ADVANCES IN PHYSIOLOGY EDUCATION

Adv Physiol Educ 50: 1–9, 2026.
First published October 24, 2025; doi:10.1152/advan.00135.2025

SOURCEBOOK OF LABORATORY ACTIVITIES IN PHYSIOLOGY

Large-scale practical cardiovascular classes with *Danio rerio*: overcoming ethical, financial, and logistical challenges associated with mammalian models

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Abstract

Traditional laboratory practicals exploring cardiovascular physiology and pharmacology rely on mammalian models, presenting ethical, financial, and logistical challenges. *Danio rerio* (zebrafish) larvae offer a compelling alternative that aligns with the partial replacement principle of replacement, reduction, and refinement (the 3Rs), while providing an opportunity for students to develop desirable in vivo skills to improve their employability. Here, we introduce an engaging set of in vivo laboratory practicals suitable for large undergraduate cohorts that utilizes larval zebrafish to investigate cardiac ion channels and receptors. The practical involves two 3-hour sessions where students measure heart rate in 72- and 96-hour postfertilization larvae in response to various treatments. The first session introduces students to handling larval zebrafish before exploring the effects of a reduced ambient temperature and application of the commonly used zebrafish anesthetic tricaine (MS-222) on both heart rate and the zebrafish startle reflex. Finally, students apply the well-known adrenergic agonist adrenaline. The second session empowers students to develop their own testable hypothesis regarding which ion channels or receptors are likely to influence zebrafish heart rate, providing them with the autonomy to select two pharmacologically active drugs from a carefully curated list [e.g. isoproterenol (β-adrenergic receptor agonist), propranolol (β-adrenergic receptor antagonist), and nifedipine (L-type calcium channel blocker)] that will enable them to address their hypothesis. Students' subsequent data for analysis allows them to develop an understanding of the conserved and divergent aspects of cardiac physiology between zebrafish and mammalian systems, and an appreciation of the importance of appropriate model selection in physiological and pharmacological research.

NEW & NOTEWORTHY The document outlines how large-scale undergraduate practical classes involving *Danio rerio* (zebrafish) can be used to teach cardiovascular physiology. It emphasizes the educational value of using live zebrafish to explore heart rate, drug effects, and homeostasis. The process supports active, inquiry-based learning, fostering engagement, critical thinking, and collaborative skills. It also addresses ethical and logistical considerations. Overall, the approach effectively combines hands-on experimental experience with core physiological concepts in an impactful educational format.

cardiovascular; partial replacement; zebrafish; 3Rs

INTRODUCTION

Exploring the cardiovascular and autonomic nervous systems is a fundamental part of any physiology and pharmacology education. To gain an understanding of human cardiac tissue, including the ion channels and receptors that regulate its activity, traditional laboratory practicals have often relied on mammalian models as a physiologically relevant alternative. However, mammalian models such as the Langendorff preparation and other isolated heart preparations (1, 2) come with significant ethical, financial, and logistical challenges. As an alternative to mammalian models, educators often turn to computational models, which can allow students to develop a good understanding of cardiac physiology (3, 4). However, computational models do not address concerns around a reduction in the number of students graduating without any in vivo experience (5), which has led to an in vivo skills gap in graduates (6–8). This necessitates the exploration of alternative, robust, and educationally valuable nonmammalian in vivo models to study cardiovascular physiology and pharmacology.

Zebrafish (*Danio rerio*) offer a compelling alternative to mammalian models, particularly in larval stages, aligning with the partial replacement aspect of replacement, reduction, and refinement (the 3Rs). In the United Kingdom, zebrafish larvae are not considered protected animals until they reach 5 days postfertilization (dpf), aligning with the point at which they are capable of independent feeding (9, 10). Although not a full replacement, this significantly reduces the ethical burden associated with their use, allowing for large-scale, noninvasive experiments. Furthermore, zebrafish husbandry is relatively inexpensive and requires

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Submitted 11 June 2025 / Revised 15 August 2025 / Accepted 20 October 2025

less specialized infrastructure compared to mammalian housing, contributing to substantial reductions in financial and logistical overheads. Here, we describe a laboratory practical using zebrafish, which provides students with hands-on experience of an in vivo lower vertebrate model to investigate the ion channels and receptors present in cardiac tissue.

Objectives and Overview

The modulation of zebrafish heart rate practical classes are suitable for large cohorts of undergraduate students and use the simple measurement of heart rate in larval zebrafish in vivo. Students apply various drugs, including those with anesthetic properties and drugs known to influence human heart rate, and observe the effects in the in vivo model, generating their own data for subsequent analysis. This practical also provides an excellent starting point for discussions around the ethics of animal use and the concepts of the 3Rs. The practical class described can be easily expanded for individual or group work projects, such as a final year capstone project.

The objectives of the described practical classes are *1*) to use zebrafish appropriately to measure a key physiological variable, heart rate, in vivo; *2*) to design appropriate experiments to investigate the effect of drugs on zebrafish heart rate; *3*) to analyze data relating to changes in heart rate and use this to evaluate the similarities and differences between mammalian and zebrafish hearts; and *4*) to introduce students to the importance of appropriate models to investigate physiological function and evaluate pharmacological compounds.

