ILAR Journal V37(4) 1995
Fish, Amphibians and Reptiles

Amphibians as Laboratory Animals

Dale DeNardo

Dale DeNardo, D.V.M., Ph.D., is an Associate Veterinarian at the University of California, Berkeley.

INTRODUCTION

Amphibians include over 4,500 species within 3 major lineages---caecilians, salamanders, and anurans (Stebbins and Cohen 1995). These lineages are linked by several unique physiological traits. The most prominent is the indirect lifestyle of many amphibians--the aquatic gill-breathing larval stage and the aquatic or terrestrial lung or skin-breathing adult stage. A second feature of amphibians is the presence of 2 types of skin glands: mucous and granular. Mucous glands keep the skin surface moist to allow transcutaneous respiration, but they also put the animals at risk of desiccation. The granular glands secrete toxins and function primarily for defense (Zug 1993).

The more than 160 species of caecilians are not diverse, all being legless and inhabiting tropical regions. This poorly understood group of amphibians is used in research relatively infrequently, and, therefore, will not be discussed further (see Wake 1994, for review of caecilian husbandry). The salamanders are represented by nearly 400 species, some of which have a four-legged aquatic larval stage, while others (primarily the Plethodontidae) pass the larval stage within eggs that are laid in moist places on land (Stebbins and Cohen 1995). Some salamanders retain the larval gills through their adult stage and depend on oxygenated water throughout their lives. While most salamanders have legs, they have become vestigial in some.

The anurans, or frog and toads, are the most successful lineage of amphibians with approximately 4,000 species distributed around the world in a multitude of habitats. The larval stage of most, but not all, anurans includes a legless tadpole or polliwog. The degree of dependency on water varies--species range from purely aquatic to completely terrestrial. Frogs are not only the most diverse group of amphibians; they also are by far the most commonly used amphibians in research. While space constraints limit detailed discussion of amphibian biology and taxonomy here, the topic is thoroughly covered elsewhere (Moore 1964-1976; Frost 1985; Duellman and Trueb 1986; Duellman 1993; Zug 1993).

SPECIES AVAILABILITY

Commercial Vendors

Only a small number of the approximately 4,500 species of amphibians are available commercially. Fortunately, it is with these few commercially available species that the vast majority of laboratory research with amphibians is conducted. Many amphibians have a seasonal availability that must be planned for or around.

"Professionalism," in terms of reliability and quality, can vary greatly among amphibian vendors. One must realize and accept the fact that vendors of amphibians do not conduct business or house animals using the high standards of most rodent vendors. However, this does not mean that one should accept unhealthy or inhumanely treated animals. Potential vendors should be assessed by obtaining information about how animals are acquired and housed. Once obtained and deemed acceptable, the accuracy of this information can usually be validated when an initial shipment of animals is received. Animals that are of poor quality upon arrival can be a result of either unhealthy animals being shipped or improper shipping of originally healthy animals. It is important to differentiate these two causes, in order to prevent obtaining animals in similar condition in the future. Problems in shipping can be corrected by using alternative packing methods or transport services, while problems originating from the vendor are usually best corrected by switching to another source.

Captive Breeding

Of the limited number of amphibians readily available commercially, only a handful of these are actually bred in captivity. Species commonly used in research and routinely cap-tive-bred include the clawed frog *(Xenopus laevis),* the African dwarf frog *(Hymenochirus boettgeri),* the fire-bel-lied toad *(Bombina orientalis),* the axolotl *(Ambystoma mexicanum),* and the tiger salamander *(A. tigrinum).* Some additional species are selectively bred at institutions. The remaining amphibians used in research are collected from wild populations by

investigators or by commercial vendors, as is the case with commonly used amphibians such as the bullfrog *(Rana catesbeiana),* grass frog *(R. pipiens),* cane toad *(Bufo marinus),* and mud puppy *(Nectutus maculosus).*

Field Collection

Many states require permits to collect amphibians. Fishing licenses may suffice in some states, while scientific collecting permits may be necessary in others. Amphibians can be collected in numerous way. Procedures concerning the collection of amphibians have been thoroughly reviewed elsewhere (Heyer and others 1994), so only a short discussion will be included here.

The most common method is to collect by hand amphibians that are secluded under rocks, logs, or other debris. Active amphibians can often be collected during rains or at night with the use of flashlights and lanterns. Slowly driving roads at night, especially during rains, can be a very productive way to capture many species of amphibians.

Numerous devices have been used to aid in the collection of amphibians. Larvae and aquatic species can be captured with nets and seines. More recently, aquatic amphibians have been collected using electroshock. When using electroshock, one must consider the potential health risk to not only the target species, but also to other species present.

Predominantly terrestrial amphibians are commonly captured using fence lines with pitfall traps. The fence lines are placed around breeding ponds, trapping individuals as they move to and from the ponds. Pitfall traps must be shaded from the sun and contain a moist substrate to prevent mortality.

