<u>Part 1</u>

I am especially interested in Colony Collapse Disorder ("CCD") as an environmental issue as well as an economic and policy issue. Within the United States honeybees contribute to the success of one-third of U.S. agriculture; furthermore these insects are responsible for countless jobs and many billions of dollars in revenue. They are the unsung heroes of harvests of numerous fruits, berries, and nuts, and therefore crucial to the long-term viability of our global economy. I have taken a special liking to these insects and, through my research; I hoped to find creative ways to enable the species to survive the current challenges to their existence. During the summer of 2010, I had a unique opportunity to conduct original research at Michigan State University in the entomology lab of Dr. Zachary Y. Huang through the High School Honors Science Program. My work focused on the impact of time on the duration of honeybees' memory in "relay learning." My field research required working in close proximity to thousands of bees to investigate degradation of memory as a possible cause of CCD.

I used statistics in order to substantiate my results. I learned how to make T-tests for P-values, and I also learned how to use StatView to record G-values. My advice to students who are undertaking a project combining mathematics and science would be to never give up on your initial goals. Although you may have to tweak your methodology, you should never give up on answering your initial questions. My research encountered several stumbling blocks related to

replicating conditions across trials, which I was able to overcome through perseverance and enthusiasm. The honeybees were not returning to the hives, and after careful observation, I realized it was due to the placement of the hives. I repeated the experiment using two different hives in locations with more landmarks and the three trials were able to have been successfully finished. Despite this small procedural change, I was ultimately able to answer some of my questions, and I know that I want to continue taking on such exciting endeavors in the future because they are required to ultimately succeed.

Part 2

1. Introduction

Apis mellifera, the domesticated honeybee, is a well-known model for determination of sensory abilities in insects. Specifically, honeybees have a highly sensitive olfactory perception, an advanced compound eye with specialized ommatidia to measure angular movement, and optic flow for distance estimation (Wells, Wenner, Abramson, Barthell & Wells, 2010; Dittmar, Egelhaaf, Sturzl & Boeddeker, 2011; Kraft, Evangelista, Dacke, Labhart & Srinivasan, 2011; Wei & Dyer, 2009). These senses are combined to accomplish foraging tasks for hive success.

1.1. *Apis mellifera Navigational Techniques in Foraging*

Greatly evolved social structure coupled with unique behavioral techniques such as cognitive mapping imparts *Apis mellifera* navigational assistance in foraging (Srinivasan, 2010). Honeybee senses are utilized in unison with path integration, landmark learning, and celestial navigation to navigate through complex routes for the purpose of foraging. Various cues of the landscape incorporated into a series of memories also aid in navigation (Giurfra, 2003; Srinivasan, 2010; Pahl, Zhu, Tautz & Zhang, 2011). This, in turn, helps them navigate with a rapid homing rate (Giurfra, 2003; Srinivasan, 2010). Honeybees can navigate back to their hives using sensory motor routines including landmarks, visual cues, the sun, and path integration to search at unpredictable sites (Menzel & Muller, 2006).

1.2. Path Integration

Bees can also gain knowledge of landmarks en route to a food source (Menzel, 2005; Menzel & Muller, 2006). Path integration, or "dead reckoning", is the ability of a forager bee to calibrate a direct path to the hive using landmarks within a give distance to both remember and navigate future flight paths. When applying path integration, honeybees use snapshot matching to guide themselves towards a destination without creating a topological map. During this process, the colors of landmarks and the distance back to the hive are used to find their path home (Menzel et al., 2005; Yu & Kim, 2011; Collett & Collett 2002; Collett & Collett, 2000). Celestial markers such as the sun may also act as a

compass along familiar routes during the process of path integration. Short-term memory is needed to make use of path integration because the memory is constantly being replaced by new estimations of distance and directions back to the hive (changing memory) (Giurfra & Capaldi, 1999).

Path integration can be used at specific locations, but no true hypothesis has been formulated connecting snapshot matching with direction. Although this concept is still vague, it is believed that the snapshot images derived from a flight path allow honeybees to form memories, which ultimately guide their foraging routines. This retention of these snapshot memories remains uncertain. It is currently unknown how much experience is needed to insure a forager bee its route home (Menzel, Geiger, Joerges, Muller, & Chittka, 1998; Fahrback & Robinson, 1995, Yu & Kim, 2011).

