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Acute ethanol ingestion impairs appetitive olfactory learning and odor discrimination in the honey bee

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Abstract

Invertebrates are valuable models for increasing our understanding of the effects of ethanol on the nervous system, but most studies on invertebrates and ethanol have focused on the effects of ethanol on locomotor behavior. In this work we investigate the influence of an acute dose of ethanol on appetitive olfactory learning in the honey bee (*Apis mellifera*), a model system for learning and memory. Adult worker honey bees were fed a range of doses (2.5, 5, 10 or 25%) of ethanol and then conditioned to associate an odor with a sucrose reward using either a simple or differential conditioning paradigm. Consumption of ethanol before conditioning significantly reduced both the rate of acquisition and the asymptotic strength of the association. Honey bees also exhibited a dose dependent reduction in arousal/attention during conditioning. Consumption of ethanol after conditioning did not affect recall 24 h later. The observed deficits in acquisition were not due to the affect of ethanol on gustatory sensitivity or motor function. However, honey bees given higher doses of ethanol had difficulty discriminating amongst different odors suggesting that ethanol consumption influences olfactory processing. Taken together, these results demonstrate that an acute dose of ethanol affects appetitive learning and olfactory perception in the honey bee.

Keywords

Alcohol; olfaction; *Apis mellifera*; associative learning; proboscis extension response

Introduction

Alcoholism and alcohol abuse are serious problems affecting millions of people worldwide, yet the mechanisms through which ethanol affects motor control, learning and sensory processing are not well understood. Model systems that can provide insight into the effects of ethanol are a valuable tool in the development of new treatments. A number of studies have shown that ethanol affects motor function in invertebrates in a manner that parallels the behavioral effects of ethanol in mammals (Davies, Pierce-Shimomura, Kim, VanHoven,

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Thiele, Bonci, Bargmann, & McIntire, 2003; Maze, Wright, & Mustard, 2006; Moore, DeZazzo, Luk, Tully, Singh, & Heberlein, 1998; Parr, Large, Wang, Fowler, Ratzlaff, & Ruden, 2001). Yet, to date, the assays used for analysis of the effects of ethanol on invertebrate model systems have largely depended on changes in locomotion, whereas addiction may have more in common with the processes involved in learning and memory (Hyman, Malenka, & Nestler, 2006; Kreek, Nielsen, & LaForge, 2004; Nestler, 2002). Previous work in mammalian systems suggests that ethanol may act upon different targets to effect locomotor behavior versus learning. For example, in humans, studies have shown that ethanol will interfere with information processing and decision making at lower concentrations than those required to affect motor function (Hernandez, Vogel-Sprott, Huchin-Ramirez, & Ake-Estrada, 2006; Mitchell, 1985). Thus, investigation of the effects of ethanol on learning and memory may be important for understanding ethanol's targets in the central nervous system and the processes of addiction.

In humans and other mammals, acute ethanol treatment influences acquisition during learning tasks as diverse as delayed match-to-sample tasks, fear conditioning, and maze learning (Bammer & Chesher, 1982; Hernandez, Valentine, & Powell, 1986; Lister, Eckardt, & Weingartner, 1987; Ryback, 1971; White, Matthews, & Best, 2000). However, little is known about how ethanol affects learning in invertebrates. In *Drosophila melanogaster*, flies with mutations in genes involved in olfactory learning and memory such as *amnesiac, rutabaga* and *fasciclinII* also show increased sensitivity to ethanol as determined by the level of ethanol required for the fly to lose postural control in the inebriometer (Cheng, Endo, Wu, Rodan, Heberlein, & Davis, 2001; Moore et al., 1998). Recently further work has characterized the effects of ethanol in a number of mutant fly lines defective for learning and memory (Berger, Kong, Dubnau, Tully, Moore, & Heberlein, 2008; LaFerriere, Guarnieri, Sitaraman, Diegelmann, Heberlein, & Zars, 2008). Using the inebriometer assay, flies with defects in learning and memory were found to have a wide range of ethanol related phenotypes including, both, increases and decreases in sensitivity and tolerance to ethanol (Berger et al., 2008; LaFerriere et al., 2008). These studies suggest a link between the mechanisms underlying learning and memory and sensitivity to ethanol; however, the effect of acute ethanol consumption on associative learning in invertebrates has yet to be studied in depth.

The honey bee can provide a valuable link between studies on the molecular mechanisms of the actions of ethanol and its influence on behavior as the honey bee is an important invertebrate model for understanding the neural and molecular mechanisms of learning and memory (Giurfa, 2003; Menzel, 2001; Müller, 2002). Honey bees can perform sophisticated learning tasks previously thought to be the exclusive domain of vertebrates such as delayed match-to-sample learning (Giurfa, Zhang, Jenett, Menzel, & Srinivasan, 2001), second order learning (Hussaini, Komischke, Menzel, & Lachnit, 2007), stimulus classification (Stach, Benard, & Giurfa, 2004; Wright, Kottcamp, & Thomson, 2008), blocking (Hosler & Smith, 2000), and contextual learning (Gerber & Menzel, 2000). Furthermore, an atlas of the honey bee brain has been developed (http://www.neurobiologie.fu-berlin.de/beebrain/), its genome has been sequenced (Honeybee Genome Sequencing Consortium, 2006), and the molecular mechanisms of learning and memory in honey bees have been well-studied (Eisenhardt, 2006; Müller, 2002). Previous studies have shown that honey bees will readily consume solutions containing ethanol and sugar (Abramson, Stone, Ortez, Luccardi, Vann, Hanig, & Rice, 2000; Maze et al., 2006), and initial studies in the honey bee suggested that ethanol may affect associative learning in invertebrates (Abramson et al., 2000). Furthermore, recent work has established a dose and time dependent response curve for the effects of ethanol on locomotor behavior in the honey bee, demonstrating that ethanol consumption has a profound effect on locomotion including reductions in walking behavior, increased grooming, and a loss of the righting reflex (Maze et al., 2006). Ethanol hemolymph (blood) levels also exhibit dose and time dependent changes after ethanol ingestion; the concentrations necessary to produce inebriation in the

honey bee are similar to the levels observed in other invertebrate model systems and for mammals (Bozic, DiCesare, Wells, & Abranson, 2007; Maze et al., 2006).

In order to develop the honey bee as a model for understanding the effects of ethanol on learning, the experiments described here investigate how acute ethanol treatment influences the ability of honey bees to learn, remember, and differentiate odors in an appetitive learning task. Using an assay developed to study olfactory appetitive learning in honey bees (Bitterman, Menzel, Fietz, & Schafer, 1983), the influence of an acute dose of ethanol on the ability of bees to learn to associate odors with appetitive rewards and to differentiate odors associated with two different reward outcomes was examined. In addition, ethanol's effect on olfactory sensory processing was examined by testing a honey bee's ability to differentiate among perceptually similar olfactory stimuli.

Materials and Methods

Subjects

Forager New World Carniolan honey bees (*Apis mellifera carnica*) were collected from the entrance of colonies maintained at the Rothenbuhler Honey Bee Research Laboratory at Ohio State University or at the School of Life Sciences at Arizona State University. Individual pollen foragers were collected in small glass vials and chilled at 4°C until immobile. Each subject was restrained in a harness comprised of a small piece of drinking straw (approximately 4 cm in length) and a strip of duct tape placed between their head and thorax allowing for free movement of the antennae and proboscis (mouthparts). Each bee was fed 20 µl of 2.0 M sucrose and was left overnight in a humid box (a plastic box with a perforated lid containing wet paper towels) at room temperature before being used in experiments. All conditioning and testing took place under standard lab conditions.

Odors

Odors used for conditioning were 1-hexanol and 1-octanol. Training odor treatments were counterbalanced throughout the experiments. 2-octanone was used as the distinct novel odor. All odors were diluted to 2.0 M in hexane. Chemicals used in the preparation of the odors were of 98% purity from Sigma-Alderich (St Louis, MO, USA). A 5 µl aliquot of odor solution was placed on a small strip of filter paper which was then placed in a 1 ml glass syringe. For odor delivery, the syringe was attached to an air source controlled by a 2-way valve regulated by a Programmable Logic Controller (Automation Direct, Cumming, GA, USA) that produced a 4 s odor pulse as described previously (Wright & Smith, 2004).