Importation

There are limited restrictions that affect the importation, exportation, and possession of amphibians in the United States. Quarantine is not required when importing amphibians into the United States. Some states, however, have restrictions for certain species. The most commonly restricted species are the clawed frogs *(Xenopus spp.)* and large toads (such as *Bufo marinus, B. horribilis,* and *B. paracnemis).* State permits may be required to own these anurans, and they may also require special housing conditions. Investigators should consult their state wildlife department for information regarding specific species restrictions.

Shipping

Regardless of the source, one's ability to successfully maintain amphibians starts with acquiring healthy animals. It is far easier to prevent than to treat disease in amphibians. Clinical disease in amphibians can be frustrating to researchers and veterinarians alike, since individuals do not often respond to treatment and infectious diseases can spread rapidly through a collection.

When choosing a vendor, one must consider reliability, location, transportation method, and, of course, condition of the animals upon arrival. Reliability refers to receiving individuals of the proper size and correct species at the appropriate time. Many species of amphibians are prone to "shipping stress," so time in transport should be minimized. Choosing a nearby vendor and using air freight or, at the very least, overnight mail, can help shorten the shipping time. Packaging should be of solid construction for physical protection and should maintain adequate moisture levels for the entire time of transit. It is often best to withhold food, but not water, for 24 hours prior to shipping, since this will reduce waste production while in transit. Shipping during extreme weather conditions, especially heat, should be avoided.

Housing for newly acquired amphibians should be functional prior to their arrival, so that the animals can be quickly unpacked. Newly arrived individuals should be given a thorough visual evaluation and housed separately from individuals already present in the colony. A minimum 30-day quarantine period is suggested. During quarantine, animals should be carefully monitored for morbidity and mortality and evaluated for endoparasites. Any clinical disease should be properly diagnosed and aggressively treated. It is best to locate recently arrived amphibians, particularly ranid frogs, in a room with low traffic since they are easily agitated. When frightened, these frogs will jump excessively and may traumatize themselves on the cage lid. Using a deep opaque cage will minimize the trauma to the frogs. Placing opaque plastic over cages housing newly acquired frogs will also minimize the amount of jumping.

ANIMAL WELFARE REGULATIONS AND POLICIES

Being "lower vertebrates," amphibians are exempt from U.S. Department of Agriculture (USDA) regulations. The *Guide for the Care and Use of Laboratory Animals (Guide)* (NRC 1996) does not provide specific recommendations for the maintenance of amphibians. However the *Guide* does state, "An appropriate environment should be provided for nontra-ditional species...Expert advice on the natural history and behavior of nontraditional species should be sought when such animals are to be introduced into a research environment" (NRC 1996, p 14 of prepublication copy). To assure that humane issues are being properly addressed, one must use available guidelines (NRC 1974; ASIH 1987; Pough 1991, 1992); possess a good understanding of amphibian biology; and apply, with adaptations, procedures used for traditional species. Institutional animal care and use committees (IACUCs) need to establish institutional guidelines for dealing with the use of amphibians, both in the field and the laboratory. These guidelines should be developed by a cooperative process, incorporating input from written guidelines,

committee members, faculty using amphibians, campus veterinarians, and consultants. Guidelines contrived in such a positive manner tend to be stronger and hold up better in this complex field where much is still not well understood.

HUSBANDRY AND HOUSING

Caging

A wide variety of caging is used for housing amphibians. Standard rodent cages with modified lids work well for smaller species, while large plastic or fiberglass tubs are used for larger species. Certain criteria should be addressed when choosing proper caging. First, the cage should be easily sanitized. Cages made of plastic or fiberglass with built-in drains seem to work best for most amphibians. Most amphibian cages need lids because some species can jump quite high and many others, including some salamanders, can climb smooth vertical surfaces. Metal screen lids can be abrasive to frogs that readily jump, especially new arrivals. Alternate smoother surfaces (such as plexiglass with holes or plastic mesh) can be used along with taller cages to decrease the likelihood of the frog abrading itself.

Amphibians can be housed singly or in groups, provided sanitary conditions can be maintained. Some amphibian species are aggressive or territorial, and should be housed individually. Additionally, care must be taken not to transfer among cages pheromones that are present in the skin secretions (Jaeger 1992).

Heating, Ventilation, and Air Conditioning

Most amphibians prefer relatively cool temperatures (10-20°C). However, it is important to know the preferred temperature for the species in question, since some montane species require much cooler temperatures (5-10°C) and tropical species may require warmer temperatures (25-30°C) (Brattstrom 1970). Overall, amphibians are more sensitive to heat stress than cold stress. A few minutes at an excessively high temperature can be sufficient to cause immediate death or stress related opportunistic infectious disease. Control systems such as temperature sensitive alarms and flow solenoids are very useful in preventing catastrophes related to physical plant failures.

Many animal rooms maintain air flow rates that are appropriate for mammals (10-15 air changes per hour), but can rapidly desiccate amphibians. This is of particular concern for species that are terrestrial, since they require moist conditions but not necessarily standing water. Reducing air flows, installing humidifiers, or simply using solid cage lids can all prevent desiccation.

