A Preliminary Investigation into the Effect of Ethanol Exposure on Body Temperature in *Apis mellifera*

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I certify that I have read this thesis and that, in my opinion, it is fully adequate in scope and quality as a thesis for an honors degree in Psychology.

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Dr. Charles I. Abramson, Thesis Director

I certify that I have read this thesis and that, in my opinion, it is fully adequate in scope and quality as a thesis for an honors degree in Psychology.

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Abstract

Comparative studies of physiological effects of toxicants with social insects can be useful to understand effects in humans. These studies can yield insight into the biochemical mechanisms that underlie addictive behavior and other substance use. The aim of the current study is to assess the physiological influence of ethanol on body temperature change in *Apis mellifera*. It is part of a project to establish honey bees as a comparative model to humans for ethanol and other toxicant studies using social insects. Bees have shown a variety of responses to alcohol that make them viable research subjects, and are an easily obtainable and ethical alternative to humans in alcohol-behavior assays. Using an infrared laser thermometer, 125 harnessed bees (25 per condition) were measured at 30s intervals to record temperature change after exposure to either 0%, 1%, 2.5%, 5%, or 10% ethanol solution. The data collected in this experiment did not show significant variation between dose conditions. This experiment demonstrates a new protocol design and an apparatus that can be used for future study of ethanol's effect on honey bee physiology. More control, better instruments, and greater sample size may yield more insight into the viability and utility of honey bee body temperature as a measures for response to toxicants. The results of this experiment give necessary preliminary data to better study the body temperature effects of ethanol in honey bees.

Introduction

There are two primary reasons for using bees as behavioral comparative subjects to humans. Firstly, the use of bees relieves human suffering in research (Alcohol Alert No.24, 1994). Secondly, bees are easy to acquire and maintain (Abramson, et. al., 2000). Humans present ethical limitations to experimentation with alcohol consumption, toxicity, and behavior. For example, it is unethical to give alcohol and perform behavioral assays on human subjects without consent. In some behavioral apparatuses that are used in bees, such as the shuttle box developed by (Abramson, et al., 1982), a shock grid is used as an aversive stimulus. These types of studies cannot be performed on humans for ethical reasons but we can use honey bees as a model to predict human results. In addition to avoiding human harm, bees can produce up to 150,000 individuals annually in some hives, making them sustainable subjects (Bodenheimer, 1937). They can also be kept in largescale research facilities and in small, personal apiaries. This makes the use of bees for comparative studies a more efficient species to gather data than humans for toxicant effect research.

Bees are social animals (Seeley, 1997). An important similarity to humans, as it allows us to study social interactions in a comparative way, such as how communication is affected by ethanol exposure. Honey bees communicate to other hive members via what is called the waggle dance, a choreographed path, shape, vibration, buzzing of wings, or some combination thereof to relay information to other hive members (Lindauer, 1971). This can include location of food sources and danger. Research has shown that this social communication is impaired after alcohol exposure. In one study, behavioral data showed that when forager bees were exposed to alcohol, they significantly reduced rest and time spent walking between rests (Mixson, et. al. 2010). In another study, the amount of waggle dancing and the response by other bees to the dancing was

significantly reduced (Bozic, J., 2006). Alcohol consumption reduces overall locomotion in bees, but it is dose-dependent: if bees are given too much alcohol, they will be impaired. Low doses stimulate an increase in locomotion, while high doses are impaired (Abramson, et. al., 2000; Maze, et. al. 2006).