Background

Zebrafish are a valued model across the biosciences from drug screening to understanding human disease, including cardiovascular disease (11–13). Zebrafish have a single circulatory system, which requires a two-chambered heart consisting of a single atrium and a single ventricle; a significant structural difference from the four-chambered mammalian heart. Despite this structural difference, the range of heart rate in an adult zebrafish [120–180 beats/min (bpm); Ref. 14] is more similar to humans than that of common mammalian models such as the mouse. Adult zebrafish electrocardiograms show distinct P, QRS, and T waves, and the QT duration suggests a similar cardiac repolarisation time to humans (14). Most of the specialized cardiac cell types, for example, pacemaker cells, are found in both human and zebrafish hearts. The overall shape of a cardiac action potential is also similar to that of humans, with the depolarizing phase of the action potential mediated by voltage-gated Na^+ channels, the plateau phase mediated by voltage-gated L-type Ca^{2+} channels, and the repolarizing phase mediated by the rapid delayed rectifier current (I_{Kr}; Refs. 15–17). These similarities can be observed pharmacologically, as the voltage-gated Na^+ channel blockers tetrodotoxin and tricaine (also known as MS-222) reduce the action potential upstroke, and the L-type Ca^{2+} channel blocker nifedipine shortens the plateau phase of zebrafish cardiac action potentials (16, 18). Although the zebrafish cardiac function is not fully developed in larval zebrafish, the ECG profile at 72 h post-fertilization (hpf) is very similar to that of adult zebrafish described above (19). Heart rate does vary with development, steadily increasing between 24 and 96 hpf, where it can reach over 200 bpm, before slowing again as they reach adulthood (16, 18). Regardless of this increase, the larval zebrafish heart rate is still lower than that of commonly used mammalian models. Unlike endothermic mammals, zebrafish are ectotherms whose heart rate can be influenced by ambient temperature, with a substantial decrease in heart rate as ambient temperature is reduced from 28°C to 18°C (19). This makes zebrafish amenable to anesthesia by rapid cooling, particularly in larval zebrafish (20); however, it is a major difference between zebrafish and mammals.

The heart rate of both humans and zebrafish is dynamically regulated by the autonomic nervous system, comprising the sympathetic and parasympathetic branches. Sympathetic stimulation, mediated by the release of norepinephrine from sympathetic neurons and activation of β-adrenergic receptors expressed by pacemaker cells and cardiac myocytes, increases heart rate and contractile strength. Conversely, parasympathetic stimulation, via acetylcholine (ACh) release from the vagus nerve, activates muscarinic receptors expressed by pacemaker cells and results in a decrease in heart rate. Some evidence suggests that this autonomic regulation can be seen early in zebrafish development, with increases in heart rate in response to the application of norepinephrine and the β-adrenergic agonist isoprenaline (also known as isoproterenol) observed from 96 hpf (18, 21, 22). The development of functional sympathetic innervation and thus the endogenous release of norepinephrine can be assessed by the observation of a decrease in heart rate in response to the β-adrenergic antagonist propranolol; in zebrafish, this is first observed at 5 dpf (21, 22). In relation to the parasympathetic system, muscarinic M2 receptors are expressed in cardiac tissue from 30 hpf (23), with the nonselective cholinergic agonist carbachol evoking bradycardia from 72 hpf. In contrast to this, responses to the nonselective cholinergic agonist ACh are not seen until 5 dpf (21). The lack of response to the nonselective muscarinic antagonist atropine until 11–12 dpf has suggested that parasympathetic innervation does not occur until this time point (21). However, mild electrical stimulation at 0.5 Hz of 5- to 7-dpf zebrafish has been observed to induce atropine sensitive bradycardia and propranolol sensitive tachycardia (24), evidencing that central autonomic regulation of cardiac function is active in larval zebrafish.

These practicals give students a valuable opportunity to observe the effects of drugs on a simple measurement in an in vivo model, something that simply cannot be achieved for logistical and ethical reasons in mammalian models. The similarities and differences between zebrafish and mammalian systems highlighted above offer excellent discussion points for students when considering zebrafish as a model organism for understanding cardiac physiology and testing cardiac drugs. This allows students to consider how aspects such as the developmental age of a model and conditions such as temperature and the use of an anesthetic can affect their results.

Learning Objectives

After completing this activity, the student will be able to do the following:

1) Explain what the term "protected animal" means in relation to performing scientific experiments using *D. rerio* (zebrafish) within the United Kingdom;
2) Design experiments to investigate the effect of drugs on heart rate in the in vivo model zebrafish;
3) Calculate and perform drug dilutions to administer drug compounds to the in vivo model zebrafish;
4) Use a dissecting microscope to observe changes in heart rate in the in vivo model zebrafish;
5) Analyze the data through data visualization and using descriptive and inferential statistics to determine the effect of drugs on heart rate in *D. rerio*;
6) Use the data collected to discuss whether tricaine is a useful anesthetic when investigating zebrafish heart rate; and
7) Use the data collected and knowledge of the mammalian heart to discuss the similarities and differences between the mammalian heart and the zebrafish heart.

Activity Level

The activity presented in its current form is suitable for all levels of undergraduate students in the general biomedical science disciplines; it acts as an early opportunity for students to engage with in vivo techniques and learn about the importance of choosing an appropriate model organism for their given question. Should the range of drugs detailed later feel inappropriate for a particular cohort of students, this can be amended to use a smaller, simpler range of drugs. Alternatively, this could also be easily adapted for students to complete as a final year capstone project or as a project for taught master's students and master's by research students. If adapting for a project, students could have more freedom in choosing the drugs that they wished to investigate.

Prerequisite Student Knowledge or Skills

Before doing this activity, students should have a basic understanding of cardiovascular and autonomic physiology, more specifically the basic complement of ion channels that are expressed in mammalian cardiac tissue and the basic receptor subtypes used by the sympathetic and parasympathetic branches of the autonomic nervous system to regulate heart rate. It is also important that students have been taught how to behave appropriately when using a model organism, to be introduced to the zebrafish as the model organism that they will be using, and to undertsand the concepts of replacement, reduction, and refinement (the 3Rs). Students should know how to use a standard laboratory micropipette, calculate drug dilutions, and use a standard laboratory dissecting microscope.

Time Required

Zebrafish must be marbled 4 days in advance of the first session if one wishes to use zebrafish at 72 hpf (this is the age when zebrafish typically hatch). Marbling refers to the technique of placing a layer of marbles at the bottom of the fish tank to prevent predation of the zebrafish eggs. Here, the eggs are protected by settling in between the marbles. The age at which initial experiments are performed can be adjusted slightly if needed to fit your timetable; however, be aware that if using fish earlier than 72 hpf, you will either have to dissect the zebrafish from their chorion or teach the students how to do this. This can be technically challenging and is only recommended if students have been taught microdissection previously.

