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Tardigrades as a Teaching Model of Learning

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This paper describes how to use tardigrades to demonstrate habituation. This experiment is designed for students with any level of experience or training in conditioning live organisms. In this experiment, tardigrades are desensitized to repeated physical touch. Tardigrades are placed under a microscope and poked with a probe until the strength of their response decreases to the point when there is no reaction for 10 consecutive trials. Once the habituation criteria are reached, a new stimulus is presented as a dishabituation control to ensure the subject responds appropriately to the new stimuli. Dishabituation is essential to show that the habituated response is still present even when a different stimulus is used to evoke that response. This experiment is easy to perform and does not require a lot of time or tools, and the effects are easily observed. The results of the study illustrate the effectiveness of this technique. Discussion questions and future research ideas are provided to aid instructors in the classroom.

Keywords: experimental design, habituation, laboratory, learning, tardigrade, teaching

One of the many plights of the classroom teacher is facilitating engaging laboratory investigations with limited budget and/or supplies. The experiment described in this paper provides an opportunity for students to receive hands-on experience in field biology, microscopy, behavior, and learning. There is flexibility in the experimental design and organism choice to meet other, more specific curriculum requirements. We believe most of the materials needed could be found in most scientific laboratories, making the exercise fairly accessible to most. The subject of the described study is the tardigrade. Tardigrades are ubiquitous and are easily collected in most locations or can be ordered from scientific suppliers (detailed in a later section). Their adaptability and small size make them relatively easy to maintain, which is a key factor for instructional laboratory exercises.

The purpose of this paper is to present a habituation exercise using tardigrades. The exercise is easy and relatively inexpensive. Several invertebrate habituation exercises have been published including those using planarians (Abramson et al., 1999; Katz, 1978), protozoans, and earthworms (Abramson, 1990). The *American Biology Teacher* contains several exercises on tardigrades. These exercises focus on the identification of different species in their natural environment, the collection process, and research methods (e.g., Rife, 2010; Shofner & Vodopich, 1993). The experiment we propose is the first publication that investigates learning in tardigrades.

This manuscript details a protocol we used to collect data regarding habituation behavior in tardigrades. Instructions are flexible and can be adjusted to match the available resources and interests. Following the provided exercise allows students to use a microscope and learn some basic laboratory skills, collect and analyze data, and discuss exercise findings and their interpretations (which can be applied to wider contexts). Real data are presented below to allow students the opportunity to compare their results to ours and practice analyzing the data we provided.

In addition to the explicit teaching opportunity this exercise provides, we believe there is enough flexibility to adapt the exercise to accommodate specific requirements. For instance, this exercise could easily be adapted to include ecological elements like species diversity, identification, and taxonomy. Experiments planned around this design could be especially fruitful if providing species comparisons, of which there are very few.

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There are few organisms as interesting as the tardigrade. Tardigrades possess exceptional capabilities that can captivate learners and provide unique research experiences in the classroom. We believe this exercise is unique and has the potential to lead to interesting future research due to the interesting capabilities of the tardigrade. In our experience, students of all ages seem to be captivated by tardigrades. Tardigrades are charismatic and seem to be growing in popularity as evidenced by their making international news in 2019 when a rover carrying thousands of the "indestructible" organisms crashed on the moon (Grady, 2019).

There are approximately 1,300 described tardigrade species, and approximately 80% of the described species are terrestrial (Greven, 2018). It is estimated that up to 10 times as many species could remain undiscovered. Student researchers discover new species fairly regularly ("Undergraduate researchers discover new species," 2019). What an exciting experience it would be for a student to discover a new species! Not to mention that the behavior and learning of tardigrades are mostly unexplored. The plethora of possibilities within this type of experimental design allows the instructor to give students the challenge and creative liberty to design their own behavior experiments. Any resulting findings have the potential to contribute to the literature and science of tardigrades.

