BIOLOGY LETTERS

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Cite this article: 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

Received: 1 April 2021
Accepted: 24 May 2021

Subject Areas:
behaviour, ecology

Keywords:
addiction, alcohol, dependence, ethanol, honeybee, withdrawal

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Electronic supplementary material is available online at https://doi.org/10.6084/m9.figshare.c.5448559.

THE ROYAL SOCIETY PUBLISHING

Animal behaviour

Discontinued alcohol consumption elicits withdrawal symptoms in honeybees

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The honeybee continues to be developed as a model species in many research areas, including studies related to the effects of alcohol. Here, we investigate whether workers display one of the key features of alcoholism, namely withdrawal symptoms. We show that workers fed for a prolonged time on food spiked with ethanol, after discontinuation of access to such food, exhibited a marked increase in the consumption of ethanol and a slight increase in mortality. We additionally show that withdrawal symptoms do not include an increase in appetitiveness of ethanol diluted in water. Our results demonstrate that workers can develop alcohol dependence, which might be especially important in the natural setting of repeated exposure to ethanol in floral nectar and for their potential as a model of alcohol addiction.

1. Introduction

Alcoholism is a major social problem and invertebrate model species are widely used for its study [1]. The honeybee is proposed as one such model due to its unique characteristics which set it apart from other invertebrates [2–5]. Indeed, honeybee workers willingly consume alcohol (i.e. ethanol) solutions in sugar, in concentrations as high as 20% [2], and under the influence of ethanol display behaviours similar to those of alcohol-intoxicated vertebrates and humans. For example, ethanol consumption affects social interactions between workers [5–9] and impairs their locomotion, foraging and learning [3,10–14]. The extent of these behavioural changes depends on the amount of ethanol consumed [12,15] and physiological consequences which follow [16]. Among honeybee workers, foragers working outside the hive seem to show the greatest resistance to the detrimental effects of ethanol [17]. This is likely because foragers are evolutionarily tuned for ethanol exposure due to its occasional encounter in nature. Indeed, floral nectar is often infested by yeast [18,19] which, through fermentation, can produce low concentrations of ethanol, probably up to 1% [20–25]. A field experiment revealed that foragers continue to collect nectar spiked with ethanol [26].

Recent studies indicate that honeybee workers not only willingly consume alcohol [26–28], but are predisposed to alcoholism. Conditioned taste aversion (CTA) leads to avoidance of an initially neutral stimulus associated with detrimental health effects. Although some forms of CTA may occur in workers [29], it is probably absent after ethanol consumption [30]. Lack or reduced ethanol CTA is characteristic of human alcoholics and strains of laboratory rodents with increased alcoholic tendencies [31,32]. Moreover, a recent study demonstrated that workers show tolerance to ethanol, expressed as a lower motor impairment in response to ethanol in individuals previously exposed to it

than those exposed for the first time [33]. These hallmarks of alcoholism suggest that workers could become addicted to alcohol (i.e. dependent) under appropriate conditions. Addiction can best be illustrated by another hallmark of alcoholism, namely alcohol withdrawal syndrome (AWS), which is a group of symptoms that occur when alcohol access is discontinued after a prolonged period of consumption [34]. Demonstrating these symptoms in honeybee workers is crucial for understanding the value of these insects in alcoholism-related studies.

Here, we investigated whether workers display AWS. We hypothesized that workers exposed to alcohol-spiked food for a prolonged time, after discontinuation, would show dependence, in contrast with workers with uninterrupted access to alcohol and those without or short prior access to alcohol. We tested multiple potential symptoms of dependence, including increased mortality as well as increased appetitiveness and consumption of alcohol.

