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Ethanol levels in honeybee hemolymph resulting from alcohol ingestion

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Abstract

Our previous work on a social insect model of ethanol-induced behavior focused on behavioral studies of honeybees (*Apis mellifera* L.). We now investigate the dependence of honeybee blood ethanol concentration on both the amount of ethanol consumed and time elapsed since ingestion. Blood ethanol level was determined using gas chromatograph using hemolymph taken from harnessed bees. Significantly increased levels of ethanol in honeybee hemolymph were detected within 15 min of feeding bees alcohol. Within 30 min, ethanol concentration increased 2.7 times. The concentration of ethanol ingested also had a significant effect on blood ethanol level. However, postfeeding times greater than 30 min did not significantly increase ethanol concentration in bee hemolymph. This study integrates with our behavioral data on the effect of ethanol on honeybees. Our laboratory and field experiments show a correlation between the time frame for behavioral changes and significant increases of blood ethanol levels shown in this study. © 2007 Elsevier Inc. All rights reserved.

Keywords: Honeybee; *Apis mellifera*; Gas chromatograph; Ethanol; Hemolymph

Introduction

Investigations of social insect models of induced ethanol behavior have concentrated on the behavioral effects of ethanol on honeybees (*Apis mellifera* L.). Previous results from this laboratory have shown several effects that are common among humans and bees. These include the ability to self-administer ethanol and disruptions in both locomotion and Pavlovian conditioning (see Abramson et al., 2007 for a review). Our most recent work has extended the behavioral analysis to more complicated aspects of honeybee behavior. Drinking ethanol will disrupt, for example, both complex decision processes in free-flying forager bees and social communication within the hive (Abramson et al., 2005; Bozic et al., 2006).

We now turn our attention to the physiology of ethanol consumption in the honeybee. The present paper provides some physiological background for the possible action of ethanol through the blood on the central nervous system. We investigate the dependence of blood ethanol concentration on postingestion time and on concentration of ethanol consumed.

In the phylum Arthropoda, some data exist on how ethanol is metabolized in insects. Alcohol dehydrogenize in the fruit fly *Drosophila* is from the type II short-chain dehydrogenises (Benach et al., 2005). It can bind both ethanol and acetaldehyde. Other studies using *Drosophila* suggest that there also exists acetaldehyde dehydrogenize (Fry et al., 2004). However, it remains controversial whether this enzyme contributes to the metabolism of ingested ethanol in fruit flies.

The majority of data on blood ethanol levels and its physiological influence is available from studies using mammals and humans (Grant et al., 2000; Jones et al., 1991; Norberg et al., 2000). Blood concentration of ethanol depends on transport from the digestive track to the blood and catabolism of ethanol in the body. Ethanol conversion to acetaldehyde is catalyzed by alcohol dehydrogenize in these organisms (Sherman et al., 1994; Umulis et al., 2005). Acetaldehyde is then oxidized to acetate by acetaldehyde dehydrogenize.

Materials and methods

Bees preparation and hemolymph sampling

Honeybees (*Apis mellifera* L.) were collected at random as they departed the hive 1 day before an experiment. Each

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bee was placed in a glass vial in an ice water bath. When a bee became inactive, it was immediately removed from the vial and placed into a restraining harness. After regaining consciousness, each subject was fed a 1.5 M sucrose solution (commercial sugar and distilled water) until it would no longer extend its proboscis; the subjects were then left until the following morning (for further details, see Abramson et al., 1997). Prefeeding ensured that all subjects had the same motivation to eat and pass food along their digestive tracks. Different sets of subjects were used for each of the experiments described.

On the following day (postfeeding day), bees were randomly assigned to treatment groups and fed with 10 μl of a test solution. Test solutions were always 1.5 M sucrose with different concentration of ethanol. Solutions were made by diluting 95% ethanol (Pharmco, Brookfield, CT ethyl alcohol, 190 proof) with distilled water to make the final concentrations for feeding bees.

