How-To-Do-It

Classical Conditioning in the Pond Snail *Lymnaea stagnalis*

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Animals, in coping with the daily problems encountered in an ever-changing environment, must show considerable malleability in their behavior. Evolution has shaped members of a particular species to be more or less alike in morphology, physiology, and behavior, all of which are under substantial genetic control. Extensive as it is, though, the genetic blueprint of an animal cannot provide specific answers to all of the daily problems individual animals face throughout their lives. Instead, the genome codes for the development of a nervous system, with its attendant properties of change and storage, which allows the animal to learn and to be capable of altering its activity on the basis of past experience.

The experimental procedure described in this paper, classical conditioning, was developed by the noted Russian physiologist Ivan Pavlov. After a neutral, ineffective stimulus (a bell) which he called the conditioned stimulus (CS), Pavlov presented meat powder to dogs (the unconditioned stimulus, or UCS) which elicited a salivation response from the dogs. After repeated paired presentations of the two stimuli: the bell (CS) and meat powder (UCS), Pavlov discovered that the bell presented alone became capable of eliciting salivation. This learning paradigm, where the animal responds to a formerly inadequate stimulus (CS) that has been associated with an adequate stimulus (UCS) was eventually called classical conditioning.

Since the publication of Pavlov's pioneering work, classical conditioning and other forms of learning have been demonstrated in many animals in a wide variety of conditions. A tremendous body of data exists in the psychological literature concerning conditioning under controlled conditions in several animals such as rats and pigeons. Learning also occurs under a variety of natural conditions, particularly when the animal's survival and/or successful reproduction are at stake. A good example of the adaptive value of conditioning is the association between an ingested food and illness in blue jays. A blue jay, getting sick after eating a poisonous butterfly, learns to associate the illness with the consumption of the butterfly and subsequently avoids similar butterflies (Brower 1969). The learning of birdsongs, imprinting in precocial birds, and the memorization of the location of flowers and their hives by bees are other examples of learning in the wild.

Several gastropod molluscs (snails and slugs) have been shown capable of classical conditioning. Recently the freshwater snail *Lymnaea stagnalis* (figure 1) has joined the list of gastropods shown capable of being conditioned (Audesirk, Alexander, Audesirk, & Moyer 1982; Alexander, Audesirk, & Audesirk 1982, 1984). The method used was nonaversive classical conditioning of feeding, in which a novel, nonfood chemostimulus (amyl acetate, CS) was paired with food (sucrose and casein digest, UCS). The snails, after several CS-UCS pairings, subsequently responded to the CS as food. This paper describes in detail a technique for classically conditioning *Lymnaea* that can be conducted by high school or college students in an introductory psychology or animal behavior laboratory course. The experimental procedure is flexible, rapid, simple, and inexpensive.

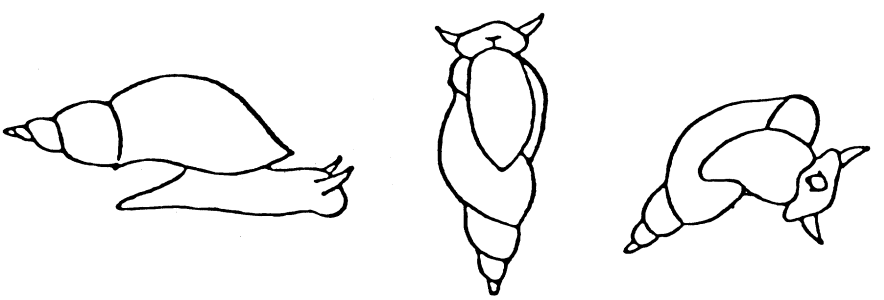
Materials

For raising and housing *Lymnaea*:

1. (1) 10 gal aquarium, with filtering apparatus
2. (3) 5 gal plastic containers for holding water
3. Piece of chalk or small amount of calcium carbonate
4. TetraMin flaked fish food or lettuce
5. Small amount of fine-grained sand
6. (1) indelible marker, black (for marking snails)
7. (1) tube of Super Glue (for marking snails)

For training *Lymnaea* (per student or group of students):

1. (6) plastic bins, 15 × 22 × 5 cm (Rubbermaid)



A B C

FIGURE 1. A. *Lymnaea stagnalis*. B. This animal is about to rasp (compare with C). C. This snail is in the middle of a rasp, the behavioral response to be observed. The mouth has opened, and the radula is about to extend through the mouth.