2. Material and methods

(a) Preparation of workers

Three different honeybee colonies were used. In the early morning of day 0, newly emerged workers were collected from frames kept overnight in an incubator (Pol-Eco Aparatura, Poland) at 34°C. These individuals were colour marked and released into their hives for a period of normal development. A week later (on day 7), marked individuals from each colony were collected and divided into four groups which ultimately were to provide individuals differing in their experience with feeding on ethanol: no exposure (group 1), short exposure (group 2), exposure withheld (group 3) and constant exposure (group 4). Each group counted 200 individuals placed in two cages (100 individuals in each) kept with ad libitum water and food (1 M sucrose solution) in an incubator at 34°C. For two weeks after collection (until day 21), a period of acclimation was applied, as pilot observations indicated that it improved general survival. On day 21, when workers reached forager age [35], the primary diet started and lasted for 3 weeks (until day 42). During that period, group 1 and 2 received normal food (1 M sucrose solution), whereas group 3 and 4 received food with the addition of 1% v/v ethanol. On day 42, the secondary diet started and lasted for 3 days (until day 45). During that period, group 1 and 3 received normal food whereas group 2 and 4 received ethanol-spiked food. One cage in each group was designated for the mortality analysis and thus during both diets, dead individuals were counted daily in these. Individuals from another cage in each group were used for testing withdrawal symptoms on day 46.

During the entire period in the laboratory (days 7–46), water and food were renewed every 12 h in all cages. To confirm that workers had constant access to alcohol during periods in-between food renewal, we analysed alcohol evaporation from feeders. On a single day after the experiment, cages with feeders filled with a 1 M sugar solution with addition of 1% v/v ethanol were placed in an incubator at 34°C. Every hour (between 08.00 and 20.00), we collected 10 µl samples of the solution from the feeders and froze them immediately in −20°C. Measurements of alcohol concentration in these samples were conducted the next morning with the use of an endpoint ethanol assay and a microplate spectrophotometer (SpectraMax iD3, Molecular Devices, USA) [12,36]. Alcohol concentration was calculated according to the assay manufacturer's instructions (Megazyme, Ireland). Evaporation of alcohol from feeders was negligible (see the electronic supplementary material, S2).

(b) Testing of workers

On day 46, workers were tested for withdrawal symptoms. First, we tested the appetitiveness of ethanol diluted in water in 30 individuals per group per colony. Workers with normal prior access to food were taken out from their cage in bouts of five individuals per group (colony after colony), harnessed and tested after a 5 min interval. Testing solutions: water, 0.313%, 0.625%, 1.25%, 2.5%, 5%, 10%, 25% v/v ethanol in water, and 1 M sugar solution without ethanol, were presented to workers in the listed order, by touching both antennae with a cotton stick soaked with a given solution, with a 5 min interval between consecutive solutions for each individual. The response of each worker, i.e. whether it extended its proboscis, was noted. Testing solutions were renewed every three bouts of individuals to limit the effects of ethanol evaporation. Second, we tested the appetitiveness of ethanol diluted in sugar in another 30 individuals per group per colony. We used an analogous procedure, except the series of testing solutions were diluted in sugar solution and thus were as follows: water, 0.313%, 0.625%, 1.25%, 2.5%, 5%, 10%, 25% v/v ethanol in 1 M sugar solution, and 1 M sugar solution without ethanol. Workers were not starved prior to both of these tests as we were interested in responses to alcohol, to which workers could react even in the absence of hunger for sugar, but the exact satiation state of workers was not controlled for. The purpose of the last testing solution in these tests, 1 M sugar solution without ethanol, was to control for the possibility of habituation. Third, we tested the consumption of food with the addition of ethanol. Individuals from the previous test were used (after a approx. 10 min break). A 1 M sugar solution with the addition of 1% v/v ethanol was prepared anew every three bouts of individuals. In the test, antennae were touched with the solution and if the proboscis was extended then a drink from an end-to-end microcapillary filled with the solution was offered. The total amount eaten was measured. Workers not extending their proboscis were given a value of 0 µl consumed.

(c) Statistical analysis

Statistics were performed using R v. 3.6.3 [37]. Mortality of workers during the secondary diet period was analysed using mixed Cox proportional hazard model (coxme function). In it, group was included as a fixed factor and colony as a random factor. Comparisons between groups were carried out using z tests with group 1 as a reference. Appetitiveness of alcohol, as ethanol diluted in water and in 1 M sugar solution, were analysed separately using generalized linear mixed models (glmer function) with a binomial distribution and logit link function. Each model included group and solution (fixed factors), as well as their interaction, and an individual nested within the colony (random factors). *Post hoc* comparisons between groups for each testing solution and between solutions for each group were carried out using Tukey tests. Consumption of the 1 M sugar solution with the addition of 1% v/v ethanol was analysed using zero-inflated generalized linear mixed model (glmmTMB function) with a Poisson distribution and log link function. The model included group (fixed factor) and colony (random factor). *Post hoc* comparisons between groups were carried out using Tukey tests. In GLMMs, to assess the general significance of a given factor, a model with and without it were compared using χ^2 tests.

