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An Integrative Brain and Behavior CURE (Course-Based Undergraduate Research Experience) Using Immunohistochemistry in the Fighting Fish *Betta splendens*

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Course-based undergraduate research experiences (CUREs) provide a variety of benefits to student learning outcomes. Here we describe an upper-level semester-long CURE that was implemented in Spring 2024 at Amherst College, a small liberal arts college, as part of the NEUR 313: Social Neuroendocrinology course. In the CURE students conducted behavioral and immunohistochemical assays in the fighting fish *Betta splendens*. Students assessed whether behavioral and neural response differed between fish exposed to social and nonsocial stimuli. The CURE exposed students to a suite of behavioral, wet lab, and data analysis techniques. In addition to completing weekly lab primers, the students' research efforts culminated in a final written paper and oral presentation where students integrated both mechanistic and eco-evolutionary thinking. The CURE was very positively reviewed by the students, and future iterations of the CURE can be easily modified to fit new research topics that further explore biological questions through a neuroethological lens.

Key words: Betta splendens, *CURE, neural activity, behavior, immunohistochemistry*

Undergraduate participation in research facilitates student retention in the sciences (Bauer and Bennett, 2003). At our institution and across the country student interest in hands-on research experiences has rapidly increased (Desai et al., 2008), outpacing faculty capacity for undergraduate research internships. The COVID-19 pandemic robbed undergraduate students of in-person laboratory training experiences, particularly experiences that involved preparation with live animals and tissue handling. These live handling experiences are relevant for students considering a variety of career trajectories, including research, medical, and veterinary career pathways. A promising response to this increased student research interest is to provide students with semester-long course-based undergraduate research experiences (CUREs). A large body of literature has detailed the benefits of CUREs for undergraduate learning and inclusion (Bangera and Brownell, 2014, Coticone and Van Houten, 2020), and specifically have demonstrated that CUREs can provide similar benefits to those of a research internship (Corwin et al., 2015). As recently described in Luth and Juo (2023), semester-long neuroscience CUREs that include primary data collection are relatively rare. Thus, we here describe a semester-long Neuroscience CURE implemented at Amherst College in the spring semester of 2024 titled Neuroscience 313: Social Neuroendocrinology.

This CURE centers around assessing behavior and neural activity in the fighting fish *Betta splendens*. *B. splendens* is "well known generally but little known scientifically" (Monvises et al., 2009; Yue et al., 2022). *Betta* have been bred for aggression (as well as bright ornamentation and physical combat ability) for at least 1,000 years (Portugal, 2023), providing students an intuitive model of selection. *Betta* is a well-suited model for research on social behavior as the species exhibits easily quantifiable and metabolically costly (Castro et al., 2006) male–male territorial combat interactions (Evans, 1985; Portugal, 2023) that result in consistent dominance relationships (Wallen and Wojciechowski-Metzlar, 1985). The hormonal mechanisms of territoriality in *Betta* have been well characterized (Ramos et al., 2021) and manipulated (Clotfelter et al., 2010; Lynn et al., 2007), but much less is known about the neural mechanisms underlying aggression. In fact, a *Betta* atlas of the telencephalon (the most rostral part of the vertebrate forebrain) was only recently published in 2018 (Magalhães Horn and Rasia-Filho, 2018). The telencephalon is remarkably conserved across vertebrates (Goodson, 2005; O'Connell and Hofmann, 2011; O'Connell and Hofmann, 2012) and in fish contains putative homologs of interest to behavioral neuroscientists such as the hippocampus, preoptic area, and nucleus accumbens (O'Connell and Hofmann, 2011; O'Connell and Hofmann, 2012). Lastly *Betta* are logistically easy to obtain and house in the lab, making them one of the more accessible vertebrate models for a CURE.

In the NEUR 313 CURE, students assessed *Betta* behavior in response to social and nonsocial stimuli. The students directly handled the animals, recorded behavioral videos, and analyzed the videos using an event logging software. The students then assessed neural activity by immunohistochemically labeling tissue for phosphorylated ribosomal protein S6 (PS6). PS6 identifies ribosomal proteins that have been phosphorylated in roughly the previous hour, corresponding to an increase in translation

CURE Schedule	
Lab 1: Introduction, Safety & Ethics Training	Lab 7: IHC Tissue Staining
Lab 2: Experimental & Hypothesis Design	Lab 8: Microscope Imaging
Lab 3: Behavior Day 1 & Ethogram	Lab 9: IHC Cell Counting & Analysis
Lab 4: Behavior Day 2 & Euthanasia	Lab 10: Final Lab Report Prep
Lab 5: Practice Sectioning & Video Scoring	Lab 11: Group Oral Presentation Prep
Lab 6: Data Analysis in R	Lab 12: Presentations & Peer Grading

Table 1. Overview of the weekly lab CURE schedule.