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2. (2) pieces of plastic or fiberglass screening, cut, molded, and sewn to fit the inner dimensions of the above bins
3. (1) piece of screening, 2 × 3 ft
4. CS training stock solution (240 ul (microliter) amyl acetate added to 1 l of aerated tap water)
5. UCS training stock solution (40 g sucrose added to 1 l aerated tap water)
6. (7) plastic measuring cups, 100 ml capacity
7. (1) timer, 1-6 min
8. (1) 5 gal plastic container
9. 5 l of aerated tap water (per day)

For testing *Lymnaea* (per student or group of students):

1. (10) glass beakers, 120-150 ml capacity
2. (1) ring stand, 8-9 inches tall, 4-5 inches in diameter
3. (1) piece of plate glass, 5 × 5 inches
4. (1) large round mirror, 4-5 inches in diameter
5. (3) pocket mirrors, 2 × 3 inches
6. (2) plastic syringes, 6 ml capacity
7. (2) plastic measuring cups, 100 ml capacity
8. CS testing stock solution (400 ul (microliter) of amyl acetate added to 1 l of aerated tap water)
9. 5 l of aerated tap water (per day)
10. (1) timer, 1-6 min
11. (1) counter (grocery story dollar/cent counter or lab hand-held counter)

Lymnaea can be raised easily in the laboratory from eggs or young animals. *Lymnaea* is a freshwater species found in lakes, streams, and ponds throughout the northern latitudes of North America and Europe. An aquarium filled with aerated tap water is adequate for raising *Lymnaea*, but several precautions should be noted. Copper is very toxic to snails; water from copper pipes could kill these animals. If the water supply is chlorinated, the water should be treated with chemicals or aerated to remove the chlorine. Aerating the water in several plastic 5 gallon containers by bubbling air through the water will remove the chlorine.

Lymnaea thrives best in slightly alkaline conditions. Additions of a small amount of chalk or calcium carbonate will assure alkalinity. A small amount of fine-grained sand also should be added because sand particles are necessary for the trituration of food. The tank should be situated out of direct sunlight. Water temperature should be maintained around 20 to 25° C. Feces should be removed and some of the water in the tank replaced periodically to remove wastes. The snails can be fed flaked fish food (TetraMin) or lettuce *ad libitum*. TetraMin results in much more rapid growth.

Experimental Procedure

An important point to be stressed is the flexibility of the experimental procedure. For purposes of description and discussion, it is assumed that the students will have two hours a day for five days to conduct the experiment. The five days do not need to be consecutive. The total number of training trials is six, three trials a day for two days. Students can work in small groups instead of individually.

Hunger motivation is important. Hunger affects performance, as seen in Audesirk et al. (1982). The snails should be starved for two days prior to the start and during the experiment. *Lymnaea* can live for more than a week without food.

One last point—gently handle the snails throughout testing and training in order to minimize disturbance.

Day 1

Assign Snails to Treatment Groups.

