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Title: Incremental Temperature Changes for Maximal Breeding and Spawning in *Astyanax mexicanus*

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NOTE: APF filmed using draft script

Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **N**

2. **Software:** Does the part of your protocol being filmed demonstrate software usage? **Maybe**

   If **Yes**, we will need you to record using screen recording software to capture the steps. If you use a Mac, QuickTime X also has the ability to record the steps. Please upload all screen captured video files to your project page as soon as reasonably possible.

3. **Interview statements:** Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees self-record interview statements outside of the filming date. JoVE can provide support for this option.

4. **Filming location:** Will the filming need to take place in multiple locations (greater than walking distance)? **No**

Protocol Length
Number of Shots: **39**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **William R. Jeffery**: The Mexican tetra, Astyanax mexicanus, is an emerging model system for studies in development and evolution. It is crucial to obtain large numbers of spawned embryos to perform the experiments. **[1]**.

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

1.2. **William R. Jeffery**: The incremental temperature changes can provide two-three consecutive spawning days with maximal numbers of high-quality embryos. **[1]**.

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

OPTIONAL:

1.3. **Li Ma**:Astynax cavefish is the only cavefish species that can breed and spawn in the lab so far. This method may be suitable to breed and spawn other cavefish species. **[1]**.

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

1.4. Please be patient and let the fish gradually adapt to this temperature system**[1]**.

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

1.5. **Li Ma**: Compared with the text description, the video system allows the operator to see the operation steps more visually **[1]**.

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

Introduction of Demonstrator on Camera

1.6. **Janet Shi**: Demonstrating the procedure will be Janet Shi, a researcher in my laboratory. **[1][2]**. NOTE: Probably don't use this

1.6.1. INTERVIEW: Author saying the above

1.6.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera

2. Monday

~~2.1. To facilitate the spawning of large quantities of high-quality fish embryos, on Monday morning between 9 and 10 AM, use a thermometer to record the room, breeding tank, and reservoir temperatures **[1]**.~~

~~2.1.1. WIDE: Talent checking temperature(s)No filming needed~~

~~2.2. Use a colorimetric test to record the ammonia, nitrate, and nitrate levels in the water **[1]** and record the pH from the monitoring system **[2]** as well as from the colorimetric test kit **[3]**.~~

~~2.2.1. Talent dipping dipstick into sample, with test kit visible in frame No filming needed~~

~~2.2.2. Shot of monitoring system pH readout No filming needed.~~

~~2.2.3. Shot of colorimetric test pH readout *Video Editor: please emphasize pH readout as necessary No filming needed.*~~

~~2.3. Then record the conductivity from the carboy monitor **[1]** and the main system monitor **[2]**. No filming need.~~

~~2.3.1. Shot of carboy monitor conductivity read out~~

~~2.3.2. Shot of main system monitor conductivity read out~~

2.4. After feeding the fish at 10 AM with a pinch of tetra flakes **[1]**, check the incubator that will be used to house fingerbowls of developing embryos **[2]** and change the water if necessary **[3]**.

2.4.1. Talent feeding fish, Please add the videos “1.2.1”, “1.2.2”, “1.2.3”, “1.2.4”, “1.2.5”, and “1.2.6”.

2.4.2. Talent checking incubatorPlease add the video “ 1.3.1 second version” and “1.3.1 third version”, and “1.3.1 first version”.

2.4.3. Talent adding water to incubator

2.5. Check the water level in all of the reservoirs **[1]** and system water to any reservoirs that are running low **[2]**.

2.5.1. Talent checking water level

2.5.2. Talent adding water to reservoir

2.6. Then set the incubator to 23 degrees Celsius **[1]**.

2.6.1. Talent setting temperature,Please videos "1.6 first version" and "1.6 second version".

2.7. At least 30 minutes after the first feeding, feed the fish in the breeding tanks with a pinch of egg yolk flakes **[1]**, enough blackworm clusters to allow each fish in the tank to consume about 5-10 worms, or both **[2-TXT]**.

