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Quantitative Analysis of Honey Bee Blood-Ethanol Levels Following Exposure to Ethanol Vapors

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The use of invertebrate models has allowed researchers to examine the mechanisms behind alcoholism and its effects with a cost-effective system. In that respect, the honey bee is an ideal model species to study the effects of ethanol (EtOH) due to the behavioral and physiological similarities of honey bees with humans when alcohol is consumed. Although both ingestion and inhalation methods are used to dose subjects in insect EtOH model systems, there is little literature on the use of the EtOH vapor-exposure method for experiments using honey bees. The experiment presented here provides baseline data for a dose EtOH-hemolymph response curve when using EtOH vapor-inhalation dosing with honey bees (*Apis mellifera*). Bees were exposed to EtOH vapors for 0, 1, 2.5, or 5 min, and hemolymph was collected 1 min post EtOH exposure. Hemolymph samples were analyzed using gas chromatography (GC) for hemolymph EtOH concentration. The ethanol-hemolymph level of the bees increased linearly with exposure time. The results provide a dosing guide for hemolymph EtOH level in the honey bee model ethanol-inhalation system, and thus makes the honey bee model more robust.

Keywords: addiction, ethanol, honey bee, inebriator, vapor ethanol

エタノール蒸気に曝露したミツバチの血中エタノール濃度の定量分析

無脊椎動物のモデルを使用することで、研究者はアルコール依存症とその影響の背後にあるメカニズムを、費用対効果の高いシステムによって調べることができるようになった。その点において、ミツバチはアルコールを摂取したときの行動や生理がヒトと似ているため、エタノールの影響を研究する理想的なモデル種である。昆虫の エタノール モデル系では摂取と吸入の両方の方法が被験者への投与に用いられているが、 ミツバチを用いた実験では エタノール 蒸気曝露法の使用に関する文献はほとんどない。この実験では、ミツバチ (*Apis mellifera*) に エタノール 蒸気を吸入投与した場合の、エタノール－血リンパの用量応答曲線の基準となるデータを提供する。ミツバチを 0、1、2.5、5 分間 エタノール蒸気に曝露し、エタノール曝露後 1 分で血リンパを採取した。血リンパサンプルはガスクロマトグラフィー（GC）を用いて血リンパ中のエタノール濃度を分析した。ミツバチの血リンパ中のエタノールレベルはエタノール曝露時間とともに直線的に上昇した。この結果は、ミツバチモデルのエタノール吸入システムにおいて、血リンパ中のエタノールレベルに基づいた投与量の指標を提供し、ミツバチモデルをより頑健なものにするものである。

キーワード：依存症、エタノール、ミツバチ、inebriator、エタノール蒸気

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Análisis cuantitativo de los niveles de etanol en sangre de abejas melíferas tras exposición a vapores de etanol

El uso de modelos animales con invertebrados ha permitido evaluar de manera eficiente los mecanismos que subyacen el alcoholismo y sus efectos. En ese sentido, la abeja melífera es una especie modelo ideal para estudiar los efectos del etanol (EtOH), debido a las similitudes conductuales y fisiológicas de las abejas melíferas con los humanos en el consumo de alcohol. Aunque se utilizan tanto los métodos de consumo como de inhalación para dosificar a los sujetos en los sistemas modelo de EtOH de insectos, existe poca literatura sobre el uso del método de exposición al vapor de EtOH para experimentos con abejas melíferas. El presente experimento proporciona datos de referencia para una curva de respuesta de dosis de EtOH-hemolinfa cuando se utiliza la dosificación por inhalación de vapor de EtOH con abejas melíferas (Apis mellifera). Las abejas fueron expuestas a vapores de EtOH durante 0, 1, 2,5 o 5 minutos, y se recolectó hemolinfa 1 minuto después de la exposición al EtOH. La concentración de EtOH en la hemolinfa de las muestras se analizó mediante cromatografía de gases (GC). Se encontró que el nivel de etanol-hemolinfa de las abejas aumentó linealmente con el tiempo de exposición al etanol. Los resultados proveen una guía de dosificación para el nivel de EtOH en la hemolinfa en el sistema de inhalación de etanol del modelo de abejas melíferas y, por lo tanto, hacen que el modelo de abejas melíferas sea más sólido.

