

Hormone preparation and use

Dr Robert K. Browne, AArk Research Officer, Royal Zoological Society of Antwerp, Belgium,
robert.browne@gmail.com

Dr Chester R. Figiel, Jr., Warm Springs Fish Technology Center, USA, chester.figiel@fws.gov

Introduction

The induction of ovulation of amphibians using hormones has been used since the early 20th century. Some amphibians have never reproduced in captivity without hormones and using hormonal induction many species can be reproduced at will.

Conservation breeding programs for the flagship species, the Wyoming toad (*Bufo baxteri*), Boreal toad (*Bufo boreas boreas*), Puerto Rican crested toad (*Peltophryne lemur*), and Chinese giant salamander (*Andrias davidianus*) are either completely or largely dependent on hormonal induction. Commercial production of African clawed toads (*Xenopus* spp.) and also numerous other species rely on hormonal induction.

One of the great advantages of hormonal induction is the production of oocytes without fertilisation by males. These can then be fertilised by the sperm of many males, used for Intracytoplasmic sperm injection (ICSI), nuclear transfer or other biological studies (see **Reproduction technology** > *In vitro* fertilisation)

Several classes of compounds are used to induce amphibians - hormones as purified compounds, compounds such as pimozide, and pituitary extracts.

The primary compound for the artificial ovulation of amphibians is Luteinising Hormone Releasing Hormone analogue (LHRHa). There are also two steroid hormones of use for amphibian induction, human Chorionic Gonadotrophin (hCG) and Progesterone. Other compounds of value to ovulation are dopamine blockers such as Pimozide and compounds such as oxytocins and vasotacin (see **Reproduction technology** > **Amphibian reproduction hormone cycle**).

Certain types of stress can be detrimental to the maturation of ovaries and must be considered during (see **Reproduction and larval rearing** > **Reproduction conditioning**). However, stress is essential to function and contributes to ovulation and spawning under natural conditions.

Pituitaries are very successful in ovulating some amphibians where purified hormones do not succeed. The disadvantage of pituitaries is that they may not be commercially available in all countries, and that they have the potential to transmit disease. Therefore, pituitaries should not be used in amphibians that are for release.

Hormones can ovulate amphibians that are not in good reproductive condition. Nevertheless, Reproduction conditioning through good husbandry, nutrition and other forms of environmental entrainment is highly to achieve good results with hormonal induction.

Hormones can be injected as aqueous solutions, preferably physiological salines, or applied topically in dimethyl sulphoxide (DMSO) or other carriers. Some compounds and particularly progesterone require solvents such as (DMSO) or alcohol to dissolve before adding to aqueous solutions.

The three methods of administering hormones are by injection, through implants, or through topical ointments. Fine needles such as 27-30 gauge and 0.5-1.0cc syringes are optimal to inject the most common volume of 100µl (0.5ml insulin syringes are particularly useful). Hormones can be dissolved in water but we recommend using sterile SAR or Phosphate buffered saline (see **Methods > Formulations**).

This document describes techniques and protocols for the use of hormones for Amphibian reproduction. It then discusses the use of pituitaries, and provides some examples of species or species groups.

Recommended reading for the use of hormones in the ovulation of amphibians:

Browne RK, Seratt J, Vance C, Kouba A. 2006a. Hormonal priming, induction of ovulation and in-vitro fertilization of the endangered Wyoming toad (*Bufo baxteri*). *Reproductive Biology and Endocrinology*. 2006; 4: 34. <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1524778>

Browne RK, Li H, Seratt J, Kouba A. 2006b. Progesterone improves the number and quality of hormone induced Fowler toad (*Bufo fowleri*) oocytes. *Reproductive Biology Endocrinology*. 2006; 4: 3. <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1373633>

Goncharov BF, Shubray OI, Serbinova IA, Uteshev VK. 1989. The USSR programme for breeding amphibians, including rare and endangered species. *International Zoo Yearbook*. 28:10-21.

Which hormone to use and when

Both theoretical and experimental evidence by 1989 showed that LHRHa was a more universal hormone for induction of oocytes from females than hCG. However, some large conservation breeding programs including have until recently used hCG (Browne *et al* 2006 a,b). The tradition of using hCG with toads evolved from the use of toads for human pregnancy testing.

