Buzzed but not elated? Effect of ethanol on cognitive judgement bias in honeybees

Names of authors

Marta Golańska, Faculty of Psychology, University of Warsaw, Warsaw, Poland 0000-0003-3305-4470

Weronika Antoł, Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Kraków, Poland 0000-0003-2047-330X

Magdalena Witek, Museum and Institute of Zoology, Polish Academy of Sciences, Warsaw, Poland 0000-0002-6172-8981

Krzysztof Miler, Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Kraków, Poland 0000-0001-7684-0629

Corresponding author

Marta Golańska

m.golanska@uw.edu.pl

Abstract

Ethanol is a neuroactive compound that alters affect and cognition in humans and other vertebrates, but its effect on affective-like states in invertebrates remain remains unknown. In this study, we tested whether exposure of honeybees to field-realistic ethanol concentrations (1%) induces 'pessimistic' or 'optimistic' cognitive judgment bias (CJB), a behavioral proxy of affect. Bees were trained in a classical olfactory conditioning paradigm and then presented with conditioned stimuli and ambiguous odor mixtures to assess decision-making changes under uncertainty. We additionally measured locomotor performance and ethanol absorption via spectrophotometric analysis of hemolymph. Ethanol levels peaked rapidly and remained elevated for at least two hours post-ingestion. Despite this, ethanol had no detectable effect on CJB responses, memory, or motor behavior. These findings suggest that acute exposure to low-dose ethanol does not induce affect-like changes in honeybees. Our study represents the first test of pharmacologically induced judgment bias in an invertebrate and contributes to understanding the resilience of insect cognition to natural neuroactive compounds. The results highlight the limits and potential of CJB paradigms for probing affect-like states across taxa and support the honeybee as a valuable model in comparative cognition and the behavioral ecology of neuroactive substance exposure.

Keywords: cognitive bias, affective state, decision-making, learning, Apis mellifera

1. Introduction

Ethanol is a neuroactive agent with a wide range of effects on human functioning. It impairs motor and cognitive capabilities (Lewis et al. 1969) and is toxic at high doses (Lieber 1991, Gable 2004). The substance also influences affect: at lower doses, ethanol acts as an euphoriant and anxiolytic (Freed 1978), while at high concentrations lowers mood (Chermack and Giancola 1997). Its chronic intake can lead to the development of mood and anxiety disorders (Saunders et al. 2019). These effects are thought to result from ethanol's modulation of several neurotransmitters and secondary messenger systems (Chastain 2006, Wand et al. 2001). Corresponding affect-like responses have also been found in other vertebrates. For example, ethanol consumption has been linked to increased aggression in rats (Van Erp and Miczek 1997) and increased anxiety in ethanol-dependent rats following deprivation (Valdez et al. 2002). These findings raise the question of whether ethanol can also modulate affect-like processes in invertebrates.

Ethanol is a naturally occurring compound in many environments, and some species have evolved mechanisms to tolerate or utilize it, despite its toxicity (Bowland et al. 2024, Miler 2025). Insects, in particular, frequently encounter ethanol in fermenting plant material. For example, fruit flies (*Drosophila melanogaster*) are attracted to ethanol-rich substrates, using it as a food source and to maintain a competitive advantage over less tolerant species (Merçot et al. 1994). The oriental hornet (*Vespa orientalis*) shows exceptional ethanol tolerance, likely a result of coevolution with yeasts (Bouchebti et al. 2024). These examples suggest that ethanol exposure can exert strong selective pressures and may have shaped behavioral and physiological adaptations across diverse taxa. Honeybees (*Apis mellifera*) also encounter ethanol in their environment, and it plays a significant role in their ecology (Abramson et al. 2025). Yeasts contribute to the fermentation processes involved in the bee bread production, a vital larval food source (Gilliam 1979). Ethanol can also be present in flower nectar collected by foragers (Kevan et al. 1988; Jakubska 2005; Schaeffer et al. 2017). Ethyl oleate, a pheromone produced from ethanol and oleic acid, regulates the division of labor in honeybee colonies

(Castillo et al. 2012). Ethanol tolerance varies across worker castes, with higher resistance and alcohol dehydrogenase levels in foragers compared to nurses (Miler et al. 2021).

Honeybees have been found to prefer low ethanol concentrations in sucrose solutions (Abramson et al. 2000; Mustard et al. 2019) and show no conditioned taste aversion to ethanol (Varnon et al. 2018). Discontinuation of chronic ethanol consumption produces tolerance and withdrawal-like symptoms in honeybees (Miler et al. 2018; Ostap-Chec et al. 2021), and their ethanol intake can be reduced with disulfiram, a treatment for alcohol dependence (Abramson et al. 2003). Ethanol impairs honeybee motor function (Abramson et al. 2000; Maze et al. 2006; Ahmed et al. 2022) and cognition, including learning performance (Abramson et al. 2000; Mustard et al. 2008). Social behaviors like trophallaxis or waggle and tremble dances, are also affected (Bozic et al., 2006; Mixson et al., 2010). In addition, ethanol has been found to increase aggression (Wright et al. 2012; Giannoni-Guzmán et al. 2014), although not all studies support this finding (Abramson et al. 2000). What is important to emphasize, most studies use high ethanol concentrations (typically 5-10%), limiting their ecological relevance, as ethanol levels naturally encountered by honeybees are likely much lower (≤ 1%; Wiens et al. 2008; Jones and Agrawal 2022).

Previous research has largely focused on the impact of ethanol on honeybee cognition in terms of classical conditioning, but honeybees show cognitive sophistication that extends far beyond simple associative learning. They exhibit contextual and conceptual learning, forms of social learning, metacognition, numerical cognition, and attentional processes (Collett et al. 1997; Leadbeater and Chittka 2007; Pahl et al. 2013; Avarguès-Weber and Giurfa 2013; Perry et al. 2017). One particularly interesting phenomenon is cognitive judgment bias (CJB), which measures decision-making under uncertainty. The CJB paradigm offers a promising tool for investigating affect-like processes as well as their evolutionary basis and function. Originally developed for rodents (Harding et al. 2004), CJB tests assess whether animals show ‘optimistic’ or ‘pessimistic’ biases when interpreting ambiguous cues. It has been adapted for invertebrates, including honeybees (Bateson et al. 2011). In these tests, animals

are conditioned to associate two stimuli with a reward and a punisher, and are later presented with the learned stimuli and novel, ambiguous ones. In honeybees, ‘pessimistic’ biases have been induced by stressors (shaking; Bateson et al. 2011; Schlüns et al. 2017), and similar effects have been found in fruit flies (Deakin et al. 2018), suggesting the effect is not limited to eusocial hymenopterans. So far, ‘optimistic’ biases have been investigated only in bumblebees (Solvi et al. 2016), and two recent works used the species of this genus to further explore the cognitive mechanisms of CJB (Strang and Muth 2023; Procenko et al. 2024). Changes in CJB are commonly interpreted as indicators of affect or emotion-like changes, following the original formulation of the test (Harding et al. 2004; Mendl et al. 2009; Neville et al. 2020; Lagisz et al. 2020 but see alternative interpretation by Baracchi et al. 2017). Whether insects possess emotional states or their analogues remains highly debated (see Baracchi et al. 2017; Perry and Baciadonna 2017). However, CJB responses in invertebrates provide a behavioral window into valence-based processing. Following Mendl and Paul (2020), we adopt a broad definition of "affect" that includes all valence-related processes (‘pleasantness’ or ‘unpleasantness’), including emotions, moods, and their possible manifestations (physiological or behavioral), whether or not they entail conscious experience. This allows for comparisons across taxa without necessarily implying that conscious experience must be involved (for a discussion, see Andrews 2024).

