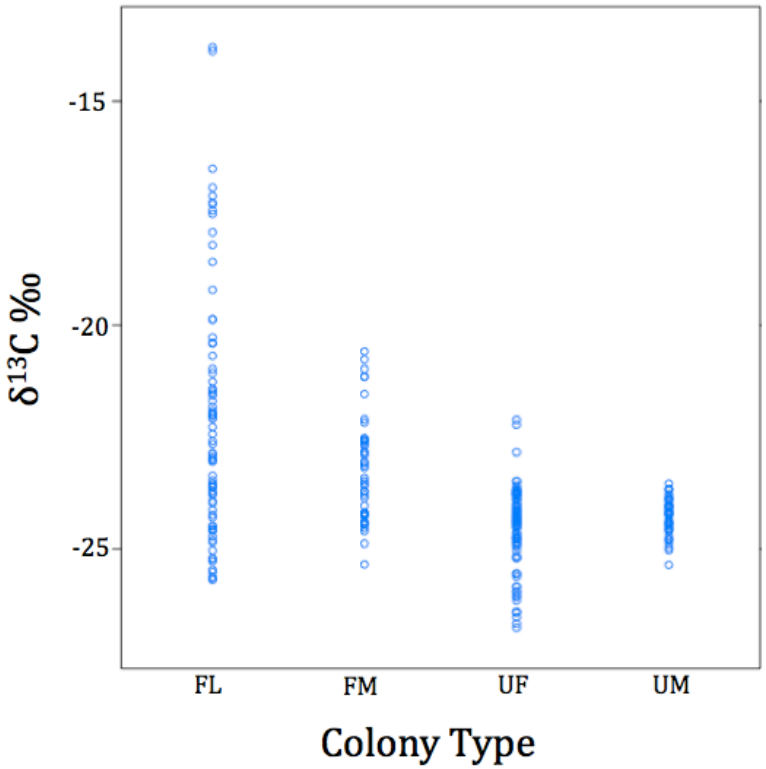

Figure 1 Carbon isotopic signature values by colony type

There was overlap in the spread of isotopic signatures between feral and managed bees, including overlap between fed managed bees and unfed feral bees (Figure 1).

Although there was overlap in the carbon isotopic signatures of the different colony types, there was a significant difference between the isotopic signature of the feral and fed-managed (UF vs. FM) bees (Linear Mixed-Effects Model, "LMM", $\chi^2 = 4.88$, $p = 0.027$). That difference did not hold when comparing feral and managed bees (UF vs. FM + UM; LMM, $\chi^2 = 1.86$, $p = 0.172$). Similarly, there was no significant difference between feral and unfed managed bees (UF vs. UM); and moreover the means of these two groups were essentially identical (LMM, $\chi^2 = 0$, $p \approx 1$).

After feeding began, isotopic signatures became heavier over time in both the fed managed and fed labeled bees, and values continued to increase for about five weeks after feeding was removed (Figure 2; up to approximately Sample Date 8). As expected, the isotopic signatures of the fed labeled individuals were heavier on average than those of the fed managed bees (across all time points, this difference was statistically significant: LMM, $\chi^2 = 6.15$, $p = 0.013$). After sample date 8, $\delta^{13}C$ values in both types of fed colonies began to decline and were ultimately indistinguishable from the unfed colonies by the end of the 15 week study.