CURE Learning Objectives	
Learning Objective	**Method** *(Relevant Primer in Parentheses)*
Reduce Anthropomorphism	read ethograms & primary literature in animal behavior (#4)
Handle Animals	net, transfer, and record fish behavior (#5)
Understand Animal Research Ethics	complete CITI Training, euthanize fish (#5)
Develop Experimental Questions & Hypotheses	search for and summarize primary literature (#3 & 4)
Learn Wet-Lab bench "Best Practices"	conduct IHC with lab coats, gloves, pipetting, proper labeling and proper disposal of waste (#8)
Learn Neuroscience Methods	cryostat (#6), IHC (#8),microscope imaging (#9)
Use Data Analysis Software	CowLog (#6) R Posit Cloud (#7), FIJI (#10)
Incorporate Written Feedback	introduction outline assignment
Contextualize Results	final written report introduction & discussion
Communicate Findings	final group oral presentation
Work Collaboratively	final group oral presentation with peer grading

Table 2. Overview of Course Learning Objectives and method of implementation.

(Magnuson et al., 2012; Butler et al., 2018). Students quantified PS6+ cells in the dorsomedial telencephalon subdivision 3 (Dm3), the teleost putative homolog of the mammalian basolateral amygdala and a region known to be involved in emotional learning in fish (O'Connell and Hofmann, 2011). Students also quantified neural activity inthe ventral part of the ventral telencephalon (Vv), the teleost putative homolog of the mammalian lateral septum and a region known to coordinate reproductive behavior in fish (O'Connell and Hofmann, 2011). Students collaborated in groups to answer previously unexplored novel research questions and presented their research findings in the form of both a final written report and a final oral presentation.

MATERIALS AND METHODS

Course Overview and Learning Objectives

The NEUR 313: Social Neuroendocrinology CURE was conducted over twelve lab sessions with each session lasting three hours led by author KJW and assisted by author GAC. Eighteen students were enrolled in the course and students were split into six teams of three for group work. The organization of the CURE schedule roughly followed the order of a typical neuroethological research project (Table 1). The primary learning objective of the course was for students to learn how to conduct independent laboratory research, including learning how to generate a hypothesis, collect and analyze data, contextualize experimental results in a written publication, and communicate results via an oral presentation (Table 2). Regarding course content, the CURE's neuroethological objective was to promote integrating mechanistic (e.g., understanding immunohistochemistry and neuronal morphology) and ecological (e.g., explaining fitness tradeoffs of territoriality and artificial selection) thinking. Importantly, this CURE also provided students a formative introduction to animal ethics, as students conducted lab and animal safety training and were provided the option to participate in animal euthanasia.

Animal Husbandry

45 male blue adult veiltail *Betta splendens* were purchased from Smithland Pet Supply in Hadley, Massachusetts (when possible, all males should be the same color to avoid potential confounding exposure effects) (Figure 1A). All subjects were housed individually with opaque barriers between the tanks to prevent aggression. The plastic tank dimensions were 6" x 10" x 6" and included a latticed plastic lid. Tanks were filled to a depth of 3" using tap water treated with API Tap Water Conditioner (Stress Coat) and dechlorinated for 24 hours. Tanks were enriched with one SunGrow Catappa Indian Almond Leaf, two Smoothedo-Pets 1" tall plastic plant, and 1 CNZ Aquarium Decor Artificial Plant. Subjects were housed on a 12:12 light cycle and fed once daily with one pellet of Aqueon Color Enhancing *Betta* food. The room was kept at 80°F, with a minimum water temperature of 77°F. As needed, space heaters were used to maintain temperature. Because of the need to maintain a high temperature, a small room is recommended. Because subjects were housed for less than one month, we did not conduct water quality tests. Subjects were first introduced to their home tank over 30 minutes of slow water exchange. Subjects were acclimated to lab conditions for two weeks prior to experimentation.

Required Equipment, Reagents, and Materials

The required large equipment to conduct this CURE was one -20°C Freezer, one 4°C Refrigerator, and a fume hood. Tissue was sectioned on a Thermo Fisher Microm HM 550 cryostat. Smaller standard laboratory equipment required included a water bath, three rockers, one combination stirrer/hotplate (three hot plates are preferred), and a pH meter. To make the solutions at least one pipette set is required (three preferred). The CURE requires at least one fluorescent microscope with a camera. We used three Nikon Eclipse 50i fluorescent microscopes in the teaching space for section localization and a Zeiss Axio Observer housed in a dedicated imaging facility to image the stained tissue. The required reagents and materials are listed in Table 3. Please note that the items listed in Table 3 do not include standard lab supplies such as gloves, Kimwipes, paper towels, or standard fish care supplies.

Behavior and Video Analysis

Each student was assigned two fish (total of 36 fish), and their team of three students (six fish) was assigned a treatment: exposure to a competitor, exposure to a mirror, or exposure to a nonsocial object. Thus, there were twelve fish per treatment. Prior to the start of the behavioral tasks students hypothesized whether their treatment would differ from others in aggression displayed and/or in time spent near the stimulus (see lab primer #3 in the Supplementary Materials). Behavior assays were conducted across two lab sessions. All behavior tasks were conducted in the subject's home tank. In the first session, each fish was exposed to all three treatments. To conduct this first behavioral task, the students moved their fish to an experimental countertop underneath a top-down camera. The lid and enrichment were removed from the tank, and an object (a 0.8" red cube attached to a clear acrylic dowel for easy placement and removal) was placed roughly one inch from the wall on the center of the short side of the tank. Subjects were allowed to freely interact with the cube for six minutes. The cube was then removed and replaced with a mirror (landscape orientation) placed along the short side of the tank for six minutes. For the third and final phase of the task, the mirror was removed and a competitor male stimulus fish (not one of the 36 subject fish) was netted from their home tank and placed in a plastic 2.5" x 5" x 5" container filled with approximately 3" of water. This plastic container had a lip and was hung over the inside edge of the short wall of the experimental tank, allowing the fish to visually interact for six minutes but not exchange olfactory or tactile cues.

