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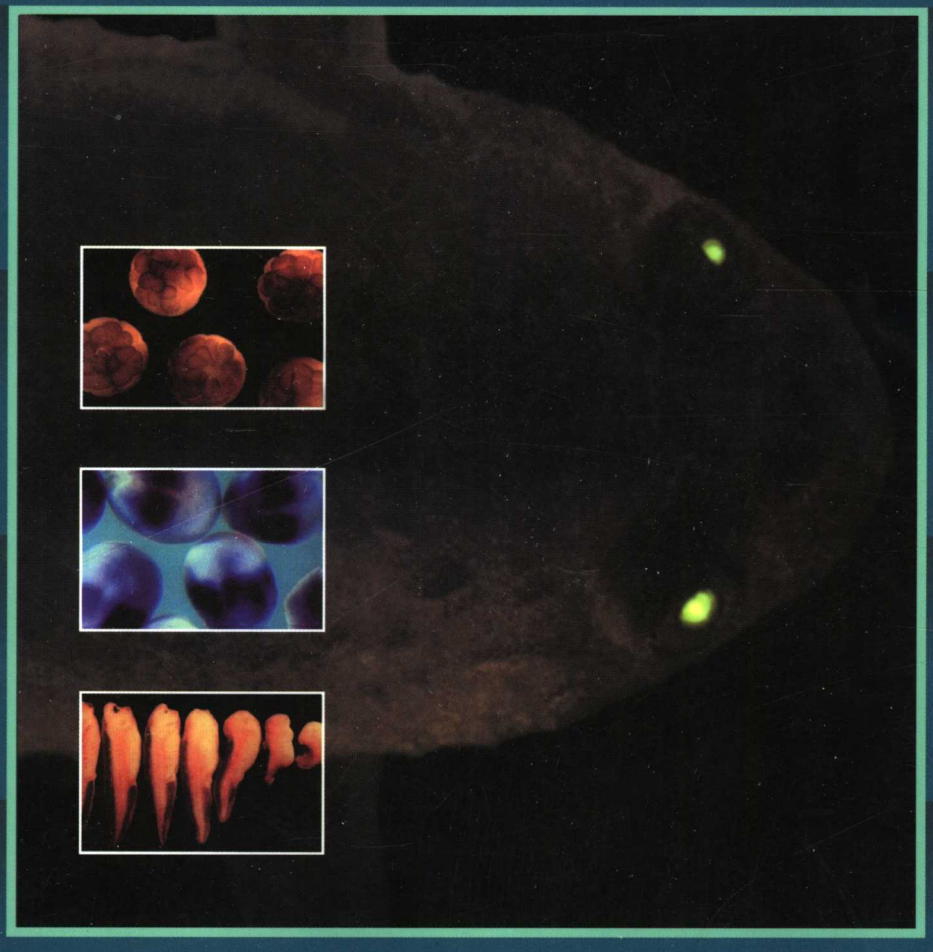
爪蟾早期发育实验指南

Early Development of *Xenopus laevis*

A LABORATORY MANUAL

（影印版）

〔美〕H. L. 西弗　R. M. 格兰杰　R. M. 哈兰　著



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内 容 简 介

爪蟾是生物学研究中的一种模式生物,在功能基因组、发育生物学、细胞生物学、蛋白质组学和神经生物学研究中占有较重要的地位,在脊椎生物的早期发育研究中扮演着非常重要的角色。本书是冷泉港实验室出版社 *Early Development of Xenopus laevis: A Laboratory Manual* 的影印本。书中的实验方案涵盖了爪蟾胚胎、成体的形态发生,爪蟾中基因表达操作,胚胎的获取和实验常规操作,微注射,免疫细胞化学,原位杂交,培养基和培养液等爪蟾早期发育研究的内容。每一个方案都经过专家的精心挑选和雕琢,实验设计严谨、准确、简洁、规范,可操作性强,值得称道。书中既包含了初学者需要了解的基础知识,也涵盖了资深研究者所需的细节。本书的版式设计侧重于方便读者使用,正文中穿插了丰富的图表作为实验设计的辅助说明,附录中还列出了爪蟾早期发育研究所需的重要资料如基本溶液、缓冲液的配方和配制方法等。

