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Raising the Mexican tetra, Astyanax mexicanus, for analysis of post-larval phenotypes and wholemount immunohistochemistry

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Professor and Chairman

Dear Editor,

Attached you will find a revised version of our manuscript, “Raising the Mexican tetra, *Astyanax mexicanus,* for analysis of post-larval phenotypes and whole-mount immunohistochemistry”.

This submission provides a protocol for breeding *A. mexicanus* (Mexican tetra) adults, raising larvae, and performing whole-mount immunohistochemistry on post-larval fish. These protocols, together, are of particular value as they set the stage for comparing the phenotypes of surface and cave morphotypes of this fish.

The reviewers of our original submission provided a thorough critique and identified a number of place where they felt the protocols could be improved and/or made more clear. As described in the accompanying point-by-point response, we have now addressed each of the issues they raised, and believe the paper is greatly improved because of their input.

We hope you will agree that the protocols will be useful to researchers in the field and is now in a form acceptable for publication in JoVE.

Thank you for your consideration,

Sincerely yours,

Clifford J. Tabin

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TITLE:

Raising the Mexican Tetra *Astyanax Mexicanus* for Analysis of Post-larval Phenotypes and Whole-mount Immunohistochemistry

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KEYWORDS:
Astyanax mexicanus, immunohistochemistry, fish husbandry, cavefish, aquaculture, whole-mount immunostaining

SUMMARY:
In this protocol, we demonstrate how to breed *Astyanax mexicanus* adults, raise the larvae, and perform whole-mount immunohistochemistry on post-larval fish to compare the phenotypes of surface and cave morphotypes.

ABSTRACT:
River and cave-adapted populations of *Astyanax mexicanus* show differences in morphology, physiology, and behavior. Research focused on comparing adult forms has revealed the genetic basis of some of these differences. Less is known about how the populations differ at post-larval stages (at the onset of feeding); although, studies may provide insight into how cavefish survive through adulthood in their natural environment. Methods for comparing post-larval development in the laboratory require standardized aquaculture and feeding regimes. Here we describe how to raise fish with a recirculating system for two-weeks, using live rotifers as a nutrient-rich food source, which does not require daily replenishment. We demonstrate how to collect post-larval fish from this nursery system and perform whole-mount immunostaining. Immunostaining is an attractive alternative to transgene expression analysis for investigating development and gene function in *A. mexicanus*. The nursery method can also be used as a standard protocol for establishing density-matched populations for growth into adults.

INTRODUCTION:
The Mexican tetra, *Astyanax mexicanus,* is a single species of fish that exists as river-dwelling populations (surface fish) and a number of cave-dwelling populations (cavefish) named for the caves they inhabit (*i.e.,* Tinaja, Molino, Pachón). A growing number of researchers are using *A.*

mexicanus to investigate the genetic and developmental bases of behavioral[1–4], metabolic[5–8], and morphological evolution[9–11]. Available resources for studies of *A. mexicanus* include a sequenced and annotated genome[12]; transcriptome[13]; developmental staging table[14]; and methods for breeding[15–17], creating transgenics[18], and editing genes[19]. Dissemination of additional tools and updated standard protocols will accelerate growth of the cavefish research community (see this methods collection[20]).

Our goal is to add to the existing repertoire of tools by providing a robust method for assessing gene activity *in situ* in post-larval *A. mexicanus*, in a manner comparable between laboratories. There are two challenges to achieving this goal. First, there is a need for standardized regimes for hatching and raising the fish between laboratories, as differences in parameters such as feeding and density affect growth and maturation, thereby impacting gene activity. Second, there is a need for a standardized yet adaptable method for examining patterns of gene activity in the post-larval fish. We address these issues here, establishing standard practices for raising fish to post-larval stages and introducing a robust whole-mount immunohistochemistry (IHC) protocol for assessing gene expression in *A. mexicanus*.

We first demonstrate how to breed the fish through natural spawning and identify fertilized eggs. Described next is how to hatch fertilized eggs (larvae) and transfer them to nursery containers, where they are maintained at a density of 20 fish per container for two weeks without recirculating or changing the water. At 5-days post fertilization, the fish have developed to post-larval stages (no longer having a yolk supply) and are provided algae-fed *Brachionus plicatilis* (rotifers) as a nutrient-rich food source that do not require daily replenishment. This method provides consistent growth parameters for larval and post-larval development.

To assess gene function, we demonstrate how to remove the fish from nursery containers and perform whole-mount IHC. The IHC method presented is adapted from protocols developed for use with *Danio rerio*[21] and is effective for examining antigens in all *A. mexicanus* tissues tested, including the brain, intestine, and pancreas. IHC is a faster alternative to generating transgenic animals for examination of gene expression and protein localization. This protocol will be useful for studies aimed at growing *A. mexicanus* and comparing the phenotypes of surface fish and cavefish at post-larval stages.

PROTOCOL:

The procedures described throughout this protocol have been approved by the Institutional Animal Care and Use Committee (IACUC) at Harvard Medical School.

1. Breeding

NOTE: There are several published methods for breeding[15–17,20] that could also be used at this step. Prior to breeding, adult fish are maintained on a 10:14 light:dark cycle at 23 °C and fed a pellet diet (see **Table of Materials)** once daily. Fish can be bred in a recirculating system with mechanical filtration and UV sterilization. Breeding can also be accomplished in static (non-

recirculating) tanks, but fish should not be left in a static tank for more than 3 days, as water quality quickly degrades.

1.1. Fill a 5 gal tank with fish-ready water (dechlorinated water adjusted to: pH = 7.1 +/- 2, conductivity = 900 +/- 150 µS, temperature = 23 °C).

1.2. Place plastic mesh (see **Materials**) in the bottom of the tank. If breeding in static tanks, affix a water heater to the side of the tank. If breeding in a recirculating system, place a water heater in the system sump.

NOTE: The plastic mesh prevents adults from consuming eggs.

1.3. Place one female and two male *A. mexicanus* fish of greater than 1 year old into the tank. Allow the fish to acclimate for 30 min.

1.4. Set the temperature of the heater to 24 °C (or 1 °C warmer than the starting temperature).

1.5. After 24 h, increase the temperature by 1 °C.

1.6. After 24 h, increase the temperature by 1 °C. Check the tanks daily for eggs by shining a flashlight into the bottom of the tank. Withhold the fish from food during this 3-day period.