It is important to note that the data from this first behavioral session was not analyzed by the class during this iteration of the CURE. Despite not analyzing the first week's behavioral data it is highly recommended that this first behavioral trial day is still conducted because it allows the students to become familiar with the behavioral task setup, basic fish handling, and the necessity of quick timing. Results highlighted here are from the second behavioral week, with the exception of one behavioral repeatability analysis conducted by author EAV after the conclusion of the course. In future iterations of the course, the first behavior session could be modified to further explore repeatability in behavior, include an additional behavioral task, or quantify another variable such as the size or color of the subject fish.

The tasks were conducted in rounds, with three students placing their two fish underneath one camera separated by an opaque barrier. Three cameras were set up; thus six fish were assessed simultaneously. This allowed us to complete the behavioral trials in the three-hour session and each team was assigned a 30-minute time slot. The second behavioral session was similar to the first, but the subject fish was only exposed to one of the three treatments (object, mirror, or competitor). Subjects were exposed for 15 minutes. After this, the fish were placed in a quiet corner of the room behind an opaque barrier for 45 minutes. Note that for subjects assigned to the competitor treatment, the assigned stimulus fish was a different stimulus than the one seen in the prior session. Following the 45 minute waiting period, students euthanized the fish for tissue collection, described below.

In the lab sessions following the behavioral procedures, students were provided a cryostat sectioning demo (Lab 5)

Required Reagents

Item	Supplier	CAT	#
70% ethanol	Fisher Sci	04-355-122	N/A
Sucrose	Fisher Sci	S5-500	480g
Sodium Phosphate Dibasic Anhydrous	Fisher Sci	S374-1	50g
Sodium Phosphate Monobasic Monohydrate	Fisher Sci	S369-500	10g
Hydrochloric Acid (HCL)	Fisher Sci	SA431-500	100mL
Sodium Hydroxide (NaOH)	Thermo Fisher	A16037.36	40g
Sodium Chloride (NaCl)	Fisher Sci	S640-3	97g
Ultrapure Tris	VWR	0497-500g	24g
Paraformaldehyde	Sigma Aldrich	P6148-500G	40g
Sodium Tetraborate Decahydrate	Fisher Sci	S248-500	39g
Normal Donkey Serum	Jackson	017-000-121	16mL
Triton X-100	Fisher Sci	BP 151-100	728uL
PS6 22115 235/236 Rabbit Antibody	Cell Signaling	2211	160uL
Alexa Fluor Red Donkey anti-Rabbit 594	Thermo Fisher	AB_141637	243uL
ProLong Gold Antifade with DAPI	Thermo Fisher	P36931	40uL
TissueTek OCT	VWR	25608-930	1

Required Materials

Item	Quantity
IBI Immunohistochemical Staining Tray	2
Glad Med. Square Storage Containers	6+
Sony HDRCX405 HandyCams	3
DNSbabi Overhead Camera Mount	3
Mr. Pen- Dry Erase Board, 14" x 11"	3
SanDisk Ultra 64GB microSDXC	3
hand2mind Plastic Mirrors, 4" x 6"	6
edxeducation Linking Cubes	6
Acrylic Rods	6
Thermo Sci 30mL Nalgene Bottles	45
Fisherbrand Immersion Thermometer	1
TruBond 380 White Slides	135
ImmEdge Hydrophobic Barrier Pen	1
Transfer Pipettes	135
Clear Laminator Sheets	18
Clear Nail Polish	6

Table 3. Required Reagents & Materials. Quantities listed are calculated for 45 fish, which translates to a class size of 18.

and analyzed their behavioral video data (Lab 5 and 6). This schedule was to allow time for the instructor to section the tissue and to prepare for IHC. During the cryostatdemonstration the instructor first mounted extra rodent brain tissue from a prior experiment. The demonstration could use other, more accessible tissue or even just OCT. Students cryosectioned 3-4 sections, then directly mounted their sections onto a glass slide.