3. Results and discussion

Here, we tested individuals that had never experienced ethanol (group 1), that had experienced ethanol for a short period (group 2), that had ethanol withheld after prolonged exposure (group 3) and that had always experienced ethanol

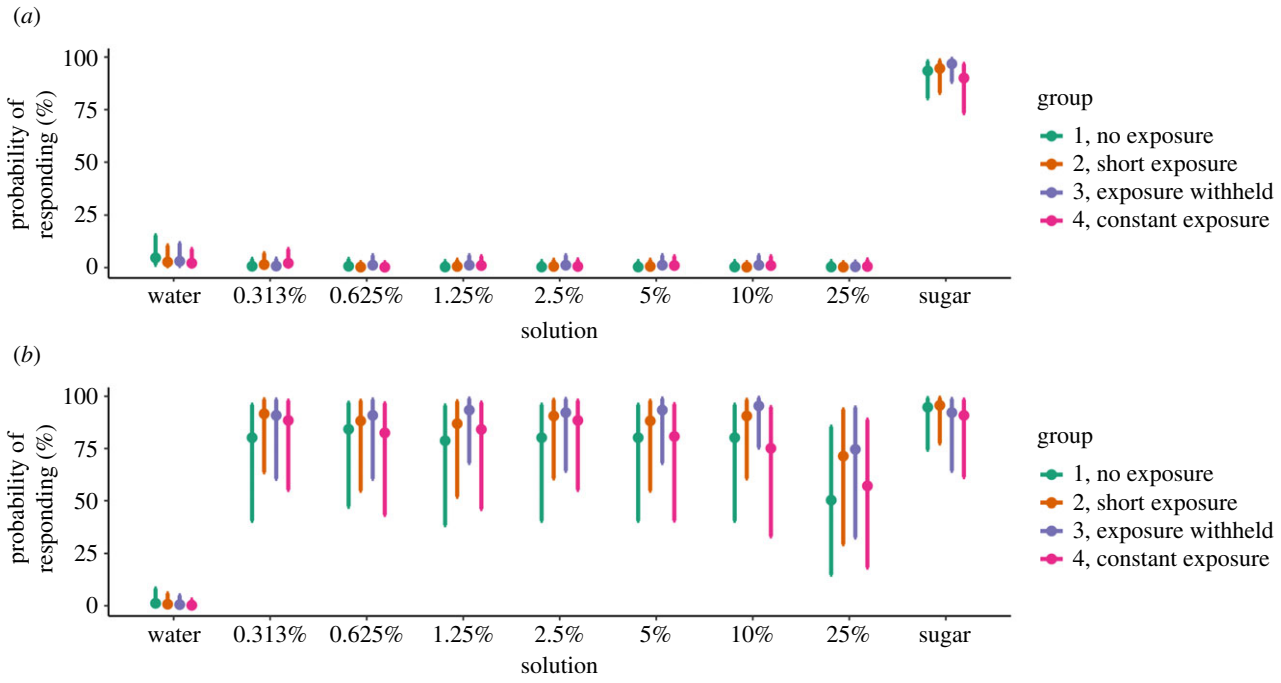

Figure 1. Probability of responding with proboscis extension in workers belonging to different groups: (*a*) shows the probability of response to water, a series of different % ethanol/water solutions, and sugar solution without ethanol; (*b*) shows probability of response to water, a series of different % ethanol/sugar solutions, and sugar solution without ethanol. Dots and whiskers represent model predictions (means and CI).

(group 4). During the secondary diet period, mortality was low in group 1 (1.5%). Compared to that, neither group 2 ($z = 1.180$, $p = 0.240$) nor group 4 ($z = 1.530$, $p = 0.130$) differed in mortality (3.0% and 3.6%, respectively). In group 3, mortality was slightly but significantly higher than in group 1 ($z = 2.160$, $p = 0.031$), as it reached 4.2%. There were no differences between colonies in mortality ($\chi^2 = 0.410$, $p = 0.522$).

