Published in final edited form as:
Cold Spring Harb Protoc. ; 2013(2): 149–155. doi:10.1101/pdb.prot071977.

Humane Anesthesia and Pain Management in Amphibian Limb Surgery of *Rana pipiens*

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Abstract

Adult *Rana pipiens* frogs are used as a model to investigate mechanisms of vertebrate organ regeneration, anti-tumor ribonucleases, zoological impacts of various pollutants, oncogenesis, neuroplasticity, and neurogenesis. In regenerative biology, the adult *Rana pipiens* frog is an important alternative to other frog models, such as *Xenopus laevis*, because it offers the opportunity to study and attempt to augment limb regeneration in an animal that spends significant time out of water and bears weight on its limbs. To elucidate regenerative processes, it is necessary to amputate the limb to study the processes associated with wound healing, blastema formation, and morphogenesis. Being able to revive the animal successfully with little or no side effects is paramount to these studies. Anesthesia and the effect it has on the frogs can vary based on the methods and post-operative care exercised during surgery. However, useful information is not readily available regarding current anesthesia methods or effective and humane analgesia use in amphibians. Amphibian patients are very sensitive to drug dosages, changes in temperature, humidity and water quality; here, special attention is given to these factors. This protocol establishes a humane anesthesia technique while maintaining physiological homeostasis during procedures in amphibians as well as a post-operative care plan addressing the clinical benefits of using analgesics in pain management. Suggestions for infection prevention are covered with a sample treatment plan to ensure that all of the animals have a positive outcome and all of the surgeries have reproducible results.

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.

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

Reagents

Amphibian Ringer's solution (200–250 mOsm) <R>

Buprenorphine (optional; see Step 7)

Ethanol (70%)

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Use benzene and additive-free ethanol only.

Oxytetracycline hydrochloride (optional; see Step 7)

Tricaine (ethyl-3-aminobenzoate methanesulfonate)

Prepare a 1% tricaine solution in amphibian Ringer's solution, adding sodium bicarbonate while the solution is stirring to buffer it to pH 7.3.

Equipment

Beaker

Dissecting board made of high-density polyethylene (Richard-Allan Scientific)

Holding tank for frogs

Nitrile gloves

Frogs are allergic to latex (Sobotka and Rahwan 1994; Gutleb et al. 2001).

Post-operative table or bench

Include basins lined with damp gauze or paper towels so that each frog gets an individual strip of gauze.

Ruler

Scale

Scalpel blade (#10 sterile, single-use; Feather Safety Razor Co.)

Stock at least one extra #10 blade per frog to ensure that a new blade is used for each frog forelimb amputation.

Screen cover for basin

Single-use needles (26G ½ precision Glide BD)

Spray bottle for 70% ethanol

Spray bottles (sterilized) for fresh amphibian Ringer's solution

Syringes (BD Luer-Lok; 1 mL or 3 mL)

METHOD

Pre-Operative Preparation

Surgical preparations take ~30 min. On the day of surgery, animals should not be fed.

1. Make the following preparations:
    - **i.** Clean table, dissection board, and all surfaces near the surgical field with 70% ethanol.
    - **ii.** Fill 1 needle per frog with 0.5 mL of 1% tricaine buffered to pH 7.3. Remove air bubbles from the syringe. Recap the syringes and place in a beaker.
    - **iii.** Weigh the frogs, record in grams, and then place frogs in holding tank.

Typically, a 40 g frog responds well to a 0.5-mL dose of 1% buffered tricaine. However, dosages may vary among specimens, so it is important to adjust for weight.

Sedation

2. Select frogs for sedation by carefully picking them up and while not squeezing them, holding each securely enough to administer the intracoelomic (IC) injection into the lower abdomen (see Fig. 1). Inject all of the frogs using a separate syringe for each frog, with needle bevel facing up. Ensure the needle has reached the IC space.

> After this step, the surgery must proceed; the steps cannot be interrupted. Anesthesia only lasts 1–2 h.

3. Ensure adequate sedation of frogs. Monitor depth of anesthesia by cessation of gular movement, closure of eyelids, lack of righting reflex, and lack of withdrawal to toe pinch.

> Effective sedation from anesthesia takes 30 min. If the frog responds to touch or rapid regular movement is still observed, the frog may need more of the 1% tricaine. See Troubleshooting.

4. Spray the frogs every 10 min with amphibian Ringer's solution.

> When frogs dry, they can drop abruptly in temperature, leaving them susceptible to infections, so it is important to maintain adequate moisture.

Surgical Amputation

Repeat Steps 5–7 for each frog. Amputations take ~5 min per frog.

5. Place one frog on dissection board with a ruler. Photograph the frog to record its size (snout to vent length in cm; see Fig. 2).

6. Amputate the limb at a desired point (depending on experiment and process under study) with the #10 blade attached to the scalpel. Discard the amputated limb into an animal waste bag.

7. Move the frog to the post-operative tank arranged with absorbent paper towel/gauze squares. Place one frog on each square so that the frog is slightly elevated with its arm raised slightly higher than its body (see Fig. 3).

