Reproduction and Larval Rearing of Amphibians

Robert K. Browne and Kevin Zippel

Abstract

Reproduction technologies for amphibians are increasingly used for the in vitro treatment of ovulation, spermiation, oocytes, eggs, sperm, and larvae. Recent advances in these reproduction technologies have been driven by (1) difficulties with achieving reliable reproduction of threatened species in captive breeding programs, (2) the need for the efficient reproduction of laboratory model species, and (3) the cost of maintaining increasing numbers of amphibian gene lines for both research and conservation. Many amphibians are particularly well suited to the use of reproduction technologies due to external fertilization and development. However, due to limitations in our knowledge of reproductive mechanisms, it is still necessary to reproduce many species in captivity by the simulation of natural reproductive cues. Recent advances in reproduction technologies for amphibians include improved hormonal induction of oocytes and sperm, storage of sperm and oocytes, artificial fertilization, and high-density rearing of larvae to metamorphosis. The storage of sperm in particular can both increase the security and reduce the cost of maintaining genetic diversity. It is possible to cryopreserve sperm for millennia, or store it unfrozen for weeks in refrigerators. The storage of sperm can enable multiple parentages of individual females' clutches of eggs and reduce the need to transport animals. Cryopreserved sperm can maintain the gene pool indefinitely, reduce the optimum number of males in captive breeding programs, and usher in new generations of *Xenopus spp.* germ lines for research. Improved in vitro fertilization using genetic diversity from stored sperm means that investigators need the oocytes from only a few females to produce genetically diverse progeny. In both research and captive breeding programs, it is necessary to provide suitable conditions for the rearing of large numbers of a diverse range of species. Compared with traditional systems, the raising of larvae at high densities has the potential to produce these large numbers of larvae in smaller spaces and to reduce costs.

_____

Robert K. Browne, Ph.D., is a Research Associate, Perth Zoo, South Perth, Western Australia. Kevin Zippel, Ph.D., is an Amphibian Program Officer for the IUCN/SSC (World Conservation Union's) Conservation Breeding Specialist Group, Apple Valley, MN.

Address correspondence and reprint requests to Dr. Robert K. Browne, c/o Perth Zoo, 20 Labouchere Road, South Perth, Western Australia, 6151, or email robert.browne@gmail.com.

Key Words: amphibian; conservation; hormones; in vitro; larvae; ovulation; reproduction technology; sperm

Introduction

> "Reproductive success for amphibians requires spermiation, ovulation, oviposition, fertilization, embryonic development, and metamorphosis are accomplished" (Whitaker 2001, p. 285).

Amphibians play roles as keystone species in their environments; model systems for molecular, developmental, and evolutionary biology; and environmental sensors of the manifold habitats where they reside. The worldwide decline in amphibian numbers and the increase in threatened species have generated demand for the development of a suite of reproduction technologies for these animals (Holt et al. 2003). The reproduction of amphibians in captivity is often unsuccessful, mainly due to difficulty in spawning and low survival rates from egg to early juvenile. Even recently collected amphibians are often surprisingly difficult to reproduce in captivity, including animals in apparently good condition. Nevertheless, technologies for the reproduction of amphibians in captivity are developing rapidly, and particular techniques for sperm cryopreservation and for the induction of ovulation are undergoing improvement. The literature reflects the development of reliable techniques for the cryopreservation of sperm and for the short-term storage of oocytes for a range of amphibians in research and conservation (Browne et al. 2002b; Buchholz et al. 2004; Sargent and Mohun 2005). The use of a wider range and combinations of hormones and improved protocols for their administration offer improved methods for the induction of ovulation (Browne et al. 2006a,b). When combined with improved methods for in vitro fertilization, these technologies offer an economical and reliable means to satisfy the increasing need for the preservation of amphibian gene lines for both conservation and biomedical research (Holt et al. 2004; Roth and Obringer 2003).

The reproductive mechanisms of amphibians are as varied as their phylogeny, microhabitats, and life histories. The diversity of reproductive modes in the Amphibia rivals that of fishes, a vertebrate class with approximately four times as many species. Overall the frequency of reproduction, number and size of oocytes, type of fertilization, stage of development at hatching, and placement of eggs are highly

variable. For instance, most caecilians and salamanders spawn annually, although some spawn biannually or intermittently. Temperate and some mountain anurans tend to spawn annually. Tropical anurans in wet habitats spawn multiple clutches throughout the year. Anurans from arid areas often aestivate and spawn immediately after flooding rains, which may not occur for years. Some amphibians spawn only one oocyte a year while some anurans such as the bullfrog (*Rana catesbeiana*) spawn many thousands. All caecilians and most salamanders utilize internal fertilization whereas most frogs fertilize externally. The stage of hatching varies from embryos to fully formed juveniles (Duellman and Trueb 1994; McDiarmid and Altig 1999). However, investigators have developed sophisticated and reliable husbandry and reproduction techniques for only some laboratory species, including *Xenopus* spp. (Frazer 1976; Halliday 1999; Reed 2005), and species used for display (Schmidt and Henkel 2004). We refer interested readers to a review of current amphibian biology and conservation, which includes reproduction protocols for a range of species (Zippel 2005).

Many pond and stream breeding amphibians reproduce by laying a moderate to large number of eggs in water (Duelmann and Trueb 1994). These amphibians include many threatened, laboratory, and display species toward which investigators have mainly directed the scientific development of larval rearing techniques (Browne et al. 2003; Frazer 1976; Schmidt and Henkel 2004). Eggs of these species typically hatch as late yolk-sac larvae, which can then metamorphose over periods from days to years.[1] A small number of other species with more specialized life cycles, notably *Dendrobates* spp., have also reproduced very successfully in captivity (Schmidt and Henkel 2004). However, there are many species that are difficult to spawn or that spawn unreliably in captivity. The induction of spawning with hormones offers the potential for reliable and unseasonal reproduction in difficult species (Browne et al. 2006a,b; Michael et al. 2004).

Many aspects of endocrinology are universal among amphibians. Although investigators have been successful in efforts to induce ovulation with hormones in many species, current protocols to induce ovulation require advanced oocytes whose production is subject to different environmental cues in different species (Whitaker 2001). Consequently, a combination of good husbandry with natural and artificial reproduction holds the most promise for the long-term management of captive amphibians (Browne 2006b). Recent advances in reproduction technologies for amphibians include the hormonal induction of oocytes and sperm, the storage of sperm and eggs, artificial fertilization, and high-density rearing of larvae to metamorphosis. In some species, it is already possible to use these improved techniques effectively to produce large numbers of genetically diverse

animals for rehabitation projects (Browne 2006b; Browne et al. 2003; Holt et al. 2004).

Increasing numbers of threatened amphibians need captive breeding programs; one third of more than 6000 species are threatened with extinction, and one quarter more are data deficient but likely also to be threatened (Young et al. 2004). The maintenance of large numbers of amphibians in captivity is expensive and risky, and over time unpredicted mortality can severely reduce genetic diversity or extirpate these populations (Ballou 1992; Holt et al. 2004). As the number of threatened amphibian species to be maintained increases, the cost and size of captive breeding programs could also increase to prohibitive levels (Holt et al. 2003, 2004). Addressing these problems will result in a rapid improvement in both amphibian husbandry and the use of reproduction technologies (Clulow et al. 1999).

Loss of genetic diversity increases with the number of generations and with smaller effective population size (Figure 1). It is necessary to maintain at least 50 males and 50 females to even begin to preserve genetic diversity for even a small number of generations. The effective population size is the number of individuals of a species that contribute to the genetic makeup of offspring. This number therefore does not include juveniles or non-reproducing adults. The maximum effective population size consists of an equal number of males and females that all contribute equally to the genetic makeup of the offspring. In captive breeding programs, the unexpected loss of genetically important individuals reduces effective population size (Ballou 1992).

The storage of sperm has the potential to increase both the security and the efficiency of captive breeding programs. It is possible to cryopreserve sperm for millennia (long-term storage) or to store it for days to weeks unfrozen in refrigerators (short-term storage). Short-term preservation of sperm can also enable multiple parentages of individual females' clutches of eggs and reduce the need to
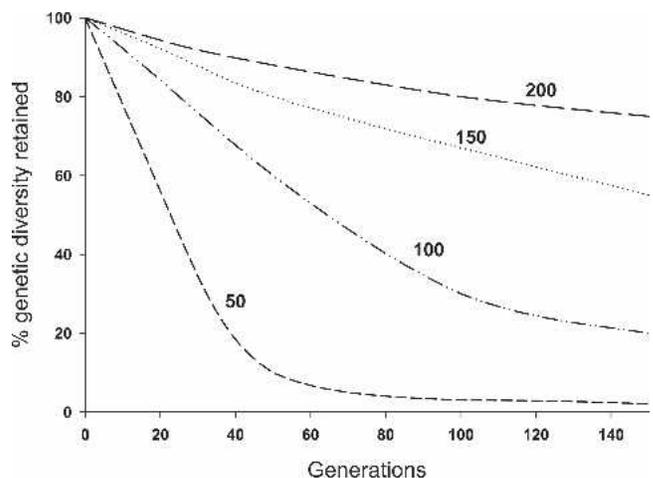

Figure 1 Relationship between effective population size, with equal numbers of males and females, and the loss of genetic diversity (heterozygosity) over 150 generations.

---

[1]Many amphibian larvae are commonly known as tadpoles. However, for consistency throughout this article, we refer to all tadpoles as larvae.

transport animals. Cryopreserved sperm can be an efficient way to store genetic diversity and affects the size of the male population by reducing the optimum number of males to be maintained as broodstock in captive breeding programs (Holt et al. 2003; Wildt 1997). Even in cases in which sperm cryopreservation proves difficult, the inter-cytoplasmic injection of the sperm into the oocyte should be successful in achieving fertilization (Holt et al. 2004; Poleo et al. 2005). The discussion of the potential for reproduction technologies such as cloning to recover genetic diversity or even to recover lost species from genetic material is beyond the scope of this article. However, amphibians have been pioneer subjects in many of these studies and are particularly suitable for the use of reproduction technologies due to external fertilization and development (Holt et al. 2004; Pukazhenthi and Wildt 2004).

The three conventional categories of larval rearing density in aquaculture systems include low-, medium-, and high-density larval rearing. Low-density systems are used when the larvae do not receive artificial feed, or, for instance, when larvae are cannibalistic and require individual rearing (e.g., Pacman frog *Ceratophrys ornate*; Budget frog *Lepidobatrachus laevis*, *Dendrobates* spp.). As larval densities increase, the amount of waste product and conflict over food and space increase. In medium-density systems, this situation often results in a wide dispensation of growth rates and stunted individuals (Alford 1999). However, after a threshold density is reached, the conflict over space ceases and, with adequate feed and the maximum larval density, appears to be limited only by the buildup of toxic waste products. In many species (e.g., bullfrog *Rana catesbeiana*) reared in medium-density systems, the optimum density of larvae is approximately three to ten per liter. Compared with traditional systems, the raising of larvae at high densities of 40 to 80 per liter can enable the production of more larvae in a smaller space and a reduction in cost. High-density rearing is particularly valuable in raising large numbers of larvae (e.g., green and golden bell frog *Litoria aurea*).

Reproductive Cycles

Age of Sexual Maturity and Breeding Condition

Before attempting to breed amphibians, and particularly in the case of hormonal induction of ovulation, it is essential to confirm that females are of breeding age and in good condition. The age of sexual maturity in amphibians is generally lower in males than in females. Males of some species regularly mature in less than 4 months (Horton 1982) whereas females often first spawn in their second year to maximize reproductive investment during risky spawning activity, when predation rates are particularly high (Duellman and Trueb 1994). However, precocious juveniles of the spotted marsh frog (*Lymnodynastis tasmaniensis*) may even reproduce when they are 40 to 80 days old (Horton 1982).

In *Xenopus tropicalis*, a standard laboratory model species, females mature at 6 months and are 65 mm in length. In most amphibians, egg numbers increase with female weight. *Xenopus* spp. peak in reproductive capability at 80 to 104 mm and can lay four clutches totaling 20,000 eggs a year (Frazer 1976; Halliday 1999; Reed 2005). In contrast, some montane aquatic species take years to metamorphose and likely several years more to reach sexually maturity (Duellman and Trueb 1994).

In species that live in environments with regular seasonal breeding opportunities, reproduction from each female can occur once yearly (semilparous). Females in other species reproduce repeatedly and opportunistically (iteroparous). An example of a semilparous species is the Mississippi gopher frog (*R. servosa*) whose females do not reproduce on every spawning event and sometimes not for several years. This behavior constitutes a bet-hedging strategy to avoid loss of complete clutches from a female due to pond drying. In *R. servosa*, the males return to ponds during most breeding opportunities (Richter et al. 2003). An example of an iteroparous species is the red-crowned toadlet (*Pseudophryne australis*). This small species reproduces by laying a few large rapidly developing eggs in stochastically ephemeral rock pools (Thumm and Mahony 2002).