Ethanol Treatment

Eighteen to 24 h after collection and restraint, each bee was tested for sensitivity to sucrose by touching its antennae with 1.5 M sucrose solution. Only bees that extended their proboscis when stimulated with the sucrose solution were included in the experiments. Each bee was fed 9 µL of solution containing 1.0 M sucrose (control) or 1.0 M sucrose with 2.5, 5, 10 or 25% ethanol. The sucrose concentration was held constant across treatments. Bees that did not consume the entire treatment were excluded from the experiments. After 2 h, the bees were tested as above with 1.5 M sucrose solution and unresponsive bees were excluded. A 2 h incubation time was used as a previous study showed that by this time both the behavioral effects of ethanol and the concentrations of ethanol in the hemolymph had reached a plateau and were fairly stable for several hours (Maze et al., 2006).

Simple appetitive olfactory conditioning

This experiment examined the ability of honey bees that had consumed 0, 5, 10 or 25% ethanol to learn to associate an odor with a sucrose reward. Each honey bee received eight acquisition trials where a 4 s pulse of odor was paired with a 0.6 µl reward of 1.5 M sucrose 3 s after the onset of odor delivery. The inter-trial interval was 5 min. A bee that learned to associate the odor with the reward would extend its proboscis in response to the odor before its antennae were stimulated with sucrose. "Nonacquiring" bees did not raise their proboscis in response to the odor, but did respond to antennal stimulation with sucrose. Each bee was fed the entire sucrose reward on each trial. At the next inter-trial interval after the final training trial, each bee was tested for its ability to recall the association between the odor and the sucrose reward. Bees were presented with the training odor (e.g.1-hexanol), an odor similar to the training odor (e.g. 1-octanol) and an odor distinct from the training odor (2-octanone) in the absence of reward. Each bee was tested with all three odors in an order that was pseudorandomized across all subjects.

Differential conditioning

This experiment examined if ethanol ingestion influenced the ability of honey bees to discriminate between odors that were differentially rewarded during conditioning. Honey bees were prepared as above, except that solutions containing a lower range of ethanol (2.5, 5, or 10%) were used. Bees were conditioned with four trials of odor paired with a reward of 0.6 µl 1.5 M sucrose (an "A" trial) and four trials where another odor was presented without reward (a "B" trial). The training trials were presented in a pseudorandom order (A B B A B A A B) to prevent the bees from being able to use the pattern of trial presentation to predict when to respond rather than the odor's identity. The inter-trial interval was 5 min. As before, a honey bee was considered to have responded if it extended its proboscis during presentation of the odor but before stimulation of its antennae with sucrose solution. The ability of the honey bees to recall the learned association was tested with unrewarded test trials either immediately after conditioning (at the next inter-trial interval) or 24 h after conditioning. Bees underwent three recall test trials: one to the rewarded odor, one to the unrewarded odor and one to a novel odor. The odors for A and B trials were counterbalanced across bees as either A = 1-hexanol and B = 1-octanol or *visa versa*. 2-Octanone was always used as the novel odor. Test trials were presented in a pseudorandomized order across subjects. Honey bees to be used in the 24 h recall test were fed 18 µL of 1 M sucrose to prevent the bees from starving. After the test trials (either immediately or 24 h later), the bees were fed to satiation with 1 M sucrose solution and the amount each bee consumed was recorded. The test trials for bees in the immediate recall group were recorded using a digital video camera (Panasonic) for measurement of the duration and latency of the proboscis extension response. Movies were downloaded to a Macintosh G4 computer and the responses were analyzed using iMovie (Apple Inc. Sunnyvale, CA).

Testing consolidation and recall

To test the effects of ethanol on consolidation and recall, honey bees were first trained using differential conditioning as described above and then given a dose of ethanol prior to testing for recall of the rewarded odor. After conditioning, each bee was fed 9 µL of a 1 M sucrose solution containing either no ethanol (control) or 2.5, 5 or 10% ethanol. Each bee was tested for its response to the rewarded and the unrewarded odors either 3 or 24 h after receiving an ethanol dose. Three hours was chosen as it corresponds to the same time as the immediate test when bees were given ethanol before training (two hour incubation plus one hour for conditioning). Previous work that measured ethanol levels in the hemolymph of bees under the same conditions used here revealed that significant levels of ethanol were maintained in the hemolymph for 12 to 24 hrs after consumption of an ethanol dose (Maze et al., 2006). Bees to be tested for recall the next day were fed 10 µL of 1 M sucrose and left overnight. Bees tested

at 24 h for their responses to the rewarded and unrewarded odors should have recovered from the ethanol treatment at this time.

Sucrose response threshold

Honey bees were restrained and fed as described above. Eighteen to 24 h later, bees were fed 9 μL of 1 M sucrose solution containing either no ethanol (control) or 2.5, 5, 10 or 25% ethanol. Bees that did not consume the entire dose were excluded from the study. Two hours after consumption of the ethanol, the sucrose response threshold (the minimum concentration of sucrose applied to the antennae necessary to produce proboscis extension) was determined as described previously (Page, Erber, & Fondrk, 1998). Briefly, the antennae of each bee were stimulated by a series of solutions containing increasing amounts of sucrose (0.1, 0.3, 1, 3, 10 and 30%). After each stimulation with a sucrose solution, the antennae were stimulated with water. The inter-stimulus interval was 2 to 3 min. A positive response was recorded when a honey bee extended its proboscis in response to stimulation of its antennae with either sucrose solution or water.

Data analysis

The response variable (proboscis extension) evaluated during conditioning, testing, and sucrose sensitivity was scored as a binary variable; these data were analyzed in a logistic regression (lreg) using the statistical software, SAS (PROC GENMOD) with *post hoc* multiple comparisons (mc). In the figures, the means are reported as the probability of responding along with the estimated standard errors of this probability (Sofroniou & Hutcheson, 2002). For the acquisition data, models were tested using 2-way interaction terms including ethanol treatment vs. conditioning trial number to test whether there were differences in the rate of acquisition. The latency and duration to proboscis extension data were analyzed using a generalized linear model in SAS (PROC GLM). In the figures, significance is indicated as: * = $P < 0.05$; ** = $P < 0.001$.

Results

Does ethanol affect acquisition during associative olfactory learning?

Ethanol administered to a honey bee two hours prior to conditioning affected its ability to learn to associate odor with a food reward. In fact, the rate of acquisition (i.e. the increase in the probability of responding during conditioning) varied in a dose dependent manner (Figure 1A, lreg, 2-way interaction: $\chi_3^2 = 123$, $P < 0.001$), such that honey bees consuming the most ethanol exhibited the lowest rate of acquisition of the learned association. The greatest rate of change in the probability of eliciting proboscis extension was observed over the first three trials (Figure 1A). At this point, approximately 91% of the 0% ethanol treated bees (control) were able to learn to associate an odor stimulus with a 1.0 M sucrose solution reward. In contrast, honey bees that had consumed ethanol learned at a much slower rate: by the third trial, only 52% of the 5% treated bees, 28% of the 10% treated bees, and 3% of the 25% treated bees responded.

As well as a significant reduction in the rate of acquisition, the asymptotic strength of the association, reflected in the point at which the probability of responding ceased to increase during conditioning, was lower on average for honey bees that had consumed ethanol (Figure 1A, lreg, main effect, $\chi_3^2 = 259$, $P < 0.001$). This difference was due in part to the fact that a larger proportion of the honey bees that had ingested ethanol prior to conditioning did not respond to the presentation of the odor on any of the trials (Figure 1B, lreg, main effect: $\chi_3^2 = 82.6$, $P < 0.001$), in spite of the fact that stimulation with a sucrose solution on the antennae elicited proboscis extension in these same bees. To test to what extent these non-acquiring honey bees influenced the data, bees that did not respond on any conditioning trial were removed from the analysis (data not shown). Ethanol treatment still affected the rate of

acquisition during the first three trials (lreg, 2-way interaction: $\chi_3^2 = 28.1$, $P < 0.001$), but the responses of the bees in the 5% ethanol treatment group were no longer significantly different from those receiving the control treatment (mc, $P = 0.249$). Bees in the 10 or 25% ethanol groups, however, acquired the learned association at a slower rate than the control bees during the first three conditioning trials (mc, $P < 0.001$).

After conditioning, the ability of the honey bees to recall the association was tested by presenting the conditioning odor (CO) without reinforcement. The proportion of honey bees that responded to the CO during the test immediately following eight forward paired conditioning trials was significantly lower for all bees that had consumed ethanol (lreg, main effect: $\chi_3^2 = 121$, $P< 0.001$) than the control bees (light bars in Figure 1C).

