

## Video Article

# Hyponeophagia: A Measure of Anxiety in the Mouse

Rob M.J. Deacon

Department of Experimental Psychology, University of Oxford

Correspondence to: Rob M.J. Deacon at [robert.deacon@psy.ox.ac.uk](mailto:robert.deacon@psy.ox.ac.uk)URL: <http://www.jove.com/details.php?id=2613>

DOI: 10.3791/2613

Keywords: Neuroscience, Issue 51, Anxiety, hyponeophagia, bait shyness, mice, hippocampus, strain differences, plus-maze,

Date Published: 17/5/2011

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Citation: Deacon, R.M. Hyponeophagia: A Measure of Anxiety in the Mouse. *J. Vis. Exp.* (51), e2613, DOI : 10.3791/2613 (2011).

## Abstract

Before the present day, when fast-acting and potent rodenticides such as alpha-chloralose were not yet in use, the work of pest controllers was often hampered by a phenomenon known as "bait shyness". Mice and rats cannot vomit, due to the tightness of the cardiac sphincter of the stomach, so to overcome the problem of potential food toxicity they have evolved a strategy of first ingesting only very small amounts of novel substances. The amounts ingested then gradually increase until the animal has determined whether the substance is safe and nutritious. So the old rat-catchers would first put a palatable substance such as oatmeal, which was to be the vehicle for the toxin, in the infested area. Only when large amounts were being readily consumed would they then add the poison, in amounts calculated not to affect the taste of the vehicle. The poisoned bait, which the animals were now readily eating in large amounts, would then swiftly perform its function.

Bait shyness is now used in the behavioural laboratory as a way of measuring anxiety. A highly palatable but novel substance, such as sweet corn, nuts or sweetened condensed milk, is offered to the mice (or rats) in a novel situation, such as a new cage. The latency to consume a defined amount of the new food is then measured.

Robert M.J. Deacon can be reach at [robert.deacon@psy.ox.ac.uk](mailto:robert.deacon@psy.ox.ac.uk)

## Video Link

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## Protocol

### 1. Introduction:

Mice and rats will initially consume only small amounts of a novel food. This is thought to be because they are liable to poisoning due to their inability to vomit.

The only exception to this is if they have previously encountered another animal which has eaten that food; if they smell it on the exhaled air from their peers, they will classify it as safe<sup>13</sup>. This is because the food odour is paired with carbon disulphide on the other rat's breath<sup>12</sup>. Indeed, carbon disulphide itself increases food consumption in both mice and rats<sup>17</sup>. The phenomenon is called "social transmission of food preferences" and can be used as a simple olfactory learning paradigm<sup>22</sup>. This has been reported to be sensitive to lesions of the hippocampus<sup>3</sup>, but other studies<sup>5</sup> failed to replicate this finding. It has been suggested that the neocortical cholinergic system, particularly the orbitofrontal component<sup>19</sup> may be more important than the hippocampal cholinergic system for social transmission. Social transmission has even been shown to track memory impairment in aged rats, probably mediated by decreased CREB (cAMP-response element-binding protein) transmission in the aged hippocampus<sup>7</sup>.

The ease with which rodents acquire a food preference by social transmission has strong implications for hyponeophagia testing. When the author was performing a hyponeophagia test with hippocampal lesioned and control rats, he accidentally put a tested hippocampal rat (which had eaten the sweet corn) into the cage of the next control rat due for testing<sup>8</sup>. It ate within 14 s, the only control not to have a latency of 300 s. Great care should therefore be taken to prevent such social transmission of food odours during hyponeophagia testing.

If the first hyponeophagia test does not result in a clear, unambiguous result, multiple further tests can be performed. Figures 1-4 illustrate examples of different apparatus. The number of test situations that can be devised is only limited by the imagination of the experimenter. Results are analysed by two-way repeated measures ANOVA. Since hyponeophagia data is generally very variable, not conforming to a Gaussian distribution and often skewed, with some animals giving very long latencies, it should first be transformed; a square root or log transformation is particularly useful for truncating the extreme longer latencies.