After feeding, a bee was set aside, still in the harness, for the required postingestion period before its hemolymph was sampled. A harnessed bee was briefly narcotized with CO_2 immediately before blood sampling. The bee's thorax cuticle was then punctured at the abdominal side of the wings' base and the blood collected in a 1-μl disposable glass microcap (Drummond Scientific Co). This procedure was successfully used in previous honeybee blood studies (Bozic and Woodring, 1997, 1998), and insures that neither the honey stomach nor the intestine is punctured during hemolymph sampling. The 1.00 μl of hemolymph taken from a bee was mixed with 9.00 μl of a 1-butanol stock solution to afford 10 μl of 0.502 mM 1-butanol solution, which was immediately frozen at −20°C until analyzed by gas chromatography.

Gas chromatography analysis

Ethanol concentration in the honeybee hemolymph was determined by gas chromatography analysis using 1-butanol as an internal standard. A standard curve was prepared with 1.000 mM 1-butanol and 5.000, 1.000, 0.500, 0.100, and 0.050 mM aqueous ethanol solutions resulting in a *R*-squared value of 0.999. The standards and honeybee blood samples were analyzed on a Hewlett Packard 5890 GC equipped with a Flame Ionization Detector (FID), Peak Simple analysis software, and a 30-m J&W Scientific capillary column (0.25 μM DB-Wax). The analysis conditions were isothermal at 80°C for 5 min with 1 ml/min of helium carrier gas and a head pressure of 16 psi.

Experiments

Time dependence

First, we wanted to determine how concentration of ethanol in blood changes over time since consumption. We used 10 μl of a test solution containing 5% ethanol, 1.5 M sucrose to feed bees ($t = 0$ min). This concentration was selected because it is known from our previous work that a 5% ethanol solution will disrupt honeybee behavior under both laboratory and field conditions. Bees were sampled at 15, 30, 60, 120, 240, and 480 min postethanol ingestion (treatments). A control group was fed 10 μl 1.5 M sucrose with no ethanol and sampled 10 min after feeding. For each test treatment, we used six to eight bees ($n = 48$ total). Half the bees for each treatment were tested on day 1 and the remaining tested on day 2.

Concentration dependence

We used a 30 min postethanol ingestion time to test the effect of ingestion of different ethanol concentration on ethanol blood levels. Bees were fed 10 μl of 0, 1, 2.5, and 5% ethanol in 1.5 M sucrose. These are the same concentrations that were used in our behavioral experiments (see review: Abramson et al., 2007). We assigned three to four bees to each treatment on two separate experimental days. Tests were run in parallel for all ethanol food concentrations ($n = 22$ total).

Data analysis

Data were analyzed using a one-way ANOVA with Tukey comparison of mean values. In addition, linear regression was used to show dose dependence of blood ethanol concentrations on concentration of ethanol in food. All statistics were applied using SPSS.

Results

Ethanol was separable based on the gas chromatography protocol used. A linear relation existed between ethanol concentration in standards and levels detected in the gas chromatography analysis (GC-mM = 0.951 Standard-mM + 0.0440; significant of regression $F = 10{,}358.01$, $df = 1.3$, $P < .0001$).

Time dependence

Onset of ethanol effects on the central nervous system of the honeybee is related to the time taken for ethanol to enter the blood. Our shortest experimental time was 15 min. At that time, we find a significant amount of ethanol in the honeybee's blood (24.6 ± 9.2 mM, Fig. 1, ANOVA: $F = 6.11$, $df = 4.21$, $P = .002$; Tukey test). Average ethanol concentration in the blood increased 2.7 times during the next 15 min. Postingestion times greater than 30 min did not significantly increase the ethanol concentration in the blood (average ranged from 64 to 77 mM: Fig. 1). We observed a relatively steady state mean ethanol value for the resting periods longer than 1 h. Based on the slope between 15 and 30 min (slope = 2.75 mM/min) extrapolated to the *x*-axis the estimated delay of onset of ethanol in the honeybee's blood is 6.1 min.