本书适合于从事发育生物学、细胞生物学、基因组学、遗传学、分子生物学、药物设计和开发以及功能基因组学研究的相关教学科研人员以及相关学科的本科生、研究生参考使用。

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Preface

Experiments using amphibian embryos have led the way in understanding signaling events in the early development of vertebrates. The power of amphibian embryology stems from the ability to obtain embryos of all stages and from the large size of the embryos. These attributes have made detailed fate maps possible, and allow embryos to be microinjected and micromanipulated relatively easily. In addition, many amphibian species develop rapidly, so that the interesting stages of axis formation and tissue differentiation are accessible in a relatively short time. Over the years, *Xenopus* has come to dominate experimental embryology in amphibians: *Xenopus* is easy to keep, and ovulates at any time of year in response to simple hormone injection.

This is an exciting time in the *Xenopus* field. During the past several years, many new techniques have been devised or adapted for *Xenopus*. These techniques include whole mount in situ hybridization and immunocytochemistry that allow visualization of gene expression in the intact embryo. Transgenic technology has for the first time led to the correctly regulated expression of promoters in *Xenopus* embryos, eliminating previous problems of mosaic and inefficient expression from DNA expression vectors. Expression cloning has led to the isolation of powerful inducing molecules. Inducible gene expression systems can control the timing of gene expression in gain-of-function assays, whereas dominant-negative proteins have been instrumental in eliminating gene function. Together, these techniques have led to sophisticated new understanding of early *Xenopus* development and continue to ensure *Xenopus* a prominent position in the group of "model organisms."

Given the growth of techniques available for work with *Xenopus*, it is time to collect a comprehensive series of protocols in one place. This book arose from a course first taught at Cold Spring Harbor Laboratory in April 1991. Various in-house protocols were cobbled together, and in subsequent years, the lab manual became more extensive, and more accurate. However, to turn this from a collection of informal protocols into something more

comprehensive and comprehensible took more than a rash promise by the authors. Much of the credit for the completion of the manual goes to the marathon efforts and cajoling of Mary Cozza and Siân Curtis at Cold Spring Harbor Laboratory Press.

H.L. Sive, R.M. Grainger, and R.M. Harland

Acknowledgments

The contribution of many people to entries in this book cannot be overstated. Many sections in the manual have been written by our colleagues, and the procedures have been road-tested and modified by many. Enrique Amaya and Kris Kroll contributed Chapter 11; Sally Moody contributed extensively to Chapter 9; Nancy Papalopulu contributed much of Chapter 2. In addition, we thank Vladimir Apekin, David Bentley, Leila Bradley, Marietta Dunaway, Tabitha Doniach, Rick Elinson, Janet Heasman, Laura Gammill, Josh Gamse, John Gerhart, Jeremy Green, Ali Hemmati-Brivanlou, Ray Keller, Chris Kintner, Anne Knecht, Peggy Kolm, Mike Klymkowsky, Paul Krieg, Martin Offield, Charles Sagerström, Bill Smith, David Turner, Daniel Wainstock, and Paul Wilson. We also thank students of the course, members of our laboratories, and lecturers in the course for suggestions and criticism.

Thanks for permission to use figures goes to Rick Elinson, Jonathan Slack, Sally Moody, Tabitha Doniach, and Ray Keller.

We thank Terri Grodzicker and Jim Watson for their support in starting the *Xenopus* course, the laboratories of Hollis Cline and Nick Tonks for special help with emergency course supplies, and Clifford Sutkavich for finding essential equipment. We would also like to thank members of the CSH community for help in this project. H.L.S. thanks Andrew Lassar for his support and for looking after two infants while she coorganized this course.

While writing and revising the manual, we have been supported by our respective institutions and the National Institutes of Health. The course was supported by the National Science Foundation, the National Institute of Child Health and Human Development, and the Howard Hughes Medical Institute.