1.7. If spawning has not been induced after 3 days, turn off the heater and allow the water to return to room temperature (RT) before moving the fish to their original tank.

2. Hatching Fertilized Eggs

2.1. Once eggs are identified in a breeding tank, remove the adults and plastic mash, and reduce the water to a depth of 10 cm using a beaker or cup.

NOTE: To estimate time of spawning, use a transfer pipette to place several eggs into a Petri dish and view them with a stereomicroscope to determine the stage[14] and estimate the time of fertilization.

2.2. Move the tank to a convenient work surface and remove any opaque eggs or feces, leaving only the translucent, fertile eggs in the tank.

NOTE: The number of fertile eggs can be recorded at this time.

2.3. Fill the tank with fish-ready water (see step 1.1) and add 6-7 drops of methylene blue to the tank as the water fills.

NOTE: The final concentration of methylene blue is approximately 1.5 ppm.

2.4. Add a heater and aquarium bubbler (attached to an air pump, with a regulator) to the tank.

2.5. Set the heater to 24 °C and adjust the airstream regulator to produce a gentle stream of bubbles.

2.6. Place a cover on the tank to help maintain the water temperature.

NOTE: The eggs should begin hatching within 24 h of the spawning time.

3. Transfer of Hatched Larvae to Nursery Containers

3.1. Add 20 g of salt (see **Materials**) to 8 L of fish-ready water (see step 1.1) and stir until dissolved. Fill each 1.5 L nursery container (see **Materials**) with 1 L of the prepared water.

3.2. Use a transfer pipette to move hatched larvae into prepared nursery containers at a density of 20 fish per container.

NOTE: A headlamp can be useful to locate and transfer the hatched larvae.

3.3. After all the visible hatched larvae have been removed, agitate the water in the tank by stirring, and/or blowing water jets into the edges and corners of the tank with the pipette.

NOTE: This will help reveal larvae that were missed on the first pass.

3.4. Dispose of unused larvae at this stage using the guidelines of the Office of Laboratory Welfare (OLAW). Add sodium hypochlorite to the tank to achieve a final concentration of 6.15%. Wait at least 5 min before pouring down the sink.

3.5. Label each nursery container with the date and time of fertilization. View nursery containers daily and continue to remove any dead larvae.

4. Preparation of Rotifer-based Fish Food

4.1 See **Table of Materials** for information on obtaining rotifers. Follow the referenced protocol to set up, maintain, and harvest rotifers[22].

4.2 Prepare fish food by adding 3 mL of algae mixture (see **Table of Materials**) to 1 L of harvested rotifers. This mixture will be added directly to the nursery containers as a food supply.

5. Feeding of Post-larval Fish

5.1 When the fish are 5 days post fertilization (dpf), add 3 mL of fish food (prepared in step 4.2) to each nursery container. At the optimal density, rotifers should be visible in dense groups at

the corners of the nursery containers, and less apparent at the center of the container. Add additional rotifer mixture until the appropriate density has been reached.

5.2 Check the containers daily for the presence of rotifers and add more if the concentration gets depleted. Continue to remove any dead larvae.

5.3 When fish reach 14 dpf, move them to a tank fit with a recirculating system at a density of 5 fish/L of water.

NOTE: The number of surviving post-larval fish can be recorded at this time.

6. Whole-mount Immunohistochemistry of Post-larval Fish

6.1. Remove post-larval fish of the desired stage from food for 24 h.

6.1.1. Pour the nursery container containing the fish through a nylon mesh strainer and place the strainer into a container with clean fish-ready water (see step 1.1).

6.1.2. Use a transfer pipette to move the fish into a 1.5 L container with clean, fish-ready water.

NOTE: It is essential to remove all of the food. Even small amounts of food in the gut are auto-fluorescent and will influence imaging.

6.2. Collect and euthanize the fish.

NOTE: The euthanasia protocol should follow OLAW guidelines and be approved by your Institutional Animal Care and Use Committee. We have noted that tricaine alone does not euthanize post-larval fish, and the fish begin to move when transferred from tricaine to fixative if the fish are not also kept on ice.

6.2.1. Prepare tricaine solution in a beaker by adding 0.4 g of tricaine-S and 0.8 g of sodium bicarbonate to 1 L of deionized water, and place it on ice.

6.2.2. Pour the water containing the fish through a nylon mesh strainer to collect the fish. Gently submerge the strainer in ice-cold Tricaine solution and leave it on ice for 10 min.

6.3. Fix the euthanized fish.

6.3.1. Use a transfer pipette with a cut tip to transfer the fish to a conical tube.

6.3.2. Remove the Tricaine solution with a transfer pipette and replace with fixative. Incubate with rocking.

NOTE: Fixative and fixation time must be determined based on the antibody used. 10% formalin solution (4% formaldehyde, see materials) overnight at 4 °C is adequate for the antibodies listed in the **Table of Materials**.

CAUTION: Formalin is toxic and flammable. Wear personal protective equipment (gloves, lab coat, and splash goggles) and handle in a chemical hood. Solutions containing formalin should be disposed as hazardous waste.

6.3.3. Use a transfer pipette to carefully remove the fixative without disturbing the fish. Add 3 mL of phosphate buffered saline-triton solution [PBS with 0.1% Triton (PBST), see **Materials**] and incubate for 15 min at RT with rocking.

6.3.4. Remove PBST and replace with fresh PBST and incubate for 15 min. Repeat this "washing" one additional time.

NOTE: Fish can be stored in PBS containing 0.02% sodium azide at 4 °C for several weeks.

6.4 Perform whole-mount immunostaining.

6.4.1 Prepare 50 mL of blocking solution [PB-0.5% Triton X, 0.2% bovine serum albumin (BSA), 1% dimethyl sulfoxide (DMSO), 0.02% sodium azide, 5% donkey serum].

CAUTION: Sodium azide and DMSO are toxic. Personal protective equipment (gloves, lab coat, and splash goggles) should be used when handling. Any solutions should be disposed as hazardous waste.

6.4.2. Use a transfer pipette to transfer the fish to a 4 mL glass vial with screw-top cap. Use a transfer pipette to remove the PBST and add 3 mL blocking solution. Incubate for 1 h at RT with rocking.

6.4.3. Use a transfer pipette to remove the blocking solution and add primary antibody diluted in blocking solution. Incubate overnight at room temperature with agitation.