Palabras clave: adicción, etanol, abeja melífera, inebriator, vapor de etanol

Approximately 14.4 million people in the United States have an alcohol-use disorder and suffer from short-term and long-term effects on learning, memory, and coordination skills such as locomotion (National Institute of Health (NIH), 2024). These individuals are at an increased risk for injury or death due to vehicular accidents, suicide, and homicide (National Institute of Health (NIH), 2024). Understanding the underlying mechanisms behind alcohol abuse and alcoholism is important in learning how the brain and metabolic system change in response to alcohol exposure (Haass-Koffler et al., 2020).

Although the invertebrate alcohol animal-models are simpler neurologically than humans, all share similar neurotransmitter systems and processes in response to ethanol (Scholz & Mustard, 2013). Consequently, understanding the cellular, molecular, and physiological mechanisms involved in alcohol abuse and alcoholism is a priority, which is where the invertebrate ethanol model systems hold great promise. The immediate goal is to determine whether the vapor method can be used in future honey bee studies as an alternative to the feeding method. The goal of this experiment is to aid in our understanding of the effects that alcohol inhalation can have compared to ingestion.

There are two methods of alcohol consumption that is used by both humans and in invertebrate research. The traditional method for alcohol consumption in humans is through drinking it; however, a newer method of consumption is through inhaling alcohol at vapor alcohol bars or through using e-cigarettes and other vapor devices with alcohol (Bompas & Parr, n.d.; Le Foll & Loheswaran, 2014; MacLean et al., 2017). The Vaportini is an invention that allows the user to inhale alcohol by creating a vapor and is legally available to adults in all 50 states (Vaportini, 2023). This method bypasses the digestive tract, allowing the individual to feel the effects almost immediately.

The honey bee (*Apis mellifera*), fruit fly (*Drosophila melanogaster*), and nematode (*Caenorhabditis elegans*) are the three most common invertebrate models for addiction research (Søvik & Barron, 2013). These three species have similar biochemical signaling processes as humans and possess characteristics that are advantageous for alcohol research (Søvik & Barron, 2013). Although *C. elegans* is neurologically less complex than the other two animal model systems (Kaletta & Hengartner, 2006), researchers can examine the role of individual neurons and how they relate to learning and addiction. Both *C. elegans* and *D. melanogaster* exhibit simpler social behavior than honey bees (Abramson et al., 2007; Quigley et al., 2018; Zhang et al., 2020). Nevertheless, *D. melanogaster* has a wealth of genetic information that relates biochemistry to development (Tolwinski, 2017), making it a cornerstone of genetic research. Finally, both honey bees and fruit flies are exposed to EtOH in their natural environments (Gibson et al., 1981; Kevan et al., 1988).

Much like humans, honey bees at times naturally consume alcohol through their diet as they forage on fermenting nectar and fruit, which is brought back to the hive (Kevan et al., 1988). There is evidence that different worker caste members are exposed to naturally occurring alcohol (Miler, Opalek, et al., 2021; Miler, Stec, et al., 2021). Honey bees willingly drink high quantities and concentrations of alcohol (Abramson et al., 2009; Sokolowski et al., 2012) and, like humans, demonstrate preferences for specific types of alcohol (bees: Abramson, Sheridan, et al., 2004; humans: Kampov-Polevoy et al., 1997). Further, bees and humans exhibit similar aggression (bees: Abramson, Place, et al., 2004; Giannoni-Guzmán et al., 2014; Stevison et al., 2014; humans: Exum, 2006; Swahn & Donovan, 2005), locomotory responses (bees: Božič et al., 2007; Maze et al., 2006; Mixson et al., 2010; humans: de Carvalho et al., 2021; Marinkovic et al., 2000; Sullivan et al., 1995), and learning changes (bees: Abramson et al., 2000, 2005, 2015; Black et al., 2021; humans: Ambrose et al., 2001; Van Skike et al., 2019) following ethanol consumption. Additionally, honey bees show an adaptive tolerance response similar to humans following the consumption of alcohol (Bennett et al., 1993; Elvig et al., 2021; Miler et al., 2018), and fail to develop conditioned taste-aversion to alcohol (Varnon et al., 2018).

Addiction is thought to share a pathway with learning and complex memory. In that respect, one of the strengths of *A. mellifera* as an EtOH model is studying the effects of alcohol on learning and memory (Collett et al., 1997; Giurfa et al., 1996, 2001; Hill et al., 1997; Srinivasan et al., 1998; Zhang et al., 1999). After consuming high quantities of ethanol over either a short or long period of time humans can suffer from learning (Ambrose et al., 2001; Van Skike et al., 2019) and memory impairments (Boening, 2001; Lee et al., 2009; White, 2003). Alcohol dose-dependent learning impairments are seen as well in honey bees (Abramson et al., 2000, 2005, 2015). One example of a simple learning task where this impairment is seen is during proboscis extension response (PER) experiments where a bee learns the association between an odor-conditioned stimulus (CS) paired with a sucrose unconditioned stimulus (US) (e.g., Abramson et al., 2000). After receiving the paired CS and US the bee should learn to predict the sucrose reward based on the presentation of the odor (Abramson et al., 2000).

