ARTICLE
Using Zebrafish Embryos to Study Pharmacological Effects on Neural Development in Hands-On Neurobiology Laboratory Activities

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Undergraduate neurobiology courses cover neural development as a major theme but there are few labs to provide hands-on experience with these topics. Here we share a 3-week set of lab activities using zebrafish embryos that allow students to see the direct effect of drug exposure on physical and emotional development. In these labs, student expose new embryos (Lab 1) to the environmental toxin lithium chloride, which inhibits anterior development and produces an eyeless phenotype in fixed larvae (Lab 2), and to psychiatric medications fluoxetine and quetiapine, which alter anxiety-like behavior measured live in grown juveniles (Lab 3). Lab worksheets ask students to investigate the signaling pathways affected by these drugs and how they might affect neural development in different ways. Student opinion surveys suggest these lab activities were successful in both providing hands-on work with zebrafish as a model organism for neural development and better understanding of how drugs can impact development of the nervous system.

Keywords: zebrafish; embryos; toxicology; neural development; anxiety; undergraduate neurobiology lab

Neural development is a key concept in Neurobiology courses, but is often difficult for students to grasp well. Topics such as neural induction, neural tube and plate formation, regional patterning of the nervous system, axon growth and guidance, and synapse formation are interesting but vast. Although these topics are helped by discussion of real-world maladies like spina bifida and holoproso-encephaly, it is difficult when these topics are frequently abstract for students. What can be helpful are accompanying laboratory activities that aid in the learning and application of class content.

The most common hands-on and virtual lab activities for foundational concepts of neurobiology are in topics such as electrical signaling across membranes, chemical signaling at synapses, and neural circuits and behavior. By contrast, there are relatively few widely known and publicized lab activities for topics related to neural development. Some examples include using *Drosophila* to focus on genetic influences on motor neuron development (Rothhaas et al., 2020) and cell cultures to study neurite growth (Pemberton et al., 2018). Other protocols utilize chick embryos, but are focused more on using those embryos to generate cell lines for cell biology labs (Haskew-Layton and Minkler, 2020). To meet the need for labs specific to neural development, we set out to design novel neurobiology lab activities utilizing zebrafish embryos to study processes directly connected to topics in neural development to pair with a Neurobiology course.

The Neurobiology course at Belmont University (NEU 4500) is a junior and senior level course that is a requirement for neuroscience majors and minors and elective offering for biology and biochemistry and molecular biology majors. Prior to taking Neurobiology, students are required to either take Principles of Neuroscience, Human Anatomy & Physiology I, or General Physiology. This course is capped at 24 students with a typical enrollment of approximately 20 students.

Zebrafish are a common model organism for the study of embryology due to their low cost, easy and prolific breeding, and wide array of molecular, genetic, and pharmacological techniques to study them (Meyers, 2018). Their large clutches of embryos develop externally and are optically clear (Bradbury, 2004). External development also allows for manipulation of the central nervous system (CNS) during development (Schmidt et al., 2013). They develop rapidly, reaching adulthood by 10-12 weeks, which allows for observation of embryonic manipulations on larval, juvenile, and adult stages relatively quickly (Kimmel et al., 1995). These vertebrate model organisms are well suited to provide a hands-on experience for undergraduate courses.

One well-known toxicological effect in zebrafish embryos involves lithium poisoning. Even with brief exposure to lithium chloride (LiCl), zebrafish hatch and develop into larvae without eyes (Van De Water et al., 2001). This eyeless phenotype is clear and easily recognized by the naked eye, translating well to an undergraduate classroom. In addition, lithium causes this eyeless phenotype (and other, less visible physical changes) through its ectopic activation of the signaling molecule, Wnt (Van De Water et al., 2001). As Wnt is involved in regional patterning of the nervous system during neural development, the eyeless phenotype directly relates to how expression of local chemical signals biases formation of the neural tube and later structures, allowing for direct translation of a lab activity to course content.

Students are also highly interested in long-term effects on behavior due to prenatal drug exposure, and in our experience neuroscience students are particularly interested in psychiatric medications. Although the prenatal effects of many drugs are well characterized in rodent

species (Thompson et al., 2009), pharmacological studies in zebrafish typically use adults (Stewart et al., 2011), and embryonic studies are more concerned with screening for possible toxic effects of these drugs (Caballero and Candiracci, 2018). Therefore, we decided to create a novel lab activity to expose zebrafish embryos to psychiatric medications, then study anxiety-like behavior *in vivo* when the exposed embryos reached a juvenile stage of development. We chose fluoxetine, a selective serotonin reuptake inhibitor (SSRI) and common antidepressant, and quetiapine, an atypical antipsychotic medication, because they are easily acquired and widely studied for their effects on affect and mood in a variety of animal models.

Over the course of two distinct classes of Neurobiology, we piloted three weeks of laboratory activities, where students 1) expose newly-generated zebrafish embryos to lithium, fluoxetine, and quetiapine, 2) analyze physical phenotypes in fixed larvae zebrafish from lithium-exposed embryos, and 3) analyze live anxiety-like behavior in juvenile zebrafish from fluoxetine- and quetiapine-exposed embryos. Timing of labs and lab worksheets were planned to correspond with topics of neural development to help students directly apply the class concepts with their experimental results and student feedback was collected to assess the success of the learning outcomes for students.

