Protocol

Obtaining *Xenopus laevis* Eggs

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Nearly a century ago, studies by Lancelot Hogben and others demonstrated that ovulation in female *Xenopus laevis* can be induced via injection of mammalian gonadotropins into the dorsal lymph sac, allowing for egg production throughout the year independent of the normal reproductive cycles. Hormonally induced females are capable of producing thousands of eggs in a single spawning, which can then be fertilized to generate embryos or used as a substrate for generation of egg extracts. The protocol for induction of ovulation and subsequent egg collection is straightforward and robust, yet some of its details may vary among laboratories based on prior training, availability of necessary reagents, or the experimental objectives. As the goal of this protocol is not to describe every single variation possible for acquiring eggs but to provide a simple and clear description that can be easily applied by researchers with no prior working experience with *X. laevis*, we focus on describing the method we use at the National *Xenopus* Resource—that is, inducing ovulation in *X. laevis* via dorsal lymph sac injection of gonadotropic hormones and the stimulation of egg laying through application of gentle pressure to the females.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

Egg-laying solution (optional; see Steps 11–20):

Marc's modified Ringer solution (MMR) <R>

Modified Barth's saline for *Xenopus* (MBS) <R>

Hormone solutions for egg laying in *Xenopus* <R>

Generally, pregnant mare serum gonadotropin (PMSG) is used for priming (see Steps 1–5), and either human chorionic gonadotropin (hCG) or ovine luteinizing hormone (oLH) is used for boosting (see Steps 6–10).

Xenopus laevis mature female frogs, preferably no younger than 18 mo old

Equipment

Incubator at 18°C

Petri dish (sterile)

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Standard *X. laevis* maintenance facility (see Protocol: **Animal Maintenance Systems: *Xenopus laevis*** [Shaidani et al. 2020a])

Syringes (1-mL) and needles (e.g., PrecisionGlide, 27 G × 3/8", from Becton, Dickinson and Company)

Temporary holding tank (see Step 9)

Use a plastic mouse cage or a large Tupperware container with an aerated cover that can be secured to prevent the frogs from escaping.

Transfer pipettes

METHOD

Priming

Prime frogs 1–7 d before planned ovulation. The priming injection on its own does not induce egg laying. Instead, it promotes consistent production of a high number of mature eggs following the boosting injection. The use of PMSC for priming has been shown to improve follicular maturity.

1. Remove PMSC stock from -20°C freezer and allow it to thaw at room temperature or 37°C .

2. Fill the 1-mL syringe with 30–50 U of 100 U/mL PMSC.

Smaller inbred J-strain frogs require less hormone and can be primed with 30 U of PMSC, whereas larger wild-type frogs can be given 50 U of PMSC.

Alternatively, hCG can be used to prime females at 0.1 of the amount used for boosting or 50 U for a wild-type female.

3. Pick up and restrain the female with one hand in such a way that the dorsal surface of the frog is rested against palm of the hand with the head of the frog pointed toward the wrist (Fig. 1A,B). Place the thumb and the middle finger along the posterior sides of the animal abdomen and use the index finger to abduct one of the frog's hind limbs (Fig. 1C).

If possible, the palm of the hand should cover the frog's eyes, which will help keep it calm. This hold makes the dorsal lymph sac pocket easily accessible while restraining the animal.

4. With the other hand, insert the needle subcutaneously through the dorsal surface into the dorsal lymph sac in the posterior medial region of the animal proximal to the lateral line "stitch marks" and slowly inject the hormone (Fig. 1C–E).

Insert the needle at a shallow angle to prevent penetrating muscle.

5. Return the frog to its permanent tank and do not feed until after the eggs have been collected.

Adult frogs can regurgitate food following hormone injection, and the presence of solid waste can reduce the durability of the laid eggs.

Boosting

Perform the boosting injection the evening before egg collection (see Step 10 for timing details). The boosting injection promotes the final steps of oocyte maturation and is necessary for induction of ovulation. Females given a boosting injection are likely to spawn, even if they have not been given a prior priming injection. The priming injection serves to ensure consistent egg-laying response to the boosting injection.

6. Remove oLH or hCG stock from -20°C freezer and allow it to thaw at room temperature or 37°C .

7. Fill a 1-mL syringe with 2 μg of oLH per 1 g of body mass (typically $\sim 200 \mu\text{g}$ of 0.4 mg/mL oLH) or with 500 U of 1000 U/mL hCG.

Smaller inbred J-strain females require less hormone and can be primed with 120 μg of oLH or 300 U of hCG. Larger wild-type females require 200 μg of oLH or 500 U of hCG.

