Failure to Find Ethanol-Induced Conditioned Taste Aversion in Honey Bees (*Apis mellifera* L.)

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Supplementary Material

Comparative Review of Conditioned Taste Aversion

Conditioned taste aversion (CTA) learning has been identified in a diverse range of vertebrates including fish (Martin et al., 2011), frogs (Greenlees et al., 2010), snakes (Burghardt et al., 1973), birds (Westbrook et al., 1980; Wilcoxon et al., 1971), marsupials (Webb et al., 2008), rodents (Braveman, 1975; Garcia et al., 1955; Garcia & Koelling, 1966; Kalat, 1975), bats (Terk & Green, 1980), hyenas (Yoerg, 1991), and humans (Arwas et al., 1989; Bernstein & Webster, 1980). Less attention has been paid to taste aversion in invertebrates, though some form of taste aversion has been documented in several taxa including gastropods (Gelperin, 1975; Sadamoto et al., 2000; Yamanaka et al., 1999), cephalopods (Darmaillacq et al., 2004), hermit crabs (Wight et al., 1990), grasshoppers (Bernays & Lee, 1988; Simoes et al., 2016), and honey bees (Wright et al., 2010).

Across species, an important feature of CTA learning is that each species appears to be biologically prepared to associate only certain types of stimuli with illness. Many species-differences are found in the types of stimuli that can support CTAs (Logue, 1979). For example, rats rapidly learn to associate taste and olfactory stimuli with illness, but do not learn similar associations with audiovisual stimuli (Garcia & Koelling, 1966). Conversely, guinea pigs learn to associate both color and taste with illness (Braveman, 1975). The important difference between these two rodents is in their foraging behavior; rats are nocturnal and forage by odor, while guinea pigs are diurnal and use both appearance and odor to forage. Other species with highly specialized diets, like vampire bats, may not display taste aversion at all (Ratcliffe et al., 2003). Thus, the behavioral ecology of the species is a good predictor for the modality of taste aversion learning (Kalat, 1977).

Honey Bees as Model Organisms

The literature clearly shows that ethanol (EtOH) consumption creates adverse effects, in terms of both behavior and health, in a diversity of animal model species (Sommer & Spanagel, 2013) as well as humans (Littrell, 2014; O'Keefe et al., 2014; Schuckit, 2014; Testino, 2008). This is also true for honey bees, making honey bees an ideal insect model for the study of EtOH-induced behavior (Abramson et al., 2007). In restrained procedures, honey bees will consume sucrose solutions containing 2% to 50% EtOH (Abramson et al., 2015; Maze et al., 2006). Ethanol consumption then affects behavior in several ways: impairing odor discrimination (Abramson et al., 2015; Mustard et al. 2008), decreasing locomotion (Abramson et al., 2000; Maze et al., 2006); decreasing the volume and quality of nectar foraging bees bring to the hive (Abramson et al., 2005; Sokolowski et al., 2012); increasing the time between foraging trips (Sokolowski et al., 2012); disrupting social behavior and communication (Bozic et al., 2006;

Wright et al., 2012), and disrupting optimal foraging strategies, leading to random flower choice (Abramson et al., 2005).

Ethanol consumption also effects the physiology of honey bees. First, it has been observed in terms of hemolymph EtOH levels using two different techniques, gas chromatography analysis (Bozic et al., 2007) and a colorimetric test measuring the reduction of nicotinamide adenine dinucleotide (NAD) by alcohol dehydrogenase (Maze et al., 2006). The Bozic et al. (2007) study used 10 µl doses from 0% to 5% EtOH, while the Maze et al. (2006) study used 9 µl dosages from 0% to 50% EtOH. Combined, these two studies produce a complete picture of honey bee hemolymph EtOH levels, measured in millimoles. From 0% to 5% EtOH, the hemolymph EtOH level plateaus at the 30-minute level for 6 to 8 hours before declining. Doses of 10% and 25% EtOH result in hemolymph EtOH levels rising over the 6 hours before showing a slow decline over the next 18 hours. The highest dose, 50% EtOH, causes hemolymph EtOH levels to plateau from 30 minutes to 24 hours, then gradually decline over the next day. Figure S1 shows hemolymph EtOH levels from both studies 30 minutes after bees have consumed EtOH solutions. While the bees are intoxicated, they may show increased heat shock protein expression (Hranitz et al., 2010). At higher doses (25 – 50%), EtOH consumption also increases captive mortality rate (Maze et al., 2006). Ethanol consumption may even have a greater effect on the overall health of a colony, as the colony's health depends on the individual workers' health and efficiency. Because of the many detrimental effects of EtOH, honey bees are expected to have evolved a strong aversion (Søvik & Barron, 2013), and it is reasonable to test if EtOH can induce CTAs.