Habituation

Habituation is a form of nonassociative learning in which an innate response to a stimulus decreases after repeated or prolonged presentations of that stimulus (Higham & Shelton, 2011). This ability is essential to survival because it allows organisms to reduce irrelevant information or environmental pressures to focus on the more demanding immediate scarcities of the constantly changing world. Tardigrades are known for their ability to survive with extreme environmental pressures present, making them ideal species for a habituation study. Although not always noted in the classroom, habituation shares many properties of associative learning including spontaneous recovery, effects of stimulus intensity, and generalization (Abramson, 1990, 1994). There are many objectives that can be acquired throughout this experiment, as listed in Table 1.

Table 1

Objectives for the Experiment

	Objectives
General	● Conduct a behavior experiment firsthand ● Introductory fieldwork ● Introductory experimental design *For example, have students analyze and discuss the data from our example exercise and design a follow-up exercise.* ● Introduce students to a microscopic world ● Basic microscopy and laboratory skills
Advanced	● Provide an opportunity for students to design and/or conduct an experiment ● An opportunity for students to collect and analyze data ● An opportunity for students to collaborate to complete an experiment ● Advanced fieldwork *You could have students strategize to pick a good location on campus to hunt for lichens. You could easily introduce GIS or other navigational skills to this exercise. There is always a very real possibility your samples may not yield subjects. A failed attempt to harvest your own tardigrades could provide an opportunity for students to problem solve a better or alternative strategy for collecting their samples. Additionally, resiliency is important in research, and we must embrace failed attempts as they often are part of the process.*
Supplemental	● Provide a means of correlating research findings with the bigger picture

Note. This table identifies some of the goals of the experiment. We have identified general, advanced, and supplemental objectives that can be gained throughout the experiment.

Materials

The materials for this exercise are readily available from biological supply houses such as Wards Scientific (www.wardsci.com) and Carolina Biological (www.carolina.com). We believe most of the required materials (except the microscope) are fairly easy to obtain, no matter where you live.

We recommend using a dissecting microscope equipped with a video camera for the most optimal experience. However, we acknowledge that not everybody will have access to such equipment. Tardigrades are also visible under standard compound microscopes but the space between the lens and the stage makes manipulating the subject challenging. If the microscope is equipped with a video camera, the entire class can see a demonstration of what is happening under the lens, in turn reducing the number of microscopes needed. We have completed a similar investigation using one video streamed from an equipped stereomicroscope and several compound microscopes with which the students took turns trying on their own. It is possible to offer rotations of related activities. Students who are not using the microscope(s) may draw what they saw under the microscope or complete a matching game in which they are told to classify (e.g., Heterotardigrada vs. Eutardigrada) or group the tardigrade species.

Tardigrades can be obtained from the field and/or from scientific suppliers like Carolina Biological (www.carolina.com; United States) or Sciento (www.sciento.co.uk; United Kingdom). The Carolina tardigrades are an aquatic species, *Hypsibius dujardini*. The sample they provide only includes tardigrades (approximately 30). In comparison, the Sciento tardigrades, another aquatic species, *Dactylobiotus dispar*, come with adult tardigrades (approximately 100), plus eggs, and enough algae to sustain the culture for four weeks. In addition to the robustness of the Sciento offering, we favor the option because it is a larger species, reaching approximately 900 µm at adulthood. The larger size makes the subject easier to find under the microscope, which benefits students without much microscopy experience. This provides an opportunity to talk about how ubiquitous tardigrades are due to their extreme tolerances and allows for comparisons between types of tardigrades (for instance, aquatic species compared to terrestrial species). Students might be interested in learning that tardigrades are so adaptable that they live on all continents, can survive living in the vacuum of space and the pressure of the deepest parts of the ocean, and can be found in our backyards.

List of Suggested Materials

Most of the following materials are necessary to complete the exercise. Acceptable replacements are allowed and may vary based on available resources. Every item on the materials list can be found for under $15, except for the microscope, which can range from $200-$1,500. Optional items are denoted by an asterisk (*).