NOTE: For example, add 1:250 dilution of anti-HuC/HuD Neuronal Protein Mouse Monoclonal Antibody (see **Table of Materials** for a list of antibodies that have been successfully used in *A. mexicanus*). A set of fish with no primary antibody added should be included at this step. Volume of antibody should be enough to cover the fish and allow for agitation.

6.4.4. Wash the fish 3 times with PBST as described in step 6.3.4. Replace PBST with secondary antibody diluted in blocking solution and incubate overnight at RT with agitation.

NOTE: Optimal primary and secondary antibody concentrations, incubation time, and incubation temperature should be determined for each antibody. Overnight at RT was effective for the antibodies listed in **Table of Materials**.

6.4.5. Wash the fish 3 times with PBST, with each wash lasting 15 min as described in step 6.3.4.

6.4.6. Transfer the fish to PBS for short-term storage before proceeding with dissecting, mounting, or sectioning the fish.

REPRESENTATIVE RESULTS:

Table 1 shows success during one year of breeding surface fish and Tinaja, Molino, and Pachón cavefish in static breeding tanks. Surface and Pachón spawns with fertilized embryos always produced hatched larvae, while Molino and Tinaja were unsuccessful some of the time (2/6 and 2/18 spawning events did not produce hatched larvae, respectively). There is variation in clutch size that does not appear to be attributed to age of the parent fish. **Table 2** shows the total number of hatched larvae resulting from some of the spawning events, and the age of the parent fish. In general, we found that surface fish produce the greatest number of larvae per spawn (average 1,550 ± 894, n = 5), followed by Pachón (average 879 ± 680, n = 6), Tinaja (average 570 ± 373, n = 11), and Molino (average 386 ± 276, n =3). The number of larvae produced is typically more than are needed per experiment or for growth into adults. We typically set up 6-18 nursery containers (120-360 larvae) and euthanize the remaining fish.

To measure the success of the nursery protocol we recorded the number of hatched larvae and surviving post-larval fish from successful spawning events. **Table 3** shows data from 1 month of breeding in recirculating tanks and includes the number of larvae transferred to nursery containers that survived to 14 dpf. During this month, the survival rate ranged from 41-81 percent, resulting in 65-293 fish available per population for experiments or growth into adults.

To determine if whole-mount immunostaining is successful, we compared the fluorescence of samples incubated with primary antibody to those incubated with secondary antibody only. The fluorescent signal is only visible in the fish incubated with primary antibody. We have used this protocol to successfully label neurons[10] (**Figure 1**) and pancreatic cells[6] at stages up to 12.5 dpf in both surface and cave morphotypes.

FIGURE AND TABLE LEGENDS:

Figure 1: Neuron labeling. Whole-mount immunostaining of *A. mexicanus.* Image of 12.5 dpf surface fish (**a**) and Pachón cavefish (**b**). Image of the mid-body region [hatched yellow outline shown in (a) and (b)] of surface fish (**c**) and Pachón cavefish (**d**) stained with pan-neuronal antibody (Hu). (**e**) Confocal image of a region of the surface fish intestine showing enteric neurons (Hu) and their projections (acetylated tubulin). For this image, the intestine was dissected out and mounted in medium containing DAPI to stain the nuclei.

Table 1: Summary of data from one year of breeding *A. mexicanus* in static tanks. Number of breeding attempts, resulting spawning events, and number of spawning events that produced hatched larvae.

Table 2: Approximate female age and number of hatched larvae from individual spawning events from the indicated populations of *A. mexicanus.*

Table 3: **Summary of data from one month of breeding *A. mexicanus* in a recirculating system and raising the larvae.** Number of breeding attempts, resulting spawning events, clutches that produced hatched larvae, average number of larvae per clutch, larvae transferred to the nursery containers, and post-larval fish present in the nursery containers after 14 days.

DISCUSSION:

Comparing gene activity between surface and cave *A. mexicanus* requires carefully controlled environmental parameters and methods that can be replicated across laboratories. Our protocol for raising *A. mexicanus* provides consistent nutritional content during post-larval development. Following this feeding regime, gene function can be confidently compared between populations using the robust immunohistochemistry protocol we present. Here we discuss the significance of this method as well as its limitations and future applications**.**

To achieve density-matched growth, we found that post-larval fish can be raised without recirculating water for two weeks in 1.5 L containers on a diet of rotifers. This protocol can also be used to raise fish on a recirculating system; however, rotifers must be added daily to compensate for those lost through the tank outflow. Newly hatched *Artemia nauplii* are commonly used as a food source in aquaculture, but we found that using rotifers has considerable advantages, including reduced price, improved biosecurity, consistent nutrition, and better water quality, each discussed below.

First, the weekly cost in consumables is 4 dollars for rotifers, compared to 14 dollars for *Artemia*. Regarding biosecurity, rotifers are raised in the laboratory under controlled conditions, while *Artemia* are collected from the wild and subject to natural variation in microbial or pathogen contents[23]. Additionally, the nutrient composition of *Artemia nauplii* are environmentally determined and therefore inconsistent. Nauplii thrive on their own energy stores after hatching; they quickly loose nutritional value as they develop and should optimally be fed to the fish within several hours. *Artemia* begin feeding at 12 house post-hatching, representing the first time that they could be nutritionally enriched; however, at this stage they have become too large for 5 dpf fish to consume. In comparison, rotifers continuously feed on marine microalgae resulting in high nutrient content regardless of when the rotifers are harvested. Rotifers are much smaller than *Artmeia nauplii* (160 *vs.* 400 microns), making them easier for the fish to capture and swallow. Post-larval surface fish and cavefish consume rotifers in similar quantities suggesting no difference in preference or ability to capture the rotifers[10].

Finally, *Artemia nauplii* begin dying in fresh water several hours after being introduced. Uneaten nauplii will decay, rapidly decreasing the water quality if they are not manually removed. Removing dead *Artemia* is time-consuming and dangerous for post-larval fish that are not much bigger than *Artemia;* therefore, they may be accidently removed or injured. Rotifers can live indefinitely in the nursery containers and provide food to the fish at all times without significantly impacting water quality.