Apis mellifera are complex learners, and function as a cohesive unit (Seeley, 1997). Honey bees respond well to basic conditioning, such as the proboscis extension response protocol developed by Bitterman et. al., (1983). In proboscis extension experiments, conditional withholding of the proboscis can be used to measure associative and discriminative learning. The proboscis extension conditioning response of bees is significantly reduced by acute alcohol consumption (Abramson, et. al., 2006). Importantly, alcohol can be used as a stimulus in all of these learning protocols. This is the pillar by which behavioral learning can be compared between humans and the honey bee. We can measure effects before, during, and after exposure in learning tasks such as the modern shuttle box protocol, proboscis extension response (PER), and sting extension response (SER). The modern shuttle box protocol was developed by Abramson (1986) specifically for bee research. It was used by Black et. al. (2018) to show the impairment of both discriminative and appetitive conditioning in response to alcohol. In one study, ethanol-water solution acted as an aversive stimulus for honey bees when compared to sucrose solution (Giannoni-Guzman, et. al., 2014), in a schedule-controlled self-administration operant conditioning protocol where free-flying foragers were trained to receive sucrose from an apparatus designed to reward head poking (into a hole to receive a reward),

Work by Sandhu (1985) showed that bees encounter ethanol in the environment in the form of fermented flower nectar and suggests the action of yeasts in this process. Looking into the stomachs of 328 bees from 7 species, and 342 nectar samples and 9 different flowers almost

770 individual yeast isolates were found. This suggests that the yeast isolates are responsible for the fermentation of flower nectar in either the flower or the bee stomach (or both, depending on the location of the yeast isolate). They have been shown to drink ethanol on their own. thereby demonstrating bees' capacity to metabolize and drink alcohol (Sokolowski, et. al., 2012). Honey bees will also continue drinking ethanol once a source is established, and even preferred to go to feeders containing ethanol-sucrose solutions (Abramson, et. al. 2004). In a choice feeder assay that gave bees the choice between 1 and 5% ethanol solution, 11 of 20 bees returned for the entirety of the experiment (12 returns to the feeders). In addition, bees will continue foraging after consuming ethanol (Abramson, et. al. 2006). In this study, a significant number of bees continued to search for nectar after alcohol consumption. This suggests commonality of alcohol consumption in nature, another reason to support the use of bees as comparative subjects. In another study, a two-feeder choice assay using proboscis extension responsiveness was used to measure preference, subjects showed a preference for 1.25%-2.5% ethanol-sucrose solutions (Mustard, et. al., 2019). These findings suggest the general preference of lower doses of alcohol in honey bees.

Studies in bees have also reported development of tolerance to alcohol after prior consumption. Bees in this study who had repeated exposure to ethanol exhibited significantly less effect than first-time exposure (Miler, et. al., 2018). This suggests the capacity for chronic alcohol tolerance in bees. Tolerance to toxicants is a common side effect of addiction (Siegel, 2005). When tolerance develops and more alcohol is needed for the same simulative effect in humans, they may seek more alcohol, which can lead to alcoholism. There are many ways to treat alcoholism, including aversive medication meant to dissuade alcoholics from continuing to drink. One of these is Antabuse, or Desilfiram. It acts as an aversive stimulus in human

alcoholics, sensitizing them to the effects of alcohol and causing an uncomfortable flushing of the face, headache, nausea, and giddiness, the symptoms were known by the French as the "mal rouge" for the trademark red flushing it caused workers who used cyanamide, a chemical that causes a similar reaction (Lipińska-Ojrzanowska, et. al., 2014). In honey bees that have developed a prior tolerance, Antabuse does not work in aversive conditioning of alcohol. In a shuttle box protocol, an electrified steel grid was designed to measure aversive conditioning, and found that bees with a prior established tolerance had impaired responses to aversive conditioning. In other words, ethanol tolerant bees were more resistant to the aversive effects of ethanol (Bennett & McKeever, 1951, Hald, et. al., 2009, Abramson, et. al., 2003).

Complex decision-making and aggression in bees is also altered by ethanol exposure. In one study, alcohol consumption reduced the amount of nectar and pollen collected while foraging, but increased visitation with regard to color of flowers bees visited after exposure. (Sokolowski, et. al., 2012). Aggression sees mixed results in bees from ethanol exposure. In some honey bee subspecies, such as *Apis mellifera scullatata*, consumption of alcohol led to an increase in aggression. When exposed to alcohol via ethanol vapor, a method established by Ammons, (2008), a hive of *Apis mellifera scutellata* not only increased the number of inflicted stings on a leather patch, they became too dangerous to continue to use in the study (Abramson, 2004). Harnessed individual bees saw no change in sting extension responses using the sting extension response (SER) protocol (Abramson, 2006).