In order to compare the responses of individual snails, each snail should be individually numbered. Remove 20 large (about 0.5 g, although larger or smaller animals can be used) snails from the tank per student, place the snails on a wet piece of paper towel, foot downward, and allow the snails' shells to dry (about 15 minutes). Clearly mark a different identifying number on the largest part of the shell (the body whorl) of each snail with a black indelible marker. After allowing the marks to dry (about 10 minutes), apply a thin coating of Su-

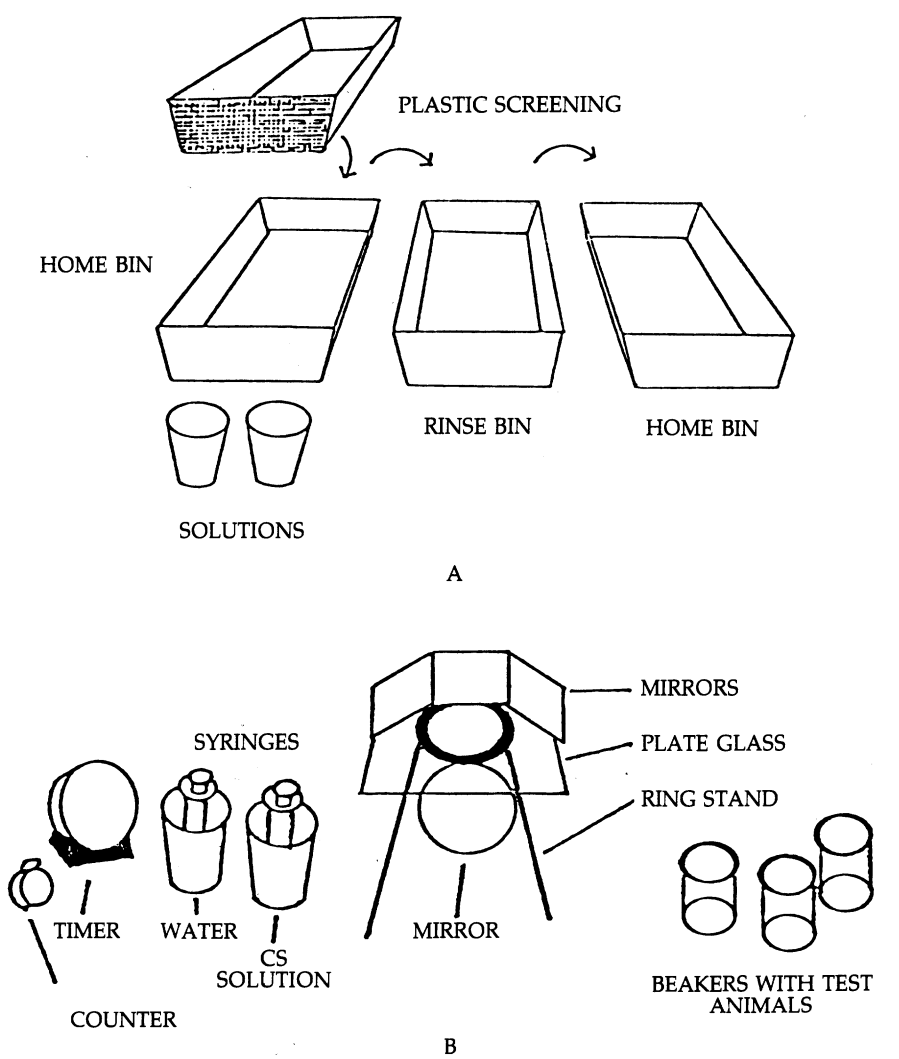

FIGURE 2. A. Training apparatus. B. Testing apparatus.

per Glue over the marks, being very careful not to get any of the glue on the snails' bodies. After this coating feels completely dry (about 15 minutes), place 10 snails in each of two plastic bins (lined with the screening) containing 200 ml of aerated tap water. Label one bin "Experimental Group" and the other "Control Group", and note which snails are in which group. Keep the bins covered with the 2 × 3 ft piece of screening to prevent snails from escaping.

Construction of the viewing platform: A viewing platform is necessary for observation of the movements and actions of the snails during testing. Glue the plate glass to the top of the ring stand (aquarium sealant or wax works well for this). Situate the large round mirror at a 45 degree angle below the top of the ring stand, and place the three pocket mirrors, taped end to end, on top of the stand (figure 2B).

Day 2

Gathering Baseline Data.