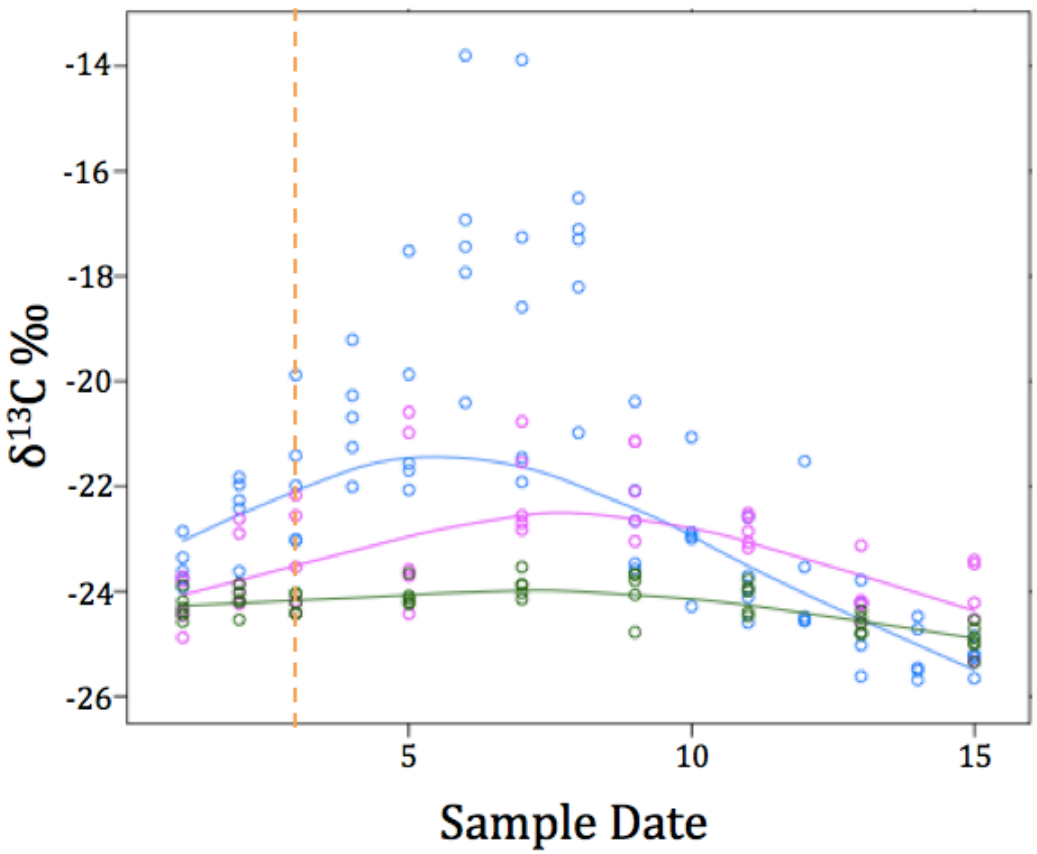

Figure 2 isotopic signatures of FM, UM, and FL colonies over the 15 week sample period. Trend lines were created using the "smooth" command in the lattice graphics package for R. Blue = Fed Labeled bees, Pink = Fed Managed bees, and Green = Unfed Managed bees. The orange dashed line represents when feeding was removed from Fed Labeled and Fed Managed colonies.

Discussion

Overview

The aims of this analysis were to: 1) determine if stable isotopes of carbon could reliably distinguish feral from managed honey bees; and 2) estimate the turnover time of carbon from supplemental feed in honey bee workers. These aims are interrelated in that reliable differentiation of feral from managed bees depends on the length of time for which the signature of enriched ^{13}C lasts after feeding of C4-enriched supplemental feed ends.

I found a significant difference between the isotopic signatures of fed managed and unfed feral bees, indicating some potential for differentiating managed honey bees receiving supplemental feed from feral honey bees using stable carbon isotopic ratios. As the temporal signal of feeding does not persist for a long period of time, however, managed and feral bees cannot be positively differentiated at any time of year. My data suggest that during the summer, managed bees receiving supplemental feed can be differentiated from feral bees for approximately 6 weeks after feeding is removed. However, due to the time frame in which the study was completed and the setup of the experiment, I did not have a sufficient number of samples to test for statistical significance in the length of time for which there remained a significantly different isotopic signature between the fed and unfed bees.

I hypothesized that due to large and well-characterized differences in ^{13}C from C3 and C4 plants, I could potentially exploit these differences to distinguish between feral and managed colonies. To some extent, this hypothesis was supported by my data in that honey bees from colonies receiving C4-based supplemental feed are statistically distinguishable from unfed bees, but it is only applicable for a limited time after feeding has been removed.

Mechanisms for Rapid Turnover of Heavy Signatures

The relatively short timescale of the heavier isotopic signals in supplementally fed honeybee tissue can most likely be accounted for by honey bee biology and/or potential limitations of the study design. In terms of bee biology, four aspects seem particularly relevant: 1) general patterns of honey bee worker development; 2) the patterns of carbon

isotope fractionation that occur during development; 3) seasonal changes in colony growth and subsequent changes in honey use; and 4) patterns of honey consumption and storage within a colony.

