Cresko Laboratory Procedures and Protocols

Cresko Laboratory

Friday, March 1, 2024

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How to use this book

This is a Quarto book that contains all of the Procedures and Protocols for the Cresko Laboratory in the Institute of Ecology and Evolution at the University of Oregon.

The book is organized into major section that contain

- General Laboratory Protocols or the lab
- More detailed Laboratory Protocols
- Husbandry protocols for vertebrate animals primarily stickleback and pipefish, but also zebrafish
- Husbandry protocols for *Daphnia*
- Bioinformatic protocols including how to get on to **Talapas**

You can scroll through the book using the index on the left, but also use the search field to find all relevant protocols.

There are also useful appendices at the end, as well as a section for the references cited throughout the book.

This book was written in Markdown using Quarto. To learn more about Quarto books visit https://quarto.org/docs/books.

Part I

General Laboratory Protocols

This section of the book contains general protocols for working in the laboratory.

1 Contact Information

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Susie Bassham	xxxx	Cell

Part II

Molecular Protocols

This section of the book contains common protocols used for molecular biology and genomics in the laboratory. These include standard protocols such as setting up creating reagents, setting up PCRs and running gels, as well as advanced protocols such as creating constructs.

2 PCR with Phusion

2.1 Introduction

- **Purpose**: This procedure describes how to set up a basic PCR with Phusion polymerase
- **Procedure Type**: Molecular
- **Species**: N/A

The following guidelines are provided to ensure successful PCR using Phusion® DNA Polymerase. These guidelines cover routine PCR. Amplification of templates with high GC content, high secondary structure, low template concentrations or long amplicons may require further optimization.

We recommend assembling all reaction components on ice and quickly transferring the reactions to a thermocycler preheated to the denaturation temperature (98°C). All components should be mixed and centrifuged prior to use. It is important to add Phusion DNA Polymerase last in order to prevent any primer degradation caused by the $3' \rightarrow 5'$ exonuclease activity.

Phusion DNA Polymerase may be diluted in 1X HF or GC Buffer just prior to use in order to reduce pipetting errors. Please note that protocols with Phusion DNA Polymerase may differ from protocols with other standard polymerases. As such, conditions recommended below should be used for optimal performance.

2.2 Materials:

- PCR tubes, plates or strip tubes (1 per reaction & 1 for positive control)
- Forward and reverse primers
- npH2O
- Phusion DNA polymerase
- 5X Phusion HF or GC Buffer
- 10 mM dNTPs
- DMSo (optional)

2.3 Solutions:

NONE

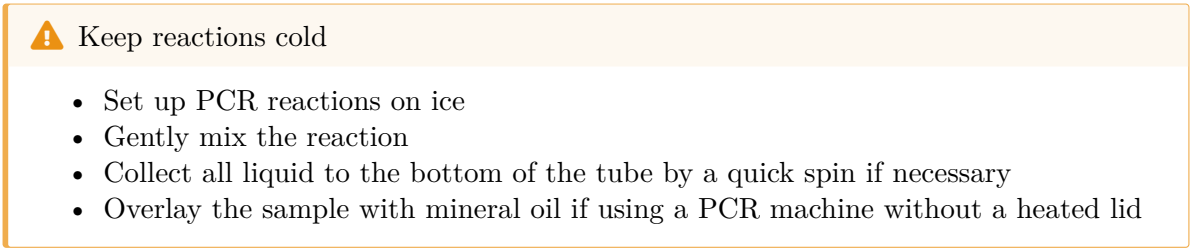
2.4 Procedure:

Prepare PCR reactions

Table 2.1: Table 1. Mixtures for PCR Reaction

	Component	20ul Reaction	50ul Reaction	Final Concentration
1	npH20	to 20 ul	to 50 ul	
2	5x Phusion Buffer	4 ul	10 ul	1X
3	10mM dNTPs	0.4ul	1.0 ul	200 uM
4	10 uM forward Primer	1.0 ul	2.5 ul	0.5 uM
5	10 uM reverse Primer	1.0 ul	2.5 ul	0.5 uM
6	Template DNA	variable	variable	<250 ng
7	DMSO (optional)	(0.6 ul)	(1.5 ul)	3%
8	Phusion polymerase	0.2 ul	0.5 ul	0.4 units/ 20 ul rxn

Mix together reagents in a 1500 ul tube for the number of reactions needed, but add an extra reaction for pipeting error and one for a negative control. For example, if a PCR will be run on 23 samples and one control, mix enough for 25 reactions.



PCR Conditions

Table 2.2: Table 2. Thermocycler regime

	Step	Temperature	Duration	Number cycles
1	Initial Denaturation	98 C	30 seconds	1 cycle
2		98 C	30 seconds	

	Step	Temperature	Duration	Number cycles
3	Amplification	Primer Anneal Temp	30 seconds	25-30 cycles
4		72 C	15-30 seconds/kb	
5	Final Extension	72 C	5 minutes	1 cycle
6	Hold	10 C	indefinite	1 cycle
7				
8				

Anneal temperature and length of amplification step

- The annealing temperature and length of time for amplification will depend upon the primers and the length of the fragment to be amplified.
- 15 seconds/kb works for most reactions. 30 seconds/kb can be used for more complex reactions.

3 cDNA basic

3.1 Introduction

- **Purpose**: This procedure describes how to synthesis cDNA for use with PCR.
- **Procedure Type**: Molecular
- **Species**: N/A

3.2 Materials:

- 2 µl Oligo d(T)23 VN (50 µM, NEB; anchored-dT primer)*
- X µl up to 5 µg total RNA
- 1 µl 10 mM dNTP
- water
- 2 µl 10x RT buffer (Invitrogen)
- 4 µl 25 mM MgCl2
- 2 µl 0.1 mM DTT – Invitrogen
- 1 µl RNase inhibitor – e.g., RNAseOUT (Invitrogen)
- 1 µl Superscript III reverse transcriptase (200 u/µl – Invitrogen)

3.3 Solutions:

NONE

3.4 Procedure:

First strand synthesis

Combine:

- 2 µl Oligo d(T)23 VN (50 µM, NEB; anchored-dT primer)*
- X µl up to 5 µg total RNA

- 1 µl 10 mM dNTP mix
- Water (if necessary) to bring total to 10 µl

Heat to 65°C for 5 min., then ice

Collect contents at bottom of tube by brief centrifugation.

Add:

- 2 µl 10x RT buffer (Invitrogen)
- 4 µl 25 mM MgCl2
- 2 µl 0.1 mM DTT – Invitrogen
- 1 µl RNase inhibitor – e.g., RNAseOUT (Invitrogen)
- 1 µl Superscript III reverse transcriptase (200 u/µl – Invitrogen)

Mix by gentle aspiration

- 25°C for 5 min.

Reaction can be scaled up to accommodate more starting RNA

Synthesis: Incubate at 50°C for 50 min.

Inactivation: 85°C for 5 min. Chill on ice, collect contents to bottom by short spin.

Destroy RNA template: 1 µl RNase H (2 u/µl), incubate at 37°C for 20 min.

Proceed to PCR. Depending on expression level, may be able to use a dilution of cDNA as template – try 1:50 dilution in EB, use 2 µl as template in a 20 µl reaction. Don't dilute your entire amount of cDNA, as some products may require a higher concentration of template.

4 2x Turbo

4.1 Introduction

- **Purpose**: This procedure describes how to create 2x Turbo PCR mix.
- **Procedure Type**: Molecular
- **Species**: N/A

4.2 Materials:

- 33,000 µl npH2O
- 2000 µl MgSO4 (100mM)
- 1600 µl 1M Tris-HCl (pH 8.6)
- 800 µl 1M KCl
- 800 µl 1M (NH4)2SO4
- 800 µl Triton-X 100 (10%)
- 400 µl DMSO (100 %)
- 120 µl dATP (100mM)
- 120 µl dGTP (100mM)
- 120 µl dTTP (100mM)
- 120 µl dCTP (100mM)
- 80 µl 100mg/ml BSA

Total = 40 ml of buffer

4.3 Solutions:

NONE

4.4 Procedure:

- Mix above reagents together
- Place in 1.5 ml ependorph tubes
- Store at -20C

5 Paraformaldehyde

5.1 Introduction

- **Purpose**: This procedure describes how to make 8% paraformaldehyde. This protocol is the one I have used and makes use of pH to get the PFA into solution relatively quickly - then you readjust the pH. It's for 8% - then you can add 1:1 2x PBS.
- **Procedure Type**: Molecular
- **Species**: N/A

5.2 Materials:

- xxx

Total = 40 ml of buffer

5.3 Solutions:

NONE

5.4 Procedure:

> ⚠ HUMAN HEALTH WARNGING
>
> Paraformaldehyde can be hazardous to your health - make sure you prepare in the fume hood.

- Add 40 g Paraformaldehyde to 450 ml distilled water (or scale for desired final volume).
- Add 1 ul of 10 N NaOH per ml of water (i.e. 500 ul for 500 ml).
- Apply medium heat while stirring at medium speed to dissolve - approx 15-20 min.
- Solution should not go above 60º C.

- Eventually, granules will fully dissolve and the solution will become translucent.

> **DO NOT LET THE SOLUTION STIR BEYOND THIS POINT**
>
> It will form a fuzzy precipitate that reduces the solution strength after filtering.

- Once the granules have dissolved and the solution clears, turn off the heat and equilibrate to pH 7.4 with approx 1.5 ml of 20% HCl (or scale, depending on target volume).
- Bring volume to 500 ml (or scaled volume) with distilled water.
- Filter while still warm to 0.45 um (or 0.2 um). Aliquot and store at -20^{o} C.

6 Alizarin Staining

6.1 Introduction

- **Purpose**: Alizarin staining of fixed adult stickleback.
- **Procedure Type**: Molecular
- **Species**:
  - Threespine stickleback, *Gasterosteus aculeatus*
  - xxx
- **Authors**
  - xxx

> ⚠ Schedule for Cleaning
>
> PLACEHOLDER

6.2 Materials:

- Alizarin red S
- KOH
- H_2O_2
- NaOH
- MESAB
- PFA

6.3 Solutions:

- **0.5% Alizarin red S Stock**:
  - To make 50 mls add 0.25g alizarin red S powder to 50 ml water.
- **0.025% Alizarin Stain**

  - To make 100 mls: Add 500µl 0.5% alizarin red S (stock) to 99.5ml 1% KOH
  - 1 Liter: Add 5ml 0.5% alizarin red S (stock) to 9950ml (1 liter) 1%KOH

- **3% H202/0.5%KOH**: Mix and keep at 4C; Before using, bring to room temperature to hold down introducing bubbles under the skin: 0.5ml 6%H202 & 0.5ml 1%KOH.
- MESAB: Tricaine: 3-amino benzoic acid ethyl ester from Sigma (Cat # A-5040). Mix in fish safe container with a stir bar:
  - 400 mg tricaine powder
  - 800 mg Na2HPO4 (anhydrous)
  - 100 ml glass distilled water
  - Adjust to ~pH 7 with a drop at a time of 1N NaOH or 1N HCl if needed but it's usually right if you weigh the sodium phosphate carefully and measure the water with a graduated cylinder.
  - For storage: Aliquot into 6 x 25 ml fish safe plastic bottles and store at 4C. Label with date made and use within a couple of weeks.
- **8% PFA**:
  - 8 g Pelleted PFA (Ted Pella, Inc.; cat# 18501)
  - 90 ml dH2O
  - 25 drops 1N NaOH
  - Heat at very low heat and stir until solution clears.
  - Add 25 drops 1N HCl. pH should be 7.0-7.2.
  - Filter and store at 4C not more than 1 week.
  - Use as 4% PFA: dilute 1:1 with 2X PBS, do not store solution more than a few hours.
- **2X PBS**
  - 1.6% NaCl
  - 0.04% KCl
  - 0.04 M PO4 pH 7.0- 7.3

6.4 Procedure:

- **Day One**
  - 2h-8h at R/T depending on size on shaker.
  - Without agitation and with lid open until eyes start to lighten and all skin pigment is gone (usually about an hour or more)
- **Day Two**
  - xxx
  - xxx

– xxx

7 Pouring and Running a Gel

7.1 Introduction

- **Purpose**: This procedure describes how to pour and run a gel to visualize DNA.
- **Procedure Type**: Molecular
- **Author**: Micah Woods
- **Date Created**: February 14, 2024

7.2 Background

Gel electrophoresis is a technique used to separate mixtures of DNA, RNA, or proteins based on their molecular size. During gel electrophoresis, an electrical field is used to help molecules travel through the pores of an agarose gel matrix. At one end of the gel, furthest away from where the samples are loaded, is a positive charge, and at the other end is a negative charge. Since DNA and RNA are both negatively charged molecules, they are pulled through the gel matrix towards the positive charge. Since proteins do not have a negative charge, researchers must first mix them with a sodium dodecyl sulfate detergent which gives them the negative charge needed to migrate towards the positive end of the gel. The speed at which molecules travel through the gel is inversely related to their molecular size. For example, small DNA molecules have a low molecular weight, enabling them to travel further through the gel matrix than a larger DNA molecule. When a gel is stained with DNA/RNA/protein-binding dye, these molecules can be seen as bands, whose location along the length of the gel represents the molecule?s size in terms of number of base pairs. When dye-stained DNA/RNA/protein is run beside a ladder with known fragment size, one can estimate the size of the assayed molecule. After the DNA/RNA/protein molecules have been separated using gel electrophoresis, the gel is imaged under UV light, visualizing the DNA/RNA/proteins present across the length of the gel.

7.3 Materials

- 500 ml Corning glass bottle labeled “Agarose Gels”
- Scale

- Weigh paper
- Microwave
- Gel comb
- Gel tray
- Pipets
- Pipet tips
- Parafilm or PCR strip tubes for mixing DNA samples and Loading Dye
- Gel box with enough buffer inside to cover gel
- Electrodes attached to power supply (Bio-Rad PowerPac)
- Gel transferring tray
- Device for visualizing gel
  - Azure Biosystems c200, located in Pacific 314
- Gel image printer
- Kimwipes

7.4 Solutions

- 0.5X TBE
- Safe View
- Molecular Biology Grade Agarose
- 6X Loading Dye
- Ladder of choice
- EB Buffer

7.5 Procedure

1. Weigh desired amount of Molecular Biology Grade Agarose - scale, weigh paper, and Agarose located on the common use bench on the north end of the laboratory - and pour it into the 500 ml Corning glass bottle labeled “Agarose Gels” (located on top of or inside the microwave on the south end of the laboratory).

   a. 1-1.2% Agarose (1 g-1.2 g) is a good starting amount of Agarose for DNA samples, though the amount of Agarose can vary depending on your particular samples and goals. The concentration of Agarose in a gel depends on the sizes of DNA fragments to be separated, with most gels ranging between 0.5%-2% Agarose.

2. Fill bottle with 0.5X TBE buffer, located in the 20-liter Nalgene located at the south sink. The volume of TBE buffer used will be based on the size of the gel mold. For example, for a 14”x10” gel tray, use 100 ml of 0.5X TBE buffer. 3. Put bottle in microwave and microwave for one minute.

3. Using the heat mittens located on top of the microwave, remove the bottle from the microwave, swirl the mixture around, and put it back in the microwave for 30 seconds. By the end of this step, the Agarose should be melted into the TBE and there should be no remaining solid Agarose particles.
4. Using the heat mittens, remove the bottle from the microwave and carry it to the fume hood.
5. Select a gel tray from the drawer under the gel boxes. Put tape around the two longest sides of the gel tray, pressing tape on firmly so it is air-tight with the tray. The distance between the top of the tape and the bottom of the tray should be at least half-an-inch tall so that it can hold all of the liquid from the Agarose Gel bottle without it spilling over the sides or leaking through any air gaps in the tape.
6. Select a gel comb from the drawer under the gel boxes. Different combs make different sized wells that can hold different volumes, so select a comb based on your specific experiment. Place the gel comb in the notches of the gel tray. The combs should not touch the bottom of the tray but be slightly above it.
7. Let the agarose solution cool for a few minutes. Using the heat mittens, swirl the Agarose Gel bottle again. Add 5 ul of Safe View (located in the Styrofoam box in the fume hood) to the Agarose Gel bottle. Gently swirl the bottle to mix in the Safe View; try to avoid creating bubbles while swirling.
8. Slowly pour the Agarose Gel mixture into the gel tray with gel comb. Be careful not to create bubbles in the gel mixture while pouring. If there are bubbles, pop or move them to the bottom of the tray, away from the comb. Slowly lift the comb up and down a few times to ensure there are no bubbles around the comb.
9. Let the gel mixture sit in the tray under the hood until it solidifies (usually takes around 20 minutes).
10. While you are waiting for the gel to solidify, clean the Agarose Gel bottle. Fill the bottle halfway with water, swirl the water, microwave for 30 seconds, swirl again and microwave for another 30 seconds. Pour out the water and set the bottle out to dry next to the microwave.
11. Once the agarose mixture has solidified, carefully remove the tape from the sides of the tray.

    > **!** This is important
    >
    > Tape should be removed from the tray while held under the fume hood. Tape should be removed slowly to prevent any splattering of residual liquid on the top of the solidified gel.