Bleeding should start tapering off and clot after 30 min. Amphibians tend to hemorrhage less than mammals and birds; however, it is still important to monitor each individual and make sure the bleeding stops.

Post-Operative Monitoring

8. Monitor the condition of each frog in the post-operative tank for 1 h. Continue to keep the frogs well hydrated, checking the skin every 5–10 min and spraying their bodies with amphibian Ringer's solution.

> Frogs should wake up ~1 h post-operative. They will be a little disoriented and will need to be monitored until they can sit upright and respond to their environment.

9. While the frogs are sedated, drain their terrarium habitat and change their habitat material so that they are returned to a clean tank. Once frogs show signs of activity, and they are no longer bleeding, place them in their terrarium habitat. Place all amputated frogs of a particular experimental group together in a clean tank (separate from the other

frogs in the colony). Do not refill the terrarium with water until the end of the day after the frogs are completely alert.

10. Monitor frogs for 1 d after surgery for signs of pain or infection. If frogs show signs of pain on reviving from tricaine anesthesia, administer 38 mg/kg buprenorphine subcutaneously every 4–6 h at the site of the dorsal lymph sac. If frogs begin to show signs of infection, one can administer 0.3 mg/mL of oxytetracycline to the tank water (Merck & Co. 1955; Sive et al. 2000).

See Discussion.

11. Examine frogs for the next 3 d to ensure wound epithelium closure at the base of the stump.

A thin film should form over the site of the amputation. See Troubleshooting.

12. Change the water with fresh amphibian Ringer's solution every day for the next 7 d.

Problem (Step 3): There is a lack of response to the standard dose of anesthesia.

Solution: Titer the dose to non-responding frogs, giving 0.2 mL every 30 min until adequately sedated.

Problem (Step 11): The wound does not appear to be closing.

Solution: This suggests a post-operative infection. Administer oxytetracycline at 0.3 mg/mL into the tank. See Discussion.

DISCUSSION

Adult *Rana pipiens* frogs are used as a model to investigate mechanisms of vertebrate organ regeneration (Smith 1967; Borgens et al. 1977), anti-tumor ribonucleases (Ardelt et al. 2007a,b; 2008; Lee and Raines 2008), zoological impacts of various pollutants (Shenoy et al. 2009), oncogenesis (Outzen and Custer 1976; Outzen et al. 1976; Ruben et al. 2007), neuroplasticity (Constantine-Paton and Capranica 1976a,b; Maden 1981), and neurogenesis (Scalia et al. 2009). This protocol provides a safe, reproducible, and humane technique to perform amputation surgery on adult amphibians, specifically *Rana pipiens*. This technique may also be applicable to other amphibian species for any type of limb surgery not limited to studying limb regeneration. To our knowledge, there are no other anesthesia, pain management, or surgery protocols for limb amputation in the literature. Wright and Whitaker (2001) and Gentz (2007) described general surgery and anesthesia techniques as well as the use of prophylactic antibiotics in amphibian surgery, but not in reference to amputation.

We used principles from veterinary surgery to create a sterile operating field to minimize infections and complications. We used laboratory-grade powder-free, non-sterile, nitrile (non-latex) gloves. We did not make use of prophylactic antibiotics, and only treated infections when clinical symptoms arose. We attribute this success under these conditions to host-defense peptides founds in *Rana pipiens* skin, specifically the ranatuerin-2 peptide which has high potency activity against both gram positive (*S. aureus, S. epidermidis, Streptococcus* Group B) and gram negative (*E. coli, E. cloacae, P. aeruginosa, K. pneumoniae*) bacteria (Goraya et al. 2000; Conlon et al. 2005). Additionally, *Rana pipiens* in particular have raneurin-2P, which shows activity against frog virus 3, a potential concern as our *Rana* are caught from wild populations that may be carriers of frog virus 3 (Chinchar et al. 2001). Theoretically, if such procedures were performed on mammals under our conditions, one would expect a higher rate of surgical site infections and post-operative infections.

Prophylactic antibiotics are not recommended, as this has the effect of desquamation of the frog skin and slime coat. Instead, we give antibiotics as needed when frogs show clinical signs of infections such as redness, abrasions, foul looking skin, or odor. Because the natural peptides present on amphibian skin both fight infection and are sensitive to environmental changes to keep the frogs healthy, antibiotics must be administered judiciously; otherwise, they may adversely impact frog health. See the discussion in Felt et al. (2012) for more information on the drawbacks and benefits of using antibiotics in amphibians.

Stevens et al. (2001) compared different analgesic therapies for amphibians. In this article, they detailed the necessity of using an anesthetic in addition to an analgesic for amphibian surgeries. The use and standardization of dosages for buprenorphine in amphibians can be found in the University of Arizona's Animal Care and Husbandry policies (Committee 2012). Gentz (2007) discussed the accepted use of tricaine solution baths for anesthesia, while Cakir and Strauch (2005) showed that tricaine is a safe anesthetic compared with the prior research standard use of benzocaine and pentobarbital. Finally, we used techniques from Wright and Whitaker (2001) for proper intracoelomic injection handling. In our experience, they offered more reproducible anesthesia than immersion methods, which can also be used.

