

Submission ID #: 71120

Scriptwriter Name: Poornima G

Project Page Link: <https://review.jove.com/account/file-uploader?src=21691648>

Title: Preparation of Conspecific Alarm Cue from Fathead Minnows

Authors and Affiliations:

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Author Questionnaire

- 1. Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**
- 2. Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**
- 3. Filming location:** Will the filming need to take place in multiple locations? **Yes**
If **Yes**, how far apart are the locations? **Two floors**
- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 24

Number of Shots: 46

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**

Answers are slightly edited in accordance with the journal guidelines

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Jessica Ward:** Our research focuses on understanding how oviparous aquatic embryos gain information from the environment.

- 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Jessica Ward:** Lots of fish have an innate response to the conspecific alarm cues, but the composition of these cues is mostly unknown. This limits our ability to quantify and standardize cue concentrations.

- 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Jewel Johnson:** This protocol standardizes the preparation of alarm cues for studies of predator recognition, risk assessment, learning, communication, and antipredator behavior.

- 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Karly Steinberg**: One potential challenge in generating consistent cues stems from biological variability that exists among donor fish, despite standardizing sex, size, and laboratory conditions.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Abby Yake**: Future studies can identify active compounds and examine variation in response across species, environments, and developmental stages.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

This research has been approved by the Institutional Animal Care and Use Committee (IACUC) at Ball State University

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

2. Dissection and Isolation of Epithelial Torso from Fathead Minnows

Demonstrator: Karly Steinberg

2.1. To begin, place the euthanized adult female fathead minnow on a sterile net [1] and rinse the fish three times with deionized or distilled water [2].

2.1.1. WIDE: Talent placing the euthanised fish on a net. **Videographer:** Please obtain CU shot as well for this shot.

TXT: Euthanasia: Immersion in ≥ 250 mg/L MS-222 buffered with NaHCO_3 to neutral pH

2.1.2. Talent adding Di water to rinse the fish.

2.2. Transfer the rinsed fish to a clean, disinfected dissection surface [1]. Using a sterile scalpel, sever the head by making a deep, approximately 0.5-centimeter to 1-centimeter transverse incision at the location of the opercular flap [2]. Then, sever the tail fin by making a deep 1-centimeter transverse incision at the caudal peduncle [3]. Remove the head and tail fin and discard them in a biohazard waste container according to the approved institutional protocols [4].

2.2.1. Talent placing a rinsed fish on a clean, disinfected dissection surface.

2.2.2. Talent using a sterile scalpel to make a transverse incision at the opercular flap and sever the head.

2.2.3. Talent using the sterile scalpel to make a transverse incision at the caudal peduncle and sever the tail fin.

2.2.4. Talent removing the head and tail fin and placing the tissues in a biohazard waste container.

2.3. Using digital calipers with a resolution of 0.01 millimeters, measure the length of the remaining torso from the posterior edge of the operculum to the caudal peduncle [1-TXT].

2.3.1. Talent measuring the remaining fish torso from the posterior edge of the operculum to the caudal peduncle with digital calipers. **TXT: Ideal torso length: $3.75 \text{ cm} \pm 0.5 \text{ cm}$**