Contents

**For color art in Chapters 2 and 9, see Color Plates between pages 102 and 103.*

CHAPTER 1

Introduction

Welcome to the world of *Xenopus*. This manual is designed to introduce developmental biologists to the use of *Xenopus* as a model system. However, it is not a comprehensive volume and should be used in conjunction with Kay and Peng (1991) and Nieuwkoop and Faber (1994). Two recent and useful studies of *Xenopus* rearing are described by Hilken et al. (1994, 1995). Another useful volume by Hausen and Riebesell (1991) contains an excellent histology of early embryos. The companion video series "Manipulating the Early Embryo of *Xenopus laevis*" presents very helpful illustrations of many of the developmental processes and procedures described here. The reader is encouraged to use these video demonstrations to complement the material presented in this manual.

At several instances in this manual, more than one protocol is presented for a single procedure. The protocol of choice generally depends on the facilities available in the laboratory and on personal preference. There is rarely one "correct" method.

Xenopus laevis is a gentle, freshwater animal that can be induced by simple hormone injection to lay eggs repeatedly. These features, coupled with the large size of the embryos, which allows micromanipulation and microinjection, and their rapid rate of development, make *Xenopus* an excellent animal for analyzing early vertebrate development. The chief disadvantages of *X. laevis* are long generation times (1–2 years) and tetraploidy. The diploid *Xenopus (Silurana) tropicalis* does not have these disadvantages. This species has a generation time of 5 months or less and so may provide a useful alternative to *X. laevis* for future research (Amaya et al. 1995).

WHERE TO OBTAIN *XENOPUS*

The two major suppliers in the United States are NASCO and Xenopus I (5654 Merkel Road, Dexter, Michigan 48130). Although adult frogs are quite expensive, late juveniles are less costly and will usually yield eggs, although in smaller numbers than adults. Frogs are usually shipped in peat moss (or equivalent), but they cannot be shipped in extreme summer heat or in frigid winters without special produres. After receiving the frogs, they should be allowed a minimum recovery period of 2 weeks prior to experimentation.

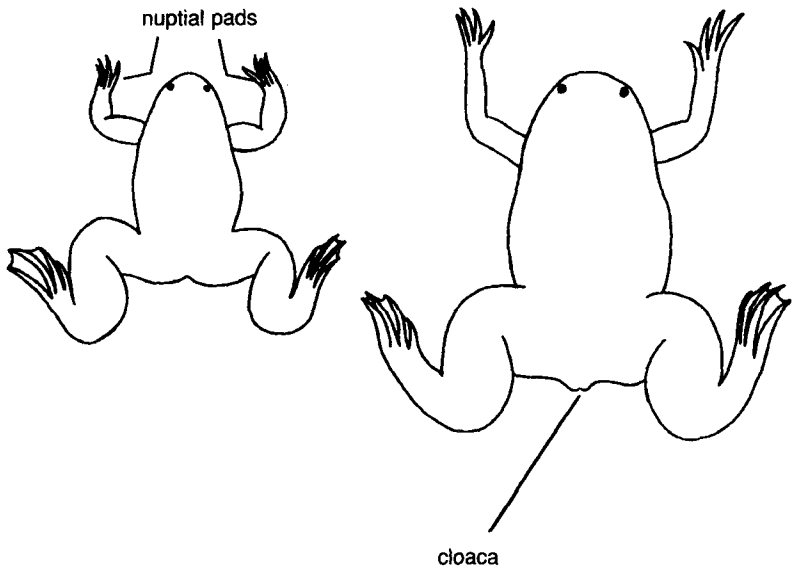

Figure 1.1 Relative sizes of male (*left*) and female (*right*) *Xenopus laevis*. Males are smaller than females of equivalent age. Males can also be distinguished from females by the absence of a cloaca, i.e., the fleshy protuberence between the legs, and by the presence of "nuptual pads," black rough regions on the inner side of the forelimb. These nuptual pads assist the male in grasping the female during mating (may not be obvious in males that are not ready to mate). In females, the cloaca becomes red and swollen when the animal is ready to lay eggs. Note the slightly rounded shape of both animals, although females may be somewhat plumper than males due to the size of their ovaries. Animals should not be excessively skinny, which could indicate illness, or excessively bloated, which can be a sign of poor health. (Courtesy of Mark Curtis.)