Does ethanol consumption affect performance during differential olfactory learning?

A large proportion of the bees (approximately 80%) given the highest dose of ethanol (25%) did not respond to the odor during conditioning making it difficult to determine if they could perform the learning task. Therefore, the 25% ethanol group was omitted from later experiments and a lower dose (2.5%) was included. Differential conditioning allowed for assessment of both, whether or not ethanol treated honey bees could learn to associate odor with reward, and whether ethanol affected the ability of bees to associate two odors with different outcomes. The ability of a honey bee to respond selectively to the rewarded odor during differential conditioning depended upon the ethanol dose consumed (Figure 2A, lreg, 2-way interaction: $\chi_3^2 = 10.8$, $P = 0.013$). While all of the bees in the experiment were capable of performing differential learning ($P < 0.001$ for all within-treatment comparisons of the rewarded and unrewarded acquisition curves), the point at which the probability of responding became significantly different during conditioning depended upon the ethanol treatment consumed prior to conditioning. Honey bees conditioned after consuming the control, 2.5 or 5% ethanol solutions were capable of differentiating the rewarded odor from the unrewarded odor by the second trial ($P < 0.001$) whereas bees that had consumed the 10% ethanol solution did not differentiate the two odors until the third trial (trial 2: $P = 0.258$, trial 3: $P = 0.026$) indicating that it was more difficult for the bees in the 10% group to perform differential learning.

As with simple conditioning, honey bees that had consumed ethanol prior to conditioning exhibited a lower probability of responding to the odor on any of the conditioning trials (for either the rewarded or unrewarded odor) than the control bees (Figure 2B, lreg main effect: $\chi_3^2 = 75.2$, $P < 0.001$). This relationship was also dose dependent: the proportion of bees that did not respond to the odor on any trial increased as a function of the ethanol dose ingested prior to conditioning (Figure 2B). However, all of the bees included in the experiment responded to stimulation of their antennae and consumed the sucrose reward on each trial. Exclusion of the non-acquiring honey bees from the analysis (as before) showed that the ability to respond selectively to the rewarded odor during differential conditioning still depended upon the ethanol dose a honey bee had consumed (data not shown; lreg, 2-way interaction: $\chi_3^2 = 10.8$, $P = 0.013$). Bees that consumed the control, 2.5 or 5% ethanol treatments responded more to the rewarded odor by trial 2 (mc, all $P < 0.010$), but bees given 10% ethanol did not differentiate the two odors until the third trial (mc, trial 2: $P = 0.234$, trial 3: $P = 0.009$).

To examine the ability of the honey bees to recall the learned association, each bee was given a recall test by presentation of the rewarded odor, the unrewarded odor and a novel odor. As observed for simple conditioning, the proportion of honey bees responding to presentation of the rewarded odor either immediately or 24 h after conditioning was significantly affected by the consumption of ethanol (Figure 2C and 2D). Immediately after conditioning, bees that had consumed the 2.5% ethanol treatment did not differ from the control group in their response to the rewarded odor (mc, $P= 0.780$), but the 5 or 10% ethanol treated bees responded less than

the control bees did to the rewarded odor (mc, 5%: P = 0.031, 10%: P < 0.001). Honey bees that were tested for recall 24 h after differential conditioning showed a similar trend: bees that had consumed the 2.5% ethanol treatment did not differ from the control in their response to the rewarded odor (mc, P= 0.127), but the 5% or 10% ethanol-treated bees responded less to the rewarded odor than the control bees (mc, 5%: P = 0.006, 10%: P < 0.001).

Does ethanol affect consolidation and recall?

The reductions observed during the recall tests for honey bees given ethanol before acquisition could be due either to deficits in learning during acquisition or to ethanol induced defects on the processes of consolidation and recall. To disentangle these possibilities, this experiment was designed to assess whether ethanol administered after conditioning but prior to the recall test affected the ability of bees to respond to the rewarded odor after differential conditioning (Figure 3). No difference in the ability to perform differential learning prior to the ethanol dose was observed between treatment groups (data not shown; for 3hr group: lreg, 2-way interaction: $\chi_4^2 = 6.20$, P = 0.184; for 24 hr group: lreg, 2-way interaction: $\chi_4^2 = 1.33$, P = 0.722). In contrast to the previous experiment, only the bees that had consumed a 10% ethanol dose exhibited a significantly lower probability of responding than control bees during the 3 h recall test (Figure 3A, mc, P < 0.001). At the 24 h test, when the bees had recovered from the effects of ethanol, the probability of responding was not significantly different from the control for any of the ethanol doses (Figure 3B, mc, all P > 0.05).

Does acute ethanol ingestion affect the ability to distinguish odors of different molecular identities?

The effect of ethanol consumption on the ability of honey bees to discriminate among different odors was examined to determine whether an acute ethanol dose affected sensory processing. Specifically, the influence of ethanol on the ability of honey bees to distinguish the CO (e.g. 1-hexanol) from two other odors that differed in perceptual similarity to the CO: a similar odor (SO; e.g. 1-octanol) and a dissimilar odor (DO; 2-octanone) was investigated (Figure 1C). After conditioning with the CO for eight trials, all bees responded more on average to the CO than to the SO or the DO (lreg, main effect: $\chi_2^2 = 8.65$, P = 0.013). Although this pattern of responding was similar across all the ethanol treatment groups (lreg, 2-way interaction: $\chi_6^2 = 8.73$, P = 0.189), multiple comparisons revealed that only bees in the control group responded significantly more to the CO than to the SO or DO (for the 0% group mc P < 0.001); all comparisons of the responses to the CO to those to the SO or the DO were not significantly different for bees in any of the ethanol treatment groups (mc P > 0.05).

The ability of honey bees to discriminate among odors after consuming ethanol was further analyzed by comparing the responses of bees to the rewarded odor, the unrewarded odor and a novel odor after bees had participated in a differential learning task (Figure 2). As with simple conditioning, the proportion of honey bees in each treatment group responding to an odor during the test immediately following conditioning depended upon the ethanol dose they had consumed prior to conditioning and on whether the test odor was the odor associated with reward, no reward, or was a novel odor (Figure 2C, lreg, 2-way interaction: $\chi_2^2 = 8,84$, P = 0.012). Honey bees in the 0, 2.5, and 5% ethanol treatment groups always responded more to the rewarded odor than to the unrewarded odor or to the novel odor. However, the response of the 10% ethanol treatment group did not differ among the test odors indicating that these bees could not differentiate the test odors and/or associate the odor with its expected outcome (reward or no reward).

The effects of ethanol on odor discrimination were also examined during a recall test 24 h after differential conditioning by testing with the rewarded odor, the unrewarded odor and a novel odor (Figure 2D, lreg, 2-way interaction: $\chi_2^2 = 6.20$, P = 0.045). The trend observed with

immediate testing was repeated 24 h later as the control bees and bees given 2.5 or 5% ethanol responded more to the rewarded odor than the unrewarded or novel odor. As with the immediate test, bees treated with 10% ethanol did not show a significantly higher level of response to the rewarded odor than to the other odors suggesting that they could either not discriminate among the test odors or they had not learned to associate the odor with its expected outcome.

Does ethanol ingestion affect a honey bee's gustatory sensitivity to sucrose?

If the consumption of ethanol affected the gustatory sensitivity of the honey bees towards the unconditioned stimulus (sucrose) during conditioning, it is possible that this could, in turn, affect the ability to acquire the learned association of odor and food reward. To identify whether sensitivity to sucrose was altered as a result of ethanol consumption, the sucrose response thresholds of honey bees given ethanol (0, 2.5, 5, 10 or 25%) were determined (Figure 4). For bees in every treatment group, the probability of eliciting proboscis extension increased as a function of the sucrose concentration of the stimulating solution (rpm lreg, 2-way interaction: χ_1^2 =65.8, $P < 0.001$). The sucrose response threshold, or the point at which the probability of responding elicited by the sucrose was significantly greater than that elicited by water, was not affected by the amount of ethanol consumed prior to conditioning (rpm lreg, 3-way interaction: $\chi_3^2 = 7.89$, $P = 0.096$). This threshold occurred between the 1% and 3% sucrose solutions for all treatment groups, as the line for the response to water and the line for the response to sucrose diverge between log (1% sucrose) and log (3% sucrose) for all groups. Gustatory sensitivity to sucrose, therefore, was not affected by ethanol consumption.