- 2.4. Lay the fish on its lateral side with the ventral surface facing the researcher [1]. Hold the fish in place with the non-dominant hand [2]. Using a scalpel or dissecting scissors, make a straight, approximately 1-millimeter-deep ventral incision from the anus toward the anterior end of the fish [3].
 - 2.4.1. Talent laying the fish on its lateral side with the ventral surface facing the researcher.
 - 2.4.2. Talent holding the fish in place with the non-dominant hand.
 - 2.4.3. Talent making 1-mm deep ventral incision from the anus toward the anterior end of the fish with a scalpel or dissecting scissors.
- 2.5. Using a small metal spatula or spoon, separate the internal organs from the body cavity and remove them [1]. Place the organs in a biohazard waste container and discard them [2].
 - 2.5.1. Talent using a small metal spatula or spoon to separate and remove the internal organs from the body cavity.
 - 2.5.2. Talent placing the organs in a biohazard waste container.
- 2.6. Blot excess moisture from the remaining body tissue with a lint-free laboratory wipe [1].
 - 2.6.1. Talent blotting excess moisture from the remaining body tissue with a lint-free laboratory wipe.
- 2.7. Next, weigh the retained body torso using an analytical or top-loading balance [1-TXT].
 - 2.7.1. Talent weighing the retained body torso on an analytical or top-loading balance.
TXT: Ideal weight: 1.25 g $\pm$ 0.25 g
- 2.8. Place the fish body on ice or in a cooler until homogenization [1].
 - 2.8.1. Talent placing the fish body on ice or in a cooler.
- 2.9. Repeat the dissection for the remaining fish [1], and pool the torso tissues from 15 fish that meet the required length and mass criteria [2].
 - 2.9.1. *Reuse 2.2.3 , 2.3.1.*
 - 2.9.2. Talent is placing a couple of more isolated torsos on the ice, containing the

initially dissected torso.

3. Preparation of Alarm Cue Extract from Fathead Minnow Epithelial Tissue

Demonstrator: Jewel Johnson

- 3.1. Place the dissected epithelial torso tissue from all 15 fish into a 1.42-liter sterile blender jar [1] and secure the jar to the blender base [2].
 - 3.1.1. Talent placing dissected epithelial torso tissue from all 15 fish into a 1.42-liter sterile blender jar.
 - 3.1.2. Talent securing the blender jar to the blender base.
- 3.2. Add 50 milliliters of deionized or distilled water to the blender jar to facilitate initial tissue breakdown [1].
 - 3.2.1. Talent adding 50 milliliters of deionized or distilled water to the blender jar.
- 3.3. Homogenize the tissue in a blender set to approximately 22,000 revolutions per minute in pulse mode [1]. Hold the pulse button down for 5 seconds [2] and release it for the next 5 seconds [3]. Repeat this pulse cycle for a total duration of 5 minutes [3].
 - 3.3.1. Talent homogenizing the tissue in the blender using pulse mode.
 - 3.3.2. Talent holding down the pulse button on the blender.
 - 3.3.3. Talent releasing the pulse button on the blender.
 - 3.3.4. Talent holding the pulse button down again.
- 3.4. Then, add an additional 350 milliliters of deionized or distilled water to the blender jar [1].
 - 3.4.1. Talent adding an additional 350 milliliters of deionized or distilled water to the blender jar.
- 3.5. Continue blending at approximately 22,000 revolutions per minute for an additional 2 minutes using the previously demonstrated pulse function [1]. Confirm that the slurry is smooth and uniform at the end of the second blending period, with no large tissue fragments visible [2].

- 3.5.1. Talent continuing to blend the slurry using the pulse function.
- 3.5.2. Close-up shot of pointing to a smooth, uniform slurry without large tissue fragments.
- 3.6. Position a stainless steel 150-mesh, 105-micrometer sieve over a 2000-milliliter sterile collection vessel [1] and load the slurry onto the sieve [2]. Stir the slurry in the sieve using a glass rod to facilitate the passage of water through the sieve without forcing tissue through the mesh [3].
 - 3.6.1. Talent positioning a stainless steel 150-mesh, 105-micrometer sieve over a 500-milliliter sterile collection vessel.
 - 3.6.2. Talent pouring the slurry onto the sieve.
 - 3.6.3. Talent stirring the slurry in the sieve with a glass rod and the water passing through the sieve into the collection vessel. **Authors:** Please use a glass rod for the shoot
- 3.7. Then, rinse the sieve with water to remove the solid tissue material after all of the water has passed through the sieve into the collection vessel [1]. Pour the collected filtrate from the vessel through the sieve a second time into another 500-milliliter sterile vessel to ensure removal of most residual particulate matter [2]. After filtration, confirm that the final cue solution in the collection vessel is homogeneous and free of visible solids. The solution may appear cloudy due to microscopic tissue particles [3].
 - 3.7.1. Talent rinsing the sieve with water to remove the solid tissue material.
 - 3.7.2. Talent pouring the collected filtrate through the sieve placed on a fresh 500-milliliter sterile vessel. **Author's NOTE:** Please check—I cannot remember if we used a 500 or 2000 ml vessel for the second pour on shoot day.
VO needs to be altered at SI stage if 2000ml jar was used for this shot
 - 3.7.3. Shot of the final homogeneous cue solution.