Lighting

Most amphibians are either nocturnal, live under leaf litter, or are extremely secretive. Therefore, lighting is not critical, and standard animal room lighting is usually adequate for most amphibians.

Diet

Amphibians, in general, are insectivorous and require live food. Most commonly, captive amphibians are fed crickets or mealworms, which are regularly available commercially. It is important to use insects that have been maintained on a balanced diet. Insects that have been fasted during transit should themselves be fed for 1-2 days prior to being offered to an amphibian. Crickets and mealworms can be fed commercially available cricket feed (Zeigler Brothers, lnc, Gardners, Pennsylvania, is one such supplier) or laying hen scratch (such as Purina® Layena®). Additional species that are used as prey items for amphibians include tubifex worms, earthworms, brine shrimp, fruit flies, and juvenile rodents (see Dierenfeld and Barker 1995 for the nutritional composition of many common prey species).

While most amphibians require prey movement to stimulate feeding, some species will feed on commercially available fish chows (available from companies such as Purina or Bioproducts, Warrenton, Oregon), which come in numerous sizes and either sink or float. Commonly used amphibian species that the author has successfully fed chows include *Xenopus spp.*, *Hymenochirus boettgeri*, *Pyxicephalus adspersus*, and aquatic *Ambystoma spp.*

Feeding often causes a frenzied response in amphibians that can lead to traumatic injury of cagemates. Problems can be minimized by avoiding overcrowding, housing only similar sized individuals together, feeding more frequently, and dispersing food evenly throughout the cage.

Tadpoles are mostly herbivorous, requiring a diet different from that of metamorphosed conspecifics. Nettle powder, boiled spinach, fish flake food, and alfalfa pellets blended in water are commonly used foodstuffs for tadpoles.

Water Quality

Water quality is probably the most critical component of amphibian housing. This is especially true for larval amphibians, although adults, including terrestrial species, are also affected by poor water quality. When assessing water quality, it is essential to evaluate both source

water and cage water.

The most common problem associated with source water is chlorine, although heavy metals and hardness can also be toxic to amphibians. Amphibians are sometimes housed in unmodified tap water, but this may be inadequate for delicate species or in certain geographical areas. Furthermore, many municipal water districts frequently alter the source of their water and the amount of additives (such as chlorine and fluorine) in the water without adequately notifying their customers. Such changes can have disastrous effects on stable amphibian colonies.

Municipal water can be made safer by using an activated charcoal filter. The charcoal needs to be changed periodically (every 2-6 months depending on use) to assure proper function. Measuring chlorine levels in the water after it has been filtered through the charcoal can help determine the life span of a particular filter. Chlorine can also be removed from water by simply letting it sit in an open container for 24 hours, but other potentially toxic chemicals may not be removed using this method.

For delicate species, especially many tadpoles, even charcoal-filtered municipal water is not sufficient. In such instances, either commercially available spring water or ion-ically balanced solutions made in-house from distilled water can be used (Table 1). Since distilled and deionized water is extremely hypo-osmotic, it should never be used without the addition of salts. Hypo-osmotic conditions can cause bloating and death in amphibians.

Even if the source water is adequate, one can still encounter problems with water quality in the cage. The primary contaminants of cage water are uneaten food and animal waste. Both overfeeding and inadequate cleaning practices often lead to poor cage water quality and, therefore, clinical disease.

The most important components to monitor in cage water are those involved in nitrogen transformation. In established systems, ammonia, as a toxic waste product, is oxidized by the *Nitrosomonas* group of bacteria to produce nitrite. Nitrite, which is also toxic, is rapidly oxidized by the *Nitro-batter* group of bacteria to non-toxic nitrate (Tucker 1993). Hazardous build-up of ammonia or nitrite may occur if either or both of these denitrifying bacteria groups are not present. This is often true with new cage setups and tanks that have been treated with antibiotics. Seeding new or antibiotic treated tanks with substrate from established healthy tanks will help create a balanced bacterial fauna.

Ammonia and nitrite levels should be monitored routinely (daily to weekly depending on species and tank history) to provide early detection of breakdowns in the nitrogen oxidation pathways. Test kits for these, as well as other critical parameters (such as total residual chlorine levels and pH) are available from wholesale and retail fish and aquaria dealers.

A critical component to maintaining optimal cage water quality is the careful evaluation of food intake. Animals should receive food in amounts that are sufficient to maintain good body weight but are not in excess. Even when food is properly portioned, cage water quality is at its worst after feeding. In addition to the presence of uneaten food, eating tends to stimulate defecation in many amphibians. For many aquatic species, it is critical to schedule cleaning soon after feeding.

Since most amphibians, including aquatic species, are air breathing, water aeration is usually unnecessary. However, water aeration may be necessary for larval forms or species that retain gills as adults. It is important to note that gas supersaturation can lead to emboli, emphysema, and even death in many amphibians (Orwicz 1985). It is critical to take advantage of all available resources--both written and human--when establishing and maintaining new species.

