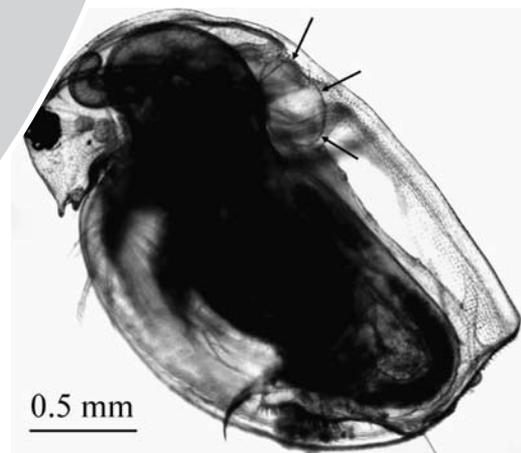


## Making the Most of the *Daphnia* Heart Rate Lab: Optimizing the Use of Ethanol, Nicotine & Caffeine

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### ABSTRACT

Students commonly test the effects of chemical agents on the heart rate of the crustacean *Daphnia magna*, but the procedure has never been optimized. We determined the effects of three concentrations of ethanol, nicotine, and caffeine and of a control solution on heart rate in *Daphnia*. Ethanol at 5% and 10% (v/v) reduced mean heart rate to ~50% and ~20% of its initial value, respectively. Recovery was rapid after removing 5% ethanol, but recovery from 10% ethanol took 20–30 minutes. Nicotine at 100  $\mu$ M reversibly increased mean heart rate by ~20%. Higher concentrations produced varied and sometimes irreversible effects. Caffeine at 0.1%, 0.5%, and 2% (w/v) had no convincing effect on heart rate. Of the three agents tested, nicotine's peculiar effects make it the least useful in an educational setting. Caffeine could be used to emphasize the need for blind observers because it does not increase heart rate in *Daphnia*. If students find that it does, their bias is revealed. Ethanol produces unambiguous effects at 5% and 10%. Heart rates recover quickly after removing 5% ethanol, which allows students to explore reversibility as an alternative to having a separate control group.

**Key Words:** Heart; caffeine; nicotine; ethanol; *Daphnia*.

*Daphnia* are small crustaceans that are inexpensive, easy to care for, and transparent. The latter property allows students to easily observe the beating heart (Figure 1). Students commonly investigate the effects of chemical agents on heart rate in *Daphnia*. Protocols are available in published laboratory manuals (see references in Gray, 1996). More can be found on the Internet. A kit for the lab is sold by Ward's Natural Science. Results of the exercise are often inconsistent and unanticipated. Gray (1996) stated "that even if data were collected from all biology sections, results were still too inconsistent to draw definite conclusions...it was not unusual for nearly half of the animals tested to show increased heart rates in alcohol while the rest showed decreases." Similarly, when this paper's first author used *Daphnia* in a course on research design, he found that several commonly tested compounds failed to generate statistically significant effects on heart rate. What compounds and concentrations should be tested to produce consistent effects that can be distinguished from those of chance?

Three commonly tested agents are nicotine, caffeine, and ethyl alcohol. Scholarly, peer-reviewed reports of how these compounds affect heart rate in *Daphnia* are scant. Baylor (1942) described effects of

nicotine but provided no sample sizes or graphs. Campbell et al. (2004) found that 2 mM caffeine caused a slight initial increase in heart rate followed by a subsequent decrease starting 30 minutes later. Foster (1997) studied a range of caffeine concentrations but only determined heart rate immediately after the caffeine was applied. Sollmann and Webb (1941) briefly mentioned the effects of 1% ethanol. Clearly, the scholarly literature provides little guidance as to what concentrations of these compounds should be tested and what effects to expect.