Students were exposed to a host of data quantification, analysis, and presentation software programs during the CURE (Figure 1B). Students scored their behavioral videos twice, once for location (near or far from the stimulus) and once for gill flaring behavior. To do so, students downloaded the event logging software CowLog, which was a software novel to all students in the course (Hänninen and Pastell, 2009). To easily determine the location of the fish, students taped a lamination sheet to their laptop monitors. The lamination sheet had a line drawn on it, and students measured the length of the tank on their laptop screen and positioned this horizontal line at the halfway mark. Students were instructed to count the fish as near or far from the stimulus based on the position of the fish's head. After scoring their subject fish for location, students repeated the scoring to quantify gill flaring. Gill flaring is an easily visible behavioral metric in which individuals extend their operculum perpendicular to their body. All score sheets (CSV files) were named to include relevant information (e.g., fish number, scorer, stimulus type) and uploaded to a shared google Drive. The instructor then combined the individual CSV files into one data spreadsheet using the R package "cowlogdata" (Wallace, 2020). Students analyzed their behavioral data (and cell count data, described below) in R using the Posit Cloud service. Using a cloud server like Posit Cloud allows students to access a controlled virtual environment that has template code, loaded packages, and data frames already provided. See Supplementary Materials for the R Markdown template file provided to students, which includes areas to define their independent and dependent variables and instructions for which statistical test to use. Students were tasked with writing out their question and variables as comments in the R markdown file, identifying their independent and dependent variables, and selecting) the appropriate analysis (e.g., t-test, linear regression based on whether their selected variables were categorical or continuous. To ensure completion, students were required to knit and submit the completed markdown file.

Euthanasia and Tissue Preparation

To assess neural activity, subjects were euthanized 45 minutes after the exposure. Students were asked prior to the lab session if they would like to participate in the euthanasia or if they would prefer to opt out. Subject fish were euthanized via immersion in an ice water bath. During this process, the subject was inside a net and thus did not touch the ice. Following the cessation of opercular movement, subjects were decapitated and the head and the instructor placed the head in 4% paraformaldehyde in 0.1M Borate Buffer for four to eight hours. It is the authors' opinion that exposing students to this aspect of animal research enforced the prior readings about animal ethics and contextualized neuroscience research more broadly.

Subsequent tissue preparation following euthanasia was conducted by the instructor. First, the brain was extracted from the head. The extraction can be difficult and must happen on the same day as the euthanasia, which is challenging, but with practice is achievable. The brain extraction technique was as follows: first extra tissue (the "lips, chin, and neck") was cut off until the brainstem is visible. Then scales on the skull were scraped off and the skull was carefully sheared off the top in a caudal to rostral direction. The brain was carefully scooped out lengthwise, cutting the brainstem as the brain was pulled up. Following extraction, the brain was placed in 30% sucrose in phosphate buffer saline overnight. The following day, the brain was frozen in Tissue Tek OCT until sectioning. Brains were sectioned at 20uM at -23C. Tissue was sectioned directly onto a glass slide in 3 series. Sections were kept towards the middle of the slide (away from edges) because the immunohistochemical protocol requires a hydrophobic barrier to be drawn around the edges of the slide. Note that if the instructor has difficulty sectioning the telencephalon (the most rostral part of the brain containing the regions of

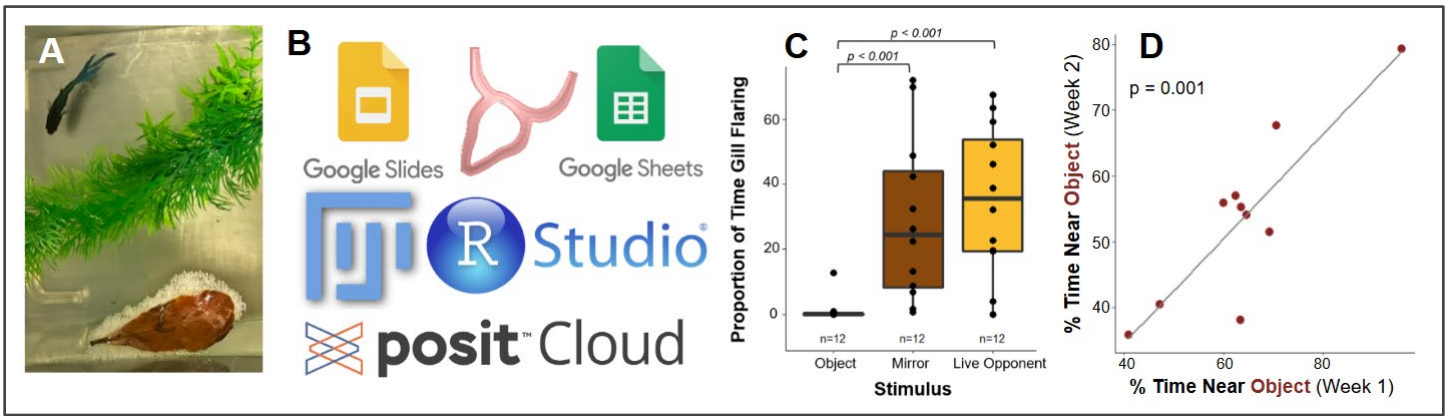

Figure 1. A *Betta splendens* in its home tank. This male has built a "bubble nest" under the floating leaf which suggests suitable conditions *(A)*. Students used a variety of data analysis software and other programs during the CURE *(B)*. When students analyzed behavior they observed a robust difference in aggression across the three exposures (object, mirror, opponent) *(C)*. Further analysis identified repeatable individual variation in behavior, thus we recommend future iterations of the course explore repeatability *(D)*.