Honey bees do exhibit some aversions to specific toxins, either as inhibition of feeding response elicited by sucrose, or by reduced consumption of sucrose solutions containing the

toxin. This suggests there may be a neurological foundation for EtOH-induced aversions. Bees have shown aversions to amygdalin (Wright et al., 2010), methionine (Hladun et al., 2012), pesticides (Abramson et al., 2006; Abramson et al., 2010), quinine (Desmedt et al., 2016; Wright et al., 2010), salicin (Desmedt et al., 2016), selenite (Hladun et al., 2012), and toxic honey made from *Tripterygium hypoglaucum* nectar (Tan et al., 2007).

In some experiments, however, it is difficult to conclude if bees are *learning* to avoid toxic substances, if feeding responses are innately inhibited by the toxin, or if overall reduced responding is an effect of the toxin. One notable exception is Wright et al. (2010), which demonstrated that bees were able to discriminate between odors associated with a sucrose and quinine solution and a pure sucrose solution. After several trials, the bees' conditioned feeding response occurred less in response to odors associated with quinine than to odors associated only with sucrose. This suggests the bees were learning to reduce consumption of the toxic substance. However, the bees did not learn to discriminate between odors associated with amygdalin and odors associated only with sucrose, suggesting they may not able to learn to avoid this specific toxin. It is likely that bees do have the ability to learn taste aversion, but that this ability is specific to each substance. In this respect, bees may be similar to humans, which also display substance-specific taste aversion learning (Riley & Tuck, 1985). Wright et al. (2010) suggested that, for bees, difference in response to toxins may be caused by separate neurological pathways. Not only do bees have the dopamine-mediated pathway found in *Drosophila melanogaster* (e.g., Honjo et al., 2009; Schwaerzel et al., 2003) but also a serotonin-mediated pathway (Wright et al., 2010).

Honey bees provide several advantages to studying EtOH-induced aversions compared to other invertebrate models of EtOH consumption such as fruit flies, *Drosophila melanogaster*,

and the nematode, *Caenorhabditis elegans*. First, compared to fruit flies and nematodes, honey bees have a much richer social and foraging repertoire, providing more options for comparisons to other species with complex behaviors, such as humans. Use of honey bee as a model organism allows many investigations in learning and behavior, while research in other invertebrate models primarily focuses on locomotion (Scholz & Mustard, 2013). Second, although honey bees will consume EtOH, they do not have a preference for EtOH (Abramson et al., 2004). Fruit flies, however are innately attracted to EtOH and show a high tolerance for EtOH consumption (Devineni & Heberlein, 2009; McKenzie & Parsons, 1972), rather than exhibiting aversion as in some rodent models (e.g., Cappell et al., 1973). There is an evolutionary fitness basis for this attraction to EtOH. Fruit fly larvae live in rotting fruit that may contain 6-7% EtOH. Adult fruit flies may be attracted to EtOH as it indicates the presence of food supply for the larvae, micro-organisms that decompose fruit (Blum et al., 2013; Fry, 2014; Schneider et al., 2012). Honey bees do not have this selective agent favoring innate attraction to EtOH. Finally, honey bees also offer greater potential to explore the physiological complexity of EtOH consumption than do fruit flies and nematodes. Honey bees have approximately 1,000,000 neurons (Witthöft, 1967), while fruit flies have only around 100,000 neurons (Shimada et al., 2005) and nematodes only 302 neurons (White et al., 1986). Fruit flies and nematodes also lack functional DNA methylation (Lyko et al., 2010) that is present in both honey bees and traditional vertebrate models of alcoholism (Søvik & Barron, 2013).