Ethanol ingestion did, however, affect the average probability of responding to any gustatory stimulus applied to the antennae during the assay (rpm lreg, main effect: $\chi_1^2 = 23.5$, $P < 0.001$). Honey bees that had ingested an ethanol solution of greater than 2.5% exhibited a dose dependent decrease in the average probability of responding on any trial when compared to control bees (mc, all comparisons: $P < 0.05$). Additionally, a significant proportion of the honey bees that had consumed ethanol simply did not respond to stimulation of their antennae with a sucrose solution (lreg, main effect: $\chi_4^2 = 117$, $P < 0.001$). The effect of ethanol on the probability of eliciting proboscis extension was dose dependent although only the 10 and 25% ethanol treatments were significantly different from the control (mc, $P < 0.001$).

Does ethanol ingestion affect a honey bee's motivation to eat?

To assess if ethanol ingestion affected a honey bee's willingness to consume the reward, and thus each bee's motivation to respond during the recall test, the amount of sucrose reward solution that each bee would willingly consume after the immediate or 24 h recall test was measured for the bees from the differential conditioning experiment (Figure 5). Consumption of ethanol prior to conditioning affected the amount of sucrose that a honey bee would consume in a dose dependent manner (glm, main effect: $F_{3,380} = 7.01$, $P = 0.001$). Honey bees fed 5 or 10% ethanol and tested immediately after conditioning showed a significant decrease in the amount of sucrose solution they would consume compared to control bees (Figure 5A) suggesting that ethanol dose directly influences the motivation to eat. However, for honey bees tested 24 h after conditioning, only the bees that had consumed the 10% solution prior to conditioning showed a significant decrease in the amount eaten compared to control bees (Figure 5B).

Does ethanol ingestion affect motor function during the response to the test odors?

Previous work has shown that the consumption of ethanol strongly influences locomotor behavior in honey bees (Maze et al., 2006). In order to confirm that the differences in the level of response to the odors during the recall tests were due to effects of ethanol on learning and memory and not due to impairment of motor function, the latency (time between onset of odor and proboscis extension) and duration of proboscis extension was determined during the

immediate recall test that followed differential conditioning (Figure 6). Ethanol consumption produced no significant difference in either the latency to (Figure 6A, glm, main effect: $F_{3,78} = 1.89$, $P = 0.705$) or the duration of proboscis extension (Figure 6B, glm, main effect: $F_{3,78} = 25.6$, $P = 0.256$) towards the rewarded odor.

Discussion

Treatment with an acute dose of ethanol impaired the acquisition of learned associations of odors with sucrose rewards by honey bees in a dose dependent manner. Furthermore, ingestion of ethanol had a significant affect on the proportion of honey bees that responded to the odor during conditioning as the proportion of nonacquiring bees increased with increasing ethanol dose. Ethanol may also reduce the motivation of bees to eat as honey bees that had been fed the higher doses of ethanol showed a significant reduction in the amount of reward solution that they willingly consumed. In addition, bees that consumed ethanol had difficulty in distinguishing among odors during a recall test. Under the conditions used, ethanol did not affect gustatory sensitivity or motor function of the proboscis extension response.

Acute ethanol treatment impairs acquisition during olfactory learning

Studies with rodents in many learning paradigms including eye blink conditioning (Hernandez & Powell, 1986), contextual and cued fear conditioning (Gulick & Gould, 2007), and aversive olfactory learning (Pautassi, Melloni, Ponce, & Molina, 2005) have shown that acute ethanol treatment impairs the acquisition of learned associations. Our results demonstrate a similar effect in honey bees as ingestion of ethanol prior to conditioning affected the ability of honey bees to learn to associate an odor with a sucrose reward. The ethanol induced deficits were dose dependent so that bees that had consumed the highest doses exhibited the greatest impairment in the rate of learning and also had difficulties in discriminating between two odors. Ethanol could be disrupting acquisition in one or more of the following ways: 1) consumption of ethanol could affect a honey bee's ability to taste sucrose thereby decreasing its sensitivity to the reward; 2) ethanol could affect the motivation state of the honey bee reducing its response level to the conditioned stimulus; 3) ethanol could disrupt the neural or molecular mechanisms underlying associative learning.

If ethanol reduced the sensitivity of honey bees to sucrose stimulation, then this would affect the perceived quality of the reward during conditioning reducing the proportion of the bees responding during acquisition (Scheiner, Erber, & Page, 1999; Scheiner, Kuritz-Kaiser, Menzel, & Erber, 2005). However, this is unlikely to explain the observed ethanol dependent reduction in acquisition as consumption of ethanol did not affect the sucrose response threshold indicating that ethanol does not interfere with the ability of a bee to perceive sucrose. On the other hand, honey bees that had ingested medium to high levels of ethanol showed a reduction in the amount of sucrose reward solution that they would willingly consume after conditioning. For bees ingesting the highest dose tested (10%), this effect persisted even 24 h after ingestion.

The observed reduction in the consumption of reward suggests that ethanol may be influencing acquisition by affecting the perceived state of hunger. Previous studies have shown that honey bees which have been recently fed sucrose solution are less likely to acquire an association during olfactory conditioning than bees that have been starved (Friedrich, Thomas, & Müller, 2004). Thus, the physiological mechanisms that mediate food intake may also modulate learning. Although the mechanisms are presently unknown, two modulators of food intake, insulin and neuropeptide F (NPF), have both been linked to ethanol sensitivity in *Drosophila*: fruit flies with reduced insulin signaling (Corl, Rodan, & Heberlein, 2005) or increased NPF signaling (Wen, Parrish, Xu, Wu, & Shen, 2005) exhibit greater sensitivity to ethanol (expressed as an increase in sedation in a locomotion assay). This pattern has also been observed in mammals, as mice with defects in neuropeptide Y (NPY) signaling (the mammalian

ortholog of NPF) exhibit more resistance to sedation and can consume greater quantities of ethanol than control mice (Thiele, Marsh, Ste Marie, Bernstein, & Palmiter, 1998)

In addition, ethanol could also potentially disrupt the ability to acquire a learned association by acting directly on neurotransmission. Recent studies have shown that ethanol interacts directly with multiple proteins including NMDA receptors (Ren, Salous, Paul, Lipsky, & Peoples, 2007; Ronald, Mirshahi, & Woodward, 2001), $GABA_A$ receptors (Wafford, Burnett, Dunwiddie, & Harris, 1990), and glycine receptors (Mascia, Machu, & Harris, 1996). Studies of the effects of ethanol on fear conditioning in rodents have suggested that one way ethanol may exert its affect on learning is via NMDA receptors in the hippocampus (White et al., 2000). Using a form of fear conditioning where odor is paired with an electric shock, it has been shown that NMDA receptors play important roles in aversive olfactory learning in *Drosophila* as a reduction in expression of NMDA R1 receptor reduces learning and memory consolidation (Xia, Miyashita, Fu, Lin, Wu, Pyzocha, Lin, Saitoe, Tully, & Chiang, 2005). NMDA and $GABA_A$ receptors are also found in the honey bee brain (Barbara, Zube, Rybak, Gauthier, & Grünewald, 2005; Grünewald & Wersing, 2008; Zannat, Locatelli, Rybak, Menzel, & Leboulle, 2006), so it is possible that the effects of ethanol on olfactory conditioning in the honey bee are also due to ethanol's direct interaction with these receptors.

As well as affecting behavior via direct interaction with specific proteins, ethanol can influence behavior and learning by affecting neuromodulator signaling pathways. For example, ethanol consumption in vertebrates leads to the release of dopamine (Phillips & Shen, 1996; Tupala & Tiihonen, 2004), and ethanol exposure has been shown to affect dopamine signaling in *Drosophila* (Bainton, Tsai, Singh, Moore, Neckameyer, & Heberlein, 2000; Lee, Kim, Dunning, & Han, 2008). In honey bees and *Drosophila*, dopamine signaling is involved in aversive olfactory learning (Kim, Lee, & Han, 2007; Schwaerzel, Monastirioti, Scholz, Friggi-Grelin, Birman, & Heisenberg, 2003; Vergoz, Roussel, Sandoz, & Giurfa, 2007) and appetitive olfactory learning (Kim et al., 2007). Furthermore, recent work suggests roles for dopamine in attention and arousal (Andretic, van Swinderen, & Greenspan, 2005; Kume, Kume, Park, Hirsh, & Jackson, 2005) and in determination of the salience of specific cues (Zhang, Guo, Peng, Xi, & Guo, 2007). In mammals, one of ethanol's primary effects is to disrupt neurotransmission in the dopaminergic neurons in reward pathways (Nestler, 2005). Similarly, ethanol could impair appetitive olfactory learning by affecting the octopaminergic reward pathways in the honey bee brain (Hammer & Menzel, 1998). However, the effect of ethanol on octopamine signaling in insects has yet to be investigated.