1. **25 tardigrades.** The tardigrades can be harvested or purchased. Tardigrades can typically be found in moss and lichen (see Figure 1). This is the most economical and accessible method of collecting subjects. However, we realize different focuses may require different approaches. For example, if one wished to compare the behaviors of two species, it would be important to order from a scientific supplier to ensure subjects are the specific species of interest and have as much control and consistency as possible. The described experiment requires 20 tardigrades. However, 25 should be harvested or purchased in case dead or unusable tardigrades need to be replaced.
2. ***Algae.** If maintaining a sample for more than a week, we recommend providing a food source, such as a culture of green algae (for example, *Chlorococcum sp.*, which is available for purchase from Sciento). This algae or similar alga may be found naturally in fresh or saltwater. If purchasing tardigrades from a supplier, algae may also be included with the purchased tardigrades. When the present experiment was conducted, Sciento provided enough algae to sustain the culture for approximately four weeks, whereas Carolina Biological provides tardigrades only.
3. **Distilled water.** Distilled or filtered spring water is used to recover and house the tardigrades. Excess water is also used to dilute the algae during the separation process. The distilled water must fill each well at least half-full to ensure the tardigrades do not dry out.
4. **Pipette.** The pipette is used to suck up the tardigrades during the separation process and is also used to create the bubbles for the dishabituation phase.
5. ***96-well plate.** The well plate is used to separate the tardigrades (Figure 2). This tool allows the tardigrades to be easily organized and individual subjects manipulated precisely.
6. **Probe.** We recommend a probe that uses an inoculating loop, which can be modified by using wire cutters and clipping off the loop to leave a straight probe instead (Figure 3). This adjustment allows for more accuracy when poking the tardigrade.
7. **Microscope slide(s).** The slide will be used when the experiment is initiated, following the separation of the tardigrades into individual wells. The tardigrades should be placed on the slide to ensure sufficient space to present stimuli.
8. ***Petri dish.** The petri dish is used to hold the tardigrades when separating them from each other into individual wells in the 96-well plate.
9. **Microscope 40X-2500X.** The microscope is needed to view the tardigrades and should have at least 40X magnification power.
10. **Data sheet.** A sample data sheet showing data collection is available in Figure 4. The datasheet contains room for students' information, observations, and data. (See Appendix 1 for ready to use datasheet.)
11. **Timer.** Any timer will work for this activity. The timer is used to measure the time between trials.

Figure 1

Lichen

Figure 2

The 96-well Plate We Used to Collect Data

Note. Column A is labeled as the experimental group and column B as the control group.

Figure 3

Probe

Figure 4

Sample Data Sheet

Name: RW_____ **Experiment:** Habituation
Date: 8/01/2020 **Time:** 9:00 am
Species: US **ITI:** 5 minutes

Subject	Trial	Response	Response Average Per Trial Block	Intertrial Interval (ITI)	Intertrial Interval Average Per Trial Block
A1	1	1 or 0	1	5:00	5:00
A1	2	1		4:55	
A1	3	1		5:05	
A1	4	1		5:10	
A1	5	0	= SUM (trial 1 + trial 2 + trial 3+ trial 4 + trial 5) / 5*	4:50	= SUM (trial 1 + trial 2 + trial 3+ trial 4 + trial 5) / 5*
A1	6	0	0	5:00	5:00
A1	7	0		5:05	
A1	8	0		5:15	
A1	9	0		4:55	
A1	10	0	= SUM (trial 6 + trial 7 + trial 8+ trial 9 + trial 10) / 5*	4:45	= SUM (trial 6 + trial 7 + trial 8+ trial 9 + trial 10) / 5*

Note. This table represents an organized way to keep track of your data. The columns represent the categories of interest and the rows represent individual trial responses. The subjects are labeled with their placement in the 96-well plate. This data sheet is filled for example purposes and a clean version is available for use in the appendix. The asterisk after the 5 indicates the number of trials per block.