2.7.1. Talent adding finger pinch of egg yolk flakes

2.7.2. Blackworm clusters being added **TEXT: See text for blackworm maintenance details**

2.8. After least one hour after the second feeding, scrub the breeding tanks as necessary **[1]** and carefully set up breeding nets without interfering with the tank air supply **[2]**.

2.8.1. Talent scrubbing tank, Please add videos "1.7 first version" and "1.7 second version".

2.8.2. Talent setting breeding net *Videographer: Shot will be used again Please add videos " 1.7" , "1.8.1", "1.8.2", "1.8.3", and "1.8.4"*

3. Tuesday

3.1. The next morning, remove the breeding nets from the bottoms of the adult system tanks between 9 and 10 AM **[1]** and use the hose attached to the carboy to gently rinse the embryos into a hand-held net **[2]**.

3.1.1. WIDE: Talent removing net *Videographer: Shot will be used again Please add videos " 1.8.5" and " 2.1.1"*

3.1.2. Talent washing embryos into net. Please add videos " 2.1.2", " 2.1.2 Li ", or "2.1.2 Janet" here.

3.2. Invert the net into a fingerbowl of clean system water **[1]** and transfer about 100 embryos per fingerbowl into 200 milliliters of clean system water supplemented with 0.00003% methylene blue **[2]**.

3.2.1. Talent inverting net into bowl

3.2.2. Talent adding embryo to new bowl, with methylene blue container visible in frame **TEXT: Split exceptionally large numbers of embryos into multiple bowls**

3.3. Stage the embryos under a light microscope to estimate the time of fertilization according to the *A. mexicanus* developmental time table **[1]**.

3.3.1. Talent at microscope, staging embryos OR LAB MEDIA: To be provided by Authors: Image of embryos under microscope NOTE: Images requested from authors do not appear to be on the project page.

3.4. After staging, place the fingerbowls into an incubator for 5-7 days **[1]** and feed the cultures with living brine shrimp **[2]**.

3.4.1. Talent placing bowl(s) into incubator

3.5. For every tank that dropped embryos, record the date and tank number **[1]** and record the number and quality of embryos dropped **[2]**.

3.5.1. Talent checking tank, recording date and tank number

3.5.2. Shot of recorded embryo number and quality in lab note or SCREEN: To be provided by authors: Screenshot of spreadsheet with recorded embryo number and quality

3.6. Record the average time of spawn according to staging table **[1]** and record the temperature of the system at the time when the fish spawned **[2]**.

3.6.1. Average time being recorded OR SCREEN: To be provided by authors: Screenshot of spreadsheet with average time to spawn

3.6.2. Average temp being recorded OR SCREEN: To be provided by authors: Screenshot of spreadsheet with temperature at spawning

3.7. Monitor the fingerbowls of embryos throughout the day **[1]**, using a Pasteur pipette to remove any dead or deformed embryos and debris, such as uneaten food or feces, **[2]** and changing the blue water as necessary **[3]**.

3.7.1. Talent checking bowl(s) Please add video " 2.1.4 Janet" and "2.1.4" at here.

3.7.2. Embryo and/or debris being removed

3.7.3. Talent changing water

3.8. At 1 PM, feed the fish in the breeding tanks as demonstrated **[1]** and set the water temperature to 24 degrees Celsius **[2]**.

3.8.1. Talent adding feed to tank *Videographer: Shot will be used again*

3.8.2. Talent setting temperature

3.9. Then remove any excess food and debris from the tanks **[1]** and scrub the tanks before resetting nets **[2]**.

3.9.1. Talent removing food/debris

3.9.2. Talent scrubbing tank

4. Wednesday-Sunday

4.1. On Wednesday and Thursday, repeat the embryo collection and evaluation as demonstrated for Tuesday **[1]**, feed the fish **[2]**, and set the water to the appropriate temperature **[3-TXT]**.

4.1.1. Use 3.1.1. Talent collecting nets

4.1.2. Use 3.8.1. Talent feeding fish

4.1.3. Talent setting temperature **TEXT: Weds: 25 °C; Thurs: 24 °C**

4.2. Check the embryos in the incubator **[1]** and replace the fingerbowl water with fresh methylene blue-treated system water **[2]**.