Student Learning Objectives (SLO)

In the development of these lab activities, the following student learning outcomes were desired:

1. To learn about signaling pathways involved in normal neural development
2. To learn how exposure to pharmaceutical drugs can affect embryonic development
3. To gain hands-on experience in working with zebrafish as a model organism for neural development

MATERIALS AND METHODS

Zebrafish Housing and Generation of Embryos

Zebrafish were maintained as previously described (Westerfield, 2000). Specifically, zebrafish adults were maintained on an Aquatic Habitats system with the following parameters: recirculating water with a pH between 6.9-7.4, conductivity (salinity) between 500-900 micro Siemens (μS), water temperature between 27.5-29°C, a 12-hour light/dark cycle and room temperature of 26°C, all of which were monitored daily. The water was exchanged with fresh reverse osmosis (RO) water with salt added (500-900μS) at a rate of 4% of the system capacity daily. Nitrates and nitrites were monitored monthly to be between 0-2ppm and 0-100ppm, respectively, and ammonia at 0ppm.

Embryos were obtained by timed matings after adults were placed in breeding tanks overnight. Tübingen long fin (TL) and AB wild-type fish strains were bred for lithium and fluoxetine/quetiapine experiments. Embryos exposed to lithium were housed in 100mm petri dishes (Grenier) with embryo water (E3: 5mM NaCl, 0.17mM KCl, 0.33mM $CaCl_2$, 0.33mM $MgSO_4$ and 10^{-5} % Methylene Blue; Westerfield, 2000) in an incubator at 28.5°C and fixed at 3 days post fertilization (dpf). Embryos exposed to fluoxetine or quetiapine were collected in 100mm petri dishes with embryo water (E3) and housed in an incubator at 28.5°C until they were 7dpf. They were then grouped by treatment into adult Aquatic Habitat tanks and raised until 7 weeks old to study live behavior in lab.

Embryo Handling for Treatment

Zebrafish embryos were spit into equal groups of embryos among petri dishes and transferred to 12-well dishes using a Bel-Air Pipette Pump 10ml Pipetter (Fisher Scientific) using borosilicate glass 5 3/4' drop pasteur pipette (Fisher Scientific).

Experimental Design

For both semesters of Neurobiology that we piloted this lab, the lab section met on Friday afternoons. The morning of Lab 1, zebrafish embryos were generated in the breeding colony and divided up into separate petri dishes filled with embryo water to allow students to work in groups of 3-4. The overall goal was to have ~10 embryos for each group per experimental condition.

In Lab 1 students incubated embryos in control or drug solutions used for Lab 2 (LiCl) and Lab 3 (quetiapine and fluoxetine). Over the weekend, the embryos hatched from their chorion and those for Lab 2 were fixed in 4% paraformaldehyde (EMS, Fisher Scientific, 32% diluted to 4% in 0.1M PBS, MilliporeSigma) at 2dpf to preserve them for as long as needed before Lab 2, where students analyzed fixed zebrafish larvae for developmental physical deformities. The remaining incubated embryos for Lab 3 were allowed to hatch and grow until they reached a juvenile stage of development (Singleman and Holtzman, 2014). Then, these juvenile zebrafish were analyzed for anxiety-like swimming behavior live during Lab 3.

Discussion of neural development in our Neurobiology lecture occurs right after the midway part of the semester so the timing of these labs was planned to fit alongside these discussions. For the first piloted, Spring 2021 semester, only Labs 1 and 2 were conducted. For the second piloted, Fall 2021 semester, we added Lab 3 and planned for zebrafish to grow for 7 weeks to reach a juvenile stage. For that semester, Lab 1 occurred early and fixed larvae for Lab 2 were saved until later in the semester. The ultimate goal was to time Labs 2 and 3 to correspond with early brain development discussion in lecture to allow for the best translation of lab activities to class content.

Lab Session 1: Embryo Treatment

In Lab 1, students become familiar with working with zebrafish embryos, then are asked to expose zebrafish embryos to various wash and drug solutions through serial incubations.

Lab 1 Preparation.

After zebrafish embryo generation, ~60 viable embryos are transferred into each petri dish containing E3, with enough petri dishes made for the number of student groups. In each of our two semesters, we had 5 student groups, so this meant a total of ~300 embryos generated and set aside for these labs.

In addition to embryo generation, each lab station should

be outfitted with a dissecting microscope, one additional petri dish, one 10mL thumbwheel pipette pump, six glass Pasteur pipettes, one 1000μL micropipetter (Fisher Scientific) with corresponding tips (Fisher Scientific), two 12-well plates, and 40mL of E3 in a conical tube. Lastly, pre-prepared drug concentrations are required as well. Although we made drug concentrations before lab, students may be asked to do so as well, if desired. We made 15mL each of 200mM and 400mM LiCl (molecular weight 42.3 g/mol; MilliporeSigma), 50μM quetiapine fumarate (molecular weight 441.5g/mol; MilliporeSigma), and 250nM fluoxetine hydrochloride (molecular weight 345.8 g/mol; Millipore Sigma) and set these communal concentrations at the front of the lab room for shared use. LiCl concentrations to generate the eyeless phenotype are already known (Robertson et al., 2014), and we chose doses for quetiapine and fluoxetine that have been previously found to be non-toxic and produce no physical deformities (Lee et al., 2013; de Farias et al., 2019).