The students require at least one 3 hour session to explore the basics of measuring zebrafish heart rate and modulating this by reducing the ambient temperature, applying the anesthetic tricaine, and the adrenergic receptor agonist adrenaline (epinephrine). However, to incorporate experimental design and allow students to develop further hypotheses, it works better as two 3 hour sessions. The second session can be used to allow students to select two or three drugs from a curated selection to allow them to test their own specific hypothesis. For example, students could choose to focus on agonists and/or antagonists that are selective for β-adrenergic receptors or for cholinergic receptors.

METHODS

Equipment and Supplies

Modulation of zebrafish heart rate.

The following should be available for a student to set up their workstation (Fig. 1):

- Three small petri dishes containing 5 mL of E3 medium and 72-hpf (for experiment 1) or 96-hpf (for experiment 2) zebrafish (nacre mutant);
- The E3 media contain 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$ at pH 7.2–7.3, dissolved oxygen >6.3 mg/L, total hardness of 65 mg/L (as $CaCO_3$), and temperature of 28 ± 1°C;
- Dissecting microscope;
- Large petri dish (to use as an ice bath);
- Dissecting seeker;
- Pasteur pipette;
- Counter;
- Stopwatch;
- Thermometer;
- Container of ice/access to an ice machine;
- Pipettes and tips (P5000, P1000, and P100);
- Waste bucket;
- An Eppendorf with 5 mM tricaine; and
- An Eppendorf of 1 mM adrenaline.

The Eppendorfs containing the appropriate stock concentrations of the remaining drugs (Table 1) are available on request from the session leader. These drugs should be kept on ice during the practical to prevent degradation.

Ethical responsibilities of staff and students.

A strain of zebrafish nacre (25) is most useful for these experiments, as it lacks melanophores and thus allows the heart to be visualized more easily. However, most other zebrafish strains, including wild-type, could be used for these experiments as the pigmentation is relatively low and does not obstruct visualization of the heart at the ages used.

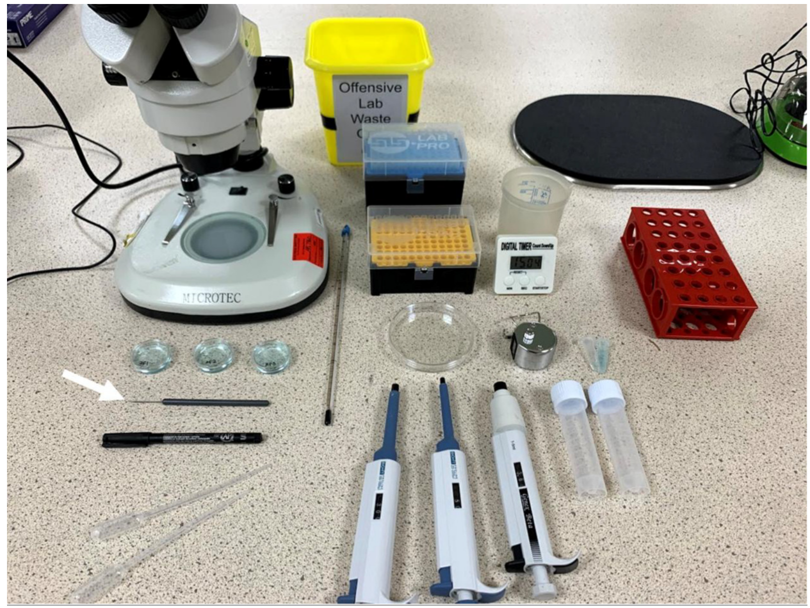

Figure 1. Equipment needed for the zebrafish experiments. Students require a standard dissecting microscope, 3 small petri dishes containing 5 mL of E3 medium, and 72-hour postfertilization (hpf; for *experiment 1*) or 96-hpf (for *experiment 2*) zebrafish (nacre mutant), a counter and a stopwatch to perform basic measurements, a dissecting seeker (highlighted by the white arrow) to enable the startle reflex to be tested, a large petri dish to use as an ice bath and a thermometer to achieve the required temperature, a Pasteur pipette to transfer zebrafish between petri dishes, pipettes and tips (P5000, P1000, and P100), and stock concentrations of tricaine and adrenaline.

The zebrafish used in the experiment are used up to 96 hpf and are therefore exempt from the UK's Animals (Scientific Procedures) Act, 1986. Regardless, students are taught to look out for any signs of distress in the zebrafish and to notify the teaching staff if they suspect that the zebrafish has been damaged. Adopters of this activity are responsible for obtaining permission for human or animal research from their home institution. For a summary of Guiding Principles for Research Involving Animals and Human Beings, please see https://www.physiology.org/mm/Publications/Ethical-Policies/Animal-and-Human-Research.

Instructions

Preparation for the practicals.

1) Production and care of zebrafish embryos:

- Zebrafish should be maintained at 28°C, ideally on a 14-hour light/10-hour dark cycle. Zebrafish can be maintained within a simple tank in tap water, which requires regular cleaning (26) or a purpose-built aquatic system that contains a circulating system that filters and aerates the water (27).
- For breeding large numbers for an experiment, Westerfield (26) provides excellent, detailed advice. Briefly, add the female to the tank with the male while adding a layer of marbles at the bottom of the tank. The following morning, the onset of light will initiate breeding, and fertilized eggs will lay within the layer of marbles. The eggs can then be collected using a net, any debris can be removed and eggs can be placed in a petri dish of E3 embryo media. Methylene blue (0.01%;