Ethanol consumption does not affect consolidation and/or recall

The reductions in response observed during the recall tests for honey bees treated with ethanol before conditioning could be due to ethanol induced effects on acquisition, or on the processes of memory consolidation and/or recall. However, when bees were conditioned first and then fed ethanol, only bees given the highest dose of ethanol showed a reduction in levels of response to the rewarded odor. Thus, when under the influence of ethanol, only bees given high doses of ethanol had difficulty remembering something they had learned while sober. The observed reduction in response for the 10% group could have been due to the effects of ethanol on the formation of the 3 hr memory, on the ability of the bees to recall the information, or due to effects on both memory formation and recall. When tested 24 h after conditioning, when the bees would have returned to a sober state (Maze et al., 2006), the level of response to the rewarded odor was the same for bees in all treatment groups. Thus, ingestion of ethanol did not affect the formation of a 24 hr memory. These results suggest that the main effect of ethanol treatment is on acquisition of the association rather than on consolidation and/or recall. A similar effect is observed in humans, as ethanol consumption appears to affect acquisition as an acute dose of ethanol impairs the ability of people to remember information presented while

they were intoxicated, but does not interfere with recall of information that was learned prior to consumption of ethanol (Lister et al., 1987; Ryback, 1971). Furthermore, in experiments with rodents, treatment with ethanol immediately after conditioning does not impair response to the conditioned stimulus (Bammer & Chesher, 1982; Gould & Lommock, 2003; Gulick & Gould, 2007). Taken together, these results show that in the honey bee, humans and rodents, ethanol consumption has a significant effect on acquisition, and suggests that the action of ethanol on processes involved in associative learning may be conserved amongst these species.

Ingestion of ethanol affects the level of arousal and/or attention

One of the largest effects of ethanol in this study was the dose dependent reduction in the number of bees that responded to a stimulus. This was observed both, in regard to response to odor presentation during conditioning, and in the low level of response to sucrose stimulation of the antennae during the sucrose response threshold assay. Nonacquiring bees still responded to antennal stimulation with sucrose and consumed the reward during each training trial; however, they did not ever extend their proboscis in response to the odor presentation during conditioning.

One possibility for the reduction in responsiveness to gustatory or olfactory stimulation is that ethanol reduced the overall level of arousal and/or attention in honey bees in a dose dependent manner. Arousal and attention are processes important in cognition that may be affected by ethanol consumption (Givens, 1997; Givens & McMahon, 1997; Koelega, 1995; Lister et al., 1987; Little, 1999). Arousal, viewed in the very general sense as the responsiveness of the animal to its environment, varies from bouts of low arousal (sleep) to high arousal during complex behavioral tasks. In insects, the level of arousal is often measured behaviorally by changes in locomotion, with a decrease in time spent walking indicating a reduction in arousal (van Swinderen & Andretic, 2003). Thus, the ethanol induced decrease in locomotion (sedation) observed in flies, nematodes and honey bees may, in part, reflect a reduction in arousal (Maze et al., 2006; Morgan & Sedensky, 1995; Rothenfluh & Heberlein, 2002; Wolf, Rodan, Tsai, & Heberlein, 2002). As well as affecting general responsiveness, ethanol may also influence the ability of subjects to pay attention to relevant stimuli (Givens, 1997; Givens & McMahon, 1997; Koelega, 1995; Lister et al., 1987). Recent work has shown that attention-like processes also occur in insects (Heisenberg, Wolf, & Brembs, 2001; Menzel & Giurfa, 2006; van Swinderen & Flores, 2007). Therefore, it is possible that the observed dose dependent reduction in response to odor with ethanol treatment is due to ethanol's effects on attention and/or arousal.

Ethanol and sensory processing

In addition to its effects on the mechanisms underlying learning and memory, ethanol may also compromise sensory processing and, thus, affect performance during discrimination tasks. Although ethanol ingestion did not influence gustatory threshold levels, higher concentrations of ethanol did affect olfaction. Different sensory systems have been shown to have different sensitivities to ethanol. For example, in humans processing of visual information is more sensitive to ethanol than auditory or tactile information (Hernandez et al., 2006). Evidence from studies of ethanol's influence on the visual system suggest that in humans high ethanol doses slow down or impair neural functions which rely on temporal integration of information from populations of neurons (Khan, Ford, Timney, & Everling, 2003; Krull, Smith, & Parsons, 1994; Maylor, Rabbitt, James, & Kerr, 1992; Pearson & Timney, 1998). Ethanol affected the ability of honey bees to differentiate odors during a generalization task following simple conditioning at all the doses greater than or equal to 5%. While honey bees given lower doses could still perform differential conditioning, indicating that when pressed they could discriminate the odors, those which consumed a high dose (10%) had greater difficulty in differentiating odors both during conditioning and testing.

The results suggest that ethanol interferes with olfactory sensory processing, perhaps by disrupting acetylcholine (ACh) and/or GABA dependent processes involved in encoding olfactory information in the antennal lobe. The insect antennal lobe is the functional analog of the vertebrate olfactory bulb, and they share many structural and physiological features (see Kay & Stopfer, 2006 for a review). ACh is a major neurotransmitter in the honey bee antennal lobe (Bicker, 1999) and adult honey bees express nicotinic acetylcholine receptors in their antennal lobes (Barbara, Grunewald, Paute, Gauthier & Raymond-Delpech, 2008). Mammalian nicotinic acetylcholine receptors may be either potentiated or inhibited by ethanol, depending on the subunit composition (Cardoso, Brozowski, Chavez-Noriega, Harpold, Valenzuela & Harris, 1999); however, the effects of ethanol on insect nicotinic acetylcholine receptors is currently unknown. In olfaction, temporal coding of olfactory information proceeds as a result of the activity of populations of neurons in the antennal lobe whose activity is synchronized by GABA-ergic inhibitory local interneurons (Friedrich & Laurent, 2001; Laurent, Stopfer, Friedrich, Rabinovich, Volkovskii, & Abarbanel, 2001; Mazor & Laurent, 2005; Stopfer, Jayaraman, & Laurent, 2003). A study using the same odors and protocols as were used in this work demonstrated that when the $GABA_A$ receptors of these neurons are blocked, honey bees fail to differentiate odors in a similar manner as we observed for ethanol (Stopfer, Bhagavan, Smith, & Laurent, 1997). Although ethanol acts to enhance the function of $GABA_A$ receptors rather than block them (Allan & Harris, 1986; Suzdak, Schwartz, Skolnick, & Paul, 1986; Ticku, Lowrimore, & Lehoullier, 1986), ethanol may disrupt olfactory processing by affecting the spike timing of the projection neurons that encode information about odor identity (Wilson & Laurent, 2005).

The actions of ethanol are dose dependent

Honey bees that received an acute dose of ethanol showed defects in acquisition, reduced levels of arousal and difficulty distinguishing between odors. All of the effects of ethanol were dose dependent: 2.5% ethanol had significant effects on arousal/motivation during acquisition, but did not significantly affect the other aspects of learning investigated here; 5% ethanol affected arousal, acquisition and motivation for consuming the reward immediately after conditioning, but not olfactory processing or recall and/or consolidation; and bees given the 10% dose of ethanol additionally showed deficits in olfactory processing, 3 hr recall/ memory consolidation, and they consumed significantly less reward solution even 24 hrs after receiving their ethanol dose. The dose dependence of the actions of ethanol is not unexpected, as it is known to affect multiple targets (Harris, Trudell, & Mihic, 2008). The results presented here suggest that specific doses of ethanol can be used to investigate its effects on the different processes involved in associative olfactory learning.