Harvesting and Maintaining Tardigrades

To harvest samples, students will need to use a sealable plastic bag. Harvesting samples may be completed as individuals, partners, or groups. Having a scraping tool can help in the collection of the lichens and moss. When collecting the samples, it is important to remind the students to not to collect too much soil with the lichens and moss, as the sample will be muddy and hard to see under the microscope. It is recommended that students disperse widely to collect a variety of mosses and lichens. The moss/lichens should be rehydrated with distilled water for 24 hours (or at least overnight) before investigation with a microscope. Samples that are collected into sealable bags can be rehydrated in the bag. The exercise may be completed in a day, a week, or longer, depending on the goals of the instructor. For example, students could learn about adaptations, biodiversity, learning paradigms, extremophiles, or a host of other topics and have tardigrades introduced one day, samples collected the following day, and left to rehydrate overnight, data collected another day, and data analyzed on a separate day. After allowing adequate rehydration time, the students will use pipettes and microscopes to locate the tardigrades in their samples.

After acquiring the tardigrades, samples should be maintained in distilled water. Since tardigrades are tolerant of a wide variety of environmental conditions, they are relatively low maintenance. Samples should be monitored daily to ensure they do not dry out (this is especially critical if you are storing samples in small and/or open containers). The tardigrades need to be fed green algae (*Chlorococcum sp.)* from a dropper every week or as needed. This alga is found in fresh or saltwater and is available for purchase from scientific suppliers. If tardigrades are kept in a closed container, the lid should be loose to provide oxygen. Tardigrades can be kept at room temperature and should be exposed to indirect ambient light during natural daylight hours.

Method

First, collect or purchase the tardigrades. Next, individual subjects must be isolated. To isolate each tardigrade, add a drop of distilled water to a microscope slide and pipette a drop of the tardigrade sample into the water on the slide. Use the pipette and microscope to gather one subject at a time. Once isolated, an individual can be moved into a well plate, Petri dish, etc. We recommend using a 96-well plate to house and organize subjects. Be careful to only collect one tardigrade at a time to avoid potentially mixing up subjects. Tips for how to solve common problems such as isolation are found in Table 2. For example, if there are two tardigrades in one well, one may receive half of the administrations of the habituation stimulus, but the other might receive the rest. This discrepancy jeopardizes the validity of the results. Next, decide how to group the subjects (for example, experimental and control groups or *H. dujardini* and *D. dispar*). Place them in the 96-well plate and record their placement on your datasheet. Also, make sure there is enough water in the individual wells, so the subjects do not dry out. Wells should be at least half-filled to avoid desiccation. Other experimental issues for troubleshooting can be found in Table 3.

Table 2

Solutions to Common Problems During Experimental Preparation

Tips
Encourage students not to collect excess dirt with their samples
Isolate subjects relatively close to when you plan to run the experiment as samples may change over time (a subject may die, or an egg may hatch)
Isolate more subjects than you think you will need (it is nice to have a couple of extras in case something goes awry)
If you are having difficulty isolating a subject, try adding more distilled water to dilute the density of your sample

Table 3

Troubleshooting Experiment Issues

Experiment Issue	Recommendation
The tardigrade does not contract	If the tardigrade fails to contract, probe the subject with more intensity. It is important to find a level that reliably elicits the curl response.
Replacing subjects	If the subject is not responding or dies, backup subjects are needed. It is a good idea to separate a few extras in the 96-well plate to account for nonresponsive subjects.
The health of your tardigrades	Purchased tardigrades should be cared for using the provided instructions. If the tardigrades are from the natural environment, be sure to feed the tardigrades algae and keep enough distilled water to keep them fully immersed. Tardigrades are resilient but need to be in good health for the experiment. Moving tardigrades indicate health.
Probing or basic microscopy problems	If time allows, students should practice the technique(s) on an extra tardigrade before beginning the exercise. Practice with the microscope to refine skills.
An uneven amount of trials in the final trial block	To solve this problem, an average would need to be taken to Account for the missing trials. In other words, if a subject completes 10 consecutive no response trials in the middle of a trial block, then the averages (both response and ITI) can be calculated for the number of trials completed. For example, for a trial block consisting of three trials, you would calculate the average by adding trial 1 + trial 2 + trial 3 and dividing by 3 (the number of trials).