Table 1. *Adrenaline (also known as epinephrine) and other optional compounds*

Drug	Known Target in Mammals	Expected Effect	Stock Concentration	Working Concentration	Supplier Details
Adrenaline (epinephrine)	Nonselective α/β-adrenergic agonist, some selectivity for β_2	Increase in heart rate	1 mM	10 µM	Sigma-Aldrich, cat. no. E4250
Isoprenaline	Nonselective β-adrenergic agonist	Increase in heart rate	10 mM	100 µM	Sigma Aldrich, cat. no. I5627
Salbutamol	β_2-adrenergic agonist	Increase in heart rate	1 mM	10 µM	Sigma-Aldrich, cat. no. S8260
Propranolol	Nonselective β-adrenergic antagonist	Decrease in heart rate*	1 mM	10 µM	Sigma-Aldrich, cat. no. P0689
Atenolol	Selective β_1-adrenergic antagonist	Decrease in heart rate*	10 mM	1 mM	Sigma-Aldrich, cat. no. A7655
Acetylcholine (ACh)	Nonselective ACh receptor agonist	Decrease in heart rate*	10 mM	1 mM	Sigma-Aldrich, cat. no. A6625
Bethanechol	Muscarinic ACh receptor agonist	Decrease in heart rate*	10 mM	100 µM	Sigma-Aldrich, cat. no. PHR2357
Nifedipine	L-type Ca^{2+} channel blocker	Decrease in heart rate*	1 mM	10 µM	Sigma-Aldrich, cat. no. N7634

Suggested stock and working concentrations are provided, along with the known molecular target and known effect in humans. *See *Expected Results* for a more detailed description of this expected effect.

Sigma-Aldrich) can be added as a fungicide. Embryos should be incubated at 28.5°C. Embryos can be sorted under a microscope to remove any unfertilized eggs; they should be cleaned daily.
- Zebrafish embryos will hatch from their chorion between 48 and 72 hpf (28), making 72 hours a useful time to start experiments as one does not have to remove the chorion through dissection.

2) Stock solutions of tricaine (5 mM; ethyl 3-aminobenzoate methanesulfonate salt; Sigma-Aldrich) and all other experimental drugs (Table 1) should be prepared in Eppendorfs in advance and frozen until the day of the practical.

Laboratory session 1.

Note that part of the aims of this session is for students to decide on exactly how they will perform the experiments. The instructions given below are more extensive than those currently given to students. Students are each given three zebrafish, each in a petri dish with 5 mL E3 medium, and perform all experiments described for *session 1* on the same three zebrafish. You can choose to allow students to use a different zebrafish for each condition; however, in the interest of reducing the number of animals used for this practical class, this is not how it is currently delivered.

1) Label each petri dish so that control, drug, and washout recordings can be attributed to the correct zebrafish.
2) Test and record the temperature of the E3 medium for your control recordings.
3) Using the dissecting microscope, identify the heart (highlighted by the white arrow in Fig. 2) and measure the heart rate for each of your 72-hpf zebrafish in E3 medium. Use the counter to keep a record of how many beats are observed over a 30-second period then double this to calculate heart rate in beats per minute. Collect three repeats in total for each of the three zebrafish.
4) Test the presence of the zebrafish startle reflex by using the dissecting seeker to gently nudge the zebrafish tail; zebrafish that are not anesthetized should swim away.
5) Assess the effect of reducing temperature to 15°C:

- Create a cold water bath by adding a small amount of ice and some cold water to the large petri dish, testing the temperature using the thermometer until your water bath is just below 15°C. Transfer one of your petri dishes to this cold water bath, ensuring no cold water enters the petri dish as this will affect the osmolarity of the solution, and leave for a few minutes to reach the required temperature of 15°C. Add more ice to your water bath if required to achieve the correct temperature.
- Once the correct temperature is achieved, measure heart rate three times and test the startle reflex before removing the petri dish containing the zebrafish back to the bench to return to your original control temperature. Repeat for the remaining two zebrafish.
- Once the zebrafish have returned to control temperature, measure heart rate three times and test the startle reflex. A warm water bath can be used to speed up this return to room temperature.

6) Using the same three zebrafish, assess the effect of 500 μM tricaine on zebrafish heart rate and immobilization:

- Dilute 5 mM tricaine to 500 μM tricaine within the petri dish containing the zebrafish. Avoiding the zebrafish, gently remove 500 μL of E3 medium from the first petri dish and add 500 μL of 5 mM tricaine.
- After 5 minutes, use the seeker to test the startle reflex. If there is no reflex, measure the heart rate three times.
- Use a pasteur pipette to transfer the zebrafish to a fresh petri dish containing 5 mL of E3 medium. After 5 minutes, use the seeker to test the startle reflex. Once the reflex has returned, measure the heart rate three times.

7) Using the same three zebrafish, assess the effect of 10 μM adrenaline (epinephrine) on zebrafish heart rate.

- Avoiding the zebrafish, gently remove 50 μL of E3 medium from the petri dish and add 50 μL of 1 mM adrenaline. After 5 minutes, measure the heart rate three times.
- Use a pasteur pipette to transfer the zebrafish to a fresh petri dish containing 5 mL of E3 medium. After 5 minutes, measure the heart rate. If it has not returned to control values, wait a further 5 minutes and measure heart rate again.

8) Any zebrafish used in experiments should be euthanized by placing them in 1% sodium hypochlorite (bleach) for

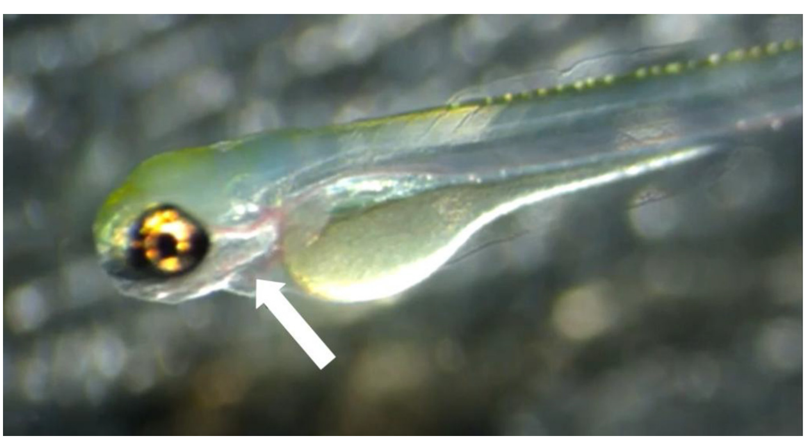

Figure 2. A 72-hour postfertilization (3-day postfertilization) nacre zebrafish. The arrow indicates the zebrafish heart. Note the ability to see the blood in this region.

at least 5 minutes. Your session lead will dispose of euthanized zebrafish.