Females are larger than males, with a prominent cloaca. Males have rough, dark pads on the inside wrist, used to clasp the female during amplexus (see Figure 1.1). Animals may be endemically infected with nematodes and can be treated prophylactically on arrival (see below, Diseases, Preventions, and Cures). Thin animals tend to be sickly and should not be used.

HOUSING AND FEEDING

Good animal husbandry is vital for maintaining a healthy frog population. This requires some effort but is generally rewarded by high-quality egg and embryo production. A healthy frog is placid, with moderately slimy skin and a nice pear shape. Jumpy frogs, frogs with dry or excessively slimy skin,

bloated frogs, and frogs that look gray and thin or reddish are not healthy and should not be used for egg collection, as this would lead to further deterioration of the animals' condition, and the resulting eggs would be generally unsuitable for experimental purposes.

Containers

Xenopus never leaves the water and so it is unnecessary to provide dry areas in their containers. Frogs may be housed in different containers depending on their age and number and the quality of the water available in the laboratory.

Standing Water Tanks

For fewer than 50 or so animals, it is a simple matter to house frogs in tanks of still water. Four females or six males can be accommodated comfortably in approximately 16 liters of water. The water should be 12–20 cm deep. Plastic tanks are the most convenient containers, since their opaque sides approximate pond conditions. Frogs are talented jumpers (up to 45 cm) and thus their tanks should be covered with a heavy lid, either Plexiglas with half-inch holes or a stainless steel mesh. An opaque pipe (12 cm long and 8 cm in diameter) provides a comfortable hiding place for the frogs. The water in standing tanks should be replaced at least three times each week.

Drip-through Systems

For a larger number of frogs, it is more cost-effective to house animals in a system that is at least partially self-cleaning. One possibility is a system in which fresh water drips in and out continuously, thus preventing accumulation of wastes. This is probably the optimal way to keep frogs, since levels of toxic wastes are kept low, and solid waste (in suspension) can be drained continuously. The disadvantages of this system are that it uses a lot of water and the quality of input water must be monitored constantly. The chlorine content, pH, and salinity of tap water can vary widely with the season and the whim of the local water authority. Alternatively, distilled water, supplemented with NaCl or Instant Ocean (available from local aquarium suppliers) to approximately 20 mM, can be used as the input water supply. However, this system can use excessive quantities of distilled water. The water should drip in at a rate of about 10–50 ml per minute depending on tank size. A faster drip rate uses more water than is necessary.

Recirculating Systems

Many investigators use a continuously recirculating system that incorporates a biological filter. The water in these systems is generally collected into a common drain, with U-tubes between the tank and the common drain to prevent mixing dirty water between tanks. The water then flows into a reservoir in which the gross particulate matter is removed by an inexpensive air-conditioner filter that is replaced on a daily to weekly basis. From the reservoir, the water is pumped through a biological (bacterial) filter to remove ammonia and nitrites. It then flows through a sand filter to remove finer particles and across a series of high-capacity UV lights to kill bacteria and other potential pathogens, before being pumped back to the tanks. The sand filter is backwashed every day, with some fresh water entering the system, such that water in the entire system is replaced every 5 days. Again, the rate of flow should be limited to less than 100 ml of water per minute.

Water quality is monitored by analyzing pH and the concentration of ammonia, nitrates, and nitrites at least once a week. Chlorine and chloramine should be undetectable, and ammonia should be less than 5 ppm; nitrates and nitrites should also be undetectable. Bacterial counts are carried out from the water that goes back into the tanks (the count should be essentially zero). This type of system requires more maintenance than the drip-through system, but uses less water—a particular advantage in laboratories where water quality is a problem and supplies of distilled water are limited. In a system that has a capacity of approximately 400 frogs and contains about 2000 liters of water, maintenance should take less than 1 hour each day. The health of frogs in such a controlled environment can be excellent.