Next, use the clean data sheet (an example is provided in Appendix 1) to begin data collection. Figure 4 has sample data input to show what the datasheet should look like after data collection. Responses are recorded as 1 if a response (curl, ball) is observed and 0 if there is no response. We suggest calculating the averages of trial blocks of five to make the data easier to interpret. We found that it is easiest to leave the two average columns blank while conducting data collection and to go back and compute the averages after. Calculate the response average per trial block by adding the responses up for each of the five trials in that block and dividing by five. The same method will be used to calculate the intertrial interval (ITI) average per trial block. The averages can also be calculated using formulas in Microsoft Excel.

Use the pipette to move the first subject onto the slide. Focus the microscope, prepare the datasheet, place the tools for easy accessibility, and begin. With the probe, gently poke any part of the body of the subject at roughly five-minute intervals. This gentle poke is the habituating stimulus (HS). It will be presented repeatedly, once per trial. After a few trials, the tardigrade should stop responding (unconditioned response; UR) to the HS (the poke). Continue these trials until the habituation criterion (10 consecutive no-responses) is reached. Finally, initiate the dishabituation phase by using the pipette to push air to make bubbles next to the tardigrade (dishabituation stimulus; DHS). The dishabituation stimulus should be presented approximately two-and-a-half minutes (half the original ITI used in training) after the last poke. The ITI is half of the paired group to keep the ITI between the stimuli approximately five minutes. When the dishabituation stimulus is presented, the student should note whether a response has occurred (it should), and then, two-and-a-half minutes later (when the regularly scheduled ITI is reached), the poke is presented a final time and the subject's response is recorded.

The dishabituation phase is included to rule out effector fatigue and sensory adaptation. In effector fatigue, the tardigrade can still sense the poke but cannot physically respond to the stimulus. In sensory adaptation, the tardigrade can make a response but can no longer sense the poke. In both effector fatigue and sensory adaptation, the tardigrade will no longer respond to the poke mimicking a learned response. Dishabituation is factored into the experimental design to rule out these alternative explanations for the behavior.

Practical Exercise Method

Twenty subjects (10 experimental and 10 controls) were used in our exercise. Each subject had a paired control. The purpose of the control was to provide information on the baseline rate of contraction and, if there is a baseline rate of contraction, to determine if it naturally decreases over time. We used a criterion of 10 consecutive no-response trials to demonstrate habituation. An experimental subject was presented with the HS (a poke) repeatedly with 5-min ITIs between HS presentations. Subjects are initially very responsive to this tactile stimulus. However, as a subject is repeatedly presented with the HS, its reaction should decrease,

eventually resulting in no response. Observing a change in a subject's response to a HS (which is initially to react defensively by curling up) to a new behavior (no response) is indicative of habituation. After an experimental subject completed its 10 consecutive no response trials, we introduced a dishabituation stimulus to ensure a different stimulus can emit the same original response. Finally, the habituated stimulus is reintroduced to confirm the subject has habituated to the HS. Its paired control would complete the same number of trials (e.g., if an experimental subject took 14 trials to reach 10 consecutive no-response trials, then its paired control would be observed for the equivalent of 14 trials). The paired control would be presented with a final probe trial to show that the control is still able to respond to a stimulus. A sample protocol is provided as supplemental material.

Practical Exercise Results

In this section, the results of the practical exercise are presented and interpreted with additional possible topics for students to discuss in class. Students can work independently or together to analyze their results. As more journals now require data to be archived, it is good practice for students to share their data with classmates. Instructors can leave it up to the students to decide which analyses are most appropriate or suggest what data to analyze. In this example, we used a correlation to show the relationship between the control and experimental groups and a chi-squared test to evaluate the distribution of responses per group. Students can additionally be asked to make a graph based on their data. Students can also practice writing their results as if it were for a journal publication. This practice can provide students experience analyzing, interpreting, and communicating results from real data.