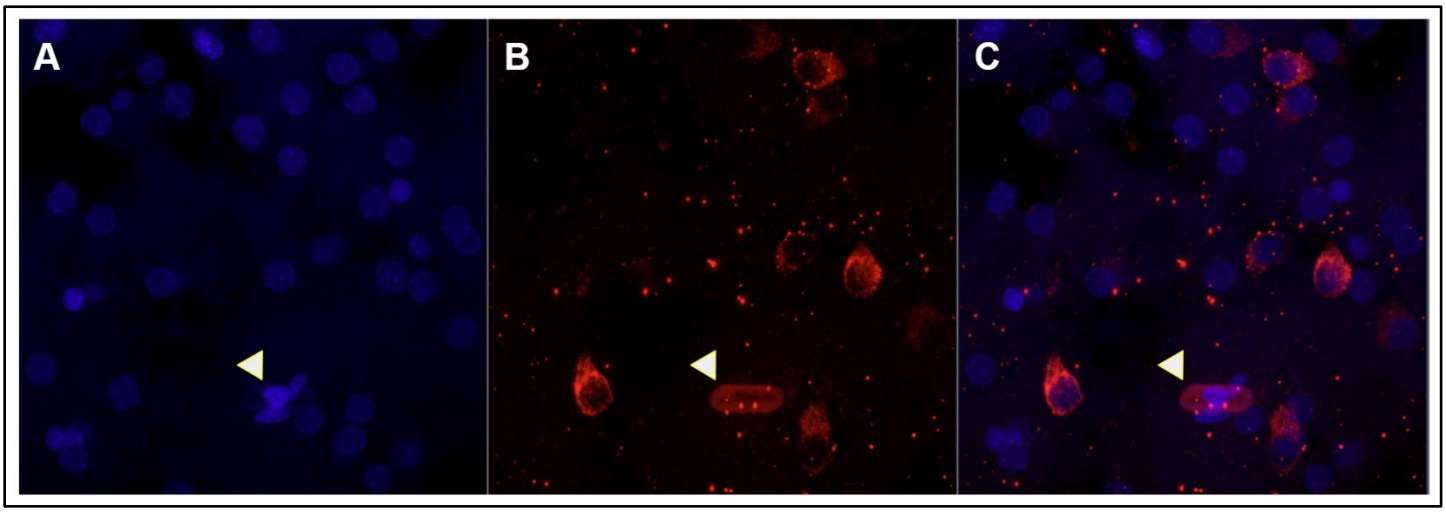

Figure 2. Betta splendens telencephalic tissue immunohistochemically stained with DAPI (blue, *A*) and PS6 (red, *B*) (combined channels in panel *C*). Note the blood cell (white arrow) has a distinct oblong morphology and homogeneous PS6 staining, whereas neurons have a "doughnut" shape and more granular PS6 staining. The PS6 "speckles" in the background can be avoided if the instructor first vortexes and centrifuges the primary antibody then pipettes only the top layer of primary antibody for use.

interest), the brain can be flipped around and sectioned caudal to rostral. Once sectioned, slides were placed on a hotplate at 40°C for 30 minutes. Slides were stored in a -80°C freezer until immunohistochemical staining.

Immunohistochemistry and Cell Counting

Each student was provided two slides of brain tissue, one from each of their assigned subject fish, for IHC. For students whose fish was not properly sectioned, they conducted IHC on reserved tissue from stimulus fish. The primary incubation was conducted by the students in the span of one three-hour lab. The following day, in lieu of lecture, the class met for 45 minutes to conduct the post-primary washes and begin the secondary incubation. The instructor conducted the final wash and coverslipped.

To conduct IHC slides were first thawed then a hydrophobic barrier was drawn around the edges of the slide using an ImmEdge pen (Vector Laboratories). All washes described in the protocol were conducted by applying liquid using a transfer pipette and placing the slide on a rocker set to low.

When the wash was complete, a corner of the slide was gently tipped onto a paper towel so that the liquid ran off. Note that when angling the slide to remove liquid, do not tip the slide more than necessary because the sections may slip. If the tissue does move around, the slide can be set on a hot plate at 30°C for five minutes to help the tissue readhere, but this method should be used sparingly as it can damage the tissue.

The IHC protocol was conducted as follows: Tissue was rinsed five times for five minutes each in 1X Tris Buffered Saline (TBS). Tissue was then washed once for five minutes in 4% paraformaldehyde in 0.1M Borate Buffer. Tissue was then washed twice for five minutes each in 1X TBS. Tissue was transferred into a block (10% Normal Donkey Serum and 0.3% Triton X-100 in TBS) and stored in a humid chamber (a closer Tupperware sandwich container with a wet paper towel) for one hour. Tissue was transferred into the primary antibody (1:500 Rabbit PS6) in diluent (5% Normal Donkey Serum and 0.3% Triton X-100 in TBS) and incubated overnight at 4°C in an IBI Immunohistochemistry Staining Tray with water in the trough to keep the environment humid. The following day, tissue was rinsed twice for 30 minutes each in TBS. Tissue was transferred into the secondary antibody (3:1000 Alexa Fluor Donkey Anti-Rabbit 594) in diluent and stored in a humid chamber at room temperature in the dark for two hours. Tissue was then washed once for 20 minutes in TBS. Lastly, ProLong with DAPI was applied to the slide and the slide was coverslipped. The following day the edges of the slides were sealed with clear nail polish and stored in the dark at room temperature until ready for imaging.