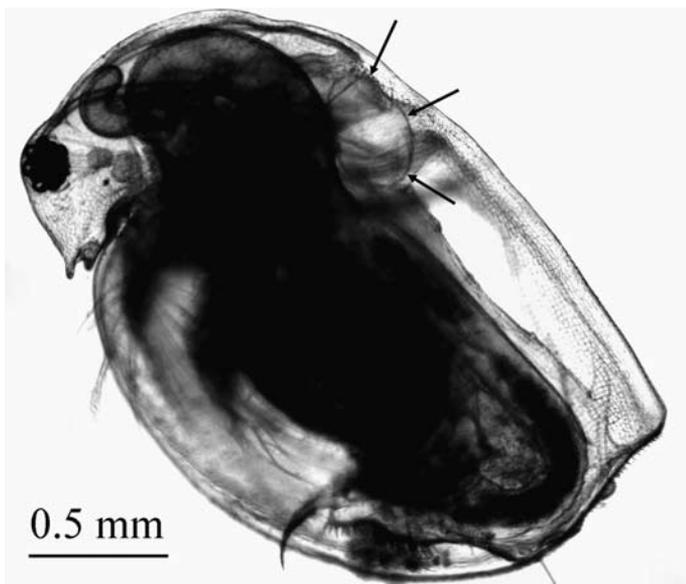
Here, we report on the effects of ethanol, nicotine, and caffeine on heart rate in *Daphnia*. Each compound was tested at three concentrations. We determined their effects, the time necessary to achieve those effects, and whether or not the effects could be reversed. Doing so required a lengthy procedure and statistical analyses that are unsuitable for high school students. We do not recommend that teachers ask students to repeat our procedure. Our intent is to help teachers modify their existing protocols to make the most of the *Daphnia* heart rate lab.

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### ○ Materials & Methods

*Daphnia magna* were shipped overnight from Ward's Natural Science (Rochester, NY). They were maintained at room temperature in the medium in which they were shipped. Animals were fed *Daphnia* growth food daily (Ward's catalog number 88W5950). Natural light provided a summer photoperiod. Feeding and overnight shipping are essential for long-term maintenance of *Daphnia* cultures. With standard shipping, cultures invariably die within a few days, even when they are fed. With overnight shipping, our cultures typically survived for 2 weeks.

The procedure was based on that of Baylor (1942). A small amount of cotton was placed into a 1.5-mL glass well. A *Daphnia* was added with enough aged tap water to immerse the animal. The cotton prevented the *Daphnia* from swimming but still allowed some movement. Heart activity was recorded directly to iMovie by a Sony Handycam camera that was mounted on a dissecting microscope and connected to an Apple computer. Heart rate was determined in iMovie by playing a 15-second portion of the recording in slow motion and counting the beats.