On the second day, test the animals to observe their naive reaction to the CS (amyl acetate) and to establish a baseline for future performance (see Table 1). Place each animal in a numbered glass beaker containing 40 ml of aerated tap water, recording the snail's number and the beaker number. After allowing the snails to acclimate (at least 15 minutes), gently place a beaker on the viewing platform, and record the beaker number. Add 5 ml of aerated tap water (a disturbance control) slowly via syringe, aiming the jet of water at the wall opposite the snail's position. Then count each *rasp* (see below) made by the snail in the next two minutes. This generates a baseline of spontaneous rasping activity.

The response to be measured is the number of stereotyped feeding movements, or rasps. A rasp consists of the following sequence of events. First, the mouth opens, and the radula, a rough tongue-like organ, extends through the mouth and scrapes along the substrate in a posterior to anterior direction. The radula then retracts into the mouth, the mouth closes, and the sequence repeats (see figure 1). Students should familiarize themselves with this response by prior observation of snails feeding in the tank.

After counting the number of spontaneous rasps, add 5 ml of the amyl acetate testing stock solution (the solution should be made and shaken vigorously just prior to use) via syringe. Count the number of rasps made in the two-minute period following the addition of the CS. After testing, pour off the water and replace with fresh aerated water. Set this snail aside and test the next snail. After all snails have been tested, place them back into their appropriate home bins. Individual snail's scores are generated by subtracting the spontaneous rasping activity (the pre-CS count) from that elicited by the CS (the post-CS count). If the CS is inhibitory, the score for that particular snail could be negative, if the CS is excitatory, the score will be positive.

TABLE 1. Testing Procedure

1. snails placed in number-coded beakers (40 ml water)
2. snails acclimate for 15 minutes
3. beaker placed on viewing stand
4. 5 ml of water added to beaker via syringe
5. rasps counted for 2 minutes (pre-CS count)
6. 5 ml of CS test stock solution added to beaker via syringe
7. rasps counted for 2-minute period (post-CS count)
8. water in beaker poured off and fresh water added to beaker
9. after all snails tested, place snails back into home bins

Days 3 and 4

Training Trials.

Training occurs on the 3rd and 4th days. The animals will receive a total of six training trials over this two-day period, three trials a day with an intertrial interval (the time between successive training trials) of 30 minutes (Table 2). Each day, the experimental animals receive three CS-UCS pairings at 30-minute intervals, alternating with three H_2O-H_2O presentations. The control group receives separate, alternating presentations of the CS and UCS. Since the control group consequently receives more handling, thus more disturbance, the experimental group receives the H_2O-H_2O presentations in order to equalize the amount of disturbance between the two groups. The final CS concentration that the snails experience is the same in training and testing, 0.004 percent. The CS solution is best kept in a stoppered bottle, since amyl acetate is somewhat volatile. The final UCS concentration that the snails experience is 0.67 percent—a concentration sufficient enough to cause rasping.

First, each student or group will need a total of six bins, two bins that house the experimental animals (labeled appropriately), and four more bins, filled with 200 ml of aerated tap water (figure 2A). Label two of the four bins "Rinse", one bin "Experimental Group", and the last bin "Control Group". Place one "Rinse" bin between the two groups labeled "Experimental Group" (one of these previously labeled and containing the experimental animals), and place the other "Rinse" bin between the two "Control Group" bins (one already containing the control animals). Label two of the measuring cups "CS", two cups "UCS", and the last three cups "H_2O". Place the timer nearby.

Pour 50 ml of the CS training stock solution into a "CS" cup, and 50 ml of the UCS into an "UCS" cup, and place these cups in front of the bin housing the experimental animals. Pour 50 ml of the UCS into another "UCS" cup, and 50 ml of aerated tap water into an "H_2O" cup, and place these cups in front of the home bin housing the control animals. The water serves as a substitute CS to equalize disturbance between the two groups. Slowly pour the CS into the bin housing the experimental animals, distributing the CS throughout the bin while avoiding pouring it directly on the snails. Then pour the water in the same manner into the bin containing the control animals.