Amphibian Endocrine System

In vertebrates including amphibians, external environmental cues are responsible for the production of primary hormones in the hypothalamus, pituitary, and gonads (Heatwole 2005; Figure 2). In female amphibians, these hormones promote gonadal maturation, spawning behavior, and spawning (Jørgenson 1975). In male amphibians, primary hormones initiate the hydration of sertoli cells, which induces spermiation (or sperm release) into the urine, and stimulate both calling and amplexus (Obringer et al. 2000). The hypothalamus produces small peptides called lutenizing hormone-releasing hormones (LHRHs[2]). These hormones stimulate the pituitary to produce the gonadotrophic hormones, follicle-stimulating hormone (FSH[2]) and lutenizing hormone (LH[2]), which then initiate structural or functional changes in the gonads. The FSH promotes mature follicles to produce progesterone, which then matures the oocytes, followed by spawning (LaMarca et al. 1985). The hormonal induction of spawning uses these hormones or artificial substitutes alone or in various combinations (Browne 2006a,b).

In nature, spawning can only occur at an advanced stage of gonadal development. As follicles develop the interstitial cells between the first growth phase (FGP[2]), oocytes (stages

---

[2]Abbreviations used in this article: DB, DeBoer's solution; FGP, first growth phase; FSH, follicle-stimulating hormone; GnRH, gonadotrophin-releasing hormone; hCG, human chorionic gonodotrophin; LH, lutenizing hormone; LHRH, lutenizing hormone-releasing hormone; LHRHa, lutenizing hormone-releasing hormone analogue; MAR, modified amphibian Ringer's; SAR, simplified amphibian Ringer's; SGP, second growth phase.
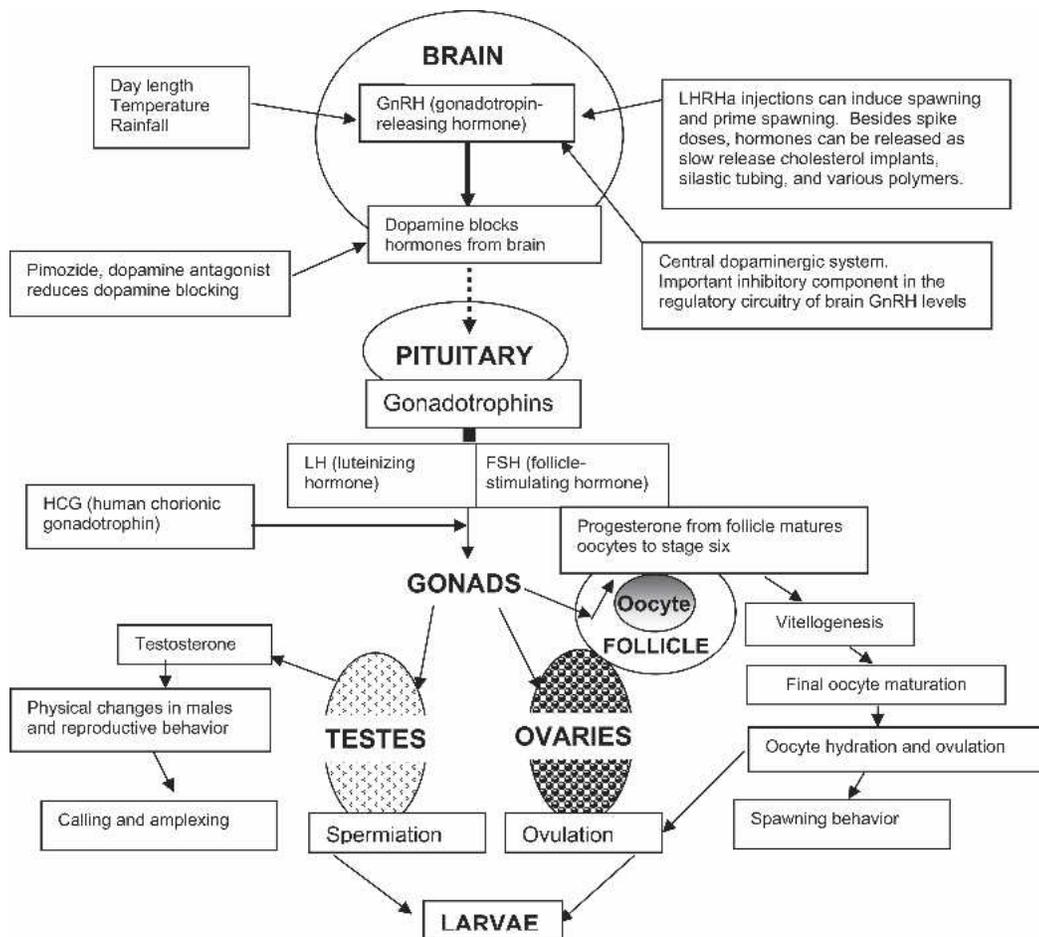
Figure 2 Hormonal pathways in amphibians. Environmental cues or the administration of luteinizing hormone-releasing hormone analogue (LHRHa) initiates the production of gonadotropin-releasing hormone (GnRH) in the brain. The influence of GnRH stimulates the pituitary to produce gonadotrophins. It is possible for the central dopaminergic system to block this pathway by reducing a dopamine antagonist such as pimozide. Gonadotrophins stimulate the testes to produce testosterone and the follicles to produce progesterone. Testosterone promotes the production of spermatozoa and reproductive behavior in males, and progesterone promotes the maturation of oocytes and ovulation.

3 and 4; Nieuwkoop and Faber 1994) secrete estradiol, which inhibits spawning. Then with suitable environmental entrainment of spawning, during the second growth phase (SGP[2]) of oocytes to final maturation (stages 5 and 6), estradiol secretion diminishes and testosterone and progesterone increase (Fortune 1983). Thus, once stimulated by the administration of ovulatory hormones or in nature through environmental entrainment, females with suitable numbers of SGP oocytes are usually compelled to spawn even without the stimulus of males. The production of SGP oocytes can be stimulated by the administration of progesterone, or by the sequential administration over days of other reproductive hormones (Browne et al. 2006a; Reynhout et al. 1975). Although investigators have studied this system most thoroughly in anurans, the hormonal systems in anurans, urodeles, and caecilians are similar (Heatwole 2005).

In the absence of hormonal induction, the recruitment of FGP oocytes to SGP oocytes (oogenesis) largely depends on temperature, nutrition, and female age. High temperatures can inhibit oogenesis, and a period of low temperatures (hibernation) may be required for ovulation (Kim et al. 1998). Jørgensen (1975, 1982) has shown that high feed levels increase recruitment of FGP oocytes to SGP vitellogenic oocytes before and after hibernation. The response of oocytes to maturating hormones can also depend on the ovulatory cycle in which Reynhout et al. (1975) have shown that smaller oocytes from recently ovulated females mature faster than larger oocytes from unspawned females. Consequently, because of the number of affecting factors and the complexity of their interaction, the success of either natural entrainment or the artificial induction of ovulation can be unpredictable.

Sexing

Sexual dimorphism is present in almost all species from all orders of amphibians except the caecilians. The most com-

mon method used to sex anurans is to identify differences between sexes in nuptial pads, tympanum size and color, throat and body color, and vocalization (Figure 3). Nuptial pads on males are usually located at the base of the thumb; however, in some taxa, the pads extend onto other digits, the lower forearm, legs, and mouth. They also are rough, sometimes have spines, and usually are dark. Nuptial pads tend to be more prominent in aquatic spawning compared with terrestrial spawning amphibians. In some species, it is difficult to detect nuptial pads.

The tympanic membrane is larger in the males of some frogs including the bullfrog, which uses it to amplify vocalizations (Purgue 1997). Throats of male frogs are generally darker and more colored than those of the females. The throats of females are often pale or white, but not in all species. Some species have dramatic color and pattern differences between males and females. Except in a very few species (e.g., *Alytes*), only males produce mating calls (Bosch 2001). Anurans also have rain and distress calls. Males can produce rain calls when they are some distance from ponds and even when there is no suitable spawning habitat. Some anuran species must be sexed by a combination of traits (Bosch and Marquez 1996). The range of reproductive characteristics in salamanders includes nuptial pads, which can form on the digits, lower forearm, and mouth. It is difficult to sex caecilians by external morphology or color; however, a sign of breeding condition unique to the caecilians is the formation of cloacal glands on the males (Duellman and Trueb 1994). The crests of many newts prominently reflect permanent or seasonal changes in the intensity and display of coloration (Dan 1983).

Conditioning Broodstock

The maturity and condition of broodstock[3] should always be the first concern for any breeding program. Adequate feed rates and optimum growth temperature contribute to the production of quality broodstock (Jørgensen 1982). However, population density, cover, and even water quality affect growth and development and, consequently, broodstock condition (Hilken et al. 1995). The entrainment of natural reproduction in captivity requires the environmental conditioning of both females and males. However, although it may be possible to compensate to some extent for problems with broodstock condition, even the use of hormonal induction may be ineffective if amphibians are not in good condition (Browne et al. 2006a,b). Variables that affect the entrainment of reproduction in various species include temperature, nutrition, and light. Water qualities including temperature, depth, and dispersion often induce the final stages of reproduction (Duellmann and Trueb 1994). In some species including *Xenopus laevis* and *Pipa parva*, vocalizations are paramount in inducing ovulation, and such calls can
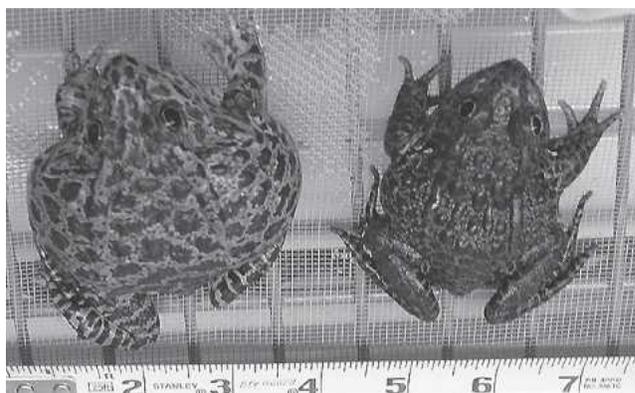
_____
[3]Broodstock and other terms are defined in the Glossary on page 234.

Figure 3 A female (left) and a male (right) Mississippi gopher frog (*Rana servosa*). In this figure, the male is smaller and darker. However, in *R. servosa*, the presence of nuptial pads (thumb pads) is the most reliable indicator of sex (not visible).

induce ovulation and even oviposition (Rabb 1973; Sughrue 1969).

Temperature

A seasonal cycling of temperature and humidity is essential for the maturation of follicles in some species. This cycling of temperature can include hibernation, aestivation, and slow and rapid temperature changes. For captive breeding, it is necessary to program the cycle to the same magnitude and duration as that in the wild (Jørgensen 1982). Temperature may also affect both ovarian maturation and the maturation of testes. Red-backed salamanders (*Plethodon cinereus*) require low temperatures to induce spermiation (Duellman and Trueb 1994, 1986). In contrast, the testes of the marbled newt (*Triturus marmoratus marmoratus*) (Fraile et al. 1989) or the northern leopard frog (*R. sylvatica*) do not mature at low temperatures (Duellman and Trueb 1994).

Brenner (1966) and Duelman and Treub (1994) have reported that for many species of anurans from temperate or cold climates with distinct seasons, a period of "hibernation" can assist the maturation of oocytes. In some species, a hibernation period in conjunction with adequate fat reserves is essential to mature oocytes (Brenner and Brenner 1969). The immune system of anurans tends to become less responsive at lower than physiologically optimum temperatures. Immunity can also take weeks to recover after low temperatures (Maniero and Carey 1997). Some pathogens including chytridiomycosis also prefer lower temperatures (Aplin and Kirkpatrick 2000; Berger et al. 2004). Independently or together, in some hibernating anurans these responses to low temperature can result in increased pathology and unacceptable mortality (Taylor et al. 1999).

Nutrition

The ingestion of large amounts of feed can accelerate the maturation of the ovaries of some amphibians (Brenner and

Brenner 1969; Jørgensen 1982). This response can be quite rapid, and the feeding to satiation of bullfrogs with high-quality feed can induce reproductive condition within 8 weeks irrespective of the season (Culley 1992). Females in particular should receive a continuous surplus of high-nutrient and high-energy foods for the last 6 to 8 weeks before reproduction. Amphibians are capable of consuming large amounts of prey in a short period if given the opportunity for instance during the insect bloom after a flood; they quickly recover their condition and then may aestivate as waters dry. Consumption of a diet in excess of an animal's energy needs can cause obesity and related disease (e.g., corneal lipidosis, fatty liver) (Whitaker 2001). Lowered feed rates can reduce obesity although the response is slow due to amphibians' low metabolic rate. In some species, no feeding during aestivation or hibernation can combine the reduction of excessive weight with the entrainment of reproduction (Brenner and Brenner 1969).