1.3. Relay landscape learning

Prior experience of a landscape allows honeybees to maintain a more rapid homing rate (Capaldi & Dyer, 1999). A novel concept, introduced by Zachary Huang (2009), examines the ambiguity of how honeybees adapt to new experiences in unique
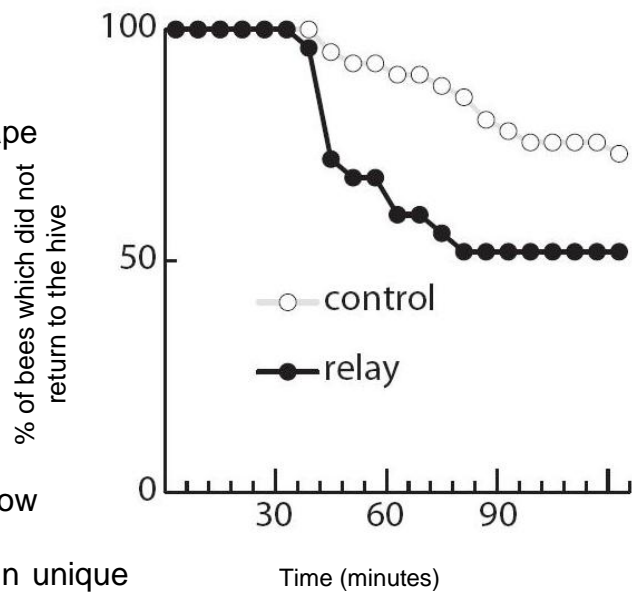
Figure 1. Relay Learning Trial. Relay groups exhibit increased ability to return to hive **(Huang, 2009; unpublished data)**

environments via the process of "relay landscape learning." Relay landscape learning is established after bees conduct reconnaissance flights, which involves returning back to the hive from a point that is familiar.

Landmarks assist the bees in gaining visual information for future flights during relay landscape learning (Wei & Dyer, 2009). Honeybees that are familiar with a specific landscape (relay bees) can return to the hive more rapidly than inexperienced honeybees (control bees), as seen in Figure 1. Initial experimental releases provided the underlying basis for assessing path integration as a means of reference of motion direction for homing navigation (Yu & Kim, 2011). Secondary releases during relay landscape learning (1km from the initial release) presented increased return success to the hive when compared to the control group (Huang, 2009; unpublished data). The ability to increase success in return was denoted as the relay effect because it allowed bees to return home at a higher rate, as if it was a relay: bees released at 2km were helped by their prior experience at 1km, if direction was held constant (Figure 2).
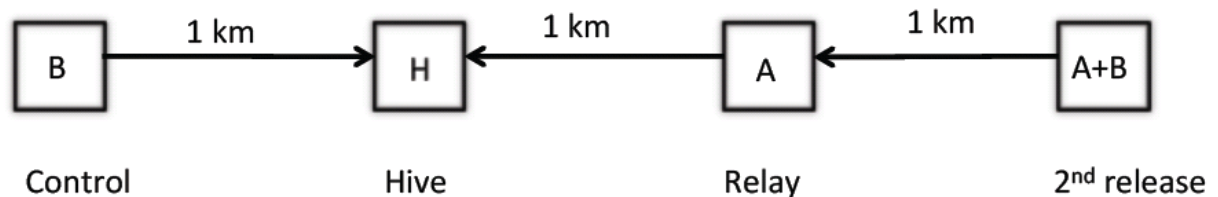

Figure 2. Relay landscape effect: Sites A and B locations are 1 km from the hive in opposite directions. Bees were collected from the hive and then released 2 km from the hive in the relay direction at A+B to determine the effects of past exposure on return rates (Huang, 2009 unpublished data).

Relay landscape learning has limitations; relay landscape learning is limited by direction or any distances greater than 3 km (Huang, 2009;

unpublished data). Previous literature has determined homing success is limited when flight distances exceed 3km (Capaldi & Dyer, 1999). The role of direction in the relay effect needs further examination.

1.4. Purpose

The consistency of path integration was tested in various directions to identify the relationship between hive navigation and snapshot memory (N vs. S; E vs. W). Also the role of snapshot image retention as an aspect of relay landscape learning where time-dependency was a factor of memory degradation was examined.