4. Preparation of Fathead Minnow Alarm Cue for Larval Behavioral Assays

Demonstrator: Abby Yake

- 4.1. Mix the filtered cue solution with a glass rod or metal spatula to ensure even mixing [1].
 - 4.1.1. Talent mixing the filtered cue solution with a glass rod.

- 4.2. Pipette 1-milliliter volumes of the cue solution into 1.5-milliliter sterile microcentrifuge tubes [1].
 - 4.2.1. Talent pipetting 1-milliliter volumes of the cue solution into 1.5-milliliter sterile microcentrifuge tubes.
- 4.3. Label the tubes clearly with the preparation date, species, and concentration [1].
 - 4.3.1. Talent labeling the tubes with the preparation date, species, and concentration.
- 4.4. Freeze the aliquots immediately at minus 20 degrees Celsius until use [1].
 - 4.4.1. Talent placing the aliquots in the freezer. *Video editor: Give a delayed transition after this step to the next step to show that some time has passed*
- 4.5. To set up the behavioral assay, remove a single aliquot of frozen alarm cue from the freezer [1].
 - 4.5.1. Talent removing a single aliquot of frozen alarm cue from the freezer.
- 4.6. Thaw the aliquot by placing the tube in a warm water bath at approximately 45 to 55 degrees Celsius for 5 minutes [1]. Once thawed, invert the tube 5 to 7 times to mix [2].
 - 4.6.1. Talent placing the tube in a warm water bath.
 - 4.6.2. Talent inverting the thawed tube 5 to 7 times.
- 4.7. Prepare the experimental alarm cue solution by adding 1 milliliter of thawed cue stock to 200 milliliters of non-conditioned, aged, and aerated water [1]. Mix with a glass rod or spatula to ensure even distribution [3].
 - 4.7.1. Talent adding 1 milliliter of thawed cue stock to 200 milliliters of non-conditioned, aged, and aerated water.
 - 4.7.2. Talent mixing the experimental alarm cue solution with a glass rod.
- 4.8. For the behavioral assay, add the alarm cue-amended water or the clean water control to the arena of the experimental setup [1]. Place a 14-day-old larval fathead minnow, *Pimephales promelas*, in the darkened refugium and allow it to acclimate for 5 minutes [2]. Remove the door separating the refugium from the bright open area [3] and record larval emergence for 5 minutes [4].

- 4.8.1. Talent adding the alarm-cue-amended water to the arena.
- 4.8.2. Talent placing a larval fathead minnow individually in a darkened refugium containing alarm cue-amended water.
- 4.8.3. LAB MEDIA: Jove-Trial-Video-Trimmed.mp4 00:02-00:06.
- 4.8.4. LAB MEDIA: Jove-Trial-Video-Trimmed.mp4 00:19-00:20.

Results

5. Results

5.1. In the presence of the alarm cue, larval emergence from the refugium was significantly lower [1] than the null expectation of 0.5 [2], indicating that the alarm cue preparation is perceived and recognized by the larvae [3].