**Figure 1.** *Daphnia magna*. Arrows indicate the wall of the heart.

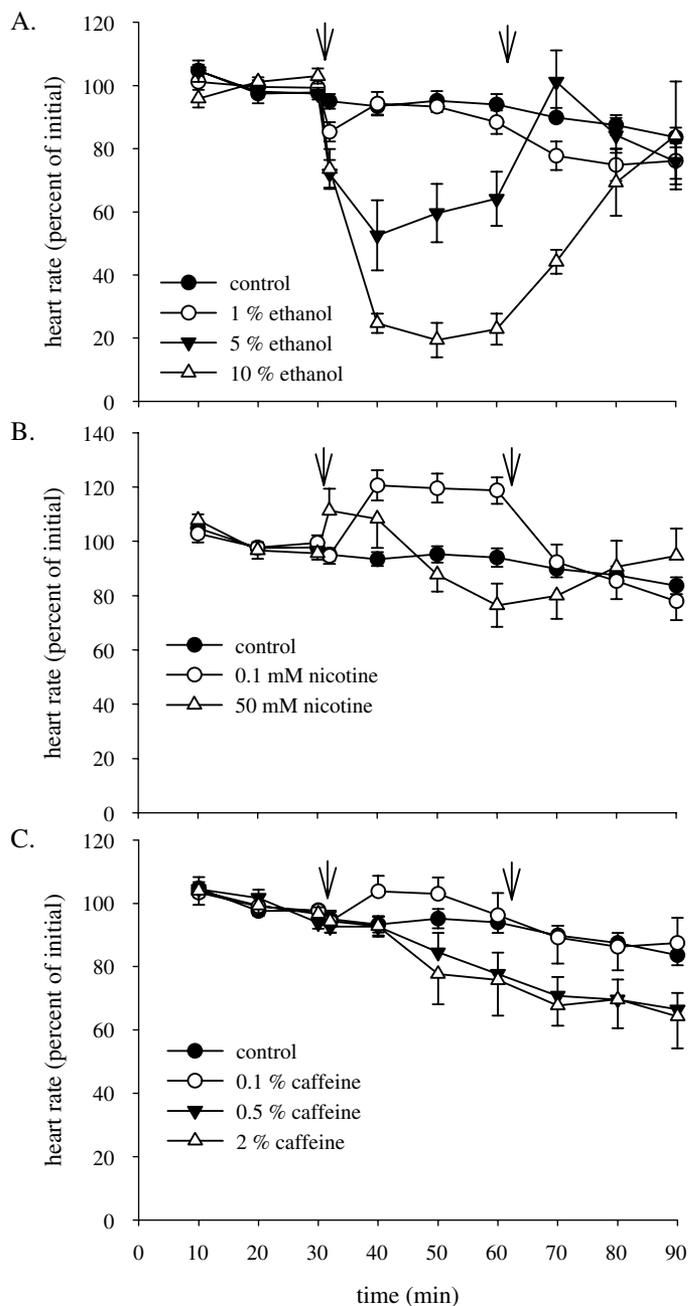
Initial experiments focused on the effects of ethanol (1%, 5%, and 10% v/v), nicotine (0.1 mM, 1 mM, and 50 mM), and caffeine (0.1%, 0.5%, and 2% w/v). All three compounds were obtained from Sigma-Aldrich (St. Louis, MO). Solutions were made with aged tap water and frozen before use. Aliquots were coded to prevent observer bias. Six animals were tested with each concentration of each compound. Eighteen animals were tested with aged tap water to serve as controls. Each animal's heart activity was recorded three times at 10-minute intervals to establish a baseline. Then, the solution bathing the animal was replaced with the test solution. Heart activity was recorded immediately and then three more times at 10-minute intervals. The solution bathing the animal was then changed four times with aged tap water to fully flush out the test agent. The heart's activity was subsequently recorded three more times at 10-minute intervals to test for recovery.

Prior to statistical analysis, heart rates for each animal were converted to percentages of the initial heart rate. Initial heart rate was calculated as the average of the heart rates determined at 10 minutes, 20 minutes, and 30 minutes. One-way analysis of variance for repeated measures was used to determine whether time alone had any effect on the heart rate of control animals. Effects of nicotine and caffeine were analyzed with separate two-way analyses of variance for repeated measures. Heart rates included in each analysis were those determined at 32 minutes (right after the test compound was applied), 40 minutes, 50 minutes, and 60 minutes (right before it was removed). The same control data were used in the analysis of both nicotine and caffeine results. Results of ethanol treatment did not require statistical analysis.

Follow-up experiments with caffeine were meant to confirm initial findings that suggested that caffeine decreased heart rate. Twelve animals were tested, six with aged tap water and six with 2% caffeine. Aliquots were coded to prevent observer bias. Heart rates were determined prior to changing the bath to the test solution and again 30 minutes later.

## ○ Results

Seventy-two *Daphnia* were used in the initial experiments. The average initial heart rate was 354 beats  $\text{minute}^{-1}$  (range: 91–521 beats  $\text{minute}^{-1}$ ). The large variation in heart rate between animals cannot be attributed to variation in their size. Linear regression showed that only 5% of the variation in heart rate could be attributed to differences in animal length.