2.0. Methodology- Preparation

The site of the experiment is located at the GPS coordinates: 42.4044° N, 84.2840° W from July 2010-August 2010. Before performing the actual experiment, the relay landscape effect was tested in order to identify the best hive location (i.e. where honeybees had the most navigational success). In this experiment, there was one experimental site, A, and one control site, B for both directions. Site A was .8km north and site B was .8km south, from the base hive (H; shown in Figure 3). For two trials, the directions east and west were used instead (shown in Figure 4). In addition, the honeybees were only released on warm (76-80°C+) and days with no overcast to avoid factors that add variables to

the experiment. The optimal temperature for honeybees to forage at is about 70-80°F.

On the first day of the experiment (Day 0), 1000 bees were collected using a BioQuip insect vacuum. All of the bees collected were forager bees that have had at least 21 days prior experience in the local area. This is inferred because the only bees used were forager bees. In other words, bees were either moving on the hive surface or flying in front of the hive. Wings and pollen also provided proof that the bee was a forager. Then on Day 0 in each trial, 500 bees each were painted blue or pink on the thorax after they were anesthetized on ice. The blue bees and pink bees represented the relay and control group, respectively. The purpose of painting the honeybees is to distinguish the relay bees from the control bees. Also, painting is done to show the difference between honeybees in the trials and other honeybees not involved in the experiment.

2.1. Release of Honeybees (Day 0-9)

Before the release, the hives were closed to prevent relay and control bees from flying into their home. On Day 0, the blue bees were released .8km north and the pink bees were released .8km south (the direction was picked randomly). On Day 1, 40 blue bees and 40 pink bees were collected from the base hive (H; shown in Figure 3). The bees were painted another color on the abdomen to distinguish between the different release days. The bees were then released 1.8 km north from the hive to determine the relay effect. After two hours, honeybees were no longer captured at the hive. Total bees retrieved between 0-

120 minutes from the North direction were recorded. This same process was completed for Day 3, Day 6 and Day 9, if possible. The hive was used instead of cages to stimulate a more natural environment for the bees. This decreased the chance of honeybee mortality.

2.2. Repetition of Trials

The experiment was repeated another two times with a different hive and a different direction. In the study field, the hives were approximately .26 km away from the hive used in the first trial. The hives used are relevant in experiments because of their location. Landmarks, which surround both hives, often aid honeybees in their flights back to the hive (Menzel & Muller, 2006). In Trial 2, 1000 bees were collected, and then painted green and silver on the thorax. On Day 0, the bees were released .8km west and .8km east. In this particular trial, the relay direction used was west. The relay effect was tested on Day's 1, 3 and 6. Testing on initial days (Day 1 and 3) is pivotal because it shows the comparison between days (i.e. the strong relay effect). In another trial, 1000 bees were collected, and then painted orange and yellow on the thorax. The honeybees were released .8km west and .8km east. In addition, west was used as the relay direction for this trial. Trial 1 tested N vs. S direction based relay learning. Trial 2 and 3 tested E vs. W direction based relay learning. The same procedure described above for Trial 1 was also used in Trials 2 and 3. All

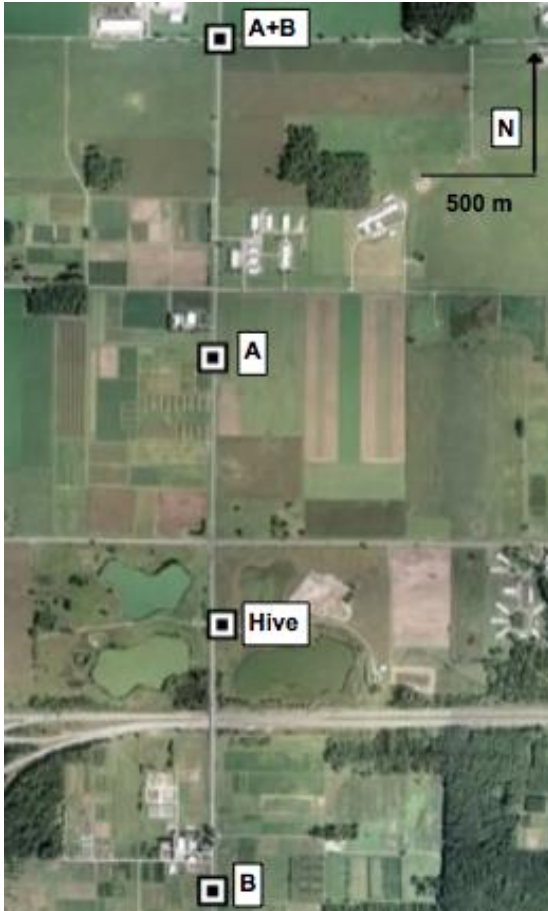statistical data, including P-values and G-values, was analyzed using StatView

(α-value=0.5).