There were differences in appetitiveness of ethanol in water solutions between colonies and individuals ($\chi^2 = 16.496$, $p < 0.001$, $\chi^2 = 161.070$, $p < 0.001$, respectively). Overall, appetitiveness did not differ between groups ($\chi^2 = 2.554$, $p = 0.466$), yet differed between solutions ($\chi^2 = 1591.400$, $p < 0.001$). Differences between solutions were consistent in all groups (group × solution interaction: $\chi^2 = 16.132$, $p = 0.883$, figure 1*a*)—sugar solution without ethanol elicited increased probability of responding (see the electronic supplementary material, S2). In terms of appetitiveness of ethanol in sugar, there were again differences between colonies and individuals ($\chi^2 = 292.66$, $p < 0.001$, $\chi^2 = 805.88$, $p < 0.001$, respectively). Overall, although the group × solution interaction was non-significant ($\chi^2 = 23.895$, $p = 0.468$), group was close to significance ($\chi^2 = 7.766$, $p = 0.051$, figure 1*b*). Indeed, *post hoc* comparisons between groups yielded significant differences for the 10% solution, for which group 3 displayed significantly higher probability than groups 1 and 4 (see the electronic supplementary material, S2). There were also significant differences between solutions ($\chi^2 = 1272.700$, $p < 0.001$), which stemmed mostly from a lower probability of responding to the 25% ethanol/sugar solution and water in all groups (see the electronic supplementary material, S2). High response to sugar solution without ethanol in both tests indicated that earlier worker responses unlikely stemmed from habituation (figure 1), although notably, performing tests with a random order of testing solutions would be better in eliminating this possibility.

Consumption of ethanol in sugar revealed differences between colonies ($\chi^2 = 12.339$, $p < 0.001$) and between groups ($\chi^2 = 53.568$, $p < 0.001$). All individuals having previously encountered alcohol responded to the solution more often than those belonging to group 1, but workers from group 3 consumed the greatest volumes (figure 2).

Voluntary consumption of sugar solutions containing ethanol is well established in honeybee workers [2,26–28]. We demonstrate that workers display AWS after discontinuation of chronic feeding on such solutions, as primarily evidenced by a marked increase in the consumption of ethanol and also a slight increase in mortality. Additionally, we demonstrate that withdrawal symptoms do not include an increase in the appetitiveness of ethanol in water. Indeed, it has been previously documented that workers do not respond to ethanol solutions in water [27]. It is clear also from our contrasting results of appetitiveness of ethanol in water or sugar solutions that ethanol alone is insufficient to elicit feeding behaviour. In turn, the possibility of an increased appetitiveness of different ethanol concentrations in sugar as another symptom of withdrawal needs to be treated with caution as our present results indicate only a trend towards that symptom. Generally, workers seem to find ethanol in sugar attractive, even when its concentration is very high in ecological terms [20–25]. Of note, psychoactive alkaloids present in floral nectar are known to elicit worker feeding preferences and are hypothesized to impose dependence [38]. Therefore, it is possible that the present results might be important in the context of alcohol encounter by foragers in natural conditions. One has to keep in mind, however, that our workers fed on artificial diets for weeks and thus their physiology and behaviour might be altered.

One can attempt to explain the present results in alternative ways. For example, a change in food type after the primary diet could possibly result in a lower propensity to feed during the secondary diet and thus lead to an increase in mortality. However, mortality of workers from group 2