Ethanol has documented effects in other organisms as well. Research has been performed on a variety of animals, including cats, mice, and rats (Ritzman & Tabakoff, 1976; Jones, et. al., 1980; Guo, et. al., 2016; Abel, 1978). Research has also established other insects as a comparative ethanol study subject to humans, namely fruit flies (Manev, et. al., 2003; Shohat-

Ophir, et. al., 2012). The methods for studying both honey bees and fruit flies overlap in some useful ways. For example, the effect of alcohol on human circadian rhythms has been compared to ethanol's effect in fruit flies (Danel, et. al. 2003; Linde & Lyons, 2011). This has been used to establish similar experimental protocols for *Apis mellifera,* such as the monitor apparatus used to measure changes in locomotion and circadian rhythms in bees in response to aqueous aluminum ingestion (Chicas-Mosier, et al., In Review for PLoS ONE).

An important note is that honey bees are ectotherms, or they are cold-blooded. According to Huey & Berrigan (2001) there is a correlation to temperature performance in ectotherms up to damagingly high or low temperatures. It is important to note this because region to region the degree that this curve is or is not damaging depends on the species and how adapted to the environment it is (Abou-Shaara, 2015). Under high temperature conditions, Yemeni bees were more tolerant than carniolan honey bees. This is vital because the environment that one gets bee subjects from will influence the base temperatures as well as the change as a result of ethanol exposure. Due to this, temperature change studies in bees that use alcohol must look at the change in the temperature compared to the baseline reading. This informed the current study's analysis of effect as a function of change from the baseline readings.

Biochemical research may give us an explanation to the underlying mechanism of temperature change in bees. Honey bee brain contains Heat Shock Protein (HSP70). The heat shock protein family functions to combat oxidative stress of various forms by acting as a chaperone protein that helps cells cope with denatured protein buildup that follow stress, such as heat. This protein is present in humans as well as mice (Daugaard, et. al. 2007). Research has shown that HSP70 increases in bees after alcohol consumption, when compared to a handling variable control indicating a stress response to alcohol consumption in bees (Hranitz, et. al.,

2010). Alcohol is a source of oxidative stress as well (Wu &Cederbaum, 2003). It does so by altering levels of metal in the body, allowing for Reactive Oxygen Species (ROS) production, which counters the body's natural defenses against alcohol and other compounds. In rats and mice, temperature increases caused by heat are regulated by this protein (Skidmore, et. al., 1995). In humans, this protein increases after exercise, presumably due to the increase in body temperature that comes from exercise (Shastry, et. al., 2002). In addition, Even, et. al. (2012) propose that humans and bees have a similar hypothalamo-pituitary-adrenal axis (HPA) within their brain that reacts similarly to oxidative stress (such as alcohol) in a way that is comparable to humans. These results give us a biochemical mode of comparison to humans. They justify our choice of body temperature as a dependent variable, and informed our hypothesis: that honey bee body temperature may increase in response to alcohol exposure.

Literature has thus supported the viability of honey bees as comparative subjects, including recorded effects on locomotion, learning, aggression, stress, foraging decisions, and social communication. Therefore, it is necessary to investigate physiological effects of ethanol in bees. The current study looks at the body temperature change in honey bees in response to ethanol consumption. This is to gain preliminary knowledge of physiologic change in response to toxicants in comparative subjects using a novel protocol and apparatus.

Methods

Subjects: Subjects will be *Apis mellifera*, sourced from Stillwater, OK. We used only forager bees greater than 21 days old to ensure similar age of subjects. Foragers are the oldest members of the hive (Visscher, P. K., & Dukas, R., 1997).