Figure 3. Google Earth © satellite view shows the release locations as portrayed in Trial 1 (N vs. S). H is representative as the base hive. A (.8km North from the hive) is the starting point for the relay group and B (.8km South from the hive) is the starting point for the control group.

Figure 4. Google Earth © satellite view shows the release locations as portrayed in Trial 2 and 3 (E vs. W). H is representative as the base hive. A (.8km West from the hive) is the starting point for the relay group and B (.8km East from the hive) is the starting point for the control group.

3. Results:

Honeybees released from the second release locations (A+B) exhibited an average decrease in retention of previous experiences by the conclusion of Day 6. By Day 9, the ability of the relay bees to return to the base hive diminished; $P=.22$. The averaged ratios in all directions (North/South or West/East) illustrate relay breakdown (Figure 6).
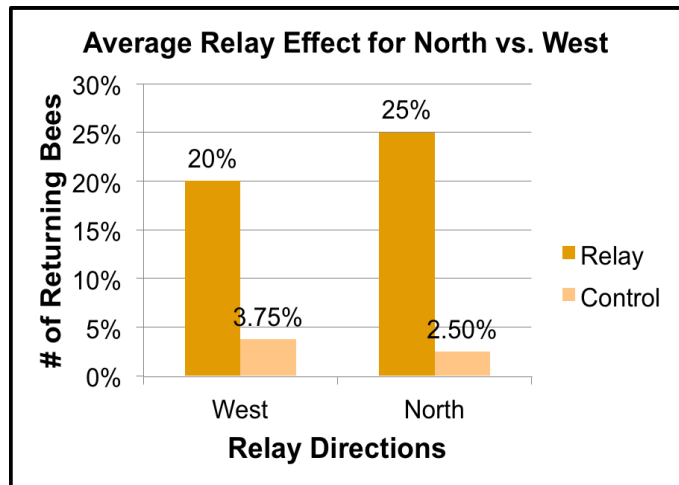

Figure 5. Comparison of the different relay directions used in the trials. The direction of the relay effect has no significant effect on the honeybee performance ($p=.45$)

The ratio of relay bees to control bees that returned to the hive showed a higher and increased relay effect. High ratios provide evidence that the relay bees recalled the landscape from the initial experimental releases on Day 0. Statistical analysis supports this trend becoming less significant as the length of relay increased (Day 1: $P=<0.001$, Day 3: $P=<0.001$, Day 6: $P=.0012$, Day 9: $P=.22$). The P-value's strongly support a decrease in the amount of bees returning to the hive (Table 1).
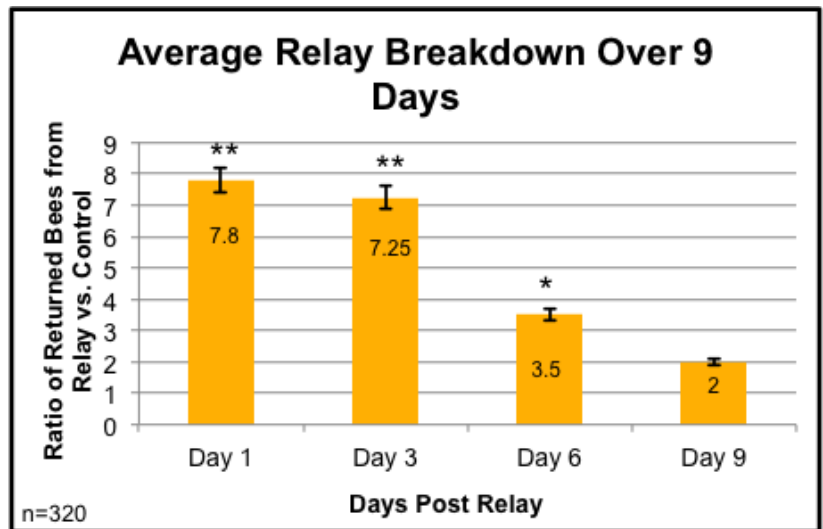

Figure 6. The average ratios of relay vs. control are shown across the various days. As the ratio decreases, relay effect decreases. ** = $p<0.001$; *, $p<0.01$

The ability to apply snapshot images was depleted between Day 6 and Day 9. Statistical significance of these trials supports the observed decrease return to the hive. The G-values tested heterogeneity of the data. G-values decreased in this trial, supporting the degradation of the relay effect. G-values (Day 1: 39.5, Day 3: 24.4, Day 6: 10.52, Day 9: 1.5) decreased over time and correlates with the observed and the expected values.