The goal of this experiment was to collect data for a dose EtOH-hemolymph response curve when using EtOH vapor-inhalation dosing for honey bees. Research shows that vapor ethanol enters the blood more rapidly than when it is consumed through the digestive system (Ammons & Hunt, 2008a; Cyders et al., 2020; MacLean et al., 2017). When comparing the traditional feeding with the newer vapor method there are advantages and disadvantages to both. Since the bee is inhaling the ethanol in the vapor method, the alcohol is absorbed through the bees' tracheal respiratory route bypassing the bees' digestive system (Ammons & Hunt, 2008a). Ethanol was found in bees' hemolymph one to two days after they ingested ethanol, which was thought to still be in the hemolymph due to inactivity and bees not drawing on stomach reserves (Božič et al., 2007; Maze et al., 2006). Unlike the feeding method, the vapor method does not require as long of a wait before the ethanol is in the bees' hemolymph and is not dependent on bees' activity level.

One disadvantage to the newer vapor method is that there is a lack of literature on the method with honey bees. This method was traditionally used with fruit flies where the flies are typically exposed until they lose consciousness. With this method researchers do not know the exact amount of ethanol the individuals are exposed to unless they run analyses on the hemolymph.

Currently, only two experiments in the United States (Ammons & Hunt, 2008a, 2008b) and three experiments in Poland (Miler, Opalek, et al., 2021; Miler et al., 2018; Miler, Stec, et al., 2021) have used the vapor ethanol-dosing method with honey bees to date. Although it is the common method used with the fruit fly (Cowmeadow et al., 2005; De Nobrega et al., 2020), these pioneering studies with honey bees did not establish a dose response curve (Ammons & Hunt, 2008a, 2008b; Miler et al., 2018; Miler, Opalek, et al., 2021; Miler, Stec, et al., 2021). The information provided here is crucial for understanding how much ethanol is absorbed by the bees when the EtOH vapor-exposure method is used since the exact dose is unknown (unlike feeding EtOH). Thus, the results presented here should be key in integrating EtOH studies using fruit flies, honey bee ingested alcohol, and the emerging honey bee inhalation work.

Methods

Subjects

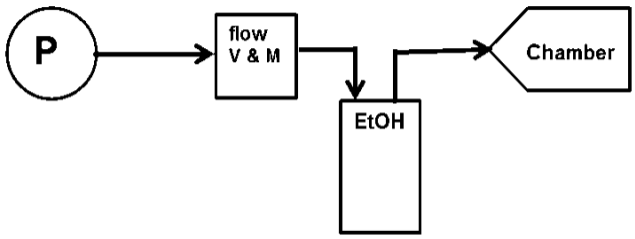
A total of 64 European honey bees (*Apis mellifera*) were collected at a feeder and placed in a cage with bee candy overnight before use in the experiment. The bee candy was used to feed the bees overnight and it was composed of a 60% sugar and 40% honey paste-like mixture in a petri dish that was covered with a piece of cheesecloth. This mixture was removed at midnight, and bees were fasted until the next morning when they were used for the experiment. This ensured that any alcohol they might have consumed when foraging in the environment was out of their systems. All bees that were collected were naïve bees and had not previously been used in experimental work.

Vapor Ethanol Procedure

Honey bees were exposed to EtOH using an inhalation "inebriator" apparatus similar to what Ammons and Hunt (2008a) use with honey bees and Krishnan et al. (2012) use with fruit flies (Figure 1). The apparatus (see Figure 1) consisted of an air pump, tubing, an Erlenmeyer flask, a rubber stopper, a pipette, a two-way splitter, and two test tubes. In this experiment, only ethanol was used for all of the bees in the ethanol concentration groups. Air from a pump (Top Fin Air-2000) was delivered to the test tubes using solvent-resistant tubing. The air supply was split and passed through two flow meters set at 15 ml/min with one air stream used for the ethanol. Both air streams entered a water bubbler comprised of a 250 ml Erlenmeyer flask with a no.10 rubber stopper to humidify the air. The vacuum flask contained a hole in the stopper where a 10 ml pipette could be placed. As the air stream passed through the pipette, the ethanol bubbled and created vapor that passed through the arm of the flask and the tubing into the test tubes. This setup was to prevent condensation on the inside of the holding tube containing the bee. Bees were exposed to 95% ethanol vapers at a flow rate of 1.5 L/min for 0, 1, 2.5, 5 or 7.5 min (treatment groups), after which the bee was transferred to a falcon tube for 1 min before hemolymph was drawn.