Tables 4 and 5 summarize the preliminary data collected in our experiment with particular reference to the experimental (n = 10) and control groups (n = 10). Students may wish to compare their results to these results. As Table 4 shows, the control group spontaneously curled or did not curl due to there not being any stimuli presented. However, when the organism is touched with the probe, a contraction was readily elicited. This result shows that the tardigrade was still able to respond to a stimulus. In contrast, Table 5 shows that the experimental group starts the same as the control but then begins to habituate as demonstrated by the 10 consecutive no-responses (0). The 0 represents that there was no response from the subject after the poke was presented. When the dishabituation stimulus is presented to an experimental subject, the organism's response is to the new stimulus. When the original habituation stimulus is reapplied, no responses are noted. All of the tardigrades in the experimental group showed habituation to the original stimulus, a response to the new dishabituation stimulus, and no response for the final presentation of the HS.

For the analysis, we used a correlation to see if there was a significant relationship between the number of trials and responses. The data suggested that as the number of trials increased, the number of responses decreased. There was a negative correlation between the number of trials and the response averages, $r(322) = -.54$, $p < .001$. There was also a difference between the experimental group's response means ($M = 0.29$, $SD = 0.37$) and the control group's response means ($M = 0.37$, $SD = 0.27$). This shows that there are more responses in the control groups as opposed to the experimental groups. Additionally, we used a chi-squared test to look for a significant association between response averages, which were categorized into separate experimental and control groups. The data suggested that there was a significant association between response averages in the experimental and control groups, $X^2(5, N = 324) = 67.17$, $p < .001$. When the dishabituation phase was presented, response averages peaked.

Table 4

Data Table for Control Group

Animal	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21-HS
1	1	1	1	1	1	1	0	0	1	0	0	1	1	0	0	0	1	1	0	0	1
2	0	0	0	0	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0	0	1
3	1	1	1	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1
4	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
5	0	1	1	1	1	1	1	0	0	0	0	1	0	1	0	0	1	1	0	1	1
6	1	0	0	1	0	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1
7	1	0	1	0	0	1	1	0	1	1	0	0	1	1	1	0	0	0	1	0	1
8	0	0	1	0	1	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	1
9	0	0	1	0	0	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	1
10	0	1	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	1	0	1	1

Note. If there was a contraction in response to the probe, a "1" was recorded; if no response occurred, a "0" was recorded. There was not a probe presented, except on trial 21 (HS).

Table 5

Data Table for Experimental Group

Animal	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	DH	HS
1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0						1	0
2	1	1	0	1	0	0	0	0	0	0	0	0	0	0							1	0
3	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0					1	0
4	1	1	1	0	0	0	0	0	0	0	0	0	0								1	0
5	1	1	1	1	0	0	0	0	0	0	0	0	0	0							1	0
6	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0					1	0
7	1	1	0	1	0	0	0	0	0	0	0	0	0	0							1	0
8	1	1	1	1	0	0	0	0	0	0	0	0	0	0							1	0
9	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0						1	0
10	1	1	1	0	0	0	0	0	0	0	0	0	0								1	0

Note. The habituation criterion for the experimental group was 10 consecutive no-response reactions to the probe. When the experimental animals reached the criteria, 2.5 min later, the dishabituation stimulus (DH) was presented, and 2.5 min after that, the original stimulus was reintroduced (HS).

Discussion

This detailed exercise provides an accessible learning model for the classroom. This simple exercise can stimulate a wide variety of discussions, for example, on the importance of learning across the phyletic scale. The exercise is also a useful tool to teach students how to make proper comparisons. For example, students could compare populations, species, or how groups may differ from one another and why. If the exercise is combined with a previously published planarian exercise (Abramson et al., 1999; Katz, 1978), students will be faced with a multitude of interesting comparative issues in both interpretation and research design such as the importance of automation. This process provides opportunities to promote discussion and debate among the students (see Table 6 for examples).