Sanitation

While it is important to keep amphibian enclosures sanitized, it is equally important to assure that no residual cleansing agents are left in the cage. Cleansing agents can be extremely toxic to amphibians. For smaller cages, standard cage washers provide a sufficient rinse. For cages that are too delicate or too large for cage washers, bleach diluted 1:32 with water is best since it is an effective disinfectant and easily rinses clean. Disinfectants containing soap are more likely to leave residues which can detrimentally affect amphibians.

Maintenance in the Field

The same husbandry parameters that are important in maintaining amphibians in the laboratory are critical to maintaining amphibians in the field. The biggest concern is the maintenance of proper temperature and moisture conditions. For short-term housing, amphibians are frequently kept in small containers, such as deli cups, inside an insulated cooler. Small containers are preferred, in order to reduce jostling of the animals. Moisture can be maintained in the containers by adding moistened substrate such as paper towels or sphagnum moss. Cool packs or ice can be placed in the cooler along with the housing containers to avoid overheating. Exposure to cool, but not freezing temperatures is well tolerated by most amphibian species. Conversely, warm temperatures can rapidly prove fatal. Animal housing containers provide a greenhouse effect and must be kept out of direct sunlight at all times.

Amphibians usually are not fed while in the field, unless the trip is an extended one. Most amphibians can be fasted for up to 2 weeks without concern. If amphibians are fed in the field, housing containers must be frequently cleaned to remove waste. Even when captive amphibians are to be fasted while in the field, it is advisable to clean the housing containers 24 hours after capture to remove the waste that is

a result of pre-capture feeding.

CAPTIVE BREEDING

While all lineages of amphibians have been bred in captivity, captive breeding is most commonly desired, and therefore achieved, in anurans. Anurans are usually bred by hormon-ally inducing females to ovulate using gonadotropin releasing hormone (GNRH) or, for some species, human chorionic gonadotropin (hCG) (Hayes 1994). It is important to realize that standard hormonal treatment does not initiate egg development, but rather induces ovulation of already present mature follicles. While some species, such as *Xenopus,* readily develop follicles in captivity, the inability to induce follicular development prohibits the establishment of long-term captive breeding colonies in many species. "Captive reproduction" for many species simply means inducing recently acquired females to ovulate and oviposit follicles that developed while the female was in the wild.

Once ovulated, ova are soon oviposited either naturally or expressed physically. Physical expression is commonly used to achieve precise time-fertilized eggs. While "stripping" eggs is routine, if performed improperly extensive bruising and pressure necrosis can occur. Fertilization in anurans is external, and can be accomplished by allowing the male to grasp, or amplex, the female. Amplexus puts pressure on the female's abdomen, which aids oviposition and aligns the cloacas to assure fertilization. The male releases sperm as the female oviposits her eggs. Manually expressed eggs can be fertilized using minced pieces of testis from a culled male or by hormonally stimulating males with GNRH and flushing the bladder to obtain sperm.

In *Xenopus,* there is often concern about the "quality" of the eggs produced. Since the majority *of Xenopus* eggs produced at research institutions are for experimentation not reproduction, "quality" refers to the ability of the eggs to remain viable after experimental manipulation (such as centrifugation and microinjection). It is currently unknown what factors influence the "quality" of eggs; however, numerous husbandry parameters are often manipulated in an attempt to improve egg quality. Such factors include diet, lighting, water salinity, water flow, frequency of cleaning, tank size, type of hormonal stimulation, frequency of egg collection, and age of females.

SAFETY CONSIDERATIONS

Toxins

The vast majority of amphibians pose little threat to investigators or care staff. The biggest threat is from the toxin glands of the skin (see Grenard 1994 for review). Species of known concern include the dart poison frogs (family Dendrobatidae), some toads (especially larger species such as *Bufo marinus* and *B. alvarius),* and some newts (such as *Taricha spp.).*

The dendrobatid frogs secrete steroidal alkaloids with both chronotropic and inotropic properties, which result in arrhythmias followed by cardiac arrest. These poisons have been used for centuries in Latin America to hunt game. The alkaloids appear to originate from the prey of the frogs (Daly and others 1994), and, therefore, captive frogs are thought to have reduced levels of toxin. Regardless, dendrobatid frogs should never be handled with bare hands.

Bufotoxins, toxins from toads of the genus *Bufo* are released from the large parotoid glands caudal to the orbits. Symptoms of bufotoxin poisoning include salivation, cya-nosis, cardiac arrhythmias, shortness of breath, and seizures.

Newts of the genus *Taricha* contain small amounts of tetrodotoxin in the skin. While the level of tetrodotoxin in newts is only a fraction of what is found in puffer fish, ingestion of *Taricha* has caused fatalities.

While amphibian toxins can cause fatalities, amphibians lack an effective delivery system. Most accidental fatalities associated with amphibian toxins are a direct result of intentional ingestion of the amphibian or its toxins. Careful hygiene should be used whenever handling amphibians, especially species that contain potentially toxic compounds. One should take particular care not to let contaminated hands come in contact with one' s eyes. If eyes do become contaminated, they should be rinsed immediately and a physician should be notified.