Drinking Straw Assay: Foragers were obtained using the feeder method. The feeder method is very simple and effective way to acquire bees for study. A design by Seeley (1995) was used. This method utilizes a jar that is turned upside down onto a flat surface such as a board or plate, then wedged with a small piece of wood such as a toothpick or matchstick. The feeder was filled with aqueous sucrose (20-40% v/v). The bees had established the feeder as a food source prior to experimentation. Catching occurred directly off the feeder and then bees were transported to a nearby laboratory. Bees were caught in 15mL falcon tubes outfitted with bee candy (40% honey, 60% sucrose) as a food source (Herrod-Hempsall, 1920). Bee candy is placed under 2.5 cm^2 cheesecloth inside the cap. Once at the laboratory, the bees were harnessed into the 'drinking' straw apparatus and fed 10 µL of ethanol-sucrose solution via a pipette. There were 5 dosage groups, each with 25 bees. Experimental solutions were 1M sucrose with 0% (control), 2.5%, 5%, 10%, and 20% ethanol. Temperature measurements were taken every (30s per bee) for 15 minutes. We used the Etekcity lasergrip 1080 infrared (IR) thermometer to measure body temperature effects. Measurements were taken in between the wings on the top of the thorax. In prior studies, the bees have been harnessed in metal bullet casings (figure 1) with a ninety-degree semi-circle cut out (Abramson & Boyd, 2001). The conductive lead of the casing would interfere with the IR thermometer gun's reading. Therefore, in this experiment, a similarly shaped harness made of plastic with a malleable putty base was used (figure 2).

Figure 1:

Figure 2:

Results

Data was analyzed using 5-1x5, 2-way ANOVAs, analyzing the interactions between dosage and time. Across doses, there was no significant increase in body temperature compared to the control (p=.589). Rather, there seemed to be an overall cooling effect, but nothing significant enough to warrant any conclusions given the current sample size. We saw an initial spike in temperature across all groups soon after exposure at t=30s. In figure 4 the first 5 minutes are shown to highlight this increase (figure 3 shows the general trend of the data based on dose across the whole 15m trial). It was not significantly correlated to ethanol exposure, however. There was a wide temperature variety in both baselines and post-exposure readings, excluding the initial increase. We saw an 8.2℃ range in base temperatures among subjects. If a bee dies during the study, the temperature of the bee diffuses and matches the surrounding background.

Figure 3:

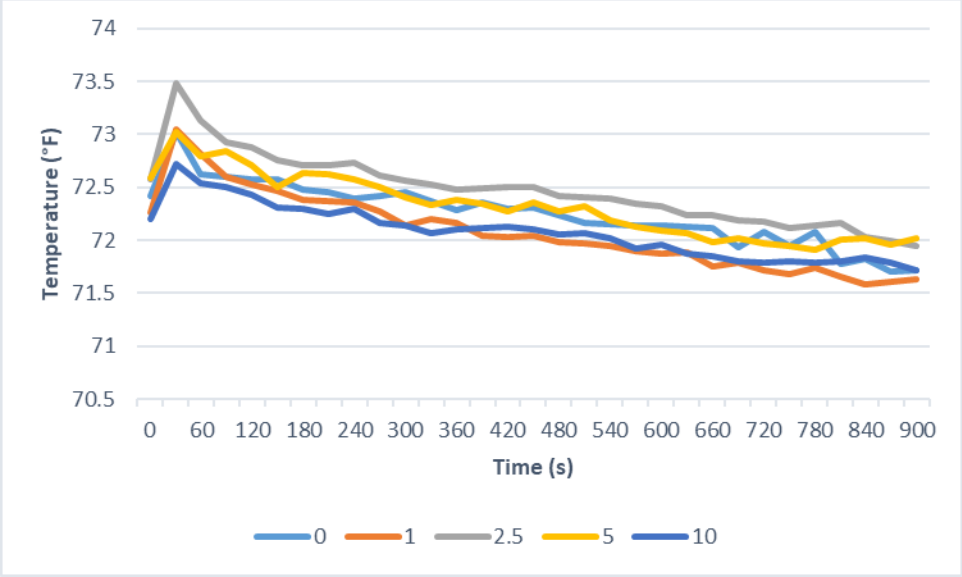
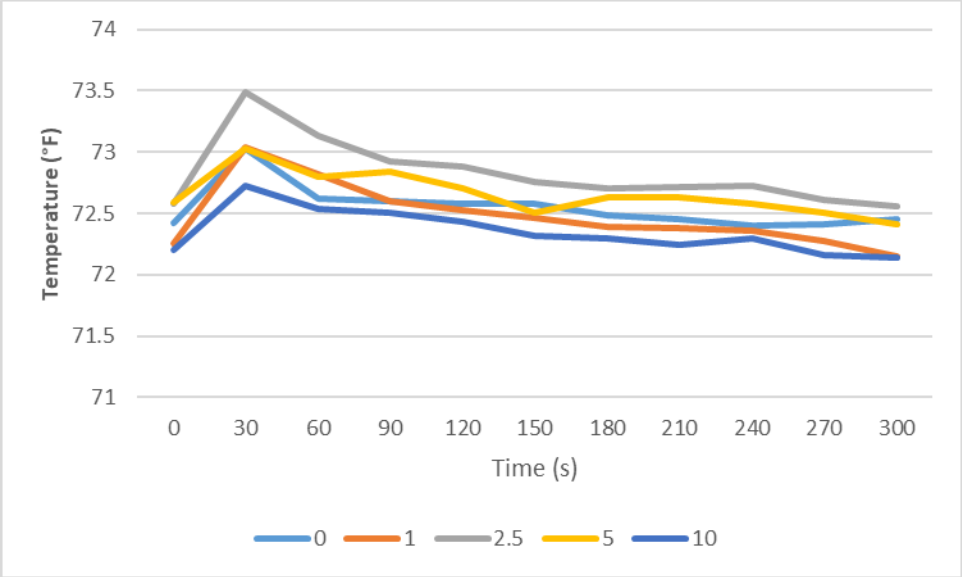


Figure 4:


74
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Temperature (°F)
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Discussion

As a preliminary investigation, this study has limitations. These include limitations with the current equipment and the potential effects of handling during feeding on the body temperature of bees. We will discuss these and suggest some protocol changes to reduce them. Suggestions for future topics of study regarding toxicant effects on honey bee physiology are also presented, along with research implications.

The type of IR thermometer used in this study varied widely in terms of accuracy, and sensitivity. The readings taken from this study will therefore need to be compared to results of future studies to understand the skew caused by the instrument. The quality of the thermometer itself can also be improved, as IR thermometers vary in sensitivity and consistency. A mounted thermometer may increase accuracy by reducing temperature fluctuations.

An important result in this study is the initial slight increase in temperature that is not correlated with alcohol, which may suggest the feeding or handling of the bees elicited the effect. Future studies may find it useful to use the protocol in this experiment, with variable concentrations of sucrose-solution as the independent variable. This could determine whether feeding or handling were the cause of this initial effect.

We also saw an 8.2℃ range in base temperatures among subjects. This, along with the wide variation seen across the experiment may be due to the ectothermic nature of honey bees. Use of larger sample sizes in future research may help get a better idea of the variation of honey bee body temperature without ethanol. Further understanding of bee body temperature without toxicant manipulation may help further studies with exposure.

We also suggest further investigation into the correlation between HSP70 protein and ethanol exposure, as this may help gain insight into the biochemical mechanisms of behavioral

learning changes in bees in response to ethanol exposure. Studies of the genetic factors that influence physiological change differences between individual bees after ethanol exposure may also be possible in future investigations.

Conclusion

In sum, *Apis mellifera* are well-supported comparative subjects. Numerous responses to alcohol have been observed in bees. These include learning, locomotion, and aggression effects. Honey bees have been used in multiple protocols, and have been used in numerous apparatuses. These effects in bees have been seen in other organisms, including humans. Though the results of this experiment did not show a significant effect in body temperature change in bees from alcohol exposure. This study demonstrates a new apparatus that can be used to understand honey bee toxicology in the future.

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