Some pharmacological substances have been shown to impact cognitive judgment bias across several, mostly mammalian, species (see Lagisz et al. 2020, for review and meta-analysis). One recent study linked optimistic-like responses to ethanol self-administration in rats (Cieslik-Starkiewicz et al. 2024), but ethanol has never been tested as a mood-manipulating agent in a CJB test. Moreover, no study has directly tested whether a pharmacological manipulation can alter CJB in an invertebrate.

In the present study, we investigated whether field-realistic ethanol concentrations (1%) induce cognitive judgment bias in honeybees. We hypothesized that ethanol-exposed bees would exhibit bias, that is, respond differently to at least one ambiguous cue compared to controls, while maintaining normal responses to learned stimuli. Additionally, we assessed locomotor behavior and ethanol

absorption via hemolymph sampling to rule out non-specific performance effects and confirm effective dosing.

2. Methods

2.1. Cognitive judgment bias test

2.1.1. Study species and collection procedure

Bees were collected from three families from the same experimental apiary located in Kraków, Poland, over 16 days. Animals were collected in one or two blocks daily between 07:30 am and 12:25 pm, with each collection lasting up to 15 minutes. Up to 30 bees were restrained per block. Only pollen-carrying bees were chosen to ensure their forager status. Bees were left for one hour to acclimate and strengthen feeding motivation. From each block, 12 bees were selected for the conditioning phase based on their overall condition, absence of proboscis or antennal defects, adequate restraint, and prompt proboscis extension upon brief antennal stimulation with sucrose solution. The remaining bees were released.

2.1.2. Olfactory conditioning

Selected bees underwent olfactory conditioning using the Proboscis Extension Response (PER) paradigm (Scheiner et al. 2013). This classical conditioning paradigm utilizes a reflexive extension of the proboscis in response to sucrose contact and is widely used for appetitive conditioning in honeybees (Giurfa and Sandoz 2012). One odor (2-octanone, >99%, Thermo Scientific) was paired with a reward (0.5 µl drop of 1.5 M aqueous sucrose solution), while a second odor (1-hexanol, >98%, Tokyo Chemical Industry) was paired with punishment (0.5 µl drop of 0.5 M aqueous NaCl solution). Each odor was delivered from a 24 ml syringe containing a cotton pad soaked with 6 µl of the odorant. The end of the syringe was placed about 0.5 cm from the bee's head, and air was expelled for 4 seconds. In the final second of odor delivery, the reward or punishment was presented, lasting 3 seconds in total (Matsumoto et al. 2012). The antennae were stimulated with the solution, and if PER was elicited, a drop was placed on the proboscis using a pipette.

Each bee underwent 16 conditioning trials (8 per odor), in a pseudorandom sequence (RRPRPRRPPRPPRPRP), where R and P stand for the odors associated with the reward and punishment, respectively, as used by Schlüns and colleagues (2017). Inter-trial intervals were 6 minutes. Bee responses during the first 3 seconds of odor presentation (before unconditioned stimulus delivery) were recorded as 1: full PER, 0: no PER, or partial extension of the proboscis. Bees were included in the experiment if they displayed correct responses in at least four of the final six trials for each odor. Lack of PER to hexanol and full PER to octanone were considered correct responses.

2.1.3. Group assignment and experimental manipulation

Bees that met the learning criteria were randomly assigned to either the control group (fed 10 µl of 1.5 M aqueous sucrose solution) or the ethanol group (fed 10 µl of 1.5 M aqueous sucrose solution with 1% ethanol). For each block, group sizes were balanced wherever possible, or when the number of bees was odd, the more numerous group was chosen randomly. The individuals were then randomly assigned to the groups. Successfully fed bees were left for one hour before testing to allow ethanol absorption into hemolymph and improve feeding motivation. Bees not meeting the learning criteria were marked to prevent reuse and released.

2.1.4. Cognitive judgment bias test

The successfully fed bees participated in the cognitive judgment bias test comprising five odor presentations (testing trials). Each odor was presented for four seconds, and PER responses were coded as 1: full PER and 0: lack of PER. No rewards or punishments were delivered, but a pipette was held nearby to mimic the conditioning context. The odors included the two conditioned odors (6:0 octanone:hexanol = positive; 0:6 = negative) and three mixtures: 4:2 (near-positive), 3:3 (neutral), and 2:4 (near-negative). Odors were delivered as in the conditioning phase. The experimenter was blind to the odor and the testing order, which was randomized per block.

Each testing trial was preceded by two reminder trials (one positive, one negative) to limit extinction of the conditioned responses after unrewarded trials (Sandoz and Pham-Delègue 2004). The

sequence was RP1RP2RP3RP4RP5 (R and P = reminder reward and punishment trials; numbers = testing trials). The full procedure lasted 44 minutes, with a 3-minute inter-trial interval. After testing, bees were marked and released, and odor identities were unblinded.

2.2. Locomotion test

Bees were collected from seven families from the same apiary over nine days (between 07:00 am and 09:30 am, in two blocks), with one bee collected per minute for up to 30 minutes. Only pollen-carrying individuals were selected, and they were restrained promptly after being caught. Bees were left for three hours to acclimate and approximate the conditioning phase duration in the CJB test. The first 15 and last 15 bees of each block were assigned to the ethanol-fed or control group, with group assignment counterbalanced across families.

Bees were fed 10 µl of either 1.5 M aqueous sucrose solution (control) or the same solution with 1% ethanol (ethanol-fed). Animals were excluded based on visible leg or mouthpart defects, poor condition, incomplete consumption, or mortality. Fifty minutes post-ingestion, bees were released from restraint and placed under a Petri dish (14.5 cm diameter, 1 cm high) for 10 minutes to acclimate. One hour after feeding, a 20-minute video of their locomotion inside the dish was recorded.

Videos were processed using AnimalTA (Chiara and Kim 2023) to extract the total distance traveled, average speed while moving, and meander (a measure of propensity to change directions while moving).

2.3. Ethanol levels in hemolymph

Bees were collected from four families from the same apiary over six days. The animals were collected for one hour, starting at 09:00 am, and assigned to one of four ethanol-fed groups, 15 individuals per group. Only pollen-carrying bees were selected, then restrained and fed 10 µl of 1.5 M sucrose solution containing 1% ethanol. Four ethanol-fed groups differed in the time of hemolymph extraction after

feeding (30, 60, 120, or 240 minutes). An additional control group (N = 15) received a sucrose-only solution and was sampled 30 minutes post-ingestion.

All bees were restrained for two hours before feeding to acclimate and mimic the CJB test procedure. The animals that did not ingest the entire solution were not used. Hemolymph was extracted using the antennal method (Borsuk et al. 2017) and collected in end-to-end 10 µl microcapillaries, then frozen until analysis.

Samples were processed using the Ethanol Assay Kit, Megazyme, following the manufacturer's protocol with some modifications. This method relies on spectrophotometric detection of NADH, a product of two enzymatic reactions involving ethanol as a substrate. For each reaction, we used the minimal volume of 3 µl of hemolymph from two samples pooled within a group, or, if the volume was sufficient, from a single individual. The absorbance at 340 nm was measured twice: before (A1) and 5 min after adding the enzyme initiating the reaction (A2). The difference A2-A1 was used to calculate ethanol concentration in the sample, based on the calibration curve with blank, performed with each reaction plate.

2.4. Statistical analysis

All analyses were performed using *R* (R Core Team, 2014).

2.4.1. CJB test

We analyzed PER responses using a binomial generalized linear mixed model (GLMM) fit by maximum likelihood with logit link (lme4 package; Bates et al. 2015). The dependent variable was PER (full PER coded as 1 and absent or partial PER coded as 0). Fixed effects included odor (continuous variable reflecting the volume of octanone in the tested odor sample, centered), group (ethanol-treated or control), and their interaction. Bee ID was included as a random effect. Interaction effects were further explored using Fisher's exact tests at each odor level, corrected using Holm's method.