While rotifers as a food source have considerable benefits, the rotifers must be provided with food daily: this can be achieved with an automatic feeder that dispenses liquid algae into the rotifer culture container (see **Table of Materials**). Rotifers must also be harvested from this set-up every 24-48 hours to maintain the health of the culture. Researchers that breed fish very infrequently (once a year, for example) and are not concerned with making comparisons between the populations at post-larval stages may prefer *Artemia* as a food source, since the encysted embryos can be hatched at any time.

We recommend tracking the number of hatched and surviving larvae to monitor the success of hatching and growth. If most of the embryos or larvae die, it may be due to bacterial or fungal contamination. It is recommended to monitor the water quality of the fish-ready water and sterilize any equipment with 70% ethanol. Nursery containers can be re-used after they are cleaned and sterilized. To minimize risk of disease, it is also critical to remove any dead fish from the nursery containers and not add rotifers before 5 dpf, when the fish begin to eat.

A. mexicanus are sexually mature at approximately one year old. This is a limitation for generating transgenic *A. mexicanus* compared to *Danio rerio* (zebrafish) that are able to breed at 10-12 weeks[24]. Immunohistochemistry (IHC) is an alternative method to investigate gene expression and protein localization. The protocol described here can be adapted at each step and used to detect antigens in any tissue of interest. It is important to note, however, that some tissues may be more difficult to visualize in surface fish due to pigmentation (a barrier that does not exist in unpigmented cavefish), which may influence the interpretation of comparative studies. To address this potential problem, surface fish pigment can be bleached after fixation using 3% hydrogen peroxide.

IHC requires successful tissue fixation, blocking, and antibody penetration. Methods for each vary depending on the tissue and protein of interest. The fixative and fixation time must preserve cell architecture while maintaining the antigen epitope. For this protocol, we use a cross-linking fixative (paraformaldehyde) and omit a denaturing fixative (such as methanol or acetone). We found that incubation in acetone diminished antibody signal for neuronal and pancreatic markers. The blocking step is essential to prevent antibodies from binding to non-target proteins in the tissue. This method uses a combination of normal serum (5%) and BSA (0.2%) in the blocking solution. The blocking solution contains antibodies and proteins that bind to reactive sites on proteins in the tissue, diminishing non-specific binding of the primary and secondary antibodies.

To achieve antibody penetration, the tissue must be permeabilized. This can be achieved with detergents or denaturing solvents but must be optimized to preserve the antigen epitope. Our protocol uses a combination of Triton and dimethyl sulfoxide (DMSO). Triton and DMSO are included during the blocking and antibody incubation steps at concentrations of 0.5% and 1%, respectively. Using this concentration, we have observed staining in the brain, pancreas, intestine, and muscle, suggesting that it is likely effective for penetration of all tissues. Fish size may also influence penetration. This protocol has not been tested on fish that are greater than

14 days old (approximately 7 mm in length). To troubleshoot staining, it is recommended to alter the fixation, blocking, and antibody penetration steps. It is also important to examine the sequence conservation of the immunogen with the *A. mexicanus* protein of interest using the available genome[25].

A. mexicanus is an excellent model to investigate evolution, as populations of the same species that have evolved in dramatically different environments can be directly compared in the laboratory. Standard husbandry protocols, both within and among laboratories, are essential to understanding the biological differences between surface fish and cavefish. Our article provides a method to examine development and gene activity in post-larval fish exposed to consistent growth parameters.

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DISCLOSURES:
The authors have nothing to disclose.

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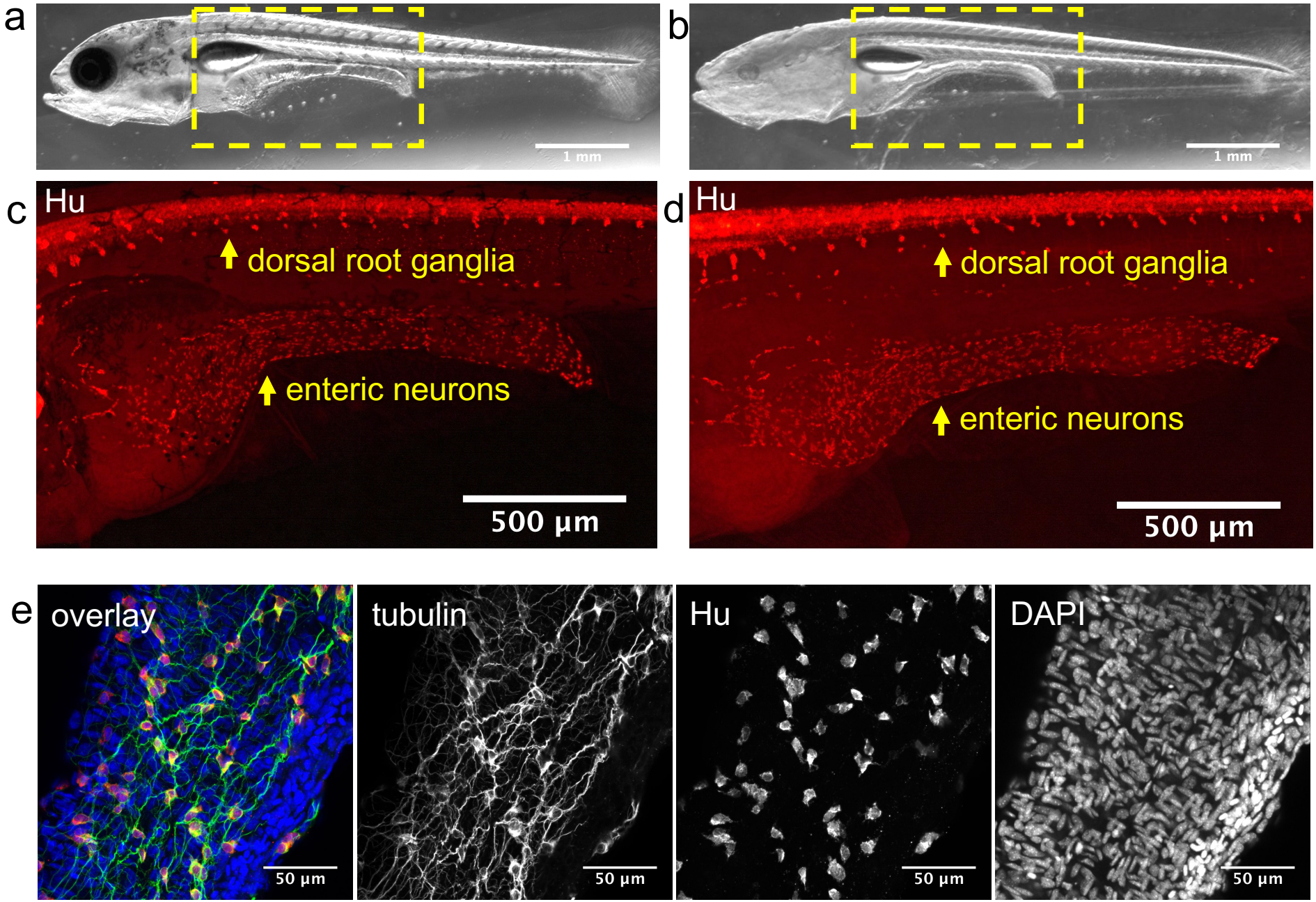