The relay direction in the North shows a slightly stronger relay effect (Figure 5). This may be due to

Relay	Control	Relay	G-Values	P-Values
Day 1	5 bees	41 bees	39.95	<0.001
Day 3	4 bees	26 bees	24.4	<0.001
Day 6	10 bees	21 bees	10.52	0.0012
Day 9	6 bees	11 bees	1.5	.22

Table 1. Average ratio of relay/control bees (# of returning bees) from Days 1-9 of landscape learning. G Tests tested heterogeneity of the data; α=0.05

landmarks surrounding this specific hive. However, the difference between the relay effects in the North versus the West is insignificant (p= .45). Direction does not impact the ability to effectively return back to the hive may not be a contributing cause of declining honeybees' memory retention. As opposed to utilizing direction to navigate during flight paths, honeybees will use static snapshot images to simultaneously calculate direction and distance back to the hive. The same mechanism of snapshot matching will occur regardless of the direction.

Homing rate calculations indicate that as time progressed, the rates of return significantly decreased. In addition, bee return rates decreased as

experimental days progressed (unreported data from this study). A decrease in homing rate from 55% to 16.67% from Day 1 to Day 6 supports a diminished memory, homing rate and navigational ability (Figures 7 and 8). The decrease in the average relay breakdown over 9 days also supports the deteriorated snapshot memory (p=.22; diminished foraging ability). The ratio of returned bees from the relay site versus the control site diminishes from 7.8 to 2.

Example of Homing Rate Calculation on a Day 1:			Rate
Relay:	$\dfrac{\text{# of returning bees from 2}^{nd}\text{ release site}}{\text{total number of bees released from 2}^{nd}\text{ site}}$ =	$\dfrac{22}{40}$ =	55%
Control:	$\dfrac{\text{# of returning bees from 2}^{nd}\text{ release site}}{\text{total number of bees released from 2}^{nd}\text{ site}}$ =	$\dfrac{1}{40}$ =	2.5%

Figure 7. Homing rate calculations of a strong relay effect (ratio of relay vs. control shows that the relay bees were able to adapt to the novel experience unlike the control bees

Example of Homing Rate Calculation on a Day 6:			Rate
Relay:	$\dfrac{\text{# of returning bees from 2}^{nd}\text{ release site}}{\text{total number of bees released from 2}^{nd}\text{ site}}$ =	$\dfrac{5}{30}$ =	16.67%
Control:	$\dfrac{\text{# of returning bees from 2}^{nd}\text{ release site}}{\text{total number of bees released from 2}^{nd}\text{ site}}$ =	$\dfrac{1}{30}$ =	3.33%

Figure 8. Homing rate calculations of a weak relay effect (ratio of relay vs. control shows that both groups had an increased difficulty applying their sensory-motor routines as time increased

4. Discussion

Honeybee success will vary upon intervals between foraging bouts. Honeybees were able to recall past exposure to the initial experimental release landscape on Day 1 and Day 3. However when the interval increased to 3 days, honeybees were unable to return to the hive. The ability to utilize path integration to return home had deteriorated and could not be retrieved for application to flight paths. Decay in memory over several days was apparent throughout three trials (n=1000). The honeybees forgot how to use their previously acquired information from their initial release because of the time between learning trials.

Distance acted as a deterrent to honeybee success (relationship instead). The A+B site was 1.8km from the base hive. To forage long distances, honeybees have to retain a strong memory. Honeybees will not be able to navigate long distances if images are not reinforced. The homing rate calculations (Figures 7 and 8) demonstrate the changing homing rate as time progressed. The inter-trial interval (ITI) increased at Day 3, implicating inhibition of relay effect and the honeybee memory (interbout trials were largely interrupted). The amount of time between consequential learning trials was too large to maintain a constant retainable memory. Errors in snapshot image matching occurred because time was a factor of the relay effect (Yu & Kim, 2011).

Contrarily, direction is insignificant in the strength of the relay effect because there are sufficient visual cues in both directions (N vs. S, E vs. W)

(Figure 5; Figure 6; Table 1). Direction plays no significant role in the relay effect (consistent effect occurs where effect is strong on Day 1).