We explored models that included date, hour, odor presentation order, and family (as fixed effects), as well as nonlinear terms for odor, but these were discarded due to poorer fit and non-significance. Model diagnostics were conducted using the DHARMA package (Hartig, F 2024), and no substantial issues were detected. Variance explained (conditional R^2) by the model was calculated as Nakagawa's R^2 (Nakagawa and Schielzeth 2013) using the package performance (Lüdecke et al. 2021).

A total of 736 observations from 148 bees were analyzed. Data for several trials for one individual were discarded due to experimenter error.

To validate the conditioning method, we analyzed learning dynamics in 274 bees that participated in the olfactory conditioning phase using Fisher's exact tests adjusted with Holm's correction.

2.4.2. Locomotion test

We analyzed the effect of ethanol on three dependent variables: the total distance traveled, average speed while moving, and tendency to meander. We assumed the threshold for considering the animal to be moving as 0.3 cm/s to account for tracking imperfections resulting in noise, with perspective correction and default smoothing applied (see Chiara and Kim 2023 for explanations of the parameters). Group differences (ethanol-fed vs. control) were tested using independent samples t-tests (for speed and distance) and the Wilcoxon rank-sum test (for tendency to meander, due to non-normality of the distribution).

Data from four individuals were discarded due to their poor physical condition. The final sample size was 281 individuals.

2.4.3. Ethanol level

We compared ethanol concentrations in hemolymph across groups (ethanol-fed that were sampled at different times post-ingestion vs. control) using a Kruskal-Wallis test followed by Dunn's post hoc tests with Holm's correction. Non-parametric tests were used due to non-normal data distribution. In total, 55 hemolymph samples from 105 individuals were analyzed.

3. Results

There was no significant effect of ethanol treatment on bees' responses in the CJB test (GLMM: group predictor: $z = -0.97$, $p = 0.333$). No evidence of judgment bias was detected (GLMM: group × odor interaction: $z = 1.95$, $p = 0.051$; Figure 1). A significant difference between treatment groups in responses to at least one ambiguous odor cue is required to support the presence of a cognitive judgment bias. In our study, the ambiguous cues were odor mixtures composed of octanone and hexanol in the following ratios: 4:2 (near-positive), 3:3 (neutral), and 2:4 (near-negative). No significant group differences were detected for any of the ambiguous cues in post hoc Fisher's exact tests (near-positive: OR 95% CI [0.18, 1.48], $p = 1$; neutral: OR 95% CI [0.50, 7.07], $p = 1$; near-negative: OR 95% CI [0.07, 13.38], $p = 1$).

Ethanol also had no detectable effect on memory, specifically, it did not lead to extinction of the conditioned responses, as no significant differences were observed between groups in responses to the conditioned stimuli (previously learned odors; positive cue (6:0): OR 95% CI [0.77, 3.21], $p = 1$; negative cue (0:6): OR 95% CI [0.01; 2.35], $p = 1$). There was, however, a significant main effect of odor type (GLMM: odor predictor: $z = 6.83$, $p < 0.001$), confirming that bees reliably discriminated among the olfactory cues. The model explained 64.6% of the variance.

In the locomotor activity test, no significant differences were observed between ethanol-treated and control bees for any of the measured parameters (Figure 2). The groups did not differ in terms of average movement speed ($M_{EtOH} = 3.19$ cm/s, $SD_{EtOH} = 1.13$ cm/s; $M_{control} = 3.30$ cm/s, $SD_{control} = 0.95$ cm/s; $t(278) = 0.90$, $p = 0.37$; 95% CI [-0.13, 0.36]), total distance covered ($M_{EtOH} = 3772.10$ cm, $SD_{EtOH} = 1512$ cm; $M_{control} = 3595.38$ cm, $SD_{control} = 1235$ cm; $t(278) = 1.07$, $p = 0.29$; 95% CI [-147.90, 501.34]) or propensity to change direction (meander; $M_{EtOH} = 255$ deg/cm, $SD_{EtOH} = 140$ deg/cm; $M_{control} = 238$ deg/cm, $SD_{control} = 75.10$ deg/cm; $W = 9814$, $p = 0.98$).

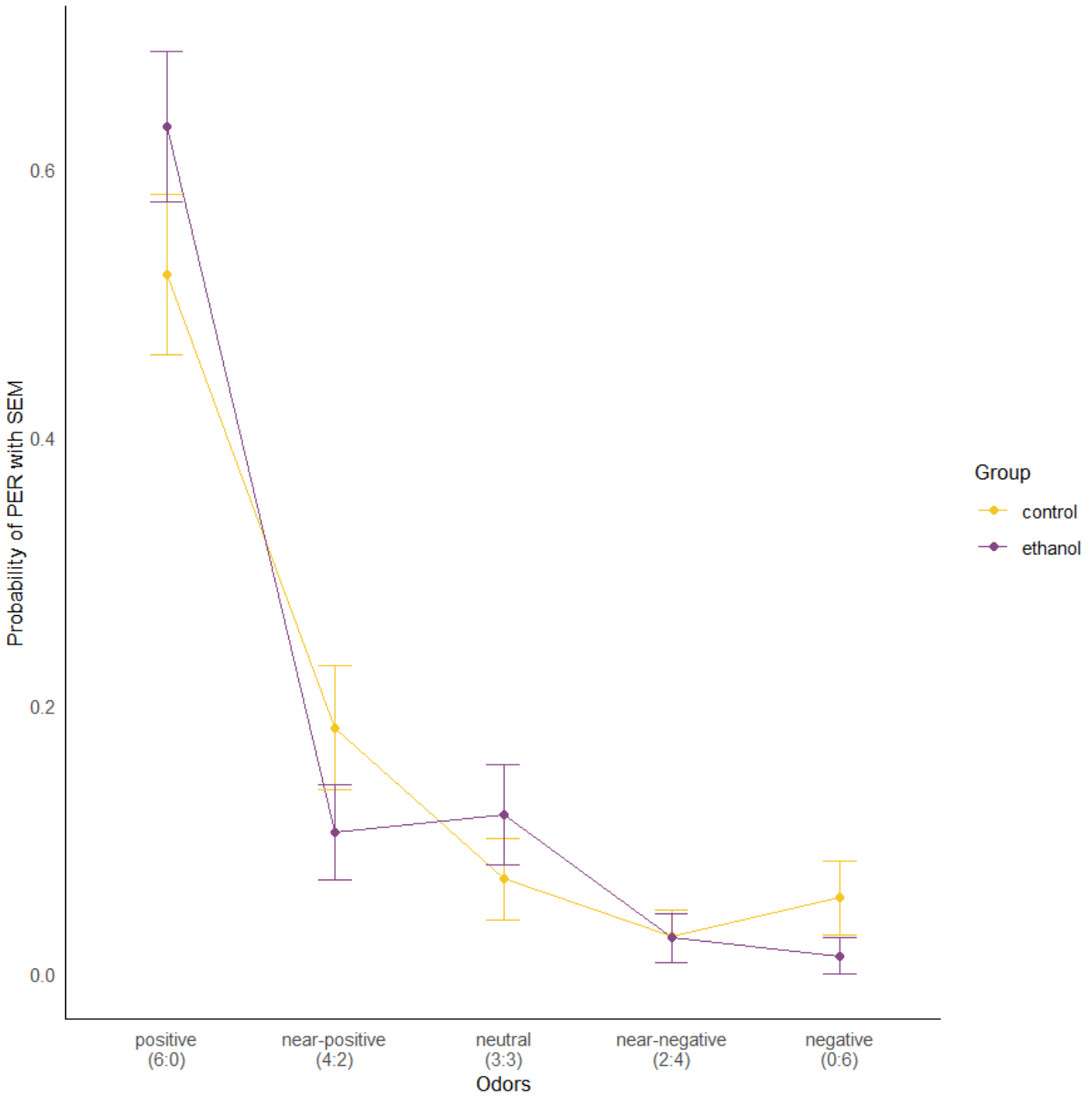

Figure 1. **Probability of proboscis extension response (PER) across odor stimuli for ethanol-treated and control bees.** The x axis shows the odors presented during the cognitive judgment bias test: the conditioned positive (6:0 octanone:hexanol), conditioned negative (0:6), and ambiguous mixtures (4:2 = near-positive, 3:3 = neutral, 2:4 = near-negative). The y-axis represents the proportion of bees showing full PER. Data are presented as means ± standard error (SE). No significant differences between groups were observed at any odor level.