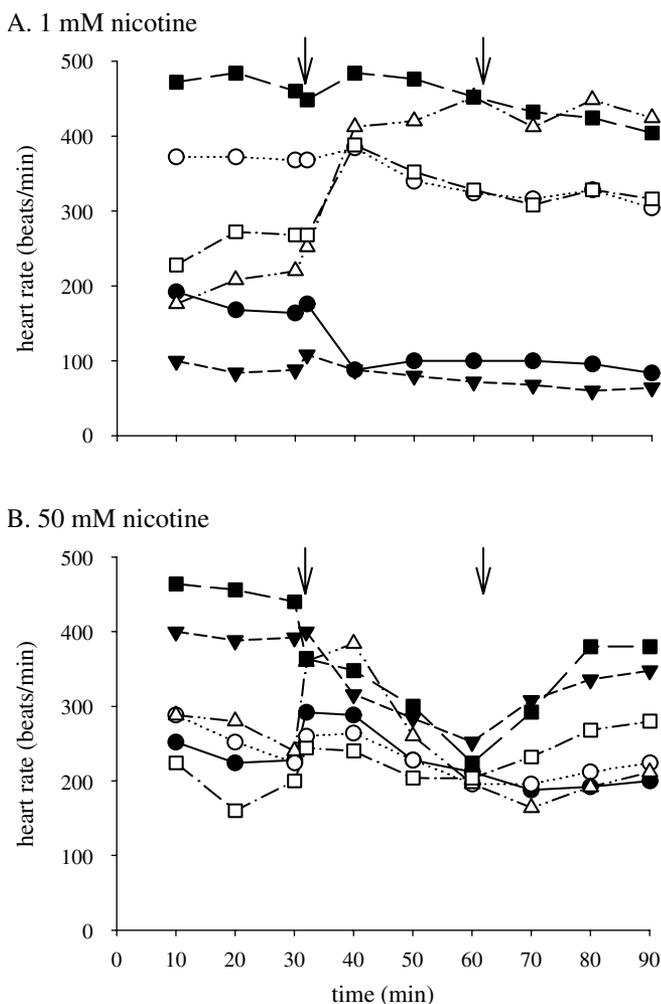
**Figure 2.** Effects of (A) ethanol, (B) nicotine, and (C) caffeine on heart rate in *Daphnia*. Arrows mark the time when the test substance was applied (between 30 and 32 minutes after the experiment began) and removed (30 minutes later). Heart rates are shown as mean percentages of the initial values. Initial values were calculated by averaging the heart rates of each animal at 10, 20, and 30 minutes. Results from 1 mM nicotine are shown in Figure 3A. The same control data are shown in A, B, and C. Bars show standard error of the mean.

In control animals, mean heart rate declined significantly from an average of 366 beats  $\text{minute}^{-1}$  to 298 beats  $\text{minute}^{-1}$  over 90 minutes (Figure 2, filled circles in A, B, and C;  $F = 7.483$ ,  $df = 9$  and  $153$ ,  $P < 0.0001$ ).

Ethyl alcohol caused a dose-dependent and reversible decrease in mean heart rate (Figure 2A). One percent ethanol may have caused a slight and transient decrease in mean heart rate immediately after it was applied, but no effect is evident at later time points. Five percent ethanol

reduced mean heart rate to just over half its initial value. This effect was fully reversible within 10 minutes of ethanol's removal. Ten percent ethanol caused mean heart rate to decrease to roughly 20% of its initial value. This effect was also reversible, but 20–30 minutes were required for full recovery. Both 5% and 10% ethanol caused obvious irregularity in heart rhythm.