During the following lab session students imaged their slides. Sections were imaged using a Zeiss Axio Observer 7 microscope with a Zeiss Plan-Apochromat 20X/0.75NA objective. DAPI labeled nuclei were imaged using the 385nm LED and the Alexa Fluor 594 (AF594) secondary antibody using the 567nm LED of the Colibri 7 illuminator. Emission was detected at 425/30nm and 592/25nm for DAPI and AF594 respectively, using a Hamamatsu Orca Flash 4.0 camera. Imaging took approximately 20 minutes per group of three students, with a five minute transition window in between each group. Importantly, the medial areas of the brain were prioritized as students were quantifying neural activity in the dorsomedial telencephalon subdivision 3 (Dm3) and the ventral part of the ventral telencephalon (Vv). For visualization purposes, an additional image was taken on a Zeiss 980 confocal microscope at high resolution (Figure 2). Images were exported as CZI files at 3891 x 3891 pixels.

In order to image all students' sections in the span of one three-hour lab, students first viewed their tissue under one of three available Nikon eclipse 50i fluorescent microscopes. Prior to lab students reviewed the *Betta splendens* brain atlas by Magalhães Horn and Rasia-Filho (2018) and were instructed on the appropriate section

Antibody Concentration Pilot Experiment (A)

Sample	PS6	594	4% PFA Wash?	Neurons Visible?
Original	1:500	3:1000	Yes	Yes
High	1:500	1:1000	No	Yes
Low	1:1000	1:1000	No	No
Control	1:500	none	No	No

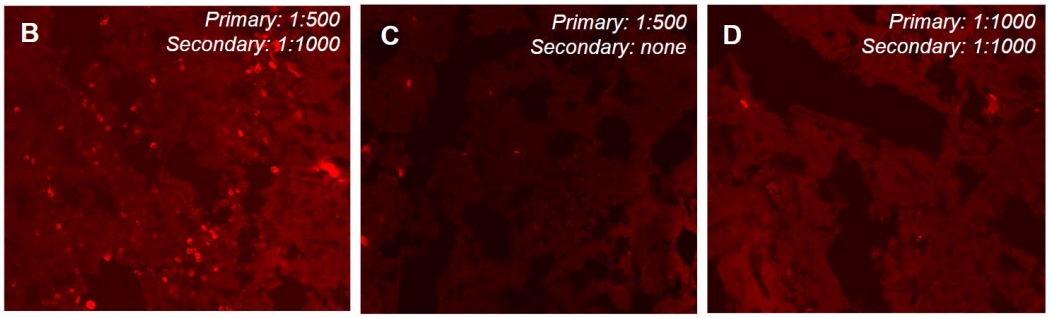

Figure 3. A summary of methods and results of a post-lab pilot can be viewed in *(A)*. A primary PS6 antibody concentration of 1:500 yields noticeable neuronal staining in *Betta splendens* telencephalic tissue *(B)* relative to a control sample *(C)*, but a lower primary concentration of 1:1000 does not yield visible staining *(D)*. The pilot demonstrated the paraformaldehyde wash step can be skipped.

morphology. Using the Nikon eclipse 50i students identified how many sections on their slide were acceptable for imaging in terms of both the quality of the section (e.g., not folded or ripped) and the appropriate morphology. Each student was instructed to image at least three consecutive sections in the correct location for scoring per subject, though some students had more or less than three depending on the quality of the sectioning. Students recorded a general assessment of where on the slide the appropriate sections were (e.g., upper right, first three in the second row), which was immensely useful during imaging.

Students used the free software FIJI (ImageJ) to quantify the number of PS6-labeled neurons on their imaged sections. Firstly, students used the FIJI ROI manager plugin to create a standardized sized ROI for their region of interest. While half the students analyzed Dm3 neuron count data (Dm3 ROI width = 625 pixels and height = 940 pixels) and the other half analyzed Vv neuron count data (Vv ROI width = 357 pixels and height = 548 pixels), all students counted both regions. Note that students counted both the left hemisphere and right hemisphere for each region, resulting in four counts per section. Students placed the ROI on their image while viewing the DAPI channel to avoid bias. Once the ROI was placed, students switched to the 594 channel and used the Point Tool to count the number of neurons in the region. Importantly, students were instructed to exclude blood cells and extra secondary labeling (see associated Lab Primer in the Supplementary Materials). Students uploaded their cell counts to a shared Google spreadsheet, and the instructor later averaged across sections and hemispheres to obtain one Vv and one Dm3 neuron count per subject fish.

RESULTS AND DISCUSSION

First, we briefly highlight behavioral results to inform future iterations of the course. Students observed individual variation as well as obvious differences in behavior by context. The amount of time spent gill flaring towards social vs nonsocial stimuli is a robust statistically significant difference ($p < 0.001$, Figure 1C), as has been previously described (Portugal, 2023; Dupeyron and Wallace, 2023). When comparing repeatability of behavior across the two behavioral sessions, individual variation in time spent near the novel object was consistent ($p = 0.001$, Figure 1D).