12. Carefully remove the comb from the gel matrix by slowly pulling it straight up.

13. Carry the gel in the gel plate to the gel boxes, remove gel box lid, and slowly lower the gel and plate into gel boxes.

14. Load ladder* of choice into the left-most well of the gel. The volume of ladder you load will vary depending on your experiment.

> **!** This is important
>
> When expelling the ladder into the well, only pipet to the first stop. Pipetting to the second stop risks expelling bubbles into the well which could interfere with the experiment.

> **!** NOTES
>
> *The ladder is made of DNA and needs a gel loading dye. When selecting a ladder to use, make sure that the ladder is not clear but has been mixed with a loading dye (should be a bright color like blue, orange, green, or purple).

15. Prepare DNA samples to be loaded into the wells of the gel. Pipet out 10 ul of DNA* from each sample onto a piece of parafilm or into a PCR strip tube, using a fresh pipet tip for each sample to avoid cross contamination.

> **!** NOTES
>
> *The volume of DNA you mix load into each well depends on the concentration of DNA and how much of DNA of your total you can afford to use for the gel, though a generally good concentration range is 10-100 ng/ul to ensure the DNA can be visualized on the gel. If you are aiming to load a specific concentration of DNA into each well, calculate the concentration of your DNA sample and find that the corresponding volume is less than 10 ul, you can add the appropriate volume of EB Buffer to bring the sample's volume up to 10 ul.

16. Add 2 ul of 6X Loading Dye* to each DNA sample. Thoroughly mix the dye and DNA sample by aspiration, but mix slowly to avoid creating bubbles. Be sure to use a fresh pipet tip in between DNA samples.

> **!** NOTES
>
> *The exact volume of loading dye you add to each sample can change depending on the amount of DNA you add, but the final dilution should be 1 part 6X Loading Dye to 5 parts DNA sample.

17. Load 10 ul of the DNA sample/dye mixture into the next available well in the gel.

> **!** This is important
>
> *When expelling the DNA sample/dye mixture into the well, only pipet to the first stop. Pipetting to the second stop risks expelling bubbles into the well which could interfere with the experiment.

18. Put gel box cover on. Attach the electrodes to the metal posts at the top and bottom of gel box; the black electrode (-) attaches to the metal post on the top of the box and the red electrode (+) attaches to the metal post on the bottom of the box.

> **!** NOTES
>
> Since DNA is a negatively charged molecule, it will run through the gel matrix towards a positive charge. A helpful way to remember electrode placement, then, is "Run to red," since the red electrode has a positive charge and should be placed furthest away from the DNA samples.

19. Turn on the Bio-Rad PowerPac and set it to your desired voltage. Press "Run." You can check to see if an electric current is running through the gel box by looking for a stream of small bubbles rising up the back of the gel box.
20. Run samples for desired time.
21. Press the "Stop" button on the Bio-Rad PowerPac and turn off the power. Remove the electrodes from either end of the gel box, loosely wind them up and place them above or next to the Bio-Rad PowerPac.
22. Remove the lid from the gel box. Carefully lift the gel tray out of the gel box, ensuring that the gel does not slide off the tray. Place the gel tray and gel onto the gel transferring tray (located behind the faucet on the south sink). Carry the gel to the gel imaging station (Azure Biosystems c200) in the Phillips Microfluidics Laboratory (Pacific 314).
23. To image the gel,

    a. Open the door of the c200 Azure Biosystems gel imaging station, and carefully slide the gel inside. The long side of the gel should be facing the opening of the imaging station. Position the gel so that it is centered inside the imaging platform, and the short sides of the gel are parallel to the sides of the imaging platform. Close the door.
    b. Select the "cSeries Capture Software" on the screen.
    c. Select the "GEL" option in the top left corner, to the right of the "GALLERY" option.
    d. Select the "BRIGHT BANDS" option in the bottom right corner.
    e. Select "CAPTURE" to take an image of the gel.

f. Once your image has been captured, you may rotate, flip, crop, or zoom in/out of the image.
g. To print the image, select "PRINT" and choose the number of copies you would like to print.

24. Open the door of the c200 Azure Biosystems gel imaging station and carefully remove the gel. Wipe down the gel platform using a Kimwipe and close the door.
25. Carry your gel and printed images back to the lab. You may either dispose of your gel in the trash or store the gel in a plastic bag in the refrigerator for future use.
26. Rise off the gel transferring tray and set it to dry behind the south sink faucets. Rinse off the gel tray and the gel comb, ensuring that there is no remaining agarose between the tines of the gel comb. Set the comb and the tray to dry on the drying rack hanging above the south sink.

7.6 Associated Papers

- Mark: "Should we add the 'things that can go wrong when running a gel' document that we discussed to this document or at least reference it??"
- Lee PY, Costumbrado J, Hsu CY, Kim YH. Agarose gel electrophoresis for the separation of DNA fragments. J Vis Exp. 2012 Apr 20;(62):3923. doi: 10.3791/3923. PMID: 22546956; PMCID: PMC4846332.
- Gel electrophoresis. Scitable by Nature Education. https://www.nature.com/scitable/definition/gel-electrophoresis-286/#:~:text=Gel%20electrophoresis%20is%20a%20laboratory,gel%20that%20contains%2

Part III

Microbiology

This ection of the book contains protocols that involve the use of microbes, mostly in the context of host-microbe interactions, but also standard protocols for cloning of nucleic acides using microbes.

8 Placeholder_Microbiology

8.1 xxx

xxxx

xxx

xxxxx

Part IV

Vertebrate Husbandry

This section of the manual contains protocols for the safe and ethical husbandry and use of vertebrate animals, particular the fish models stickleback, zebrafish and syngnathids.

9 Autoclaving Fish Room

9.1 Introduction

- **Purpose**: For sanitizing nets and scrub pads that have been used in the fish room.
- **Procedure Type**: xxx
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
  - Bay pipefish, (*Syngnathus leptorhyncus*)
  - Gulf pipefish (*Syngnathus scovelli*)
  - Zebrafish, (*Danio rerio*)
- **Author**: xxx
- **Date Created**: xxx

> ⚠ NOTES
>
> xxxx

9.2 Materials

- xx
- xx
- xx

9.3 Procedure

1. Place dirty nets and scrub pads in autoclave wire bin
2. Put bin into autoclave, and close the door (close door by pushing main door against hinge on left of door, then push door shut and seal by turning handle clockwise to just tight, DO NOT OVER TIGHTEN).
3. Change menu to B using keypad
4. Hit #1 twice, this will start the autoclave.
5. Sign sheet on outside of entrance door

6. Wait 45 minutes and remove nets
7. Put nets in clean nets tub.
8. Initial Check list on outside of door. **Gravity 45 min OK to Remove**

10 Tank Cleaning 20G

10.1 Introduction

- **Purpose**: This procedure describes how to clean 20 gallon glass tanks.
- **Procedure Type**: Husbandry
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: Mark Currey
- **Date Created**: April 8, 2008; revised December 6, 2018 by M. Currey

> **!** This is important
>
> Tank cleaning is to be done **ONLY Monday - Friday**.

10.2 Materials

- Scrub pad or sponge
- Cart
- Old clothes (this can be messy)
- Personal protection equipment (Splash proof glasses or face shield)
- Gloves
- Sink

10.3 Solutions

- **Bleach solution**: Make a 10% bleach solution in a used 1 gallon bleach bottle. To do so: Add 2.7 L of water. Add 0.3 L of bleach and gently stir. Dispense into appropriately labeled squirt bottle for use.

- **Sodium thiosulfate**: Make a 5% solution of sodium thiosulfate in the carboy located on counter above the fridge. To do so: Add 500 g of sodium thiosulfate. Fill carboy to the fill line indicated on front of carboy (~ 5L) with water. Mix aggressively. Dispense into appropriately labeled squirt bottle for use.

> Chemical Hygiene
>
> When using bleach and/or sodium thiosulfate. Eye protection is required. Please use splash proof glasses or a face shield when using bleach and sodium thiosulfate.

10.4 Procedure

- **Complete bleaching and cleaning of tank.** This needs to be done to each tank every 2 months.

    1. Remove fish from tank and put them into a clean tank. Tanks that are emptied of fish need to be cleaned and sterilized before another batch of fish can be introduced.
    2. Drain the tank and remove it from the rack. Clean air diffuser as instructed below. Clean the tank and all parts thoroughly with a scrub pad, taking care not to damage the silicon water seals on the inside (algae should be left if very gentle rubbing will not remove it.
    3. Squirt about 10-20 mls of bleach into the tank. Wash the bleach water thoroughly around the inside of the tank by hand using a pad or sponge exposing all inside portions of the tank to bleach.
    4. Rinse the tank thoroughly with hot tap water. **Rinse the tank with sodium thiosulfate, and then rinse it again with hot water. Put a few thiosulfate crystals into the tank and leave it.**
    5. Reassemble the tank and put it back on the rack. Fill with system water and using a dry erase marker record date/time on the front of the tank.
    6. Allow water to recirculate for about **30 minutes** before adding fish. If adding fish, **Watch fish for 15 min** after adding them to look for any signs of distress.

- **Initial check list**
- **Air diffuser cleaning**:

    1. Remove dirty air diffusers from tanks and rinse with tap water to remove excess algae and debris.
    2. Place in 10% bleach solution for 15-30 minutes. Please see {#sec-Plastic_Glassware_cleaning-bleach} for bleach and sodium thisolfate instructions.
    3. Rinse with hot water for 5 and then place into 3% sodium thiosulfate for 5 minutes.
    4. Rinse with hot water for 5 minutes.

5. When cleaned, air diffusers are placed back into aquaria, observe fish for 15 min for signs of distress.

10.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

11 Artemia Decapsulation

11.1 Introduction

- **Purpose**: This procedure describes standard practices for decapsulating brine shrimp.
- **Procedure Type**: Husbandry
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: Mark Currey
- **Date Created**: Adapted April 3, 2008 by M. Currey; Updated February 12, 2024 by M. Currey

11.2 Materials

- 15 oz can of dried Artemia cysts (approximately 430 g)
- 4.3 L ~6% laundry grade bleach
- Rock Salt (NaCl)
- 125 ml 40% Lye (NaOH) solution
- 30.0 g Sodium thiosulfate (Na2S2O3)
- 16 L Hatching Cone with aeration
- 125 micron mesh bag (Aquatic Eco-Systems PMB3, 125 micron x 18”)
- Several 3-5 L beakers
- (1-2) Squirt bottles - squeeze type

11.3 Solutions

> ⚠ NOTES
>
> Solutions should be prepared in advance.

Chemical Hygiene

When using bleach and/or NaOH. Eye protection is required. Please use splash proof glasses or a face shield and gloves during this protocol.

- Bleach, ~6% laundry grade
- 25 ppt Salt Solution
    1. Combine: 50 g Rock Salt (NaCl) To 2.0 L with tap water
    2. Stir to dissolve completely.
- 40% Lye (NaOH) solution
    1. Combine: 200 g Lye (NaOH) To 500 mL with tap water
    2. Stir to dissolve completely.
    3. Store in refrigerator (4°C)
- Buffered Salt Solution
    1. Combine: 2L, 25 ppt Salt Solution
    2. 125 mL 40% Lye Solution, pre-chilled to 4°C
- 1.0% Sodium Thiosulfate
    1. Combine: 30 g sodium thiosulfate To 3.0 L with tap water
    2. Stir to dissolve.
- Saturated Brine
    1. Combine: ~25g Rock Salt to 4.0 L with tap water
    2. Aerate to dissolve.

11.4 Procedure

1. **Cyst hydration**: Hydrate one full can of dried cyst in 5 L of tap water in a hatching cone with aeration for 1 hour at room temp. Examine the cyst under a dissecting scope with top lighting before proceeding. Dry cysts are dimpled, resembling a deflated basketball, whereas fully hydrated cysts are completely spherical in shape. The cysts must be fully hydrated prior to the de-capsulation step. If cysts are not completely spherical after 1 hour, continue the hydration process (for a maximum of 2 hours), checking the progress of the cysts under a microscope every 15 min.
2. **Filter and rinse cysts**: Collect the hydrated cyst in a 125 um mesh bag and rinse with cool tap water.

3. **Transfer cysts back to the cone**: Add the Buffered Salt Solution to the cone and aerate (save back a filled squirt bottle of salt solution to help transfer cysts to cone). Transfer cysts into cone.
4. **De-capsulation**: Add the bleach (4.3 L) to the cone and continue aeration. Watch the cysts turn from brown to grey to orange, When the cysts are 90% orange, stop the reaction by quickly siphoning the cysts through a 125 um mesh bag and rinsing well with cool tap water.
5. **Neutralization residual chlorine**: To neutralize any residual chlorine transfer the mesh bag to a clean 4 L beaker and pour the 1.0% Sodium Thiosulfate (3L) into the bag. Soak the cysts in the sodium thiosulfate solution for ~1 min, then rinse the cysts with de-ionized tap water. Rinse until discharge turns clear.
6. **Dehydration for long-term storage**: Transfer the cysts back to the cone with 4 L of saturated brine and aerate until salt is dissolved. Transfer dehydrated cyst to (5 or 6) 1 L Nalgene bottles filled with 200 - 300 grams of salt. Add enough salt so that it does not dissolve when de-capsulated brine is added. Fill the bottles with de-capsulated brine. Store in refrigerator. The de-capsulated brine will store for at least 1 month. Hatch brine as you would capsulated brine (see Hatching and Feeding Brine SOP).

11.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

12 Brine Shrimp Enrichment

12.1 Introduction

- **Purpose**: This procedure describes how to enrich brine shrimp with high-carotenoid marine algae (or other enrichments).
- **Procedure Type**: Husbandry
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: Mark Currey
- **Date Created**: May 6, 2008; February 2, 2024 updated by M. Currey

12.2 Materials

- De-capsulated Brine Shrimp
- Rock Salt
- 105 m mesh shrimp collector
- Baking Soda
- Squirt Bottle
- Enrichment material

#Solutions

- none

12.3 Procedure

1. Hatch and harvest the brine shrimp (see brine shrimp hatching and feeding SOP).
2. Transfer into a specimen cup with clean seawater to resuspend them. Fill the specimen cup with new seawater until it reaches 1L.

3. **Dilute the brine shrimp.** Assuming an 80% hatch rate from the 1 g of brine shrimp eggs about 200,000 nauplii will hatch. For further culturing the recommended density is 1,000-2,000 brine shrimp/L. As many will be lost in the culturing process shooting for a higher density will result in more alive at the end. Add between **300-400 mL** of the brine shrimp to a 5-gallon (18L) conical hatching jar (made to 30-35 ppt) ensuring that the brine shrimp are suspended in the specimen cup and not settled at the bottom.

    - Provide a lid to reduce evaporation and proper aeration. Make sure the tube extends as far down as possible to eliminate the possibility of a "dead zone"

4. **Feed the brine shrimp.** Brine shrimp do not eat for the first 48 hrs. of their life cycle as they absorb nutrients from their yolk sac. After 48 hr. feed the brine shrimp High-Carotenoid Marine Algae (ordered from brineshrimpdirect.com). Add 1 tsp. of Hi-C Algae to a blender with 0.5L of seawater and blend until smooth. Add this solution to the conical hatching jar for the brine shrimp to feed on. Feed this solution to brine shrimp daily.

    - Store leftover Hi-C Algae in 50 mL Falcon Tubes sealed with parafilm in the freezer

5. **Conduct daily water changes.** Since a filtration system cannot be implemented due to the small nature of the brine shrimp, **20-30%** water changes should be conducted daily. Remove air from the system and open the stopcock to allow water to exit the container. Filter the water through a fine mesh net to catch any brine shrimp as it exits the conical hatching jar. Immediately transfer those brine shrimp to a specimen container and re-add them to the system. Wipe down and food that has settled along the edge and lid and re-fill the conical hatching jar with clean seawater. Reintroduce the air.

6. **Feed out the brine shrimp.** It takes around 8-12 days for the brine shrimp to reach their full size of 7mm (this amount of time will change depending on how optimum the conditions are for the brine shrimp). Once large enough, drain a portion of the container through a fine mesh net and immediately transfer into a specimen cup with clean seawater. Drain this water through a net with larger holes to remove food from the solution (brine shrimp will be large enough at this time to not fall through the holes) into a new specimen cup. Use a turkey baster to allocate food evenly to all of the tanks.

Depending on the amount of fish that must be fed the culture may be fed out over a period of multiple days

7. If the conical hatching jar is completely fed out, restart the culture with new nauplii. For best results stagger the start of the conical hatching jars so brine shrimp will always be growing while you feed out others.

12.4 Associated Papers

- xxx
- xxx
- xxx
- xxx

13 Bleach and Sodium Thiosulfate

13.1 Introduction

- **Purpose**: This procedure describes how to use bleach and sodium thisosulfate to clean plastics and glassware in the Pacific fish facility.
- **Procedure Type**: Husbandry
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: Mark Currey
- **Date Created**: April 10, 2008; updated April 4, 2012 by M. Currey

13.2 Materials

- Gloves
- Eye protection
- Apron
- Gloves
- Old clothes (I have ruined many shirts doing this)

13.3 Solutions

- **Bleach solution**: Make a 10% bleach solution in a 2 gallon bucket. Add 4.5 L of water. Add 0.5 L of bleach and gently stir.
- **Sodium thiosulfate**: Make a 5% solution of sodium thiosulfate in a separate 2 gallon bucket. Add 5 L of water (to line) and 250 g (marked on dispenser) of sodium thiosulfate. Mix aggressively.