TABLE 2. Training Procedure

Time (min)	*Experimental Group*	*Control Group*
0,30,60	CS-UCS, rinse, transfer	water-UCS, rinse, transfer
15,45,75	water-water, rinse, transfer	CS-water, rinse, transfer

After this, pour the UCS into the experimental animals' bin and the UCS into the control animals' bin. Work at a pace so that the UCS is added to the experimental bin roughly 15 seconds after the addition of the CS. Set the timer for two minutes and leave the experimental animals in the CS-UCS mixture and the control animals in the presence of the UCS for two minutes.

After two minutes, lift the screens (and thus the snails) gently out of the home bins (figure 2A) and place the screens briefly (about 15 seconds) into the "Rinse" bins. Then lift the screens out again and place the screens into the appropriate new home bins (figure 2A). Empty, rinse, replace, and refill the rinse bins and old home bins with 200 ml of fresh aerated water in preparation for the next presentation. Fifteen minutes after the UCS presentation, pour 50 ml of the CS training stock solution, followed 15 seconds later by 50 ml of aerated water (a substitute for UCS) into the bin containing the control snails. At the same time, pour 50 ml of aerated water (a substitute CS) followed 15 seconds later by 50 ml of water (a substitute UCS) in the bin housing the experimental group. Rinse and transfer both groups two minutes later. Empty, rinse, and refill the rinse and old home bins with 200 ml of fresh water.

Alternate between these two types of presentations until the experimental group has received three CS-UCS presentations and the control group receives three separate presentations of the CS and UCS. After this has been accomplished, training is done for the day.

Day 5

Post-training Testing.

On the fifth day of the experiment test each snail again to observe the effect of training on the response to the CS. The testing procedure for this test (the post-training test) is exactly the same as the testing procedure prior to training (the pre-training test—see Table 1). The snails should be placed in numbered beakers and the snail and beaker numbers should be recorded by an assistant so that the students doing the testing are unaware of the groups to which the snail belongs.

Alternative Experiments

Some students may wish to investigate the influences of other variables that affect learning. The following are a few suggestions for expanding the objectives of this experiment.

Influence of food-deprivation: some students can compare the response of a group of well-fed snails (allowed to feed on 1 g TetraMin for five minutes daily) to that of food-deprived snails (snails that have been starved one week prior to and during the experiment). Hunger influences learning as seen in Audesirk et al. (1982).

Influence of the number of training trials: a group of students could compare the response of snails given one CS-UCS presentation versus that of snails trained with 10 or 20 training trials.

Extinction: some students could observe the length of time required for snails to 'forget' the learned response. Also, these students could see if differences in extinction rates occur between groups of snails given different numbers of training trials, or between groups of snails given the same number of training trials, but with the trials presented over a different amount of time (see below).

Influence of time between training trials: students could compare the effects of 10 training trials in one day versus 10 training trials spread out over a five-day period. The time interval between trials affects conditioning in *Lymnaea* as seen in Alexander et al. (1982).

Different control groups: different control groups could be used and compared with each other and with the experimental group. Students thus can discover the importance of control groups in learning. For further discussion of this topic, see Audesirk et al. (1982).

Manipulating the temporal relationship between the CS and UCS: some students can investigate this important parameter of learning, the temporal relationship between the two stimuli of classical conditioning. They can compare the responses of snails trained under a forward delay conditioning procedure (where the CS precedes and overlaps with the UCS) to snails trained under a backward delay conditioning procedure (the UCS precedes and overlaps with the CS). For further discussion see Alexander et al. (1982).

Statistical Analysis

After generating means and standard deviations of rasps for experimental and control groups before and after training, students can plot the results and analyze the data. Students can use the t-test for comparing the response of the experimental group to that of the control group before and after training. In addition, a paired t-test can be run to compare the experimental group's response after training to that observed before training. If snails are repeatedly tested, the results could be analyzed using a three-way analysis of variance with repeated testing.

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