The cell counting proved more challenging than originally expected. Even in FIJI in a dark room after image adjustment, it was difficult to distinguish blood vs neurons. The adjustments from the antibody pilot described below may somewhat alleviate this issue in the future, as a lower secondary antibody concentration may facilitate easier counting. Furthermore, students should be given as much time as possible to learn to distinguish blood from neurons from background secondary antibody. In the future, images from prior years' tissue can be used as practice counting to standardize the students' expectations and/or tissue could be double-labeled with a neuron-specific marker. Despite these challenges students successfully counted enough PS6-labeled cells to statistically compare the treatments, but no effect of exposure (object, mirror, competitor) was observed in the brain region quantified, suggesting future iterations should quantify other regions of interest, many of which are visible on the same section of tissue as the Dm3 and Vv. Upon learning these results, the instructor explained that null results are a common part of an authentic research process. The questions the students were exploring in the CURE were not previously explored in the scientific literature, thus null results still provide valuable information.

The behavioral and neuromolecular components of this lab have a great deal of flexibility as to the experimental design, questions, and assessments. In the future students could count different regions or use a label more likely to yield significant differences. Students could easily measure fish size, which often predicts dominance in *Betta* (Dupeyron and Wallace, 2023). Students could compare sexes, color morphs, or record color before and after contests (Portugal, 2023) as color correlates to mate choice and immunity (Clotfelter et al., 2007).

Post-Lab Student Pilot Identified a Safer Protocol

Before describing the graded assignments of the CURE, here we briefly acknowledge an additional methodological detail. Following the conclusion of the lab, two students (DF and SZ) made adjustments to the IHC protocol. There were two goals for this post-lab piloting experiment. The primary goal was to determine if the paraformaldehyde wash step was required for successful labeling. During the CURE the paraformaldehyde wash step was conducted by the instructor in a fume hood for safety reasons, which created a bottleneck that disrupted the flow of the three hour lab.

Secondly, the antibody concentrations were reduced to potentially lower the amount of antibodies needed to be purchased for the CURE. Thus, tissue from the second cryosectioned series was used from three subject fish to conduct the pilot. The IHC protocol was followed as previously described, with changes as follows (Figure 3A): for all three pilot slides the paraformaldehyde wash was not conducted. On the "high pilot" slide, the tissue was incubated in 1:500 primary and 1:1000 secondary. On the "low pilot" slide, the tissue was incubated at 1:1000 primary and 1:1000 secondary. On the "control pilot" slide, the tissue was incubated at 1:500 primary and no secondary. The "high pilot" modifications were successful, as neuronal labeling was still easily visible (Figure 3B) relative to the control pilot that did not undergo a secondary antibody incubation (Figure 3C). Further reducing the primary concentration was unsuccessful, as the "low pilot" did not yield visible neuronal labeling (Figure 3D). Thus in future iterations of the course the instructor will remove the paraformaldehyde step and reduce the secondary antibody concentration.

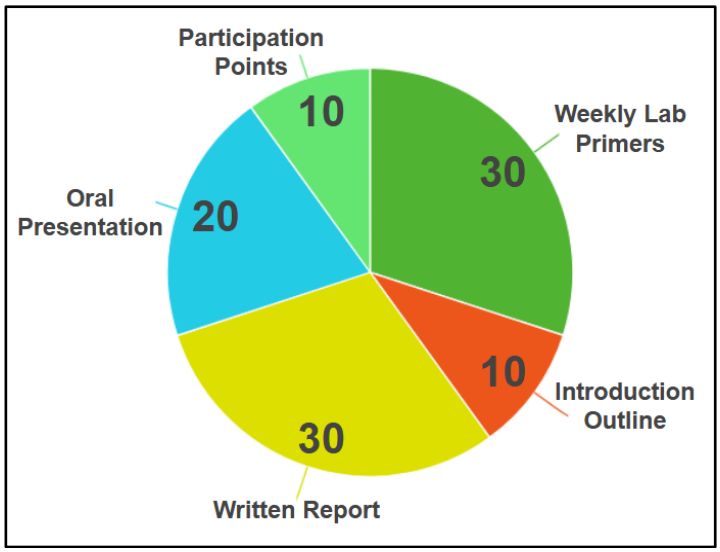

Figure 4. Breakdown of graded assignments for the *Betta* CURE.

Graded Assessments

The students' lab grade consisted of three main components: a series of lab primers that were completed before the respective lab session, an individually written final lab report, and a final group oral presentation (Figure 4). The combination of both written and oral components to the research process is known to facilitate active learning (Coticone and Van Houten, 2020). Students completed a pre-lab assignment before each lab that introduced students to the day's activities. All lab primers are available in the Supplementary Materials. The multiple lab primers throughout the semester provided students the components to build a conceptual framework for their project, which they detailed in the later written assignments.

The detailed instructions for the introduction outline are available in the Supplementary Materials. The instructions emphasized that a scientific introduction should "set the stage" for the experiment by including background, experimental justification, and hypothesis. Importantly, the Introduction Outline required five primary literature article citations. This facilitated student practice in writing in-text citation and a bibliography. The introduction outline was due three weeks before the final written report, and the instructor provided a rubric for the assignment as well as feedback on the introduction outline that was relevant for the final written report. Progressive writing assignments like those implemented here have been demonstrated to increase student critical thinking (Gutilla Reed et al., 2024). Prior literature has demonstrated the benefit of assignment scaffolding but has cautioned that to see positive effects, these structures must be high-quality (Kang et al., 2014). The instructor believes that the assignment scaffolding via instructions, rubrics, and checklists was high quality given students successfully completed the assignment goals.