8. Inject the frog as described in Steps 3 and 4.

9. Prepare a temporary holding tank with system water for the frog.

FIGURE 1. Single hand manual frog restraint for gonadotropin injection and egg collection. (A) View from the dorsal surface with the index finger in-between both legs, one of the legs being held between the index and the middle fingers, the thumb located around the waist on the other side of the frog. (B) View of the hold from the ventral surface with the index finger between the legs, the middle finger and the thumb located on the waist on the opposite sides of the frog, and the tip of the little finger pressed against the throat to secure the head against the base of the palm of the hand, thus aiding to cover the eyes. (C) The frog in fully restrained hold with one of the legs pulled back against the abdomen and a needle ready for injection through the dorsal surface. (D) *X. laevis* female adult with the general location of the dorsal posterior lymph sacs, the injection target, outlined in red. (E) A more relaxed hold on the female with neither leg fully restrained against the abdomen and with a needle ready for injection through the dorsal surface.

10. Place the frog in the holding tank, and place the tank into an 18°C incubator.

Ovulation will begin ~8–12 h postinjection and should continue for ~5 h. Do not feed the frogs while waiting for them to lay eggs.

The onset of ovulation can be delayed by several hours by keeping the female at a lower temperature of 15°C.

Obtaining Eggs

11. Remove the temporary holding tank with the frog from the 18°C incubator.

The steps below describe a way of handling a female frog to promote egg laying and subsequent collection of a batch of eggs into a Petri dish. As an alternative, at this point the system water in the frog holding tank can be replaced with an egg-laying solution like 1× MMR or 1× MBS. The viability of eggs laid into system water is lost rapidly but can be maintained for at least 1 h if laid into egg-laying solution. This allows for eggs to be collected throughout the day, without the need to handle the females. The animals tolerate being placed in the egg-laying solution well; however, further handling to promote egg laying should be avoided as it does stress the females. This approach is potentially less reliable at producing a large number of viable eggs at exactly the desired time.

12. Obtain a sterile Petri dish for egg collection.
13. Similar to the hold used for restraining the animal during hormone injection, gently but firmly pick up the female from the dorsal side with the dominant hand, allowing its head to be concealed by the palm (Fig. 1A,B).

The frog should be in the prone position.

FIGURE 2. Manual two-handed restraint of a female ready for egg expulsion. (A) Initial hold with the female held in the dominant hand. The face of the frog rests against the inside of the base of the palm of the hand. One of the legs is held between the index and the middle finger while the thumb is near the pit of the contralateral arm. The other leg is being restrained between the index and the middle fingers of the other hand. (B) Both hands are used to progressively pull both legs out and forward toward the head. (C) Fully restrained female with both legs held against the abdomen. The hold is loose enough to allow the female to wiggle and flex its leg and abdominal muscles, yet firm enough to prevent it from breaking loose.

14. Place the thumb and middle finger along the posterior side of the female with the little finger against the throat of the animal to restrain it further.
15. Using the index finger, abduct one of the frog's hind limbs to reflect it rostralward. At the same time abduct the second limb using the other hand (Fig. 2A–C).

This will expose the cloaca/vent and allow for the best control when attempting to position the female. The thumb, ring, and index finger should be available to massage the dorsal and ventral lower trunk.

16. Position the female vertically, with the cloaca over the Petri dish.

This helps prevent the eggs from running along the body of the female.

17. Use the dominant hand's ring and middle finger tips to apply pressure to the belly and the other hand's thumb to apply pressure to the back of the animal.

18. Gently shift the pressure in an anterior to posterior direction to aid the expulsion of eggs.

Simply restraining the female in the described hold, without applying additional pressure, is often sufficient for egg expulsion. The hold itself should be tight and stiff enough to prevent the female from breaking loose. Eggs will come out as she flexes her leg and abdominal muscles attempting to escape. If no eggs come out when simply holding the animal, gently increase the applied pressure being sure not to hurt the animal. Monitor the animal for stress indicators such as sudden excess of skin surface mucus. Females should only be squeezed once every hour and no more than three times in a single day.

19. Transfer the frog to a clean tank overnight and allow it 24 h to recover prior to returning it to the system.

*When dealing with albino females in particular, even gentle pressure may result in mild bruising around the eyes and the part of the abdomen handled directly. This bruising will disappear within hours and has no long-term ill effects on the health of the female. *X. laevis* are hardy, although care should be taken not to apply such pressure that it leads to injury.*

20. Immediately after putting the female back, use a transfer pipette to remove any system water that may have dripped into the egg dish during collection.