Chemical Hygiene

When using bleach and/or sodium thiosulfate. Eye protection is required. Please use splash proof glasses or a face shield when using bleach and sodium thiosulfate.

NOTES

These solutions need to be changed once a week. (see below for directions)

13.4 Procedure

- **Cleaning of Dishes**: (Do the steps below in the order displayed)

  1. Put away all clean dishes that are on drying shelve.
  2. Transfer sodium thiosulfate soaked dishes into sink and rinse well. After they have been rinsed place the dishes on drying racks to dry.
  3. Transfer bleach soaked dishes into sodium thiosulfate solution and let soak overnight.
  4. Rinse dirty (dishes) glass and plastic ware, **NO NETS**, in the sink.
  5. Place rinsed dishes into bleach and let soak overnight.

- **Changing solutions**:

  1. Wear gloves and protective eyewear.
  2. Empty bleach/sodium thiosulfate containers of all dishes.
  3. Empty used solutions into sink.
  4. Rinse container with water and allow to drain.
  5. Fill with water and the proper amount of bleach/sodium thiosulfate to make the concentrations as stated above. (The directions for this with amounts of water and chemical should be written on container)
  6. Initial Check list

13.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

14 Food Storage and Sources

14.1 Introduction

- **Purpose**: This procedure describes sources of the various fish foods that are used in the Pacific Stickleback Facility and standard practices for storing these foods.
- **Procedure Type**: Husbandry
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
- **Author**: Mark Currey
- **Date Created**: August 25, 2011; updated 2023 by M. Currey

14.2 Procedure

> ⚠ NOTES
>
> - Label all foods with received date and expiration date (see below for how to determine expiration date).
> - If one or more component is missing (being ordered) it is ok to leave it out of the mix. Please alert Mark C. to order the missing component.

1. **Brine Shrimp**:
   - Source: Brine Shrimp Direct, www.brineshrimpdirect.com
   - Expiration: Good **indefinitely** if frozen in tightly sealed container.
     - Upon receiving label with received date.
     - Store unopened tins in -20°C freezer
     - After de-capsulation label with date de-capsulated and date of expiration (**30** days from de-capsulation).
     - Store de-capsulated shrimp at 4°C.
2. **Selco (brine shrimp supplement)**:
   - Source: Aquatic Ecosystems, http://www.aquaticeco.com/

- Expiration: Good **indefinitely** if kept unopened at room temperature.
  - Upon receiving label with received date.
  - Once opened label with opened date and expiration date of 1 year post opening
  - Store at room temperature.

3. **Golden Pearl Larval Diet**: 100-200, 300-500, and 800-1000 micron

   - Source: Brine Shrimp Direct, www.brineshrimpdirect.com
   - Expiration: Good **1** year if kept unopened in the fridge
     - Store in -4°C fridge.
     - If stored unopened label with expiration date **1 year** from receiving date.
     - Upon opening change expiration date to **6 months** from date opened.
     - Store at room temperature.

4. **Hikari dry foods**: Micro Pellets

   - Source: Pet Mountain, www.petmountain.com, That Pet Place: http://www.thatpetplace.com
   - Expiration: If un-opened, expiration date is labeled on container by manufacturer. Once opened and placed in the fridge the food is good for **6 months**.
     - Keep out of direct sunlight, high heat, and humidity.
     - Store at 4°C.

5. **New Life Spectrum dry foods**: Optimum saltwater flakes, Growth Formula

   - Source: Jehmco, www.jehmco.com
   - Expiration: If un-opened, expiration date is labeled on container by manufacturer. Once opened and placed in fridge the food is good for **6 months**.
     - Keep out of direct sunlight, high heat, and humidity.
     - Store at 4°C.

6. **Zeigler Larval dry food**: AP100 (150-250 microns)

   - Source: Aquatic Ecosystems, http://pentairaes.com
   - Expiration: Good **2 years** if kept unopened and in freezer. Once opened and placed in fridge the food is good for **12 months**.
     - Upon receiving label with received date and expiration date (2 years from received date).
     - Store at 4°C.

7. **Otohime Fish Diet**: S1 and S2

   - Source: http://reedmariculture.com/product_otohime_fish_diet.php#tab_tech
   - Expiration: If un-opened, expiration date is labeled on container by manufacturer. Once opened and placed in freezer the food is good for **6 months**.

  - Upon receiving label with received date and expiration date (1 years from received date).
  - Store at 4°C.

8. **Ziegler Zebrafish Diet**:

   - Source: http://www.zeiglerfeed.com/research-diets/adult-zebrafish-diet/
   - Expiration: If un-opened, expiration date is labeled on container by manufacturer. Once opened and placed in freezer the food is good for **6 months**.
     - Upon receiving label with received date and expiration date (1 years from received date).
     - Store at 4°C.

9. **Frozen Mysid and Blood Worms**:

   - Source: Online and local pet stores
   - Expiration: Expiration date printed on front label.
     - Upon receiving label with received date
     - Store at -20°C.

14.3 Associated Papers

- xxx
- xxx
- xxx
- xxx

15 PLastic Tank Cleaning

15.1 Introduction

- **Purpose**: This procedure describes how to clean plastic tanks. Fish will live in these tanks (4.5 or 10.5 liter) for 2-3 months at which point the tank will be emptied and sterilized using the dishwasher then stored for further use. If the fry remain in these tanks for longer then 3 months, or there is a build-up of waste or algae in the tanks, then follow this procedure.
- **Procedure Type**: Husbandry
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: Mark Currey
- **Date Created**: April 10, 2008

15.2 Materials:

- 2.8 L or 9.5 L Aquaneering tanks
- matching lid
- fry baffle - note: When placing new fish into the system use 400 micron (smaller) mesh baffles.
- Blue tubing.

15.3 Solutions:

> Chemical Hygiene
>
> When using bleach and/or Sodium Thiosulfate. Eye protection is required. Please use splash proof glasses or a face shield and gloves during this protocol.

- Bleach solution: Make a 10% bleach solution in a 2 gallon bucket. Add 4.5 L of water. Add 0.5 L of bleach and gently stir.
- Sodium thiosulfate: Make a 3% solution of sodium thiosulfate in a separate 2 gallon bucket. Add 5 L of water (to line) and 150g (marked on dispenser) of sodium thiosulfate. Mix

15.4 Procedure:

1. Obtain clean fry tank and install lid and baffle.
2. Put fish from dirty tank into clean tank. To do this remove dirty fish tank from rack and carefully pour off 1/3 of tank water. Then pour the rest of the water and fish into clean tank. If there is lots of waste in the tank transfer fish with a net to leaving waste in dirty tank.
3. Put clean tank of fish back on rack and start water.
4. Wash dirty tank, lid, and baffle using the dishwasher (see Dishwasher SOP).
5. Clean blue tubing with a squirt of bleach followed by a squirt of sodium thiosulfate and then rinsed thoroughly with hot water.

15.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

16 Sick and Dead Fish

16.1 Introduction

- **Purpose**: This procedure describes how to do daily health checks on stickleback and pipefish.
- **Procedure Type**: Husbandry
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: Mark Currey
- **Date Created**: April 14, 2008; updated January 01, 2015 by Mark Currey

16.2 Materials

- Fish Morgue consisting of a sealable plastic bag located in the freezer.
- Small container for fish transport
- Net

16.3 Solutions

- Mesab, a.k.a. MS222, tricaine or 3-aminobenzoic acid ethyl ester

16.4 Procedure

1. Check for sick and dead fish **daily** by looking through all tanks. This is best done when feeding. Be sure to look along the sides both at the top and bottom of the tank and near the outflow baffle.
2. When done initial and record sick and dead fish on the daily checklist. Symptoms of Sick and Distressed fish are posted in the fish room.

- If dead fish is found:
    1. Make note of the number of fish, tank space, and what stock the fish was from on the daily check list.
    2. With a clean net, remove fish and place into fish morgue. (The fish morgue can be found in the chest freezer. It is a small bucket labeled “Fish Morgue” on the lid).
- If sick/dead fish are found:
    1. If sick - make a note on the tank and contact supervisor.

> ! This is a callout IMPORTANT
>
> **If there are more than 3 sick or dead fish in any one tank please contact Mark Currey 541-505-0006 or Bill Cresko 541-285-5446 immediately.**

16.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

17 Mysid Culture

17.1 Introduction

- **Purpose**: This procedure describes standard practices for culturing mysids to feed to the pipefish. We will use 10.5 L tanks on the dedicated pipefish Aquaneering rack. The basic premise is that each tank will be collected once a month and reset with a subset of the collected shrimp and the rest of the collected shrimp fed to the pipefish, kind of like a sour dough culture.
- **Procedure Type**: Husbandry
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: Tiffany Thornton, Mark Currey
- **Date Created**: 2023

17.2 Materials

- 10.5 L Aquaneering tanks/rack plumbed to the common pipefish sump.
- 6" Brine shrimp nets.
- Plastic containers for collecting.

17.3 Procedure:

1. **Feeding**
   - Feed each mysid tank with newly hatched brine shrimp supplemented with Selcon twice daily. Ideally this is done when feeding the stickleback.
2. **Water Change**
   - 2-3 times per week. The mysid tanks are connected to the pipefish water system so will experience a water change along with the pipefish. See pipefish water change SOP.

> **i** This is a callout NOTE
>
> Mysids will only be collected Monday through Friday.

3. **Mysid Collection and Tank Reset (Daily - except on weekends)**
    - Turn off water to the oldest tank as noted by the date placed on the front of the tank.
    - Take the 10.5 L tank to the sink.
    - Obtain a clean 10.5 L tank and remove the green baffle and place a screen in the slots at the rear of the tank. Place this tank in the empty slot and start the water. Record the date with a piece of tape on the front of the tank.
    - Using the clip light for illumination, a white 6" brine shrimp net, and two plastic containers collect all of the mysids from the tank and place into the plastic containers. Place ~ 20 mysids into container #1. Collect the rest of the mysids and place in container #2.
    - Put all the mysids from container #1 into the empty "reset" tank.
    - Feed the mysids in container #2 to the adult pipefish.

17.4 Associated Papers

- xxx
- xxx
- xxx
- xxx

18 Stock Number Assignment

18.1 Introduction

- **Purpose**: To track stickleback crosses, families, lines and collections.
- **Procedure Type**: xxx
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
  - Bay pipefish, (*Syngnathus leptorhyncus*)
  - Gulf pipefish (*Syngnathus scovelli*)
  - Zebrafish, (*Danio rerio*)
- **Author**: Mark Currey
- **Date Created**: February 12, 2024

##Materials N/A

##Sloutions N/A

> ⚠ NOTE
>
> If you have not been given access to the stickleback database please ask Mark C to do this.

18.2 Procedure

##Wild Collections

1. Go to the stickleback website, https://sticklebackdb.uoregon.edu/sticklebackdb/ (if you don't have access please see note above).
2. Click on the "stocks" button from the tool bar.
3. Click on "New Stock from Capture".
4. Find "capture population" from drop down list. If it is a new location add it by clicking the "add new population" button.

5. Enter capture date, line, and stock name information. The convention for wild collections is “wild caught” - “location”(e.g. Wild Caught - Cushman Slough).
6. Highlight protocol information.
7. Click the “create” button.
8. Record information in the crossing book.

##Lab Crosses

1. Go to the stickleback website, https://sticklebackdb.uoregon.edu/sticklebackdb/ (if you don’t have access please see note above).
2. Click on the “stocks” button from the tool bar.
3. Click on “New Stock from Breeding”.
4. Add stock name information. The convention for crosses is “stock - line” (e.g. Stock - Cushman Slough) or if it’s an experimental cross, “line - experiment information” (Cushman - CRISPR - FGF16 CNE KO).

> ⚠ NOTE
>
> If it is a new experiment (e.g. a new CRISPR target) you will need to create a name by simply typing it in.

5. Add line information from drop down list.
6. Add maternal ID or add a new maternal individual.
7. Add paternal ID or add a new paternal individual.
8. Add the number of eggs and number of fertilized embryos.
9. Add protocol information.
10. Add any Label notes or comments
11. Click the “create” button.
12. Record information in the crossing book.

19 UV Filter Bulb

19.1 Introduction

- **Purpose**: Properly maintain and change UV bulbs in recirculating system in 310 Pacific Hall.
- **Procedure Type**: Husbandry
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: xxx
- **Date Created**: xxx

19.2 Materials

- New UV bulbs
- Stickleback system – 120 Watt
- Pipefish system – 40 Watt
- Gloves
- High vacuum grease
- Replacement Seals
- Cloth for cleaning bulb housing
- Bucket

19.3 Solutions

- NA

19.4 Procedure

> ⚠ NOTES
>
> - Always wear gloves when handling UV bulbs as oils from your hands can ruin the bulb.
> - If bulb is not on it is probably because the prongs are incorrect.
> - Take bulb back out and change the way the bulb is plugged in.
> - Bulbs should be replaced once per year.

1. Turn off power to UV filter by unplugging power cord
2. Remove bulb by unscrewing plastic bulb bolt (grey threaded bolt at end of filter).
3. Unplug bulb noting prong placement.
4. Plug in new bulb with same prong placement as old bulb.
5. Screw in bolt and UV.
6. Turn power back on by plugging filter back in.
7. Check to see that UV is working by looking for light through “viewing window” at the end of the filter.
8. Update changed/next change needed sticker on outside of unit.

19.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

20 Weekday Feeding & Health Checks

20.1 Introduction

- **Purpose**: This procedure describes how to conduct weekday feedings and health checks for stickleback and pipefish
- **Procedure Type**: Husbandry
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: Mark Currey
- **Date Created**: December 15, 2023; modified February 29, 2024

20.2 Materials

- Adult stickleback food
- Juvenile stickleback food
- Brine shrimp
- Squirt bottle
- De-capsulated brine shrimp
- Rock salt
- 105 m mesh shrimp collector
- Baking soda
- Frozen mysids
- 1000 ml beaker labeled “Pipefish Only”
- Net
- Container to hold fish while being euthanized
- Postmortem fixing equipment
- Morgue

Adult and Juvenile mixes can be found here: {#sec-recipe-dry_fish_food}

20.3 Solutions

- Euthanasia strength Mesab (located in Mark's fridge in 324 Pacific)

20.4 Procedure

> ⚠ NOTES
>
> Fish will be fed 2x per day on weekdays, once in morning and another in the afternoon ideally spread as far apart as schedules will allow.

1. **Conduct health checks** of all fish in Winter Room and Summer Room according to the steps described in {#sec-husbandry-fish_health_check}.
2. Prepare brine cone for feeding by following the steps described in {#sec-husbandry-brine_gen_hatch_feed}.

> ⚠ NOTES
>
> When feeding brine shrimp in the morning only use half of the shrimp to leave the other half do evening feeding. When feeding brine shrimp in the evening please reset the brine cone {#sec-husbandry-brine_gen_hatch_feed}.

3. **Feed Stickleback**
    1. Winter Room
        - 20g tanks
            1. Feed 1 scoop of adult stickleback food.
    2. Summer Room
        - 20g tanks
            1. Feed 1 scoop of adult stickleback food.
        - 4.5 and 10.5 liter tanks
            1. **Larval** 4.5 liter tanks: feed small squirt of brine shrimp.
            2. **Juvenile** 10.5 liter tanks: feed half of a scoop of juvenile food.

> ℹ NOTES
>
> If there is not sufficient adult or juvenile food to conduct feedings, see {#sec-recipe-dry_fish_food} for instructions on making more food.

4. Feed Pipefish

> ⚠ NOTES
>
> Please move any male that has given birth into a community (20g) adult pipefish tank

AM - 20g tanks (adults and juveniles) 1. Frozen mysids 2. Squirt of brine shrimp

- 10.5 liter tanks (mysids, pregnant males, and juvenile pipefish) 1. Squirt of brine shrimp

PM - 20g tanks (adults and juveniles) 1. Frozen mysids 2. Squirt of brine shrimp

- 10.5 liter tanks (mysids, pregnant males, and juvenile pipefish) 1. Squirt of brine shrimp 1. Frozen mysids 2. **Live mysids** {#sec-husb-mysid_culture}

> ⚠ NOTE FOR PREPARING AND FEEDING FROZEN MYSIDS
>
> Partially fill the 1000 ml beaker labeled “Pipefish Only” located on the pipefish rack with pipefish water. Place a dime sized piece of frozen mysids (bottom shelf in freezer) in the beaker with water, and swirl water to thaw mysids. Once thawed, feed to the adult pipefish.

20.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

21 Stickleback Adult Euthanasia

21.1 Introduction

- **Purpose**: This procedure describes best practices for euthanizing adult threespine stickleback.
- **Procedure Type**: Husbandry
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
- **Author**: Mark Currey
- **Date Created**: May 6, 2008; revised November 29, 2017 and 2023 by Mark Currey

21.2 Materials

- Euthanasia strength Mesab (see below)
- Container to hold fish while being euthanized.
- Post mortem fixing equipment
- Morgue

21.3 Solutions

> ⚠ NOTES
>
> `Tricaine must be pharmaceutical-grade. We use tricaine purchased from Pentair, manufactured`

- **Mesab Stock Solution (4g/L From Zebrafish Book 4th edition) (tris buffered)**:
  - 4 g tricaine powder
  - 979 ml DD water
  - ~21 ml 1 M Tris (pH 9).
  - Adjust pH to ~7.