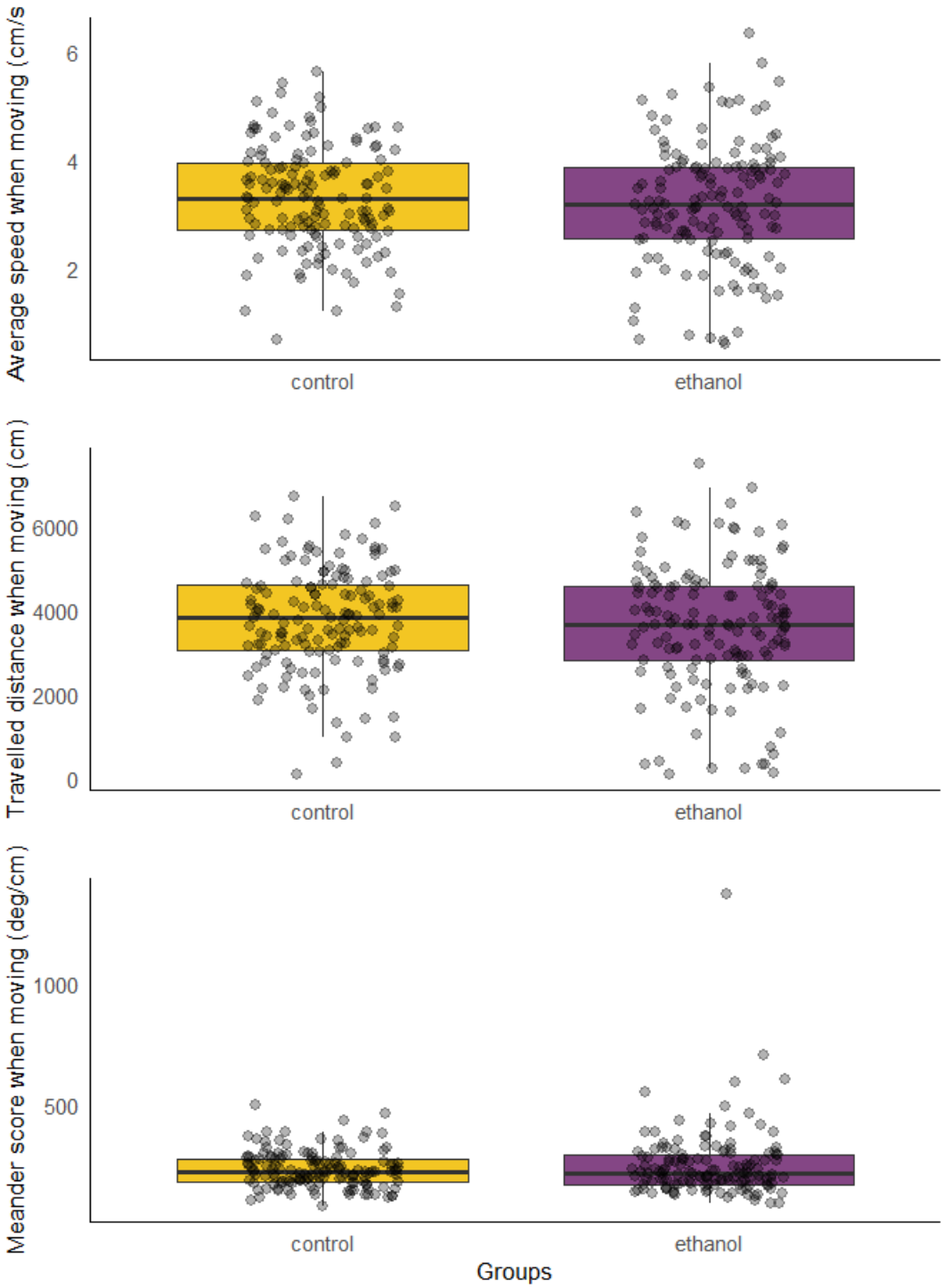

Figure 2. **Locomotor activity of ethanol-fed and control groups.** Boxplots show the total distance traveled, average speed while moving, and meander (a measure of directional change). Whiskers indicate values within 1.5 of the interquartile range, boxes represent interquartile ranges, horizontal lines indicate medians, and dots represent individual data points. No significant differences were found between groups for any parameter.