An additional benefit of investigating EtOH-induced CTAs in honey bees is to improve the comparative study of taste aversion learning. Although there is substantial evidence to suggest that taste aversion learning is a highly conserved process, eusocial insects are notably unrepresented in the literature. It is surprising that taste aversion learning is not often studied in

hymenoptera, given the prevalence of behavioral research on ants and bees. Additionally, while taste aversion learning research has been conducted in invertebrates, many such experiments use non-traditional methods that often employ repeated associations between CS and US (e.g., Darmaillacq et al., 2004; Wright et al, 2010; Yamanaka et al., 1999). Although these experiments clearly show some aversion or change in behavior, it is not clear if the animals would respond similarly in a traditionally defined taste aversion learning experiment with a single association of CS and US across a long interstimulus interval. In order to connect the vertebrate and invertebrate literature in taste aversion learning, more invertebrate experiments following the classic method are needed. Honey bees are an optimal species for such experiments because of what is already known about their ability to rapidly learn (Hammer & Menzel, 1995; Menzel, 1999).

Sucrose Solutions Preparation

Sucrose solutions used in this study were made by diluting 95% EtOH (Pharmco, Brookfield, CT Ethyl EtOH, 190 proof) with sucrose and distilled water. All solutions were made 2 M sucrose and contained either 0%, 2.5%, 5%, 10% or 20% EtOH, all of which bees will consume (Abramson et al., 2000; Bozic et al., 2007; Hranitz et al., 2010). The solutions were unscented, or scented with either 4 µl/ml of cinnamon or lavender oil (Gilbertie's, Southampton, NY).

Conditioned Stimulus Preparation

The CS were delivered manually through a syringe. For cinnamon and lavender CS, the odors were transferred to a 1 cm^2 piece of filter paper (Whatman #4, GE Healthcare Bio-Sciences, Pittsburg, PA) by dipping a wooden dowel in the odor and lightly applying the odor to the filter paper. The paper was then secured to the plunger of a 20 ml plastic syringe with an

uncoated metal thumbtack, thereby making an odor cartridge. A syringe to deliver the air-puff CS was prepared in the same manner except that the filter paper was unscented.

Repeated Measures Logistic Regression

In general, regression analysis is used to investigate how the independent variables are related to the dependent variable. This allows for an understanding of the magnitude, direction, and significance of the effects of each independent variable. Once the effects of the independent variable are identified, regression analysis can also be used to predict values of the dependent variable from specified values of the independent variables. In our case, the dependent variable is the proboscis extension response of the bees (either unconditioned response or conditioned response), while the independent variables in our main analysis (Table 2) are group, dose, and trial.

Our repeated measures logistic regression is a specialized form of regression that is well-suited to the nature of our data. Our regression is specialized in two major ways. First, the repeated measures aspect of our regression means that our analysis controls for possible correlation between two measurements from the same subjects across multiple points in time. As our bees participated in 12 trials, it is therefore possible that each bee's responses across the 12 trials are correlated simply because those responses all come from the same individual. Our analysis accounts for any repeated measures effects within subject via an exchangeable dependence structure. This conservative approach makes no assumptions about the manner in which repeated measures (responses on trials 1 to 12) may be correlated. Instead, it assumes that all repeated measures are equally correlated. For example, possible correlation between responses on trials 1 and 2 are treated the same as possible correlation between responses on trials 1 and 3. Ultimately, this technique statistically controls for the possibility that

measurements from the same subject are correlated, much as a dependent samples *t*-test or repeated measures analysis of variance also controls for within-subject correlation.