  - Aliquot in 50ml tubes, label with MESAB Stock Solution 4g/L, and store in a -20 freezer.
  - This makes 1 liter of solution.

- **Euthanasia Solution (300 mg/L)**:

  - Make a solution of tris buffered **Stock Solution** as described above. (Or obtain an aliquot from the freezer)
  - Make a solution of 3.0 ppt Instant Ocean by adding 0.3 grams of Instant Ocean to 100 ml of DD water.
  - Combine 7.5ml of stock solution into 100 ml of 3 ppt Instant Ocean.
  - Store solution in an amber bottle to protect it from light and add mixed and expiration dates (6 months after being mixed) to the bottle.

21.4 Procedure

1. Place fish in mesab and wait 10 minutes.
2. **Describe how death will be confirmed**:

   - Ten minutes after opercular movement a tap test will be performed by gently tapping on the table next to the container holding the fish. If the fish is still alive this will trigger a startle response, wait 5 more minutes and repeat tap test. If no response, proceed to fixing for post mortem experiments or place fish in the morgue.

21.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

22 Pipefish Embryo & Larval Euthanasia

22.1 Introduction

- **Purpose**: This procedure describes best practices for euthanizing embryonic and larval pipefish.
- **Procedure Type**: Husbandry
- **Species**:
  - Bay pipefish, (*Syngnathus leptorhyncus*)
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: Mark Currey
- **Date Created**: 2023

22.2 Materials

- Euthanasia strength Mesab (see below)
- Container to hold fish while being euthanized
- Post mortem fixing equipment
- Morgue

22.3 Solutions

> ⚠ NOTES
>
> `Tricaine must be pharmaceutical-grade. We use tricaine purchased from Pentair, manufact`

> 🔥 This is a callout CAUTON
>
> When handling Mesab please wear gloves and wash your hand after use.

- **Mesab Stock Solution (4g/L, From Zebrafish Book 4th edition) (tris buffered)**:

  - 4 g tricaine powder
  - 979 ml DD water
  - ~21 ml 1 M Tris (pH 9)
  - Adjust pH to ~7
  - Aliquot in 50ml tubes, label with MESAB Stock Solution 4g/L, and store in a -20 freezer
  - This makes 1 liter of solution

- **Euthanasia Solution (300 mg/L)**:
  - Make a solution of tris buffered **Stock Solution** as described above. (Or obtain an aliquot from the freezer)
  - Combine 7.5ml of stock solution into 100 ml of fish water

22.4 Procedure

1. Procedure for embryo euthanasia:
    1. Place embryos into a MS-222 euthanasia solution.
    2. Leave embryos in solution until movement has stopped, ~ 10 minutes.
    3. If fish is to be used for experiments, proceed with fixation or preparation for the experiment.
    4. If the fish are to be disposed of, place embryo in 95% ETOH for 5 minutes and then into morgue for disposal.

> ⚠ NOTES
>
> `This step is necessary as a startle response is not obvious in unhatched fish.`

2. Procedure for larval euthanasia:
    1. Place larva into a MS-222 euthanasia solution.
    2. Wait 10 minutes and perform a tap test to look for a startle response. If there is a response wait 5 minutes and repeat startle response test. Repeat this step every 5 minutes as needed. If there is no response move to fixing for post mortem experiments or place in morgue.

22.5 Associated Papers

- xxx
- xxx

- xxx
- xxx

23 Dishwasher Use

23.1 Introduction

- **Purpose**: This procedure describes how to use the dishwasher to clean plastics, glassware, and nets in the Pacific fish facility.
- **Procedure Type**: Husbandry
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: J. Crandall
- **Date Created**: April 2016

23.2 Materials

- Dishwasher soap

23.3 Solutions

- none

23.4 Procedure

> **NOTE**
>
> Bulkheads, PVC fittings, and PVC pipefish should be cleaned with bleach and sodium thiosulfate (see xxxxxSOP).

- Loading the dishwasher:

1. If there are clean dishes from a previous cycle, put them away in their respective locations. If some are still wet, set them out to dry on the drying rack before putting them away.
2. RINSE ALL DIRTY DISHES VERY WELL, especially those with algae/food residue. the dishwasher will sanitize, but will not effectively clean, the dishes. Scrub dishes with a scrub pad to remove buildup, if needed.
3. Small tank lids should be stacked on the bottom rack, but not on the raised portion of the rack, as this impedes the rinser from spinning properly.
4. Plastic tanks, Ziploc containers should be placed on the top rack.
5. Nets can be placed on both racks.

> **NOTE**
>
> BEFORE RUNNING THE DISHWASHER, SLIDE BOTH RACKS IN AND MAKE SURE THE RINSER ON THE BOTTOM OF THE TOP RACK CAN SPIN FREELY

> **NOTE**
>
> Once per week (ON THURSDAY) include temperature tape

- Running the dishwasher:

  1. Add ~1/2 scoop of dish detergent (found under the sink) to the well in the door of the dishwasher. Close the lid to the well.
  2. Close and latch the door to the dishwasher. The display will light up, and after booting up it should read "User 1." Press the RUN/CANCEL button to begin the cycle. If it instead displays a list of programs, use the down arrow key to navigate down the list to "User 1." When the "User 1" program is highlighted, press the RUN/CANCEL button to begin the cycle. If the screen displays something other than "User 1," press the DISPLAY button to show the list of programs, scroll down to "User 1," and press RUN/CANCEL.
  3. Should the program ever need to be cancelled mid-cycle, pressing the RUN/CANCEL button once will result in the cycle canceling and the water draining.

- Cleaning large tank lids

  1. The small frontal lids to the large glass tanks should be loaded into the dishwasher. The large lids to the large glass tanks should be hosed off thoroughly with very hot water, and set on the rack to dry.

23.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

24 Brine Shrimp Culture

24.1 Introduction

- **Purpose**: This procedure describes the hatching and feeding of brine shrimp as well as how to reset the brine cone.
- **Procedure Type**: Husbandry
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: Mark Currey
- **Date Created**: May 6, 2008; updated February 5, 2024 Mark Currey

24.2 Materials

- Brine Shrimp
- Rock Salt
- 105 m mesh shrimp collector
- Baking Soda
- Squirt Bottle

24.3 Solutions

- Selcon

24.4 Procedure

1. Remove air bubbler, place a light at the bottom of the cone, and allow the shrimp to collect at the bottom

2. Collect Brine Shrimp by draining the brine through the two system brine shrimp collector.

> **NOTES**
>
> a. Only collect the bottom portion of the cone (only the orange 4-5 inches)
> b. It will take awhile for the brine shrimp to separate from the cysts. The brine shrimp colletion system can be placed on a stand while water is run through it from the sink.
> c. Do not use the spary nozzle to force the brine shrimp through net 1 as it will destroy the shrimp.

3. Reset Brine Cone: Fill cone with DI water to 10 L. Put airline and heater (set at 80°F) into cone. Add **300 ml of rock salt**, **1 scoop (5 ml) of baking soda**, and **1 ml of Selcon**. Obtain brine shrimp from refrigerator. Measure out **30 ml of dry brine cysts** and add it to the cone.

   > **NOTE**
   >
   > The amount of brine shrimp needed will vary depending on the number of juvenile fish that need to be fed. If there is a need for more brine shrimp add more brine shrimp cysts to cone and leave a note for the next person. Increase the amount in 30 ml increments.

4. If enriching with a High-Carotenoid Marine Algae see Brine Shrimp Enrichment SOP.
5. Wait 24 hours and repeat.

24.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

25 Incubator & Stickleback Fry

25.1 Introduction

- **Purpose**: This procedure describes best practices for the use of the embryo incubator.
- **Procedure Type**: Husbandry
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
- **Author**: Mark Currey
- **Date Created**: April 1, 2008

25.2 Materials

- Petri Dishes
- Incubator
- Fry food
- Checklist

25.3 Solutions

- Embryo Medium (found above incubator)

25.4 Procedure

> **!** This is important
>
> Researchers are responsible for monitoring and caring of their research animals while in the incubator. This includes water changes, removing ?deads?, fixing, euthanizing and any other procedures regarding the fish. If the fish are to be raised past hatching please contact Mark, prior to hatching, to make arrangements for putting fish into the system. Also, please see Mark prior to initial use of incubator for brief training.

Procedure:

1. Raise Embryos (check daily):

    - Embryos will develop at approximately 2.5 times zebrafish time [see http://zfin.org/zf_info/zfbook/st
    and hatch at approximately 7 days. 48hr stickleback embryos have completed most major morphogenetic processes, and the melanic pigment cells are just starting to migrate. A beating heart can be seen after ~72hr. Check petri dishes daily and remove deads and those with arrested development. Change embryo medium at 3-4 dpf and more if warranted.

2. Hatching, Begin Feeding Brine Shrimp, and Move to System (t = 7-8 days post fertilization):

    - After hatching at 7-8 days post fertilization, remove chorions and allow young to absorb yolk (2-3 days). At approximately day 2 post hatching take petri dish containing fry out of incubator and place dishes on a table. To each petri dish add approximately the same volume of system water, as there is embryo medium; this will double the volume of liquid in the dish. This acclimates the fry to the system water. Allow the fry to acclimate for 15-20 minutes, the longer the better. Then transfer fry to small aquaria on the system. Feed newly hatched brine shrimp 1-2 times daily according to directions posted in the fish room.

3. **Initial checklist and add what type of check was done to checklist.**

25.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

26 Room Light Programming

26.1 Introduction

- **Purpose**: This procedure describes how to set and or change the lighting regime in 310 A,B,C.
- **Procedure Type**: xxx
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
  - Bay pipefish, (*Syngnathus leptorhyncus*)
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: xxx
- **Date Created**: xxx

> ⚠ NOTES
>
> To check if the lighting is programmed correctly use a data logger to track the light levels over the course of at least 24 hours.

26.2 Materials

2. Older PC located in the closet outside the animal facility.
3. Liason software and manual.

26.3 Procedure

Standard approach

- Programming of fish room lighting regimes is done using the wall unit Grafik Eye 3000 and a PC.
- The unit is programmed in terms of scenes.

- Each scene specifies a lighting event, e.g. turning on the lights in one room over a 1/2 hour time period.
- Scenes are set at the control unit following the directions in the manual.
- Four scenes have been set up to turn off or on lighting in the main room and the breeding room.

Scene 1: **Main room ON** with 30 min dawn.

Scene 2: **Main room OFF** with 30 min dusk.

Scene 3: **Breeding room ON** with 30 min dawn.

Scene 4: **Breeding room OFF** with 30 min dusk.

- Once the scenes are programmed you must tell the unit when to activate the scenes.
- This is done with a PC and Grafik Eye Liason software.
- If the software needs to be installed do so.
- Connect the PC to the control unit.

In EDIT MODE,

1. Select control unit (I think that this unit is GRX3105).
2. Go to schedule (Not program).
3. Drag scenes (upper right box) to desired times.
4. Drag control unit (lower right box) to each scene.
5. Be sure and copy schedule to the weekend.
6. Go to FILE and establish communications via Online Options.
7. After communications have been established go back to edit mode.
8. Go to Online Options and select transfer data. This should program the wall unit when to activate scenes.

Alternate Version:

1. Open program located on PC desktop
2. Select create or edit
3. Under file and select sticklePAC310.LIA and OPEN
4. Under Online options select ESTABLISH COMMUNICATIONS
5. Under timeclock select SCHEDULE EVENTS
6. Drag scenes (upper right box) to desired times
7. Drag control unit (lower right box) to each scene
8. Be sure and copy schedule to the weekend
9. Go to FILE and establish communications via Online Options.
10. After communications have been established go back to edit mode

11. Go to Online Options and select transfer data. This should program the wall unit when to activate scenes.

27 Animal Facility Cleaning

27.1 Introduction

- **Purpose**: Procedure for keeping animal facility clean and disinfected
- **Procedure Type**: xxx
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
  - Bay pipefish, (*Syngnathus leptorhyncus*)
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: xxx
- **Date Created**: xxx

> Chemical Hygiene
>
> When using bleach and/or sodium thiosulfate. Eye protection is required. Please use splash proof glasses or a face shield when using bleach and sodium thiosulfate.

27.2 Materials

- Mop and broom
- Sponges
- Bleach

27.3 Solutions

- **Bleach solution**: Make a 10% bleach solution in a 2 gallon bucket. Add 4.5 L of water. Add 0.5 L of bleach and gently stir.

27.4 Procedure

- Once per week the floors will be swept and mopped using a 10% bleach solution.
- Once per week counter tops will be cleaned with sponge and a 10% bleach solution.
- Broken tanks are to be taken to the dumpster located at the northeast corner of Pacific Hall.
- Dishes will be done daily (see Plastic and Glass Cleaning SOP.

28 Stickleback Adult Anesthesia

28.1 Introduction

- **Purpose**: This protocol descibes how to anesthitize adult threespine stickleback
- **Procedure Type**: Anesthesia
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
- **Author**: Mark Currey
- **Date Created**: February 28th, 2024

28.2 Materials

- Petri dishes or fish container

28.3 Solutions

- MS-222 Stock solution of (4g/L, From Zebrafish Book 4th edition)

> ⚠ NOTES
>
> Tricaine must be pharmaceutical-grade. We use tricaine purchased from Pentair, manufactured

Mesab Stock Solution (4g/L) (tris buffered)

```
-   4 g tricaine powder
-   979 ml DD water
-   \~21 ml 1 M Tris (pH 9).
-   Adjust pH to \~7.
-   Aliquot in 50ml tubes, label with MESAB Stock Solution 4g/L, and store in a -20 freezer.
-   This makes 1 liter of solution.
```

Anesthesia (168 mg/L) - Make a solution of tris buffered Stock solution MS-222 solution as described above. (Or obtain a pre-made solution from freezer) - Make a solution of 3 ppt Instant Ocean by adding 0.3 grams of Instant Ocean to 100 ml of DD water. - Combine 7.5ml of stock solution into 100 ml of 3 ppt Instant Ocean. - Store solution in an amber bottle to protect it from light and add mixed and expiration dates (6 months after being mixed) to the bottle.

28.4 Procedure:

1. Place fish into 168 mg/l MS-222 solution and wait for the fish to slow down.
2. Image or do other experiments quickly to minimize fish exposure to MS-222. Pay attention
3. Revive by placing in fish water and moving fish gently through the water to pass water o

28.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

29 Stickleback Density Standards

29.1 Introduction

- **Purpose**: This document describes stickleback and pipefish density standards that are used in the Cresko Lab.
- **Procedure Type**: Husbandry
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: Mark Currey
- **Date Created**: January 01, 2020, updated February 05, 2024

29.2 Materials

- N/A

29.3 Solutions

- N/A

29.4 Procedure:

> **i** NOTES
>
> - Fry and early juveniles stickleback are kept in short day conditions 12 hours of light/day
> - Adult stickleback are kept in the long day room with 12 hours of light/day.
> - All pipefish are kept in the long day room with 12 hours of light/day.
> - These standards for fish densities are what we in the Cresko lab have determined

> optimal after 20 years of keeping threespine stickleback and 8 years of keeping pipefish in recirculating aquaculture systems.

Stickleback Fry (9dpf - 2 months)

- Fry are kept in 4.5 L tanks at an ideal density of 20 fish per container.
- Fry can be kept in densities of up to 40 fish per tank.
- If fish densities are near 40 per tank fish are transferred or thinned after 1 month of age.

Stickleback Juveniles (2 months - 3 months) Early Grow Out

- Juvenile fish are transferred from fry tanks into 10.5 L tanks and kept at a density of 20 fish per container.

29.5 Stickleback post-Juvenile (3 months - 1 year) Late Grow Out

- Fish older are then 3 months are transferred into 20 gallon tanks (if not already in a 20 gallon) and grown to 1 year of age in the Winter room.

29.6 Stickleback Adults (1 year - 1.5 years) Breeding:

- Adult fish are transferred to 20 gallon tanks in the long day room.
- Fish can be kept at a density of 40 fish per tank if space is needed.
- Adult fish become sexually mature 4-6 weeks after being in long day conditions and will stay in breeding condition for 1-3 months.

29.7 Pipefish

- Pregnant males are placed in 10.5 L tanks and moved back to 20 gallon tanks when the haev given birth.
- Young pipefish (0-3 months) are grown in 10.5 L tanks at the density they are born at.
- Adult pipefish are kept in mixed gender 20 gallon tanks at no more than 15 fish per tank.

