Zoo Biology 29 : 774–782 (2010)

BRIEF REPORT

Hormonal Induction of Spermiation, Courting Behavior and Spawning in the Southern Bell Frog, *Litoria raniformis*

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We trialled the efficacy of various exogenous hormones to induce spermiation, courtship behavior, and spawning in the "endangered" southern bell frog, *Litoria raniformis*. Intralymphatic administration of Lucrin®, a synthetic nonapeptide luteinizing hormone releasing hormone (LHRH), was used successfully to induce courting behaviors and ejaculation of spermatozoa in males. Various hormones, including Lucrin®, another synthetic LHRH analog ([des-Gly^{10}, D-Ala^{6}]-LHRH), human chorionic gonadotropin, progesterone, and a dopamine receptor antagonist failed to promote oviposition and spawning in females. This and earlier studies indicate that in the efficacy of hormonal induction in amphibians varies between taxa, hormones, and genders. The lack of response in females may limit the use of reproduction technology in the southern bell frog and closely related species of Australian bell frogs. Zoo Biol 29:774–782, 2010. © 2010 Wiley-Liss, Inc.

Keywords: luteinizing hormone releasing hormone (LHRH); human chorionic gonadotropin (hCG); dopamine antagonist; gonadotropin (GtH); captive breeding; amphibian population decline

Grant sponsors: Australian Government's Water for the Future Program, and the NSW Government's Rivers Environmental Restoration Program.

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Received 9 December 2009; Revised 30 March 2010; Accepted 27 April 2010

DOI 10.1002/zoo.20331
Published online 14 June 2010 in Wiley Online Library (wileyonlinelibrary.com).

INTRODUCTION

Of the 6,285 species of amphibians listed in the IUCN redlist, 1,895 species (30%) are endangered or threatened with extinction [www.iucnredlist.org, 2009]. In response to this crisis in amphibian biodiversity, the amphibian conservation action plan [Gascon et al., 2007] recommended captive breeding and genetic resource banking for threatened species. Several zoos in Australia, including Taronga Zoo in Sydney [McFadden et al., 2008], Perth Zoo in Western Australia, and the Amphibian Research Centre in Victoria, have embarked on breeding programs to aid the recovery and reintroduction of endangered frogs into the wild.

However, despite the large amount of information known regarding a few laboratory species, such as the African clawed frog (*Xenopus laevis*), the reproductive biology of the majority of amphibian species remains poorly understood. Amphibians have the highest diversity in reproductive strategies of all terrestrial vertebrates. This high diversity in reproductive strategies dictates a similar diversity in the physiological control of reproduction, including hormonal control of sexual behavior, spermiation, spawning, and oviposition [Michael et al., 2004].

One of the more remarkable characteristics of amphibians is the change of ovarian cyclicity in correlation with variation in environmental or seasonal conditions, especially the seasonal climatic cycle of temperature and rainfall [Sretarugsa et al., 2001]. In tropical countries, where there is a pronounced wet and dry seasons, the breeding and nonbreeding periods are clearly separated [Sretarugsa et al., 2001]. In temperate regions, amphibians may reproduce throughout the year with ovulation triggered through environmental factors, such as the nutritional state, temperature, and rainfall [Jørgensen, 1982]. In other anurans, a period of hibernation can be essential for ovulation [Tchou and Wang, 1963].

Follicular development and ovulation in female frogs, spermatogenesis and spermiation in male frogs, and ultimately, spawning events are under neuroendocrine control (Fig. 1). However, when maintained in captivity, many anuran species fail to spawn. Some of these failures may be a consequence of poor general husbandry or, more specifically, because the necessary environmental cues to stimulate breeding cannot be replicated.

Several reproductive hormones have been demonstrated to induce ovulation and spermiation in anuran species, acting either at the level of the pituitary or directly upon the gonads. Gonadotropin releasing hormones (GnRH), also called luteinizing hormone releasing hormone (LHRH), is the hypothalamic hormone controlling vertebrate reproductive physiology. Following the identification of the decapeptide amino acid sequence of porcine GnRH [Schally et al., 1971] and the realization that the structure was conserved across all mammals, interest then focused on developing modifications to the sequence to produce greater potency and increased resistance to degradation.