The second specialized component of our regression is the logistic aspect. While the standard linear regression predicts values of the dependent variable on a straight line from negative to positive infinity, logistic regression constrains the analysis from 0 to 1, and ultimately predicts the probability of a binary outcome. Logistic regression is thus a perfect fit for dichotomous measures such as the presence or absence of proboscis extension in our experiment. The logistic curve itself is also a good match for learning experiments, as learning often fits nonlinear functions (e.g., Rescorla & Wagner, 1972; Stepanov & Abramson, 2005). While linear regression, and related techniques such as analysis of variance, may provide close approximations, they do not fit the nature of binary data as well as logistic techniques. Linear regression may also predict nonsensical values, such as -1 or 2 responses in a trial. This error is not possible with logistic regression, as it is specifically intended to analyze binary data.

Repeated measures logistics regression is only available through the Generalized Estimating Equations (GEE) family of statistical analysis, while the more familiar linear regression is in the General Linear Model (GLM) family of statistical analysis. The differences between the statistical mechanisms of GEE and GLM are beyond the scope of this paper. However, the general interpretation of GEE and GLM regressions remain consistent. The general form of logistic regression can be written as:

$$logit(R) = \log\left(\frac{p(R=1)}{p(R=0)}\right) = \sum_{i=0}^{n} \beta_i X_i$$

This equation indicates that the *logit* of the dependent variable, *R* (standing for response), can be interpreted as the log odds of *R*, or as the sum of the effects of each independent variable. For the independent variables (*i* through *n*), each *ß* is a parameter estimate indicating the impact of

changes in independent variable on the dependent variable, while *X* is a specific value of that independent variable.

For our analyses in Table 2 the equation can be written in long form as:

$$logit(R) = \beta_0 + \beta_1 Same + \beta_2 Air + \beta_3 Dose + \beta_4 Trial + \beta_5 Same{\times}Dose + \beta_6 Air{\times}Dose + \beta_7 Same{\times}Trial + \beta_8 Air{\times}Trial + \beta_9 Dose{\times}Trial + \beta_{10} Same{\times}Dose{\times}Trial + \beta_{11} Air{\times}Dose{\times}Trial$$

Note that the first *β* parameter estimate has no corresponding independent variable. This is the intercept, or the prediction of *logit(R)* when all independent variables are 0 (i.e., group = 0, dose = 0, trial = 0). The parameter estimates for each independent variable thus refer to how much *logit(R)* changes from the intercept. As group is a categorical variable with no inherent numerical structure, one group was picked to be included in the intercept, or counted as "group 0." We selected the different-stimulus group to be included in the intercept as we primarily wanted to make two group comparisons. First, comparing the different-stimulus group to the same-stimulus group satisfied our main research question, and second, comparing the different-stimulus group to the air-control group satisfied a necessary experimental control question. In our regression analysis, the same-stimulus group parameter estimate refers to the disparity between the same-stimulus and different-stimulus groups, while the air-control group parameter estimate refers to the disparity between the air-control and different-stimulus groups. We can therefore easily see if these groups differ in their effects on the proboscis extension response.

Logistic regression is typically displayed in the format as seen in Table 2 as this presents the simplest way to compare the effects of each independent variable. Positive parameter estimates indicate a variable increases the probability of responses, while negative parameter estimates indicate a variable decreases the probability of response. The absolute value of the

parameter estimates indicates the magnitude of the effect. Given the information in such tables, regression equations can be solved to find predictions for specific combinations of independent variables. The *ß* parameter estimates can be taken from the table, while the *X* values can be used to adjust the equation for the specific prediction. While solving the regression equation leads to an unintuitive *logit(R)* unit. The result can be easily transformed into a probability measure using the equation:

$$p(R = 1) = \frac{e^{logit(R)}}{1 + e^{logit(R)}}$$

For example, the information in Table 2 could be used to predict the responses of the same-stimulus group at the 10% EtOH dose on trial 8. The regression equation can also be used to predict what would occur at levels of the independent variable not used in the experiment, such as the probability of response at 15% EtOH, or on a 13th trial. As logistic regression is constrained between 0 and 1, it will never predict an impossible outcome.