Light

Brenner (1966) and Duellman and Trueb (1994) have reported that photoperiod affects the reproductive cycles of some caudates and anurans. For example, bullfrogs (Horseman et al. 1978) and the skipping frog (*Rana cyanophlyctis*; Pancharatna and Patil 1997) need long photoperiods for full ovarian maturation without excessive atresia of oocytes. In the large-scale reproduction of *Xenopus* spp. for research, Reed (2005) and Schultz and Douglas (2003) recommend a 12/12 hr light/dark cycle to maximize reproduction success and fecundity. However, for most species, the photoperiod requirements are not known (Whitaker 2001). Therefore, it is advisable to follow the natural day/light cycle when providing light for captive species, particularly with species from high latitudes.

Aestivation and Hibernation

To avoid climate extremes, amphibians can aestivate to avoid dehydration or hibernate to avoid cold. Many amphibians from dry climates with erratic rainfall bury themselves between substantial rainfalls and then emerge to reproduce when flooding occurs (Duellman and Trueb 1994). They aestivate by the formation of a cocoon composed of skin and mucus as a mechanism to prevent dehydration (Withers 1995). Whitaker (2001) has shown that the cycling of reproduction follows rainfall in some species and that aestivation protocols can be a useful tool to bring some amphibians into breeding condition.

A good example is the aestivation protocol for the Puerto Rican crested toad (*Bufo lemur*), as Paine et al. (1989) have described. As the dry season begins in Puerto Rico, the investigators put *B. lemur* on a water-soaked substrate of peat and sphagnum mosses at a temperature of 22°C. Staff allowed the substrate with the toads in their burrows to slowly dry over a period of 1 month. They fed the toads weekly and gave them water in a bowl until the moss became dry, after which they maintained the animals for another month. At the end of 60 days, they added water to saturate the moss over 2 days, and increased the air temperature to 27°C. The males were then transferred to rain tanks and played mating calls for 2 days before the gravid females were added. Simulated rainfall and the recordings of mating calls were continued for an additional 3 or 4 days. Whitaker (2001) has reported that Puerto Rican crested toads amplex more successfully when they are held in groups, and they normally amplex soon after the females are introduced. We direct interested readers to the Hibernaculum section in the accompanying article in this issue, "Facility Design and Associated Services for the Study of Amphibians" (Browne et al. 2007).

Spawning in Rain Tanks

Species that respond to rainfall as a cue to induce calling in males and spawning in females can be amenable to the use of simulated rain events for promoting reproduction. The rain tank should have a design and a program to produce intermittent simulated heavy rainfall. The simulated rain should cycle with dry periods with a suggested duration of 30 to 60 minutes two or three times a day. Rainfall events normally produce a cooling of water; however, ambient water temperatures of flow-through systems may be lower than those found in temperate or tropical aquatic systems. For this reason, recirculating systems are superior to flow-through systems for the control of water temperatures and to reduce fluctuations of water quality.

A recirculation rain tank system consists of an aquarium or tub that is partially filled with water, with an elevated terrestrial section accessible from the water (Figure 4). The rain tank must have an overflow drain set at a suitable height from the tank bottom. A rain bar runs along the top of one of the longer sides of the rain tank, which is connected to a submersible pump. The pump is placed in a separate drainage tub, which receives the overflow water from the tank. The drainage tub must be big enough to enable the submersion of the pump when water levels decrease as pipes fill and until overflow begins. It is possible to connect the pump to a timer to cycle "rainfall" from the rain bar. One can then place an aquarium heater in the drainage tub to control water temperature. It is important to place the rain bar so that the terrestrial area stays relatively dry. There should not be any furnishings or substrates in the tank that can become suspended and block pipes, pumps, or the holes in the rain bar. Plastic plants and fine gravel substrate are ideal. It is essential to provide lighting and sun lamps on the natural daylight cycle. For small arboreal species from tropical forests and others that thrive in "natural" terrariums, a cycling from a dry environment to a wet and humid environment may induce spawning. Small sheltered pools, raising water levels, or the provision of large plastic leaves provide specialized spawning sites.
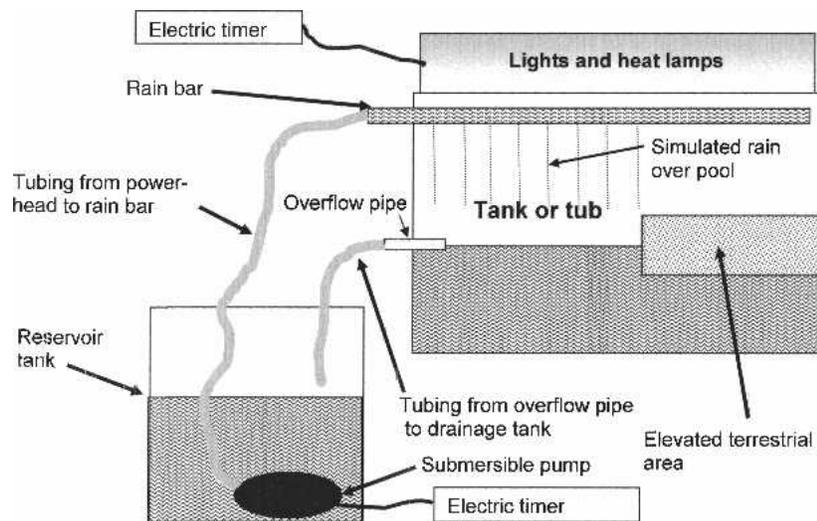
Figure 4 A schematic diagram of a rain tank for the spawning on terrestrial amphibians. A tank with part aquatic and part terrestrial habitat is provided with a rain bar and a drain. A submersible pump (powerhead) in a reservoir, which receives the tank overflow, cycles water from the reservoir through the rain bar to the tank overflow. It is possible to use an aquarium heater in the aquatic habitat of the tank or reservoir to maintain moderate temperatures, or a cooler in the reservoir to maintain low temperatures.

Induction of Oocytes

Investigators have induced ovulation in toads and frogs through the use of hormones with varying success since the early 20th century. They have found that it is advisable to select the most mature and healthy animals for hormonal induction. However, even apparently gravid females may not ovulate when administered hormones. These hormones can consist of pure material from chemical supply companies or pituitary extracts from the same or other species. Two techniques have great potential to improve current protocols for the ovulation of amphibians: (1) the sequential administration of hormones over days to weeks, and (2) antidopaminergic compounds (Browne et al. 2006a).

Hormones

The best-studied anurans with respect to hormonal induction of oocytes or sperm are the laboratory species the African clawed frogs (*X. laevis* and *X. tropicalis*) and the northern leopard frog (*R. pipiens*) (Whitaker 2001). However, hormones are regularly used to induce ovulation in the amphibian pet trade and recently, due to the conservation crisis, to induce ovulation and spermiation in threatened species. The several classes of substances used to ovulate amphibians include hormone compounds, pituitary extracts, and antidopaminergic drugs such as pimozide (Browne et al. 2006a).

In anurans, the following three hormones are used for artificial ovulation: (1) luteinizing hormone-releasing hormone analogue (LHRHa[2]), (2) human chorionic gonodotrophin (hCG[2]), and (3) progesterone. LHRHa is an analogue of gonadotrophin-releasing hormone (GnRH[2]), and it stimulates the release of LH. Under the stimulation of LHRHa or hCG, follicles that surround the eggs produce progesterone, which then causes the final maturation of the eggs. Amphibians harvested out of season, or captive frogs without adequate environmental entrainment, do not have mature follicles. To ovulate these amphibians, it may be necessary to administer progesterone in combination with other ovulatory hormones, or with pituitary extracts. In some difficult situations, to achieve ovulation, it may be necessary to prime the females with a low dose of hormones for a period of days to weeks before administering a larger, final ovulatory dose.

Many analogues of LHRHa are commercially available; however, Arimura et al. (1974) have reported that only D-Ala des-Gly10 ethylamide (Sigma Aldrich, St. Louis, MO, L4513) is effective in inducing ovulation (Browne et al. 2006b; Michael et al. 2004; Toro and Michael 2004; Wright 2001). In a limited number of amphibians, other investigators have shown that hCG is effective in stimulating ovulation (Browne et al. 2006a,b; Hollinger and Corton 1980; reviewed in Whitaker 2001).

Pituitaries

The use of pituitaries to induce ovulation should be a purely experimental procedure that investigators use only when they will not release the broodstock, their progeny, or future progeny from either (Chakraborty et al. 2005). Hypophysation in its strictest sense is the process of inducing breeding through injection of pituitary extract from pituitary glands. Anuran pituitaries are commercially available for purchase

in the form of a dried powder; however, there is limited information about the interspecific differences in the efficacy of pituitaries. Wright and Whitaker (2001) have documented the removal of the brain of a cane toad (*Bufo marinus*) (Plates 25.8 A-C dorsal view, and Plate 25.9 A-B, ventral view). After the removal of the brain, the pituitaries are visible at the junction of the spinal cord with the brain. Investigators may use the pituitaries immediately, store them at 4°C in 100% ethanol, or lyophilize them. There are descriptions in the literature of homogenizing pituitaries in 1 mL of simplified amphibian Ringer (SAR[2]; Rugh 1962; Table 1) and injecting them intracoelomically with a 23-gauge needle (Browne et al. 1998). There are several methods to inject frogs, toads, and other amphibians, including intracoelomically and in the dorsal lymph sac. It is important to use methods that provide proper restraint and the least stressful handling of animals. Diagrams of standard handling methods (without mandatory gloves for clarity) and explanatory information are available online (http://www.bioteck.org/methods/handling/Handling.htm).

When investigators have deemed the experiments necessary, they have used pituitaries most commonly to induce ovulation in northern leopard frogs (*R. pipiens*). Of the ranids, the most studied ovulation under laboratory conditions is that of *R. pipiens*. There are seasonal changes in the potency of pituitaries and in female sensitivity to pituitary extracts. The sensitivity of females to pituitary extracts increases with the administration of progesterone to mature oocytes. For this reason, the number of pituitaries and the amount of progesterone used with pituitaries will correspondingly vary with the season (Bagnara and Stackhouse 1973; Table 2). For example, in October or November pituitary extracts will be sufficient for inducing ovulation in only one mature frog. Using the same amount during December and until March, two frogs may be induced to ovulate; and if used in March and April, as many as three frogs may be induced to ovulate. In addition, the earlier females are induced with pituitaries before the natural spawning

Table 1 Simplified amphibian Ringer's (SAR) solution[a,b]

Compound	Molecular weight	Gram/ liter	Gram/ 3 liter
113.0 mM NaCl	58.44	6.40	19.20
1.0 mM CaCl$_2$	110.98	0.15	0.45
2.0 mM KCl	74.55	0.15	0.45
3.6 mM NaHCO$_3$	84.01	0.30	0.90

[a]Adjust pH to 7.2 with NaOH. Some Ringer's solutions use lactate; however, it is advisable to avoid the use of lactate with amphibians due to problems with lactate metabolism.
[b]Adapted from Rugh R. 1962. Culturing of amphibian embryos. In: Rugh R, ed. Experimental Embryology: Techniques & Procedures. Minneapolis: Burgess Publishing. p. 50-51.

Table 2 Doses of progesterone used to ovulate *Rana pipiens* when combined with pituitaries[a]

Month	Pituitaries alone (n)	Pituitaries (n)	Pituitaries and progesterone (mg)
Sept-Oct	10-12	2	5.0
Nov-Dec	6-8	2	2.5
Jan-Feb	4-5	1	2.5
Mar-Apr	1	1	0

[a]Doses shown are calculated as female pituitaries; two male pituitaries are equivalent to one female pituitary. It is necessary to inject both the pituitary and progesterone into the coelomic cavity approximately 48 hours before scheduled fertilization.

season, the more progesterone is needed to achieve ovulation. With cane toads, the use of pituitaries to induce ovulation has also proved far superior to the use of purified hormones (Browne et al. 1998).

Doses and Administration of Hormones and Other Compounds

The dose of hormones used to ovulate anurans is approximately 130 IU hCG per 10 g body weight, or 20 μg LHRHa per 10 g body weight. However, the response to "priming" and the response time vary dramatically between species. It is often necessary to highly dilute hormones and in particular LHRHa in solution and then to store them. A protocol for the dilution of LHRHa is available online (http://www.bioteck.org/methods/formulations/LHRHa.htm). The three methods of administering hormones are by injection, by implants, or through topical ointments. The traditional method is to administer hormones by injection into the coelemic cavity or dorsal lymph sac after dissolving the hormones in sterile phosphate-buffered saline or in SAR. Intracoelemic injection requires skill, to avoid injection into organs. This method involves insertion of a 23-gauge needle from the posterior of the coelemic cavity at a shallow angle.