5.1.1. LAB MEDIA: Figure 2B. *Video editor: Highlight the black point for “Alarm cue”.*

5.1.2. LAB MEDIA: Figure 2B. *Video editor: Highlight the dashed horizontal line at 0.5.*

5.1.3. LAB MEDIA: Figure 2B. *Video editor: Highlight the black point for “Alarm cue”.*

5.2. In the absence of the alarm cue, larval emergence from the refugium [1] did not differ from chance [2].

5.2.1. LAB MEDIA: Figure 2B. *Video editor: Highlight the black point for “Control”.*

5.2.2. LAB MEDIA: Figure 2B. *Video editor: Highlight the dashed horizontal line at 0.5.*

1. Euthanasia

Pronunciation link: <https://www.merriam-webster.com/dictionary/euthanasia>

IPA: /ˌjuːθəˈneɪzə/

Phonetic Spelling: yoo·thuh·nay·zhuh

2. Epithelial

Pronunciation link: <https://www.merriam-webster.com/dictionary/epithelial>

IPA: /ˌepəˈθiːliəl/

Phonetic Spelling: eh·puh·thee·lee·uhl

3. Opercular

Pronunciation link: <https://www.merriam-webster.com/dictionary/opercular>

IPA: /oʊˈpɜːkjələr/
Phonetic Spelling: oh·pur·kyuh·ler

4. Caudal

Pronunciation link: <https://www.merriam-webster.com/dictionary/caudal>
IPA: /ˈkɔːdəl/
Phonetic Spelling: kaw·duhl

5. Peduncle

Pronunciation link: <https://www.merriam-webster.com/dictionary/peduncle>
IPA: /pɪˈdʌŋkəl/
Phonetic Spelling: puh·dung·kuhl

6. Calipers

Pronunciation link: <https://www.merriam-webster.com/dictionary/caliper>
IPA: /ˈkælɪpərz/
Phonetic Spelling: kal·uh·perz

7. Ventral

Pronunciation link: <https://www.merriam-webster.com/dictionary/ventral>
IPA: /ˈventrəl/
Phonetic Spelling: ven·truhl

8. Homogenization

Pronunciation link: <https://www.merriam-webster.com/dictionary/homogenization>
IPA: /həˌmɑːdʒənəˈzeɪʃən/
Phonetic Spelling: huh·maa·juh·nuh·zay·shuhn

9. Analytical

Pronunciation link: <https://www.merriam-webster.com/dictionary/analytical>
IPA: /ˌænəˈlɪtɪkəl/
Phonetic Spelling: an·uh·lit·ih·kuhl

10. Homogenize

Pronunciation link: <https://www.merriam-webster.com/dictionary/homogenize>

IPA: /hə'mɑ:dʒəˌnaɪz/

Phonetic Spelling: huh·maa·juh·nyze

11. Filtrate

Pronunciation link: <https://www.merriam-webster.com/dictionary/filtrate>

IPA: /'fɪlˌtreɪt/

Phonetic Spelling: fil·trayt

12. Microcentrifuge

Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>

IPA: /ˌmaɪkroʊ'sentrəˌfjuːdʒ/

Phonetic Spelling: my·kroh·sen·truh·fyooj

13. Acclimate

Pronunciation link: <https://www.merriam-webster.com/dictionary/acclimate>

IPA: /ə'klaɪˌmeɪt/

Phonetic Spelling: uh·kly·mayt

14. Refugium

Pronunciation link: <https://www.howtopronounce.com/refugium>

IPA: /rɪ'fjuːdʒiəm/

Phonetic Spelling: ruh·fyoo·jee·uhm

15. Pimephales

Pronunciation link: <https://www.howtopronounce.com/pimephales>

IPA: /pɪ'mɛfəliːz/

Phonetic Spelling: pih·mef·uh·leez

16. Promelas

Pronunciation link:
<https://www.howtopronounce.com/promelas>

IPA: /proʊ'mɛləs/

Phonetic Spelling: proh·mel·uhs

17. Aliquot

Pronunciation link: <https://www.merriam-webster.com/dictionary/aliquot>

IPA: /'æli,kwɑ:t/

Phonetic Spelling: al·ih·kwaht

18. Dissection

Pronunciation link: <https://www.merriam-webster.com/dictionary/dissection>

IPA: /daɪ'sɛkʃən/

Phonetic Spelling: dye·sek·shuhn

19. Scalpel

Pronunciation link: <https://www.merriam-webster.com/dictionary/scalpel>

IPA: /'skælpəl/

Phonetic Spelling: skal·puhl

20. Biohazard

Pronunciation link: <https://www.merriam-webster.com/dictionary/biohazard>

IPA: /'baɪoʊ,hæzərd/

Phonetic Spelling: by·oh·haz·erd