LHRHa is capable of stimulating the entire complex of reproductive processes and producing healthy offspring. Evidence shows that LHRHa is a universal stimulant to female reproduction, providing it is administered at a time when the individual has undergone gamete maturation and is ready to respond to stimulus. However, there has been considerable variance in sensitivity between species, some being acutely sensitive and showing a response after a single injection of LHRHa (2mg/kg; 2µg/g) and others responding only after repeated (up to five times) application of large doses (up to 8mg/kg; 8µg/g). However, for the induction of sperm from male toads hCG is often preferred to LHRHa.

Pituitary gland extracts have been widely used in the induction of frogs in the USA. The use of pituitary gland of the same or a closely related species have several shortcomings; 1) to obtain the pituitary the donors must be sacrificed, 2) it could be difficult, or impossible, especially with rare species to obtain pituitary of the same or a related species, 3) even an effective preparation an incorrect level may produce negative results. The use of standard mammalian gonadotropins such as hCG have proved effective only in a limited number of species, and can also result in an immune response that involves the use of increasingly higher doses for repeated spawning or spermiation.

Sensitivity to hormones is a function of several factors including species specificity, reproductive condition (physiological state of gonads and neuroendocrine system). For effective induction, doses, varying response between individuals, and the number and intervals between injections may vary. The doses may need to be varied during the course of the experiment according to the behaviour of the mating pair. Means to assess the reproductive condition of females are discussed in (see **Reproduction and larval rearing > Reproduction conditioning**).

Luteinizing Hormone Releasing Hormone analogue (LHRHa) Sigma L4513

Of the different types of LHRHa available Sigma L4513 (D-Ala6, des-Gly10, ethylamide LHRHa) is the only one that has been found effective with amphibians. LHRHa vials from Sigma contain 1mg of powder that should be dissolved in 2ml of phosphate buffered saline or SAR; resulting in a concentration of 0.5mg/ml (500 µg/ml). This stock solution can then be stored indefinitely in Eppendorf tubes at -80°C in 100 µl aliquots, which will give one dose of 50µg for example at 2 µg/g for a 25g male toad.

A milligram (mg) is one thousandth of a gram (1×10^{-3} g), a microgram (µg) is one millionth of a gram (1×10^{-6} g), and a picogram (pg) is one billionth of a gram (1×10^{-9} g).

Human Chorionic Gonadotropin (hCG) Sigma C1063, Intervet Inc.Choluron®

Human Chorionic Gonadotropin (hCG) comes as a powder and must be dissolved into an aqueous solution before administration by injection. The most convenient units are vials at 2500 or 5000 International Units (IU). Store hCG in the fridge at 4°C for short periods or frozen for months at -80°C.

Table 2. Standard vial amount from *Sigma hCG; #Intervet Inc.Choluron®. IU = international units. The volumes are products of 100µl plus 20, 40, or 80µl for vial loss.

Vial (IU)	Water (µL)	No. 250 IU doses	IU/100µL
*2500	1020	10	250
*5000	2040	20	250
#10000	4080	40	250

Priming

Priming is useful in preparing females for the final induction of oocytes especially where the state of 'Reproductive conditioning' is uncertain. Typical priming doses are 10% of the doses used to finally induce ovulation. For extended priming administer LHRHa ten days and three days before the final ovulatory dose. Priming administered about 24hrs before administration to bring oocytes to stage 6 may also be advantageous even with well conditioned females. However, females in optimal reproductive condition may be very sensitive to hormones. In these cases priming should not be used as even very low doses of hormones may produce ovulation.

When the fertilisation of oocytes is critical, males may be induced about 12hrs after the administration of priming doses and placed with females in spawning tanks.

Typical induction protocol for females

From an overview of the traditional and recent studies the optimal dosage of hormones for ovulation is 2-8 µg/g. So for a 50 g toad is 60µg LHRHa, or 20µg LHRHa with 500IU hCG could be used (IU, International Units). Progesterone used with LHRHa or hCG offers considerable potential for improving traditional methods (see Progesterone this doc). Females typically ovulate about 12-24hrs after an ovulatory dose of hormones. However, this varies with factors including species, female age, reproductive condition, and temperature.

Table 1. The different doses of hormones and the amount per gram of amphibian weight in a study of the Fowler toad (*Bufo fowleri*; Browne et al 2006b).

Compound	Administered dose	Amount per gram of toad weight
LHRHa	4 µg	0.12 µg
LHRHa	20 µg	0.61 µg
LHRHa	60 µg	1.8 µg
hCG	500 IU hCG	10 IU

hCG alone : A recommended ovulatory dose for females is 10IU hCG per gram. It simplifies the process to standardize the volume for storage to 100µl (Table 1). For medium sizes toads this approximates to 500IU for a 50g female (200 µl). Males are smaller and only need 300 IU (Browne et al. 2006a,b).