Of all amphibian models, *X. laevis* is the most studied for the induction of oocytes. A priming dose of 10 IU hCG 12 to 72 hours before an ovulatory dose of 100 to 200 IU hCG induces formation of oocytes in *X. laevis*. Ovulation doses may be lower with older females wherein 100 IU produces ovulation after 6 to 8 hours and 200 IU after 4 hours. *X. tropicalis* requires a much lower dose than *X. laevis*. For natural amplexus, doses of 200 IU hCG for females and 100 IU hCG for males are given in the evening, resulting in pairs laying eggs overnight. It is essential to keep reproducing *Xenopus* and their eggs at temperatures between 20 and 25ºC (Halliday 1999; Reed 2005). We provide recommended doses for the induction of oocytes in Fowler's toads in Table 3.

Table 3 Recommended doses for the induction of oocytes in 45-g female Fowler's toads (*Bufo fowleri*)[a,b]

Compound[c]	Administered dose	Amount per gram of toad weight
hCG	500 IU hCG	15 IU
LHRHa	4 μg	0.12 μg
LHRHa	20 μg	0.61 μg
LHRHa	60 μg	1.8 μg
Progesterone	5 mg	0.15 mg
Pimozide™	0.25 mg	0.0076 mg

[a]Toads administered 20-60 μg of LHRHa with progesterone produced superior ovulation to 20 μg of LHRHa alone, or 20 μg of LHRHa with Pimozide™, or 500 IU of hCG with 4 μg of LHRHa.
[b]Adapted from Browne RK, Seratt J, Li H, Kouba A. 2006. Progesterone improves the number and quality of hormonally induced Fowler's toad (*Bufo fowleri*) oocytes. Reprod Biol Endocrinol 4:3.
[c]hCG, human chorionic gonadotrophin; LHRHa, luteinizing hormone-releasing hormone analogue.

Priming

Priming is the use of sequential doses of hormones to induce ovulation. Wyoming toads (*Bufo baxteri*) held warm without hibernation at 28°C have required several primings over several days to achieve ovulation through hormonal induction (Browne et al. 2006b). Fish have endocrine systems similar to those in amphibians (Ng et al. 1986). In tests of more than 40 fish species, extended administration of hormones using implants (compared with single or multiple-pulse doses) improved reproductive output (Constantinos and Zohar 2000). The anovulatory (priming) dose is usually much lower (10-50%) than the ovulatory dose. We recommend spacing the priming dose 12 to 72 hours before the ovulatory dose. The dose will vary with the species, their maturity, and environmental influences (Browne et al. 2006a,b).

Antidopaminergic Compounds

Investigators have also trialed a number of other compounds, including antidopaminergic compounds, in combination with hormones. These have resulted in improved egg production and quality in fish (Mikolajczyk et al. 2004; Mishra and Joy 2004). In cyprinid fish and in catfish, the most potent method for inducing ovulation is to combine antidopaminergic compounds like Pimozide™ (Orap®, Sigma Aldrich P1793; 1-[1-[4,4-bis(4-fluorophenyl)butyl]-4-piperidinyl]-1,3-dihydro-2H-benzimidazol-2-one) with LHRH analogues. With some fish species, combining Pimozide™ with other compounds has promoted ovulation when the compounds used alone have failed (Peter and Yu 1999).

The central dopaminergic system is an important inhibitory component in the regulatory circuitry of brain GnRH. Stress-induced corticosteroids can inhibit the release of gonadotropin to the extent that in some species Pimozide™ is necessary to achieve high rates of ovulation and egg quality (Kumarasiri and Seneviratne 1988). The use of Pimozide™ may also have similar effects in anurans (Browne et al. 2006a).

Sampling and Storage of Oocytes

After successful induction, investigators can decide which of the following courses of action to follow: (1) to allow the females to spawn spontaneously, (2) to strip the oocytes, or (3) to excise the oocytes. With oocytes that have spawned spontaneously, it may be possible for the amplexing males to provide fertilization or, alternatively, the investigator can provide artificial fertilization. Stripped or excised oocytes require artificial fertilization.

Spontaneous Spawning

It is important to place females in an enclosure that is an appropriate size for the species. The following approximate dimensions should be adequate:

- For small species (<5 g), provide an area 20 × 20 cm;
- For medium-sized species (5-15 g), provide an area 30 × 40 cm; and
- For large species (>15 g), provide an area 40 × 70 cm.

The depth of the water and the accessories in the spawning enclosure differ depending on whether the oocytes will be fertilized naturally or artificially (Figure 5). If males in amplexus will be used to fertilize oocytes, the water depth must provide for natural behavior. Alternatively, artificial fertilization will require spawning in a shallow depth of SAR. It is essential to carefully observe oviposition with some species (e.g., pipids) in which adults reportedly eat their eggs (Summers et al. 2007).

Sampling Oocytes Through Palpitation

If females ovulate but do not spawn the oocytes, it may be advisable to strip oocytes from females of some species (Whitaker 2001). The use of this method is successful for some species including ranids. However, it does not appear to be suitable for others, such as toads, perhaps because of the conformation of the toad egg mass as paired strings. Paired strings of eggs are seldom successfully stripped. If ovulation is successful, species that do not spawn normally will express a few oocytes in the cloaca approximately 12 hours after induction. In these egg-bound females in which palpitation does not release the oocytes, it is possible to obtain the mature spawn mass directly from the oviduct (Browne et al. 1998).

Figure 5 A hormonally induced Wyoming toad (*Bufo baxteri*) nestled among recently spawned strings of oocytes. Note spawning substrate (i.e., plant material) (R.K.B., unpublished).

Natural Fertilization

Natural fertilization requires sufficient depth and extent of artificial pond water to enable the natural performance of amplexus or other behaviors that are necessary for the animals to successfully deposit fertilized eggs. Too shallow water can result in some anuran females drowning. The use of artificial pond water produced from distilled water with added salts avoids potential problems with pathogens and contaminants. One method of producing simplified artificial pond water is to use 5% (v/v) SAR in distilled water. Water in spawning containers should be quite deep with some species that deposit eggs at depth on artificial substrates. It is advisable to provide artificial substrates, possibly plastic plants, in which eggs can lodge and to provide resting sites at the surface of the water to prevent females from drowning.

Short-term Storage of Oocytes

It is possible to extend the period over which oocytes remain fertilizable by spermatozoa by using saline solutions to increase osmolarity or low temperatures (Browne et al. 2001). Females may spawn oocytes directly into saline for delayed fertilization (Browne et al. 2006a,b) or oocytes may be obtained directly from the oviduct through palpitation or excision (Browne et al. 1998). To obtain oocytes through spawning, place females without males in appropriately sized plastic boxes with a shallow depth of saline (e.g., for 40-g toads, 1.5 cm of SAR in a plastic box 45 × 30 cm; Table 1). It is then possible to extend the fertility of the oocytes by storing them in SAR (Browne et al. 2001) or DeBoer's solution (DB[2]; Table 4) at three times the standard concentration (Hollinger and Corton 1980). We refer

interested readers to the following additional resources for information about the storage in saline of particular species oocytes:

- African clawed frog (*Xenopus* spp.; DB, Holliger and Corton 1980);
- Cane toad (*B. marinus*; SAR, Browne et al. 2001);
- Fowler's toad (*Bufo fowleri*; SAR, Browne et al. 2006b);
- Wyoming toad (*B. baxteri*; SAR, Browne et al. 2006a); and the
- Striped marsh frog (*Limnodynastes tasmaniensis*; SAR, Edwards et al. 2004).

The optimum temperature for the storage of the oocytes is less than the optimum temperature for housing adults (e.g., the cane toad, 15°C [Browne et al. 2001]; Fowler's toad, 10°C [Browne et al. unpublished]).

Counting Eggs and Larvae

Techniques used to count eggs include the displacement method, image analysis, and direct counting (http://www.bioteck.org/methods/counting/Counting.htm). The **displacement method** is fast, and it enables investigators to reduce inaccuracies from different egg size and gel thickness in different parts of the spawn mass. The method involves pooling five subsamples of approximately 50 eggs and then counting and measuring their volume (usually in a measuring cylinder). The investigator then measures the volume of the total egg mass (including the subsamples) and calculates the total number of eggs as follows:

$$\text{Total number} = \text{total volume} \times \text{subsample count}/\text{subsample volume.}$$

The displacement method is not suitable for some hormonally induced spawn masses, which may be of low quality and vary significantly in egg and gel size.

Table 4 DeBoer's solution at three times concentration for storage of oocytes[a,b]

Compound	Molecular weight	Gram/ liter	Gram/ 3 liter
110.0 mM NaCl	58.44	6.40	19.20
1.3 mM KCl	74.55	0.10	0.30
0.44 mM CaCl^2	110.98	0.05	0.15

[a]Mix salts in water and adjust pH to 7.2 with 8.4% (1 M) NaHCO$_3$.
[b]Adapted from Wold DP, Hedrick JL. 1971. A molecular approach to fertilisation. II. Viability and artificial fertilisation of *Xenopus laevis* gametes. Dev Biol 25:348-359.

Image analysis utilizes a dedicated computer program to count the number of eggs from an image. The investigator takes a digital image with a camera of the spread-out egg mass placed against a contrasting background (e.g., light gray for dark eggs and dark gray for light eggs). Inaccuracies with this method can occur when eggs overlap and make it difficult to identify individual eggs. Visual identification is particularly difficult when the eggs are in a globular mass rather than in a string. It is advisable to dissociate the eggs (see the description of degelling eggs in Artificial Fertilization, Egg Gel-Extract Solution), which then enables accurate counting. It is also possible to independently distribute early larvae in a tray for counting by image analysis.

Direct counting methods involve counting the number of eggs from a printed image or counting with a plastic disposable pipette during hatching or when transferring early larvae. Both methods produce an accurate count of eggs or larvae but can be tedious, particularly with extensive research that involves counting large numbers of eggs. As with image analysis, direct counting can be difficult when the images of eggs overlap. The use of a disposable pipette is appropriate for counting small numbers of eggs or for counting and moving early larvae. It is possible to cut the end of a disposable plastic pipette to produce a tube of maximum diameter. One can then aspirate and count the eggs or larvae (up to just below the diameter of the tube) and place them in a new container. It is common to use pipetting to count and separate dead eggs and larvae from developing larvae.

Sampling and Storage of Sperm

The following two important principles apply when working with amphibian sperm: (1) sperm concentrations must be high ($\geq 10^5$ mL) to achieve fertilization; and (2) the viability of sperm when activated at room temperature declines over a period from minutes to 1 hour, or after several hours when the immotile sperm are held in solutions of high osmolarity. In the text below, we describe factors to consider in the application of these principles.

Sampling of Testicular Sperm

One samples testicular sperm by macerating the testes into an equivalent volume of SAR. It is possible to sample sperm from the testes of recently dead threatened anurans for several hours after death if the amphibian is kept in ice slurry. A diagrammatic protocol showing the excision of testes from a natural mortality of a Fowler's toad is available online (http://www.bioteck.org/methods/Testes%20Excision/testes%20excision.htm). Although the sperm are immotile due to the high level of osmolarity, it is still necessary to cool the suspensions immediately to between 0° and 4°C. The motility of toad sperm that have been stored in SAR at 21°C ceases over the next 4 to 6 hours (Browne et al. unpublished).

Induction of Spermiation

It is possible to induce spermiation and amplexing behavior in male amphibians by using hCG or LHRHa (Figure 6). Many analogues of LHRHa are commercially available; however, Obringer et al. (2000) and Waggener and Carroll (1998) have reported that only D-Ala6, des-Gly10 ethylamide is effective in inducing spermiation. Doses for hCG are 300 IU per 35-g toad, or 8 IU per g of toad. After the administration of hormones, spermiation in urine occurs between 30 minutes and several hours, peaks in toads at approximately 6 hours, and may continue for up to 12 hours. With some anurans, especially toads, it is possible to collect sperm in urine without cannulation by stimulating the animal to urinate as follows: (1) Carefully remove the toad from its box and dry the superficial water with a tissue. (2) Hold the toad by one thumb and index finger, anterior to the pelvic girdle, above a 150-mm-diameter Petri dish. (3) Gently massage the anterior sides of the toad. This technique usually promotes urination within 60 seconds, and the volume can be several milliliters (Browne et al. 2006a). With species that do not urinate, it is possible to collect sperm by using a pipette to irrigate the cloaca or by cannulation into the efferent ducts (Waggener and Carroll 1998). Once collected by either method, it is essential to keep the samples in individual 0.5- or 1.5-mL Eppendorf tubes or in cannulation tubes, in ice slurry, and to check them for sperm concentration.