### 2. Apparatus:

Figures 1-4 are presented as examples of hyponeophagia test set-ups. The specific combinations of novel food and environment are not critical, though the food well shown in Figure 1 is obviously best suited to containing liquids, although a niblet of sweet corn could also be placed in it.

In general, it is a good idea to have most of the floor of the apparatus covered with the food. Not only do animals appear to find this unusual surface aversive, this technique also eliminates confounds due to exploration before finding the food. Thus a sedative drug might appear anxiogenic because it increased the latency to find and eat the food because it depressed locomotion, not because it was anxiogenic. For example, one publication advised putting the food in the centre of an open field<sup>2</sup>. Not only does this result in the feeding latency being confounded by exploratory time, it also introduces an additional facet of anxiety, i.e. open field central area aversion, a classic measure of open field-mediated anxiety, which has been related to avoiding predation risk in natural habitats<sup>14</sup>. However, in some situations this might actually be a desired feature of the experiment. Another disadvantage of the experiment described in<sup>2</sup> was that the bait was standard lab chow in the centre of the open field. This has the double disadvantage that it is not novel, and also is not *extremely* palatable, unlike foods such as sweet corn or sweetened condensed milk. We have always found that using baits of extreme palatability not only increases the conflict over whether to eat or avoid the novel food, but also the animals do not need to be excessively food deprived before the test; only mild deprivation is necessary, so this aspect of the protocols presented here reflects the "Refinement" aspect of the 3Rs of Russell and Burch<sup>20</sup>.

## 2.1 Procedure:

Ration the food of the mice overnight. Remove all food from the cage hopper the evening before testing then give 1 g/mouse of ordinary diet, which is approximately a third of what they would normally eat. If they are group housed make sure that the pieces are small so that all the mice can eat equal shares. Test them the next morning and replace their food as soon as testing is finished.

Set up the chosen hyponeophagia test apparatus, also a row of small cages in which mice from the group home cage are placed individually before and during testing. (This avoids the increase in anxiety in the remaining mice left in the group cage, which can occur if you progressively remove mice from a group, also social transmission of food preferences. It also assists in test standardisation (between group and singly housed animals) and ensures that the animals are optimally alert.

Place a mouse in the test apparatus. Measure the latency to eat. This is defined as eating/drinking continuously for 2-3 s. Often the mice will sniff or lick the food briefly at first, but only later start to eat continuously without inhibition.

Other measures can also be taken. The time spent eating during a set period, e.g. 2 min after feeding first starts, and possibly also the amount of food consumed during this period, can also be measured. Brain lesions sometimes affect these measures differentially<sup>4</sup>.

## 2.2 Specific details of running a hyponeophagia test with the apparatus shown in Figure 1:

A translucent plastic jug (approximate volume 1.5 l, 15 cm diameter with a spout protruding a further 2 cm) is the test apparatus. Use full cream sweetened condensed milk (Nestle Carnation brand is excellent) diluted 50:50 with water (to facilitate handling it) as the novel food. To contain the mouse on the base and facilitate investigation of the milk, place the jug upside down with the spout forming a small alcove over the food well. This focuses the attention of the mouse on to the well and the milk, so minimising the effects of treatments that might affect perception of, or orientation to, the food source.

The mouse is placed facing away from the well and the jug is gently lowered into position, taking care not to trap the tail of the mouse. The latency from being placed in the apparatus to start *properly* drinking is taken. Sniffs at the milk do not count; at least two seconds of drinking should occur. Count any faecal boli and note any urination (but note that these measures will be influenced by the time in the apparatus). The cut-off time is 2 min. If a mouse has not drunk by then, remove it from the apparatus and test another one or two mice, then retest the one that failed to drink about 3 min later. This time is probably optimal for anxiety levels to decrease while still preserving a state of habituation to the apparatus. This test-rest-retest-rest-retest cycle can be continued until the mouse eats or a set number of trials have been performed. A maximum of 3 trials should result in most mice drinking without making the experiment too long. The test parameter is the (cumulative) latency to drink.