Heart rate was significantly affected by nicotine (interaction of time and concentration:  $F = 5.380$ ,  $df = 9$  and  $96$ ,  $P < 0.0001$ ). At 0.1 mM, nicotine reversibly increased mean heart rate by roughly 20% (Figure 2B, open circles between arrows). At 1 mM, the heart rate of two animals increased, whereas that of a third decreased (Figure 3A). Neither effect was reversed upon removal of the nicotine. At 50 mM, nicotine caused an initial increase in mean heart rate followed by a subsequent decline over the following 30 minutes (Figure 2B, open triangles). The effect of 50 mM nicotine on individual animals' heart rates depended on the initial heart rates of the animals. *Daphnia* with high initial heart rates experienced a slow decline during their 30-minute exposure to 50 mM nicotine followed by partial recovery upon its removal (Figure 3B, solid squares and triangles). *Daphnia* with lower initial heart rates experienced an initial increase in heart rate followed by a subsequent decrease



**Figure 3.** Heart rates of individual *Daphnia* before, during, and after exposure to nicotine at (A) 1 mM and (B) 50 mM. Arrows mark the time when the test substance was applied (between 30 and 32 minutes after the experiment began) and removed (30 minutes later). The data shown in Figure 3B are the same as in Figure 2B (50 mM), where they are shown as means with error bars.

(Figure 3B; compare the position of symbols at 30 minutes and 32 minutes to see the initial increase). The overall effect of 50 mM nicotine was to reversibly reduce the variation between animals in their heart rates.

Statistical analysis indicates that caffeine affected heart rate (interaction of time and concentration:  $F = 2.993$ ,  $df = 9$  and  $96$ ,  $P = 0.0035$ ). Mean heart rate declined in the presence of 0.5% and 2% caffeine at the same time that mean heart rate in control animals remained roughly constant (Figure 2C). With a caffeine concentration of 0.1%, mean heart rate increased. In spite of the finding of statistical significance, we doubt that caffeine had any real effect. The increase in mean heart rate seen at 0.1% was attributable to the contribution of just one animal, others were unaffected, and much of the decrease seen at 2% was attributable to another whose heart rate declined more than that of others. If those two animals are removed, statistical significance is lost ( $P = 0.0511$ ). Also, although mean heart rate in control animals was stable between 30 and 60 minutes into the procedure, mean heart rate fell significantly in control animals across the entire 90-minute period. The stability during the 30–60 minute window used in the statistical analysis may have been a fluke. Finally, the decline in mean heart rate in the presence of 0.5% and 2% caffeine began before the caffeine was present (Figure 2C). Between 10 and 30 minutes into the procedure, mean heart rate fell by 10% in the group that would receive 0.5% caffeine and by 6% in the group that would receive 2% caffeine. The decline in the presence of caffeine may simply be a continuation of that trend. Follow-up experiments with caffeine were meant to determine whether caffeine has any real effect.

In follow-up experiments, mean heart rate fell by 20% (standard error = 6%) over 30 minutes in animals exposed to 2% caffeine. In control animals, mean heart rate fell by 16% (standard error = 8%). The difference was not statistically significant ( $t = 0.385$ ,  $df = 10$ ,  $P = 0.7082$ ).

## ○ Recommendations for Teaching Laboratories

Ethanol is a highly suitable agent to test in a teaching laboratory focused on heart rate in *Daphnia*. Both 5% and 10% ethanol cause rapid decreases in heart rate that are large in magnitude. Neither concentration caused any mortality in our study. If students are to attempt to reverse the effect of the treatment, 5% ethanol is the concentration to use. Full recovery occurs just 10 minutes after several thorough rinses to remove the ethanol (Figure 2A). Recovery from 10% ethanol takes two to three times as long.

In addition to its large, reversible effect, there is another good reason to test ethanol. It causes marked irregularity in heart rhythm. Teachers can exploit this action of ethanol to emphasize the importance of making casual observations. Such findings can lead to new areas of investigation. Teachers can emphasize this fact before students conduct the laboratory exercise. Students can be queried for casual observations during the exercise or later during classroom discussion. Attentive students will note the irregular rhythm caused by ethanol.