Intraperitoneal or intralymphatic administration of LHRH analogs has been used to successfully induce ovulation and spermiation in some anurans [Browne et al., 2006a,b; Mann and Bidwell, 2001; McCreery and Licht, 1983; Michael et al., 2004; Sotowska-Brochocka, 1988; Waggener and Carroll, 1998], and they seem to function by stimulating secretion of endogenous GtH from the pituitary [McCreery and Licht, 1983; Obringer et al., 2000; Sotowska-Brochocka, 1988]. Release of gonadotropic hormones from the pituitary is also under dopaminergic influence. Dopaminergic projections of hypothalamic origin [Sébert et al., 2008] inhibit release

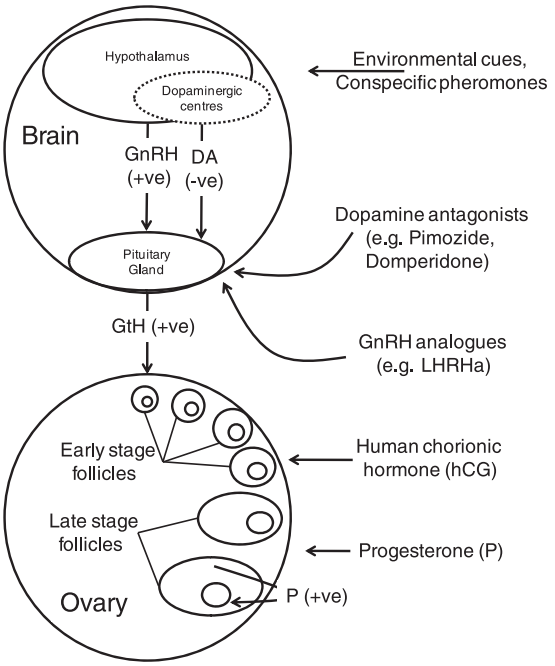

Fig. 1. Neuroendocrine factors that control gametogenesis and some of the compounds that have been used to induce ovulation (and spermiation) and spawning in frogs. DA, dopamine; GnRH, gonadotropin releasing hormone; GtH, gonadotropic hormones; LHRHa, luteinizing hormone releasing hormone analog.

of GtH from the pituitary. In fish, blockade of D2 dopamine receptors or disruption of dopaminergic nerves can increase GtH production [Chang et al., 1984; Osornio et al., 2004], and the administration of exogenous GnRH in combination with a D2 antagonists (e.g. pimozide, domperidone, or metoclopramide) has become routine for the purpose of inducing ovulation in fish [Zohar and Mylonas, 2001]. Similar physiology is evident in frogs [Minucci et al., 1993; Sotowska-Brochocka, 1988] and the D2 receptor antagonist pimozide in combination with non-species-specific [des-Gly^{10}, D-Ala^{6}] LHRH (LHRHa; Sigma L4513) has been successfully used to induce spawning in *Bufo fowleri* [Browne et al., 2006a].

Administration of hormones that act directly on the gonads has also been used to successfully induce spawning in some anurans. Human chorionic gonadotropin (hCG) has been used to induce spermiation in *B. marinus* and Australian hylids (*Litoria chloris* and *L. aurea*) [Clulow et al., 1999]. In *X. laevis*, hCG is routinely used to induce ovulation, spermiation, and spawning behaviors [e.g. ASTM, 2004]. However, *Xenopus* seems to be unusual in this aspect because it is one of a minority of anuran species that respond to hCG. Progesterone also is involved in the final oocyte maturation. Late-stage follicles secrete large quantities of progesterone [Fortune, 1983; Medina et al., 2004]. Administration of progesterone has also been used to improve LHRHa-induced spawning in *B. fowler* [Browne et al., 2006a].

This study examines the feasibility of the use of hormones to promote spermiation, courtship behaviors, and spawning in southern bell frogs (*L. raniformis*, Keferstein), a species that normally breeds with the onset of spring rains [Pyke, 2002]. Historically, the

southern bell frog was a widespread and locally abundant species occurring throughout southern New South Wales (NSW) and Victoria on mainland Australia and southern islands including King Island, Flinders Island, and northern and eastern Tasmania. However, more recently, local extinctions have reduced numbers in the South Eastern Highlands and the western slopes of NSW, the ACT, central Victoria and parts of Tasmania and South Australia [DEC, 2005; Mahoney, 1999; Osborne et al., 1996; Pyke, 2002; Sadlier and Pressey, 1994; Wassens, 2008]. The species is now listed as endangered in all Australian states [DEC, 2005; DSE, 2003; Heard et al., 2008; Schultz, 2008].