As in other tests where odour could play a significant role, non-experimental mice should be placed in the apparatus before testing starts. Follow a standardised cleaning protocol between all mice. Remove urine/faeces, wipe the food well and the plastic base with moist followed by dry cloth/tissue. Refill the food well with milk (about 0.2 ml, to the rim of the well).



**Figure 1.** A plastic domestic jug converted for use as a hyponeophagia testing unit. Sweetened condensed milk can be placed in the small food well located inside the spout, which conveniently directs the attention of the mouse straight to it. The lead weight glued to the inverted base of the jug helps to stabilise it against any movement of the mouse against the walls.



**Figure 2.** An observation chamber containing two white (anxiety-provoking) white inserts for hyponeophagia testing. Sweet corn could be used as the novel food.



**Figure 3.** Another hyponeophagia test unit. The novel food is monkey treat mix.



**Figure 4.** Another hyponeophagia test unit. The food presented is mixed chopped nuts.

### 3. Running multiple hyponeophagia experiments:

The first hyponeophagia test may not provide an unequivocal result. This often happens and is frequently due to the baseline (control) values being too high or low, resulting in ceiling or floor effects. Floor effects were encountered when testing hippocampal lesioned mice<sup>10</sup>; it appeared that the control value had to be sufficiently high to observe reduced hyponeophagia.

It can be difficult to judge the required aversiveness of the test situation. If the mice have been tested several times already (not necessarily on anxiety-related tasks) their emotionality level will be lower than if they are naive to behavioural testing and a more anxiogenic test will be required. This can often be provided by an elevated test environment (as in Figure 4). Aversive noise could be added to the test situation, e.g. a radio playing at a loud volume or a white noise generator. Brighter lighting would also increase test environment aversiveness.

So, if the first hyponeophagia result is not significant, try several more test situations and perform a repeated measures ANOVA. This is a very powerful experimental design.

### 4. Expected results:

Hippocampal lesions produce a strong decrease in hyponeophagia in mice<sup>10</sup> and recent unpublished work has shown that this is, as in the rat<sup>1</sup>, due to the ventral region of the hippocampus. Mice lacking the KATP channel subunit Kir6.2 showed increased hyponeophagia in one test but less in another<sup>9</sup>, a confusing result but one which serves to emphasize that differences in test environment can have a marked effect on the result, and also points out the usefulness of performing multiple tests. 129S2/SvHsd mice were more hyponeophagic than C57BL/6JolaHsd<sup>6</sup>. Indeed, a comparison of several inbred mouse strains showed marked inter-strain differences<sup>21</sup>.

## Discussion

The latency figures in hyponeophagia testing are typically highly variable. The technique described here, of imposing a set time period for the animal to eat, then retesting later if it fails to eat, serves to minimise the risk of ultra-long latencies and saves the experimenter time.

One advantage of hyponeophagia over anxiety tests such as the plus-maze, is that if the results of the first test fail to achieve statistical significance, another test can be run using a different novel test environment and food, as the type of anxiety being measured appears to be the same and not qualitatively changed by the previously run tests, unlike in the plus-maze<sup>16</sup>. Repeated measures ANOVA can then be used to assess the test series.

It is now known that several different aspects of emotionality are covered by the umbrella term "anxiety"<sup>11,15,18</sup>. Mice showing changes in hyponeophagia will not necessarily show changes in open field, elevated plus-maze or light-dark box tests. So the brain systems underlying performance on these tests must be at least somewhat different. For example, the hypothalamus and amygdala may well participate in hyponeophagia as they are concerned with (among other things) regulation of food intake.

## Disclosures

No conflicts of interest declared.

## Acknowledgements

The Wellcome Trust for providing Open Access funding to Oxford University. Robert Deacon is a member of Oxford OXION group, funded by Wellcome Trust grant WT084655MA.

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