



Illness-dependent conditioned prey avoidance in an amphibian



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ARTICLE INFO

Article history:

Received 10 September 2013

Received in revised form 8 January 2014

Accepted 17 January 2014

Available online 27 January 2014

Keywords:

Amphibians
Avoidance learning
Illness behavior
Taste

ABSTRACT

Conditioned taste avoidance (CTA) helps prevent consumption of dangerous foods. It results from the pairing of a novel food or taste with subsequent aversive consequences, such as illness. Previous studies of CTA in amphibians have produced conflicting results. Establishing the presence or absence of CTA in amphibians is needed to clarify the phylogeny of this phenomenon. This experiment evaluated the ability of the fire-bellied toad *Bombina orientalis* to avoid a novel food item previously paired with subsequent illness or unpalatable taste. Mealworms, a novel prey item for the subjects, were coated with a solution of either 2% HCl or 3% CuSO₄ to make them unpalatable or nauseating, respectively. Lengthy and obvious signs of illness such as face wiping and retching followed the consumption of mealworms coated with CuSO₄, whereas consumption of mealworms coated with HCl only resulted in distinct and short lived aversive reactions at the time of consumption. The results showed that consumption of mealworms tainted with CuSO₄, but not HCl, rapidly induced prey avoidance. This response was specific to mealworms; the usual food (crickets) was not avoided. The results suggest that CTA following illness is not restricted to amniote vertebrates.

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1. Introduction

Important differences in emotion and learning are proposed to have emerged after the phylogenetic divergence of amniote and anamniote vertebrates. For example, handling-induced tachycardia and hyperthermia are exhibited in reptiles, mammals and birds but not amphibians or fish (Cabanac and Gosselin, 1993; Cabanac and Laberge, 1998; Cabanac and Cabanac, 2000; Cabanac and Aizawa, 2000). Conditioned taste avoidance (CTA) is the avoidance of a novel food item following the pairing of that food with negative consequences (Garcia et al., 1955; Parker, 2003). It is an important phenomenon adapted to prevent the consumption of toxic or dangerous foods and can manifest as a result of the pairing of a food item with subsequent consequences, such as pain and nausea. CTA represents a trait whose evolutionary origin is unclear. It could have evolved in amniotes (Paradis and Cabanac, 2004) or the trait could have evolved prior to the origin of amniotes in amphibians (Mikulka et al., 1981), fishes (Mackay, 1974; Little, 1977; Martín et al., 2011) or invertebrates (Kita et al., 2011). Although prey avoidance has been observed in amphibians in response to certain consequences,

such as a bee sting (Brower and Brower, 1962), unpalatability (Sternthal, 1974), and consumption of toxic prey (Greenlees et al., 2010), there is a debate about the ability of amphibians to learn to avoid a novel prey following its association with illness.

In past laboratory studies of CTA involving amphibians, novel food items were paired with intraperitoneal injections of lithium chloride (LiCl) in attempts to induce nausea. Paradis and Cabanac (2004) showed that four species of reptiles avoided food paired with LiCl but two species of amphibians did not. Thus, they asserted that CTA of nausea-paired foods does not exist in amphibians. This conclusion conflicts with the results of Mikulka et al. (1981), who showed that *Bufo americanus* could develop a prey avoidance based on pairing with LiCl. Interestingly, no outward signs of illness (retching or vomiting) were observed following intraperitoneal injections of LiCl in both studies. Thus, it is unclear whether the amphibians used in the above studies were sensitive to LiCl at all, especially when considering that amphibians are known to exhibit retching and vomiting when exposed to emetic drugs (Naitoh et al., 1991). It is also uncertain whether Mikulka et al. (1981) demonstrated CTA by inducing nausea or some other aversive effect. In that respect, it may be of note that Mikulka et al. (1981) used a concentration of 0.5 M LiCl to produce prey avoidance, which is hypertonic to body fluids, while Paradis and Cabanac (2004) used an isotonic concentration of 0.15 M LiCl. This discrepancy offers a potential explanation for the drastically differing observations between the two studies. It is possible that the avoidance observed

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by Mikulka et al. (1981) could be attributed to an osmotic shock caused by the hypertonic injection.

Consequently, to test the potential of amphibians to learn to avoid nausea-paired foods, it was of interest to use a potent emetic drug. Copper sulphate (CuSO_4) is known to cause retching and emesis (vomiting), both signs of nausea, in many amphibian species (Naitoh et al., 1991), and our preliminary studies indicated that this was also true in the species used in our experiments, the fire-bellied toad *Bombina orientalis*. On the other hand, hydrochloric acid (HCl) is a chemical with an unpalatable taste that produces an immediate, acute aversive reaction, including recoil and tongue-wiping (E. To, personal observations). HCl has been used to induce prey avoidance in amphibians in discrimination situations, without note of clear signs of illness (Sternthal, 1974; Mikulka et al., 1980). In the present study, we used mealworms, a novel food, paired with CuSO_4 or HCl to compare the effects on prey avoidance of treatments inducing illness or unpalatability, respectively. This was done to find out if the phenomenon of conditioned prey avoidance could depend on illness in amphibians. Here, we use the terms 'conditioned taste avoidance' and 'conditioned prey avoidance' interchangeably despite the fact that our experimental design could not ascertain whether avoidance is based solely on gustation or other sensory modalities, such as olfaction and vision. We hypothesized that fire-bellied toads would exhibit CTA when mealworms are associated with both nausea and unpalatability, but that avoidance would develop quicker and last much longer following the nausea treatment, because nausea conveys important information about food integrity. Thus, the toads should reject mealworms (novel food item) but not crickets (non-novel food item) following the pairing of a mealworm with either a CuSO_4 or HCl solution but not with water (the vehicle), and this avoidance should arise faster in toads that receive CuSO_4 -paired mealworms than those that receive HCl-paired mealworms. The toads should also begin to accept untaunted mealworms more rapidly following pairings with HCl than with CuSO_4 .