Sperm Motility and Short-term Storage

When personnel handle sperm samples for more than a few minutes, it is essential to maintain their temperature as close to 0°C as possible whether the sperm are active or inactive.
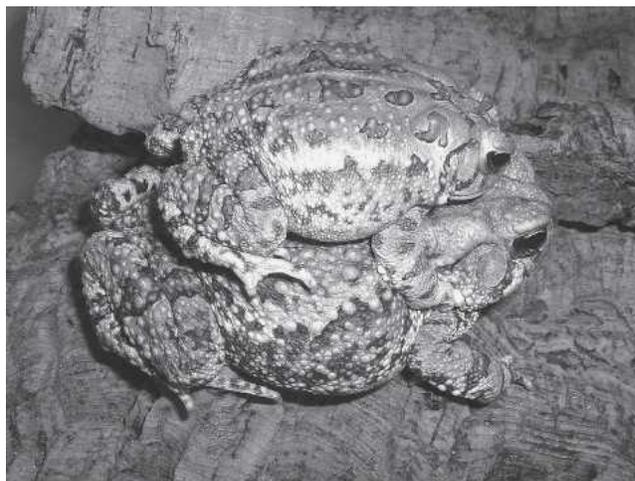

Figure 6 A pair of Fowler's toads (*Bufo fowleri*) amplex after the male has been given 300 IU of human chorionic gonadotrophin by injection into the intracoelemic cavity (R.K.B., unpublished).

There are no reports to date of cold shock with amphibian sperm where viability is reduced at near zero temperatures (0°C). Instead, sperm motility ceases at near zero temperatures, where sperm can be held for up to weeks. It is possible to create these temperatures by using ice slurry in fresh water. To avoid freeze damage, we advise against placing the containers with samples on untempered ice and instead, recommend tempering the ice in fresh water to bring its temperature close to 0°C (Browne et al. 2002d).

In amphibians with external fertilization, sperm becomes motile in response to the low osmolarity of fresh water (5 mOsmol kg^{-1}). To maintain the viability of sperm samples, it is necessary to hold the samples in an immotile state in solutions of high osmolarity that exceed 220 mOsmol kg^{-1}, including SAR or cryoprotective additives, and preferably at 0° to 4°C (Browne et al. 2002c). At 0° to 4°C, sperm is immotile irrespective of solution osmolarity (Browne et al. 1998). In the case of hormonally induced sperm that has been expressed in urine and is motile, it is necessary to use the sperm for fertilization immediately, to centrifuge and place it in solutions of high osmolality, and to extend its storage life by holding it at 0° to 4°C. To maintain the viability of sperm sampled from testicular macerates in solutions of high osmolality, including SAR or cryodiluents, it is also necessary to cool the holding temperature to between 0° and 4°C. After storage, one can activate the sperm motility by lowering osmolarity and/or by warming to the normal ambient temperatures of fertilization. In response to high and low osmolarity, the motility of amphibian sperm is reversible, which allows additional flexibility in techniques for storage (Browne et al. unpublished).

Sperm Concentration, Motility, and Fertilization

High concentrations of amphibian sperm are necessary to achieve fertilization, partially because the gel layer blocks the majority of sperm (Reinhart et al. 1998). The minimum concentration of sperm for successful artificial fertilization is between 10^5 and 10^7 mL^{-1} (Browne et al. 1998; Edwards et al. 2004; Sargent and Mohun 2005). Sperm macerate at a concentration of approximately 10^8 mL^{-1}, therefore a dilution of approximately 1:100 will result in optimum fertilization. The concentration of sperm when sampled through hormonal induction in urine is less than 10^6 mL^{-1}. During cryopreservation, it is customary to dilute sperm samples with cryodiluents and later, after thawing, to further dilute the samples to achieve activation. Thus, it is necessary to concentrate sperm from urine by centrifugation. One can measure the sperm concentration of each sample with a Neubaeur hemocytometer (measuring to the nearest 0.1 × 10^6mL^{-1}) using combined counts of approximately 200 sperm. The easiest counts are approximately 10^5mL^{-1}, and we advise diluting the samples to suit.

Complex interactions occur between sperm motility, osmolarity, fertilization, and sperm quality. The type of mo-

tility that most anuran sperm exhibit is a vigorous forward motion between 25 and 45 μm s^{-1} (Table 5). Although this speed is slower than that of fish sperm, the duration of motility of most amphibian sperm, which is approximately 1 hour, is far greater than that of most fish. The period of motility extends as the osmolarity of the solution increases; however, as the osmolarity increases, the number of motile sperm diminishes. Sargent and Mohun (2005) reported that the maximum motility of *Xenopus* spp. sperm occurs at an approximate 40 mM NaCl concentration, and the duration of the motility of cane toad (*B. marinus*) sperm can extend from 1 hour at the osmolarity of pond water (5 mOsmol kg^{-1}) to 5 hours at 40 mOsmol kg^{-1}. This effect of higher osmolatities in maintaining the duration of motility may be more pronounced when sperm are compromised by short-term storage or cryopreservation.

Centrifugation of Sperm Samples

It is possible to increase sperm concentration by centrifugation in 1.5-mL Eppendorf tubes for 5 minutes at 300 × g. During procedures when possible, personnel must keep all samples and containers at 0° to 4°C. Because centrifugation and the handling of sperm inevitably reduce viability, it is essential to complete the procedures efficiently to reduce the loss of sperm viability (Browne et al. unpublished).

Transport of Amphibian Oocytes and Sperm

We advise against transporting oocytes due to their delicacy and short storage life (Browne et al. 2001). To achieve artificial fertilization, we prefer and recommend transporting sperm to oocytes. It is also better to store sperm as testicular macerates rather than in whole testes. For maximum storage time, it is necessary to keep testes or sperm suspensions in ice slurry. For example, storage time at 4°C

Table 5 The sperm of some amphibian species are quite vigorous at speeds up to 48 μm s^{-1}; however, families like the myobatrachids have sluggish sperm that sometimes have little forward motility

Species	VCL[a]	Reference (see text)
Six different species	23-48 μm s^{-1}	Van der Horst et al. 1995
Three hylids	25-30 μm s^{-1}	Clark 1997
Two myobatrachids	3-7 μm s^{-1}	Clark 1997

[a]VCL, curvilinear velocity.

is less than half that in an ice slurry at 0ºC. Before storage, it is necessary to remove blood and extraneous tissue from the testes. If small testes are transported, it is essential to keep them on a piece of paper that is moist, but not moist enough to form a film of water over the testes. If treated gently, fertilized eggs generally transport well. It is possible to sample sperm from the testes of recently dead amphibians for hours after death if personnel have held the animals' sperm in ice slurry (Browne et al. 2002b).

Cryopreservation of Sperm

The optimum freezing rates and the best cryodiluents to preserve amphibian sperm are not well known. Nevertheless, numerous investigators have reported success with a variety of species (Browne et al. 1998, 2002b; Costanzo et al. 1998, Mugnano et al. 1998), and others (Buchholz et al. 2004) have used cryopreserved *Xenopus* spp. sperm commercially in biomedical gene banks. Most studies of the cryopreservation of amphibian sperm have involved sperm from testicular macerates. The cryopreservation of toad sperm from hormonal induction has been more difficult than with sperm from testicular macerates. For toad sperm sampled through hormonal induction, even with the best known cryopreservation protocols, recovery after thawing produces a low percentage of motile sperm with only limited viability (Browne et al. unpublished). However, toad sperm is unique in that the mitochondria are located in an exposed collar at the base of the sperm head, and there is a flagellum with a thin membrane between an axoneme and an axial rod (Lee and Jamieson 1993). These characteristics could make toad sperm more vulnerable to damage during the rigors of cryopreservation. Nevertheless, we have observed that cryopreserved Fowler's toad sperm obtained through hormonal induction has successfully fertilized eggs (Browne et al. unpublished). It is possible to store sperm from a variety of species, including cane toad sperm, in an unfrozen state for some time and then to cryopreserve it (Browne et al. 2002b,c). During cryopreservation procedures, it is necessary to keep the samples as cool as possible to retain sperm motility, except for the allowance of time at higher temperatures for the diffusion of any permeating cryoprotectants into the sperm (Browne et al. 1998).

Cryodiluents

A cryodiluent is primarily an organic solution that protects sperm during cryopreservation. Cryodiluents used in the cryopreservation of amphibian sperm are a mixture of non-penetrating and penetrating cryoprotectants. The main non-penetrating cryoprotectants are sugars (10% w/v) and egg yolk (5-10% v/v), and penetrating cryoprotectants are glycerol or dimethyl sulphoxide (5-10% v/v) in the final sperm suspension (Browne et al. 1998, 2002a,d; Costanzo et al. 1998; Mugnano et al. 1998). Salts exist in final mixtures of cryodiluent and testicular macerates but in lower amounts than in tissue. High salt levels appear to cause damage (Browne et al. 2002d). Sargent and Mohun (2005) have reported success using sugars and egg yolk alone in the cryopreservation of sperm from *X. laevis* and *X. tropicalis*.

Preparation of Egg Yolk/Sucrose Cryodiluent

Sargent and Mohun (2005) also successfully used egg yolk/sucrose diluent to cryopreserve *X. laevis* and *X. tropicalis* sperm. They emulsified the egg yolk in an equal volume of distilled water and then diluted one part emulsion to four parts water. After they added sucrose (800 mM) and sodium bicarbonate (20 mM), they centrifuged the mixture for 20 minutes at 13,000 rpm and then stored the aliquots of supernatant frozen at –20°C. Finally, they mixed this cryodiluent with an equal volume of testicular macerate.

Preparation of Samples for Freezing

For the cryopreservation of sperm from testicular macerates, Browne et al. (1998) and Sargent and Mohun (2005) have reported adding cryodiluent during the testicular maceration stage. The process involved preparing the cryodiluent at twice the concentration required for cryopreservation, weighing the testes and placing them in a Petri dish, and placing a similar weight of cryodiluent over the testes, which investigators then macerated with a pair of tweezers. With hormonally induced sperm, it is necessary to concentrate the sperm through centrifugation enough for it to become diluted 1:1 with cryodiluent and then, on thawing, to dilute it another three times to activate motility. Sperm suspensions at high concentrations are then mixed 1:1 with cryodiluent. The resulting sperm suspensions in cryodiluent from both testicular macerates and hormonal induction are kept in Eppendorf tubes in ice slurry before loading into cryo-straws.

Straw Size, Labeling, and Record-keeping

Smaller volumes and greater surface areas result in more uniform cooling and thawing. Most studies have utilized 0.25- or 0.5-mL cryo-straws to store amphibian sperm, although Sergent and Mohun (2005) have reported using 500-µl Eppedorf tubes. It is imperative to label the straws clearly and to keep accurate records of samples.

Freezing Protocols

In Sargent and Mohun's (2005) studies with *X. laevis* and *X. tropicalis* mentioned above in which egg yolk/sucrose was the cryodiluent, they reported successful cryopreservation of sperm using a cooling ramp of 10°C per minute. The

efficacy of cooling was the same whether they used a programmable cooler or ethanol dry ice slurry for freezing. They froze the samples in 500-μl Eppedorf tubes and kept the bases in the ethanol dry ice slurry. Earlier, in studies with other species, Browne et al. (1998) had shown that more complicated cooling ramps produced in programmable coolers were necessary.

Thawing Protocols

Thawing rates do not affect the recovery of cryopreserved amphibian sperm in the limited number of species studied (Browne et al. 1998; Sargent and Mohun 2005). Reports have described simply placing cryo-straws on a bench to thaw or, if faster rates are required, placing them under running water at <30°C. If the sperm suspensions are kept more than a few minutes before use, it is necessary to maintain them between 0° and 4°C.

Artificial Fertilization

In this section, we provide essential protocols for the in vitro manipulation and fertilization of anuran eggs. Three commonly used physiological salines are simplified amphibian's Ringer (SAR; Table 1), DeBoer's solution (DB; Table 4), and modified amphibian's Ringer (MAR^2; Table 6), and egg gel-extract, which may be necessary for artificial fertilization.

Egg Gel-Extract Solution

Egg gel-extract is an additive to assist the fertilization of degelled eggs. Reports of numerous studies have either confirmed or disputed the existence of components in the egg gel of amphibians that "capacitate" sperm, thus enabling fertilization. Studies of the normally occurring three layers of gel have identified specific compounds that affect sperm motility and aid fertilization; however, studies of fertilization have not always confirmed the effect of these com-

Table 6 Modified amphibian Ringer's solutions[a]

Compound	Molecular weight	Gram/liter
100.0 mM NaCl	58.44	5.80
1.8 mM KCl	74.55	0.15
1.0 mM MgCl$_2$	203.30	0.20
2.0 mM CaCl$_2$	110.98	0.30
5.0 mM Na-Hepes	260.30	1.30

[a]Modified from Heasman J, Holwill S, Wylie CC. 1991. Fertilization of cultured *Xenopus* oocytes and use in studies of maternally inherited molecules. Methods Cell Biol 36:214-228.

pounds. To create the fertilization medium, investigators have extracted these compounds by "agitating" spawn in a physiological saline. Freshly laid spawn have been incubated in 0.3 × MAR with pH 7.8 at the ratio of 8 mL MAR to 3 g spawn for 45 minutes at 20°C in a Petri dish on a rocker plate at approximately 15 cycles/minute. Heasman et al. (1991) have reported that it is possible to recover approximately 60% of the originally added volume.