population	attempts	spawning events	clutches
surface	94	23	23
Molino	110	6	4
Pachón	167	13	13
Tinaja	242	18	16

population	parent age	hatched larvae
surface	1 year	989
surface	1 year	1050
surface	3 years	2214
surface	3 years	432
surface	3 years	1768
surface	4 years	2852
Pachón	1 year	1194
Pachón	1 year	1933
Pachón	1.5 years	371
Pachón	3 years	480
Pachón	4 years	1190
Pachón	4 years	110
Tinaja	9 months	259
Tinaja	9 months	253
Tinaja	10 months	1100
Tinaja	11 months	857
Tinaja	1 year	713
Tinaja	1 year	853
Tinaja	1 year	542
Tinaja	1.5 years	360
Tinaja	1.5 years	58
Tinaja	1.5 years	1100
Tinaja	4 years	185
Molino	2.5 years	460
Molino	2.5 years	619
Molino	3 years	81

population	attempts	spawning events	clutches	clutch size	larvae transferred to nursery cups	post-larval fish at 14dpf	survival (%)
surface	4	3	2	576 & 1728	360	174	48
Molino	4	2	1	228	159	65	41
Tinaja	4	1	1	1952	175	93	53
Pachón	4	1	1	1696	360	293	81

Target	host species	company	catalog number	concentration
insulin	Guinea pig	Dako	A0564	1:200
glucagon	Sheep	Abcam	ab36215	1:200
acetylated tubulin	Mouse	Sigma	T6793	1:500
HuD/HuC	Mouse	Life Technologies	A-21271	1:500
nitric oxide synthase (nNOS)	Rabbit	Abcam	ab106417	5µg/mL
choline acetyltransferase (ChAT)	Rabbit	Abcam	ab178850	1:2000
seratonin (5HT)	Rabbit	Immunostar	20080	1:500

Name of Material/ Equipment	Company	Catalog Number
methylene blue	Kordon	B016CBHZUS
heater	Finnex	4711457836017
airstone	Lee's Aquarium & Pet Products	10838125202
salt	Instant Ocean	51378014021
nursery container	IPC	21545-002
transfer pipette	VWR	414004-002
compact culture system(CCS) starter kit with Brachionus plicatilis (L-type) rotifers	Reed Mariculture	na
RGcomplete	APBreed	817656016572
Programmable Auto Dosing Pump DP-4	Jebao	DP-4
Tricane-S	Western Chemical	MS 222
sodium bicarbonate	Sigma-Aldrich	S5761-500G
nylon mesh strainer	HIC (Harold Import Co.)	735343476235
Formalin solution, neutral buffered, 10%	Sigma-Aldrich	HT501128-4L
10X PBS	Invitrogen	AM9625
Triton-x 100	Sigma-Aldrich	T8787-250ML
sodium azide	Sigma-Aldrich	S2002-25G
bovine serum albumin	Sigma-Aldrich	A9647-100G
glass vial with screw-top cap 4mL	Wheaton	224742

plastic mesh screen for breeding tank	Pentair	N1670
vinyl-coated disk magnets	Kjmagnets	D84PC-AST
New Life Spectrum Thera-A pellet fish food	New Life International	na

Antibodies

insulin antibody from guinea pig	Dako	A0564
glucagon antibody from sheep	Abcam	ab36215
acetylated tubulin antibody from mouse	Sigma	T6793
HuD/HuC antibody from mouse	Life Technologies	A-21271
nitric oxide synthase (nNOS) antibody from rabbit	Abcam	ab106417
choline acetyltransferase (ChAT) from rabbit	Abcam	ab178850
seratonin (5HT) from rabbit	Immunostar	20080

Comments/Description
antifungal
100W Digital Control Heater
disposable air stone
Sea Salt
40 oz or 1.5 L clear containers
plastic bulb pipettes
fish food
32 oz bottle of rotifer food
automatic feeder for rotifers
fish anesthetic
for tricane solution
3-inch diameter
fixative
buffer, dilute to 1X using distilled water
detergent
anti-bacterial
blocking reagent
staining vial

Cut into a rectangle 6mm larger on all edges than the dimensions of the bottom of the breeding tank. Cut a 6mm square from each corner of the rectangle. Bend the edges of the screen down along all four edges.Place a pair of 6mm vinyl-coated disk magnets on either side (top and bottom) of the mesh on each corner. The screen should be as snug as possible to the sides of the tank. The screen can be removed from the tank with a metal fish net.

Adult fish food. A list of retailers for this product is available on the company website

1:200
1:200
1:500
1:500

5µg/mL

1:2000
1:500

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Author(s): CLIFF TABIN, MISTY RIDDLE, BRIAN MARTINEZ, MEGAN DAVEY

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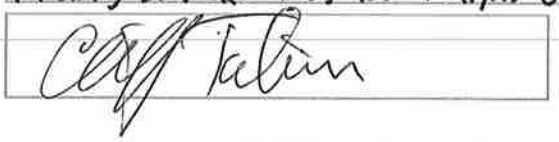
CORRESPONDING AUTHOR:

Name: CLIFF TABIN

Department: GENETICS DEPARTMENT

Institution: HARVARD MEDICAL SCHOOL

Article Title: raising A. mexicanus for analysis of post-larval phenotypes

Signature: 

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We thank the reviewers for carefully reading the manuscript and for providing constructive edits and suggestions. Below is a point-by-point response to the concerns that were raised.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Line 9: Please provide the address for the affiliation.
3. Keywords: Please provide at least 6 keywords or phrases.
4. Please expand the Summary to briefly describe the applications of this protocol.
5. Please spell out each abbreviation (RO, PBS, DMSO, DAPI, etc.) the first time it is used.
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We have made all of the edits (1-6) suggested above.