2. Methods

2.1. Animals

The test subjects consisted of 38 *B. orientalis* which had not been previously exposed to mealworms in our care. They were purchased from a local supplier (National Reptile Supply, Mississauga, ON) and held at a temperature of 21 °C under a photoperiod of 12:12-h light:dark (lights on at 7:00 h) for a period of six months before the experiment. The toads were housed in groups in plastic tanks (28 cm × 18 cm × 18 cm) with gravel substrate, broken clay pots, and flat stones for cover. Prior to the experiment, toads were fed live crickets once per week and received ad libitum access to water. Food was withheld for one week leading up to the experiment and feeding was restricted to handfed mealworms and crickets for the duration of the experiment. The experimental procedures were approved by the University of Guelph animal care committee under the guidelines of the Canadian Council on Animal Care.

2.2. Procedures

The experiment involved two stages: acquisition trials, where chemical-mealworm pairings took place, and consumption tests, where prey avoidance/consumption was measured. In acquisition trials, toads were placed into a 20 l glass tank with two mealworms trapped under an inverted glass Petri dish, allowing visual exposure to the prey but not consumption. After 1 min of visual exposure, the toads were presented with a mealworm dipped in the appropriate

solution (depending on treatment group). This presentation was made by the experimenter using forceps. Toads were monitored for 5 min for any signs of distress such as retching, face-wiping, or flattening of the head or body (Naitoh et al., 1991), and their onset latency and duration.

The toads were sorted into six groups: CuSO_4 -continuous ($n = 7$), CuSO_4 -interrupted ($n = 7$), HCl-continuous ($n = 6$), HCl-interrupted ($n = 6$), H_2O -continuous ($n = 6$), and H_2O -interrupted ($n = 6$). Toads designated continuous underwent four straight weekly acquisition trials followed by three straight weekly consumption tests. Toads designated interrupted followed the same schedule except one additional consumption test occurred between the first and second acquisition trials in order to evaluate effects after a single trial. Groups were balanced for body mass (average mass = 4.91 g, between group SEM = 0.003). Toads designated CuSO_4 received mealworms dipped in a 3% CuSO_4 solution during acquisition trials. This concentration reliably induced behavioral signs of illness without inducing mortality. It was chosen from a preliminary dose-response study (E. To, unpublished observations). Toads designated HCl received mealworms dipped in a 2% HCl solution during acquisition trials, as in Sternthal (1974). Toads designated H_2O received mealworms dipped in distilled water during acquisition trials. Distilled water was the vehicle used to dissolve CuSO_4 and HCl for the other treatments.

Consumption tests occurred one week after the first (interrupted groups) or fourth acquisition trials (all groups), or a week after the previous consumption test. In these tests, the toads were given the opportunity to consume two mealworms from an accessible Petri dish for 2 min followed by the opportunity to consume two crickets presented similarly for 2 min. Latency to strike and number of mealworms and/or crickets consumed were recorded by the experimenter (E.S.K. To). A digital timer was used to record latencies and monitor the maximal 2 min duration. To ensure that any malaise induced by consumption of mealworms during this phase (e.g. a disgust reaction due to pairing of mealworms and nausea) did not affect the consumption of crickets, the second consumption test for each toad was altered so that crickets were presented before mealworms. No difference in cricket consumption between consumption tests was noted. In all subsequent consumption tests, mealworms were again offered first to eliminate the possibility of confounding selective mealworm avoidance with satiation from cricket consumption.

2.3. Statistics

Since the data were not normally distributed, it was analyzed using nonparametric statistics. Fisher's exact probability tests were used to evaluate if there were differences in the number of toads that consumed 0, 1 or 2 mealworms after the different treatments administered during acquisition trials. Upon a significant Fisher's exact test ($\alpha = 0.05$) including the three treatments, each treatment pair was compared to determine between-treatment differences. This test was also used to assess potential differences in mealworm consumption between interrupted and continuous groups of toads. A Kruskal-Wallis test was used to find out if treatments had an effect on strike latency, and upon significance ($\alpha = 0.05$) it was followed by Dunn's post test to establish between-treatment differences. The Wilcoxon matched pairs test was used to examine potential strike latency differences in the same group of toads at different time points, while the Mann-Whitney test was used to assess if there was a difference in strike latency between interrupted and continuous groups of toads. Statistical analyses and graphs were done in Prism version 5.04 (GraphPad Software Inc., San Diego, CA, USA), except for Fisher's exact tests, which were computed online at <http://vassarstats.net>.

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