Laboratory session 2.

1) Using the same protocols as laboratory *session 1*, first measure the heart rate for each of your 96-hpf zebrafish in E3 medium. This is important as you are using three new zebrafish and they are now 24 hours older, so you must establish a new baseline heart rate.

2) Once you have developed your hypothesis regarding zebrafish cardiac ion channels and/or receptors, choose two appropriate drugs from Table 1 to test this hypothesis. Assess the effect of your two chosen drugs on zebrafish heart rate using the methods from laboratory *session 1*. You can also apply two drugs together if this helps to test your hypothesis. The drugs (Table 1) are not an exhaustive list of what can be given to students but are those often used for convenience and relevance to our students.

3) Any zebrafish used in experiments should be euthanized by placing them in 1% sodium hypochlorite (bleach) for at least 5 minutes. Your session lead will dispose of euthanized zebrafish.

Troubleshooting

Students usually identify the heart easily; however, they can struggle to determine exactly what to focus on to measure heart rate. As the blood travels through both chambers, for each heartbeat there are movements that can be observed in the atrium and ventricle. Students are advised to pick a chamber to focus on when counting to ensure they do not count each beat twice. Although not required for students to achieve the learning objectives, you can use a camera for improved counting accuracy. This is advisable if developing this experiment as a capstone or master's research project. You could use a camera to record the heart, then count heartbeats at a slower playback speed. This can be achieved in two ways. First, if you have mobile phone mounts (for example, the Phonelink Adapter; Brunel Microscopes, Ltd.), students can attach a phone to the microscope eyepiece and record the heart. Counting can be achieved by watching at a slower playback speed. Second, if you have a no mobile phone policy for students during practicals, you can purchase cameras, for example, the Brunel Eyecam Plus 5 M (Brunel Microscopes, Ltd.) to insert into the microscope.

If a student cannot evoke a startle reflex in control conditions, they should be assured that this is a possibility, as although the tactile startle reflex generally develops by 48 hpf (29), there can be interindividual variation in the development, with some zebrafish not exhibiting a startle reflex (30). This is no reason to abandon this zebrafish. If the tricaine does not appear to be inducing a loss of the startle reflex, students should first consider whether they have pipetted the correct amount. Subsequently, they should check whether they have overstimulated the zebrafish with excessive light and thus an increase in ambient temperature or through excessive movement of the fluid and use of the dissecting seeker. These considerations should also be made if the heart rate does not return to baseline levels following removal of the anesthetic. Variations in responses are expected, and this can prove a useful point to discuss the variability of real biological data. If zebrafish responses become more variable over the course of the application of drugs, this can be a useful discussion point for the balance between collecting good-quality data and reducing the number of animals used.

Recommended working concentrations of drugs are given based on their potency and considerations given to the maximum concentrations considered safe for undergraduate students to work with. The working concentrations suggested for drugs all evoke responses where expected based on the expression of receptors and development of the autonomic nervous system (see *Expected Results*). Higher working concentrations could be used if instructors deem this safe for their students on consultation with the material safety data sheets. It should be noted that the atenolol and ACh stocks are made up in E3 media, so the 1:10 dilution should not affect the osmolarity of the solution or introduce another factor that could affect heart rate. This could be used as a point at which you can introduce the concept that a solvent used to dissolve a drug could influence the experiment and that where this is the case appropriate solvent controls should be used.

Zebrafish can become more active at 96 hpf, swimming around the petri dish more, which can make it more difficult to visualize the heart for a long enough time for counting. Students can be encouraged to use tricaine as an anesthetic or create an ice bath to lower the body temperature of the zebrafish and thus reduce zebrafish movement. If expanding this into a project, students could be taught to mount zebrafish in 3% methylcellulose to prevent excessive movement and increase the accuracy of heart rate measurements. To achieve this, prepare the 3% methylcellulose (Sigma; M-0387) in E3 medium and place on a shaker to dissolve overnight. Transfer a small amount of 3% methylcellulose onto a shallow glass depression slide (SLS Select Slides Single Cavity; MIC3450). Transfer the zebrafish on top of the methylcellulose and cover with E3 medium, with or without your test drug, to fill the depression. Gently press the zebrafish into the methylcellulose and adjust it to the correct orientation to view the heart (31). To change the solution, simply remove the solution from the top of the methylcellulose, replace it with the new solution, and leave it to equilibrate for 2 minutes.

At 96 hpf, the zebrafish heart rate also increases (18), making it more difficult to count. If not using a camera, it can be easier to encourage students to select drugs that are more likely to slow down the heart rate. One could complete both experiments using 72-hpf zebrafish if both sessions are run on the same day; however, one is likely to see responses to fewer of the drugs (see *Expected Results*). As a single batch of zebrafish embryos can be 200 or more, to reduce the use of animals and prevent excess waste of zebrafish, the sessions could be run on the same or concurrent days.

Safety Considerations

For animal welfare, we advise students to use a low intensity of light from the dissecting microscope.

Due to the potentially toxic nature of some of the drugs, stock solutions are kept in small quantities in Eppendorf tubes, and students are required to ask staff for the drugs when ready to perform their experiments. Recommended working concentrations of drugs are given.

Before any students in the United Kingdom undertake laboratory work of the type described in this paper, they must fill out forms relating to the identification of hazards relating to the planned experiments. These Control of Substances Hazardous to Health (COSHH) forms are a ubiquitous feature of experimental work in UK universities, and students are well versed in these safety protocols and acceptable ways of working.

Students are therefore required to provide confirmation that they have read the COSHH form for this experiment before they can take part. Additionally, students are trained how to take gloves off appropriately to ensure the skin is never in contact with the external aspect of the gloves.