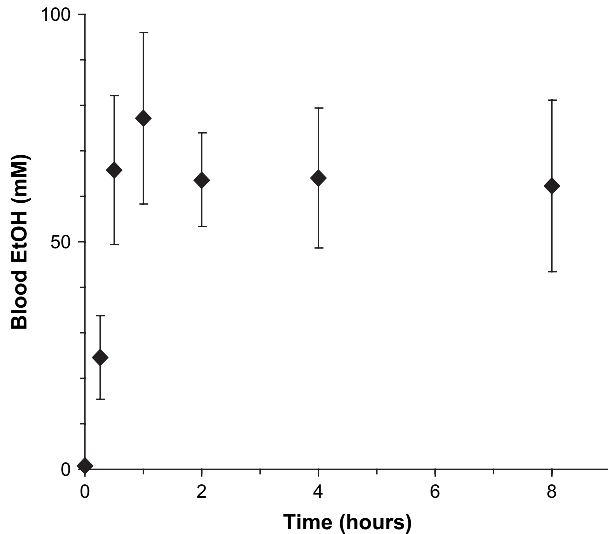

Fig. 1. Effect of resting time after feeding bees with 10 μl of 5% ethanol sugar solution on ethanol blood concentration. Mean values and standard error are plotted for each data point.

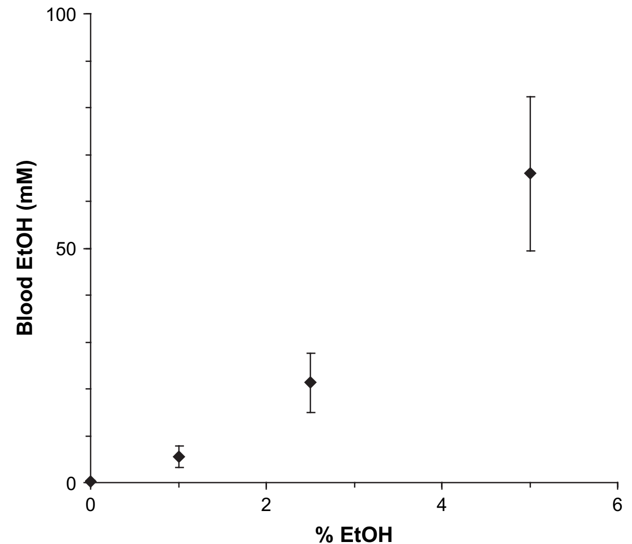

Fig. 2. Effect of concentration of ethanol in sugar solution on blood ethanol concentration 30 min after feeding. Mean values and standard error are plotted for each data point.

Concentration dependence

Ethanol concentrations in food had a significant effect on ethanol concentration in the blood of honeybees 30 min after consumption of the ethanol solution ($F = 7.58$, $df = 3.18$, $P = .0017$). The largest differences were observed at lower concentrations of ethanol in sugar solution (Fig. 2). A positive correlation was found between an increase in blood ethanol concentration and the amount of ethanol consumed ($F = 24.56$, $df = 1.20$, $P < .0001$); amount consumed was equal to the ethanol concentration $\times$ 10 μl droplet.

Discussion

This study correlates well with our behavioral data on the effect of ethanol on honeybees. Our laboratory and field experiments were in the same time frame as the significant increases of blood ethanol level observed in our sample. Just 15 min postingestion there were elevated ethanol levels. In foraging situations, the elevated levels are likely to increase even faster due to the anatomy and physiology of the honeybee. Nectar consumed by a forager is regurgitated from its honey stomach to be processed into honey by hive mates and stored in wax cells for future use. Of course, foragers must use some of the nectar collected for metabolic purposes to stay alive. This occurs by passing nectar from the honey stomach into the intestines where sugar is absorbed (Morse and Flottum, 1990). Nectar flow from honey stomach to intestine is highly regulated in honeybees, and inversely related to hemolymph sugar levels (Rocess and Blatt, 1999). Activities that increase sugar metabolism have been shown to increase the rate at which nectar passes from the honey stomach to the intestine (Rocess and Blatt, 1999). So, higher energetic demands of bees increase flow of nutrients from the honey stomach to the intestine, and from the intestine to the hemolymph. We suspect that ethanol absorption is correlated with that of sugars and, like mammals, increased flow from the stomach to the intestine increases the rate of absorption of ethanol. The key to this flow in honeybees is metabolic demand, which is elevated by flight activity.