Zoonoses

While extremely rare, there is potential for transfer of infectious agents from amphibians to man. Prominent organisms include bacterial agents such as *Pseudomonas, Salmonella,* and *Mycobacterium,* and parasites including tapeworms (Grenard 1994).

USE IN RESEARCH

Amphibians have long been used as subjects for studies in ecology, ethology, and evolution (Stebbins and Cohen 1995). More recently, declining amphibian species throughout the world have stimulated an interest in these animals as environmental sentinels (McVey and others 1992).

Amphibians are frequently used as models in comparative medicine, with the predominant model species being the African clawed frog

(Xenopus laevis). Xenopus is used in studies of development and differentiation at both cellular and molecular levels. Additionally, amphibians are frequently used in biomedical research to study vision, hearing, respiratory physiology, endocrinology, analgesia, toxicology, tissue freezing, and tissue regeneration. Numerous peptides of frog origin have received considerable attention as potential therapeutics for bacterial infections, allergies, asthma, central nervous disorders, and analgesics (for review see Grenard 1994).

IDENTIFICATION TECHNIQUES

Identification of amphibians can be quite difficult, and, as a result, many techniques have been tried (see Donnelly and others 1994, for review). The preferred technique will depend on the species in question and the requirements of the particular study.

Cage Numbering

The most common and easiest way of identifying amphibians is by using numbered cage cards. Confusion may exist with this method if multiple animals are removed simultaneously or if cage cards get detached from their respective cages. Since water is usually present around amphibians, indelible ink and waterproof cage cards should be used.

Pattern Marking

In species where there is individual variation in color pattern or surface morphology, drawings or photographs of the subjects can be used to distinguish individuals. While non-invasive, this technique is laborious and is generally restricted to studies using relatively small numbers of animals. Additionally, patterns may change with age in some species. Even small ontogenetic changes might make animals unidentifiable using this technique.

Toe-clipping

Toe-clipping is commonly used to identify amphibians. While in general this technique is quite safe, some species are prone to bacterial and fungal infections at the clip site. Unlike other animals, amphibians have the ability to regenerate their toes; therefore, toe-clipping is often an impermanent identification technique. The rate at which regeneration occurs depends on the species in question. For lengthy studies, toes may need to be re-clipped periodically. In some species, toe-clipping can be a permanent identification, since the regenerated toes are distinguishable from the original ones. Alternately, regeneration can be inhibited with beryllium nitrate (Heatwole 1961). There is some evidence that toe-clipping may negatively effect survival of free-ranging amphibians (Clarke 1972).

Tattooing and Dye Injecting

Amphibians are sometimes marked by injecting ink subdermally. Standard tattooing equipment with vibrating needles can only be used on larger specimens. Smaller individuals can be dye marked by injecting acrylic paint via a hypodermic needle. The paint is injected as the needle is withdrawn, leaving a bar of color. Using multiple color and bar positions, numerous animals can be uniquely coded (Woolley 1973). It is best to use areas of minimal pigmentation for injection sites. In larval forms, the tail fin is the site of choice. Unlike other species, amphibian tattoos or dye marks are usually not permanent and frequently last only a few weeks to months.

Branding

Both chemical (Wolf and Hedrick 1971) and cold (Farrell and Johnson 1973; Daugherty 1976) branding techniques have been used in amphibians. Because of the potential for chronic pain associated with branding techniques and the paucity of information regarding analgesics in amphibians, other methods of identification should first be considered. Heat branding is not advised, since it can cause excessive trauma. If branding is to be performed, it is advisable to anesthetize the patient. This is especially true for younger individuals.

Chemical brands created using a solution of 0.5% amido Schwartz in 7% acetic acid usually last at least a year (Wolf and Hedrick 1971). The solution is applied after mucous secretions are wiped away. Aquatic species can be returned to water after one minute, provided air-breathing species are properly propped to prevent drowning until fully recovered from anesthesia. The dye in the solution provides a temporary identification until the scar tissue is formed in response to the acid.

Tagging

Amphibians can be marked with color coded waist or knee tags (Emlen 1968; Elmberg 1989). The tags can be commercially available fish tags or strung colored beads. The advantage of such techniques is that individuals can be identified without being handled. However, disadvantages are that the tagged animal could be more conspicuous to predators (if free-ranging) and that complications could occur associated with tags getting caught on other objects.

Transponder Implantation

Passive integrated transponders (PIT tags) are becoming more popular in research, and are beginning to be used in amphibians. Sterile implants the size of a grain of rice are injected either subcutaneously or intracoelomically using a specialized syringe. A reader is then passed over the animal to reveal its unique identification number. In addition to being relatively expensive, microchip identification can be inconvenient, since the reader is required whenever an animal needs to be identified. Additionally, many amphibians have the ability to extrude foreign material from their bodies, especially if the material is placed subcutaneously.