The exercise can also be adapted to fit numerous needs. For example, this exercise could be used as part of a formal experiment during an organized laboratory section. In other classroom applications, with a microscope equipped with video capabilities, one person could conduct the experiment in front of the class while classmates record the data. The instructor could comment on the technique and results in real-time, providing an interactive and engaging learning exercise. Students could compare their records (what may register as a response to one student might be categorized as a no-response to another, introducing the concept of operational definitions). Lastly, the exercise can be used as part of a distance learning project, which becomes exceedingly important as schools move online due to various public health outbreaks or pandemics, such as COVID-19 during 2020.

Table 6

Class Discussion Questions

Example Discussion Questions
Do the tardigrades curl on their own or only when threatened?
Can you think of any other stimuli that could cause the same curl response?
Based on your results, do you think tardigrades can learn to habituate? Why or why not?
What is the biological significance of habituation?
What is the relationship between habituation and sensitization?
Does the strength of the subject's response change over trials? How is this measured? Is this the best method to measure this parameter?
How do your results compare with those of your classmates?
What question(s) was this exercise designed to answer? What is a relevant measure that cannot be addressed using data collected from this exercise?
Describe another learning paradigm and how it could be investigated in tardigrades.
Propose a follow-up study that expands upon the findings of this exercise.

Note. This table provides sample questions to help aid in-class discussion. These questions are a range of different levels of questions and are helpful to implement to help keep the class discussion focused on the activity.

Conclusion

Anecdotal feedback from students who have engaged in this exercise has indicated that the students find the exercise informative. Perhaps one of the biggest revelations is that students were surprised that these animals can learn. We hope to capture this sense of wonder to encourage students to learn more about comparative psychology. Moreover, students anecdotally report enjoying watching the tardigrades navigate their environment. Some tardigrades explore more than others and have various speeds. Under the microscope, students can watch them grasp on to algae and curl into a ball. Students were fascinated by how fast the tardigrades were able to habituate and were surprised when the subjects responded to the dishabituation trial. Students were also excited to do a hands-on experiment instead of just reading about it. This hands-on learning immersed them in research methods and design. Students reported liking the independence of conducting their own experiment, taking data, directly observing the changes in behavior for themselves, and comparing their results. We are especially pleased that students commented positively that the exercise taught them the importance of habituation.

Future Directions

Experiments can be designed to study other aspects of habituation. These include spontaneous recovery, stimulus intensity, habituation of dishabituation, stimulus generalization, variations in the intertrial interval, and the effects of repeated training. However, there are more interesting topics that could be investigated that take advantage of tardigrades' unique abilities such as anhydrobiosis.

Future research should explore anhydrobiosis with habituation to look at memory retention. To do this, you would need to run the current experiment and let the water evaporate to induce anhydrobiosis. Next, rehydrate the tardigrades and present the same experiment again to observe if anhydrobiosis (or length thereof, for example) affects habituation (or memory retention) or if trials required to habituate increases or

decreases after rehydration (or if performance is different between individuals that have underdone differing lengths of anhydrobiosis).

Another area that could be explored is the potential effects of chemical signaling on the performance of subsequent individuals. You would need to collect data using the habituation exercise with one subject, then remove that individual from the well or slide and put a new tardigrade into the same water. Collect data again to see if the new subject habituates faster than the one before him, which may be indicative of indirect communication via chemical messengers (e.g., hormones).

Cross-species comparisons with different species or subspecies can also be researched for comparative exploration. It is valuable to compare different species to investigate potential geographical differences in behavior. Different populations may have different environmental pressures, leaving room for unique adaptations to occur.

Finally, students can try to develop other conditioning techniques associated with classical and instrumental/operant conditioning. One promising procedure for aquatic invertebrates is to train them to seek water. Planarians, for example, can be trained to move several centimeters to find water (Chicas-Mosier & Abramson, 2015).

In conclusion, students gain hands-on learning experiences in experimental design, the use of inquiry-based experimentation, observation skills, and statistical analysis by completing this experiment. They were able to complete their own experiment, with the supervision of their instructor. This exercise opens up critical thinking and interest in experimentation since students observe habitation firsthand and learn the importance of experimental controls. Working with tardigrades captures the imagination of students and may ignite an interest in comparative psychology.

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