Lab 1 Student Protocol.
Working in groups of 3-4, students followed instructions on the Lab 1 worksheet (Supplementary Material 1). In short, students practiced transferring zebrafish embryos using thumbwheel pipette pumps before serially incubating groups of embryos in both control and drug solutions.

Because the first lab was a setup lab for future analysis of drug treatments, the questions students needed to answer on the Lab 1 worksheet focused on developing hypotheses for these experiments. This involved walking students through how to research literature on embryonic zebrafish drug exposure in order to formulate specific hypotheses for both physical defects in larvae and behavioral phenotypes in juveniles.

Lab Session 2: Lithium Effects on Eye Development
In Lab 2, students take fixed larvae and analyze their physical phenotypes for any potential developmental deformities resulting from embryonic lithium exposure. The 400mM lithium-exposed embryos should have obvious loss of eyes to compare to the 200mM and 0mM LiCl control groups.

Lab 2 Preparation.
After Lab 1, embryo petri dishes were placed on ice to anesthetize the embryos and they were transferred to a microcentrifuge tube. The E3 water was replaced with 4% PFA and the microcentrifuge tubes were placed at 4°C overnight, and then washed and stored in PBT (PBS with 0.1% Tween 20). Each lab station is outfitted with the student group's fixed larvae, one dissecting microscope and petri dish for each student, a 10mL thumbwheel pipette pump and glass Pasteur pipette, and 20mL of E3 in a conical tube.

Lab 2 Student Protocol.
Working in their original groups from Lab 1, but at individual dissecting microscopes, students followed the directions on their Lab 2 worksheet (Supplementary Material 2). In brief, students characterized the physical phenotypes of the fixed larvae, then dug into the mechanism behind the lithium eyeless phenotype. Students learned about Wnt signaling in the developing nervous system, its interaction with other signaling molecules like Frizzled, GSK-3β, and β-catenin, and how lithium affects these signaling pathways through a guide article (Van De Water et al., 2001). Then, students looked up information related to how Wnt is important for posterior development in order to draw conclusions on why lithium prevents the eyes (and other unseen neuronal structures) from forming during development. In all, these questions are designed to help students connect class material on regional patterning of the developing nervous system to the hands-on effects seen in their zebrafish.

Lab Session 3: Psychiatric Medication Effects on Juvenile Anxiety-like Behavior
In Lab 3, students analyze the live swimming behavior of juvenile fish they previously incubated in quetiapine or fluoxetine for anxiety-like behavior. Unlike the eyeless phenotype, there was no previous research on juvenile anxiety-like behavior following embryonic exposure to psychiatric medications, so there was no stereotypical behavior expected.

Lab 3 Preparation.
After Lab 1, embryos were raised on an Aquatic Habitat zebrafish system until 7 weeks old. Larvae were transferred from the adult tank to a breeding tank for behavioral analysis. Each lab station is given a standard zebrafish breeding tank (Aquatic Habitats breeding tank, 8"x 3.5"x 4") filled with water and with thin lab tape dividing the tank into four equal quadrants (Figure 1). With labroom lights on full, each tank is placed on one of the black-topped lab tables. One half of the tank is made darker because of the dark table surface underneath and a black plastic lid covering the top, occluding light. The other half of the tank is made lighter because of no lid and a white laminated sheet of paper underneath to maximize light. In addition to a dark and light side, the tank is divided in half from top to bottom. In general, anxious behavior is operationally defined as swimming in the darker and deeper sections of the water tank during experimentation. Other than the tank setup, students were provided with two large beakers filled with E3 water to serve as before and after housing tanks and one 3" quick-net aquarium fish net per group (Penn-Plax).

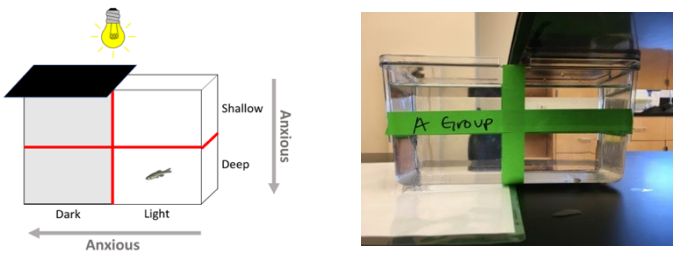

Figure 1. Water Tank Configuration for Anxiety-Like Behavior. *Left)* Schematic of how the tank is split between light and dark and deep and shallow swimming, with anxiety-like behavior as swimming towards the dark, deep quadrant. *Right)* Example of actual setup, arrow pointing to juvenile zebrafish.