RESULTS AND DISCUSSION

Expected Results

Students measuring heart rate of larval zebrafish by eye in vivo found that the heart rate at 72 hpf was 156 ± 25 bpm ($n = 120$), and this decreased significantly (73 ± 27 bpm, $n = 120$; $P < 0.001$) when the ambient temperature was reduced to 15°C (Fig. 3*A*). Heart rate was capable of a full recovery to baseline when zebrafish were returned to the original control temperature. At 72 hpf, 80% of zebrafish displayed a startle reflex ($n = 120$), whereas only 26% displayed a startle reflex at 15°C ($n = 120$); as with heart rate, a full recovery was observed when returned to control temperatures.

Tricaine (MS-222) is a voltage-gated Na^+ channel blocker and a commonly used anesthetic in zebrafish. It was expected to abolish the startle reflex and decrease heart rate (9). Student experiments showed a decrease in the number of zebrafish exhibiting a startle response from 78% in control conditions to 24% in 500 μM tricaine ($n = 117$), with the vast majority of zebrafish (71%; $n = 116$) showing a recovered startle reflex on return to E3 media. A small but significant decrease in heart rate was observed by students (control, 160 ± 26 bpm, $n = 117$; tricaine, 155 ± 27 bpm, $n = 117$; return to control, 167 ± 26 bpm, $n = 116$; $P < 0.01$; Fig. 3*B*).

When turning attention to drugs known to regulate heart rate in humans, as expected, students observed a reversible increase in heart rate on application of the nonselective adrenergic agonist adrenaline (control, 168 ± 29 bpm, $n = 78$; 10 μM adrenaline, 194 ± 36 bpm, $n = 78$; return to control, 168 ± 27 bpm, $n = 71$; $P < 0.0001$; Fig. 3*C*). Moving to the optional drugs that can be chosen by students based on their specific hypothesis, the isoprenaline, a nonselective β-adrenergic agonist causes an increase in heart rate (18), while the effects of salbutamol, a β_2-adrenergic agonist, are more varied due to the expression of two different β_2-adrenergic receptor genes in the zebrafish heart that have been shown to have complex effects on heart rate (22). The potential effects of salbutamol provide a platform to discuss the possible cardiac side effects of its use in the treatment of asthma. It also allows for a separate discussion about how many animals, including zebrafish, have multiple genes for a single protein compared to humans having a single gene, and what this means for studying these species.

The application of the nonselective β-adrenergic antagonist propranolol and the selective β1-adrenergic antagonist atenolol is not expected to elicit a change in heart rate at 96 hpf. Responses have previously been observed from 5 dpf (21, 22), suggesting sympathetic regulation is not active until this point. However, at 96 hpf, the increase in heart rate observed in response to adrenaline can be reduced by the coapplication of either propranolol or atenolol. These differences can be used as useful discussion points for choosing the correct developmental time points for studying a certain function within a given organism and for a discussion around the mechanism of action of antagonists in the presence and absence of endogenous and exogenous agonists.

Moving to the drugs modulating the parasympathetic control of cardiac function, the nonselective cholinergic agonist acetylcholine (ACh) and the muscarinic cholinergic agonist bethanechol may be expected to decrease heart rate. However, given the varied developmental time points (3–5 dpf) at which responses in heart rate to cholinergic agonists have been observed (21, 23), it is not surprising that many students do not observe responses to these agonists. Again, this does provide an opportunity to discuss the importance of choosing a model organism at an appropriate time point for your given hypothesis.

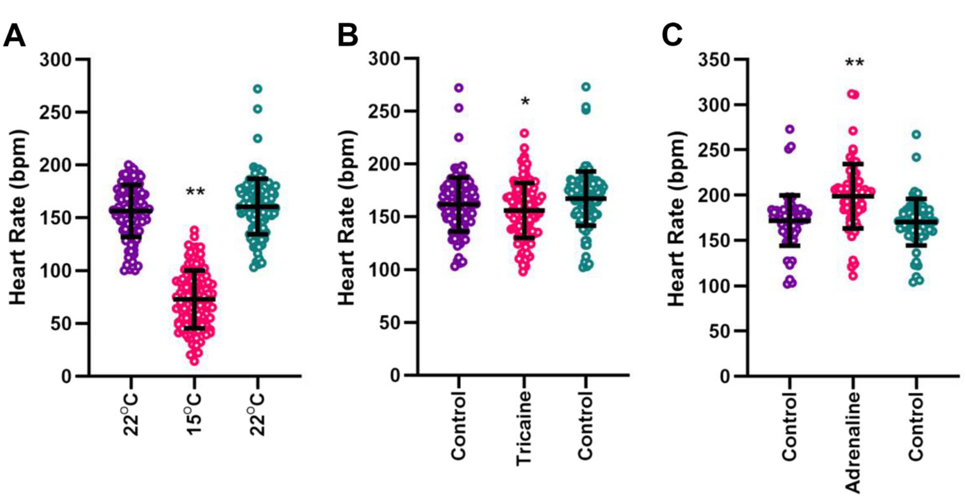

Figure 3. Modulation of embryonic zebrafish heart rate. *A*: decreasing temperature from 22 °C to 15 °C reduces heart rate in 3-day postfertilization (dpf) zebrafish ($n = 119$); bpm, beats per minute. *B*: tricaine (500 μM) elicits a small decrease in heart rate in 3-dpf zebrafish ($n = 113$). *C*: adrenaline (10 μM) increases heart rate in 4-dpf zebrafish ($n = 68$). Data shown as mean ± SD. *$P < 0.05$, **$P < 0.001$, Friedman test and Dunn's multiple comparisons.

Finally, the L-type Ca^{2+} channel blocker, nifedipine, usually produces a decrease in heart rate (32). However, an increase in heart rate is sometimes observed, which could be analogous to the reflex tachycardia seen in humans in response to high doses of nifedipine (33). This response allows for a discussion around the complexities of in vivo studies and the importance of considering how drugs may impact the vasculature as well as the heart.

Misconceptions.

As mentioned when troubleshooting, there are misconceptions from students about zebrafish being defective if they do not exhibit a startle reflex under control conditions. Although the tactile startle reflex generally develops by 48 hpf (28), there can be interindividual variation in the development, with some zebrafish not exhibiting a startle reflex (30).

Evaluation of Student Work

Inquiry applications.