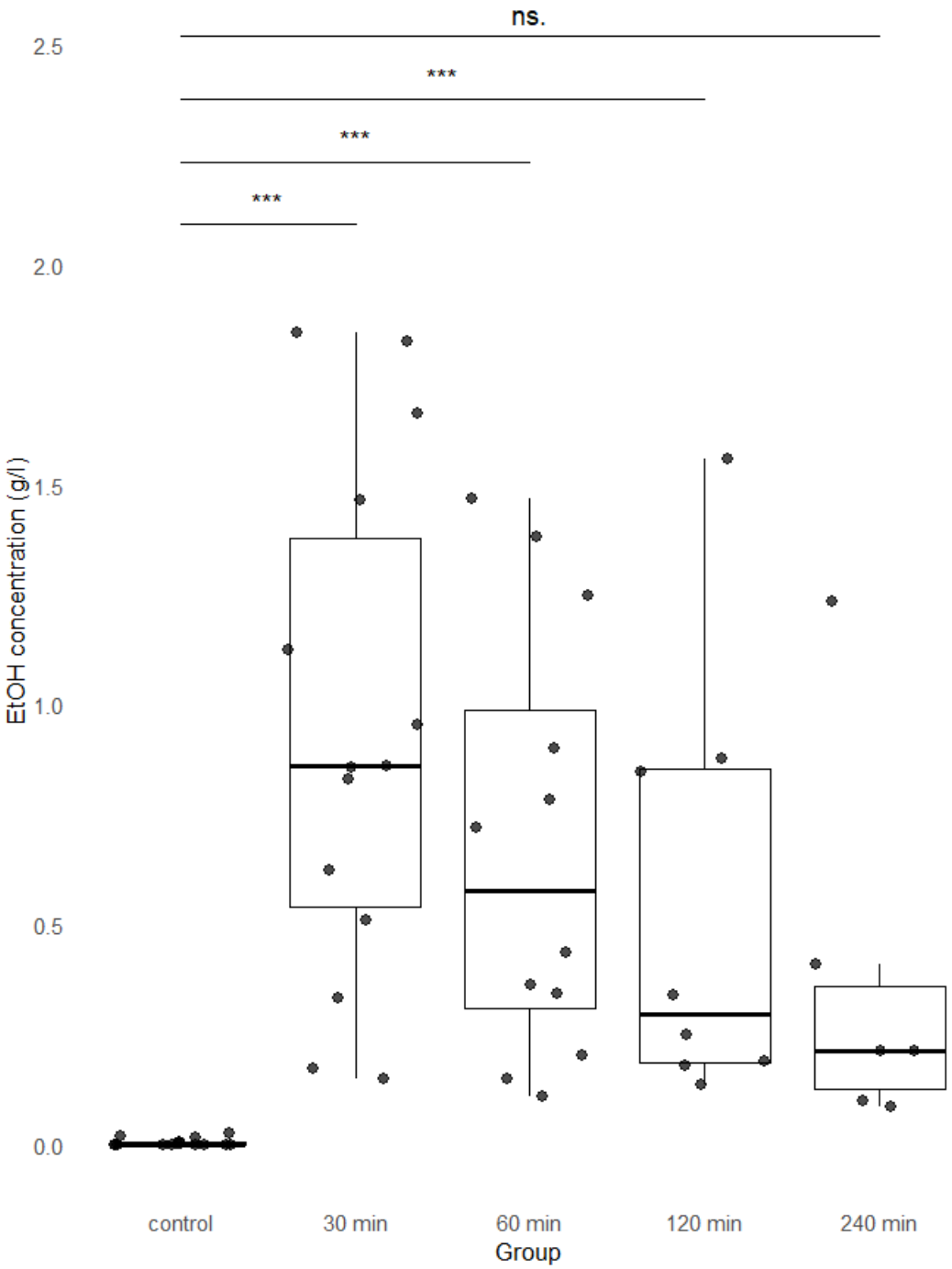

Figure 3. **Hemolymph ethanol concentration in bees sampled at different times post-ingestion.** Groups were sampled at 30, 60, 120, and 240 minutes after feeding on a 1% ethanol solution. A control group received no ethanol. Boxplots show medians (horizontal lines), interquartile ranges (boxes), and values within 1.5 of the interquartile range (whiskers). Dots represent individual samples. Asterisks indicate significant differences compared to the control group (** = $p < 0.01$, *** = $p < 0.001$). Ethanol levels were elevated at 30-120 minutes but declined by 240 minutes.

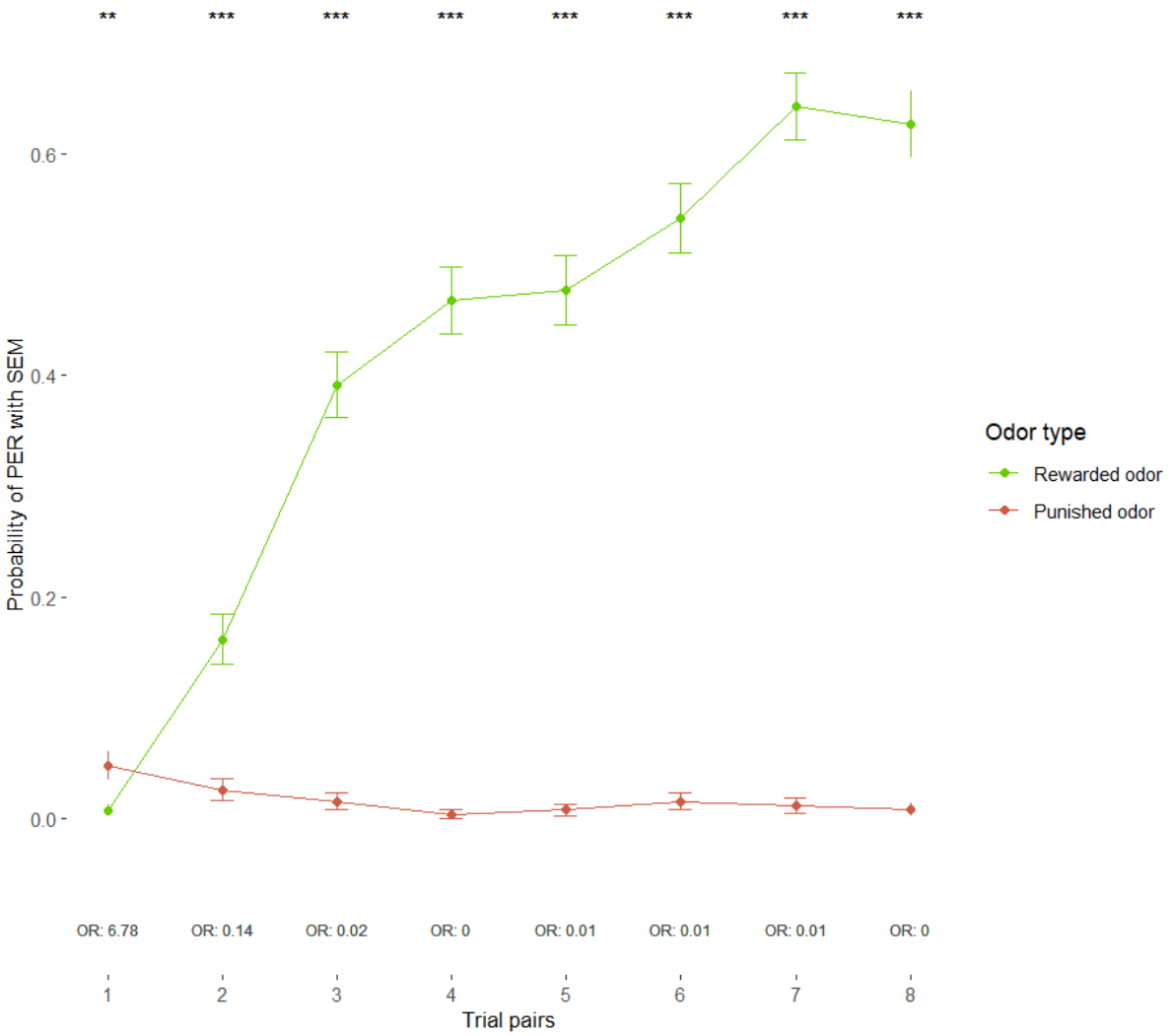

Figure 4. **Acquisition of the conditioned responses across trials during olfactory conditioning.** The proportion of bees showing proboscis extension response (PER) is shown for punished and rewarded trials across the conditioning sequence. Data are presented as mean ± SE. Odds ratios (OR) and significance levels are indicated for each pairwise comparison (** = $p < 0.01$, *** = $p < 0.001$). The figure illustrates successful learning, with higher PER to rewarded cues over time

Ethanol levels in the hemolymph differed significantly across sampling groups (including control), depending on the time elapsed since ingestion ($\chi2$ (df = 4, N = 55) = 35.4, $p < 0.001$). Post hoc comparisons showed significant differences between the control group and the ethanol-fed groups sampled at 30 minutes ($p < 0.001$), 60 minutes ($p < 0.001$), and 120 minutes ($p = 0.004$) post-ingestion. At 240 minutes post-ingestion, ethanol levels no longer differed significantly from controls ($p = 0.077$; Figure 3). No significant differences were found among the three ethanol-fed groups sampled at 30, 60, and 120 minutes ($p = 1$ for all comparisons), suggesting relative stability of ethanol levels during the behavioral testing period.

Analysis of bees' responses during the conditioning phase confirmed effective acquisition. In total, 54% of bees met the learning criteria for inclusion in the CJB test. Pairwise comparisons between reward and punishment trials at each subsequent presentation showed significant differences in PER responses for all comparisons ($p = 0.004$ for the comparison of the first pair, $p < 0.001$ for all subsequent pairs; Figure 4). The first trial pair showed higher PER rates to the punished odor, likely due to trial order effects (see Discussion). The direction of the effect was reversed for the subsequent pairs, consistent with the assumptions.