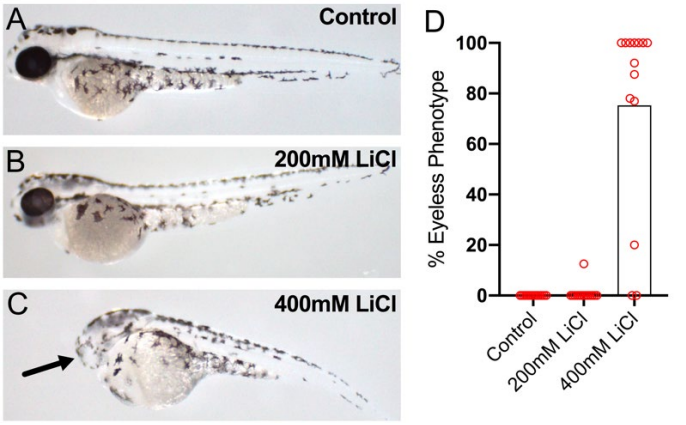

Figure 2. Sample Results from Lab 2. *A-C)* Representative pictures of zebrafish larvae following embryonic incubation in *A)* 0mM LiCl control solution, *B)* 200mM lithium chloride (LiCl), or *C)* 400mM LiCl. Arrow pointing to lack of eye. *D)* Embryos incubated in 400mM LiCl, but not 200mM LiCl develop the eyeless phenotype. Red dots depict individual student group data.

Lab 3 Student Protocol.
Instead of working in their original groups testing their original fish, groups of 3-4 students were randomly assigned to analyze half of the class-wide fish from one of the three experimental conditions (control, quetiapine, fluoxetine), blind. Student groups tested one fish at a time, following the instructions on the Lab 3 worksheet (Supplementary Material 3). Students had to present the instructor with their group's data by the end of lab time. The instructor then gave all students the class-wide data for all three drugs in raw form.

To complete the third and final lab worksheet, students were asked to attempt data analysis and interpretation for the class-wide data as homework. They were to come to the next lab having looked at the data, compiled graphs that they felt represented the class-wide data appropriately, and be ready to discuss with the class. We began the next week's lab period by having a group brainstorming session on how to best understand the "story" of the class-wide data. The results of this brainstorming session are depicted in Figure 3.

Pedagogical Study Design
The assessment of student learning outcomes and student opinions about the lab activities was reviewed by the Belmont University Institutional Review Board (IRB) and approved for exempt status. Student data was collected across two successive semesters of Neurobiology taught by the same professor.

Out of 45 total students (23 in Spring, 22 in Fall), 27 students (*N* = 16 for Spring, *N* = 11 for Fall) elected to complete the anonymous surveys via Qualtrics for a 60% response rate. Students in the Spring semester only completed the first two labs with zebrafish embryos and eyeless larvae. Students in the Fall semester completed the third lab as well, assessing anxiety-like behavior in juvenile zebrafish. In addition to providing their opinions on the success of the student learning outcomes, students rated their opinions of the lab activities in whole using a 5-choice Likert Scale (strongly agree---strongly disagree) for the following statements:

1. These labs helped me understand neural development concepts in lecture better
2. I am interested in doing research using zebrafish
3. The labs were set up to help and not restrict my learning
4. I enjoyed the activities of these labs
5. I would recommend the use of these labs for future semesters
6. The instructions provided were clear
7. These labs helped me feel more competent in searching for research literature and applying it to my research data
8. These labs improved my research abilities including data collection and analysis
9. I understand the practical implications of this research

Lastly, students also answered open-ended questions related to what they learned from the lab activities and what things could be improved for the lab activities.

RESULTS AND DISCUSSION

Lab 2 Results: Lithium Produces Eyeless Phenotype
Across two semesters, students analyzed the percentage of zebrafish larvae that were missing eyes following embryonic incubation in control, 200mM, and 400mM LiCl solutions (Figure 2). The majority of zebrafish embryos exposed to 400mM LiCl developed the eyeless phenotype, while most embryos exposed to 200mM LiCl did not (Figure 2D). A few student groups obtained very low prevalence of the eyeless phenotype in the 400mM LiCl group, however these were all in the first piloted, Spring 2021 semester. After some troubleshooting, we believe this was due to excessive dilution of the drug wells during embryo transfer. In the second piloted Fall 2021 semester, we changed the protocol to pipette drug directly onto the embryos, and all groups got near-unanimous eyeless findings.

Studies in amphibian embryos show that Wnt signals induce development of the posterior sections of the nervous system. Thus, ectopic activation of Wnt promotes posteriorization and inhibits anterior development, particularly of the head (Kim et al., 2000). Lithium enhances Wnt signaling by mimicking its actions as a Gsk3β inhibitor (Hedgepeth et al., 1997), so lithium exposure should act as a widespread agonist of Wnt signaling pathways and posteriorization. This explains the lack of eyes and probable lack of other anterior structures that are more difficult to see. We don't have student dissecting microscopes with camera attachments so we cannot ask students to do more quantitative measurements in the fixed larvae. Previous research, however, shows shrunken heads in lithium-exposed embryos (Van De Water et al., 2001), so taking pictures and measuring head area using software like ImageJ or Fiji is certainly possible with other setups.

Other than the eyeless phenotype, there were other physical changes that students reported with the drug treatment groups. The majority of these were paler skin, suggestive of reduced melanocyte expression, and inward curvature of the tails, suggesting underdevelopment of the ventral axis of the spinal cord. These changes were

sporadic and inconsistent across student groups, but consistent with previously reported effects on skin pigmentation (Jin and Thibaudeau, 1999) and tail curvature (Siebel et al., 2014). For any non-eyeless effect, students were still asked to research how the Wnt pathway may be involved in these effects.