ANESTHESIA

Chemical Anesthesia

As a result of the absorptive properties of amphibian skin, transcutaneous anesthesia is very effective and, therefore, the most common route employed. Tricaine methanesulfonate (MS-222), 0.02-0.05% in water (Crawshaw 1992), is the most widely used anesthetic agent. Major drawbacks of MS-222 are that it is acidic (and therefore may irritate the amphibian's skin) and a potential carcinogen. Neutralizing MS-222 with 5mM of sodium bicarbonate will hasten anesthesia and reduce skin irritation. Additionally, gloves should be worn whenever working with MS-222. As an alternative to water immersion, MS-222 can be injected into the dorsal lymph sac or intracoelomically at 100mg/kg in *Rana pipiens* (Nace and Richards 1972) and 250-400 mg/kg in *R. cates-beiana* (Letcher 1992). However, one must be cautious when using these routes of delivery, since there seems to be significant intra- and interspecific variation (Letchef 1992).

Benzocaine, 0.005-0.03% in water (Crawshaw 1992), is becoming popular as an alternative transcutaneous anesthetic agent. Although benzocaine is known to cause methemoglob-inemia in mammals (Lagutchik and others 1992; Rodriquez and others 1994), this has not been reported in amphibians.

Both MS-222 and benzocaine are commonly used by placing the patient in baths containing the drug. Since benzocaine dissolves poorly in water, it should first be dissolved in a small amount (approximately 5-10 ml) of alcohol before being added to the water bath (Crawshaw 1992). To avoid drowning, lung-breathing amphibians should be anesthetized in shallow baths of water and propped to prevent submersion.

Animals are left in the bath until motor activity ceases, and then removed. For longer procedures, it may be necessary to drape the amphibian with a sponge or paper towel that has been moistened with the anesthetic-containing water. For recovery, the animal is placed in fresh water. Recovery is hastened by periodically changing the water to remove the anesthetic as it leaches from the animal.

Gas Anesthetics

Some amphibians can be anesthetized in a closed receptacle containing methoxyflurane (Arena and Richardson 1990). A cotton wad soaked with 0.5-1.0 ml of methoxyflurane should be placed in a 1 liter container. The animal should not be allowed to contact the anesthetic-containing cotton. Induction is relatively rapid (approximately 2 minutes) and anesthesia lasts about 40 minutes after removal from the anesthetic chamber. Recovery can be lengthy (up to 7 hours) and is aided by rinsing the animal in fresh water. Because of the effectiveness of transcutaneous and induction chamber anesthetics, anesthetic vaporizers, while safe, are rarely used in amphibians.

Hypothermia

The use of cold as an anesthetic, while widely used, is greatly debated. Little data exists to refute or support the anesthetic properties of hypothermia (see Martin, p 186 of this issue). The limited data available indicates that nerve conduction velocities in amphibians do decrease with decreasing temperatures (Hutchinson and others 1970). However, it is unknown whether hypothermia induces conduction block. Additionally, the great variation in preferred body temperature among amphibian species may lead to variation in the effectiveness of hypothermia as an anesthetic. As a result of the uncertainty surrounding the use of hypothermia as an anesthetic, it is advisable to use alternative means of anesthesia when possible.

SURGERY

Surgical technique follows that for most other species. Although amphibian skin is known to contain antimicrobial compounds (see Grenard 1994 for review), surgeries should be conducted in an aseptic manner. While a complete sterile environment may not be required, all instruments and other materials entering the body should be sterile.

Closure of incisions can sometimes be problematic for novice surgeons, with dehiscence common. Small incisions (such as those made for implant placement) can be effectively closed using cyanoacrylate. The glue should be carefully applied to the incision site while tissues are held in apposition with forceps. A fine-tipped instrument should be used to apply the glue to assure that excessive amounts of tissue are not covered.

Larger incisions should be closed with nonorganic suture material that is either long-lasting or nonabsorbable. The use of an everting pattern

(such as horizontal mattress) seems to minimize the frequency of dehiscence (DeNardo personal observance). Excessively tight sutures may lead to local tissue necrosis and dehiscence of the incision site. After surgery the incision sites should be monitored closely for signs of necrosis, dehiscence, and fungal infection. If not expelled by the amphibian, sutures should be removed after 4 weeks.

EUTHANASIA

Amphibians can be euthanized in numerous ways. The method of choice will depend upon the species in question, the research needs, and the limitations set forth by the institution.

Physical Methods

As with anesthesia, the use of hypothermia and freezing for euthanasia is controversial. While widely used with apparently good results, freezing is not recommended for euthanasia because of a lack of data (Cooper and others 1989; Andrews and others 1993). However, precooling amphibians prior to euthanasia is supported to ease handling and increase proficiency of physical methods.

Recommended physical methods include blunt cranial concussion (Cooper and others 1989) and decapitation followed by double pithing (AVMA 1993). The effectiveness of such methods are highly dependent on the abilities of the individual performing the procedure, so it is imperative to assure individuals have been properly trained.

Chemical Methods

Whenever possible, chemical means of euthanasia should be chosen over physical methods. Overdoses of anesthetic agents are most commonly used. The author has successfully used 60 mg/kg sodium pentobarbital, 0.10-1.0% MS-222, and 0.10% benzocaine. Regardless of the chemical agent and route chosen, a physical method of euthanasia should always follow chemical euthanasia of amphibians, since death can sometimes be difficult to determine.