This practical allows students to experience facilitated inquiry, where there are broad guidelines for the research question and methods, but the students need to specify the exact question in choosing their drugs and design the finer details of the method. They are given the question "Are zebrafish a useful model for investigating cardiac physiology?' and told to focus on the similarities in changes of heart rate in response to drugs that have known actions in humans. The first session is very guided, and then the second allows for the development and testing of their own hypotheses. They are given the freedom to choose whether to perform the second day's experiments with tricaine anesthesia or at a lower temperature, and whether they wish to apply drugs alone or together.

The activity could be made more student-centered by allowing them to choose which aspect of zebrafish physiology or behavior they wish to investigate to determine whether they are a useful model. They could be allowed to research the experimental drugs from scratch rather than choosing from a defined list, or consider manipulating ion concentrations in the E3 medium to assess the effect on homeostasis.

Wider educational applications.

As discussed in *Expected Results*, this practical series allows one to explore numerous topics including the mechanism of action of antagonists, the localized expression of specific receptor subtypes, and mechanisms underlying side effects of clinical drugs, the complications arising from using an in vivo model, and the importance of using appropriate developmental ages for studying different systems. Any of these topics could be expanded on to create additional problem-based learning studies.

DATA AVAILABILITY

Data will be made available upon reasonable request.

ACKNOWLEDGMENTS

We thank Louise Robson for support and guidance when initially developing this practical, the Biological Services aquarium team at the University of Sheffield for breeding the zebrafish, and the School of Biosciences teaching technical team for yearly preparation of these practicals. We also thank the students who have engaged positively with this practical and the principles that it teaches.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L.F.C. conceived and designed research; L.F.C. performed experiments; L.F.C. analyzed data; L.F.C. interpreted results of experiments; L.F.C. prepared figures; L.F.C., N.S.F., J.L.R., and A.S. drafted manuscript; L.F.C., N.S.F., J.L.R., and A.S. edited and revised manuscript; L.F.C., N.S.F., J.L.R., and A.S. approved final version of manuscript.

REFERENCES

1. **Bell RM**, **Mocanu MM**, **Yellon DM.** Retrograde heart perfusion: the Langendorff technique of isolated heart perfusion. *J Mol Cell Cardiol* 50: 940–950, 2011. doi:10.1016/j.yjmcc.2011.02.018.
2. **Liao R**, **Podesser BK**, **Lim CC.** The continuing evolution of the Langendorff and ejecting murine heart: new advances in cardiac phenotyping. *Am J Physiol Heart Circ Physiol* 303: H156–H167, 2012. doi:10.1152/ajpheart.00333.2012.
3. **Dewhurst DG**, **Ullyott RT.** Computer simulated demonstration of the actions of drugs on the isolated perfused mammalian heart (Langendorff preparation). *Altern Lab Anim* 19: 316–322, 1991. doi:10.1177/026119299101900305.
4. **Samsel RW**, **Schmidt GA**, **Hall JB**, **Wood LD**, **Shroff SG**, **Schumacker PT.** Cardiovascular physiology teaching: computer simulations vs. animal demonstrations. *Adv Physiol Educ* 266: S36–S46, 1994. doi:10.1152/advances.1994.266.6.S36.
5. **British Pharmacological Society.** A survey of integrative physiology/pharmacology teaching undertaken by the BPS and the Physiological Society. *pA2 Online* 3: 10–11, 2004.
6. **The Association of the British Pharmaceutical Industry.** Sustaining the skills pipeline in the pharmaceutical and biopharmaceutical industries (Online). https://www.abpi.org.uk/media/ou0hnrf2/sustain-skills-pipeline.pdf [2025 Apr 24].
7. **The Association of the British Pharmaceutical Industry.** Skills needs for biomedical research (Online). https://www.abpi.org.uk/media/bufdzm3b/skills-biomedical-research.pdf [2025 Apr 24].
8. **The Association of the British Pharmaceutical Industry.** Bridging the skills gap in the biopharmaceutical industry (Online). https://www.abpi.org.uk/publications/bridging-the-skills-gap-in-the-biopharmaceutical-industry-2022/ [2025 Apr 24].
9. **GOV.UK.** Animals (Scientific Procedures) Act 1986 c.1 (Online). https://www.gov.uk/government/publications/the-operation-of-the-animals-scientific-procedures-act-1986 [2025 Apr 24].
10. **Strähle U**, **Scholz S**, **Geisler R**, **Greiner P**, **Hollert H**, **Rastegar S**, **Schumacher A**, **Selderslaghs I**, **Weiss C**, **Witters H**, **Braunbeck T.** Zebrafish embryos as an alternative to animal experiments—a commentary on the definition of the onset of protected life stages in animal welfare regulations. *Reprod Toxicol* 33: 128–132, 2012. doi:10.1016/j.reprotox.2011.06.121.
11. **Bowley G**, **Kugler E**, **Wilkinson R**, **Lawrie A**, **van Eeden F**, **Chico TJA**, **Evans PC**, **Noël ES**, **Serbanovic-Canic J.** Zebrafish as a tractable model of human cardiovascular disease. *Br J Pharmacol* 179: 900–917, 2022. doi:10.1111/bph.15473.
12. **Choi T**, **Choi TI**, **Lee YR**, **Choe SK**, **Kim CH.** Zebrafish as an animal model for biomedical research. *Exp Mol Med* 53: 310–317, 2021. doi:10.1038/s12276-021-00571-5.
13. **Patton EE**, **Zon LI**, **Langenau DM.** Zebrafish disease models in drug discovery: from preclinical modelling to clinical trials. *Nat Rev Drug Discov* 20: 611–628, 2021. doi:10.1038/s41573-021-00210-8.
14. **Milan DJ**, **Jones IL**, **Ellinor PT**, **MacRae CA.** In vivo recording of adult zebrafish electrocardiogram and assessment of drug-induced QT

prolongation. *Am J Physiol Heart Circ Physiol* 291: H269–H273, 2006. doi:10.1152/ajpheart.00960.2005.