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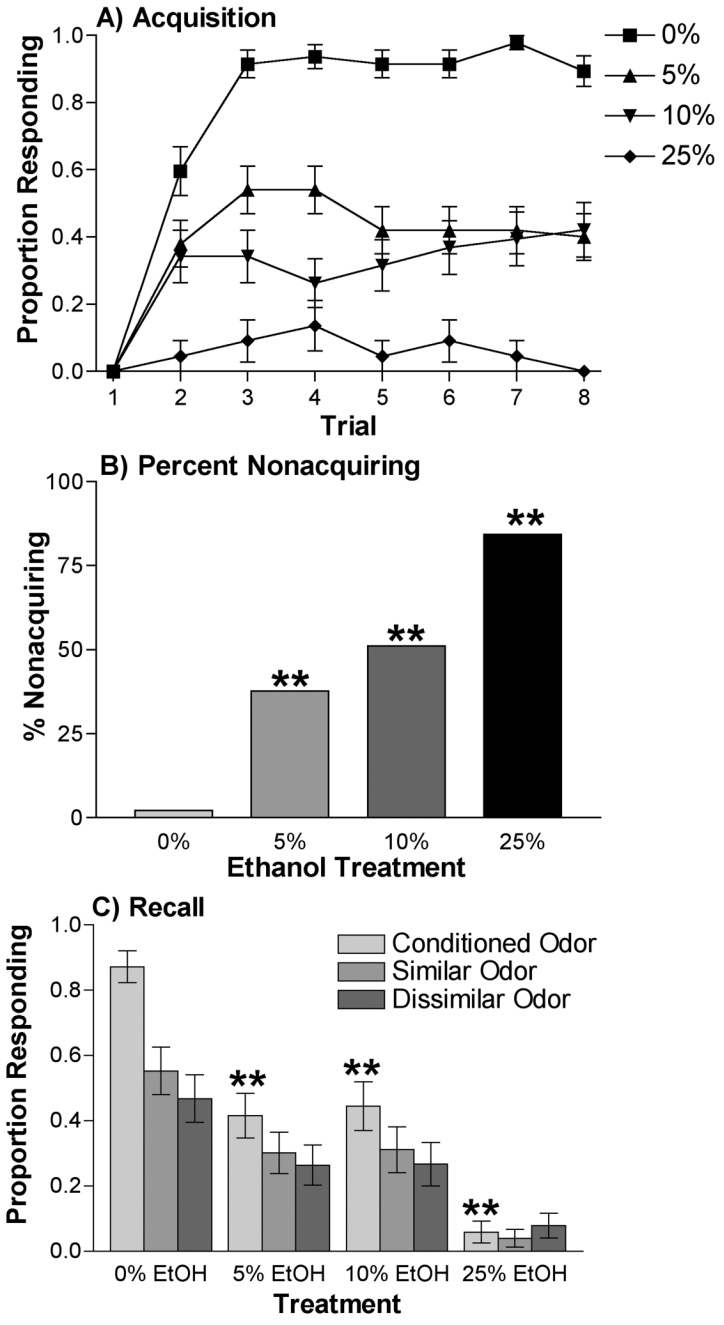

Figure 1.

Ethanol consumption significantly affects both the rate of acquisition and the asymptotic plateau achieved during appetitive olfactory conditioning. (A) The proportion of honey bees responding to the conditioned odor during eight training trials is shown for bees that consumed different doses of ethanol prior to conditioning. (B) The percentage of "nonacquiring" bees in each treatment group that never responded to the conditioned odor before presentation of the reward during conditioning. Consumption of ethanol significantly increased the proportion of bees that did not respond during conditioning. (C) Ethanol ingestion influenced both, the average response to the conditioned odor (CO) and the generalization from the CO to the other test odors. Consumption of ethanol significantly reduced the proportion of bees responding to

the conditioned odor. Only honey bees in the control group were able to differentiate the CO from the similar odor or the dissimilar odor ($P < 0.001$). Bees consuming 5, 10 or 25% ethanol solutions did not respond significantly more to the CO than to the similar or dissimilar odor (all $P > 0.05$). $N_{control} = 47$; $N_{5\%} = 53$; $N_{10\%} = 45$; $N_{25\%} = 51$. (Mc, **$P < 0.001$ compared to control.)

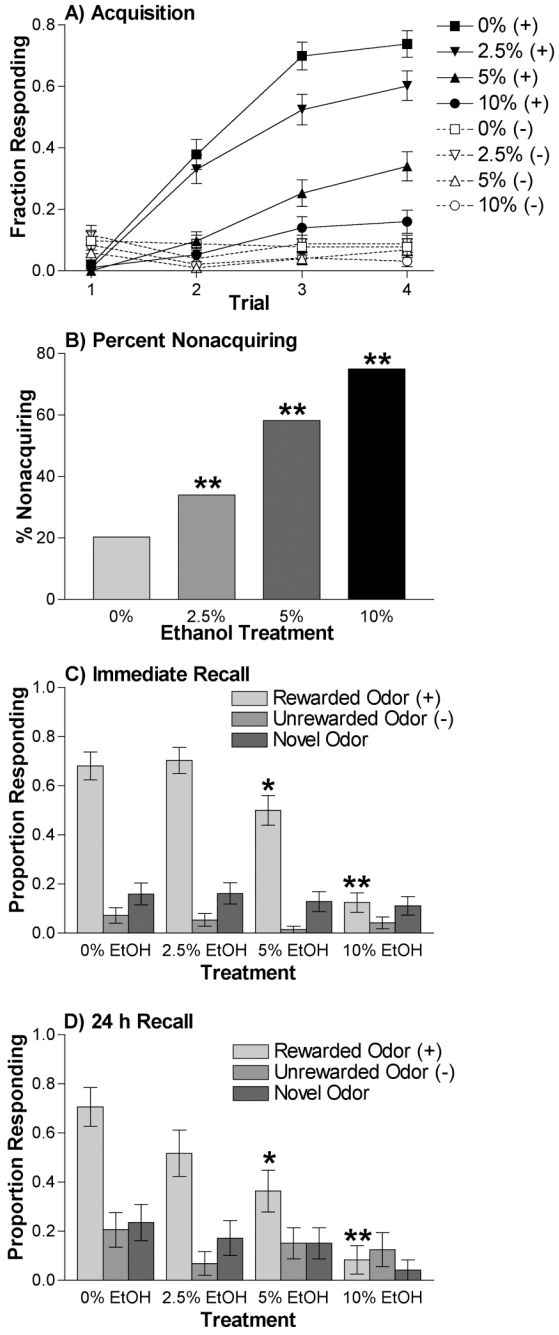

Figure 2.

Effects of ethanol on acquisition during differential conditioning and the proportion of honey bees that respond to the test odors immediately or 24 h after conditioning. (A) The proportion of honey bees that responded to the rewarded (+) or unrewarded (−) odor during conditioning is shown for bees that consumed ethanol prior to conditioning. Honey bees in the 0, 2.5 and 5% treatment groups were able to differentiate the odors by the second trial with each odor, whereas bees given 10% ethanol did not respond differentially to the two odors until the third trial with each odor. (B) The percentage of nonacquiring bees that never responded to odor during conditioning for each treatment group was dependent on the amount of ethanol consumed before conditioning. $N_{control} = 103$; $N_{2.5\%} = 103$; $N_{5\%} = 103$; $N_{10\%} = 96$. A

significantly lower proportion of honey bees that consumed either 5% or 10% ethanol responded to the rewarded odor compared to control bees when tested either immediately (C) or 24 h (D) after conditioning. Honey bees that were treated with sucrose alone or with 2.5 or 5% ethanol could distinguish the rewarded odor from the unrewarded and novel odors at both the immediate and 24 h time points ($P < 0.05$ for all), whereas bees consuming 10% ethanol could not ($P > 0.05$). For immediate recall, $N_{control} = 66$; $N_{2.5\%} = 72$; $N_{5\%} = 68$; $N_{10\%} = 69$; for 24 h recall, $N_{control} = 34$; $N_{2.5\%} = 29$; $N_{5\%} = 33$; $N_{10\%} = 24$. (Mc, * $P < 0.05$, ** $P < 0.001$)