For more details on this method see Liang and Zeger, (1986), Hardin and Hilbe (2003) and Ziegler, Kastner and Blettner (1998). Applications of this method to learning in bees can be seen in Hartz et al. (2010), Mustard et al. (2008), Riddell & Mallon, (2005), Simone-Finstrom et al., (2010), and Wright et al., (2010). See Malone, Iacono, and McGue (2002), Quinn and Fromme (2011), Stein et al. (2011), and Suh et al. (2014) for applications of this technique in alcohol research.

Individual Dose Analysis

As EtOH dose had a substantial effect, we conducted additional analyses directly comparing CR of the same-stimulus and different-stimulus groups at each dose. This analysis considered dose to be a categorical variable. The air-control group was not included for this analysis, as the previous analysis (Table 2) showed no differences between the different-stimulus

and the air-control groups. Table S1 shows the results of the individual dose analyses. The different-stimulus group was included in the intercept, thus the same-stimulus group, and same-stimulus group x trial interaction refer to disparity between the same-stimulus and different-stimulus groups.

At 0% EtOH, the group effect was significant (estimate = 1.635, $p = 0.018$), the trial effect was significant (estimate = 0.228, $p = 0.000$), and the group x trial interaction was not significant (estimate = -0.132, $p = 0.079$). Although the group x trial interaction was slightly outside of the significant range, the direction of all the effects continues what was observed in our previous analysis (Table 2). At 2.5% EtOH, the group effect was significant (estimate = 1.074, $p = 0.013$), the trial effect was significant (estimate = 0.323, $p = 0.000$), and the group x trial interaction was significant (estimate = -0.274, $p = 0.001$). Again, the same general trends are observed as those seen in Table 2. This analysis also demonstrates, that at 2.5% EtOH, bees in the same-stimulus group did not show the robust acquisition curves of bees in the different-stimulus group. Additionally, note that the high level of response on the first trial again indicates that the bees did not learn a conditioned aversion. The 5% dose analysis shows surprising results. At 5% EtOH, the group effect was not significant (estimate = 0.067, $p = 0.875$), the trial effect was significant (estimate = 0.222, $p = 0.000$), and the group x trial interaction was not significant (estimate = -0.073, $p = 0.184$). The results of the analysis for this dose are interesting and suggest there is little difference between groups at 5% EtOH. This finding is supported by comparing Figures 1 and 2. However, it is important to note that the same-stimulus group did not show reduced responding compared to the different-stimulus group as would be predicted by taste aversion learning.

The 10% and 20% EtOH doses appear to substantially inhibit responding, making group comparisons difficult. At 10% EtOH, the group effect was not significant (estimate = 0.700, p = 0.136), the trial effect was significant (estimate = 0.222, p = 0.000), and the group x trial interaction was significant (estimate = -0.085, p = 0.037). Although the group effect was not significant at 10% EtOH, the group x trial interaction is significant. The direction of the effects support what can be seen in Table 2, and in comparing Figures 1 and 2; bees in the same-stimulus group do not show an acquisition curve at 10% EtOH while bees in the different-stimulus group do. Although the group differences are less pronounced than at 2.5% EtOH, the analysis suggest similar overall effects at 10% EtOH. Finally, at 20% EtOH, the group effect was not significant (estimate = 0.443, p = 0.536), the trial effect was significant (estimate = 0.105, p = 0.001), and the group x trial interaction was not significant (estimate = -0.041, p = 0.373). At this dose, almost all responding is inhibited for both groups, and no differences are observed.