30 Stickleback Crosses

30.1 Introduction

- **Purpose**: The procedure describes the creation of stickleback crosses via invitro fertilization.
- **Procedure Type**: Husbandry
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
- **Author**: Mark Currey
- **Date Created**: created March 4th, 2004, updated February 2nd, 2024

30.2 Materials:

- 25 and 90mm disposable sterile petri dishes (standard)
- Fine scissors and forceps
- Wide-blade entomology forceps
- Squeeze wash bottles
- Stress coat (standard from pet store)
- Sterile flat razor blades
- Sterile disposable large bore transfer pipettes (VWR cat# 691)
- Dissecting microscope

30.3 Solutions

- Testes Solution {#sec-recipes_testes_solution}
- MS222 Tricaine Methanesulfonate {#sec-recipes_Mesab}
- 95% ethanol
- Embryo medium {#sec-recipes_embryo_medium_solution}

30.4 Procedures

Squeeze Female

Gravid females can be identified by those that have large rounded abdomens and a slightly extended cloaca. If you can see individual eggs in the abdomen this is an indication that the eggs are old.

1. Cover fingers with stress coat and gently squeeze gravid female.
2. If eggs do not emerge with very slight pressure the female is not quite ready or the eggs are too old.
3. Remove eggs by slightly squeezing the abdomen starting just posterior of the pectoral girdle towards the cloaca expelling the eggs into a 25mm sterile petri dish.
4. Note the stock number of the female on the petri dish.

The small size of petri dish allows the sperm to be concentrated on the eggs.

Dissect Testes From Male

5. Euthanize male by placing him into a small container containing a lethal volume of MS222.
6. Clean a large, 30cm by 60cm or similar size, glass sheet, fine scissors, and forceps with 95% ethanol.
7. When male is motionless and not responding in accordance with the Stickleback Adult Euthanasia procedure {#sec-husbandry_stickleback_adult_euthansisa}, remove and the fish and place on the glass sheet. Sever the spinal cord behind skull using a razor to be sure of euthanization.
8. Use scissors to make incision from the cloaca anteriorly to the pelvic girdle making the incision along the mid line of the fish.
9. Make another cut to each side of the fish from the cloaca dorsally approximately 15mm so that the body cavity is easily accessed.

Incisions should be made just deep enough to cut the skin and body wall muscle while being wary to not cut into the stomach and intestines that are located just below the skin surface.

10. Locate paired testes and vas deferens.

Testes are variable in shape and coloration, but are usually long and pigmented, usually having the same pigmentation as the skin surface, and sit in the dorsal part of the coelom near the kidneys. The vas deferens are threadlike and are usually as long as the testis to which it connects.

11. Use fine forceps to grab vas deferens, sever near the cloaca, and remove one (or often) both testes.

Doing so keeps the testes intact, allowing them to contain viable sperm for up to 1 month at 4°C in Testes Solution.

Storing of Testes

12. If only a single cross is to be made with a male, proceed to step 4.
13. Otherwise, place testes into 15 ml tube filled with cold Testes Solution and label tube with make stock number and date.
14. Store testes at 4°C.
15. To store testes for extended periods, change out medium once a week.

Prepare Testis

16. If multiple crosses are to be performed from a single pair of male's testes, place both testes on the inside lid of the 25mm petri dish into which a females eggs have been squeezed.
17. Using a sterile razor to macerate testis.

Under a dissecting scope swimming sperm should cause a 'sparkling' refraction.

18. Add approximately 1.0 ml of sterile embryo medium to testes prep with disposable pipette, mix, and then add to the eggs one drop at a time, using same disposable pipette, dispersing drops between petri dishes so that all eggs are covered with sperm mixture. Label petri dish(s) with male stock number and time/date of fertilization.
19. Leave sperm on the eggs for about 5-10 minutes.
20. Add embryo medium to cover the embryos.
21. Place in incubator at 20°C.

Separation of Embryos and Second Cleaning (t = 2 hr)

The animal pole of the embryo is established at the site of sperm entry, and at 20°C the first cell syncitium is usually visible within 45 minutes. The first cell division usually occurs approximately 25 minutes later, and the two cell stage is fully visible approximately 1.5 - 2 hr post fertilization. During this time, the chorion thickens and attaches to the petri dish as well as to other embryos.

22. After 2 hours post fertilization, use the wide blade entomological forceps to detach the embryos from one another, as well as to dislodge embryos from the bottom of the petri dish.
23. Remove all of the unfertilized eggs to limit mold contamination, and record the total number of eggs and embryos.
24. Rinse embryos 3 to 4 more times then distribute embryos to 90mm petri dishes (30-50 embryos per dish) filled half way with fresh embryo medium.
25. Enter cross information into database, print labels for dishes, and place embryos into incubator at 20°C.

Raise Embryos (check daily)

Embryos will develop at approximately 2.5 times zebrafish time [see http://zfin.org/zf_info/zfbook/stages/index.html], and hatch at approximately 7-9 days. 48 to 52 hpf stickleback embryos have completed most major morphogenetic processes, and the melanic pigment cells are just starting to migrate. A beating heart can be seen after ~72hr.

26. Check petri dishes daily and remove embryos that have arrested development.
27. Change of embryo medium at 3-4 dpf and more if water has fouled.
28. Record health checks on incubator checklist.

30.5 Place embryos into aquaculture system

29. Prepare small 4.5 L fry tank by adding 400 micron baffle and tuirning in the system water to the tank while letting it flush for 15 minutes.

If the plumbing line has set stagnent for awhile flush the line by turning on the valve and expressing system water into a small (not the tank) container.

30. Once embryos have hatched and thier yolks have absorbed (~9 dpf) add aquaculture system water to dish and let sit 15 minutes.
31. Gently add the fry to the preppared tank making sure all fry have been added.

31 Stickleback Dry Fish Food Mix

31.1 Introduction

- **Purpose**: This procedure describes standard practices for mixing of dry food to feed all life stages of threespine stickleback in the Pacific Stickleback Facility.
- **Procedure Type**: Husbandry
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
- **Author**: Mark Currey
- **Date Created**: August 25, 2014; updated January 26, 2023 by Mark Currey

31.2 Materials

- Plastic measuring cup
- Clean food container

31.3 Solutions

- none

31.4 Procedure

> ⚠ NOTES
>
> At each new mixing of dry food (approximately once per week) food containers will be cleaned (washed in the dish washer) and spoons cleaned with 70% ETOH. The beaker that holds feeding spoons will also be replaced with clean beaker.

1. Mix equal parts of the following, store at 4C in fish facility food fridge. Label with an expiration date of 1 month from mixing.

Fry Mix: 1. 100-200 micron golden pearls 2. Ziegler AP100

Juvenile Mix: (label with a yellow dot or yellow tape) 1. Aquanix cool mix flakes (or other flake food) 2. 200-300 micron golden pearls 3. Otohime C1 pellets

Adult Mix: 1. Otohime S1 2. Ziegler zebrafish diet 3. Hikari micro pellet 4. Golden pearls 500-800 micron 5. Aquanix cool mix flakes (or other flake food)

> NOTES
>
> If we are out of one of the types of food it is ok to omite it from the mix.

31.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

32 Testes Storage

32.1 Introduction

- **Purpose**: This procedure describes how to store stickleback testes for fertilization up to 1-2 months post extraction.
- **Procedure Type**: Husbandry
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
- **Author**: Mark Currey
- **Date Created**: May 6, 2008; updated January 15, 2015 by Mark Currey

32.2 Materials

- 15 ml falcon tube
- 4°C refrigerator
- Gentamycin (antimycotic) (Stock - 10mg/ml)*
- Cell Culture anti-biotic/mycotic from Gibco-BRL (15240-096) 100x Concentration*

> ⚠ NOTES
>
> *Both of these reagents are located in separate boxes in the scientific -20° C freezer. They are partitioned into 100 l aliquots.

32.3 Solutions

- Ginzberg's Ringers {#sec-recipe_ginzbergs}
- Testes solution {#sec-recipe_testes_solution}

Ginzberg's Ringers - Mix solids into 750 ml of npH2O - 6.6g NaCl - 0.25g KCl - 0.3g $CaCl_2$ - 0.2g $NaHCO_3$ - Bring to 1 liter total volume with npH_2O. - Store at 4° C. **Testes solution** (100ml) - Add 100 l of Gentamycin and 100 l of Anti-biotic/mycotic to 100ml of Ginzburg's Ringers solution. Store at 4°C.

32.4 Procedure

1. Make solutions
2. Dissect testes {#sec-husbandry_stickleback_cross}.
3. Place testes in 15 ml tube with ~10 ml of testes solution.
4. Label tube with stock number and date of testes dissection and place tube with testes in 4°C refrigerator.
5. Change testes solution once per week.

> ⚠ NOTES
>
> Testes can be used up to ~1-2 months when stored this way.

33 Sentinel Fish

33.1 Introduction

- **Purpose**: This protocol describes the setup, care, and processing of sentinel fish for the Pacific Stickleback fish room.
- **Procedure Type**: xxx
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
  - Bay pipefish, (*Syngnathus leptorhyncus*)
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: xxx
- **Date Created**: xxx

> ⚠ NOTES
>
> xxxx

33.2 Materials

- Dietrich's fixative {#sec-general_recipe_dietrichs_fix}
- 50 ml tubes
- Straight razor

33.3 Solutions

- Prepare Dietrich's Fixative (100 ml) as follows & {#sec-general_recipe_dietrichs_fix}
- Store fixative at room temperature.
  - 30 ml Ethanol (95%)
  - 10 ml Formalin (Formaldehyde 37% solution, histological grade, contains 10-15% methanol, Sigma # F1635)
  - 2 ml Glacial Acetic Acid
  - 58 ml Distilled Water

33.4 Procedure

Location and monitoring of sentinel tanks

1. Sentinel fish are placed in tanks in both the Summer and Winter rooms.
2. A subset of a stock cross can be used, allow fish to live in the water system for at least 2 weeks before sampling.
3. Every 6 months four fish from each tank are fixed with Deitrich's fixative.
4. Take fish to the Histology lab for Hematoxylin and Eosin staining and sectioning.
5. Prepared slides are given to Dr. Kathy Snell for diagnosis.

Fixing of Fish and Sectioning

1. Fish are euthanized using MS222.
2. Cut 2 cm segments starting just behind the eye to 2 cm caudally.
3. Put these segments into Dietrich's fix for at least 24 hours.
4. Parafin embedded sagital sections need to be done.
5. Request cross sections through the gills and into the gut.

Fish to Dr. Kathy Snell

1. Arrange drop off of slides to Dr. Snell located in Huestis Hall, snellk@uoregon.edu

34 Embryo Bleaching for Disinfection

34.1 Introduction

- **Purpose**: This protocol allows for the removal of ecto-parasites from embryos produced from wild-caught stickleback. Use this protocol whenever new fish are added to a pre-existing stickleback rearing system, or whenever stocks are transferred between labs.
- **Procedure Type**: Husbandry
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
- **Author**: Mark Currey
- **Date Created**: August 25, 2011, update February 29, 2014

34.2 Materials

- Wash bottle and petri dishes
- Timer

34.3 Solutions

- 6% sodium hypochlorite (standard bleach)
- Working stock of bleach, mix: 500 µl of bleach 1 liter of embryo medium {#sec-general_recipe_embryo_medium}

34.4 Procedure

1. Raise embryos according to standard crossing and rearing protocols {#sec-husbandry_sti
2. At 48-60 hours post-fertilization (@ 20C - at this point most organogenesis is complete
3. Fill petri dish with working stock solution of bleach. Swirl and let sit for 1.5 min. D
4. Drain bleach solution from embryos, and wash them three times with fresh embryo medium

5. Replace embryo medium daily. If crosses are done in the field, the embryos can be shipp
6. When the embryos arrive at the lab, rinse the outside of the containers well before mov

34.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

35 Stickleback Embryo and Larval Euthanasia

35.1 Introduction

- **Purpose**: This procedure describes best practices for euthanizing embryonic and larval threespine stickleback.
- **Procedure Type**: Vertebrate Husbandry
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
- **Author**: Mark Currey
- **Date Created**: April 10, 2010, update January 1, 2014

35.2 Materials

- Container to hold fish while being euthanized.
- Post mortem fixing equipment
- Morgue (if needed)

35.3 Solutions

- Mesab Stock Solution {#sec-general_recipe_mesab}
- Euthanasia strength Mesab (see below)
- **Euthanasia Solution (300 mg/L)**:
  - Make Mesab stock solution or obtain an aliquot from the freezer.
  - Make a solution of 3.0 ppt Instant Ocean by adding 0.3 grams of Instant Ocean to 100 ml of DD water.
  - Combine 7.5ml of stock solution into 100 ml of 3 ppt Instant Ocean.
  - Store solution in an amber bottle to protect it from light and add mixed and expiration dates (6 months after being mixed) to the bottle.

35.4 Procedure

Procedure for embryo euthanasia

1. Place embryos into a MS-222 euthanasia solution.
2. Leave embryos in solution until movement has stopped, ~ 10 minutes.
3. If fish is to be used for experiments, proceed with fixation or preparation for the expe
4. If the fish are to be disposed of, place embryo in 95% ETOH for 5 minutes and then into

> **NOTES**
>
> The last step is necessary as a startle response is not obvious in unhatched fish.

Procedure for larval euthanasia

1. Place larva into a MS-222 euthanasia solution.
2. Wait 10 minutes and perform a tap test to look for a startle response.
3. If there is a response wait 5 minutes and repeat startle response test.
4. Repeat this step every 5 minutes as needed.
5. If there is no response move to fixing for post mortem experiments or place in morgue.

35.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

36 Pipefish Crossing

36.1 Introduction

- **Purpose**: The procedure describes how introduce wild caught gulf pipefish into our aquaculture systems.
- **Procedure Type**: husbandry
- **Species**:
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: Mark Currey
- **Date Created**: February 29, 2024

> **NOTES**
>
> Wild pipefish will arrive very stressed out so they need to be handled carefully.

36.2 Materials

- Empty 20 gallon Tank
- Clean 5 gallon bucket

36.3 Solutions

- none

36.4 Procedure

1. When pipefish arrive remove them from packaging and let float them in system water for 15-30 minutes to accilmate to the temperature.
2. Poor contents of each bag of pipefish into a 5 gallon bucket.

3. Slowly acclimate the new pipefish by adding 1/4 - 1/2 gallon of system water to the 5 gallon bucket over the course of a few hours. Do this until most of the water in the bucket is system water.
4. Move pipefish (not water) to clean 20 gallon tank.
5. Feed lightly for 2-3 days as the fish acclimate to captivity. Slowly introduce frozen mysids along with live mysids.

37 Pipefish Crossing

37.1 Introduction

- **Purpose**: The procedure describes how to set up pipefish for crossing.
- **Procedure Type**: husbnadry
- **Species**:
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: Mark Currey
- **Date Created**: February 29, 2024

> NOTES
>
> Pipefish are housed communally or kept as pairs and allowed to naturally spawn.

37.2 Materials

- Empty 20 gallon Tank

37.3 Solutions

- none

37.4 Procedure

Setting up crosses

1. House pairs of adults or groups of mixed sex pipefish in a 20 gallon tank.
2. Check tanks each day to look for pregnant males.

3. Place pregnant males singly in 4.5 L tanks containing plastic grass with a 850 mesh baffle installed.
4. Once the male has given birth move him to a 20 gallon tank.

38 Pipefish Water System Maintenance

38.1 Introduction

- **Purpose**: This procedure describes maintenance of the pipefish recirculating system. The pipefish system is a ~ 500 gallon recirculating salt water system. Water parameters: 25C, 25ppt salinity, no ammonia, no nitrites, and nitrates below 40 ppm.
- **Procedure Type**: Husbandry
- **Species**:
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: Mark Currey
- **Date Created**: February 29, 2024

38.2 Materials

- Clean 20 gallon tanks
- Clean tank hardware
- Plastic plants
- Gravel vacuum

38.3 Procedure

20 gallon adult pipefish tanks

> ⚠ NOTES
>
> All 20 gallon pipefish tanks are cleaned and reset at least once a month.

- *Complete take down and cleaning*

1. After transferring fish to a clean tank, take down the old tank as described in {#sec-vert_husb-aquarium_cleaning}, but leave the bulkhead on and keep the substrate in the tank.
2. Put the strainer catch over the drain in the sink to prevent substrate from clogging the drain. Tilt the tank vertically so the substrate pools in the back.
3. Reset tank as described in the S{#sec-vert_husb-aquarium_cleaning}, but add at least 2 clean plastic plants to the tank for enrichment.
4. Circulate clean tanks 30 minutes before transferring fish.

> ⚠ NOTES
>
> If there are not enough open tanks to completely clean and reset 3-5 per week, use the gravel vac to clean all occupied tanks at least 1x per week:

- *Gravel Vac cleaning*

  1. Use an empty bucket (with drain drilled in) to catch the water siphoned out of each tank.
  2. Rinse gravel vac and tube with RO water.
  3. Turn off water and air flow to the tank being cleaned.
  4. Create a siphon with the gravel vac to begin draining the tank water into the bin. Keep a section of the gravel vac hose near the end pinched to control suction by pinching down to stop and releasing slightly to restart. Agitate substrate by "fluffing" up and down with the gravel vac.

> ⚠ CAUTION
>
> Do not allow water level to be drained below 5-6 inches in the sump. Immediately stop suction if a pipefish enters the gravel vac. Check the bin for any pipefish that may have been accidentally sucked into the gravel vac before moving to the next tank.