For smaller amphibians adjust the dose proportionately to body weight within a volume of 50-100µl. For instance for small 5g female dilute the 500IU/100ml dose into 900ml and administer 100µl.

Injections should be intraperitoneal or through the dorsal lymph sac (see **Reproduction technology > Handling and injecting frogs and toads**). Once females receive the final ovulating dose spawned oocytes may be fertilised by induced males or females can spawn into Simplified Amphibian Ringers for *in vitro* fertilisation (see **Reproduction technology > In vitro fertilisation**). If the oocytes are to directly fertilised induced males should be introduced to the females (see below). The pairs should be placed in an appropriate depth of water. Be careful to have a solid place for females to rest otherwise they can drown when amplexing.



Left: Dwarf African clawed frog

Typical induction protocol for males

With toads inject 35g males with 300 IU of hCG preferably also with 20µg LHRHa. Males produce sperm within about three hours and sperm production lasts about 8-

12 hrs. Because the female begins laying eggs around 10-24 hours after her injection, a synchronized injection scheme is important to optimize fertilization (Browne et al. 2006a,b).

Progesterone P 8783

Progesterone P 8783 comes in a 1g bottle of powder. Progesterone has a poor solubility in aqueous solution. At 25°C, the maximum solubility is about 7µg/ml.

In post spawning Fowler toads (*B. fowleri*) the use LHRHa and hCG in various combinations with progesterone showed that only treatments with progesterone produced ovulation.

Overall, results suggest that progesterone 100 µl at 7µg/ml with a dose between 20µg and 60µg of LHRHa may be optimal for the induction of ovulation in toads with poorly developed stage 6 oocytes.

A good article about progesterone solubility is; Nandi I, Bateson M, Bari M, Joshi HN. 2003. Synergistic effect of PEG-400 and cyclodextrin to enhance solubility of progesterone. APS PharmSciTech. 4 (1) Article 1 (<http://www.pharmscitech.org>).

Pimozide Sigma P 1793

Pimozide blocks estradiol produced by the gonads preventing the release of luteinising hormone from the pituitary. Hence, pimozide circumvents the need for environmental entrainment to mature and ovulate amphibians.

Pimozide (Sigma P 1793) comes in 500 mg bottle of powder. Pimozide can be dissolved in dimethyl sulphoxide (Me₂SO; Sigma D 2438) at 18mg/ml.

The use of pimozide with amphibians is at an early experimental stage. Browne *et al.* 2006 with the Fowler toad *B. fowleri* showed no effect of pimozide on improving ovulation.

However, pimozide had proved very successful with some fish species. Optimal doses for carp *Cyprino carpio* are 10 µg/kg LHRHa with 5 mg/kg pimozide. Indian catfish *Heteropneustes fossilis* can be induced to spawn by LHRHa alone in a dosage range of 0.15-0.2 mg/kg body weight or in combination with pimozide at a very low dosage of 0.05 mg/kg.

Examples of hormonal induction with various species

Goncharov BF, Shubrayv OI, Serbinova IA, Uteshev VK. 1989. The USSR programme for breeding amphibians, including rare and endangered species. International Zoo Yearbook. 28:10-21.

In 1983 the USSR established a working group to provide technologies for the conservation of amphibians.

1. to develop methods for maintaining amphibian species of interest;
2. to develop methods for obtaining healthy offspring in captivity;
3. to develop the most economical methods for egg incubation and rearing, that is, the maximum yields for the minimum expense;
4. to carry out practical measures for breeding and rearing of sufficient numbers of animals to ensure captive conservation of the chosen species;
5. to carry out practical measures for the establishment of new populations in nature based on animals bred and reared in captivity.

In this program 37 species in 17 genera were successfully induced with LHRHa:

Onychodactylus fisheri

Salamandrella keyserlingii

Mertensiella caucasica

Triturus alpestris, *T. cristatus*, *T. helveticus*, *T. karelini*, *T. montandoni*, *T. vittatus*, *T. vulgaris*

Bufo bufo, *B. calamita*, *B. danatensis*, *B. marinus*, *B. melanostictus*, *B. viridus*

Bombina bombina, *Bo. maxima*, *Bo.orientalis*, *Bo.variegata*

Hyla arborea, *H. japonica*, *H. savignyi*

Litoria caerulea

Osteopilus septentrionalis

Ceratophrys cranwelli, *C. ornate*

Discophus antongilli

Kaloula pulchra

Microhyla ornata, *M. pulchra*

Pelobates fuscus, *P. syriacus*

Pelodytes caucasicus

Xenopus borealus, *X. laevis*

Rana adspersa, *R. macrocnemus*, *R. nicobariensis*, *R. pipiens*, *R. temporaria*

African Clawed Toads Xenopus sp.