Water

Water quality is very important, particularly with respect to pH, chlorine, and ammonia content. The water must be completely dechlorinated before use, which can be achieved by exposure to the air for several days in standing tubs. However, many water authorities also add chloramine to the water. This compound is extremely stable, but can be removed by running the water through a carbon filter (obtainable as a cartridge type from most aquarium dealers, e.g., Barnstead). The chloramine content of the water determines how often the cartridge should be changed, but it should be changed before chloramine is detected in the output water. Chlorine and chloramine levels can be monitored with easy-to-use kits (Hach Co.). Deaminating liquids (e.g., Prime) can be purchased from a local aquarium supplier and used according to the manufacturers' instructions for removal of chloramine and ammonia. In addition,

water should be filtered through a dirt/rust or particle filter, also obtainable through aquarium dealers. Add NaCl or Instant Ocean to a final concentration of 20 mM. Alternatively, use rock salt at 1 g/liter of tap water, which is less expensive than NaCl.

Frogs can also be maintained in distilled or reverse-osmosis water supplemented with Instant Ocean to 20 mM; NaCl alone is not sufficient. This is advisable if the quality of the tap water in the laboratory is poor or variable. Frogs must not be kept in unsupplemented distilled water. This will cause their skin to flake and may lead to the development of stress-related diseases. The pH of distilled and reverse-osmosis water should be adjusted to 6.5 with SeaKem Neutralization Buffer (available from That Fish Place, 237 Centerville Road, Lancaster, Pennsylvania 17603), with soda lime, or with NaOH. A conductivity of 1.0 ms/cm ± 0.1 units is optimal.

pH

A pH of 6.5 is optimal for *Xenopus*. At low pH (below 7.0), ammonia waste is present as ammonium ions, which are nontoxic, but as the pH increases, free ammonia, which is toxic, forms rapidly. Even in the absence of ammonia, a change of 1 pH unit (e.g., from 6.5 to 7.5) can lead to the loss of the frogs' protective mucus, a higher susceptibility to pathogen attack, and other stress-related conditions.

Light and Temperature

Frogs must be kept on a regular light/dark cycle. They respond well to 12–14 hours of light and 12–10 hours of dark. Daylight spectrum fluorescent lighting can be used, but some investigators believe that egg quality improves when frogs are exposed to sunlight-equivalent light levels. Despite normally living in dark ditches, *Xenopus* appears to enjoy light conditions. However, it is important to have dark areas, such as opaque plastic pipes, so that the frogs can escape the light if they want to.

Temperatures of 16–20°C are optimal for *Xenopus* at all stages of development. Above 25°C, egg quality declines precipitously. After fertilization, it should be noted that the higher the temperature, the faster the rate of development. The effect of temperature on development is discussed in more detail in Appendix 2.

Food

Adult frogs must be fed a minimum of three times each week, several hours before changing the water. Frogs feed well on floating food (e.g., Trout Chow

Pellets; Purina), which is inexpensive and convenient. An alternative includes Frog Brittle (NASCO). If frogs have previously been fed liver, it may take them 1 week or more to eat the pellets. They can be encouraged to accept the new type of food by housing a frog that already eats pellets with those that do not in order to initiate a feeding frenzy. Each adult frog will eat 5–10 pellets per feeding. Feeding regimes for tadpoles are discussed below (see Raising Tadpoles and Frogs).

Seasonal Variation

Many investigators report seasonal variation in the quality of embryos, even for animals that have been kept for years or even bred without seeing seasonal light/dark changes. Variation may be due to changes in water quality or more significantly water temperature. High summer temperatures (above 26°C) will adversely affect egg production. Injection of frogs with 50 units of pregnant mare serum (PMS) a few weeks before inducing ovulation may help to reduce the effects of high temperatures. Whole serum is not commercially available, but an alternative to whole PMS is chorionic gonadotropin (CG) obtainable from Sigma. Injecting the frogs with 50 units of human chorionic gonadotropin (hCG) also may help to maintain egg production.