Egg viability will decline rapidly if eggs are exposed to a significant amount of system water. If collected eggs are not going to be used immediately, it may be useful to flood the egg collection dish with 1× MMR or 1× MBS, which will prevent them from drying out and will help maintain their viability for at least an hour.

DISCUSSION

The method described here is well-established (Wlizla et al. 2017, 2018) and allows for a simple and efficient way to collect eggs from female *X. laevis* at desired times. These eggs can then be used directly

in experiments or fertilized via in vitro fertilization (IVF) to generate embryos (see Protocol: **Obtaining *Xenopus laevis* Embryos** [Shaidani et al. 2020b]). Developing a correct technique for restraining *X. laevis* females for hormone injection and egg collection requires some practice but is essential for decreasing distress caused to the animal and for collecting the best-quality eggs. Restrained frogs appear to struggle less against the hold when their eyes are covered. For those not experienced in holding frogs, a good initial practice may be to place a moist paper towel on a tabletop, put the female on the paper towel, and gently wrap her head to cover her eyes while keeping the posterior dorsal surface still accessible for injection. This way the hard tabletop surface can be used to aid in immobilizing the animal.

Another aspect of this procedure that requires some practice is determining the amount of pressure that should be applied to the animal during egg expulsion. Too little pressure might not produce any eggs, whereas too much may cause the eggs to be damaged or cause injury to the animal. If simply holding the female is not productive, some pressure will be necessary. The pressure should be applied in a way in which it shifts in a rostral to caudal direction so that it is not simply a squeeze, but instead a massage intended to push the eggs toward and out of the cloaca. As an alternative to handling the female, following the boosting hormone injection, she can be placed in a tank containing egg-laying solution. The female will lay eggs at a slower rate but they will maintain their viability for at least 1 h and can be collected directly from the tank; however, it is likely that the total amount of eggs produced will not be as high without the additional manual pressure. Common egg-laying solutions include 1× MMR or 1× MBS, both of which work equally well for maintaining egg viability. The choice of egg-laying solution will depend on prior user experience and experimental design aspects beyond the scope of this protocol.

A final point to address is the use of mammalian gonadotropic hormone injection into the dorsal lymph sac of the *X. laevis* female as a reliable and effective way for induction of ovulation. hCG has been historically used for this purpose; however, during a shortage of commercially available hCG, we determined that other gonadotropins, including PMSG for priming and oLH or human luteinizing hormone for boosting, can stimulate spawning as effectively (Wlizla et al. 2017). Although using hormones not sourced in animals may be the more humane option when considering animal welfare, the awareness of these alternatives is useful when dealing with supply disruptions or when trying to find the most economical option.

RECIPES

Hormone Solutions for Egg Laying in Xenopus

Hormone	Concentration in 1× PBS
Human chorionic gonadotropin (hCG; from National Hormone and Peptide Program, Sigma-Aldrich, Merck Animal Health [Chorulon brand], or BioVendor)	1000 U/mL
Ovine luteinizing hormone (oLH; from National Hormone and Peptide Program)	0.4 mg/mL
Pregnant mare serum gonadotropin (PMSG; from BioVendor)	100 U/mL

Dissolve phosphate-buffered saline (PBS) tablets (Sigma-Aldrich P4417) in type 1 ultrapure water, at a concentration of 1 tablet per 200 mL. Sterilize by autoclaving. Prepare individual hormone solutions in 1× PBS at the concentrations listed above. Store all hormone solutions at 20°C until needed for use.

Marc's Modified Ringer Solution (MMR)

1 M NaCl
20 mM KCl
10 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
20 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
50 mM HEPES free acid

Prepare a 10× stock that consists of the reagents listed above, adjusting the pH to 7.4–7.8 with NaOH and sterilizing by autoclaving. Store the stocks indefinitely at room temperature. Dilute stock as necessary with type 1 ultrapure water (ASTM International 2018) without further pH adjustment.

Modified Barth's Saline for *Xenopus* (MBS)

Make a 10× MBS stock:

800 mM NaCl
10 mM KCl
10 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
50 mM HEPES free acid
25 mM NaHCO_3

Prepare a 10× stock that consists of the reagents listed above, adjusting the pH to 7.8 with NaOH and sterilizing by autoclaving. Prepare a separate 0.1 M stock of CaCl_2 , and sterilize by autoclaving. Store the stocks indefinitely at room temperature.

To make 1× MBS, dilute the 10× MBS stock with type 1 ultrapure water (ASTM International 2018), and add CaCl_2 stock to a final concentration of 0.7 mM.

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