Figure 1

Apparatus Schematic for Inhalation EtOH-dosing Used in This Study



Note. P = aquarium air pump; flow V & M = adjustable airflow valve and air flow meter; EtOH = ethanol bubbler.

Hemolymph Etraction Procedures

Bees were sedated by placing them at -18°C for 1.5 min before collecting hemolymph following the method of Božič et al. (2007). Once a bee's timer went off, the bee was placed in a -18°C freezer for 1.5 min or until the bee was fully sedated. Then, the bee was removed from the freezer and a wing was amputated. Using forceps to hold the bee and a glass pipette the researcher collected 1 full microliter of hemolymph. The 1 µl of hemolymph was placed in a 0.5 ml PCR tube with 9 µl distilled water and held at -80°C.

Gas Chromatography Analysis

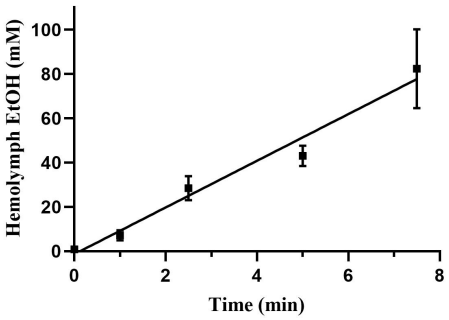
A Shimadzu GC-2014 gas chromatography (GC) analyzer equipped with a Flame Ionization Detector (FID) and a 30 m Restek column (0.25 µM Rtx-Wax) was used to determine bee hemolymph EtOH concentration. The column conditions were isothermal at 80ºC, with a sample runtime of 3 min. Acetone (1 mM) was used as an internal standard and eluted at 2.25 min, while ethanol eluted at 2.67 min. To prepare samples for GC, 5 µL of the bee sample (consisting of 1 µL bee hemolymph and 9 µL water) was mixed with 5 µL of 2 mM acetone in a 100 µL GC vial insert, giving a final acetone concentration of 1 mM and a final dilution factor of the bee hemolymph of 20. On each day of analysis, a calibration curve based on a linear fit of ethanol/acetone standards was used to determine the response factor between the ethanol and acetone peaks.

Results

Hemolymph mM EtOH levels are presented as mean and standard error for each dosing-time in Figure 2. Theoretically blood-EtOH level should be directly related to EtOH dose and dose to vapor inhalation time when using a constant EtOH vaper concentration. Thus, a linear model should predict blood-EtOH level in our experiments (EtOH = a·(Time) + b). The linear-model theory was tested by using the blood-EtOH levels to calculate a least-squares linear regression, and the regression tested for only for significance (slope significantly different from zero), but also for a Lack-of-Fit to a linear model. Both tests are ANOVAs (Draper & Smith 1998; JMP 2024; Montgomery 2001), and we used SAS-JMP software to perform the analysis (JMP 2024). The Lack-of-Fit test was possible because we had a fixed-effect experimental design with a different set of several bees tested at each EtOH dose. The Lack-of-Fit test was used to determine whether a polynomial regression model offers a significantly better fit to the dataset than simply the equation of a line.

Figure 2

EtOH Inhalation-Chamber Time vs Honey Bee Hemolymph mM EtOH.



Note. Mean and standard error are shown, as well as the least squares regression line fit to the data.

A least square linear regression was performed on the data set (N = 64 bees; with n = 12 for each treatment time, except time 5 min (n = 18). The significance of the regression was tested via an ANOVA (Sokal, & Rohlf, 1995) and lack-of-fit of the linear model via another ANOVA (SAS-JMP, 2023). The regression was significant ($F(1,63) = 56.17$, $p < .0001$), while the lack of fit was not significant ($F(3,60) = 0.64$, $p = .59$). Thus, a linear model appears to be a good predictor of blood EtOH level. The linear regression results depicted blood EtOH levels increasing by approximately 10 mM per min the bee was in the EtOH vapor chamber (*regression line*: [mM EtOH] = 10.38·[Time] – 1.58).