The capstone assignment of the CURE was a final written report. In this final written report students described their experiment in the format of a scientific publication with an introduction, hypothesis, methods, results, figures, discussion, and bibliography. To provide students an authentic research experience, the instructor explained that the final written report would be graded *like a scientist reviewing a manuscript for publication*. The instructor explained that the grading rubric of the final written report was intentionally less detailed than previous assignments. This was to promote students thinking critically about the content they had been exposed to throughout the semester and independently translate it to a novel assignment. The specific language of this instruction and the grading rubric is available in the Supplementary Materials. Students were encouraged to review their primary literature articles for guidance on how to write their own reports.

Lastly, on the final lab day students presented their findings via PowerPoint in their assigned groups of three students. The content of the final presentation was an abbreviated version of the written report: background, hypothesis, results, discussion. Originally the final oral presentation was scheduled to have one day of in-class preparation time for students to coordinate for their oral presentation the following week. But the instructor decided that students needed more time on their final written report thus the lab on Week 11 was used as additional writing time instead of group oral presentation prep time. Therefore students prepared and presented their final oral presentations all in the same three-hour lab session. While unintentional, this "rapid presentation prep" activity was a highly useful experience in group work. When given only two hours to prepare a presentation (6 presentations in the final hour, 8 minutes each), the students quickly "divided and conquered" the sections of the final presentation and communicated their progress and needs to each other throughout the two hours. This shortened timescale did not negatively affect students' ability to put together a comprehensive and professional presentation. The student presentations were creative, thoughtful, and engaging. This final oral presentation was partially peer graded. Students were given a peer grade sheet (see Supplementary Materials) where they graded in three categories:

context (did the presentation logically flow?)
style (were the slides and figures clear?)
excitement (did the group tell a compelling story?)

This final presentation on the last day of class served as a way for students to highlight all the work they had completed throughout the semester to achieve their research objectives.

Student Experiences

The CURE structure exposed students to a variety of techniques that students found challenging, engaging, and valuable. Based on informal communications with the instructor, students felt that conducting one long-term project over the course of the semester facilitated high engagement and motivation in the course, reflecting trends seen in other CUREs (Wiseman et al., 2020). In addition to live animal handling and wet lab techniques built into the course learning objectives, students learned the important skill of distinguishing local versus cloud storage practices by collecting shared data and importing data into their own analysis environment.

The most challenging assignment for the students was the Introduction Outline. It is the instructor's opinion that the use of the word “outline” (and the instruction that bullet points were sufficient) undermined the depth of conceptual thinking the students were tasked to demonstrate in the assignment. Students tended to simply list vaguely relevant dwgoarticles rather than explain their logical argument and how the literature supported the argument. While this assignment was difficult for students, it provided an excellent opportunity for feedback that was successfully implemented in the final written report and oral presentation. Following the introduction outline feedback from the instructor the students able to independently synthesize primary literature articles to support their claims and interpret their results instead of just “data dump”.

Notably, students asked their own experimental questions within a set larger framework, which led to excitement and curiosity that was evident in the final oral presentation. For example, some students chose to assess whether the presence of “bubble nests”, a male reproductive behavior in this species (as *Betta splendens* exhibit paternal care) influenced behavior and neural activity either across treatments or variation with a treatment. During final presentations and lab reports students presented background literature on bubble nests, leading to a useful integration of neurobiological and evolutionary thinking (Wallace and Hofmann, 2021). Students asked questions to their peers during the Q&A portion of the oral presentations, and the instructor did not need to intervene in the process, which was rewarding for all involved.

Overall, student evaluations of the lab were highly positive. Students remarked that the CURE felt like a genuine research experience that taught the entire scientific process, thus reflecting the intended learning objectives. Their responses in course evaluations indicated that the CURE did indeed fulfill a similar role to that of a lab research internship. The instructor plans for future iterations of the course to directly assess student learning and attitudes towards the course via surveys. Taken together, this CURE is an excellent research experience to inspire and train the next generation of neuroscientists.

Ethical Statement and Contributions

All procedures were approved by the Institutional Animal Care and Use Committee of Amherst College (Protocol 2024-1). The IACUC oversees compliance with the Guide for the Care and Use of Laboratory Animals, Eighth Edition, published by the National Research Council of the National Academies (National Research Council, 2011). Amherst College is an OLAW-accredited institution (Office of Laboratory Animal Welfare D16-00534 A3925-01). KJW designed and conducted the laboratory course as principal investigator. GAC provided assistance in the laboratory course, including experimental design, as a teaching assistant. ODF and JZ collected data for the antibody concentration pilot experiment. EAAV collected and analyzed additional behavioral data. R data analysis code template is available at https://github.com/kellyjwallace/JUNE-Betta-CURE-2024 or by request to author KW.

Supplementary Materials Available

Betta CURE Lab Primers
Betta CURE Intro Outline & Written Report Instructions
Betta CURE Final Oral Presentation Peer Grading Sheet
Betta CURE Data Analysis Template R Markdown

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