In our foraging experiment (Abramson et al., 2005; Bozic et al., 2006), we found dramatic effects of ethanol consumption occurring within 15 min postingestion when an artificial feeder contained ethanol at the foraging site. The frequency of social behavior naturally occurring inside of the hive also changed because of ethanol consumption. Most prominent was disruption of waggle dance communication, which has been shown to correlate with the dopamine system in honeybees (Bozic and Woodring, 1998). Dopaminergic, GABA, and neuropeptide neuron transmission and modulation have been recognized to control in animals centrally generated motor patterns (Marder and Bucher, 2001). A fertile area for future research is the neurophysiologic background of ethanol effects on fixed motor patterns generated by the central nervous system.

In comparison to vertebrate models, ethanol blood concentration was high and remained elevated for an extended period of time (Matthews et al., 2001). These results were reflected in a study that complements the work presented here (Maze et al., 2006). The Maze study used an enzyme assay to estimate ethanol in bee blood. Its focus was higher concentrations of ethanol (5–50%), and looked at blood alcohol levels starting 30 min after ingestion (then at 6, 12, 24, and 48 h). Our study focus was on lower concentrations of ethanol (1–5%) that are more likely to be encountered naturally by honeybees. We also looked more closely at onset (15 min;

then at 30 min, 1, 2, 4 and 8 h), and considered chronic alcoholism in addition to a single meal. As expected, 50% ethanol produces much higher blood alcohol levels (over 100 mM) than does 1% ethanol (approximately 5 mM) in a bee's diet. However, the blood alcohol levels we report for 5% ethanol consumption are consistent with those reported earlier based on the variation among bees observed in both studies (SE bars of the studies meet at about 50 mM). At the lower ethanol concentrations we used, ethanol level in the blood can be seen to be an accelerating function of ethanol amount ingested. This is probability related to the maximum rate a bee can process ethanol (Fig. 2). The Maze study does not report a drop in blood ethanol until 12 h postingestion. The correlation in results from both studies suggests that these results are either artifacts of an analytical technique or artifacts of sampling bees from a particular hive.

With respect to time, notably, both studies show that ethanol remains extremely elevated in the hemolymph for a prolonged period. This is most likely due to the rate of nectar passing from the honey stomach into intestine, which is under control of blood glucose level (Rocess and Blatt, 1999). That is, unlike mammals that deal with excess glucose by sequestering it after it has entered the blood stream, honeybees sequester the sugar by holding it in the honey stomach so that it never reaches the hemolymph (Rocess and Blatt, 1999). Honeybee metabolic rate in resting bees is about 1/25 that of foragers in flight (Rothe and Nachtigall, 1989; Wood et al., 2005), Thus, the nectar flow from the honey stomach during our experimental time would be expected to be low since we used resting bees. Although data do not exist on whether ethanol can be digested before passing into the blood, it has been shown that alcohol dehydrogenase and acetaldehyde dehydrogenase are among the proteins secreted by honeybee feeding glands (Santos et al., 2005). Further, there are several different alleles of alcohol dehydrogenize in honeybees (Martins et al., 1977). The transport model from stomach to intestine links ethanol with sucrose absorption and predicts differences in both blood alcohol levels and duration of inebriation base on metabolic rate and nectar sugar concentration. Obviously, much more work is required in this area and how enzyme system relates to the ecology of honeybees.

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References

Abramson, C. I., Aquino, I. S., Silva, M. C., and Price, J. M. (1997). Learning in the Africanized honey bee: *Apis mellifera* L. *Physiol. Behav. 62*, 657–674.

Abramson, C. I., Sanderson, C., Painter, J., Barnett, S., and Wells, H. (2005). Development of an ethanol model using social insects V: honey bee foraging decisions under the influence of alcohol. *Alcohol 36*, 187–193.

Abramson, C. I., Wells, H., and Bozic, J. (2007). A social insect model for the study of ethanol induced behavior: the honey bee. In R. Yoshida (Ed.), *Trends in Alcohol Abuse and Alcoholism Research* (pp. 197–218). Hauppauge, NY: Nova Science Publishers.