10.5 L pipefish fry tanks

> ℹ NOTE
>
> Fry are generally large enough to be transferred to clean 10L tanks at 2 months post-birth

1. Set up new 10.5 L tanks band place a clean small plastic plant in the tank for enrichment.
2. Uneaten food will build up quickly. Use a turkey baster and muck bucket to spot clean 2x per week or as needed.
3. Replace the back 850 screen baffle in tanks when they appear clogged.

Changing water in the system

> **NOTE**
>
> 10-20% of the system water should be changed every week.

1. Drain system water by attaching a hose to one of the 20 gallon tanks supply placing the other end of the hose in the corner room drain. Turn on valve and allow water to drain until the water level is 3-5 inches above the basket that supplies the system pump (located left side of largest sump under 20 gallon rack).
2. The makeup water vat should be full. Check makeup water salinity with a refractometer. Target salinity is ~25 ppt. Adjust salinity by adding salt or RO water if necessary. Put the end of the makeup water hose into the small sump.
3. Turn makeup water pump on until makeup water drops to the drain line marked on the outside of the makeup water vat, then turn off. Lift the makeup water hose out of the sump and rest on the rim of the makeup water vat.
4. To reset the makeup water vat, use the RO located in the northeast corner of the room to fill the vat up to the fill line. This take about 15 minutes.
5. Use a 5 L beaker to measure 2.5 L of instant ocean and pour into makeup water vat. Recheck salinity after several minutes and adjust as necessary.

38.4 Associated Papers

- xxx
- xxx
- xxx
- xxx

39 Syngnathid Euthanasia

39.1 Introduction

- **Purpose**: This procedure describes best practices for euthanizing embryonic and larval pipefish
- **Procedure Type**: Vertebrate Husbandry
- **Species**:
  - Bay pipefish, (*Syngnathus leptorhyncus*),
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: xxx
- **Date Created**: xxx

> ⚠ NOTES
>
> xxxx

39.2 Materials

- Euthanasia strength Mesab (see above)
- Container to hold fish while being euthanized.
- Post mortem fixing equipment
- Morgue

39.3 Solutions

- MS-222 Stock solution of (4g/L, From Zebrafish Book 4th edition)??
  - Tricaine must be pharmaceutical-grade
  - We use tricaine purchased from Pentair, manufactured by Western Chemical and FDA approved.
  - Tricaine (3-amino benzoic acid ethyl lester also called ethyl m-aminoboenzoate) comes in a powdered form.

  - Purchase the smallest amount possible because tricaine expires quickly.

- Mesab Stock Solution (4g/L) (tris buffered):
  - 4 g tricaine powder
  - 979 ml DD water
  - ~21 ml 1 M Tris (pH 9).
  - Adjust pH to ~7.
  - Aliquot in 50ml tubes, label with MESAB Stock
  - Solution 4g/L, and store in a -20 freezer.
  - This makes 1 liter of solution.

- Euthanasia Solution (300 mg/L):
  - Make a solution of tris buffered Stock Solution as described above.
  - (Or obtain an aliquot from the freezer)
  - Combine 7.5ml of stock solution into 100 ml of fish water.

39.4 Procedure

- Procedure for embryo euthanasia:
  - Place embryos into a MS-222 euthanasia solution.
  - Leave embryos in solution until movement has stopped, ~ 10 minutes.
  - If fish is to be used for experiments, proceed with fixation or preparation for the experiment.
  - If the fish are to be disposed of, place embryo in 95% ETOH for 5 minutes and then into morgue for disposal.

> ⚠ NOTES
>
> This last step is necessary as a startle response is not obvious in unhatched fish

- Procedure for larval euthanasia:
  - Place larva into a MS-222 euthanasia solution.
  - Wait 10 minutes and perform a tap test to look for a startle response.
  - If there is a response wait 5 minutes and repeat startle response test.
  - Repeat this step every 5 minutes as needed.
  - If there is no response move to fixing for post mortem experiments or place in morgue.

40 Pipefish Adult Euthanasia

40.1 Introduction

- **Purpose**: This procedure describes best practices for euthanizing adult pipefish.
- **Procedure Type**: Husbandry
- **Species**:
  - Bay pipefish, (*Syngnathus leptorhyncus*)
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: Mark Currey
- **Date Created**: February 29, 2024

40.2 Materials

- Container to hold fish while being euthanized.
- Post mortem fixing equipment
- Morgue

40.3 Solutions

> ⚠ NOTES
>
> Tricaine must be pharmaceutical-grade. We use tricaine purchased from Pentair, manufactured by Western Chemical and FDA approved. Tricaine (3-amino benzoic acid ethyl lester also called ethyl m-aminoboenzoate) comes in a powdered form. Purchase the smallest amount possible because tricaine expires quickly.

- Euthanasia strength Mesab (see below) and {#sec-general_recipe_mesab}
- **Mesab Stock Solution (4g/L From Zebrafish Book 4th edition) (tris buffered)**:
  - 4 g tricaine powder

  - 979 ml DD water
  - ~21 ml 1 M Tris (pH 9).
  - Adjust pH to ~7.
  - Aliquot in 50ml tubes, label with MESAB Stock Solution 4g/L, and store in a -20 freezer.
  - This makes 1 liter of solution.

- **Euthanasia Solution (300 mg/L)**:

  - Make a solution of tris buffered **Stock Solution** as described above. (Or obtain an aliquot from the freezer)
  - Make a solution of 3.0 ppt Instant Ocean by adding 0.3 grams of Instant Ocean to 100 ml of DD water.
  - Combine 7.5ml of stock solution into 100 ml of 3 ppt Instant Ocean.
  - Store solution in an amber bottle to protect it from light and add mixed and expiration dates (6 months after being mixed) to the bottle.

40.4 Procedure

1. Place fish in euthanasia strength mesab and wait 10 minutes.
2. To confirm death wait ten minutes after opercular movement has stopped and perform a tap test. TO do this gently tap on the table next to the container holding the fish. If the fish is still alive this will trigger a startle response, wait 5 more minutes and repeat tap test. If no response, proceed to fixing for post mortem experiments or place fish in the morgue.

40.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

41 Pipefish Adult Anesthesia

41.1 Introduction

- **Purpose**: This procedure dscribes how to anesthetize adult pipefish
- **Procedure Type**: Anesthesia
- **Species**:
  - Bay pipefish, (*Syngnathus leptorhyncus*)
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: xxx
- **Date Created**: xxx

41.2 Materials

- Petri dishes or fish container

41.3 Solutions

> ⚠ NOTES
>
> `Tricaine must be pharmaceutical-grade. We use tricaine purchased from Pentair, manufactured`

- MS-222 Stock solution {#sec-general_recipe_mesab}

Mesab Stock Solution (4g/L) (tris buffered)

```
-   4 g tricaine powder
-   979 ml DD water
-   \~21 ml 1 M Tris (pH 9).
-   Adjust pH to \~7.
-   Aliquot in 50ml tubes, label with MESAB Stock Solution 4g/L, and store in a -20 freezer.
-   This makes 1 liter of solution.
```

Anesthesia (168 mg/L) - Make a solution of tris buffered Stock solution MS-222 solution as described above. (Or obtain a pre-made solution from freezer) - Make a solution of 3 ppt Instant Ocean by adding 0.3 grams of Instant Ocean to 100 ml of DD water. - Combine 7.5ml of stock solution into 100 ml of 3 ppt Instant Ocean. - Store solution in an amber bottle to protect it from light and add mixed and expiration dates (6 months after being mixed) to the bottle.

41.4 Procedure:

1. Place fish into 168 mg/l MS-222 solution and wait for the fish to slow down.
2. Image or do other experiments quickly to minimize fish exposure to MS-222. Pay attention
3. Revive by placing in fish water and moving fish gently through the water to pass water o

41.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

Part V

Vertebrate Experiment

This section of the manual contains protocols for the safe and ethical experimental use of vertebrate animals, particular the fish models stickleback, zebrafish and syngnathids.

42 Alizarin Staining

Syngnathid fishes

42.1 Introduction

- **Purpose**: This procedure describes how to live alizarin stain pipefish
- **Procedure Type**: Amimal Experimental
- **Species**:
  - Bay pipefish, (*Syngnathus leptorhyncus*),
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: xxx
- **Date Created**: xxx

> ⚠ NOTES
>
> xxxx

42.2 Solutions

- Alizarin stock solutions: 0.5g?Alizarin red in 100ml or in 50ml in sterile water?(SIGMA cat# A5533 Alizarin Red S, certified).?? ?
- Staining Solution: For 1 Liter:?
  - 990 ml embryo medium?(See embryo medium recipe).?
  - Add 10 ml 0.5% or 1% Alizarin Stock in sterile water?for final concentrations of 0.005% or 0.01%.
  - Adjust to pH 7.5 with NaOH ? For 50 ml (enough for 100mm diameter petri dish):?
- 49.5 ml Embryo medium?
- Add 500 ?l 0.5% or 1% Alizarin Stock in sterile water?
- Adjust to pH 7.5 with NaOH

42.3 Procedures

- Place fish into a container containing stain for 1-2 hours for larvae to overnight for juveniles or adult fish in the dark. We have found that fish do not experience adverse effects from being exposed to stain. Monitor fish every 30-60 minutes if possible. To de-stain, rinse thoroughly with embryo medium by placing fish into container of embryo medium without stain for 30 minutes; background continues to go down with time. Move on to DASPEI live staining if desired (see DASPEI live staining SOP) or anesthetize until the fish reaches a light plane of anesthesia (i.e. movement has slowed down enough that the fish can be safely handled) and observe/image.?
- Bone fluorescence will decrease over time, so plan on imaging the same day if possible.?
- Keep fish in the dark as much as is reasonably convenient.?
- After fish has been observed/imaged place in a container of fish water. Monitor fish every 5-10 minutes until the fish is revived. Once fish is revived place back on the fish system and monitor during daily health checks. If the fish is to be fixed for post mortem experiments place fish directly into euthanasia MS222 solution and follow the euthanasia SOP.

42.4 Associated Papers

43 Calcein staining

Syngnathid fishes

43.1 Introduction

- **Purpose**: This procedure describes how to live stain pipefish with calcein
- **Procedure Type**: Animal Experimental
- **Species**:
  - Bay pipefish, (*Syngnathus leptorhyncus*),
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: xxx
- **Date Created**: xxx

> ⚠ NOTES
>
> xxxx

43.2 Materials:

- Petri dishes and/or 1 L tanks
- Calcein (Molecular Probes; cat. C481)

43.3 Solutions:

- MS-222 Anesthesia solution (see fish anesthesia and euthanasia SOP)?
- Artificial sea water
- 10% NaOH
- Stain Solution:
- 0.005 to 0.05% calcein in sea water.
- Adjust pH to 8.2 with NaOH
- Make fresh, keep in dark.

43.4 Procedures:

- Place fish into a container containing stain for 1-2 hours for larvae to overnight for juveniles or adult fish in the dark. We have found that fish do not experience adverse effects from being exposed to stain. Monitor fish every 30-60 minutes if possible. To de-stain, rinse thoroughly with embryo medium by placing fish into container of embryo medium without stain for 30 minutes; background continues to go down with time. Move on to DASPEI live staining if desired (see DASPEI live staining SOP) or anesthetize until the fish reaches a light plane of anesthesia (i.e. movement has slowed down enough that the fish can be safely handled) and observe/image.?
- Bone fluorescence will decrease over time, so plan on imaging the same day if possible.?
- Keep fish in the dark as much as is reasonably convenient.?
- After fish has been observed/imaged place in a container of fish water. Monitor fish every 5-10 minutes until the fish is revived. Once fish is revived place back on the fish system and monitor during daily health checks. If the fish is to be fixed for post mortem experiments place fish directly into euthanasia MS222 solution and follow the euthanasia SOP.

43.5 Associated Papers:

44 DASPEI staining

Syngnathid fishes

44.1 Introduction

- **Purpose**: This procedure describes how to live stain pipefish with DASPEI
- **Procedure Type**: xxx
- **Species**:
  - Bay pipefish, (*Syngnathus leptorhyncus*),
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: Susan Bassham
- **Date Created**: 25 October 2021

> ⚠ xxxx
>
> xxxx

44.2 Materials:

- Petri dishes and/or 1 L tanks
- DASPEI (Sigma Aldrich; cat. D3418)

44.3 Solutions:

- MS-222 Anesthesia solution (see fish anesthesia and euthanasia SOP)?
- Artificial sea water

- Staining Solution: 0.005% DASPEI in sea water

44.4 Procedures:

- Stain larvae in Petri dishes and juveniles/adults in 1 L tanks containing stain solution for 5 to 75 min in the dark; stagger so that no fish stain longer than this before imaging. Rinse for 20 to 60 min in container of sea water without DASPEI to reduce background.?
- Keep fish in the dark as much as is reasonably convenient through procedures. Monitor fish every 15-30 minutes.?
- Anesthetize until the fish reaches a light plane of anesthesia (i.e. movement has slowed down enough that the fish can be safely handled) and observe/image fluorescence immediately.
- After fish has been observed/imaged place in a container of fish water. Monitor fish every 5-10 minutes until the fish is revived. Once fish is revived place back on the fish system and monitor during daily health checks. If the fish is to be fixed for post mortem experiments place fish directly into euthanasia MS222 solution and follow the euthanasia SOP.??
- Staining solution can be stored at 4C and reused.

44.5 Associated Papers

adapted from DOI: 10.1007/s10162-002-3022-x

45 Fin clipping

Syngnathid fishes

45.1 Introduction

- **Purpose**: This procedure describes how to take fin clips from live pipefish.
- **Procedure Type**: Husbandry
- **Species**:
  - Bay pipefish, (*Syngnathus leptorhyncus*),
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: Mark C. Currey
- **Date Created**: 06 April 2010

> ⚠ xxxx
>
> xxxx

45.2 Solutions:

- MS-222 Anesthesia solution (see fish anesthesia and euthanasia SOP)
- Melafix (antifungal and antibacterial agent)

45.3 Materials:

- Beakers of system water
- Forceps and scissors
- Bucket of ice
- 1.5ml tubes

45.4 Procedure:

- To minimize fish?s exposure to MS-222, do all labeling of tubes and vials prior to fin clipping.
- Place fish in beaker containing MS-222 anesthesia dose in system water (168 mg/L).
- Once the breathing of the fish slows take fish out of water and remove caudal fin blade ? carefully avoiding the peduncle - with scissors and forceps. Note: To observe slowed breathing watch the operculum movement. This movement will slow and the fish will turn on its side.
- Place fin in labeled 1.5 ml tube on ice.
- Put fish into clean system water and move gently to force water over gills.
- Once fish is revived and swimming upright, put fish into new container with Melafix (diluted in system water according to manufacturer?s recommendations) for 15 minutes.
- Return fish to aquaculture system.

46 Syngnathid wild collections

46.1 Introduction

- **Purpose**: This procedure describes how to collect wild pipefish. Please note that new collectors should go with an experienced collector the first time to learn how to handle collecting devices and how to handle pipefish and by-catch.
- **Procedure Type**: Field
- **Species**:
  - Bay pipefish, (*Syngnathus leptorhyncus*),
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: Mark Currey
- **Date Created**: September 10, 2012

46.2 Materials

- Minnow Traps
- Seine
- Dip nets
- Field Notebook
- 500 and 1000ml Nalgene bottles
- Bucket
- Net
- Mouse cage
- Gloves

> ⚠ OPTIONAL
>
> - Waders
> - Kayak

46.3 Solutions

- 95% ETOH
- Mesab (see fish Euthanasia SOP)

46.4 Procedure

- **Minnow trap collecting**
  - Place traps in areas with cover.
  - Tether trap to shore or other non-movable object.
  - Leave traps up to but not over 24 hours. Traps may need to be checked more frequently depending on the tidal influence of water body being investigated.
  - Pull traps looking for endangered species such as salmonids, Oregon chub, and/or Bull Trout. If an endangered species is noticed open trap under water and release all of the fish.
  - If no endangered species are noticed, remove trap from the water and empty contents in a bucket filled 1/3 with water from where the trapping is taking place.
  - Remove, count, and record all pipefish and non-pipefish species. This will be used for ODFW reporting at the end of the year.
  - Record (in field notebook); collecting location, GPS coordinates, water temp, water type, water condition, substrate condition, vegetation condition, pH (if possible), D.O. (if possible), and other environmental measurements.
  - Use dip net and/or siene to capture fish, return all non-targeted species immediately back into the water.

> ⚠ TRAP PLACEMENT
>
> Care should be taken when setting traps in tidally influenced waters as traps that are set above the low water line have the potential to leave fish without water resulting in fish fatality.

- **Trap Disinfection**:
  - Let minnow traps and other collecting equipment air dry for ~ 1 week between collecting locations.
  - Further steps may be needed depending on the invasive species encountered. Updates will come soon.

> ⚠ TRAP DISINFECTION
>
> When trapping between watersheds or between different water bodies within a watershed that ODFW has requested that we disinfect our traps and collecting equipment (see comments for current ODFW fish take permit) please do the following.

- **Euthanize and Storage of Collected Fish**:
  - Euthanize fish using MS222 (see Fish Euthanasia and Anesthesia SOP)
  - Collect fish by pouring fish through a net. MS222 can be collected by pouring solution into a secondary container.
  - Place fish into a Nalgene container and fill with 95% ETOH. Do not fill bottle over half full with fish as fluids coming out of fish can dilute ETOH to the point that DNA/Tissue degradation can take place.
  - Return to lab and assign stock #.
  - Alternatively, euthanized fish can be flash frozen by placing in liquid nitrogen and stored in dry ice if high quality DNA is required.