REFERENCES

Arena PC, Richardson KC. 1990. The relief of pain in cold-blooded vertebrates. SCAW Newslett 12(3):8-11.

[ASIH] American Society of Ichthyologists and Herpetologists. 1987. Guidelines for Use of Live Amphibians and Reptiles in Field Research. American Society of Ichthyologists and Herpetologists, The Herpetologist's League, and Society for the Study of Amphibians and Reptiles.

[AVMA] American Veterinary Medical Assocation. 1993. 1993 Report of the AVMA Panel on Euthanasia. J Am Vet Med Assoc 202(2):229-249.

Brattstrom BH. 1970. Amphibia. In: Comparative Physiology of Thermoregulation, Vol. 1. New York: Academic Press. p 135-166.

Brothers AJ. 1977. Instruction for the care and feeding of axolotls. Axolotl Newslett, Indiana University 3:9-16.

Clarke RD. 1972. The effect of toe-clipping on survival in Fowler's toads (Butb woodhousei fowleri) Copeia 1972:182-185.

Cooper JE, Ewbank R, Platt C, Warwick C. 1989. Euthanasia of Amphibians and Reptiles. London: Universities Federation for Animal Welfare and World Society for the Protection of Animals.

Crawshaw GJ. 1992. Medicine and diseases of amphibians. In: Schaeffer DO, Kleinow KM, Krulisch L, editors. The Care and Use of Amphibians, Reptiles and Fish in Research. Greenbelt, MD: Scientists Center for Animal Welfare. p 41-48. (Available from Scientists Center for Animal Welfare, Golden Triangle Building One, 7833 Walker Drive, Ste. 340, Greenbelt, MD 20770. Tel: 301/345-3500; Fax: 301/345-3503.)

Daly JW, Garraffo HM, Spande TF, Jaramillo C, Rand AS. 1994. Dietary source for skin alkaloids of poison frogs (Dendrobatidae)? J Chem Ecol 20:943-955.

Daugherty CH. 1976. Freeze-branding as a technique for marking anurans. Copeia 1976:836-838.

Dierenfeld ES, Barker D. 1995. Nutrient composition of whole prey commonly fed to reptiles and amphibians. In: Willette-Frahm M, editor. Proceedings of the Second Annual Conference of the Association of Reptile and Amphibian Veterinarians, Sacramento, California, October

27-29, 1995. p 3-15.

Donnelly MA, Guyer C, Juterbock JE, Alford RA. 1994. Techniques for marking amphibians. In: Heyer WR, Donnelly MA, McDiarmid RW, Hayek LC, Foster MS, editors. Measuring and Monitoring Biological Diversity: Standard Methods for Amphibians,. Washington, DC: Smithsonian Institution Press. p 277-284.

Duellman WE. 1993. Amphibian Species of the World: Additions and Corrections. Lawrence, KS: University of Kansas.

Duellman WE, Trueb L. 1986. Biology of Amphibians. New York: McGraw-Hill.

Elmberg J. 1989. Knee-tagging--a new marking technique for anurans. Amphibia-Reptilia 10:101-104.

Emlen ST. 1968. Territoriality in the bullfrog, Rana clamitans. Copeia 1968:240-243.

Farrell RK, Johnson SD. 1973. Identification of laboratory animals: Freeze marking. Lab Anim Sci 23:107-110.

Frost DR, editor. 1985. Amphibian Species of the World: A Taxonomic and Geographical Reference. Lawrence, KS: Allen Press.

Grenard S. 1994. Medical Herpetology: Amphibians and Reptiles--their Influence on, and Relationship to, Human Medicine. Pottsville, PA: NG Publishing.

Hayes TB. 1994. Breeding anurans for research purposes: Artificial manipulation of reproductive cycles with hormone injection. Proceedings of the Northern California Herpetological Society Conference on Husbandry and Captive Propagation of Reptiles and Amphibians 1993:53-56.

Heatwole H. 1961. Inhibition of digital regeneration in salamanders and its use in marking individuals for field studies. Ecology 42:593-594.

Heyer WR, Donnelly MA, McDiarmid RW, Hayek LC, Foster MS, editors. 1994. Measuring and Monitoring Biological Diversity: Standard Methods for Amphibians. Washington, DC: Smithsonian Institution Press.

Holtfreter J. 1931. Uber die eufzucht isolierter teile des amphibian keimes Il. Arch F Ent Mech 124:404-465.

Humason GL. 1967. Animal Tissue Techniques. 2d ed. San Francisco, CA: WH Freeman.

Hutchinson NA, Koles ZJ, Smith RS. 1970. Conduction velocity in myeli-nated nerve fibers of Xenopus laevis. J Physiol 208:279-289.

Jaeger RG. 1992. Housing, handling, and nutrition of salamanders. In: Schaeffer DO, Kleinow KM, Krulisch L, editors. The Care and Use of Amphibians, Reptiles and Fish in Research. Beltsville, MD: Scientists Center for Animal Welfare. p 25-29. (Available from SCAW, Golden Triangle Building One, 7833 Walker Drive, Ste. 340, Greenbelt, MD 20770. Tel: 301/345-3500; Fax: 301/345-3503.)