The summary now reads:
" In this protocol, we demonstrate how to breed A. mexicanus adults, raise the larvae, and perform whole-mount immunohistochemistry on post-larval fish to compare the phenotypes of surface and cave morphotypes."

7. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below:
1.1: Please provide more details, e.g., the fish species, number of fish in the tank, size of the breeding tank, etc.

We have added more details to the protocol steps. Since the breeding method was brought up by several reviewers, we now include additional steps at the start of our protocol that describe how to breed the fish. We also reference several alternative methods.

4.1: Is the rotifer culture prepared in step 3.2?

The distributor of the rotifers provides detailed instructions on how to set up the rotifer culture (including a video on their website). We therefore referenced their protocol at this step. Step 3.2 is to create the solution that

will be added to the nursery cups. We edited the text from step 3 to 4.1 to make it more clear. It now reads:

"3. Prepare the rotifer-based fish food

3.1 See table of materials for obtaining rotifers. Follow the referenced protocol to set up, maintain, and harvest rotifers [1].

3.2 Prepare fish food by adding 3mL algae mixture (see table of materials) to 1 liter of harvested rotifers. This mixture will be added directly to the nursery cups as a food supply.

4. Feeding post-larval fish

4.1 When the fish are five days post fertilization (dpf), add 3mL fish food to each nursery cup…."

5.4.1: Please list an approximate volume to prepare.
5.4.3, 5.4.5: Please give an example of antibody (including concentration) that will be used in the protocol.
8. 1.6: Please convert temperature to °C instead of °F.
9. Lines 158-159: Please remove the google doc link; instead please upload the file as a Table or Supplemental Information.
10. Please include single-line spaces between all paragraphs, headings, steps, etc.
11. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
12. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.
13. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We have addressed the concerns listed above.

14. Discussion: Please also discuss any limitations of the technique, the significance with respect to existing methods, and any future applications of the technique.

We have added additional text to the discussion to highlight limitations, significance, and future applications. For example:

…"Rotifer culture is inexpensive and easy to maintain but requires daily feeding…"

"...Fish size may influence penetration; this protocol has not been tested on fish that are greater than 14 days old"
...Standard husbandry protocols, both within and among laboratories, are essential to understanding the biological differences between surface fish and cavefish. Our article provides a method....
...The protocol described here can be adapted at each step and used to detect antigens in any tissue of interest....

15. References 2-5 and 10: Please provide volume (issue) and page numbers. Please provide complete citation information for reference 21.

We edited the references.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This is a clearly written and straightforward MS and I am happy to recommend its acceptance. I have a few suggestions, but they are all minor and should the authors prefer to reject them, that is just fine too.
1) The title might usefully be changed to help the ignorant, like me, know what Astyanax is! (For example "Raising the blind cave fish (Astyanax...) for ...; or, "Raising the Mexican tetra Astyanax...) for ...).

We changed the title to include "Mexican tetra"

2) Point 5.3.1 (line 137): I assume the tricane solution is already ice-cold (otherwise the ice bath won't do much in 10 min.). Perhaps clarify?

We changed the text to: *Gently submerge the strainer in ice-cold tricane solution and leave on ice for 10 min.*

3) Line 141: definition of PBS (similar queries apply elsewhere).

In the updated protocol, we define PBST at the first use and refer the reader to the materials section.

4) Line 143: Personally I prefer such concentrations in grams/L (but if you leave it as it is I think 0.02% is clearer).
5) Line 148. The vial is 5 ml here and 4 ml in the Table of Materials.

We changed line 148 to the correct description, 4mL.

6) Line 161: be explicit about what "washing" means.

We changed the text for clarity. It now reads:

5.3.3 Remove the fixative and replace with phosphate buffered saline-triton solution (PBST, see materials). Incubate for 15 minutes at room temperature with rocking.
5.3.4 Remove PBST and replace with fresh PBST and incubate for 15 minutes. Repeat this "washing" one additional time.

7) Line 169: 5.4.7. Maybe explain why you DAPI stain?

We changed the text to eliminate this optional step.

8) Line 212-217. Figure 1.
a. Identify the (false?) colours in the overlay rather than have us work it out. I assume the blue is DAPI?
b. Add a reference to Hu expression in fish (and maybe use anti-HuC/HuD in the text to agree with Table 2?).
c. The mention of elav-4 is a bit confusing to me (I thought HuD was ELAVL-4, for example). Maybe just omit?
d. Mention tubulin in the legend.
e. If there are published images from sections that can be compared to your Fig. 1, it would be convincing to reference them.

We have added a reference to our article showing Hu expression in *A. mexicanus* and additional panels to figure 1. We have also updated the figure legend using the reviewer's suggestions. Please see the revised figure and legend.

9) Line 232: fungal not fugal.
10) Line 249-250. I'm not clear what the phrase "while BSA similarly competes with the primary antibody for binding" is getting at. Maybe rephrase.

We rephrased this paragraph. The text now reads:
The blocking step is essential to prevent antibodies from binding to non-target proteins in the tissue. This method uses a combination of normal serum (5%) and BSA (0.2%) in the blocking solution. The blocking solution contains antibodies and proteins that bind to reactive sites on proteins in the tissue, diminishing non-specific binding of the primary and secondary antibodies.

11) Add somewhere that reagent penetration is not a problem (and how you know: and also mention how big these post-larval fish are, and what exactly is post-larval?).
12) What ages of fish does this method work for?

We have added the following text to discuss reagent penetration and fish age/size:
Triton and DMSO are included during the blocking and antibody incubation steps at a concentration of 0.5% and 1% respectively. Using this concentration we have observed staining in the brain, pancreas, intestine, and muscle

suggesting it is likely effective for penetration of all tissues. Fish size may influence penetration; this protocol has not been tested on fish that are greater than 14 days old (approximately 7mm in length).