Our novel use of anesthesia also merits further discussion, as it helps the researcher to proficiently and accurately amputate the limb at specific points while reducing trauma to the specimen. Tricaine injection is superior to tricaine soaks, as the amount of tricaine injected is a more precise dose, allowing the researcher to avoid underdosing (leading to the animal awakening during surgery) or overdosing (leading to the animal never recovering from anesthesia). Both of these are problems with the imprecise absorption when using tricaine soaks. We attempted the use of benzocaine in this protocol, but all the frogs exhibited seizures and died within 2 wk. Further studies (including extensive necropsies) will be necessary to fully understand the etiology of this delayed toxicity, but we caution researchers against the use of benzocaine in *Rana pipiens*, as we never observed similar seizures in any of our animals not treated with benzocaine.

RECIPE

Amphibian Ringer's Solution (200–250 mOsm)

6.6 g NaCl

0.15 g KCl

0.15 g $CaCl_2$

0.2 g $NaHCO_3$

Add the reagents to 1 L of distilled water. The solution may be stored for up to 7 d, or longer if it is autoclaved and stored in sterile containers.

An alternative formulation consists of 6.5 g NaCl, 0.42 g KCl, and 0.25 g $CaCl_2$ added to 1 L of distilled water. Up to 10 g of glucose may be added to either formulation, but this reduces the shelf life of unsterilized solution to 24 h.

Source: Wright (2006).

Acknowledgments

We thank Ghann's Cricket Farm, Inc. (http://www.ghann.com) for the use of their cricket care and feeding advice. We thank Kelly G. Sullivan for assistance (including photography of Fig. 1), and Clara Bieck and Nikola Kojic for their help in maintaining our experimental Rana colony. This work was supported by the Telemedicine and Advanced Technology Research Center (TATRC) at the U.S. Army Medical Research and Materiel Command (USAMRMC) through award W81XWH-10-2-0058, and NIH through grant 1R01AR055993.

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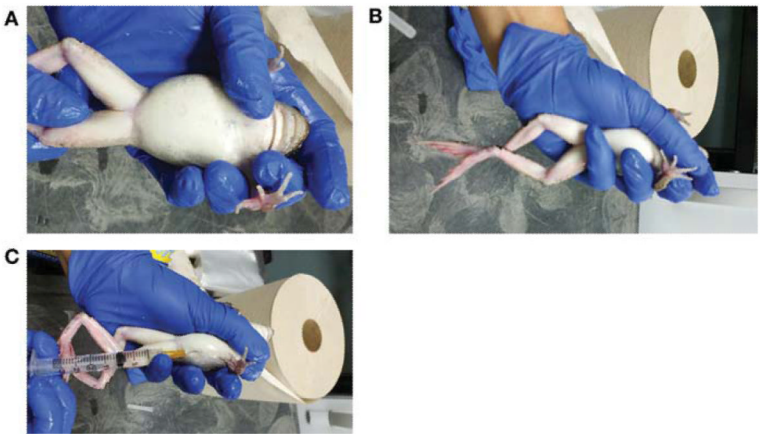

FIGURE 1.

Injecting *Rana pipiens* with tricaine anesthetic. (*A*) The proper way to restrain the frog without injuring internal organs is a loose grip so that one can inspect the frog before any procedure. The eyes are covered and the arms are isolated, which limits movement and exposes the abdomen. (*B*) Proper handling ensures that the abdomen is not being squeezed, but that the frog's arms are gently restrained. A firm but gentle grip around the frog's "waist" keeps the legs under control during an injection. (*C*) The proper way to administer an I.C. injection into the lower abdomen. The needle is in the I.C. space but angled upward so that the needle doesn't damage delicate organs. Bleeding should not occur during the injection. Note that the needle can be seen through the skin. The organs are not close to the needle and the frog is not harmed during this procedure.

FIGURE 2.
Measuring limbs after anesthesia, just before cutting. It is necessary to obtain measurements of the frog's arm and of the vent-to-snout length before amputation. Knowing the vent- to-snout length helps if you need to age the animal. When amputating limbs, it is necessary to remove roughly the same amount of tissue each time and to measure limbs so as to have a reference point to compare regeneration rates among controls and treated animals.

FIGURE 3.
Post-operative *Rana pipiens* after limb amputation with arm in elevated position. After amputation, the frog bleeds for a few minutes. Damp paper towels moistened in fresh amphibian Ringer's solution are folded into small squares with an extra fold tucked under the arm to raise it slightly. This usually stops the bleeding within 5 min. The frogs will stay sedated for 45 min-1 h; during this time, they are kept moist by spraying them with amphibian Ringer's solution and changing the moist paper towel squares as needed to keep the wound moist and clean. The animals must be monitored until they are completely awake and aware of their surroundings so that they can be returned to their aquarium.