15. **Baker K**, **Warren KS**, **Yellen G**, **Fishman MC.** Defective "pacemaker" current (Ih) in a zebrafish mutant with a slow heart rate. *Proc Natl Acad Sci USA* 94: 4554–4559, 1997. doi:10.1073/pnas.94.9.4554.
16. **Nemtsas P**, **Wettwer E**, **Christ T**, **Weidinger G**, **Ravens U.** Adult zebrafish heart as a model for human heart? An electrophysiological study. *J Mol Cell Cardiol* 48: 161–171, 2010. doi:10.1016/j.yjmcc.2009.08.034.
17. **Hassel D**, **Scholz EP**, **Trano N**, **Friedrich O**, **Just S**, **Meder B**, **Weiss DL**, **Zitron E**, **Marquart S**, **Vogel B**, **Karle CA**, **Seemann G**, **Fishman MC**, **Katus HA**, **Rottbauer W.** Deficient zebrafish ether-à-go-go–related[1] gene channel gating causes short-qt syndrome in zebrafish reggae mutants. *Circulation* 117: 866–875, 2008. doi:10.1161/CIRCULATIONAHA.107.75222.
18. **De Luca E**, **Zaccaria GM**, **Hadhoud M**, **Rizzo G**, **Ponzini R**, **Morbiducci U**, **Santoro MM.** ZebraBeat: a flexible platform for the analysis of the cardiac rate in zebrafish embryos. *Sci Rep* 4: 4898, 2014. doi:10.1038/srep04898.
19. **Dhillon SS**, **Dóró É**, **Magyary I**, **Egginton S**, **Sík A**, **Müller F.** Optimisation of embryonic and larval ECG measurement in zebrafish for quantifying the effect of QT prolonging drugs. *PLoS One* 8: e60552, 2013. doi:10.1371/journal.pone.0060552.
20. **Chen K**, **Wang CQ**, **Fan YQ**, **Xie YS**, **Yin ZF**, **Xu ZJ**, **Zhang HL**, **Cao JT**, **Han ZH**, **Wang Y**, **Sun DQ.** The evaluation of rapid cooling as an anesthetic method for the zebrafish. *Zebrafish* 11: 71–75, 2014. doi:10.1089/zeb.2012.0858.
21. **Schwerte T**, **Prem C**, **Mairösl A**, **Pelster B.** Development of the sympatho-vagal balance in the cardiovascular system in zebrafish (Danio rerio) characterized by power spectrum and classical signal analysis. *J Exp Biol* 209: 1093–1100, 2006. doi:10.1242/jeb.02117.
22. **Steele SL**, **Yang X**, **Debiais-Thibaud M**, **Schwerte T**, **Pelster B**, **Ekker M**, **Tiberi M**, **Perry SF.** In vivo and in vitro assessment of cardiac β-adrenergic receptors in larval zebrafish (Danio rerio). *J Exp Biol* 214: 1445–1457, 2011. doi:10.1242/jeb.052803.
23. **Hsieh DJ**, **Liao CF.** Zebrafish M2 muscarinic acetylcholine receptor: cloning, pharmacological characterization, expression patterns and roles in embryonic bradycardia. *Br J Pharmacol* 137: 782–792, 2002. doi:10.1038/sj.bjp.0704930.
24. **Lister JA**, **Robertson CP**, **Lepage T**, **Johnson SL**, **Raible DW.** *nacre* encodes a zebrafish microphthalmia-related protein that regulates neural-crest-derived pigment cell fate. *Development* 126: 3757–3767, 1999. doi:10.1242/dev.126.17.3757.
25. **Mann KD**, **Hoyt C**, **Feldman S**, **Blunt L**, **Raymond A**, **Page-McCaw PS.** Cardiac response to startle stimuli in larval zebrafish: sympathetic and parasympathetic components. *Am J Physiol Regul Integr Comp Physiol* 298: R1288–R1297, 2010. doi:10.1152/ajpregu.00302.2009.
26. **Westerfield M.** *The Zebrafish Book: a Guide for the Laboratory Use of Zebrafish (Danio rerio)* (5th ed.). University of Oregon Press, 2007.
27. **Avdesh A**, **Chen M**, **Martin-Iverson MT**, **Mondal A**, **Ong D**, **Rainey-Smith S**, **Taddei K**, **Lardelli M**, **Groth DM**, **Verdile G**, **Martins RN.** Regular care and maintenance of a zebrafish (Danio rerio) laboratory: an introduction. *J Vis Exp* 69: e4196, 2012. doi:10.3791/4196.
28. **Kimmel CB**, **Ballard WW**, **Kimmel SR**, **Ullmann B**, **Schilling TF.** Stages of embryonic development of the zebrafish. *Dev Dyn* 203: 253–310, 1995. doi:10.1002/aja.1002030302.
29. **Saint-Amant L**, **Drapeau P.** Time course of the development of motor behaviors in the zebrafish embryo. *J Neurobiol* 37: 622–632, 1998. doi:10.1002/(sici)1097-4695(199812)37:4<622::aid-neu10>3.0.co;2-s.
30. **Fitzgerald JA**, **Kirla KT**, **Zinner CP**, **Vom Berg CM.** Emergence of consistent intra-individual locomotor patterns during zebrafish development. *Sci Rep* 9: 13647, 2019. doi:10.1038/s41598-019-49614-y.
31. **Liang JO**, **Ahmed S**, **Akusoba C**, **Amacher S**, **Bell A**, **Brick J**, **et al.** Zebrafish in the classroom (Online). 2007. http://www.zfic.org [2025 Apr 24].
32. **Salgado-Almario J**, **Vicente M**, **Molina Y**, **Martinez-Sielva A**, **Vincent P**, **Domingo B**, **Llopis J.** Simultaneous imaging of calcium and contraction in the beating heart of zebrafish larvae. *Theranostics* 12: 1012–1029, 2022. doi:10.7150/thno.64734.
33. **Dustan HP.** Calcium channel blockers. Potential medical benefits and side effects. *Hypertension*, 13: I137–I140, 1989. doi:10.1161/01.hyp.13.5_suppl.i137.