Typically *X. laevis* are induced by a priming dose of 10IU hCG 12-72 hrs before the ovulatory dose of 100 - 200 IU hCG. A priming dose of 20 IU can ovulate. With older females 100 IU produces ovulation after 6-8 hours, whereas 200 IU produces ovulation after 4 hours. For fertilisation through amplexus in the evening only the ovulatory dose of 200 IU hCG for females is given and 100 IU hCG for males. High temperatures of 30°C will prevent mating and low temperatures of 16°C cause developmental abnormalities.

Pituitaries (with the Northern Leopard frog *Rana pipiens*)

Hypophysation in its strictest sense is the process of inducing breeding through injection of pituitary extract from pituitary glands of amphibians of the same species. Anuran pituitaries as a dried powder can be purchased from supply houses or can be sampled directly from frogs or toads. The advantage of commercial pituitaries is that they may be obtained aseasonally, and that their purchase saves the trouble of harvesting them. The disadvantage of commercial pituitaries is that interspecific differences in their efficacy is not well known, and that pituitaries sampled from a local population have less chance of spreading disease. *Rana pipiens* pituitaries and ovulation kits can be obtained from Connecticut Valley Biological Supply Company http://www.ctvalleybio.com/product_search.html.

The earlier before the normal spawning time the greater number of pituitaries and amount of progesterone are needed to induce ovulation (Table 2).

Table 2. The doses of progesterone when combined with pituitaries use to ovulate *Rana pipiens*. NB: Doses shown are calculated as female pituitaries; two male pituitaries are equivalent to one female pituitary. Both the pituitary and progesterone should be injected into the coelomic cavity about 48 hours before scheduled fertilization.

Month	Pituitaries Alone	OR Pituitaries	+ 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	-

The following description is based on *R. pipiens*, whose ovulation has been most routinely conducted under laboratory conditions. However, pituitaries are readily collected following the technique described by Rugh (1965) and used immediately or stored frozen for days.

Breeding through amplexus

If breeding is to be accomplished by amplexus, both male and female should be treated. Follow the above table for pituitary dosage for females. Males will respond to half the pituitary dose; progesterone is not needed. The injected animals should be placed in an aquarium with 200-250 mm (8-10 in.) of dechlorinated water at 20-22°C. The animals should be placed above plastic screening with a coarse mesh 9.5 mm approximately 50 mm (2 in.) above the floor of the aquarium to protect the egg masses. Depending on the season, fertile eggs should be available in 1848h if the aquarium is placed in an undisturbed area. Note that the precise time of fertilization is not known when this technique is used.

Fekete DM, Brockes JP. 1988. Evidence that the nerve controls molecular identity of progenitor cells for limb regeneration. *Development* 103: 567-573.

Spanish newt, *Pleurodeles waltl* embryos were obtained either from Xenopus Ltd (UK) or from breeding pairs maintained in the laboratory. In either case, females were induced to spawn by subcutaneous injections of 1 mg/ml "of LHRHa (Sigma L4513) at 0.1mg/kg body weight (0.1µg/g). Up to 400 fertile eggs could be obtained from a single female during the breeding season (November-March).

HUSBANDRY MANUAL PUERTO RICAN CRESTED TOAD (*Peltophryne lemur*) 2006/07 UPDATE. Andrew Lentini .
http://www.caza.ca/media/Pdf/Conservation/member%20resource%20page/PRCT_Husbandrymanual2006-07draft%5B1%5D.pdf

Two different hormones are used to induce reproduction. LHRHa for females and hCG for males. LHRHa is diluted to make a 100 microgram/ml solution and frozen in 1 ml syringes prior to use. The shelf life of the dry product is 2 years. Toads are injected using a dose of 0.1 µg/g subcutaneously (intraperitoneal injection can also be used).

Although there were no statistical differences in peak sperm concentrations in response to LHRH and hCG, we still recommend using hCG because of the timing of the spermiation response and the enhanced behavioural response seen. The timing of the peak appears to be sooner with hCG (3 to 6 hours) compared to LHRH (24 hours). The hCG dose that we recommend for males is 10 IU/g subcutaneously. Research shows that sperm production peaks between 6 and 24 hours following injection.