46.5 Associated Papers

- xxx
- xxx
- xxx

47 Stickleback wild collections

47.1 Introduction

- **Purpose**: This procedure describes how to collect wild threespine stickleback.
- **Procedure Type**: Field
- **Species**: Threespine stickleback, (*Gasterosteus aculeatus*)
- **Author**: Mark Currey
- **Date Created**: September 10, 2012

47.2 Materials

- Minnow Traps
- Field Notebook
- 500 and 1000ml Nalgene bottles
- Bucket
- Net
- Mouse cage
- Gloves
- Crossing materials (see stickleback crossing SOP)

> ⚠ OPTIONAL
>
> - Waders
> - Kayak

47.3 Solutions

- 95% ETOH
- Mesab (see fish Euthanasia SOP)

47.4 Procedure

- **Minnow trap collecting**

    1. Place traps in areas with cover.
    2. Tether trap to shore or other non-movable object.
    3. Leave traps up to but not over 24 hours. Traps may need to be checked more frequently depending on the tidal influence of water body being investigated.
    4. Pull traps looking for endangered species such as salmonids, Oregon chub, and/or Bull Trout. If an endangered species is noticed open trap under water and release all of the fish.
    5. If no endangered species are noticed, remove trap from the water and empty contents in a bucket filled 1/3 with water from where the trapping is taking place.
    6. Remove, count, and record all stickleback and non-stickleback species. This will be used for ODFW reporting at the end of the year.
    7. Record (in field notebook); collecting location, GPS coordinates, water temp, water type, water condition, substrate condition, vegetation condition, pH (if possible), D.O. (if possible), and other environmental measurements.

> ⚠ TRAP PLACEMENT
>
> Care should be taken when setting traps in tidally influenced waters as traps that are set above the low water line have the potential to leave fish without water resulting in fish fatality.

- **Trap Disinfection**:

    1. Let minnow traps and other collecting equipment air dry for ~ 1 week between collecting locations.
    2. Further steps may be needed depending on the invasive species encountered. Updates will come soon.

> ⚠ TRAP DISINFECTION
>
> When trapping between watersheds or between different water bodies within a watershed that ODFW has requested that we disinfect our traps and collecting equipment (see comments for current ODFW fish take permit) please do the following.

- **Euthanize and Storage of Collected Fish**:

1. Euthanize fish using MS222 (see Fish Euthanasia and Anesthesia SOP)
2. Collect fish by pouring fish through a net. MS222 can be collected by pouring solution into a secondary container.
3. Place fish into a Nalgene container and fill with 95% ETOH. Do not fill bottle over half full with fish as fluids coming out of fish can dilute ETOH to the point that DNA/Tissue degradation can take place.
4. Return to lab and assign stock #.
5. Alternatively, euthanized fish can be flash frozen by placing in liquid nitrogen and stored in dry ice if high quality DNA is required.

- **Wild crosses introduction into the lab**:
  - Crosses fish (see stickleback crossing SOP).
  - Bleach embryos (see embryo bleaching SOP).
  - Introduce into fish system (see stickleback crossing SOP).
  - If embryos are to be fixed and used for investigation fix embryos (see Embryo and larval euthanasia SOP).

47.5 Associated Papers

- xxx
- xxx
- xxx

48 Dechorionating Zebrafish Embryos

48.1 Introduction

- **Purpose**: This procedure describes how to dechorionate zebrafish embryos.
- **Procedure Type**: Vertebrate experiment
- **Species**:
  - Zebrafish (*Danio rerio*)
- **Author**:
- **Date Created**:

48.2 Materials

- Two pairs of Dumont #5 forceps
- Fire-polished wide-bore Pasteur pipet
- If using enzymatic dechorionation:
  - 6X flyfishing tippet
  - Capillary tube
- Microscope

48.3 Solutions

- E3 medium (quantities for 5 L of 60X stock listed after each ingredient in parentheses)
  - 5 mM NaCl (86 g)
  - 0.17 mM KCl (3.8 g)
  - 0.33 mM $CaCl_2$ (14.5 g $CaCl_2$ x $2H_2O$)
  - 0.333 mM $MgSO_4$ (24.5 g $MgSO_4$ x $7H_2O$)
  - 0.00001% (w/v) Methylene Blue (to be added to 1X solution)
- 2 mg/ml Pronase in E3 medium (Pronase Solution made from solid Powder (Roche 165921))

48.4 Procedure

1. Chorions can be removed easily using two forceps (it is critical that the tips are sharp and that their ends can touch). When raised in E3 medium at 28.5°C, zebrafish embryos develop normally outside of their chorions. Manually dechorionate embryos, using the following procedure:

    a. While using one pair of forceps to hold the chorion, make a tear in the chorion with the other pair of forceps.
    b. Hold the chorion in a region opposite of the tear, and gently push the embryo through the opening by passing the chorion and the embryo between the tips of the other pair of forceps.

> ⚠ IMPORTANT
>
> Embryos younger than 15 hpf are particularly fragile and great care must be taken not to damage them.

2. Alternatively, embryos can be enzymatically dechorionated in bulk using the following procedure:

    a. Incubate the embryos in the Pronase Solution at room temperature for a brief time (typically 1-10 minutes, depending on the embryonic stage). Constantly check the condition of the chorions by gently depressing them with a poker (made by attaching a loop of 6X flyfishing tippet to a capillary tube) and looking at them under a microscope. When the chorions no longer return to a spherical shape, the Pronase treatment is complete.
    b. Thoroughly wash the embryos (3 to 4 times) in E3 mediun to remove the Pronase solution. Using the fire-polished Pasteur pipet, pipet the embryos back and fourth in the E3 medium. The Pronase-treated chorions should break open, releasing the embryos.
    c. Dechorionated embryos should be handled using fire-polished Pasteur pipets to minimize damage.

48.5 Associated Papers

-

49 Fish Blood Collection

49.1 Introduction

- **Purpose**: Describe procedures for the collection of fish blood
- **Procedure Type**: Vert Experiment
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
  - Bay pipefish, (*Syngnathus leptorhyncus*)
  - Gulf pipefish (*Syngnathus scovelli*)
  - Zebrafish, (*Danio rerio*)
- **Author**: Ann Petersen
- **Date Created**: August 1, 2011

> ⚠ HUMAN SAFETY MEASURES
>
> - Wear gloves at all times when handling blood collection equiptment. While this will not prevent accidents, it may reduce the risk of contamination.
> - Never attempt to re-sheath needles. Always discard them into a sharps container immediately after use.
> - Never overfill the sharps container.
> - Place the sharps container in the area you are working to avoid transporting needles outside of your work area.
> - Ensure the work area is clean.
> - Wash hands after handling fish blood.

49.2 Materials:

- Gloves to be worn when handling fish, anesthesia water, blood samples
- Disinfectant for cleaning after blood sample
- Scalpal or sharp knife for tail ablation
- Sterile needles or vacutainer needles from 14-30 gauge
- Syringe for collecting samples

- Hematocrit tubes
- Critoseal
- Sharps container for needles
- Whatman 903 Specimen collection paper

49.3 Solutions

- Euthanisia MS-222 (tricaine-methanosulfanate) {#sec-husb-adutl_sb_euthanasia}

49.4 Procedure

1. Place fish in beaker containing MS-222 euthanasia dose (300 mg/L).
2. Wait for fish to slow and become unresponsive to taps on the container to ensure it is fully euthanized
3. Sever the caudal peduncle with a scalpel blade or sharp knife
4. Fill a heparnized hematocrit tube/capillary tube with the blood as it flows from the caudal vein. Plug one end of the hematocrit tube with critoseal. Spin on microhematocrit centrifuge to separate plasma from red blood cells. Measure hematocrit volume %, Use a pipette or break the capillary to extract the plasma or packed cells as needed.
5. Alternatively, collect a 25 L drop of blood at the tail and allow it to drop onto Whatman specimen collection filter paper for some endocrinological downstream applications. Blood spotted filter paper must be dried for 4 hours on the bench away from heat and light, and then may be stored at -20C in a sealed bag with desiccant packs.
6. Dispose of fish soma and tail in fish morgue.
7. Clean and disinfect area where blood was collected.

50 Needle Pulling for Injections

50.1 Introduction

- **Purpose**: This procedure describes how to pull needles for injections.
- **Procedure Type**: Vert Experiment
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
  - Zebrafish, (*Danio rerio*)
- **Author**: Susie Bassham
- **Date Created**: March 1, 2024

> ⚠ NOTES
>
> xxxx

50.2 Materials

- filament capillaries (1.2OD/0.69ID)
- needle puller
- glass

50.3 Solutions

50.4 Procedure

- use zebrafish filament capillaries (1.2OD/0.69ID)
- needle puller
- Program 1, range from 140 to 200 PSI by 20PSI steps
- Mark Appropriately

51 Whole Mount *In situ*

51.1 Introduction

- **Purpose**: This procedure describes how to perform insitus on whole mount embryos.
- **Procedure Type**: Vert Experiment
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
  - Bay pipefish, (*Syngnathus leptorhyncus*)
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: Susan Bassham
- **Date Created**: March 1, 2024

> ⚠ Chemical Warning
>
> Wear PPE when handling PFA.

51.2 Materials

- ice
- distilled water
- primary antibody (eg. mouse anti-fish)
- secondary antibody (eg. goat anti-mouse)
- tertiary antibody (ie mouse PAP)

51.3 Solutions

- 8% PFA {#sec-general_recipe_8%PFA}
- BT fix, 4% paraformaldehyde in PBS
- 0.1 M sodium citrate buffer, pH 6.4
- citrate buffer containing 0.1% sodium azide
- acetone

- blocking solution (2% normal goat serum in wash solution -- PBS containing 1% BSA, 1% DMSO, 0.2% triton-X 100)

51.4 Procedure

Fixation

1. Fix embryos in BT Fix for 4 to 6 hours on ice or at 4°C (see notes below).

Modified Antigen-Retrieval

2. Wash in 0.1 M sodium citrate buffer, pH 6.4, 5 min.
3. Replace with fresh citrate buffer containing 0.1% sodium azide.
4. Incubate overnight in 35-40°C water bath. (see note below).

Acetone Permeabilization

5. Wash in DW 5 min.
6. Freeze in acetone at -20°C for 7 min. to permeabilize the tissue (see note below).
7. Wash in DW 5 min.
8. Wash in 0.1 M citrate buffer 5 min.

51.5 Immuno-histochemistry

9. Treat with blocking solution (2% normal goat serum in wash solution -- PBS containing 1% BSA, 1% DMSO, 0.2% triton-X 100) to block nonspecific binding sites for 30 min.
10. Treat with primary antibody (eg. mouse anti-fish) diluted in blocking solution for 5 hours at RT or overnight at 4°C. A shaker may be used to facilitate process.
11. Wash for about 2 hours with frequent changes of wash solution.
12. Treat with secondary antibody (eg. goat anti-mouse) diluted in wash solution overnight at 4°C or for 5 hours at RT.
13. Repeat wash step.
14. If required, treat with tertiary antibody (ie mouse PAP) diluted in wash buffer overnight at 4°C or for 5 hours at RT.
15. Repeat wash step.
16. If required, develop enzyme-mediated stain.

Composition of fixative

I simultaneously fixed fish in three different aldehyde fixatives: BT fix, 4% paraformaldehyde in PBS, or 4% paraformaldehyde in 0.1 M phosphate, pH 7.3. - Those fish fixed in 4% paraformaldehyde in phosphate buffer were poorly and incompletely stained. - Those fish fixed in BT fix or in 4% pf in PBS demonstrated robust staining throughout the head and trunk of the fish (the staining in the BT-fixed fish may have been slightly better). - I also tested the components in the BT fix. The 4% sucrose in this fix was required for high-quality staining. - In contrast, the quality of the staining in fish fixed in BT fixative without calcium was comparable to that of fish fixed in BT fix containing 0.15 mM calcium chloride.

Modified Antigen Retrieva

- First introduced in the early 1990s, antigen-retrieval methods have been widely adopted by the clinical community as a way to recover antigenicity in paraffin sections.
- Several authors have demonstrated that heat is one of the most important variables in antigen retrieval (Brain Res 632, 105-113, 1993; Pathol Int 44, 759-764, 1994; APMIS 102, 295-307, 1994). In my initial experiments,
- I performed overnight incubations in citrate buffer at one of 4 temperatures: 4C, room temperature, 37C or 44C.
- The 5-day embryos incubated overnight at 4C or room temperature were poorly and incompletely stained, whereas those incubated at 37C and 44C were stained robustly throughout the head and trunk of the fish.
- However, the background staining in embryos incubated at 44C was quite high.
- This background staining was not present in embryos incubated at 37C.
- Since these initial experiments, I have been using embryos incubated overnight at 4C as my negative control.
- The quality of the staining in the negative control varies tremendously from experiment to experiment, whereas there is little or no experiment-to-experiment variation in the quality of staining in my positive controls using the above protocol.

##Papers

- Antigen Retrieval Techniques: Immunohistochemistry and Molecular Morphology*, S-R Shi, J Gu and CR Taylor, Eds., Eaton Publishing, Natick, MA, 2000.

52 Stickleback Backfilling of Hindbrain Neurons

52.1 Introduction

- **Purpose**: This procedure describes how to perform backfilling of stickleback hindbrains.
- **Procedure Type**: Vert Experiment
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
- **Author**: Susie Bassham
- **Date Created**: January 01, 2020

52.2 Materials

- Obsidian
- Rhodamine

52.3 Solutions

- MS-222 Anesthesia solution {#sec-sb_adult_anesthisia}
- MS-222 Euthanasia solution {#sec-husb-adutl_sb_euthanasia}
- Stickleback embryo medium {#sec-general_recipe_embryo_medium}
- DiO lipophilic carbocyanine dye
- Antibiotic (Cell Culture anti-biotic/mycotic from Gibco-BRL (15240-096) 100x Concentration)

52.4 Procedure

1. Sedate Larval fish in MS222 in sterile filtered seawater + antibiotic (Cell Culture anti-biotic/mycotic from Gibco-BRL (15240-096) 100x Concentration).

2. Use a obsidian glass flake, an extremely sharp scalpel, an incision will be made from the dorsal side, extending ventrally just through the spinal cord.
3. Use a fine insect pin to apply dried crystals of rhodamine dextran, DiO, or similar reagent to the cut spinal cord.
4. Continuously sedate larvae. Euthanized with MS222 and fixed 5 to 16 hours after surgery prior to imaging.

52.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

53 Stickleback Creation of Cybrids

53.1 Introduction

- **Purpose**: This procedure describes how to generate cytoplasmic hybrids (cybrids). Cytoplasm Transfer (CT) protocols have been established for a long time in vertebrates with the first human born from cytoplasm transfer in 1997. Methods follow similar protocols to somatic cell nuclear transfer in zebrafish and include the donation of approximately 20% of the donor egg cytoplasm into recipients at the single cell stage.
- **Procedure Type**: Vert Experimental
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
- **Author**: Emily Beck
- **Date Created**: January 01, 2024

53.2 Materials

- Petri dish with specialized trough rig
- Injection rig
- Transfer pipette

53.3 Solutions

- Embryo medium

53.4 Procedure

1. Collect eggs and sperm from one mitochondrial line (recipient) and fertilize {#sec-husbandry_stickleback_crossing}.
2. Collect eggs from alternate (donor) mitochondrial line.
3. Use microinjector to draw 20% of cytoplasm from donor egg {#sec-vert_exp_embryo_injections}.

4. Use microinjector to inject recipient embryo at single cell stage, ~ 1 hour post fertilization.
5. Rear animals until 10 days post fertilization, euthanize {#sec-vert_husb-euthanasia_syngnathid} animal, and use a restriction digest to test for heteroplasmy.

53.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

54 Stickleback Embryo Injections

54.1 Introduction

- **Purpose**: This procedure describes how to inject embryos with injection rig.
- **Procedure Type**: Vert Experimental
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
- **Author**: Mark Currey
- **Date Created**: January 01, 2024

54.2 Materials

- Petri dish with specialized trough rig
- Injection rig

54.3 Solutions

- Embryo medium

54.4 Procedure:

> **i** background
>
> (from: Zebrafish Book 4th addition chp 5) This is an easy method for holding embryos while injecting CRISPR elements, DNA, lineage tracer dyes, etc., without removing their chorions. The embryos are held in wedged-shaped troughs. Each trough can hold approximately 35 embryos (with chorions). Embryos can be aligned by gently tamping them down with forceps. As the pipette penetrates the chorion, the embryo is forced against the rear vertical wall of the trough. The exact positioning of the pipette tip within the

> embryo is achieved by slight movement of the pipette with a micromanipulator or by movement of the stage. If the pipette tip is thin and long enough, it can be withdrawn from the chorion without dragging the embryo out of the trough. A problem with thin pipettes, however, is that they lack the tensile strength to penetrate the chorion and bend when forced onto the chorion surface. Thicker pipettes do not easily slip out of the chorion, although the embryo can be held with forceps as the pipette is slowly withdrawn.