It should be mentioned that in the first piloted semester, students also exposed embryos to a higher dose of quetiapine to look for physical defects alongside lithium. Most quetiapine-treated embryos did not produce defects and those that did showed a wide variety, without consistency, in what changes were produced. Although we deleted this from the lab procedures once we added the third lab, instructors are encouraged to try any drug of interest in their own lab and use it as an opportunity for students to study unknown findings.

Lab 3 Results: Embryonic Fluoxetine and Quetiapine Affect Juvenile Swimming Behavior

Students analyzed the anxiety-like behavior of juvenile fish while swimming at baseline and during a brief net exposure. Although students gathered data on both swimming entries and time spent in each quadrant of the test tank, both measurements gave similar results for drug effects (data not shown). Students reported that measuring time in each quadrant live was very difficult with only 3-4 students/test group. Therefore, we have decided to omit collecting time measurements in future semesters and only focus on quadrant entries to measure anxiety-like swimming behavior. Each student group measured swim data from approximately 10 zebrafish from only one treatment group but were given the full class-wide data to attempt data analysis and interpretation for their Lab 3 Worksheet.

At the start of the following lab period, we conducted a group brainstorming session to create the best story to explain the class-wide data. Students first talked with their tablemates, then the instructor facilitated a class-wide discussion on what results and interpretations best explained what the zebrafish did the previous week. Figure 3 contains the result of this group brainstorming session as one possible set of results faculty can expect from this lab. Collectively, we felt pie charts and subsequent bar graphs best visualized how fluoxetine and quetiapine affected swimming behavior at both baseline and during the presence of the net.

During 4 minutes of baseline swimming, all zebrafish primarily entered the bottom quadrants of the tank, with similar entries into both the light and dark halves of the bottom (see pie graphs in Figure 3B). Because light had a minimal effect, we decided to only compare top vs. bottom entries overall at baseline. Our data suggested that embryonic fluoxetine increased bottom entries, suggesting increased anxiety-like behavior at baseline.

Following baseline swimming, anxiety-like behavior was again measured during 30 seconds of net presentation. Here, light did have an effect (see pie graphs in Figure 3C), so we compared light vs. dark entries in the bottom, where zebrafish did the vast majority of their swimming. Compared to baseline, control and fluoxetine-treated zebrafish swam more to the bottom away from the net, with most swimming

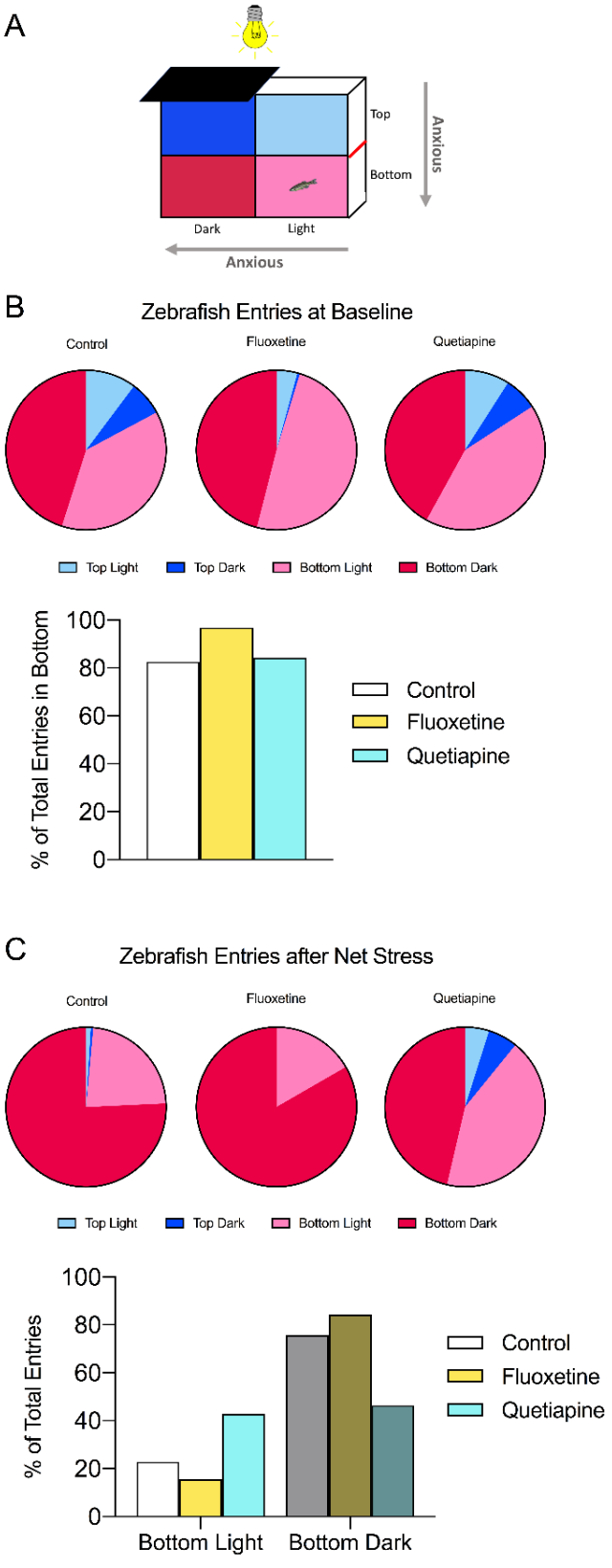

Figure 3. Sample Results from Lab 3. *A)* Test tank schematic. *B)* At baseline, juvenile zebrafish prefer the bottom, independent of light, but embryonic fluoxetine increases this preference. *C)* During net stress, zebrafish swim more into the safest quadrant (bottom dark), however quetiapine zebrafish have an even split between light and dark bottom quadrants.