Taken together, the 0%, 2.5%, and 10% dose analyses support the findings of our previous analysis (Table 2) that considered EtOH dose as a continuous variable. The 2.5% and 10% doses also strongly support the finding that bees do not show EtOH-induced taste aversion learning. By 20% EtOH, group differences disappeared as all responding was greatly inhibited by the high level of EtOH. The 5% dose however, does not fit the trends observed in our continuous EtOH analysis in Table 2, or the other individual dose analysis in Table 3. The distinction in the 5% dose suggests either that this dose is functionally distinct from other doses, or that the distinction is simply due to subject variability. It is possible that this dose of EtOH is enough to inhibit the initial response of the same-stimulus 5% group, but not enough to prevent them from learning or sobering during the conditioning procedure. Or, perhaps some form of state-dependent learning takes place at this dose. Regardless of cause of the 5% dose similarity

between groups, it is important to note that EtOH-induced taste aversion learning is still not observed at the 5% dose, as the acquisition observed in the same-stimulus group was never inhibited compared to that of the different-stimulus group. Until additional experiments can provide clarity on why the groups are so similar at the 5% EtOH dose, we suggest that continuous EtOH dose analysis (Table 2) is the more parsimonious and informative approach.

Pharmacodynamics of Ethanol

A major consideration of future investigations may be the pharmacodynamics of ethanol (EtOH). The pharmacodynamics of EtOH have been thoroughly studied in vertebrates, where the most important direct actions for EtOH involve the neurotransmitters GABA, glutamate, serotonin, and dopamine, all of which are also implicated in learning, memory or motivation (Di Chiara, 1997; Lovinger, 1999; Malenka et al., 2009). GABA is the primary inhibitory neurotransmitter in the vertebrate central nervous system. When GABA binds to $GABA_A$ receptors, the result is an inhibitory post-synaptic potential that quells signal transmission (Olsen & DeLorey, 1999), and leads to a reduction in many processes associated with aversion such as fear, stress and anxiety (Kalueff & Nut, 2007). Ethanol acts directly on $GABA_A$ receptors, causing an increase in neuron tonic inhibition (Santhakumar et al., 2007). Differences in $GABA_A$ receptor subunit composition creates functional diversity in response to EtOH (Kumar et al., 2009). Inclusion of the δ subunit appears critical for EtOH influence on the channel, leading to individual and species variation in EtOH effects (Santhakumar et al., 2007). For example, $GABA_A$ receptors have been shown to be associated with human alcoholism (Edenberg et al., 2004; Enoch, 2008; Soyka et al., 2008), and certain mutations of these receptors eliminate EtOH-induced taste aversion learning in rodents (Blednov et al., 2011).

GABA receptors have been found in invertebrates, including insects, but less is known about invertebrate pharmacodynamics (Ashby et al., 2012; Buckingham et al., 2005; Lunt, 1991). Insect GABA receptors have properties similar to both $GABA_A$ and $GABA_C$ vertebrate receptors (Buckingham et al., 2005). Presumably, insects have a class of GABA receptors enhanced by EtOH, whose action further inhibits behavior, as observed in vertebrates. Indeed, this would lead to the diminished flower fidelity of free-flying honey bees under the influence of alcohol (Abramson et al., 2005), and the reduced responding observed in PER experiments (Abramson et al., 2015; Mustard et al., 2008).

While EtOH acts as an agonist for GABA, it simultaneously acts as an antagonist for glutamate, the major excitatory neurotransmitter of the central nervous system. This occurs primarily through the NMDA receptor, which is also a key element for synaptic plasticity and memory (Li & Tsien, 2009). Stimulation of NMDA receptors contribute to changes in the density of receptors on post-synaptic membranes, which, in turn, affects the neuron's excitability in response to stimuli (Ryan & Grant, 2009).

Although EtOH generally inhibits the NMDA receptor (Malenka et al., 2009), it also has agonistic effects. The cellular events underlying the enhanced functioning of NMDA receptors from prolonged EtOH exposure include changes in the regulation of NMDA subunit expression, localization, post-translational modifications and interactions with other receptors (Nagy, 2008). Ethanol-induced changes in NMDA receptor density leads to EtOH tolerance, dependence and withdrawal symptoms. Up-regulation of NMDA receptors reduce EtOH's effect, and subsequent neurochemical changes produce behavioral symptoms associated with EtOH dependence. The altered balance between excitatory and inhibitory processes also produces withdrawal symptoms (Nagy, 2008).