RAISING TADPOLES AND FROGS

Given sufficient space and time, raising *Xenopus* is not difficult. Tadpoles require approximately 1 liter of water each, whereas fully grown frogs need up to 4 liters each.

Hundreds of tadpoles can be generated by fertilizing several dishes of eggs in vitro as described in Chapter 5. Alternatively, unused embryos from several days of experiments can be pooled and reared through metamorphosis to adulthood. It is important not to overcrowd dejellied embryos (see Protocol 6.1). Overcrowding leads to abnormal development, usually as a result of gastrulation defects thought to be caused by anoxia, and dejellied embryos should be separated from each other by at least one embryo diameter at all times. Swimming tadpole-stage embryos should be transferred to a tank with the appropriate volume (1 liter per individual) of good-quality water containing 20 mM NaCl.

The most efficient method of generating large numbers of tadpoles is to induce natural matings between several females and males. Each male is

injected with approximately 300 units of hCG and each female is injected with approximately 800 units of hCG (concentration 1000–2000 units/ml; Sigma) and placed in a large tank (80 liters) containing good-quality water supplemented with 20 mM NaCl at a depth of approximately 15 cm. Equal numbers of males and females should be used during mating. After 6–8 hours, the males clasp the females around the hips (amplexus) and the eggs are fertilized as they emerge from the cloaca. It is important that the frogs are not disturbed during mating, which may result in the release of amplexus and a decrease in fertilization efficiency. For this reason, it is generally preferable to inject the frogs in the afternoon so that they will have most of the night to mate undisturbed. After 24 hours or so, the males release the females and mating ceases. At this time, the energy-depleted frogs are removed from the tank to prevent them from eating their own eggs. After 3–4 days, the embryos will hatch and begin to colonize the sides of the tank at the water's edge. Unfertilized eggs must be removed promptly since they will decay and allow bacteria and fungi to contaminate the water. If the fertilization frequency is particularly low (<20%), healthy embryos should be screened as soon as possible and transferred to fresh water supplemented with penicillin and streptomycin or gentamycin (0.05 mg/ml). This procedure is time consuming and very laborious, but worthwhile if embryos are in limited supply.

After 1 week, the tadpoles should be free-swimming and ready to begin feeding, as evidenced by the rhythmic opening of their mouths. The tadpoles thrive on very fine food. They can be fed routinely on nettle powder mixture, i.e., a combination of nettle powder, active dry yeast, and powdered bone meal, mixed at 7:2:1, respectively. These ingredients can be obtained at health food stores. Tadpole Brittle (NASCO) can also be used according to the manufacturer's instructions. Food should be delivered to the tank as a water suspension through a pipette. This method decreases the amount of surface scum that forms when the food is added directly to the tank. It is important not to overfeed the tadpoles, because they will become anoxic, presumably due to food-clogged gills. Death due to overfeeding is common, particularly in young tadpoles. In general, tadpoles should clear the water within 2 hours of feeding. Ideally, they should be fed daily, with a weekly supplement of fresh whole milk added dropwise until the water in the tank is slightly cloudy. The milk and bone meal add calcium and phosphate to the water, which should eliminate skeletal deformities in the metamorphosing frogs. Although not essential, oxygenating the water with submerged aerators appears to enhance the growth rate of tadpoles. With proper feeding, the water in tadpole tanks need only be changed once every 1–2 weeks.

Tadpoles do not develop synchronously, even among siblings. Some metamorphose after 8 weeks, whereas others require 4–6 months to mature.