entries into the bottom dark, or safest quadrant (Figure 3C). This resembles stress-induced swimming changes (Egan et al., 2009), suggesting the net produced a stress response. By contrast, quetiapine-treated zebrafish swam like at baseline, showing a lack of net or stress reactivity.

Going into this lab, we did not know what to expect in terms of student results. Because of this, we thought it would be a good exercise in the Lab 3 Worksheet to have students interact with a lot of data without preconceived notions of what results they "should" be getting, as this is how real science often operates. However, this is also difficult to do and students did struggle to come up with cohesive stories and find research to support their interpretations. We do like that the unknowns baked into this lab allow students the opportunity to come up with potential hypotheses that explain their effects and future studies that could directly test their predictions. This opens up labs to focus on interpretation and explanation of results and proposals for future research; more faculty guidance may benefit students' abilities to feel comfortable doing so. We have brainstormed modifying this lab to be most concerned with data analysis and creating a story of the results. Then, following a class brainstorming session, we plan to ask students to create an interpretation for their results and find research that explains and supports their own discussions for a follow-up worksheet.

We chose these drugs and these behavioral measures because of the drug availability in our departments and our relative expertise in animal behavior. Depending on the chemical inventories for a faculty and their expertise, this lab could be modified in any direction. Different behaviors in various contexts could be piloted and further labs to measure mRNA or protein expression in juvenile zebrafish to relate to their swimming behavior could be implemented to directly test some of the hypotheses that students generate. Overall, we feel that allowing students to be creative and academic in being scientific detectives is important when trying to explain results. Although not having clear answers can be frustrating to students, it teaches the type of critical thinking and creativity that translates well to scientific discovery.

Survey Results

Following completion of all lab worksheets during each semester, students were invited to complete anonymous surveys about how the labs achieved their proposed student learning objectives (SLOs) and student opinions about the lab activities and their usefulness. Student opinions on student learning outcomes were largely positive (Figure 4) and similar across semesters. In both semesters, most students strongly agreed that the labs satisfied the SLOs, with a small minority of students who disagreed with these assessments. Student attitudes were also gauged through a variety of opinion statements students could agree to (Figure 5). Like opinions to the SLOs, student attitudes were largely positive with some negative opinions in both semesters. Again, student opinions were similar despite the additional lab activities for the Fall 2021 semester with the juvenile zebrafish.

Student opinion surveys are helpful in understanding the

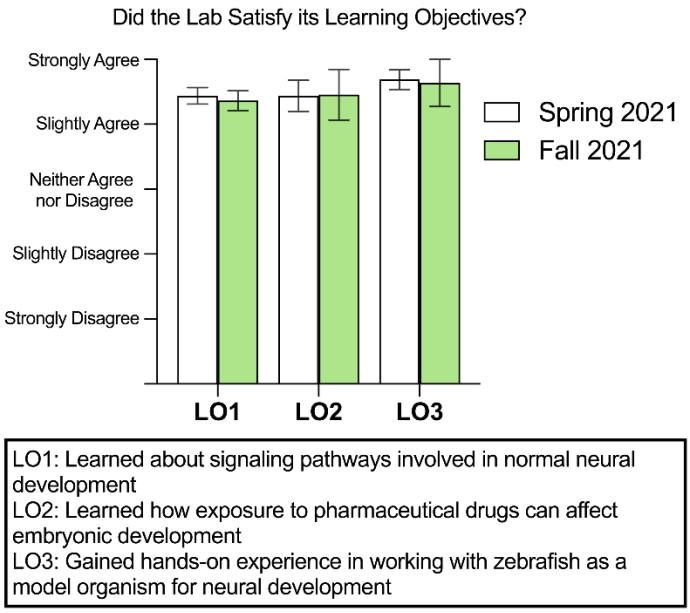

Figure 4. Student attitudes towards learning outcomes (LOs). Students in both semesters agreed that learning outcomes were attained. Error bars represent S.E.M.

student experience with a lab, but do not correlate with actual gained knowledge and learning (Price and Randall, 2008). Lab Worksheets were graded by the instructor primarily for completeness of thought and full attempts at researching ideas and hypotheses, not accuracy. Because of this, scores were uniformly high (~95% in both semesters), and also poor indicators of true student learning. One other mode of formal assessment could come from exam scores. The class material covered by these labs were tested on a Unit Exam on neural development and repair. In the semester directly before we implemented these labs, average scores on that Unit Exam were 74%, while average scores for the same exam were 84% and 86% for the Spring 2021 and Fall 2021 semesters, respectively. Although this shows a gain in performance, the specific questions that these labs associated with were small in comparison to the whole exam, so it's assumed that many other factors than these labs contributed to enhanced student learning. Because of a lack of strong formal assessment, we relied on student opinions to change and better plan the labs for future semesters, as detailed below.