Much less is known about the role of NMDA receptors in invertebrate behavior, but some of the subunit diversity seen in vertebrates also exist in invertebrates (Huang et al., 2015). In honey bees, inhibition of the NMDA NR1 receptor subunit is known to impair memory formation (Müssig et al., 2010). It is assumed that chronic EtOH consumption would lead to similar effects as seen in vertebrates.

The serotonin receptor, 5-HT, also plays a prominent role in alcohol abuse in humans (Lovinger, 1997). Like GABA receptors, 5-HT receptors have different roles, many related to mood and motivation (Buhot, 1997). The binding of serotonin to 5-HT receptors can lead to changes in various cellular functions, such as changes in the neurons activity or in the expression of genes (Buhot, 1997). The net result can be either inhibition or the excitation of a neuron, depending on the cell, and a cascade of neuronal events. This can ultimately effect many types of motivations, including the desire for alcohol consumption (Lovinger, 1997).

In vertebrates, EtOH affects both serotonin levels and the activity of some 5-HT receptor types (Mukherjee et al., 2008). For example, increased activity of 5-HT_{1B} receptors is related to EtOH intoxication (Crabbe et al., 1996). Other changes in 5-HT receptors may be caused by chronic exposure to alcohol, including increased density of 5-HT_2 receptors. The 5-HT_2 receptor also appears to be an important element in anxiety associated with alcohol withdrawal (Lal et al., 1993). Ethanol also enhances the signals generated by the 5-HT_3 receptor that stimulate the release of other of neurotransmitters (Lovinger, 1997). Nevertheless, some research reports a decrease in serotonin transporter (5-HTT) that carries serotonin across the interneuron gap (Burnett et al., 2012), but the reverse has also been reported (Shibasaki et al., 2010). The variability in results seems to reflect the heterogeneity in genetic backgrounds, which appears to influence epigenetic processes involving the interaction of 5-HTT alleles with an individual's

environment (Thompson & Kenna, 2016). Indeed, this may explain the drug related serotonergic and plasticity reported in the literature (Renoir et al., 2012).

Invertebrates have 5-HT receptors homologous to the 5-HT_1, 5-HT_2 and 5-HT_7 vertebrate receptors (Thamm et al., 2013; Vleugels et al., 2015). The role of serotonin in invertebrate is similar to vertebrates and is implicated in modulating memory, appetite, and behaviors related to aversive situations (e.g., Bicker, 1999; Sitaraman et al., 2012; Wright et al., 2010). Although the pharmacodynamics are less studied in invertebrates, there are many similarities to the vertebrate counterpart (Vleugels et al., 2015). In terms of aversions in insects, serotonin is implicated in learning to associate odors with toxins (Wright et al., 2010). When given 5-HT receptor antagonists, honey bees showed improved ability to discriminate between odors associated with sucrose and odors associated with quinine (Wright et al., 2010). Ethanol acts as a 5-HT agonist, at least in vertebrates (Lovinger, 1997; Wallis et al., 1993), and based on this, and the finding of Wright et al. (2010), expectations might be for enhancing EtOH-induced CTA. However, enhanced 5-HT activity also increases the release of dopamine that is linked to appetitive rewards (Imperato & DiChiara, 1986; Kornetsky et al., 1988).

