

Corwin Lab Axolotl Protocols

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Reagents

Axo Water

Distilled water, dechlorinated tap water, or commercially available spring water can be used. Deionized distilled water is not recommended due to expense and lack of trace nutrients.

400% Holtfreter's Stock (HFR)

KCl	2.30 g
CaCl ₂ ·2H ₂ O	4.29 g
MgSO ₄ ·7H ₂ O	8.90 g
NaCl	126.72 g

Packets can be prepared ahead of time and stored in Ziploc bags at room temperature in a dry place. Dissolve salts in axo water and adjust volume to 8L. For 100% HFR, dilute 1:4 in axo water; for 50% HFR, dilute 1:8; for 20% HFR, dilute 1:20.

0.07% Benzocaine Stock (10X)

Ethyl p-aminobenzoate (Sigma # E-1501)	0.7 g
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Dissolve in 1L distilled water (takes several days at room temperature with stirring). Store at room temperature.

Anesthetic HFR Solution (0.007% Benzocaine)

400% HFR	500 ml
benzocaine stock (10X)	200 ml
HEPES (free acid)	2.384 g
ddH ₂ O to 2L final volume	

Check pH and adjust to 6.8 - 7.0 with 1N NaOH. Autoclave if desired (not necessary for anesthesia of less than 18 hours or for amputations) and store at room temperature.

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Brine Shrimp

NaCl	22.5 g
brine shrimp eggs	1.5 g

(Bonneville Artemia International Inc., Salt Lake City, UT has given best results. Various brands from pet shops have been less satisfactory.)

Packets can be prepared ahead and stored in small Ziploc bags at room temperature. 18-24 hours before feeding, mix with 1L axo water in glass or clear plastic container until salt has dissolved. Aerate and incubate 6-12 inches from incandescent light source.

Hatching Axo Larvae

Keep eggs in the solution in which they were shipped, at room temperature and out of direct sunlight, until hatching begins.

As axos hatch, transfer them to 20% HFR using a wide-bore pipet. (We cut the tips off plastic transfer pipets using a razor blade or scalpel.) For eggs containing healthy axos which have not hatched within 24 hours of the rest of the batch, gently tear open the egg membrane using two No. 5 forceps to release the axo. (Otherwise axo will be permanently bent.) Transfer to 20% HFR.

The next day, transfer the larvae to 50% HFR. Axos need to eat daily at this stage, but if there are too many brine shrimp they will kill the axo larvae. (There will be a lot of brine shrimp in the gills of the dead larvae.) Therefore, feed a few brine shrimp at a time, and clean out the dish when the axos' bellies are full. Be sure egg shells have been removed from the brine shrimp.

After 7-10 days, increase the amount of brine shrimp given, and decrease feeding frequency to every other day.

Older Larvae

Place larvae into containers of fresh 50% HFR when they are received. (We use shallow 4-10 cup Rubbermaid refrigerator dishes without the tops.) Larvae less than 1.5 cm long can all be placed in the same container. If brine shrimp are available, feed new larvae as soon as they have recovered from shipping (when movements become rapid). Otherwise, start a fresh batch of brine shrimp immediately and feed the next day.

Feed every 2–3 days, being careful to remove brine shrimp egg casings before placing in axo dishes. Clean a few hours after feeding, when bellies are full. Remove any dead (unresponsive and no heartbeat) larvae as soon as possible. "Sick" larvae can sometimes be saved by transferring to small containers of 100% HFR. They can be returned to 50% HFR after recovery (usually takes several days).

When axos get to be 1.5–2 cm long, separate them to avoid nipping and cannibalism. They can be housed in Rubbermaid 12-oz. shallow containers without lids, plastic deli dishes, or other similar containers which have not been used for toxic chemicals. These can be kept in stackable plastic or wire closet organizing bins which provide airflow space between levels, to save bench space. As the axos grow, transfer to larger containers. They should have sufficient space to swim around, and the level of 50% HFR should be sufficient that they can stay completely submerged without having to duck their heads.