Multiple Founder Stock Males

Artificial fertilization is a useful technique when investigators require amphibians of a known genetic history and/or from synchronous fertilization. It is possible to fertilize induced oocytes with the sperm from many males. With crypreservation, even after many years, these males may be founder stock, and with short-term storage of sperm, the males can be from remote locations. It is possible to achieve maximum rates of fertilization through dry fertilization, by the direct application of sperm samples in urine, Ringer solutions, or cryodiluents, or from testicular macerates to oocytes. After 10 minutes, personnel gently flood the oocytes and sperm with an aqueous solution of 5% (v/v) SAR with distilled water.

Egg Development and Larval Rearing

The larvae of amphibians live a multitude of life styles in almost every freshwater aquatic habitat on earth. However, most laboratory model species to date have been pond breeders with simple life histories. In the text below, we emphasize the larval rearing of these and other pond-breeding amphibians. Other species show various degrees of parental care that complicate larval rearing protocols. Parental care, which is more common in terrestrial than aquatic amphibians, characterizes most salamanders, 10% of anurans, and most caecilians. The spectrum of care extends from randomly depositing single eggs in ponds, to guarding foam-protected egg masses, to carrying the eggs on their backs or in pouches, to transporting hatched larvae (Alford 1999; Duellman and Trueb 1994).

Larval Husbandry

The environmental conditions under which amphibian larvae are hatched and reared can profoundly affect diverse aspects of larval and even adult biology. Larval density, temperature, light, pH level, dissolved oxygen, and diet have major effects on development, internal and external larval morphology, intestinal commensals, and the eventual adult anatomy (McDiarmid and Altig 1999). Practicality will limit the approximation of natural conditions that can be made in captivity. Although studies have shown that the larvae of many species are highly adaptable to artificial

conditions, as described above, the larvae of some less well-known amphibians are still best reared in conditions simulating those found in nature. The Amphibian Taxon Advisory Group (AZA 2007) recommends that individuals or organizations that make advances with amphibian husbandry present the information as a Taxon Management Account for evaluation and distribution.

The optimum larval rearing system for any batch will depend on the species, numbers of larvae to be reared, and group size. One objective that is common to all larval rearing protocols is to maintain the optimum growth rate to minimize the larval period. These combined results will produce the largest and fittest metamorphs with the least size difference (Alford 1999; Browne et al. 2003; Schmidt and Henkel 2004). At each stage of development, from eggs to metamorphosis, it is important to provide conditions that maximize growth and condition. There is more diversity in the life history of larval anurans than in caecilian and caudate larvae. Even in species from the same habitats such as pools, it may be necessary to use different larval rearing techniques to achieve optimum survival and growth.

Handling

Amphibian larvae are very sensitive, and even latex gloves that are suitable for most biomedical applications may result in amphibian mortalities (Sobotka and Rahwan 1994). It is acceptable to handle small, recently hatched larvae with plastic pipettes opened to maximum tube diameter. This method is also convenient for counting larvae as personnel transfer them from one container to another. When pouring larvae through nets, to prevent physical and physiological damage to larvae, we advise always suspending the net in water that has a composition and temperature similar to the water of origin.

Hatching Eggs

The quality of both the eggs and the egg gel will vary with broodstock condition and spawning methods. It is possible to hatch good-quality egg masses or strings in aquariums with appropriate aeration that is held at a temperature similar to the species' natural environment. Personnel may distribute the larvae at swim-up either singularly or between medium- or high-density trays.

It is important to note, however, that the artificial induction of oocytes may compromise the hatch rate and gel quality. Due to the instability of the egg matrix, these eggs are generally hatched in containers such as Petri dishes (Figure 7). The nutrients released from decomposing eggs or gel can result in lethal bacterial blooms, which necessitates separating developing eggs from bad eggs and decomposing gel. This separation is best done by pipetting the eggs or egg clumps into a fresh tray with a plastic pipette with a tube diameter large enough to avoid damage to the eggs. Bacte-

Figure 7 Petri dishes with hatching eggs produced by hormonal induction and then artificially fertilized (R.K.B., unpublished).

rial blooms can develop rapidly at warm temperatures, especially when sperm in cryoprotectants such as egg yolk are used to fertilize oocytes. Bacterial blooms appear as a white/yellow turbidity that spreads from decomposing eggs. To avoid this development, it is essential to inspect the eggs twice daily, especially during the first few days after fertilization.

Rearing Densities

Early studies showed that as rearing densities increased the larval period, the size range of metamorphs and the length of the metamorphosis period also increased (Hoff et al. 1999). Nevertheless, it is possible to raise larvae of many species without these effects at very high densities. Certain amphibian larvae including those that are highly cannibalistic or slow growing are not amenable to high-density rearing. Some species appear to prefer individual rearing (*Dendrobates* spp.; ceratophryinines) whereas other species are not amenable to high-density rearing for no apparent reason (R.K.B., personal observation).

Traditionally, investigators have raised most larvae in aquariums at medium densities of fewer than five larvae per liter. Many toad larvae are small, and it is possible to satisfactorily raise up to 20 per liter in medium-density systems (Alford 1999; Figure 8). Such medium-density rearing is ideal when small numbers of metamorphs are required, or for species unsuitable for high-density rearing. Medium-density rearing in aquariums requires daily inspection and regular water changes. The period between water changes depends among other factors on feed rates, type of feed, and water temperature (Culley 1992). The disadvantages of medium-density rearing are extended larval periods, the necessity of a large area for a similar number of larvae, and potentially smaller and thinner metamorphs. In contrast, high-density rearing generally requires reliable 12-hour water changes to maintain water quality and avoid mass mortalities (Browne et al. 2003).

Figure 8 Fowler's toad (*Bufo fowleri*) larvae raised at medium density and fed fish flakes, spirulina, and water weed (R.K.B., unpublished).

Figure 9 An experimental array to test the high-density rearing of Fowler's toad (*Bufo fowleri*) larvae consisting of pairs of trays vertically descending in the figure at 100, 150, and 200 larvae per 600 mL (R.K.B., unpublished).

Single Rearing

It is possible to rear amphibian larvae in small containers or separated on trays by partitions. Under these conditions, personnel can feed the larvae to satiation without the effects of either interference or exploitative competition and can achieve high growth. Maximum growth is possible with high concentrations of feed, 12-hour water changes, and shallow water to assist natural aeration. However, there are less demanding methods for the single rearing of the larvae of *Dendrobates* spp. (Schmidt and Henkel 2004).

Medium-density Rearing

The equipment for medium-density rearing can be as simple as an aquarium with an air stone. If large numbers of larvae are required, it is advantageous to equip tanks with flow-though water systems to house several thousand large larvae. For the rearing of amphibian larvae, it is important to maintain a very high quality of water (Ultsch et al. 1999).

High-density Rearing

The high-density rearing method has produced high-quality metamorphs for a range of anurans including *Litoria*, ranids, myobatrachids, and bufonids. High-density rearing depends on the use of shallow water to maintain oxygen, densities >40 larvae per liter, frequent water changes to remove waste and bacteria, and the provision of finely ground high-quality feed to ensure satiation. We advise holding the larvae in trays that have a water depth approximately three times the height of the larvae: 1.5 to 3 cm deep. A 30 × 45 cm tray and a 1.5-cm water depth would house 60 to 120 medium-sized larvae (Figure 9). Personnel should feed the larvae a fine, particulate, high-quality feed such as ground fish pellets with spirulina powder. Every 12 hours, it is essential to remove the larvae from the tray, wash the tray, change the

tray water, and then replace and feed the larvae (Browne et al. 2003).

Metamorphosis

Metamorphosis is the change from a larval to a juvenile stage (Nieuwkoop and Faber 1994). This change is most profound with terrestrial amphibians, especially anurans. The structure of the mouth changes dramatically, the metamorphs do not eat, lungs replace gills for respiration, the tail becomes absorbed in anurans, and locomotion is poor (Ultsch et al. 1999). The modification of organs as larvae metamorphose is not the same for all species. However, once the front legs of an anuran larva have emerged, swimming becomes difficult, feeding ceases, air breathing commences, and soon after metamorphosis is completed (McDiarmid and Altig 1999). Therefore, in anurans at least, front leg emergence is the most practical partition between the end of the larval period and the beginning of metamorphosis (Browne et al. 2003).

Once the front legs emerge, it is important to stop feeding anuran larvae and to place them in a sloped enclosure, which creates a transitional water (2/3) and terrestrial (1/3) environment (Figure 10). The terrestrial portion should be a substrate on which metamorphs can emerge from their aquatic environment. The two types of substrates that work well are sand and small gravel. Gravel does not stick and provides a sense of security for the emerging juveniles. It is necessary to wash the substrates regularly and to change the water at least every 2 days. Gravel is easy to wash by spraying self-draining tanks whereas sand enclosures re-

Figure 10 A metamorphosis enclosure with a shallow sloping bed of fine gravel for Wyoming toad (*Bufo baxteri*) larvae (R.K.B., unpublished).

quire removing the metamorphs and larvae to clean the enclosures.

Postmetamorphosis

The postmetamorphic juveniles of most terrestrial amphibians require small insects or other invertebrates for sustenance. The amount of feed the juveniles ingest will depend on the density of prey. Consequently, it is necessary to place the metamorphs that are nearing the tail bud stage in small containers with low headroom and an insect-proof lid if the metamorphs are feeding on flying insects. Unfortunately, juveniles of some anurans drown easily, therefore it is better to supply water through wet paper toweling and to provide a few dry leaves for shelter and for insects to climb. To provide a continuous supply of *Drosophila* fruit flies, it is advantageous to place a colony jar that has the mouth covered with a fine mesh. The mesh enables the escape of *Drosophila* and prevents the early juveniles from entering the jar. It is appropriate to feed large juveniles (>20 mm) on *Drosophila* and juvenile crickets. Smaller feeder insects such as collembollans are also suitable for small metamorphs. It is acceptable to house the metamorphs of aquatic species in the same tanks as those described above for the medium-density rearing of larvae (Figure 11. The larvae and juveniles of some species are cannibalistic, therefore it is essential to raise and metamorphose these animals individually in either flow-through grids or individual enclosures.

Figure 11 Recently metamorphosed early juvenile Wyoming toads (*Bufo baxteri*) (R.K.B., unpublished).

Caecilians

Approximately 30% of caecilians are viviparous, and metamorphosis to gill loss occurs at or near birth. The neonates are miniature versions of the adult and have the same environmental and dietary needs. O'Reilly (1996) has suggested keeping gravid females of aquatic species at lowered water levels of 3 cm because neonates that retain gills after birth can drown in deeper water. Some investigators have proposed that parental care might be important in some taxa. Wake (1994) observed in a *Typhlonectine* sp. that "female caecilians lift young on their backs to the surface of the water to take their first breaths," and "neonates rub their snouts along the flanks of their mother similar to discus fry feeding on skin secretions" (personal communication, Andy Snider, Fresno Zoo, Fresno, CA, 2000). Cohorts of neonates have failed to thrive when separated from their parents (K.Z., personal observation). Therefore, when possible, we advise leaving the neonates with the parents. Beyond these observations, the distribution of parental care of neonates across the viviparous caecilians is unknown.

Some oviparous species are direct developing, with metamorphosis occurring in the egg before hatching, whereas others are primarily aquatic and secondarily terres-

trial. All of the egg clutches that have been observed in the wild have been located on moist soil near water. Therefore, housing for breeding adults should consist of moist soil near water. Investigators have described hatching wild-collected eggs of *Ichthyophis glutinosus* (Breckenridge and Jayasinghe 1979; Breckenridge et al. 1987) and *Grandisonia* sp. (O'Reilly 1996) in the laboratory and successfully rearing the larvae on worms or worm pieces. Hofrichter (2000) and O'Reilly (1996) advise giving the larvae a mixed aquatic and terrestrial habitat. O'Reilly (1996) has also reported conditioning wild-caught larval *Epicrionops* by wiggling the food at the larvae's snouts. The larvae would initially take only amphipods but took chopped worms after the conditioning. Hofrichter (2000) has documented that larval development took 10 to 14 months for two *Ichthyophis* species. Maternal attendance of egg clutches exists in oviparous caecilians both with and without direct development; however, the significance of this behavior is unknown. Hofrichter (2000) has suggested that parental attendance of eggs is possibly for the protection of developing eggs through the removal of infertile eggs, defending eggs from predators, and providing antiseptic compounds.

Caudates

Sixty-five percent of caudates belong to the subfamily Plethodontinae, and most of these amphibians directly develop. Several desmognathines also have direct development, and several salamandrids are viviparous. Many species are larviform, spending their entire lives in a larva-like condition. Thus, with caudates, larval rearing and early juvenile rearing can be concurrent. As with caecilians, parental attendance of eggs is common in caudates possibly for the same reasons. A unique behavior among amphibians is the prodding or fanning of aquatic eggs by pairs of caudates. In light of the parental behavior of caudates, it is important to allow these animals to attend the egg mass but also to observe them carefully because adult caudates sometimes eat viable eggs.