4. Discussion

Our study investigated whether ethanol in field-realistic concentrations influences cognitive judgment bias (CJB) in honeybees. We found no evidence that ethanol affected bees' responses to ambiguous cues, suggesting an absence of judgment bias. Additionally, we observed no detectable influence on bees' decision-making regarding conditioned stimuli or in general motor function (based on the locomotion test). Ethanol ingestion was confirmed by spectrophotometric analysis of hemolymph samples, which showed elevated levels for at least two hours after ingestion. Together, these findings suggest that acute exposure to low doses of ethanol does not produce detectable affect-like changes in honeybee behavior.

Our findings contrast with previous investigations using similar CJB paradigms, which have demonstrated that invertebrates can show affect-like states. For instance, Bateson et al. (2011) and Schlüns et al (2017) found that honeybees exposed to a physical stressor (shaking) exhibit pessimistic biases. However, this study is the first to investigate whether pharmacological intervention may induce cognitive judgment bias in invertebrates. The lack of effect suggests that the ethanol does not impact affect-like states in bees, that the CJB paradigm may be less sensitive to ethanol effects, or that a different dose or duration of exposure may be needed to produce measurable changes.

Several factors might explain the absence of an effect. One possibility is that ethanol's influence does not manifest at low, field-realistic concentrations. However, previous studies have shown that even 1% ethanol can influence honeybee behavior (e.g. Bozic et al. 2006; Ahmed et al. 2022). Higher concentrations of ethanol might induce CJB, and further investigation is warranted, but such studies should also account for the known confounding effects of ethanol on learning and motor performance. From the perspective of bee ecology and apiculture, investigating higher ethanol concentrations is also relevant. Although we used the term 'field-realistic' for ethanol concentrations used in the study, as suggested by Ostap-Chec and colleagues (2025), concentrations might be considerably higher in some environments, especially human-modified ones like orchards, where bees may encounter fermenting fruit. It remains unclear how often and under what circumstances bees supplement their diet with fallen fruit (Abramson et al. 2025), and how this might affect their decision-making or welfare. Another possibility for the absence of an effect is that a single, acute dose may be insufficient to induce affective changes, whereas chronic exposure might yield different results. It is also possible that inter-individual variability obscured a small existing effect (Van Der Goot et al. 2021).

Despite the null results, our study has broader significance. To our knowledge, this is the first attempt to use a pharmacological agent to influence affective state in an invertebrate, providing a valuable reference point for future investigations. These findings also inform efforts to develop the honeybee as a model for studying alcohol-related behavior and neurobiology, suggesting that its utility

may be limited for modeling ethanol's effects on affective processing. More generally, our results also introduce the possibility that CJB paradigms may not always capture subtle or pharmacologically induced affect-like states in invertebrates. Future work could benefit from combining behavioral assays with physiological or neurochemical markers.

Our findings also lend credence to the possibility that honeybees have evolved resistance to environmental sources of ethanol. Natural ethanol production dates back to the late Cretaceous, when yeast gained the ability to ferment sugars in fleshy fruits of early angiosperms (Benner et al. 2002). Adaptation to ethanol would have likely been advantageous, particularly for foragers, that are likely especially exposed to the substance. In fact, they are known to be the most ethanol-resistant caste and possess elevated alcohol dehydrogenase levels (Miler et al. 2021). However, as shown by our hemolymph data, ethanol remains in the bee's system for hours post-ingestion, suggesting that metabolism alone does not fully mitigate its effects. This persistence is likely due to the gradual passage of food from its storage in the crop to the stomach, where it is digested (Crailsheim 1988). Thus, even a single ethanol-containing meal could potentially influence a honeybee throughout a foraging bout, an activity that demands substantial cognitive engagement, including navigation, decision-making, predator avoidance, and learning. However, despite ethanol's potent, cross-species impact on neurotransmission (Phillips and Shent 1996), honeybees seem to resist many of its deleterious effects at field-realistic levels.

We detected no effect of ethanol not only on cognitive judgment bias but also on other behaviors previously shown to be sensitive to ethanol effects. Our results suggest that resistance to field-realistic ethanol concentrations extends across multiple functional domains, from whole-body activity (locomotion) and finer motor control (proboscis extension), to body coordination (propensity to change directions) and memory retrieval (expression of learned responses), and finally to decision-making under uncertainty. Some previous studies align with our findings, showing no effect of ethanol on bee learning (Abramson et al. 2000) or flight performance(Ostap-Chec et al. 2025). However, other

works report behavioral changes even at 1% ethanol, including altered flight kinematics (Ahmed et al. 2022), delayed return to the hive, reduced trophallaxis and self-grooming, as well as changes in social communication (Bozic et al. 2006). It is possible that low concentrations of ethanol impact aspects of body movement that bees can compensate for, making the effects undetectable at coarser scales (e.g. speed). In addition, particularly for social behaviors like waggle dance communication, the impact of low ethanol concentrations might not reflect a disturbance in the organism, but rather, a functional response to a particular environmental condition. It is possible that some consequences of ingesting low ethanol concentrations are only apparent on the group level and might reflect collective decision-making, for example, to reduce foraging when many individuals are carrying ethanol-containing food at the same time. For instance, Bozic and colleagues (2006) reported effects of colony-level inebriation when multiple foragers consumed ethanol simultaneously. Future studies should explore how low-dose ethanol concentrations influence social dynamics and collective behavior in honeybees.

The lack of observable behavioral changes is unlikely to be due to insufficient ethanol absorption. As in earlier studies (Bozic et al. 2006; Maze et al. 2006), we confirmed that ethanol was absorbed rapidly and persisted in the hemolymph during behavioral testing. The learning dynamics (Figure 4) and the acquisition rate observed during conditioning validate the effectiveness of our procedures. The initially higher response to the punished odor likely reflects trial order effects (as the punished cue was introduced later), but this pattern reversed in subsequent trials, confirming successful learning.

5. Conclusion

This study investigated the impact of ethanol on cognitive judgment bias in honeybees. Contrary to our expectations, field-realistic concentrations of ethanol had no discernible effect on bees' responses to ambiguous cues. In addition, ethanol did not impair motor activity or expression of conditioned responses. Ethanol presence was confirmed in the hemolymph for prolonged periods post-ingestion. We propose that honeybees may be behaviorally and physiologically adapted to tolerate low ethanol

levels. Our findings highlight the need to consider both evolutionary adaptations and methodological sensitivity when evaluating affect-like states in insects.

6. Funding information

This work was supported by the Faculty of Psychology, University of Warsaw, from the funds awarded by the Ministry of Science and Higher Education in the form of a subsidy for the maintenance and development of research potential in 2024 (501-D125-01-1250000 zlec. 501 1000 191). The research was also supported by the National Science Centre, Poland [grant number Sonata UMO-2021/43/D/NZ8/01044].