We have edited the text in several locations to make the distinction between larval (has yolk) and post-larval (no yolk):

…"Less is known about how the populations differ at post-larval stages (at the onset of feeding) although it could provide insight into how cavefish survive to adulthood in their natural environment…"

"…At 5-days post fertilization, the fish have developed to post-larval stages (no longer having a yolk supply) and are provided algae-fed Brachionus plicatilis (rotifers) as a nutrient rich food source…"

Reviewer #2:
Manuscript Summary:
The article by Riddle et al describe methods to grow larval stages and immunohistochemistry procedures to label and visualize different cell types in the absence of huge collection of transgenic lines for Astyanax mexicanus, a fish model system used for evodevo studies. Authors should address below mentioned minor points and a few details in the discussions before it is suitable for publication at JOVE.

Minor Concerns:
Tricaine/MS222 is misspelled throughout the paper, please correct that.
Please rephrase sentence on line no 227 to fix double use of word therefore
Line 135: Authors suggest place the larvae in Tricaine on ice for 10min, please provide a note why it is necessary to place them on ice.

We have corrected the misspelling and added the following note:
Note: The euthanasia protocol should be discussed with your Institutional Animal Care and Use Committee. We have noted that tricaine alone does not euthanize post-larval fish; the fish begin to move when transferred from tricaine to fixative if the ice step is not included.

Lines 158-160: Antibody details should include Vendor details. End users can determine the optimal dilutions but it is important to know the correct company to order specific antibodies.

The antibody details have been included in Table 4 of the revised submission.

Line 178 and that para: It was not clear to me whether these are individual pair wise breedings or multiple fish were set up in a single breeding cross. Authors should clarify on how these breedings were set up. They should also mention what are the ages of the breeding fish. It is well known from zebrafish studies that their fecundity decreases as

they age, if age is not an issue for cavefish, Authors should highlight that because then it is an important advantage for using cavefish in developmental studies. It is important to show std dev next to average clutch size in table 1 or show data only from age matched populations and if the deviation is huge please add a note on explaining the reasons briefly.

We now include additional steps at the start of our protocol that describe the breeding method. We use one female and two males for our breeding set-up. We do not have enough data to confidently state whether fish age influences clutch size. Our breeding fish range in age from 1 year to 4 years old. Other labs have bred fish that are over 10 years old. There is variation in clutch size but it does not appear to be based on age.

To address the reviewers concern, we separated the data presented in Table 1 into two tables. Table 1 shows only the success of breeding. Table 2 shows the total number of hatched larvae from individual spawning events with the age of the parent indicated. We cite the average and standard deviation for clutch size in the text. It is currently unclear why there is variation in clutch size.

The updated text now reads:
"Table one shows the success during one year of breeding surface fish and Tinaja, Molino, and Pachón cavefish using the protocol we described. Surface and Tinaja clutches with fertilized embryos always produced hatched larvae in surface and Pachón, while Molino and Tinaja were unsuccessful some of the time (2/6 and 2/18 clutches did not produce hatched larvae, respectively). There is variation in clutch size that does not appear to be attributed to age of the parent fish. Table 2 shows the total number of hatched larvae resulting from some of the spawning events, and the age of the parent fish. In general, we found that surface fish produce the greatest number of larvae per spawn (average 1,550 ±894, n=5), followed by Pachón (average 879 ±680, n = 6), Tinaja (average 570 ±373, n = 11), and Molino (average 386 ±276, n =3). The number of larvae produced is typically more than are needed per experiment or for growth into adults. We typically set up 6-18 nursery containers (120-360 larvae) and euthanize the remaining fish."

I have animal care and use committee (ACUC) related concern with cavefish larval rearing protocol. Different institutes have their ACUC committees who decide what is a best practice to grow zebrafish/cavefish/other fish models at their institutes after consultation with their veterinary doctors. This generic protocol might not work for every institute. I know several institutes where zebrafish and cavefish fry has to go on circulating water systems lot before day 14/15 of development. It will be useful if Authors should include a disclaimer or a note at the start of the larval rearing protocol stating researchers should check with their institute's animal care and use committee regarding correct procedures on handling and rearing larval fish. This is important

because authors report that three out of four cave populations have only 50% survival with this protocol.

We have included the following note at the start of the protocol:

"Note: The procedures described throughout this protocol should be discussed with your Institutional Animal Care and Use Committee."

This protocol could be carried out on a recirculating system. We have added this alternative to the discussion.

"*This protocol can also be used to raise fish on a recirculating system, however the rotifers must be added daily to compensate for those lost through the tank outflow."*

Reviewer #3:
Manuscript Summary:
The current manuscript by Riddle et al. provides details on rearing of Astyanax from larval through 14-day-old juvenile stages. Astyanax is growing as a model organism, and more labs are turning to this system due to the pronounced differences between epigean and hypogean forms. This current issue of JoVE will facilitate the adoption of the model by more labs, and this manuscript is a particularly important contribution, as fish are most precious and fragile at larval and juvenile stages. Riddle et al. present a simple, yet extremely useful approach to care for fry at these stages, based on methods used in zebrafish research. The authors provide additional information for performing immunohistochemistry in fry as old as 12.5 days post fertilization. The manuscript is well written, informative, and extremely useful. I have only minor comments, meant to add clarity to the manuscript; I fully support the manuscript as written, and my comments should be considered mere suggestions.

Major Concerns:
Part 1, Hatching fertilized eggs
1. Line 81: The reference for the article in this issue describing breeding is Stahl et al. Also, the Borowsky 2008 Cold Spring Harbor Protocols manuscripts should be cited.

We have included the references.

2. Line 89: Can you give parameters or a reference for "fish-ready water"? It may be clearer to state, "RO or dechlorinated water adjusted to: pH = 7.1 +/- 2, conductivity = 900 +/- 150 µS, and temperature = 76 +/- 2°F", or whatever parameters and/or ranges are typically used in the Tabin laboratory.

We have changed this line to include more detail.

Part 2, Transferring hatched larvae to nursery cups

1. Line 96: The authors should define nursery cup. Is this a reference to a literal kitchen cup, or are 1.5 L plastic fish tanks used?

We have included more detail to make it clear that this is a 1.5L container. We have replaced the word "cup" with "container".

2. Line 97: Is the type of salt a required parameter, or will any salt do? Is Instant Ocean typically used?

We use Instant Ocean. JoVE prohibits use of commercial language in the protocol. We now include a note to direct the reader to the materials list which includes the information.