Caudate larvae do not necessarily begin feeding immediately after hatching; they subsist for some time on the yolk stores, which are often visible through their thoracoabdominal wall as a yellow or white mass. Depending on their enclosure temperature and metabolism, salamanders might not require food for weeks, especially species reared at cooler temperatures. To avoid problems with early feeding and consequent water quality issues, we advise feeding small amounts initially, and at least until the larvae have absorbed their yolks. It is then advisable to gauge subsequent feeding by how much food the larvae ingest.

Caudate larvae will feed on a variety of meat-based food items. For exceptionally small hatchlings (the smallest plethodontid larvae), microworms are an appropriate initial food. Potential choices for slightly larger larvae include Grindal worms, daphnia, or brine shrimp nauplii. The next larger size class of food includes blackworms, whiteworms, bloodworms, tubifex worms, isopods, and mosquito larvae.

The largest larvae take bigger items like chopped earthworms or fish. Diversity is key; axolotl larvae raised exclusively on brine shrimp or white worms can suffer from a number of nutritional deficiencies (Nace 1974).

All known caudate larvae are carnivorous, and many have cannibalistic tendencies. If they are raised in large groups, size differences develop among the growing larvae. Often larger specimens will then consume the smaller, thus biasing the population toward those that exhibit early rapid growth. However, it is possible to keep many caudates including aquifer plethodontids in groups with no cannibalism or growth dispensation (K.Z., personal observation). With growth dispensation, interference and exploitative competition results in a broadening of the range of size in a batch of larvae and possibly increased mortality of "stunted" specimens. Investigators can avoid growth dispensation through high-density rearing (Browne et al. 2003); however, there are no reports to date of the testing of this technique on the larvae of amphibians other than anurans.

For research of individual animals, to increase survival or to reduce genetic bias, the preferred rearing protocol is individual maintenance. In such a case, it is appropriate to raise larvae in containers that have holes covered with mesh in the sides. These containers could vary from small plastic cups for small larvae to larger food containers as larvae grow. It is customary to keep these containers in a tray with approximately 5 cm of water. Personnel should circulate the water in the tray to increase the rate of water exchange in the individual containers. As the larvae grow, personnel should move them into successively larger containers. For those species that metamorphose, once the gills are nearly resorbed, it is then appropriate to move the larvae into a tank with shallow water and a gradual slope onto land For caudates that remain aquatic, sit is customary to house similar-sized larvae communally after they are at least half grown. Although there may be cases in which limbs are bitten off, lost limbs do often regenerate.

Although all caecilian and caudate larvae are carnivorous, most anuran larvae are mostly filter feeders or browsers, and a few are carnivorous and sometimes cannibalistic. A good initial feed for most larvae is a nutritious commercial fish food flake with a high algae/plant content. It is important to avoid spinach, cabbage, and kale due to their toxins (Berns 1965; Borland 1943). For taxa with more carnivorous tendencies, we recommend providing a progression of food items as suggested for caudates. For filter feeders, it is possible to make suspensions from ground fish feed or Sera Micron sprinkled on the water surface. Note that as with caudate larvae, anuran larvae do not necessarily begin feeding immediately after hatching, and early feeding might lead to loss of water quality.

Concluding Thoughts

Research on the reproduction of amphibians has mainly included investigative models and amphibians for display or

consumption. Although amphibian reproduction technologies have traditionally favored easily bred species, the current crisis of these animals' imminent extinction now demands the development of new reproduction technologies for amphibians, which will provide a vast range of research strategies. These strategies vary from natural reproduction to the in vitro indefinite preservation of a species' natural genetic diversity. Although the application of best practice in husbandry, including the provision of species-specific microhabitats, should enable the natural reproduction of many species, past experience with difficulties in reproduction indicates that some challenging cases require advanced in vitro reproduction technologies. Over time the provision of genetically competent amphibians from captive breeding facilities will greatly benefit from methods that utilize cryopreserved sperm or nuclei. To date investigators have used sperm induction and ovulation, short-term storage of eggs and sperm, and indefinite storage of sperm through cryopreservation with <1% of amphibian species. Nevertheless, the field has an exciting future in continuing the recent advances we have described in this article. Increased research and knowledge will assuredly promote the conservation of amphibians and benefit these animals and humans alike.

Acknowledgments

References

Alford RA. 1999. Ecology: Resource use, competition, and predation. In: McDiarmid RW, Altig R, eds. Tadpoles: The Biology of Anuran Larvae. Chicago: University of Chicago Press. p 240-278.

Aplin K, Kirkpatrick P. 2000. Chytrid in Southwest Australia: Historic Sampling Documents the Date of Introduction, Rates of Spread and Seasonal Epidemiology, and Sheds New Light on Chytrid Ecology. Proceedings of Getting the Jump on Amphibian Disease, held in Cairns, Australia. p 24.

Arimura A, Vilchez-Martinez JA, Coy DH, Coy EJ, Hirotsu Y, Schally AV. 1974. [D-Ala6, Des-Gly-NH210]-LH-RH-ethylamide: A new analogue with unusually high LH-RH/FSH-RH activity. Endocrinology 95:1174-1177.

AZA [Association of Zoos and Aquariums]. 2007. Taxon Advisory Groups. Available online (http://www.aza.org/ConScience/ConScienceTAGFact/).

Bagnara JT, Stackhouse HL. 1973. Observations on Mexican *Rana pipiens*. Am Zool 13:139-143.

Ballou JD. 1992. Potential contribution of cryopreserved germ plasm to the preservation of genetic diversity and conservation of endangered species in captivity. Cryobiology 29:19-25.

Berger L, Speare R, Hines HB, Marantelli G, Hyatt AD, McDonald KR, Skerratt LF, Olsen V, Clarke JM, Gillespie G, Mahony M, Sheppard N, Williams C, Tyler MJ. 2004. Effect of season and temperature on mortality in amphibians due to chytridiomycosis. Aust Vet J 82:434-439.

Berns MW. 1965. Mortality caused by kidney stones in spinach-fed frogs (*Rana pipiens*). BioScience 15:297-298.

Borland JR. 1943. The production of experimental goiter in *Rana pipiens* tadpoles by cabbage feeding and methyl cyanide. J Exp Zool 94:115-37.

Bosch J. 2001. Female reciprocal calling in the Iberian midwife toad (*Alytes cisternasii*) varies with male call rate and dominant frequency: Implications for sexual selection. Naturwissenschaften 88:434-437.

Bosch J, Marquez R. 1996. Discriminate functions for sex identification in two midwife toads (*Alyes obstetricans* and *A. cisternasii*). Herpetological J 6:105-109.

Breckenridge WR, Jayasinghe S. 1979. Observations on the eggs and larvae of *Ichthyophis glutinosus.* Ceylon J Sci 13:187-202.

Breckenridge WR, Nathanael S, Pereira L. 1987. Some aspects of the biology and development of *Ichthyophis glutinosus* (Amphibia: Gymnophiona). J Zool Lond 211:437-49.

Brenner FJ. 1966. Influence of light and temperature on reproduction and hibernation in amphibians and reptiles. Yrbk Am Phil Soc p 319-322.

Brenner FJ, Brenner PE. 1969. The influence of light and temperatures on body fat and reproductive conditions of *Rana pipiens.* Ohio J Sci 69: 305-312.

Browne RK, Clulow J, Mahony M. 2001. Short-term storage of cane toad (*Bufo marinus*) gametes. Reproduction 121:167-173.

Browne RK, Clulow J, Mahony M. 2002a. The effect of saccharides on the post-thaw recovery of cane toad (*Bufo marinus*) spermatozoa. Cryo Letters. 23:121-128.

Browne RK, Clulow J, Mahony M. 2002b. The short-term storage and cryopreservation of spermatozoa from hylid and myobatrachid frogs. Cryo Lett 23:29-136.

Browne RK, Clulow J, Mahony M, Clark A. 1998. Successful recovery of motility and fertility of cryopreserved cane toad (*Bufo marinus*) sperm. Cryobiology 37:339-345.

Browne RK, Davis J, Pomering M, Clulow J. 2002c. Storage of cane toad (*Bufo marinus*) sperm for 6 days at 0ºC with subsequent cryopreservation. Reprod Fertil Dev 14:267-273.

Browne RK, Mahony M, Clulow J. 2002d. A comparison of sucrose, saline, and saline with egg-yolk diluents on the cryopreservation of cane toad (*Bufo marinus*) sperm. Cryobiology 44:251-257.

Browne RK, Pomering M, Hamer AJ. 2003. High density effects on the growth, development and survival of *Litoria aurea* tadpoles. Aquaculture 215:109-121.

Browne RK, Seratt J, Li H, Kouba A. 2006a. Progesterone improves the number and quality of hormonally induced Fowlers toad (*Bufo fowleri*) oocytes. Reprod Biol Endocrinol 4:3.

Browne RK, Seratt J, Vance C, Kouba A. 2006b. Hormonal induction with priming and in vitro fertilisation increases egg numbers and quality in the Wyoming toad (*Bufo baxteri*). Reprod Biol Endocrinol 4:34.

Buchholz D, Li F, Shi Y. 2004. Cryopreservation of *Xenopus* transgenic lines. Mol Reprod Dev 67:65-69.

Chakraborty C, Nandi S, Jana S. 2005. Prion disease: A deadly disease for protein misfolding. Curr Pharm Biotechnol 6:167-177.

Clark, AK. 1997. The effects of ionicity in diluents on the motility of cane toad (*Bufo marinus*) sperm. B. Sc. (Hons) thesis. University of Newcastle, Australia.

Clulow J, Mahony M, Browne RK, Pomering M, Clark A. 1999. Application of assisted reproduction technologies (ART) to endangered anuran amphibians. In: Cambell A, ed. Declines and Disappearances of Australian Frogs. Canberra, Australian Capital Territory: Environ Australia. p 219-225.

Constantinos CM, Zohar Y. 2000. Use of GnRHa-delivery systems for the control of reproduction in fish. Rev Fish Biol Fisheries 10:463-491.

Costanzo JP, Mugnano JA, Wehrheim HM. 1998. Osmotic and freezing tolerance in spermatozoa of freeze-tolerant and -intolerant frogs. Am J Physiol 44:713-719.

Culley DD. 1992. Managing a bullfrog research colony. In: Schaeffer DO, Kleinow KM, Krulisch L, eds. The Care and Use of Amphibians, Reptiles and Fish in Research. Bethesda: Scientists Center for Animal Welfare. p 30-40.

Dan C. 1983. Reproduction twice a year of the crested newt in captivity. In: Townson S, ed. Breeding Reptiles and Amphibians. London: British Herpetological Society. p 209-211.

Duellman WE, Trueb L. 1994. Biology of Amphibians. Baltimore: The Johns Hopkins University Press.

Edwards DL, Mahony MJ, Clulow J. 2004. Effect of sperm concentration, medium osmolality and oocyte storage on artificial fertilisation success in myobatrachid frog (*Limnodynastes tasmaniensis*). Reprod Fertil Dev 16:346-354.

Fortune JE. 1983. Steroid production by *Xenopus* ovarian follicles at different developmental stages. Dev Biol 99:502-509.

Fraile B, Paniagua R, Rodrigues MC, Saez J. 1989. Effects of photoperiod and temperature on spermiogenesis in marbled newts (*Triturus marmoratus marmoratus*). Copeia 1989:357-363.

Frazer JFD. 1976. Anura (frogs and toads). In: UFAW Handbook. 5th ed. Edinburgh: Churchill Livingston. p 516-524.

Halliday TR. 1999. Amphibians. In: The UFAW Handbook on The Care and Management of Laboratory Animals. Vol 2, 7th ed. p 90-102.

Heasman J, Holwill S, Wylie CC. 1991. Fertilisation of cultured *Xenopus* oocytes and use in studies of maternally inherited molecules. Methods Cell Biol 36:214-228.

Heatwole H. 2005. Amphibian biology. In: Endocrinology. Vol 6. Chipping Norton: Surrey Beatty & Sons.

Hilken G, Dimigen J, Iglauer F. 1995. Growth of *Xenopus laevis* under different laboratory rearing conditions. Lab Anim 29:152-62.

Hoff KS, Blaunstein AR, McDiarmid RW, Altig R. 1999. Behaviour: Interactions and their consequences. In: McDiarmid RW, Altig R, eds. Tadpoles: The Biology of Anuran Larvae. Chicago: University of Chicago Press. p 215-239.

Hofrichter R, ed. 2000. Amphibians: The World of Frogs, Toads, Salamanders and Newts. Buffalo: Firefly Books.

Hollinger TG, Corton GL. 1980. Artificial fertilisation of gametes from the South African clawed frog (*Xenopus laevis*). Gamete Res 3:45-57.