To understand specific areas that students were positive and critical about, we asked open-ended questions about their learning and possible areas for improvement. In response to the question, "What are specific things that you LEARNED through these labs?", the majority of students from the first-piloted Spring 2021 class described learning about the Wnt pathway and its involvement in neural development. For the second-piloted Fall 2021 class, most students described learning how drugs to zebrafish embryos could affect later anxious behavior. Because students in both classes took the survey after their final labs, respectively, it seems their answers were biased by what they most recently did.

This recency bias was present in the second, open

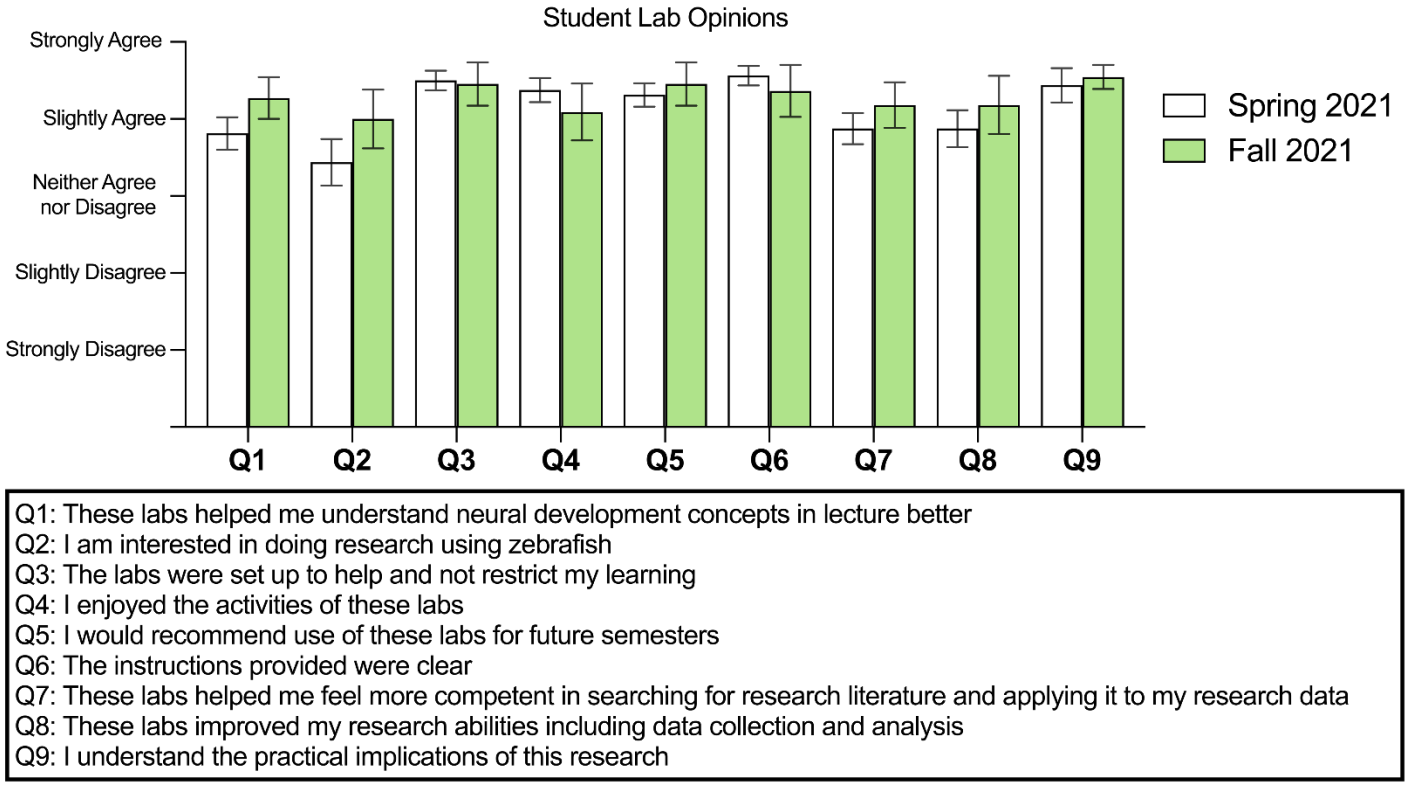

Figure 5. Student Lab Opinions. Students were asked how much they agreed or disagreed on the following attitudes about the lab activities and their usefulness. Error bars represent S.E.M.

ended question, "What are specific things that could be IMPROVED about these labs?" Between the Spring and Fall semesters, we used answers from the Spring 2021 class to improve the labs for the Fall. For example, some students mentioned being frustrated by not getting an eyeless zebrafish larvae, which is why we edited the protocol so more students would get reliable lithium effects. A few students said they wished they could do further experiments with live zebrafish when they were older, which is why we brainstormed on how to do a lab measuring behavior of juvenile zebrafish so students could treat embryos and measure live behavior in the same semester. Responses in the second-piloted Fall 2021 semester similarly are helping us to edit the juvenile anxiety lab to be more successful in the future as well. Multiple students said there were too many measurements for the number of students in each lab group, which is why in the future we are going to focus on students just collecting quadrant entries data, and not quadrant time data as well. One student stated that the lab tape we used was too thick and too hard to see the fish if they were behind it, so we plan to use Sharpie instead to mark thinner lines to divide the quadrants. Overall, we weren't sure how well the anxiety lab would go as it was entirely new to us and areas outside of our relative expertise. One faculty is an expert in zebrafish embryology and one is an expert in rodent behavior, so this lab was both a union of our interests and creativity but also dependent on reading previous research on how to best model anxiety in young zebrafish. Given this, we were pleased with how smoothly the lab went and will continue to monitor the lab moving forward for potential additions or editions, based on student experience and interest.