"Stressed" axos (recently operated or amputated, sick, or after long periods of anesthesia) should be kept in 100% HFR during recovery.

Feeding

The day before feeding, prepare brine shrimp as specified in **Reagents**.

Remove airstone from 18 to 24-hour-old brine shrimp. Allow 10–15 minutes for shells and unhatched eggs to settle. Brown casings will be seen floating on brine surface and at bottom of container. Pink brine shrimp move in jerks and congregate close to the light source.

Wet a fine fishnet and place in an empty container below the level of the brine shrimp container. Position rigid end of a siphon tube in the shrimp container near the swimming shrimp congregation and away from egg casings. Start siphon by applying suction to the other end of the tube until fluid level extends below the bottom of the shrimp container. Allow fluid to flow through the net into the empty container so the brine shrimp collect in the net. Avoid transferring egg casing material. When sufficient shrimp have been collected, stop the siphon.

Avoid feeding egg casings to newly hatched larvae (less than 3 months old) because they can not process the casing material. For small larvae, we transfer shrimp to a second container of salt solution (22.5g/L axo water) and allow any egg casings to settle a second time

in front of the lamp before collecting the shrimp for feeding.

Rinse the shrimp collected in the fishnet with axo water to remove the salt, then transfer a small amount to each axo dish using a plastic transfer pipet or spatula. When all axos have been fed, rinse inverted net to remove any remaining shrimp. Clean siphon of shrimp and salt deposits. Clean brine shrimp container by rinsing with tap water and wiping any residue with a paper towel. Invert to drain, and turn off incandescent light.

Allow several hours for axos to feed (bellies should be swollen with pink mass in gut), then discard any uneaten brine shrimp and clean the dishes.

Larger axos (older than 4 months) can be fed small pieces of liver. Clean dishes 30 minutes to 2 hours after the axo finishes feeding. (If disturbed too soon, axos will regurgitate, but if left too long the uneaten liver will really stink.)

Cleaning

Transfer axos to holding dish containing 50% HFR, avoiding debris as much as possible. This can be done using a wide-bore transfer pipet to suck up the axo head-first (fit should not be tight, but also not loose enough to allow the axo to turn around). Alternately, pour off as much dirty HFR and debris as possible and then gently pour the axo into the holding dish. (Axos larger than 3 cm or so may try to jump out; be prepared for this. If the axo escapes, keep it wet with 50% HFR until it can be scooped up and returned to a dish.) For very small axos, pour dirty axo water through a clean brine shrimp net to contain any escapees.

Discard any dirty HFR remaining in the dish, and scrub out residue with a clean paper towel. Use a separate paper towel for each axo dish, and clean the newest axos last to avoid transferring any diseases. Refill the axo dish with fresh 50% HFR and return the axos. Wipe out the holding dish after each use.

Between cleanings, check for evaporation (a salt ring around the dish above the surface of the HFR). If this occurs, clean as above but rinse dish with axo water (not HFR) to dissolve the salt before adding fresh HFR.

Anesthesia

Prepare anesthetic HFR as specified in **REAGENTS** section. Larvae are sensitive to both

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the pH of the solution and the concentration of benzocaine. For small larvae (less than 6 months after hatching), further dilute anesthetic HFR solution with 100% HFR.

The axo should be completely immobile within 10-15 minutes of immersion (it can be turned onto its back without twitching). It should recover completely within 20 minutes of removal from the anesthetic solution, and its heart rate should remain steady at approximately 1 beat/sec throughout the period of anesthesia. If the heart rate drops, discontinue anesthesia, remove axolotl to 100% HFR and allow it to recover.

Keep the axo in 100% HFR for a period of time (several hours for short periods of anesthesia, or during the period of regeneration or healing if axo has been operated on.) Feed and

clean according to usual schedule. Axos can be fed as soon as they have recovered from anesthesia.

If euthanasia is necessary, add 0.4 g NaCl to 100 ml stock Benzocaine (0.07%). When salt has dissolved, place axo in solution until heart stops beating completely.

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