First, the length of development and lifespan of honey bee workers most likely affected the length of time for which a heavier signature persisted in my data. Bees have a 21-day developmental period after the egg is laid before they emerge from brood cells. In the summer, once they emerge they have an average lifespan of approximately 28 days. As worker bees age, their roles in colony maintenance change (a process called "age-based polytheism") (Graham 1992). The younger bees work inside the hive, cleaning, handling food, and building and maintaining comb and brood cells, and begin to forage at the very earliest when they are 18 days old (Sakagami 1953; Winston & Fergusson 1985). It is likely that most of the bees collected for this analysis were relatively young, and likely pre-foragers. Bees were collected from a frame inside the hive for sampling, and specifically avoided choosing individuals for analysis that had wing damage, a sign of aging. If supplemental feed was transferred into brood cells relatively soon after it was provided to the hive, the three weeks of development plus an additional two to three weeks working inside the hive amount to an equivalent length of time for which the distinct isotopic signature persisted. This temporal scale also accounts for lag in the spike of carbon isotopic signatures in the bees. Even with immediate consumption of the sugar solution, the three-week developmental period causes a time lag in detection of heavier isotopic signals. Thus, the increase in heavier isotopic signatures was seen after larvae provided with the heavier solution in brood development had emerged.

Second, there is little information available on the fractionation of the carbon isotopes in the developing individual honey bee. Sources of carbon in growing tissue could originate from pollen, nectar, sugar solution, or most likely, a combination of the three, provisioned to honey bee eggs in individual brood comb cells via worker bees. Brood cells are sealed with wax after provisioning and egg laying, and thus no new nutritive provisions are added or consumed by the developing bee. It is likely that chitin and keratin in the honey bee exoskeleton have different fractionation processes than other tissues or hemolymph (the equivalent of insect blood). Diet-tissue fractionation in black fly larvae vary between 1 and 2‰ (Overmyer et al. 2008), but the mechanism by which it fractionates is unknown. Using this as a baseline, future studies should examine the extent of honey bee diet-tissue fractionation in various tissues.

Third, the fastest carbon isotopic turnover in a honey bee colony likely occurs in the summer. This is due to shorter worker lifespan coupled with an increase in the production of offspring (and thus high resource use). Minimal foraging occurs in the winter due to the lack of floral resources and cold temperatures, and the focus of the workers is spent keeping the hive alive and warm. In the spring and summer, the colony is much more active, foraging and creating honey stores and producing new worker bees. The lifespan of bees in the summer tends to be two to three times shorter than that of bees in the winter (Graham 1992), creating a much more rapid turnover of bee individuals in a colony. Summer is the height of the colony growing season, when the most brood are produced to accommodate for the decline in foraging resources present in the spring. With shorter lifespan and high levels of resources required to produce more cohorts of offspring, it is likely that isotopic turnover in the colony was at its fastest during the time of this study,

and also that significant differences between colonies receiving supplemental feed and unfed colonies could be seen for an extended length of time during other points of the year.

Finally, immediate consumption and transfer of supplemental feed to brood cells could affect the temporal turnover. When bees consume nectar and supplemental feed, they regurgitate the solution, now enriched with enzymes, back into wax honeycomb cells in the hive in the process of creating honey. These honey stores are capped (sealed with wax) and then reentered and either consumed for energy or distributed to brood cells when eggs are laid, as a mixture of honey and pollen is created to give the larvae resources to grow and develop. Because this study took place in the summer, it is possible that when adult bees gathered the sugar water solution, most of it was either: 1) being transferred directly or very quickly to brood cells in the colony; or 2) consumed immediately by the workers as an energy source. In this case, little of the sugar water solution would be left in the hive for the consumption of future larvae, thus leading to a short turnover time of the ^{13}C signal from feeding.

In addition to possible biological mechanisms that could be responsible for the short duration of supplemental feeding signals, it is possible that methodological shortcomings could have also contributed to this result. The bees were stored in 90% ethyl alcohol from collection until pinned and dried, and there is a possibility that this alcohol could have weakened the carbon isotopic signatures of the honey bees that were created in the individuals from their food source. The feral bees were stored in the alcohol for a longer period of time, so if there was an effect, it could have been amplified in the feral bees. In addition, the feral colonies in this study were established from swarm traps. While swarm traps were placed in relatively isolated areas of the Chattahoochee-Oconee National Forest,

some of the feral swarms could potentially have originated from managed colonies. If there is indeed a longer-lasting signal of feeding, it may have gotten obscured by having colonies with a signal of feeding in my "unfed feral" category.

Applications of Isotope Analysis in Honey Bees

Although the use of stable carbon isotopes may not be applicable in differentiating feral and managed honey bees in all situations, this technique may hold promise for applications in more broad ecological studies. If it is known that managed colonies are receiving supplemental feed, relative distributions and habitat and foraging location preferences could be identified with the use of carbon isotopic signatures of bees foraging in a specific area.