7. References

Abramson CI, d'Isa R, Wells H (2025) Physiological and behavioral pharmacology of ethanol in honey bees. J Comp Physiol A. https://doi.org/10.1007/s00359-025-01743-8

Abramson CI, Fellows GW, Browne BL, et al (2003) Development of an Ethanol Model Using Social Insects: II. Effect of Antabuse® on Consumatory Responses and Learned Behavior of the Honey Bee (*Apis Mellifera* L.). Psychol Rep 92:365–378. https://doi.org/10.2466/pr0.2003.92.2.365

Abramson CI, Stone SM, Ortez RA, et al (2000) The Development of an Ethanol Model Using Social Insects I: Behavior Studies of the Honey Bee (*Apis mellifera* L.). Alcohol Clin Exp Res 24:1153–1166. https://doi.org/10.1111/j.1530-0277.2000.tb02078.x

Ahmed I, Abramson CI, Faruque IA (2022) Honey bee flights near hover under ethanol-exposure show changes in body and wing kinematics. PLOS ONE 17:e0278916. https://doi.org/10.1371/journal.pone.0278916

Andrews K (2024) "All animals are conscious": Shifting the null hypothesis in consciousness science. Mind Lang 39:415–433. https://doi.org/10.1111/mila.12498

Avarguès-Weber A, Giurfa M (2013) Conceptual learning by miniature brains. Proc R Soc B Biol Sci 280:20131907. https://doi.org/10.1098/rspb.2013.1907

Baracchi D, Lihoreau M, Giurfa M (2017) Do Insects Have Emotions? Some Insights from Bumble Bees. Front Behav Neurosci 11:157. https://doi.org/10.3389/fnbeh.2017.00157

Bates D, Mächler M, Bolker B, Walker S (2015) Fitting Linear Mixed-Effects Models Using **lme4**. J Stat Softw 67:. https://doi.org/10.18637/jss.v067.i01

Bateson M, Desire S, Gartside SE, Wright GA (2011) Agitated Honeybees Exhibit Pessimistic Cognitive Biases. Curr Biol 21:1070–1073. https://doi.org/10.1016/j.cub.2011.05.017

Benner SA, Caraco MD, Thomson JM, Gaucher EA (2002) Planetary Biology--Paleontological, Geological, and Molecular Histories of Life. Science 296:864–868. https://doi.org/10.1126/science.1069863

Borsuk G, Ptaszyńska AA, Olszewski K, et al (2017) A New Method for Quick and Easy Hemolymph Collection from Apidae Adults. PLOS ONE 12:e0170487. https://doi.org/10.1371/journal.pone.0170487

Bouchebti S, Gershon Y, Gordin A, et al (2024) Tolerance and efficient metabolization of extremely high ethanol concentrations by a social wasp. Proc Natl Acad Sci 121:e2410874121. https://doi.org/10.1073/pnas.2410874121

Bowland AC, Melin AD, Hosken DJ, et al (2024) The evolutionary ecology of ethanol. Trends Ecol Evol S0169534724002404. https://doi.org/10.1016/j.tree.2024.09.005

Bozic J, Abramson CI, Bedencic M (2006) Reduced ability of ethanol drinkers for social communication in honeybees (Apis mellifera carnica Poll.). Alcohol 38:179–183. https://doi.org/10.1016/j.alcohol.2006.01.005

Castillo C, Chen H, Graves C, et al (2012) Biosynthesis of ethyl oleate, a primer pheromone, in the honey bee (Apis mellifera L.). Insect Biochem Mol Biol 42:404–416. https://doi.org/10.1016/j.ibmb.2012.02.002

Chastain G (2006) Alcohol, Neurotransmitter Systems, and Behavior. J Gen Psychol 133:329–335. https://doi.org/10.3200/GENP.133.4.329-335

Chermack ST, Giancola PR (1997) The relation between alcohol and aggression: An integrated biopsychosocial conceptualization. Clin Psychol Rev 17:621–649. https://doi.org/10.1016/S0272-7358(97)00038-X

Chiara V, Kim S (2023) AnimalTA : A highly flexible and easy-to-use program for tracking and analysing animal movement in different environments. Methods Ecol Evol 14:1699–1707. https://doi.org/10.1111/2041-210X.14115

Cieslik-Starkiewicz A, Piksa M, Noworyta K, et al (2024) Unveiling the power of optimism: Exploring behavioral and neuromolecular correlates of alcohol seeking and drinking in rats with biased judgement. Prog Neuropsychopharmacol Biol Psychiatry 135:111124. https://doi.org/10.1016/j.pnpbp.2024.111124

Collett TS, Fauria K, Dale K, Baron J (1997) Places and patterns -- a study of context learning in honeybees. J Comp Physiol [A] 181:343–353. https://doi.org/10.1007/s003590050120

Crailsheim K (1988) Regulation of food passage in the intestine of the honeybee (Apis mellifera L.). J Insect Physiol 34:85–90. https://doi.org/10.1016/0022-1910(88)90158-8

Deakin A, Mendl M, Browne WJ, et al (2018) State-dependent judgement bias in *Drosophila* : evidence for evolutionarily primitive affective processes. Biol Lett 14:20170779. https://doi.org/10.1098/rsbl.2017.0779

Freed EX (1978) Alcohol and Mood: An Updated Review. Int J Addict 13:173–200. https://doi.org/10.3109/10826087809039273

Gable RS (2004) Comparison of acute lethal toxicity of commonly abused psychoactive substances. Addiction 99:686–696. https://doi.org/10.1111/j.1360-0443.2004.00744.x

Giannoni-Guzmán MA, Giray T, Agosto-Rivera JL, et al (2014) Ethanol-Induced Effects on Sting Extension Response and Punishment Learning in the Western Honey Bee (Apis mellifera). PLoS ONE 9:e100894. https://doi.org/10.1371/journal.pone.0100894

Gilliam M (1979) Microbiology of pollen and bee bread: the yeasts. Apidologie 10:43–53. https://doi.org/10.1051/apido:19790106

Giurfa M, Sandoz J-C (2012) Invertebrate learning and memory: Fifty years of olfactory conditioning of the proboscis extension response in honeybees. Learn Mem 19:54–66. https://doi.org/10.1101/lm.024711.111

Harding EJ, Paul ES, Mendl M (2004) Cognitive bias and affective state. Nature 427:312–312. https://doi.org/10.1038/427312a

Hartig, F (2024) DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models. R Package Version 047. https://github.com/florianhartig/dharma

Jakubska A (2005) Why do Pollinators Become “sluggish”? Nectar Chemical C Onstituents from Epipactis Helleborine (L.) Crantz (orchidaceae). Appl Ecol Environ Res 3:29–38. https://doi.org/10.15666/aeer/0302_029038

Jones P, Agrawal AA (2022) Caffeine and ethanol in nectar interact with flower color impacting bumblebee behavior. Behav Ecol Sociobiol 76:103. https://doi.org/10.1007/s00265-022-03208-3

Kevan PG, Eisikowitch D, Fowle S, Thomas K (1988) Yeast-Contaminated Nectar and its Effects on Bee Foraging. J Apic Res 27:26–29. https://doi.org/10.1080/00218839.1988.11100777

Lagisz M, Zidar J, Nakagawa S, et al (2020) Optimism, pessimism and judgement bias in animals: A systematic review and meta-analysis. Neurosci Biobehav Rev 118:3–17. https://doi.org/10.1016/j.neubiorev.2020.07.012

Leadbeater E, Chittka L (2007) Social Learning in Insects — From Miniature Brains to Consensus Building. Curr Biol 17:R703–R713. https://doi.org/10.1016/j.cub.2007.06.012

Lewis EG, Dustman RE, Beck EC (1969) The Effect of Alcohol on Sensory Phenomena and Cognitive and Motor Tasks. Q J Stud Alcohol 30:618–633. https://doi.org/10.15288/qjsa.1969.30.618

Lieber CS (1991) Hepatic, Metabolic and Toxic Effects of Ethanol: 1991 Update. Alcohol Clin Exp Res 15:573–592. https://doi.org/10.1111/j.1530-0277.1991.tb00563.x

Lüdecke D, Ben-Shachar M, Patil I, et al (2021) performance: An R Package for Assessment, Comparison and Testing of Statistical Models. J Open Source Softw 6:3139. https://doi.org/10.21105/joss.03139

Matsumoto Y, Menzel R, Sandoz J-C, Giurfa M (2012) Revisiting olfactory classical conditioning of the proboscis extension response in honey bees: A step toward standardized procedures. J Neurosci Methods 211:159–167. https://doi.org/10.1016/j.jneumeth.2012.08.018