-

1. Injecting:

    1. Warm the Petri dish to the temperature you prefer for injection (embryos can tolerate 18°C for about 1 hour, and the slower division rate allows more time to inject at the 1 and 2 cell stage).
    2. Remove the plastic mold.
    3. Position the dish on a microscope stage and adjust the angle of the pipette so that you can aim directly into the trough.
    4. Transfer embryos into the troughs.
    5. Using forceps, align the embryos all in the same orientation. Add enough culture medium so that the level reaches the plastic edges of the slits. Gently tamp the embryos down into the trough.
    6. Using controls on the micromanipulator, force the pipette through the chorion and the yolk cell, entering the embryonic cell(s) from the yolk cell. (You might prefer to enter the embryonic cell directly; in this case, you would choose a different orientation of the embryo in the trough.)
    7. As the pipette goes through the yolk, move the Petri dish a bit so that the pipette is close to the plastic cover.
    8. Inject the desired volume of solution and then withdraw the pipette with a slow steady motion. The embryo will catch on the plastic cover and drop back into the trough.
    9. Transfer the embryos into a dish with embryo medium and maintain at 20°C for further development.

54.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

55 Germ Free Stickleback

55.1 Introduction

- **Purpose**: This procedure describes the creation of germ-free threespine stickleback.
- **Procedure Type**: Vert Experiment
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
- **Author**: Mark Currey
- **Date Created**: January 01, 2024

> ⚠ NOTES
>
> xxxx

55.2 Materials

- filter sterilizer
- 3 sterile 50 ml beakers with foil tops
- Hood
- Individually wrapped, sterile transfer pipettes

55.3 Solutions

- embryo medium {#sec-general_recipe_embryo_medium}
- 100mg/ml Ampicillin
- 50mg/ml Kanamycin
- 8mg/ml Amphotericin

> ℹ NOTES
>
> All stored at -20C

NOTES

Make the following solutions fresh for each use.

Antibiotic Stickleback Medium

- 250ul Ampicillin 100mg/ml (100ug/ml final)
- 25ul Kanamycin 50mg/ml (5ug/ml final)
- 7.8ul Amphotericin B 8mg/ml (250ng/ml final)
- 250 ml Stickleback medium
- Filter sterilize

0.003% Bleach solution

- 125ul 6.0% bleach solution
- 250ml SM
- Filter sterilize

0.2% PVP-I solution (Polyvinylpyrrolidone-iodine [0.01% free iodine] Western Chemical Inc.)

- 5ml 10% PVP-I stock
- 245ml SM
- Filter sterilize

55.4 Procedure

1. Make crosses as in {#sec-husbandry_stickleback_crossing} except spray the outside of the euthanized male with 95% ethanol prior to dissection.
2. Incubate eggs at 20°C until eggs reach 2 cell stage ~2 hours post fertilization, remove non-viable embryos from 2 cell stage eggs, and rinse viable embryos with fresh antibiotic stickleback medium 2X.
3. About 6 hours post fertilization, transfer viable embryos into 50 ml beakers

NOTES

To increase the probability of maintaining sterility wear standard latex laboratory gloves for subsequent steps of protocol.

4. Transfer the following into the hood to minimize number of times entering and exiting the hood (per 100 embryos cleaned) • 3 sterile 50ml beakers with foil tops • Individually wrapped, sterile transfer pipettes • 1L filter sterilized stickleback medium • 0.003% bleach • 0.2-0.4% PVP-I (some flasks may be contaminated with 0.2% PVP-I, but more fish are likely to die in the 0.4% PVP-I) • 10 sterile flasks – 250 or 500cm2 with screw topped caps (in hood, add 48ml sterile • stickleback medium per 250cm2 flask or 98ml per 500cm2 prior to adding embryos) • 25ml or 50ml pipettes, sterile, individually wrapped Pipette aid • Embryos in 50ml covered beaker • Large beaker for collecting liquid waste
5. Transfer embryos to clean beaker
6. Pour off all but ~10ml SM carefully into waste container
7. Transfer remaining 10ml SM with eggs into clean 50ml beaker
8. Add ~20ml sterile SM to old beaker to get remaining eggs
9. Transfer remaining eggs to beaker
10. Bring volume in new beaker up to 50ml with sterile SM
11. Rinse embryos 3X with 50ml filter sterilized SM
12. Immerse embryos in ~50ml 0.2-0.4% PVP-I solution for 10 minutes
13. Rinse embryos with sterile SM 1X
14. Transfer embryos to fresh beaker as in step b
15. Rinse embryos in sterile SM additional 2X
16. Immerse embryos in 0.003% bleach for 10 minutes.
17. Rinse embryos in sterile SM 1X
18. Transfer embryos to fresh beaker.
19. Rinse embryos additional 2X in sterile SM
20. Transfer 20-40 embryos to flasks containing sterile SM using sterile, individually wrapped pipettes
21. Incubate embryos at 20°C in incubator in the Cresko Lab, room 324 Pacific.

**Post sterilization care 22. At 9 dpf, upon yolk absorption, feed larva and change and test water for
contamination. 23. On the daily checklist record the number of fish dead or not moving in response
to external stimuli. In tissue culture hood, remove dead or not responsive (sick) fish. Euthanize sick fish with tricaine {#sec-husbandry_euthanasia_stickleback}. 24. In tissue culture hood, remove 70% of fish water with sterile pipette 25. Replace water with sterile stickleback medium 26. Add one scoop of irradiated Ziegler dry food to flask 27. Plate 50ul to 100ul of water on TSA plates to check for contamination of bacteria.

56 Live stickleback alizarin staining

56.1 Introduction

- **Purpose**: This procedure describes how to live stain stickleback with alizarin.
- **Procedure Type**: Vert Experimental
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
- **Author**: Mark Currey
- **Date Created**: January 01, 2024

56.2 Materials

- Large Petri Dish
- xxx
- xxx
- xxx

56.3 Solutions

- Alizarin stock solutions: 0.5g Alizarin red in 100ml or in 50ml in sterile water (SIGMA cat# A5533 Alizarin Red S, certified).
- Staining Solution:
  - 990 ml embryo medium {#sec-general_recipe_embryo_medium}
  - Add 10 ml 0.5% or 1% Alizarin Stock in sterile water for final concentrations of 0.005% or 0.01%.
  - Adjust to pH 7.5 with NaOH
- For 50 ml (enough for 100mm diameter petri dish):
  - 49.5 ml Embryo medium
  - Add 500 µl 0.5% or 1% Alizarin Stock in sterile water
  - Adjust to pH 7.5 with NaOH

- MESAB anesthesia strength {#sec-husbandry_euthanasia_stickleback}
- MESAB euthanisia strength {#sec-sb_adult_anesthisia}

56.4 Procedure

1. Place fish into a container containing stain for 1-2 hours for larvae to overnight for juveniles or adult fish in the dark. We have found that fish do not experience adverse effects from being exposed to stain. Monitor fish every 30-60 minutes if possible. To de-stain, rinse thoroughly with embryo medium by placing fish into container of embryo medium without stain for 30 minutes; background continues to go down with time. Move on to DASPEI live staining if desired (see DASPEI live staining SOP) or anesthetize until the fish reaches a light plane of anesthesia (i.e. movement has slowed down enough that the fish can be safely handled) and observe/image.
2. Bone fluorescence will decrease over time, so plan on imaging the same day if possible.
3. Keep fish in the dark as much as is reasonably convenient.
4. After fish has been observed/imaged place in a container of fish water. Monitor fish every 5-10 minutes until the fish is revived. Once fish is revived place back on the fish system and monitor during daily health checks. If the fish is to be fixed for post mortem experiments place fish directly into euthanasia MS222 solution and follow the euthanasia SOP.

56.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

57 Live stickleback calcein staining

57.1 Introduction

- **Purpose**: This procedure describes how to live stain stickleback with calcein.
- **Procedure Type**: Vert Experimental
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
- **Author**: Mark Currey
- **Date Created**: January 01, 2024

57.2 Materials

- Petri dishes and/or 1 L tanks
- Calcein (Molecular Probes; cat. C481)

57.3 Solutions

- MESAB anesthesia strength {#sec-husbandry_euthanasia_stickleback}
- MESAB euthanisia strength {#sec-sb_adult_anesthisia}
- Embryo medium
- 10% NaOH
- Stain Solution:
  - 0.005 to 0.05% calcein in sea water.
  - Adjust pH to 8.2 with NaOH
  - Make fresh, keep in dark.

57.4 Procedure

1. Place fish into a container containing stain for 1-2 hours for larvae to overnight for juveniles or adult fish in the dark. We have found that fish do not experience adverse effects from being exposed to stain. Monitor fish every 30-60 minutes if possible. To de-stain, rinse thoroughly with embryo medium by placing fish into container of embryo medium without stain for 30 minutes; background continues to go down with time. Move on to DASPEI live staining if desired (see DASPEI live staining SOP) or anesthetize until the fish reaches a light plane of anesthesia (i.e. movement has slowed down enough that the fish can be safely handled) and observe/image.
2. Bone fluorescence will decrease over time, so plan on imaging the same day if possible.
3. Keep fish in the dark as much as is reasonably convenient.
4. After fish has been observed/imaged place in a container of fish water. Monitor fish every 5-10 minutes until the fish is revived. Once fish is revived place back on the fish system and monitor during daily health checks. If the fish is to be fixed for post mortem experiments place fish directly into euthanasia MS222 solution and follow the euthanasia SOP.

57.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

58 Live stickleback DASPEI staining

58.1 Introduction

- **Purpose**: This procedure describes how to live stain stickleback with DASPEI.
- **Procedure Type**: Vert Experimental
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
- **Author**: Susie Bassham
- **Date Created**: October 25, 2021; adapted from DOI: 10.1007/s10162-002-3022-x

58.2 Materials

- Petri dishes and/or 1 L tanks
- DASPEI (Sigma Aldrich; cat. D3418)

58.3 Solutions

- MESAB anesthesia strength {#sec-husbandry_euthanasia_stickleback}
- MESAB euthanisia strength {#sec-sb_adult_anesthisia}
- Embryo Medium

- Staining Solution:
  - 0.005% DASPEI in sea water

58.4 Procedure

1. Stain larvae in Petri dishes and juveniles/adults in 1 L tanks containing stain solution for 5 to 75 min in the dark; stagger so that no fish stain longer than this before imaging. Rinse for 20 to 60 min in container of sea water without DASPEI to reduce background.

2. Keep fish in the dark as much as is reasonably convenient through procedures. Monitor fish every 15-30 minutes.
3. Anesthetize until the fish reaches a light plane of anesthesia (i.e. movement has slowed down enough that the fish can be safely handled) and observe/image fluorescence immediately.
4. After fish has been observed/imaged place in a container of fish water. Monitor fish every 5-10 minutes until the fish is revived. Once fish is revived place back on the fish system and monitor during daily health checks. If the fish is to be fixed for post mortem experiments place fish directly into euthanasia MS222 solution and follow the euthanasia SOP.
5. Staining solution can be stored at 4C and reused.

58.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

59 Live fin clipping in Stickleback and Pipefish

59.1 Introduction

- **Purpose**: This procedure decribes how to take live fin clips in stickleback and pipefish
- **Procedure Type**: Vert Experimental
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
  - Bay pipefish, (*Syngnathus leptorhyncus*)
  - Gulf pipefish (*Syngnathus scovelli*)
- **Author**: xxx
- **Date Created**: xxx

59.2 Materials

- Beakers of system water
- Forceps and scissors
- Bucket of ice
- 1.5ml tubes

59.3 Solutions

- MS-222 Pipefish Anesthesia solution {#sec-husbandry_anesth_pipefish}
- MS-222 Stickleback Anesthesia solution {#sec-sb_adult_anesthisia}
- Melafix (antifungal and antibacterial agent)

59.4 Procedure

1. To minimize fish's exposure to MS-222, do all labeling of tubes and vials prior to fin clipping.
2. Place fish in beaker containing MS-222 anesthesia dose.

To observe slowed breathing watch the operculum movement. This movement will slow and the fish will turn on its side.

3. Once the breathing of the fish slows take fish out of water and remove caudal fin - carefully avoiding the peduncle - with scissors and forceps.
4. Place fin in labeled 1.5 ml tube on ice.
5. Put fish into clean system water and move gently to force water over gills.
6. Once fish is revived and swimming upright, put fish into new container with Melafix (diluted in system water according to manufacturer's recommendations) for 15 minutes.
7. Return fish to aquaculture system.

59.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

60 Stickleback Bacterial Monoassociation

60.1 Introduction

- **Purpose**: This protocol is used to determine whether the microbial community of one strain of stickleback can colonize the gut of another strain that is evolutionarily different. This protocol has been adapted for use in stickleback from an established, approved zebrafish protocol.
- **Procedure Type**: Vert Experiment
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
- **Author**: xxx
- **Date Created**: xxx

60.2 Materials

- xxx
- xxx

60.3 Solutions

- xxx
- xxx

60.4 Procedure

The bacterial inoculums should be determined empirically for each species used. For many bacteria 104 Colony Forming Units (CFUs) per milliliter of stickleback medium will suffice for full colonization of the intestine and minimal toxicity to the fish. Dead larvae in the flask will be removed as this will often result in increased bacterial growth that can ultimately be fatal to the remaining larvae. The method below is used for several proteobacteria strains.

1. Raise fish in a germ-free environment as described here: {#sec-vert_exp_gremfree_SB}
2. Incubate bacteria for 16 hours in 50 ml of growth medium in 250 ml flask at 25°C, 200 RPM. These bacteria will originate from the intestine of a healthy stickleback to ensure that the bacteria are not pathogens.
3. Measure the Optical Density$_{600}$ (OD_{600}) of the culture.
4. Calculate the original culture density, assuming 1 OD_{600} = 109 CFU / ml.
5. Dilute the culture to pre-determined concentration as needed in filter sterilized stickleback medium, and inoculate flask in tissue culture hood. The fish will be exposed to the bacteria in the water after their digestive system is open and they have been shown to consume food, which occurs between 9 and 13 days post fertilization. The inoculums will be determined in a manner similar to those already established for zebrafish and used regularly in the Guillemin laboratory. Germ-free fish water is inoculated between 1000 and 1000000 bacteria per milliliter so the concentration the concentration can be chosen that results in healthy fish surviving to three days post-exposure.
6. Plate dilutions of the original bacterial culture in order to verify the CFU/ml of the inoculum.
7. Monitor fish daily for signs of illness, including lack of motility, response to external stimuli, rapid movement of opercular (gill) cover and any other sign of non-normal behavior.
8. At the end of the experiment, euthanize fish by established and approved protocols {#sec-husb-adutl_sb_euthanasia}.

61 Stickleback Salinity Treatment

61.1 Introduction

- **Purpose**: Protocols for treating threespine stickleback with salinities that mimic natural freshwater, brackish and oceanic conditions.
- **Procedure Type**: Vert Experiment
- **Species**:
  - Threespine stickleback, (*Gasterosteus aculeatus*)
- **Author**: xxx
- **Date Created**: xxx

61.2 Materials

- Glass containers of fish water of varying salinity ranging from 2 - 32 ppt
- Glass 4L fish containers (not plastic due to endocrine disruptive properties)

61.3 Solutions

- MS-222 Anesthesia solution {#sec-sb_adult_anesthisia}
- MS-222 Euthanasia solution {#sec-husb-adutl_sb_euthanasia}

Different concentrations of Instant Ocean will be used.

- Instant Ocean saltwater mix at concentrations of:
- 2 ppt “Freshwater” environment (40g Instant Ocean/ 20L Nanopure H20)
- 15 ppt “Brackish” environment (300g Instant Ocean/ 20L Nanopure H20)
- 30 ppt “Ocean” environment (600g Instant Ocean/ 20L Nanopure H20)

61.4 Procedure

1. Follow stickleback crossing and rearing SOP {#sec-husbandry_stickleback_crossing} to generate 100-200 fertilized embryos.
2. Raise fish in incubator until hatching as in {#sec-vert_husbandry_incubator_use}.
3. At hatching move 20 larval fish to a 10.5 L static tank containing the appropriate salinity water as listed above.
4. On 9 dpf feed enough artemia so that it is consumed by the juveniles within 15-30 minutes.
5. Feed and change water every other day. Check on fish at least twice EVERY DAY, and use the care checklist to denote daily health check, feeding, and water change. Look for sick or stressed fish (low rate of opercular fluttering, bad color, floating upside down, etc.) 6. Check water quality on two random tanks in each treatment once every two days before water has been changed. If ammonia and/or nitrite are detected then change water more frequently, check water quality daily, and denote this on checklist. Ideal ammonia and nitrite levels are 0 ppm.
6. At designated time of interest up to 40 days post-fertilization euthanize fish with MS222.
7. Fix appropriately in liquid nitrogen, 4%PFA, or Bouin's.

1. Anesthetize using above concentration of MS-222 and allow fish to lay motionless with only slight opercle movement. This should take approximatley 10 minutes.
2. Place fish in liquid N2 for 2 minutes. This instantaneously freezes the fish.
3. Thaw into RNAlater-ICE.

Note: If downstream application requires instantaneous, non stressed measurement of gene transcription, remove fish from water, rapidly blot, and sacrifice directly into liquid nitrogen or RNAlater.