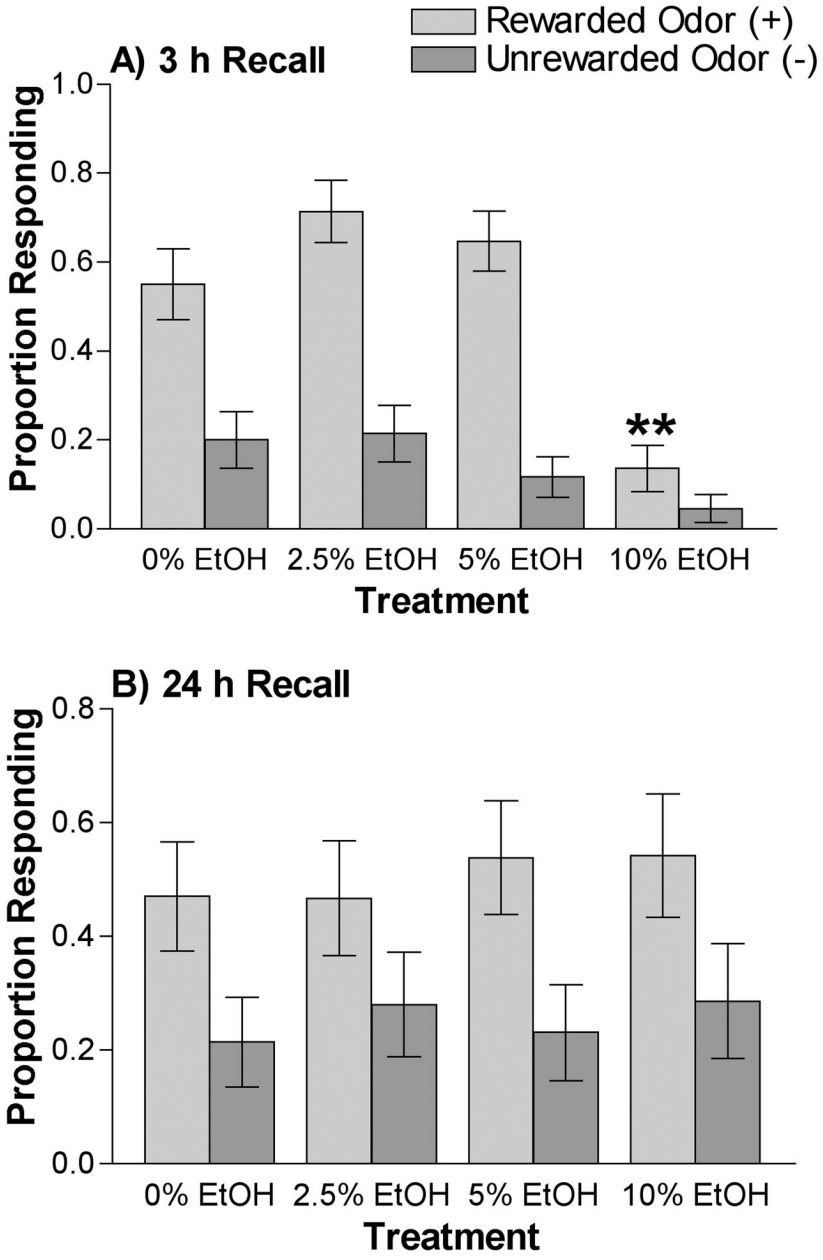

Figure 3.

Effects of ethanol on consolidation and recall. Bees were differentially conditioned to two odors and then fed solutions containing 1 M sucrose and 0, 2.5, 5 or 10% ethanol. The proportion of honey bees responding to the rewarded (+) or the unrewarded (−) odor 3 h (A) and 24 h (B) after consuming ethanol is shown. A significantly lower proportion of bees fed 10% ethanol responded to the rewarded odor 3 h after ethanol consumption compared to control bees. However, bees in all treatment groups responded similarly 24 h later. (**P < 0.001 compared to control.) For 3 h recall, $N_{0\%} = 40$; $N_{2.5\%} = 42$; $N_{5\%} = 51$; $N_{10\%} = 44$; for 24 h recall, $N_{0\%} = 32$; $N_{2.5\%} = 32$; $N_{5\%} = 26$; $N_{10\%} = 24$.

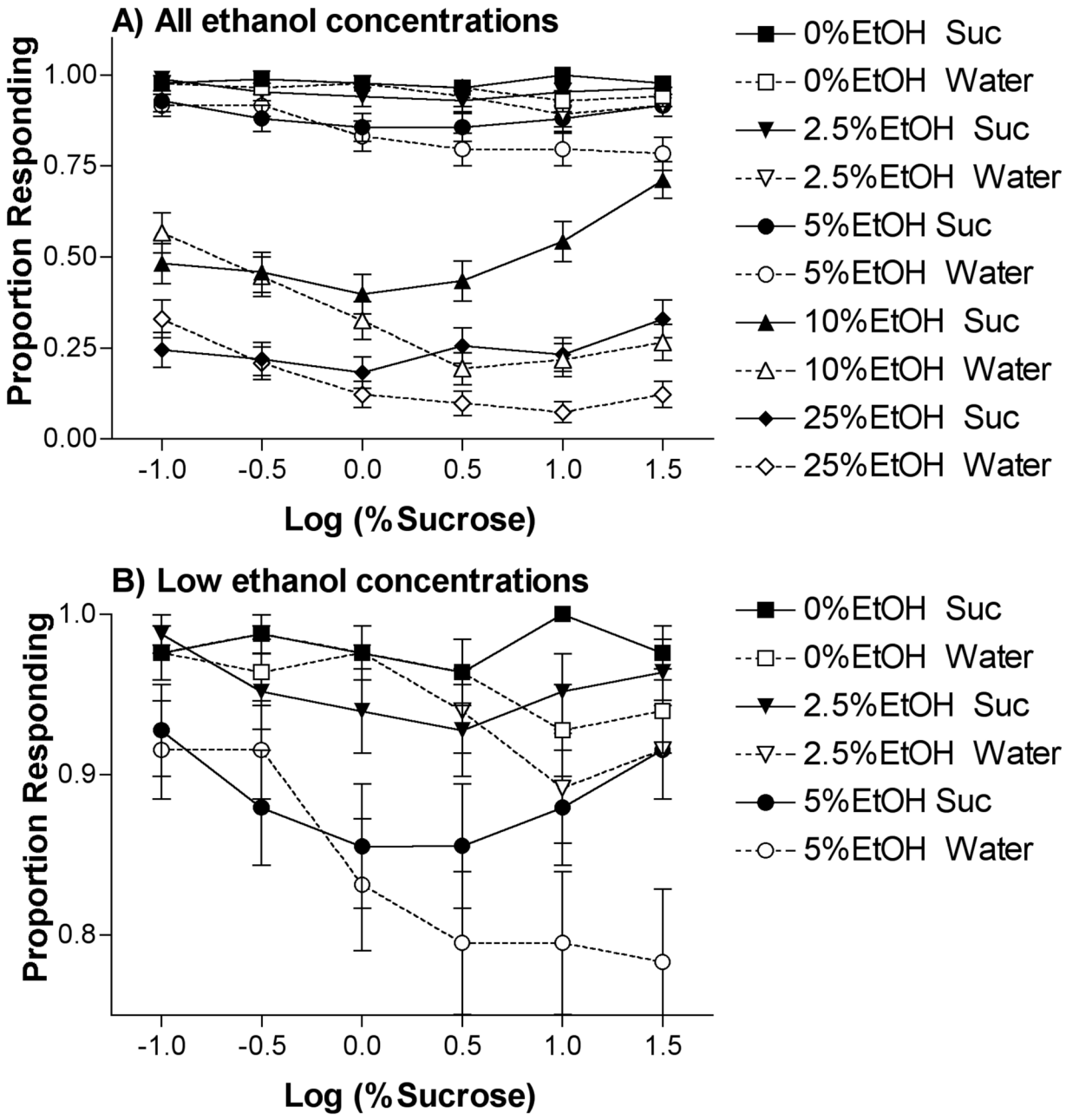

Figure 4.

Treatment with ethanol did not affect the sucrose response threshold. The proportion of honey bees extending their proboscis in response to antennal stimulation with either water or a series of solutions with increasing sucrose (suc) concentrations after ethanol consumption is shown. (A) The sucrose response threshold for bees consuming no ethanol (0% EtOH) compared to those consuming ethanol prior to testing. (B) The proportion of bees responding to water and solutions of increasing sucrose concentration for bees that had consumed no ethanol (0% EtOH), 2.5% or 5% ethanol. These data are the same as in A shown on an expanded scale. Based on the divergence of the water and sucrose response lines for each treatment group, honey bees in all treatment groups could distinguish sucrose solutions of 3% or greater from

water. The proportion of bees that responded during the assay was on average lower for bees fed 10 or 25% ethanol. $N_{control} = 83$; $N_{2.5\%} = 83$; $N_{5\%} = 83$; $N_{10\%} = 83$; $N_{25\%} = 82$.