Lagutchik MS, Mundle TG, Martin DG. 1992. Methemoglobinemia induced by a benzocaine-based topically administered anesthetic in eight sheep. J Am Vet Med Assoc 201(9): 1407-1410.

Letchef J. 1992. Intracoelomic use of tricaine methanesulfonate for anesthesia of bullfrogs (Rana catesbeiana) and leopard frogs (Rana pipiens). Zoo Biol 11(4):243-251.

Maruska EJ. 1994. Procedures for setting up and maintaining a salamander colony. In: Murphy JB, Adler K, Collins JT, editors. Captive Management and Conservation of Amphibians and Reptiles. Ithaca, NY: Society for the Study of Amphibians and Reptiles.

Mattison C. 1982. The Care of Reptiles and Amphibians in Captivity. Poole, Dorset, UK: Blanford Press.

McVey M, Born T, Hanson E, Trenhan P. 1992. Biological Populations as Indicators of Environmental Changes. Washington, DC: Environmental Protection Agency, Office of Policy, Planning, and Evaluation.

Moore JA., editor. 1964-1976. Physiology of the Amphibia, 3 Volumes. New York: Academic Press.

Nace GW, Richards CM. 1972. Living Frogs. 2. Care. Carolina Tips 35:41-43.

[NRC] National Research Council. 1996. Guide for the Care and Use of Laboratory Animals. Washington, DC: National Academy Press.

[NRC] National Research Council. 1974. Amphibians. Guidelines for the Breeding, Care, and Management of Laboratory Animals. Washington, DC: National Academy Press.

Orwicz K. 1985. The effects of gas supersaturation on amphibians. In: Gray RL, editor. Proceedings of Northern California Herpetological Society and Bay Area Amphibian and Reptile Society Conference on Captive Propagation and Husbandry of Reptiles and Amphibians. Davis, CA: Northern California Herpetological Society. p 153-162.

Pough FH. 1991. Recommendations for the care of amphibians and reptiles in academic institutions. ILAR News 33(4):S 1-S21.

Pough FH. 1992. Setting guidelines for the care of reptiles, amphibians and fishes. In: Schaeffer DO, Kleinow KM, Krulisch L, editors. The Care and Use of Amphibians, Reptiles, and Fish in Research. Greenbelt, MD: Scientists Center for Animal Welfare. p 7-14 (Available from SCAW, Golden Triangle Building One, 7833 Walker Drive, Suite 340, Greenbelt, MD 20770. Tel: 301/345-3500; Fax: 301/345-3503.)

Rodriquez LF, Smolik LM, Zbehlik AJ. 1994. Benzocaine induced methemoglobinemia: Report of a severe reaction and review of the literature. Ann Pharmacother 28(5):643-649.

Stebbins RC, Cohen NW. 1995. A Natural History of Amphibians. Princeton, NJ: Princeton University Press.

Tucker CS. 1993. Water analysis, in: Stoskopf MK, editor. Fish Medicine. Philadelphia: WB Saunders. p 166-197.

Wake MH. 1994. Caecilians (Amphibia: Gymnophiona) in captivity. In: Murphy JB, Adler K, Collins JT, editors. Captive Management and Conservation of Amphibians and Reptiles. Ithaca, NY: Society for the Study of Amphibians and Reptiles. p 223-228.

Wolf DP, Hedrick JL. 1971. A molecular approach to fertilization. II. Viability and artificial fertilization of Xenopus laevis gametes. Devel Biol.25:348-359.

Woolley HP. 1973. Subcutaneous acrylic polymer injections as a marking technique for amphibians. Copeia 1973(2):340-341.

Zug GR. 1993. Herpetology: An Introductory Biology of Amphibians and Reptiles. San Diego, CA: Academic Press.

TABLE I Various artificial water solutions used for housing amphibians in captivity (modified from Maruska 1994).

Holtfreter's Solution
(Holtfreter 1931)

3.5g NaCl
0.05g KCl
0.1g $CaCl_2$
0.2g $NaHCO_3$
1 liter dH20
[Author's note: Some tadpoles are best maintained in 1/10 strength Holtfreter's solution (e.g. use IOL of dH20 rather than 1L)]

Amphibian or Ringer's Solution
(Humason 1967)

6.6g NaCl
0.15g KCl
0.15g $CaCl_2$
0.2g $NaHCO_3$
1 liter dH20

Artificial Pond Water
(Mattison 1982)

Stock A:
175g NaCl
35g $CaCl_2$
2 liters dH20

Stock B:
15g $NaHCO_3$
2 liters dH20

Steinberg's Solution
(Brothers 1977)

20mi 17.0% NaCl
10mi 0.5% KCl
10mi 0.8% Ca(NO3)2e4H2O
1Omi 2.05% MgSO4°7H2O
4mi 1.00N HCl (for pH7.4)
560mg Tris buffer
946ml dH20

For use, add 20mi each of stocks A and B to 5 liters of pond water.