In addition, the carbon isotopic signatures of nectar in different flowers and plants vary geographically, with climate and rainfall having a major effect on which isotopes of carbon they absorb from the atmosphere. With increased drought stress, plants close their stomates (leaf pores) in order to conserve moisture. This leads to less discrimination against ^{13}C and a subsequent heavier isotopic signature in the plant relative to a similar plant in a tropic environment receiving adequate moisture (Peterson & Fry, 1987). Because of this, there may be potential in situations of drought or extreme moisture for the use of IRMS to differentiate feral and managed bees.

An example of a study where this concept could apply could take place in areas with high rainfall such as tropical rainforests, where it is assumed that the feral honey bee populations are foraging on C3 plants that do not experience drought stress, producing relatively light carbon isotopic signatures due to the presence of ample moisture. In such a

context, if it is known that beekeepers are currently feeding their managed bees with C4-based supplemental feed, it may be possible to determine whether or not any bee is from a managed colony.

Although there is overlap in the isotopic signature values of managed and feral bees, there may be potential for differentiation of a bee as feral or managed if its isotopic signature value falls outside of a specific range. For example, in this study the heaviest isotopic value of a feral bee is -23.48 and the lightest signature of a fed managed bee is -25.6. Although the ranges of isotopic signals overlap, with further research it may be possible to establish a range of values on either side of this overlap zone (likely region-specific) that can be used to selectively identify a feral versus a managed bee. My labeling experiment, however, presents a counter-argument to this idea: the isotopic signals of the fed managed and fed labeled bees became indistinguishable from unfed managed bees in the same amount of time in my experiment, so a larger gap in isotopic signatures may not impact the timeframe over which there is a distinct signature.

Future Directions

Future research is necessary in several areas including 1) seasonal isotopic turnover; 2) the relationship between duration of feeding and duration of signal persistence; and 3) stable carbon isotope fractionation in honey bee tissue development.

The variation in carbon turnover in bee colonies between seasons should be explored in the future to help determine the applicability of using stable isotopes to differentiate feral and managed bees. Due to the increased lifespan of bees in the winter months in addition to the fact that fewer brood are produced during those months, it is

likely that isotopic signatures from C4-based supplemental feed would be traceable to a specific colony for longer periods of time and signals would persist as honey stores are not being used as rapidly. There is a significant relationship between the carbon isotopic signatures of mosquito tissue after feeding individual larvae different amounts of ^{13}C labeled glucose (Hood-Nowonty 2006). The ^{13}C levels in the mosquitoes decreased over time, and comparing the change in ^{13}C levels over the lifetime of honey bees could lead to better understanding of isotopic fractionation in insects overall.

Exploring the relationship between duration of feeding and the duration of heavy isotopic signal persistence could provide further insight on the effect of continued feeding on the turnover rate of carbon in honey bee tissue. Following three weeks of supplemental feeding, the data indicate that there was a distinct signal between fed and unfed honey bees for approximately six weeks. As most beekeepers provide supplemental feed for longer than three week periods, exploring if longer feeding periods are correlated with a longer persistence of distinguishable isotopic signatures post-feeding could be useful in terms of differentiating feral and managed honey bees for longer periods of time.

In addition, as so little is known about the fractionation of carbon in developing honey bee tissues, further research should be done labeling pollen with ^{13}C to determine the extent to which pollen-derived carbon is involved in exoskeleton formation and to develop an understanding of carbon turnover from pollen. While interesting, such a study would likely be very expensive, since labeling carbon with ^{13}C would most likely require plant growth in an environment with enriched levels of ^{13}C-labeled carbon dioxide. An alternative methodology could provide honey bees with ^{13}C labeled amino acids that mimic pollen. Although this would be considerably cheaper, it is dependent on finding compounds

with similar properties to pollen that bees would willingly consume, and that would provide sufficient nutrition for developing bee brood. This would also provide insight on the temporal turnover of pollen in the colony from the honeycomb into the brood cells.

In conclusion, I was able to detect a signal of supplemental feeding in the bee tissue, consistent with isotope studies in a range of systems including bees (Brosi et al., 2009). Although stable carbon isotopic ratios cannot be used to differentiate feral and managed bees in all situations, this study gives insight into the temporal turnover of ^{13}C in honey bee colonies. There is great potential for using isotopes in diet and foraging studies, especially with more work on understanding carbon turnover in honey bees and other insects.

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