Nicotine would be a difficult compound to work with in a *Daphnia* heart rate lab. Baylor (1942) found that *Daphnia* exposed to “ $10^{-4}$ ” nicotine exhibited a 17% decrease in heart rate. If he meant  $10^{-4}$  M, and not  $10^{-4}$  v/v (0.01%), then our results contradict his: we found that heart rate increased at that concentration. Testing an agent that increases heart rate presents a problem. Students already have difficulty counting heart beats in *Daphnia* because the heart rate is so high. If nicotine causes it to increase, would students still be able to determine heart rate? Nicotine at 0.1 mM could be useful if students do not count beats by eye. Baylor (1942) used a stroboscope for this purpose, a technique advocated by Foster (1997).

Concentrations of nicotine above 0.1 mM should not be used if “clean” results are desired. Effects at 1 mM are unpredictable and irreversible (Figure 3A). At 50 mM, nicotine reduces heart rate, but only if it is high to begin with, and the full effect takes at least 30 minutes to

occur (Figure 3B). Another reason to avoid high concentrations is that nicotine is toxic and easily absorbed across the skin. We worked with small volumes, 1.5 mL aliquots. At 50 mM, 1.5 mL contains 75  $\mu\text{mol}$  (12 mg) of nicotine (its molecular weight is 162  $\text{g mol}^{-1}$ ). That is almost three times the quantity absorbed when chewing tobacco, estimated at 4.5 mg by Benowitz (1997). So if a student spills just 1.5 mL of 50 mM nicotine on his or her skin, and does not wash it off quickly, sufficient nicotine will be absorbed for the student to feel its effects. Teachers may work with concentrations of nicotine that high. According to their material safety data sheet (MSDS), Fisher Scientific estimates that their tobacco extract contains almost 0.5% nicotine, which corresponds to 31 mM, so millimolar concentrations can be obtained by extracting tobacco into water – a procedure commonly used by teachers preparing for the *Daphnia* heart rate lab. If students are to work with a nicotine solution, its concentration should be low (0.1 mM) and known. This makes the exercise safe for students but not for teachers, who must prepare the test solution from pure liquid nicotine. Fisher Scientific's MSDS states that their pure nicotine solution “may be fatal if absorbed through the skin.” It is probably best not to test nicotine at all.

Our initial experiments suggested that caffeine decreases heart rate in *Daphnia*. The same trend was observed in follow-up experiments: mean heart rate fell further in caffeine-exposed animals than in controls, but the difference was not statistically significant. We conclude that either caffeine has no effect on heart rate at the concentrations we tested or it causes a slight decrease that is hard to detect statistically. Foster (1997) reported increased heart rate in response to caffeine, but she mostly tested lower concentrations than we did, and she only evaluated heart rate immediately after applying the caffeine. Her highest concentration, however, was the same as our lowest, 0.1%. At that concentration, we found that mean heart rate decreased by 3.5% immediately after caffeine was applied (not evident in Figure 2C because of overlapping symbols). She reported a 53% increase. We cannot explain this discrepancy.

One good reason to have students test caffeine is that they will go into it with an expectation. Caffeine elevates heart rate in humans, so they will expect the same with *Daphnia*. Teachers can exploit this to illustrate observer bias. If students find that caffeine elevates heart rate, their bias is revealed. Testing caffeine also makes the exercise more realistic – scientists often find that their treatments have no effect. Provided that students also

test 5% or 10% ethanol, they will not become discouraged by what they perceive as “failed” experiments with caffeine. We recommend using caffeine at 0.5%. We abandoned several experiments because 2% caffeine came out of solution. One 100-mg tablet of caffeine dissolved in 20 mL of water yields the recommended 0.5%. If students are to be kept blind to the treatment, avoid yellow tablets, because they yield yellow solutions. If your goal is to probe for observer bias, use yellow tablets.

Much can be achieved with the *Daphnia* heart rate lab. Students can learn about stimulants and depressants. Pharmacologic agents can be tested to illustrate the actions of neurotransmitters and hormones, agonists and antagonists, and second messengers. Outside of physiology, students can learn about observer bias, control groups, and the importance of testing for reversibility. Advanced placement or college students can employ various experimental designs requiring different statistical tests of null hypotheses. Regardless of the exercise's intent, it helps the teacher to know what to expect.

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