Holt WV, Abaigar T, Watson PF, Wildt DE. 2003. Genetic resource banks for species conservation. In: Holt WV, Pickard AR, Rodger JC, Wildt DE, eds. Reproductive Science and Integrated Conservation. Cambridge: Cambridge University Press. p 267-280.

Holt WV, Pickard AR, Pranther RS. 2004. Wildlife conservation and reproductive cloning. Reproduction 127:317-324.

Horseman ND, Smith CA, Culley DD Jr. 1978. Effects of age and photoperiod on ovary size and condition in bullfrogs (*Rana catesbeiana* Shaw) (Amphibia, Anura, Ranidae). J Herpetol 12:287-290.

Horton P. 1982. Precocious reproduction in the Australian frog *Limnodynastes tasmaniensis*. Herpetologica 38:486-489.

Jørgensen CB. 1975. Factors controlling the annual ovarian cycle in the toad *Bufo bufo* (L.). Gen Comp Endocrinol J 25:264-273.

Jørgensen CB. 1982. Factors controlling the ovarian cycle in a temperate zone anuran, the toad *Bufo bufo*: Food uptake, nutritional state, and gonadotrophin. J Exp Zool 224:437-443.

Kim JW, Im WB, Choi HH, Ishii S, Kwon HB. 1998. Seasonal fluctuations in pituitary gland and plasma levels of gonadotropic hormones in *Rana*. Gen Comp Endocrinol 109:13-23.

Kumarasiri WSAAL, Seneviratne P. 1988. Induced multiple spawning of Chinese carps in Sri Lanka. Aquaculture 74:57-72.

LaMarca MJ, Westphal LM, Rein DA. 1985. Gonadotrophins and the timing of progesterone-induced meiotic maturation of *Xenopus laevis* eggs. Dev Biol 109:32-40.

Lawson DP. 1993. The reptiles and amphibians of Korup National Park Project, Cameroon. Herpetol Nat Hist 1:27-90.

Lee MSY, Jamieson BMG. 1993. The ultrastructure of the spermatozoa of bufonid and hylid frogs (Anure, Amphibia): Implications for phylogeny and fertilisation biology. Zoologica Scripta 22:309-323.

Maniero GD, Carey C. 1997. Changes in selected aspects of immune function in the leopard frog, *Rana pipiens*, associated with exposure to cold. J Comp Physiol-B, Biochem, System, Environ Physiol 167:256-263.

McDiarmid RW, Altig R, eds. 1999. Tadpoles: The Biology of Anuran Larvae. Chicago: University of Chicago Press.

Michael SF, Buckley C, Toro E, Estrada AR, Vincent S. 2004. Induced ovulation and egg deposition in the direct developing anuran *Eleutherodactylus coqui*. Reprod Biol Endocrinol 2:6.

Mikolajczyk T, Chyb J, Sokolowska-Mikolajczyk M, Szczerbik P, Socha M, Epler P. 2004. Potentiation of the action of sgnrha on LH secretion in goldfish by aromataze inhibitor fadrozole. In: Proceedings of the Fifth International Symposium of Fish Endocrinology, Castellón, Spain.

Mishra A, Joy KP. 2004. Ovarian monosacharides (glucose and fructose): Hormonal effects and their role in final oocytes maturation and egg quality in catfish *Heteropneustes fossilis*, Block. Ind J Exp Biol 42: 1084-1090.

Mugnano JA, Costanzo JP, Beesley SG, Lee Jr RE. 1998. Evaluation of glycerol and dimethyl sulphoxide for the cryopreservation of spermatozoa from the wood frog (*Rana sylvatica*). Cryo-Lett 19:249-254.

Nace GW. 1974. Amphibians: Guidelines for the Breeding, Care, and Management of Laboratory Animals. Washington DC: National Academy Press.

Ng TB, Lee YH, Chan ST. 1986. Pituitary extract of the ricefield eel *Monopterus albus* (Synbranchidae, Teleostei) exhibits gonadotrophic activity in the classes Mammalia, Aves, Reptilia and Amphibia. Comp Biochem Physiol A 84:371-381.

Nieuwkoop PD, Faber J, eds. 1994. Normal Table of *Xenopus Laevis* (Daudin, 1956): A Systematical and Chronological Survey of the Development from the Fertilized Egg Until the End of Metamorphosis. New York: J. Garland Publishing Inc.

Obringer AR, Brien JK, Saunders RL, Yamamoto K, Kikuyama S, Roth TL. 2000. Characterization of the spermiation response, luteinizing hormone release and sperm quality in the American toad (*Bufo americanus*) and the endangered Wyoming toad (*Bufo baxteri*). Reprod Fertil Dev 12:51-58.

O'Reilly JC. 1996. Keeping caecilians in captivity. In: Strimple PD, ed. Advances in Herpetoculture. Des Moines: Crown Craft Printing/ International Herpetological Symposium, Inc. p 39-45.

Paine FL, Miller JD, Crawshaw G, Johnson B, Lacy R, Smith CF, Tolson PJ. 1989. Status of the Puerto Rican crested toad *Peltophryne lemur*. Int Zoo Yrbk 28:53-58.

Pancharatna K, Patil MM. 1997. Role of temperature and photoperiod in the onset of sexual maturity in female frogs, *Rana cyanophlyctis*. J Herpetol 31:111-114.

Peter RE, Yu KL. 1999. Induced spawning of the Indian catfish, *Heteropneustes fossilis*, by GnRH analogue alone or in combination with dopamine-affecting drugs. J Appl Aquacult 9:23-32.

Paleo GA, Godke RR, Tiersch TR. 2005. Intracytoplasmic sperm injection using cryopreserved, fixed, and freeze-dried sperm in eggs of Nile tilapia. Marine Biotech 7:104-111.

Pukazhenthi BS, Wildt DE. 2004. Which reproductive technologies are most relevant to studying, managing and conserving wildlife. Reprod Fertil Dev 16:33-46.

Purgue AP. 1997. Tympanic sound radiation in the bullfrog *Rana catesbeiana*. J Comp Physiol-A: Neuroethol Sensory Neural Behav Physiol 181:438-445.

Rabb GB. 1973. Evolutionary aspects of the reproductive behavior of frogs. In: Vial JL, ed. Evolutionary Biology of the Anurans: Contemporary Research on Major Problems. Columbia: University of Missouri Press. p 213-227.

Reed BT. 2005. Guidance on the housing and care of the African clawed frog—*Xenopus laevis*. London: Royal Society of the Prevention of Cruelty to Animals, Research Animals Department.

Reynhout JK, Taddei C, Smith LD, LaMarca MJ. 1975. Response of large eggs of *Xenopus laevis* to progesterone in vitro in relation to eggs size and time after previous hCG-induced ovulation. Dev Biol 44:375-379.

Reinhart D, Ridgway J, Chandler DE. 1998. *Xenopus laevis* fertilisation: Analysis of sperm motility in egg jelly using video light microscopy. Zygote 6:173-182.

Richter SC, Young JE, Johnson GN, Seigel RA. 2003. Stochastic variation in reproductive success of a rare frog, *Rana servosa*, implications for conservation and maintaining amphibian populations. Biol Cons 111: 171-177.

Roth TL, Obringer AR. 2003. Reproductive research and the worldwide amphibian extinction crisis. In: Holt WV, Pickard AR, Rodger JC, eds. Reproductive Science and Integrated Conservation. Cambridge: Cambridge University Press. p 359-375.

Rugh R. 1962. Culturing of amphibian embryos. In: Rugh R, ed. Experimental Embryology: Techniques & Procedures. Minneapolis: Burgess Publishing. p 50-51.

Sargent MG, Mohun TJ. 2005. Cryopreservation of sperm of *Xenopus laevis* and *Xenopus tropicalis*. Genesis 41:41-46.

Schmidt F, Henkel FW. 2004. Professional Breeders Series: Poison Frogs. Frankfurt: Chimaira.

Schultz TW, Douglas DA. 2003. Housing and husbandry of *Xenopus* for oocyte production. Lab Anim 32:34-39.

Sobotka JM, Rahwan RW. 1994. Lethal effect of latex gloves on *Xenopus laevis* tadpoles. J Pharmacol Toxicol Methods 31:59.

Sughrue M. 1969. Underwater acrobats. Brookfield Bandarlog 37:6–10.

Summers K, McKeon CS, Hetling H, Hall J, Patrick W. 2007. Social and environmental influences on egg size evolution in frogs. J Zool 271: 225-232.

Taylor SK, Williams ES, Thorne ET, Mills KW, Withers DI, Pier AC. 1999. Causes of mortality of the Wyoming toad. Wild Dis 35:49-57.

Thumm K, Mahony M. 2002. Hatching dynamics and bet-hedging in a temperate frog, *Pseudophryne australis* (Anura: Myobatrachidae). Amphibia-Reptilia 23:433-444.

Toro E, Michael SF. 2004. In vitro fertilisation and artificial activation of eggs of the direct-developing anuran *Eleutherodactylus coqui*. Reprod Biol Endocrinol 2:60.

Ultsch GR, Bradford DF, Freda J. 1999. Physiology: Coping with the environment. In: McDiarmid RW, Altig R, eds. Tadpoles: The Biology of Anuran Larvae. Chicago: University of Chicago Press. p 189-214.

Van der Horst G, Wilson W, Channing A. 1995. Amphibian sperm: Phylogeny and fertilisation environment. In: Jamieson BGM, Ausio J, Austio JJL, eds. Advances in Spermatozoal Phylogeny and Taxonomy. Paris: Muséum National d'Histoire Naturelle. p 166, 333-342.

Waggener WL, Carroll EJ Jr. 1998. A method for hormonal induction of sperm release in anurans (eight species) and in vitro fertilisation in *Lepidobatrachus* species. Dev Growth Diff 40:19-25.

Wake MH. 1994. Caecilians (Amphibia: Gymnophiona) in captivity. In: Murphy JB, Adler K, Collins JT, eds. Captive Management and Conservation of Amphibians and Reptiles. Ithaca: SSAR Publications. p 223-228.

Whitaker BR. 2001. Reproduction. In: Wright KM, Whitaker BR, eds. Amphibian Medicine and Captive Husbandry. Malabar FL: Krieger Publishing Company. p 285-307.

Wildt DE. 1997. Genome resource banking: Impact on biotic conservation and society. In: Karow AM, Crister J, eds. Reproductive Tissue Banking. New York: Academic Press. p 399–439.

Withers PC. 1995. Cocoon formation and structure in the aestivating Australian desert frogs, *Neobatrachus* and *Cyclorana*. Aust J Zool 43:429-441.

Wright KM, Whitaker BR, eds. 2001. Amphibian Medicine and Captive Husbandry. Malabar FL: Krieger Publishing Company. p 285-307.

Young BE, Stuart SN, Chanson JS, Cox NA, Boucher TM. 2004. Disappearing Jewels: The Status of New World Amphibians. Arlington VA: NatureServe.

Zippel K. 2005. AmphibiaWeb: Information on Amphibian Biology and Conservation [web application, 2006]. Berkeley: AmphibiaWeb. Available online (http://amphibiaweb.org/declines/zoo/index.html).

GLOSSARY

aestivation: cessation of activity and a slowing of metabolism in some animals during a hot or dry period

amplexus: the mating posture of a pair of frogs or toads, in which the male clasps the female from behind during egg release and fertilization

anovulatory: a low dose of hormones administered before an ovulating dose to mature oocytes

antagonist: a compound that prevents the physiological expression of another compound

aquatic salamander: obligate or facultative perennibranchs

bet hedging: a reproductive strategy wherein eggs are laid in a series of batches over time to avoid total loss of reproductive effort through habitat drying

breeding condition: adult amphibians with gonads capable of producing mature sperm or oocytes

broodstock: adult amphibians used for reproduction

clutch: a group of simultaneously laid eggs deposited in the same location

cryodiluent: a compound solution that protects sperm during cryopreservation

cryoprotectant: a constituent of cryodiluents, which protects sperm during freeze/thaw

entrainment: a state in which further stages of a process are predetermined

ephemeral: lasting a short time; temporary

follicle: the part of an ovary that surrounds an oocyte

hibernation: cessation of activity and a slowing of metabolism in some animals during cold periods

hormonal induction: the induction of spermatogenesis or ovulation through the administration of hormones

infusoria: minute organisms located in decomposing infusions of organic matter

lyophilization: a method of drying tissue without destroying its physical structure; material is frozen and then warmed in a vacuum so that the ice sublimes

metamorph: an amphibian larva

montane: a section of a mountainous region below the timberline, which has cool, moist temperatures and in which evergreen trees predominate

oocyte: an unfertilized egg

oogenesis: the completion of the final stage of the generation of mature oocytes

oviposition: the spawning of oocytes

ovulation plateau: the stage of gametogenesis after which ovulation occurs

photoperiod: the period of light generally expressed as hours of light and dark per 24 hours

rehabitation: the establishment of populations in previously occupied or created habitat in their natural geographic range