Benach, J., Winberg, J. O., Svendsen, J. S., Atrian, S., Gonzalez-Duarte, R., and Ladenstein, R. (2005). Drosophila alcohol dehydrogenase: acetate-enzyme interactions and novel insights into the effects of electrostatics on catalysis. *J. Mol. Biol. 345*, 579–598.

Bozic, J., Abramson, C. I., and Bedencic, M. (2006). Reduced ability of ethanol drinkers for social communication in honeybees (*Apis mellifera carnica* Poll.). *Alcohol 38*, 179–183.

Bozic, J., and Woodring, J. (1997). Effect of activity on the haemolymph sugar titres in honey bees. *J. Apic. Res. 36*, 33–39.

Bozic, J., and Woodring, J. (1998). Variations of brain biogenic amines in mature honeybees and induction of recruitment behavior. *Comp. Biochem. Physiol. A 120*, 737–744.

Fry, J. D., Bahnck, C. M., Mikucki, M., Phadnis, N., and Slattery, W. C. (2004). Dietary ethanol mediates selection on aldehyde dehydrogenase activity in *Drosophila melanogaster*. *Integr. Comp. Biol. 44*, 275–283.

Grant, S. A., Millar, K., and Kenny, G. N. (2000). Blood alcohol concentration and psychomotor effects. *Br. J. Anaesth. 85*, 401–406.

Jones, A. W., Jonsson, K. A., and Neri, A. (1991). Peak blood-ethanol concentration and the time of its occurrence after rapid drinking on an empty stomach. *J. Forensic Sci. 36*, 376–385.

Marder, E., and Bucher, D. (2001). Central pattern generators and the control of rhythmic movements. *Curr. Biol. 11*, R986–R996.

Martins, E., Mestriner, M. A., and Contel, E. P. (1977). Alcohol dehydrogenase polymorphism in *Apis mellifera*. *Biochem. Genet. 15*, 357–366.

Matthews, D. B., Overstreet, D. H., Rezvani, A. H., Devaud, L. L., and Morrow, A. L. (2001). Effects of sweetened ethanol solutions on ethanol self-administration and blood ethanol levels. *Pharmacol. Biochem. Behav. 68*, 13–21.

Maze, I. S., Wright, G. A., and Mustard, J. A. (2006). Acute ethanol ingestion produces dose-dependent effects on motor behavior in the honey bee (*Apis mellifera*). *J. Insect Physiol. 52*, 1243–1253.

Morse, R. A., and Flottum, K. (1990). *The ABC & XYZ of Bee Culture*, 40th ed., Medina, OH: The A.I. Root Company.

Norberg, A., Gabrielsson, J., Jones, A. W., and Hahn, R. G. (2000). Within- and between-subject variations in pharmacokinetic parameters of ethanol by analysis of breath, venous blood and urine. *Br. J. Clin. Pharmacol. 49*, 399–408.

Rocess, F., and Blatt, J. (1999). Hemolymph sugars and the control of the proventriculus in the honey bee *Apis mellifera*. *J. Insect Physiol. 45*, 221–229.

Rothe, U., and Nachtigall, W. (1989). Flight of the honey bee. *J. Comp. Physiol. B 158*, 739–749.

Santos, K. S., dos Santos, L. D., Mendes, M. A., de Souza, B. M., Malaspina, O., and Palma, M. S. (2005). Profiling the proteome complement of the secretion from hypopharyngeal gland of Africanized nurse-honeybees (*Apis mellifera* L.). *Insect Biochem. Mol. Biol. 35*, 85–91.

Sherman, D. I., Ward, R. J., Yoshida, A., and Peters, T. J. (1994). Alcohol and acetaldehyde dehydrogenase gene polymorphism and alcoholism. *EXS 71*, 291–300.

Umulis, D. M., Gurmen, N. M., Singh, P., and Fogler, H. S. (2005). A physiologically based model for ethanol and acetaldehyde metabolism in human beings. *Alcohol 35*, 3–12.

Wood, W. A., Heinrich, B., and Stevenson, R. D. (2005). Honeybee flight metabolic rate: does it depend on air temperature? *J. Exp. Biol. 208*, 1161–1173.