Conclusions

Overall, the purpose of these labs was to build hands-on activities that would help student learning during the section of the class on neural development. Because the Biology Department currently has a strong zebrafish breeding colony and faculty with extensive experience in embryology, we collaborated using our collective strengths. We think these labs were a large success for the Neurobiology course because informal conversations with students and formal survey responses in both course evaluations broadly and the surveys used for these activities in particular suggested that students believed that these lab activities aided in their course learning. One student summarized the classes' sentiments by stating specifically, "I liked having concrete examples that we could see, not just words in a textbook." This was the design of these labs, to offer hands-on activities that could pair with the self-described most abstract and difficult sections of Neurobiology, neural development, and make learning more accessible. Although the specific gains in learning from these labs are still to be determined, we are encouraged by the excited student response we received.

For an undergraduate environment, the running of these labs with students is easy and straightforward. These labs required no prior student training and all skills can be taught

in the lab periods themselves, making any combination of them transferrable to classes at other institutions. Although the LiCl effects are well-characterized and thus easy to replicate, access to or purchase of any drugs could be used to introduce novel experiments, like we did with embryonic exposure to quetiapine and fluoxetine. For institutions with additional equipment, longer and more extensive discovery-based labs could be designed to allow students to investigate other hypotheses with multiple methods for data collection: behavior, genetic manipulations, histology, in situ hybridization, etc.

While zebrafish are an attractive model organism for students to work with (Fields et al., 2009), we recommend that lab instructors without formal zebrafish training collaborate with a faculty member or scientist who is familiar with zebrafish husbandry for best success. For institutions without a housing colony for zebrafish, this may be a barrier that prevents application of these labs, but there are some possibilities. Breeding store-bought zebrafish without a controlled water environment is difficult and unadvised, however embryos can be purchased from Carolina Biological Supply Company and other lab suppliers. This option might be cost restrictive for departments with limited budgets but is an option for those wanting to do the first two labs with short survival times for embryos. Otherwise, acquiring zebrafish embryos usually requires the use of zebrafish housed in a specific system; collaborating with neighboring zebrafish research labs in the area, if possible, could be a fruitful option. Embryo generation and raising embryos is cheap and relatively easy in these labs, but not all programs will have neighboring facilities or the relationships with them to allow this.

If faculty are able to adapt these labs, it is important to understand the time and effort required to prepare them. Although more detail is given above in Materials and Methods, the approximate time that is needed to generate and collect embryos from a zebrafish system is 1 hour to set up breeding adult pairs in tanks off of the main system, and then 2-3 hours the following day to collect embryos from tanks and organize them into groups in a petri dish for the first lab. Following Lab 1, the care of embryos requires specific conditions that a zebrafish faculty member or scientist will be familiar with so the students can observe the embryos following treatment. Filtering and storing incubated embryos and then fixing them for later analysis requires 2-3 hours over multiple days, depending on how many student groups there are. For embryos treated with drugs that will be raised to juvenile age, they will need to be grown in water tanks with no running water and fed baby zebrafish food (multiple vendors, including Techniplast) starting around 8 dpf. Up until about 1 month of age, growing zebrafish need to be transitioned slowly to a free running water system and grown-up food, requiring daily care. However time-consuming, this care of growing zebrafish embryos is routine for an established zebrafish lab, due to extensive breeding and genetic line creations.

Although we created these labs for a class under 25 students, these activities could be ramped up for larger classes as well. Zebrafish proliferate large numbers of embryos, but even with the numbers we generated, the embryos could be separated into smaller groupings to spread among more student groups with a lab class up to 50 students. More realistically, large neurobiology courses may have multiple, smaller lab sections. In this scenario, the biggest limiting factor would be faculty time with generating embryos the morning of each given lab day. After Lab 1, fixed larvae and grown juveniles could be stored together and divvied out among various lab classes readily, though each lab session would magnify the hours needed for embryo generation on the front end.

On a positive note, we found these lab activities to be encouraging and invigorating as faculty members. Anecdotally, students were excited to work with the embyros, observe the eyeless phenotypes, and learn how to code behaviors of live zebrafish. One of the joys for us was working with students to both offer them a lab experience that mirrored class concepts more closely but also give them a chance to research areas of neuroscience that they find really interesting. We felt that these labs in particular had the most conversation and buzz across our department throughout the semester. We are excited to offer these labs again currently and also are excited to continue to edit, add, and refine these labs to better the student experience. We hope other faculty may be able to do the same in modifying these labs towards the equipment, supplies, faculty expertise, and student interest at their institutions.

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