Finally, the neurotransmitter dopamine also plays a major role in EtOH consumption, and is known to be involved in learning, motivation, and attention (Di Chiara, 1997; Wise, 2004). Dopamine has at least six receptors in two receptor families, the D1-like family, and the D2-like family (Surmeier & Kitai, 1993). Unlike GABA and NMDA receptors, dopamine receptors do not directly affect the activity of ion channels. Instead, they act indirectly, and thus dopamine is often called a neuromodulator (Di Chiara, 1997). For example, when dopamine binds to D1 receptors, it may enhance excitatory effects that occur when glutamate binds to NMDA (Cepeda et al., 1993). Dopamine pathways in the nucleus accumbens are known to be sensitive to EtOH

and may be instrumental in the development of alcohol dependence (Di Chara, 1997). For example, rats injected with EtOH show increased dopamine release in the nucleus accumbens shell, leading to chronic EtOH self-administration. (Lyness & Smith, 1992). Ethanol consumption similarly increases dopamine release in the nucleus accumbens in humans (Boileau et al., 2003). Dopamine activity in the nucleus accumbens shell may also account for individual differences in EtOH consumption. In rats, EtOH induces stronger dopamine release in the nucleus accumbens shell for rats with EtOH preferences than those that avoid EtOH (Bustamante et al., 2008). Considering the neuromodulator effects of dopamine, it is also possible that dopamine modulates the effects of EtOH on other neurotransmitter pathways.

Dopamine receptors are also seen in invertebrates (for a review of invertebrate dopamine receptors, see Mustard et al., 2005), and similar behavioral findings are observed. In fruit flies, dopamine pathways are required to express, but not learn, preferences for stimuli associated with EtOH (Kaun et al., 2011). Dopamine is also implicated in aversive conditioning in insects. In fruit flies, three distinct dopamine pathways, all projecting from the mushroom body, contribute to aversive olfactory conditioning (Aso et al., 2012). Additionally, dopamine antagonists have shown to reduce the effectiveness of aversive conditioning in honey bees (Agarwal et al., 2011). With respect to CTA in honey bees, dopamine receptor antagonists reduce learned avoidance of odors associated with quinine (Wright et al., 2010).

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Table S1

Group Conditioned Response Comparison 0% EtOH

Parameter	Estimate	Standard Error	95% Confidence Intervals		*p*-value
Intercept	-0.570	0.242	[-1.044	-0.097]	0.018
Same-stimulus group	1.635	0.429	[0.795	2.476]	0.000
Trial	0.228	0.051	[0.127	0.329]	0.000
Same x Trial	-0.132	0.075	[-0.279	0.015]	0.079

Group Conditioned Response Comparison 2.5% EtOH

Parameter	Estimate	Standard Error	95% Confidence Intervals		*p*-value
Intercept	-0.506	0.324	[-1.141	0.130]	0.119
Same-stimulus group	1.074	0.432	[0.227	1.921]	0.013
Trial	0.323	0.077	[0.171	0.474]	0.000
Same x Trial	-0.274	0.083	[-0.436	-0.111]	0.001

Group Conditioned Response Comparison 5% EtOH

Parameter	Estimate	Standard Error	95% Confidence Intervals		*p*-value
Intercept	-0.904	0.304	[-1.499	-0.308]	0.003
Same-stimulus group	0.067	0.423	[-0.762	0.895]	0.875
Trial	0.222	0.042	[0.139	0.305]	0.000
Same x Trial	-0.073	0.055	[-0.181	0.035]	0.184

Group Conditioned Response Comparison 10% EtOH

Parameter	Estimate	Standard Error	95% Confidence Intervals		*p*-value
Intercept	-2.065	0.312	[-2.676	-1.453]	0.000
Same-stimulus group	0.700	0.470	[-0.220	1.620]	0.136
Trial	0.145	0.031	[0.085	0.206]	0.000
Same x Trial	-0.085	0.041	[-0.166	-0.005]	0.037

Group Conditioned Response Comparison 20% EtOH

Parameter	Estimate	Standard Error	95% Confidence Intervals		*p*-value
Intercept	-2.777	0.503	[-3.763	-1.792]	0.000
Same-stimulus group	0.443	0.717	[-0.962	1.849]	0.536
Trial	0.105	0.033	[0.041	0.169]	0.001
Same x Trial	-0.041	0.046	[-0.130	0.049]	0.373

Note. The different-stimulus group is included in the intercept. The same-stimulus group is abbreviated in the interactions as same.