During metamorphosis, the water level in the tank should be no more than 30 cm deep because the newly metamorphosed froglets may have difficulty reaching the surface to breathe. Froglets cannot eat tadpole food and so must be fed small Trout Chow Pellets (Purina, pellet size 4) or Frog Brittle (NASCO). The small pieces of food sink to the bottom where the froglets prefer to eat. There is no need to separate froglets and sibling tadpoles. They can coexist for several months until all the tadpoles metamorphose. In fact, it can be rather convenient to have a mixture of tadpoles and froglets in the tank because the tadpoles tend to keep the water clean. Once one third of the tadpoles have metamorphosed, it is no longer necessary to feed the remaining tadpoles nettle powder mixture since the froglets break up the Trout Chow sufficiently for the tadpoles to filter the scraps. This simplifies the feeding regimen to:

- nettle powder mixture only, up to the emergence of the first froglets
- nettle powder mixture and small Trout Chow Pellets, until one third of the froglets have emerged
- Trout Chow Pellets or Frog Brittle exclusively beyond this time

Given sufficient space, froglets reach sexual maturity after 1 year and full size after 3 years.

DISEASES, PREVENTIONS, AND CURES

The two most common diseases in *Xenopus* are bacterial septicemia (the culprit bacterium varies widely) and nematode infestation. Fungal infections may also occur. In general, veterinarians are not familiar with *Xenopus,* and thus, treatment must often be carried out by the investigator. Many diseases are stress-induced and commonly occur after induction of ovulation or egg collection. It is therefore imperative to minimize stress and to observe animals carefully during and after these procedures. Females should be handled with care and isolated for 24 hours after egg collection in water supplemented with 20 mM NaCl and 5 μg/ml gentamycin (i.e., 10x lower than the dose given to embryos). As an additional precaution, oxytetracycline, which targets bacteria different from those killed by gentamycin, can be included to a final concentration of 50 mg/ml, replaced every day. (Never leave a frog in a bucket of dirty water containing excess food or especially rotting eggs. After a few days, it will get septicemia and die a horrible death.)

It should be noted that the treatment of a disease with drugs may not remove the cause of the disease. Bacterial, nematode, and fungal infections often arise from pathogens endemic to the frogs that become a problem only

when frogs are stressed. Such stresses can include overcrowding, a change in pH or water quality, or careless frog handling. It is important to establish the root cause of a problem before it inflicts significant damage on the colony.

Frogs should have a moist but not excessively slimy skin. The skin should not be visibly flaking (although they do shed some skin normally) and the pigmentation should not be patchy (normal pigmentation is mottled, but the mottling should cover the entire body). Animals should be fat but not bloated (the difference is easily recognized when the frogs are handled) or excessively thin. The skin should not be red, which may indicate subcutaneous hemorrhaging.

Nematode Infection (Capillariasis)

The symptoms of nematode infection include sloughing of skin, patchy pigmentation, grayish and thin skin, and weight loss. Redness is not usually apparent. Examination of skin scrapings will reveal nematode presence. This infection is often precipitated by stress.

Many frogs carry latent nematode infections. Animals can be treated prophylactically with Ivermectin (PRO-VET; available from local aquarium suppliers) on arrival. Apply treatment as soon as symptoms are recognized. Infected animals must be isolated, since both eggs and adult nematodes are shed into the water. Administer Ivermectin via injection into the dorsal lymph sac, as described in Protocol 5.2. Deliver two injections of 2 μg/g of body weight 2 weeks apart, each in a volume of approximately 100 μl. Although the drug can also be administered by oral gavage, it is difficult to carry out without severely stressing the frogs. This treatment is very effective, and frogs will recover if it is administered promptly. If the frogs are very thin with fragile skin, recovery can take several months (see Cromeens et al. 1987; Stephens et al. 1987).

Red Leg (Bacterial Septicemia)

The symptoms of red leg are cutaneous hemorrhages, especially on flexor surfaces of thighs and foot webs, dull discoloration of skin, subcutaneous edema, and neurological disorders (trembling, initially of limbs). This disease can be caused by a number of gram-negative bacteria, primarily *Enterobacteria*, particularly *Aeromona*, *Pseudomonas*, and *Citrobacter*, and is precipitated by stress.

Infected individuals must be isolated since the disease is highly contagious. Treatment can be effective if administered promptly. The most convenient antibacterial treatment is to add oxytetracycline to the water, to a final concentration of 100 μg/ml. Change the water every day and administer