4.2.1. Talent opening incubator same as previous

4.2.2. Talent adding water to bowl Please add the video :1.3.1 third version".

4.3. Then clean the breeding tanks as needed **[1]** and reset the nets **[2]**.

4.3.1. Use 2.8.2. Talent setting net The same shot will be used again.

4.4. On Friday, after feeding the fish, set the water temperature to 23 degrees Celsius **[1]** and check the embryos in the incubator **[2]**.

4.4.1. Talent setting temperature

4.4.2. Shot of embryos in incubator

4.5. On Saturday and Sunday, feed the fish **[1]**.

4.5.1. Talent adding feed to tank

Results

5. Results: Representative Surface and Cave Fish Population Breeding Data

5.1. In this figure, the breeding data from July 2017 through March 2020 **[1]** for the Rio Choy **[2]** and Texas surface fish **[3]** and the Los Sabinos **[4]**, Tinaja **[5]**, and Pachón cave fish are shown **[5]**.

5.1.1. LAB MEDIA: Figure 2
5.1.2. LAB MEDIA: Figure 2 *Video Editor: please emphasize Rio Choy surface fish graph*
5.1.3. LAB MEDIA: Figure 2 *Video Editor: please emphasize Texas surface fish graph*
5.1.4. LAB MEDIA: Figure 2 *Video Editor: please emphasize Los Sabinos cavefish graph*
5.1.5. LAB MEDIA: Figure 2 *Video Editor: please emphasize Tinaja cavefish graph*
5.1.6. LAB MEDIA: Figure 2 *Video Editor: please emphasize Pachon cavefish graph*

5.2. The data were analyzed by breeding week **[1]** and the average number of embryos collected per day during a single breeding week **[2]**.

5.2.1. LAB MEDIA: Figure 2 *Video Editor: please emphasize x-axis*
5.2.2. LAB MEDIA: Figure 2 *Video Editor: please emphasize y-axis*

5.3. The data indicates that breeding was continuous throughout the year in Rio Choy and Texas surface fish and in Pachón cave fish **[1]**.

5.3.1. LAB MEDIA: Figure 2 *Video Editor: please emphasize data lines in Rio Choy, Texas, and Pachon graphs*

5.4. The quantity and quality of most Rio Choy surface fish was between low and high **[1]**, while the quantity and quality of most Texas surface fish and Pachón cave fish was between low and medium **[2]**.

5.4.1. LAB MEDIA: Figure 2 *Video Editor: please add horizontal lines across lowest peaks(s) and highest peaks(s) in Rio Choy graph*
5.4.2. LAB MEDIA: Figure 2 *Video Editor: please emphasize horizontal lines across lowest peak(s) and medium peak(s) in Texas and Pachon graph*

5.5. The occurrence of spawning was not continuous in Tinaja or Los Sabinos cave fish **[1]**, with spawning being low or non-existent during the late summer to autumn in these fish **[2]**.

5.5.1. LAB MEDIA: Figure 2 *Video Editor: please emphasize Tinaja and Los Sabinos graphs*

5.5.2. LAB MEDIA: Figure 2 *Video Editor: please emphasize lack of data lines between 7-10 sections in both graphs*

5.6. Although the lowest levels of spawning were recorded for Los Sabinos cave fish, the quality of embryos was the best **[1]**. Overall, the surface fish exhibited a better spawning quantity and quality than cave fish **[2]**.

5.6.1. LAB MEDIA: Figure 2 *Video Editor: please emphasize Los Sabinos graph*

5.6.2. LAB MEDIA: Figure 2 *Video Editor: please emphasize Rio Choy and Texas graphs*

Conclusion

6. Conclusion Interview Statements

6.1. Before the breeding, good feeding is very important. **[1]**.

6.2. For in vitro fertilization animals exhibiting spawning behavior, may be removed from tanks and used for crosses during the temperature induced breeding regime. **[1]**.

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

6.3. Whether used for scientific research, teaching, or biotechnology, A. mexicanus is an excellent model system for exploring the fascinating questions surrounding the evolution of development. **[1]**.

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