Bees that were released in the beginning of each trial (Day 1 and Day 3) were more successful in navigating than the bees released on later days of each trial (Day 6 and Day 9). In order for the bees to efficiently return to the hive, they used path integration and/or landmarks. Initially, honeybees used their short-term memory or working memory but this ability was diminished after the amount of days increased. The relay bees that were previously able to return to the hive could not return as time lapse between releases progressed; returning rates decreased daily and the bees navigated back to the hive at a slower rate (at Day 6, 16.67% relay returned versus 3.33% control bees) (Figures 7 and 8).

5. Conclusion

Relay bees rely heavily on previous landscape information to return to the hive. Honeybees can only travel efficiently if they have had sufficient, consistent contact with complex landmarks and other visual images (Pahl, Zhu, Tautz & Zhang, 2011). On brief reconnaissance (relay) flights, bees navigated with high accuracy determining honeybee homing rates were enhanced by previous knowledge of the landscape. Sensory motor routines were crucial in the ability of the bees to pinpoint the hive location with accuracy after foraging (Wei & Dyer, 2009; Pahl, Zhu, Tautz & Zhang, 2011). The homing rates of the relay bees were higher because prior experience had reduced the uncertainty of the

location. If bees are to be successful on their foraging trips, the time between consequential learning trials must be reduced (Wei & Dyer, 2009).

Landmarks were crucial in the capability of the honeybees to return to the hive. The landmarks surrounding both hives assisted honeybee navigation by serving as reference markers (Collett & Collett, 2000). On Days 1 and 3, relay bees were able to calibrate a direct path back to the hive. There was an inability of the relay bees to apply their sensory motor routines to navigational tasks after Day 3. The honeybees did not retain their short-term memory; the time window impaired their ability to navigate using snapshot imaging (Hammer & Menzel, 1995). The control bees could never return to the hive as rapidly as relay bees because they were inexperienced with the location in the North and West. *Apis mellifera* could learn from past experience by the recollection of landmarks. In short, if landmarks remain constant but time span increases, honeybees will still lose orientation for future flights back to the hive (Wei & Dyer, 2009).

6. Implications

The ability to retain snapshot images decayed rapidly over a period of 9 days. Working memory could not be combined with snapshot images to successfully return home. In reality, honeybee pollination would be impaired if snapshot images were unable to be retained. Pollination is crucial for the continuation of crops and flowers and honeybees are primary pollinators within the United States. Without foraging, the balance of ecosystems, the economy,

and the agricultural industry would suffer. Bees act as an indicator of environmental quality. 15-30% of the U.S. food supply comes from animal pollination. *Apis mellifera* pollination contributes $15-$20 billion towards the U.S. agriculture industry annually (Johnson, 2010; Greenleaf, Williams, Winfree & Kremen, 2007). Knowing more information regarding honeybee navigational skills is vital to agriculture.

This study is also crucial because anthropogenic disturbances (i.e. deforestation) may decrease the honeybee population and this in turn will limit foraging. It is important to note which factors can also limit foraging (inability to conduct path integration) because the memory loss will affect the ecosystem and its natural patterns (Menzel, 1999; Cruse & Wehner, 2011). If honeybees did not forage, most of the crops worldwide would not exist (Johnson, 2010). This study may also explain why some honeybees do not return back to hive. If bees do not return to the hive, it may be that the bees never reinforced their previously learned snapshot imaging routines.

7. Future Work

In the future, the specific biological mechanisms, which lead to the degradation of short-term memory, should be further investigated. Future repeated experiments should focus on snapshots used in path integration, and their relation to long-term memory past 3 days. Snapshot memories play a large role in honeybee navigational success and it should be accessed how

honeybees navigate with global landmarks as opposed to local landmarks. It would be also relevant to identify how bees cope with other navigational challenges such as camouflaged landmarks or even an absence of terrestrial cues (Dittmar, Egelhaaf, Sturzl & Boeddeker, 2011).

It may be helpful to correlate the lack of a retainable working memory with Colony Collapse Disorder (CCD). Memory degradation as a function of time and distance may not be associated only with path integration but also microbial pathogens. It is believed that a combination of a fungi and a virus may be the culprit of Colony Collapse Disorder. Increased application of pesticides could enhance honeybee susceptibility to disease (Johnson, 2010; Ellis, Evans & Pettis, 2009; VanEngelsdorp et al., 2009). The correlation of disease and lack of honeybee return to the hive would lead to a greater insight into the diminishing honeybee population. In summation, all factors that have the potential to limit foraging should be investigation to support ecosystem homeostasis.