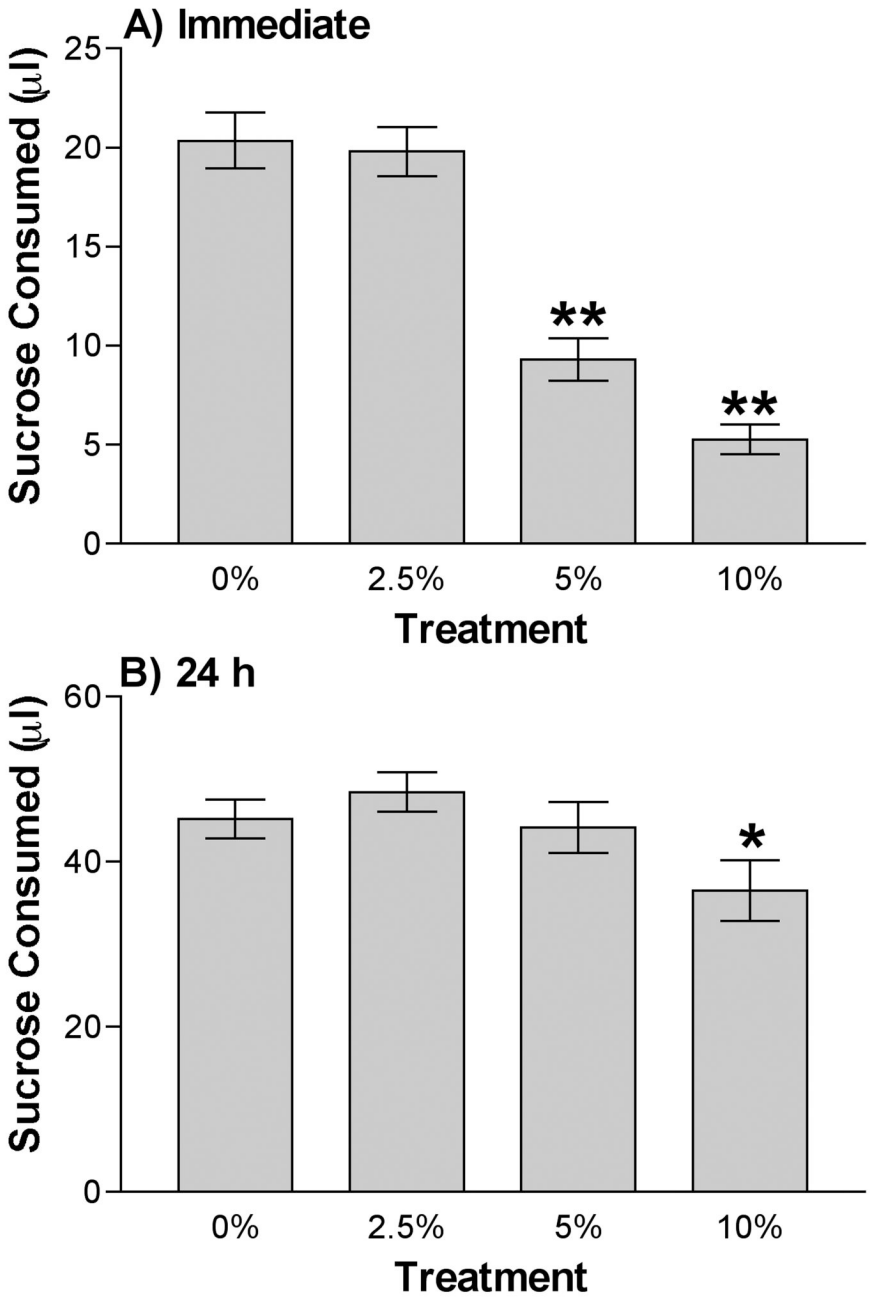

Figure 5.

The amount of sucrose reward solution consumed after testing depended on the ethanol dose honey bees consumed before conditioning. (A) Bees that were treated with 5% or 10% ethanol consumed significantly less than control bees when fed to satiation 3 h after receiving the ethanol dose (fed after the immediate recall test). (B) Only bees that had been treated with 10% ethanol consumed significantly less sucrose solution than control bees 26 h after treatment as determined after the 24 h recall test. Values shown are the mean +/− SEM. (* $P < 0.05$, ** $P < 0.001$). For immediate group: $N_{control} = 66$; $N_{2.5\%} = 72$; $N_{5\%} = 68$; $N_{10\%} = 69$; for the 24 h group: $N_{control} = 34$; $N_{2.5\%} = 29$; $N_{5\%} = 31$; $N_{10\%} = 19$.

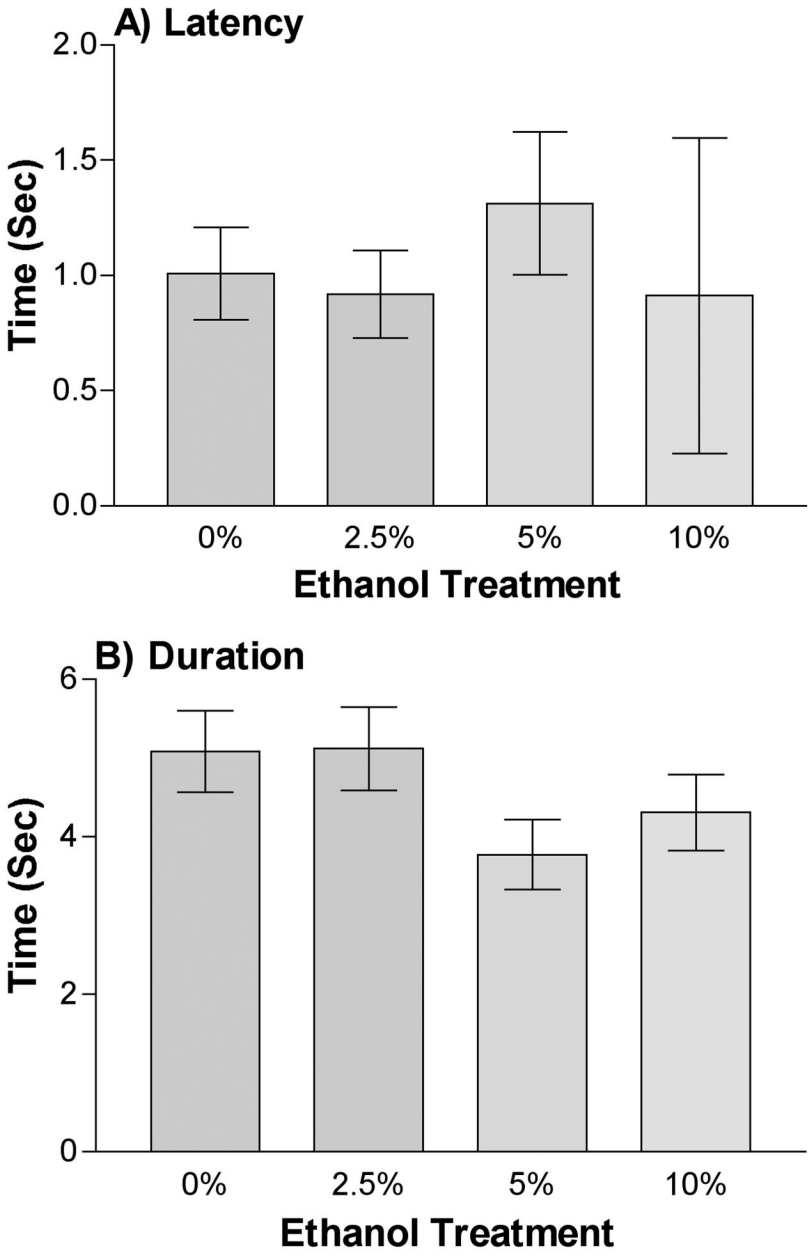

Figure 6.

Consumption of ethanol does not influence the latency to or duration of the proboscis extension response to the rewarded odor during the immediate test. (A) The time elapsing between the onset of odor presentation and proboscis extension (latency) does not differ between control bees and bees consuming ethanol. (B) The duration of proboscis extension during presentation of the rewarded odor does not differ between the control and ethanol treated bees. Values shown are the mean +/− SEM. $N_{control} = 22$; $N_{2.5\%} = 32$; $N_{5\%} = 25$; $N_{10\%} = 7$.