3. Line 104-106: Many OLAW policy, as well as many IACUC's, require addition of sodium hypochlorite, to ensure that development ceases and animals are fully euthanized before discarding. It may be helpful for labs, especially those that do not typically work with fish, if this was included.

We now include the guidelines of OLAW:
"Dispose of unused larvae at this stage using the guidelines of the Office of Laboratory Welfare (OLAW); add sodium hypochlorite to the tank to achieve a final concentration of 6.15%. Wait at least 5 minutes before pouring down the sink."

Part 5, Whole mount immunohistochemistry of post-larval fish
1. Line 158-159: The online excel file is an incredibly useful resource, and that the authors have made this available to users for editing is very attractive. Will the authors add information to the cells that are currently blank? Adding information to missing parameters such as fixation time, protocol details, etc., will not only be helpful, but will inspire others to utilize fully the excel file provided.

Unfortunately JoVE does not allow reference to the online excel file. We have included the completed list as Table 4.

Minor Concerns:
The authors should check their references, as there are some errors in them

We have corrected the references.

Reviewer #4:
Manuscript Summary:
The manuscript by Riddle et al described methods "how to raise larval and post-larval Astyanax mexicanus" and "how to collect post-larval fish and perform whole mount immunostaining". The manuscript is well written and very informative, particularly for someone starting a new Astyanax research lab. I recommend this manuscript for publication in JoVE.

Major Concerns:
None

Minor Concerns:
It would be better to describe more details.
Line 89 What is the final concentration of methylene blue?
Line 92 Please add "24.4 degrees Celsius".
Line 97 How many grams of sea salt? What is the Ph and conductivity of the nursery water?
Line 146 "(1XPBS, 0.5% Triton X, 0.2% BSA….) "
Line 166 "Volume of the antibody solution should be enough to… "
Line 169 What is the concentration of DAPI and phalloidin-fluorophore conjugate?
Line 172 "perform tissue cryo-sectioning"
DAPI and phalloidin-fluorophore conjugate should be added to the table of materials.

The details and edits suggested by the reviewer have been added.

Reviewer #5:
Manuscript Summary:
In the manuscript "Raising Astyanax mexicanus for analysis of post-larval phenotypes and wholemount immunohistochemistry" by M. Riddle et al. the authors describe a methodology to optimize the growth of Astyanax mexicanus larvae in laboratory conditions for phenotypic studies and histological processing. A. mexicanus is an original model for evolutionary studies, however as an emergent model it lacks standardized methods for husbandry in comparison to the zebrafish. Importantly, the protocol described by the authors is inexpensive, time saving and most importantly standardizable, in order to compare larvae from different populations, growth under the same conditions of density and food available. I recommend this article for publication in Journal of Visualized Experiments if the comments listed below are addressed.

Major Concerns:
1.- Although the authors discussed about their preference to use rotifers, as they are inexpensive and easy to culture, their point on choosing rotifers over Artemia is not clear. It is necessary to give more details in this section in terms of nutritive values of each food source, contamination of the water by decomposition, larval survival, etc. Do the larvae originating from surface versus cave or from different cave populations have different food preference?

We have added a lengthy discussion on the benefits of using rotifers as a food source, addressing each of the points made by the reviewer. The text reads:

"Newly hatched Artemia nauplii are commonly used as a food source in aquaculture but we found that using rotifers has considerable advantages for

raising A. mexicanus, including: reduced price, improved biosecurity, consistent nutrition, and better water quality. We discuss each of these below.

Price: weekly cost in consumables is 4 dollars for rotifers compared to 14 dollars for Artemia.

Biosecurity: rotifers are raised in the laboratory under controlled conditions while Artemia are collected from the wild and subject to the natural variation in microbial or pathogen content[22].

Nutrition: the nutrient composition of Artemia nauplii are environmentally determined and therefore inconsistent. Nauplii thrive on their own energy stores after hatching; they quickly loose nutritional value as they develop and should optimally be fed to the fish within several hours. Artemia begin feeding at 12 house post-hatching, representing the first time they could be nutritionally enriched; however at this stage, they have become too large for 5 dpf fish to consume. In comparison, rotifers actively consume marine microalgae resulting in high nutrient content regardless of when the rotifers are harvested. Rotifers are much smaller than Artmeia nauplii (160 microns versus 400 microns) making them easier for the fish to consume. Post-larval surface fish and cavefish consume rotifers in similar quantities suggesting no difference in preference or ability to capture the rotifers[10].

Water quality: Artemia nauplii begin dying in fresh water several hours after being introduced. Uneaten nauplii will decay, rapidly decreasing the water quality if they are not manually removed. Removing dead Artemia is time consuming and dangerous for post-larval fish that are not much bigger than Artemia and therefore may be accidently removed or injured. Rotifers can live indefinitely in the nursery containers and provide food to the fish at all times without significantly impacting water quality. "

2.- Since cavefish are depigmented in contrast to surface fish, the processing for wholemount immunohistochemistry and hence the results obtained may be different at larval stages. I think this must be discussed in the article. In addition, Figure 1 needs to be better explained. A scheme of a larvae to indicate the origin/level of sectioning of this sample and axes for orientation are necessary. Is it a cave or a surface larva? Why is only one morph shown? I think it is important and necessary to show that the method works equally well on both morphs.

We have added multiple panels to figure 1 to include the detail requested by the reviewer. In addition, we have added the following text to the discussion:

…"The protocol described here can be adapted at each step and used to detect antigens in any tissue of interest. It is important to note however that some tissues may be more difficult to visualize in surface fish due to pigmentation; a barrier that

does not exist in unpigmented cavefish, and could influence the interpretation of comparative studies. To address this potential problem, surface fish pigment can be bleached after fixation using 3% hydrogen peroxide…."

Minor Concerns:
Line 81.- "Carry out breeding as described (see Duboue et al, this methods collection)." It is necessary to explain more, i.e. volume of the breeding tanks, number of adults, sex ratios, etc. This information is essential to ensure the obtaining of larvae.

We now include additional details on breeding the fish.

Line 83.- "4 Inches." Units need to be in cm.
In Line 92: "79°F" and then in line 143: "4°C." Units need to be systematically on °Celsius.
Line 97.- What is the meaning of RO water? Which Salt? Instead of "Teaspoons" I think "grams" would be more precise.
Line 169.- DAPI and/or Phaloidin diluted on PBST? What are the concentrations?

These concerns have been addressed in the updated manuscript.