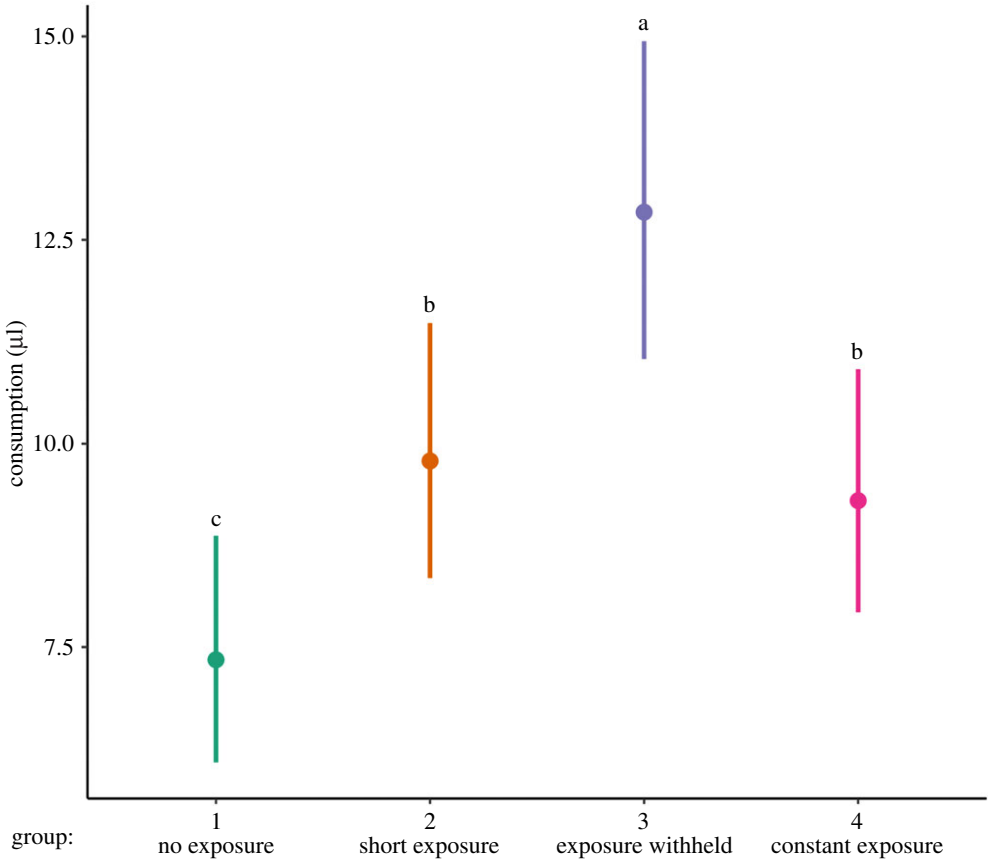

Figure 2. Consumption of a sugar solution with the addition of 1% ethanol in workers belonging to different groups. Dots and whiskers represent model predictions (means and CI). Letters indicate statistical differences at $p < 0.04$.

did not increase significantly despite a change in diet, like in workers from group 3. Also, we did not observe any apparent loss of appetite following a change in diet (see electronic supplementary material, S2). Moreover, workers could possibly learn cues associated with feeding during the primary diet (e.g. ethanol odour in those fed ethanol-spiked food) and thus show less aversive reactions to ethanol during testing. However, then workers in group 4 can be expected to perform similarly to those in group 3, yet they did not. Therefore, AWS seems best to comprehensively explain our results.

To understand alcohol abuse, the utilization of animal models is essential. Our results further develop the honeybee in this context [1–17,26–28] and support previous studies showing that workers are predisposed to alcoholism [30,33]. Studies on the occurrence of alcohol dependence in other invertebrates initiated discoveries in terms of the mechanisms behind alcoholism [39]. Physiological bases of alcohol dependence in the honeybee thus deserve particular research attention, especially considering comparative aspects (e.g. to *Drosophila*, [40]). Considering the unique characteristics of the honeybee, the prospective benefits of its use in research devoted to alcoholism may be unparalleled.

Ethics. Our protocols comply with standard welfare practice in our field.

Data accessibility. The dataset supporting this article has been uploaded as the electronic supplementary material, S1 [41].

Authors' contributions. M.O.-C. collected the data, participated in data analysis and drafted the manuscript; M.O. and D.S. collected the data and critically revised the manuscript; K.M. conceived of the study, designed the study, carried out the statistical analyses, coordinated the study and helped draft the manuscript. All authors gave final approval for publication and agree to be held accountable for the work performed therein.

Competing interests. We declare we have no competing interests.

Funding. We received no funding for this study.

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