Discussion

The results from the bee ethanol-hemolymph levels provide information on the vapor ethanol exposure method. The results from the regression suggest that ethanol exposure accounts for a significant proportion of the variation in the ethanol hemolymph levels for the vapor bees. These findings indicate that there was a positive relationship between the ethanol exposure levels with the ethanol hemolymph concentrations. Similarly, Maze et al. (2006) and Božič et al. (2007) found a positive correlation between the amount of ethanol ingested and the bees' hemolymph ethanol concentrations. However, ingested EtOH must pass through the gastro-intestinal tract of the bee before entering the hemolymph and having a neurologic effect. In fact, Hemolymph EtOH levels rose sharply for approximately 30 min post alcohol ingestion by honey bees before leveling. Further, when bees were fed EtOH, ethanol levels in the hemolymph did not increase linearly with EtOH-dose consumed for the lower percentages of EtOH fed to bees (Božič et al., 2007).

Thus, the advantages of the vapor EtOH dosing method are that there is not a delay in ethanol entering the hemolymph and the fact that hemolymph EtOH levels increase linearly with exposure time. Nevertheless, gastrointestinal stimulus by ingested alcohol may be a component leading to human alcoholism, and this could be explored using a combination of EtOH inhalation and ingestion using the honey bee model. Also, with ingestion the exact quantity of EtOH consumed is known.

It appears that equivalent dosing may be possible via fed-dosing and vapor-dosing using the information we present here. High EtOH dosing via the ingestion-dose method produces quite prolonged EtOH absorption into the bee's hemolymph (Božič et al., 2007; Maze et al., 2006), which should not occur in vapor-dosed bees since this method bypasses the gastrointestinal system. The differences between the absorption by the feeding and vapor bees may prove useful in future studies, and the region of overlap a useful control for future alcohol research.

Conclusion

Our experiment is the first to provide a dose-response curve for the alcohol inhalation method with honey bees. Bee hemolymph EtOH levels rose at about 10 mM per minute bees were exposed to EtOH vapor (linear model). A higher order polynomial [e.g., (Blood-EtOH) = $a\cdot(\text{Time})^2 + b\cdot(\text{Time}) + c$] did not increase the accuracy of predicting blood-EtOH concentration. Thus, our results presented here are truly linear in nature. In comparison, when dosing EtOH via feeding, the result is a second-order polynomial-fit rather than a simple line predicting blood-EtOH level. Božič et al. (2007) show when feeding bees EtOH that: (Blood-EtOH) ≈ $190\cdot(\mu\text{l EtOH})^2 + 40\cdot(\mu\text{l EtOH}) - 0.3$.

In comparison, when dosing EtOH via feeding, the result is a second-order polynomial-fit rather than a simple line predicting blood-EtOH level. Božič et al. (2007) shows when feeding bees EtOH that: (Blood-EtOH) ≈ $190\cdot(\mu\text{l EtOH})^2 + 40\cdot[\mu\text{l EtOH}] - 0.3$.

Use of the regression line provided here for vapor inhaled EtOH and the polynomial of Božič et al. (2007) for feeding EtOH allows dosing comparison between feeding EtOH and inhaling EtOH, and ultimately comparison of ingestion to inhalation EtOH induced behavioral effects. This allows for comparisons of ingestion to inhalation dosing behavioral data. There are several important directions for future research that include addictive effects of inhalation verses ingestion, and key apparatus used by research using the fruit fly model may be key in this area when applied to the honey bee model system.

Fruit fly researchers use an apparatus, an "*inebriometer*," to record when a fly loses balance (Cohan & Hoffmann, 1986; Park et al., 2017; Singh & Heberlein, 2000). Revised versions of this apparatus use automated tracking methods that have a light beam sensor, which automatically records when the fly loses balance (Ogueta et al., 2010). A similar apparatus called the "*inebri-actometer*" is an automated system that uses infrared signals to measure the activity of individual flies (Parr et al., 2001). Additionally, other tracking programs have been used to quantify real-time group activity following ethanol consumption (Ramazani et al., 2007; Scaplen et al., 2019). A future experiment using an *inebriometer* or an *inebri-actometer* like apparatus could provide better insight into the progression of effects on locomotion in honey bees.

Bees will drink large quantities of ethanol even though it negatively impacts their locomotion, learning, and memory (Abramson et al., 2000, 2004, 2005, 2009). Honey bees also show significant levels of tolerance to ethanol in a free-flying study (Stephenson et al., 2021). However, researchers do not fully understand the genetic components of ethanol-seeking behavior. Researchers are starting to understand the ways that ethanol works on the honey bee system, but much is still unknown.

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