Maze IS, Wright GA, Mustard JA (2006) Acute ethanol ingestion produces dose-dependent effects on motor behavior in the honey bee (Apis mellifera). J Insect Physiol 52:1243–1253. https://doi.org/10.1016/j.jinsphys.2006.09.006

Mendl M, Burman OHP, Parker RMA, Paul ES (2009) Cognitive bias as an indicator of animal emotion and welfare: Emerging evidence and underlying mechanisms. Appl Anim Behav Sci 118:161–181. https://doi.org/10.1016/j.applanim.2009.02.023

Mendl M, Paul ES (2020) Animal affect and decision-making. Neurosci Biobehav Rev 112:144–163. https://doi.org/10.1016/j.neubiorev.2020.01.025

Merçot H, Defaye D, Capy P, et al (1994) Alcohol Tolerance, ADH Activity, and Ecological Niche of Drosophila Species. Evolution 48:746–757. https://doi.org/10.1111/j.1558-5646.1994.tb01358.x

Miler K (2025) Ethanol and pollinators: expanding Bowland et al.’s framework. Trends Ecol Evol 40:115–116. https://doi.org/10.1016/j.tree.2024.11.019

Miler K, Kuszewska K, Privalova V, Woyciechowski M (2018) Honeybees show adaptive reactions to ethanol exposure. Sci Rep 8:8707. https://doi.org/10.1038/s41598-018-27117-6

Miler K, Stec D, Kamińska A, et al (2021) Alcohol intoxication resistance and alcohol dehydrogenase levels differ between the honeybee castes. Apidologie 52:230–241. https://doi.org/10.1007/s13592-020-00812-y

Mixson TA, Abramson CI, Božič J (2010) The Behavior and Social Communication of Honey Bees (*APIS Mellifera Carnica* Poll.) under the Influence of Alcohol. Psychol Rep 106:701–717. https://doi.org/10.2466/pr0.106.3.701-717

Mustard JA, Edgar EA, Mazade RE, et al (2008) Acute ethanol ingestion impairs appetitive olfactory learning and odor discrimination in the honey bee. Neurobiol Learn Mem 90:633–643. https://doi.org/10.1016/j.nlm.2008.07.017

Mustard JA, Oquita R, Garza P, Stoker A (2019) Honey Bees (*Apis mellifera*) Show a Preference for the Consumption of Ethanol. Alcohol Clin Exp Res 43:26–35. https://doi.org/10.1111/acer.13908

Nakagawa S, Schielzeth H (2013) A general and simple method for obtaining R^2 from generalized linear mixed-effects models. Methods Ecol Evol 4:133–142. https://doi.org/10.1111/j.2041-210x.2012.00261.x

Neville V, Nakagawa S, Zidar J, et al (2020) Pharmacological manipulations of judgement bias: A systematic review and meta-analysis. Neurosci Biobehav Rev 108:269–286. https://doi.org/10.1016/j.neubiorev.2019.11.008

Ostap-Chec M, Bajorek D, Antoł W, et al (2025) Honey bees are resilient to the long-term presence of alcohol in their diet. Ecotoxicology. https://doi.org/10.1007/s10646-025-02903-x

Ostap-Chec M, Opalek M, Stec D, Miler K (2021) Discontinued alcohol consumption elicits withdrawal symptoms in honeybees. Biol Lett 17:20210182. https://doi.org/10.1098/rsbl.2021.0182

Pahl M, Si A, Zhang S (2013) Numerical Cognition in Bees and Other Insects. Front Psychol 4:. https://doi.org/10.3389/fpsyg.2013.00162

Perry CJ, Baciadonna L (2017) Studying emotion in invertebrates: what has been done, what can be measured and what they can provide. J Exp Biol 220:3856–3868. https://doi.org/10.1242/jeb.151308

Perry CJ, Barron AB, Chittka L (2017) The frontiers of insect cognition. Curr Opin Behav Sci 16:111–118. https://doi.org/10.1016/j.cobeha.2017.05.011

Phillips TJ, Shent EH (1996) Neurochemical Bases of Locomotion and Ethanol Stimulant Effects. In: International Review of Neurobiology. Elsevier, pp 243–282

Procenko O, Read JCA, Nityananda V (2024) Physically stressed bees expect less reward in an active choice judgement bias test. Proc R Soc B Biol Sci 291:20240512. https://doi.org/10.1098/rspb.2024.0512

Sandoz J-C, Pham-Delègue M-H (2004) Spontaneous Recovery After Extinction of the Conditioned Proboscis Extension Response in the Honeybee. Learn Mem 11:586–597. https://doi.org/10.1101/lm.81504

Saunders JB, Degenhardt L, Reed GM, Poznyak V (2019) Alcohol Use Disorders in ICD-11: Past, Present, and Future. Alcohol Clin Exp Res 43:1617–1631. https://doi.org/10.1111/acer.14128

Schaeffer RN, Mei YZ, Andicoechea J, et al (2017) Consequences of a nectar yeast for pollinator preference and performance. Funct Ecol 31:613–621. https://doi.org/10.1111/1365-2435.12762

Scheiner R, Abramson CI, Brodschneider R, et al (2013) Standard methods for behavioural studies of *Apis mellifera*. J Apic Res 52:1–58. https://doi.org/10.3896/IBRA.1.52.4.04

Schlüns H, Welling H, Federici JR, Lewejohann L (2017) The glass is not yet half empty: agitation but not Varroa treatment causes cognitive bias in honey bees. Anim Cogn 20:233–241. https://doi.org/10.1007/s10071-016-1042-x

Solvi C, Baciadonna L, Chittka L (2016) Unexpected rewards induce dopamine-dependent positive emotion–like state changes in bumblebees. Science 353:1529–1531. https://doi.org/10.1126/science.aaf4454

Strang C, Muth F (2023) Judgement bias may be explained by shifts in stimulus response curves. R Soc Open Sci 10:221322. https://doi.org/10.1098/rsos.221322

Valdez GR, Roberts AJ, Chan K, et al (2002) Increased Ethanol Self-Administration and Anxiety-Like Behavior During Acute Ethanol Withdrawal and Protracted Abstinence: Regulation by Corticotropin-Releasing Factor. Alcohol Clin Exp Res 26:1494–1501. https://doi.org/10.1111/j.1530-0277.2002.tb02448.x

Van Der Goot MH, Kooij M, Stolte S, et al (2021) Incorporating inter-individual variability in experimental design improves the quality of results of animal experiments. PLOS ONE 16:e0255521. https://doi.org/10.1371/journal.pone.0255521

Van Erp AMM, Miczek KA (1997) Increased aggression after ethanol self-administration in male resident rats. Psychopharmacology (Berl) 131:287–295. https://doi.org/10.1007/s002130050295

Varnon CA, Dinges CW, Black TE, et al (2018) Failure to Find Ethanol-Induced Conditioned Taste Aversion in Honey Bees (*Apis mellifera* L.). Alcohol Clin Exp Res 42:1260–1270. https://doi.org/10.1111/acer.13761

Wand G, Levine M, Zweifel L, et al (2001) The cAMP–Protein Kinase A Signal Transduction Pathway Modulates Ethanol Consumption and Sedative Effects of Ethanol. J Neurosci 21:5297–5303. https://doi.org/10.1523/JNEUROSCI.21-14-05297.2001

Wiens F, Zitzmann A, Lachance M-A, et al (2008) Chronic intake of fermented floral nectar by wild treeshrews. Proc Natl Acad Sci 105:10426–10431. https://doi.org/10.1073/pnas.0801628105

Wright GA, Lillvis JL, Bray HJ, Mustard JA (2012) Physiological State Influences the Social Interactions of Two Honeybee Nest Mates. PLoS ONE 7:e32677. https://doi.org/10.1371/journal.pone.0032677