METHODS

A small colony of *L. raniformis* was maintained at the DECCW laboratory in Sydney, Australia. The frogs included several adults (Wild, 2–4 years old), captured in the Riverina region of NSW between early 2006 and late 2007, and several younger animals (domestic), reared from tadpoles of Victorian origin obtained from the Melbourne Museum in January 2006.

Adult *L. raniformis* were collected and maintained under permit S11480 issued by the NSW National Parks and Wildlife Service Permit. All pharmacological procedures, described here, were conducted within the auspices of the Department of Environment, Climate Change & Water (DECCW) Animal Care and Ethics Animal Research Authority project approvals 070618/04 and 080331/05.

General Husbandry

All frogs were maintained in either temperature-controlled rooms or incubators at the following temperatures: summer at 23–26°C, autumn at 16°C, winter at 10–13°C with no feeding, and spring at 16°C. The frogs were housed in glass aquaria (60 × 30 cm) with chlorine-free water to a depth of 10 cm. The tanks were furnished with rock platforms, artificial weeds, and a recirculating water filtration system. The frogs were exposed to a UV-A light source for basking for a 3–8 hr period each day, depending on the season. Densities of these frogs did not exceed two adults or four juveniles per tank, and males were maintained separate from females. All frogs were monitored and fed three to five times per week, using live crickets with higher frequencies of feeding with the addition of mealworms before and during breeding trials. All food items were coated with a calcium ($CaHPO_4$) and multivitamin powder (Repti-vite, Aristopet Pty Ltd, Australia).

Breeding trials were performed in a separate temperature-controlled room in a larger aquaria (140 × 40 cm) that were similarly furnished. One end of these tanks was modified to provide a terrestrial zone with peat moss substrate, vegetation, and rocks. UV-A lighting was provided above the terrestrial zone. At the commencement of trials, males and females were introduced to the breeding tanks. This also coincided with various environmental changes; the breeding tanks were "flooded" with fresh chlorine-free water; the temperature was increased to at least 26°C; duration of UV-A lighting was increased from 6 to 8 hr; and the water filtration outlet was adjusted to simulate rain over the tanks. Hormones, if used, were administered just before introductions.

Description of the Hormone Administration Protocol

The hormones used in breeding trials are listed in Table 1. All hormones, with the exception of progesterone and Ovaprim®, were either formulated or diluted in sterile deionized water to an extent that ensured only low volumes were administered

TABLE 1. Compounds Used in Hormone Induction Trials

Preparation	Source	Compound	Working concentration	Diluent
Lucrin®	Abbott, Australasia	Leuprorelin oxo-Pro-His-Trp-Ser-Tyr-[d-Leu]-Leu-Arg-Pro-NHEt	200 μg/ml	Sterile (0.2 μm filtration) Milli-Q® water
LHRHa	Sigma L4513	[des-Gly10, d-Ala6]-LHRH pGlu-His-Trp-Ser-Tyr-[d-Ala]-Leu-Arg-Pro-NHEt	1 mg/ml	Sterile (0.2 μm filtration) Milli-Q® water
Ovaprim®	Syndel Laboratories, Canada	Salmon GnRH analog, domperidone	20 μg/ml, 10 mg/ml	No dilution—supplied in propylene glycol
Pregnyl®	Organon (Australia) Pty Ltd	Human chorionic gonadotropin (hCG)	3,000 IU/ml	Sterile saline
Progesterone	Sigma P8783	Synthetic progesterone	10 mg/ml	Sterile (0.2 μm filtration) 20% cyclodextrin

(<200 μl). Because progesterone has low solubility in water, it was dissolved in 20% cyclodextrin [Yaksh et al., 1991]. Ovaprim® was used without dilution. All hormones were administered as injections into the dorsal subcutaneous lymph sinus using 0.5 ml/29 guage insulin syringes. Early trials indicated that a dose of 20 μg of the synthetic LHRH, leuprorelin (Lucrin®), induced calling behavior and spermiation in male frogs. These frogs also attempted to enter amplexus with female frogs. In all trials described here, males were administered Lucrine® at this same dose. Females received between zero and three doses of hormone, which included one or two priming doses to stimulate (or prime) the final maturation of oocytes, and an ovulatory dose 48–96 hr later (Table 2).

RESULTS AND DISCUSSION

All attempts to induce reproduction in wild-caught adult *L. raniformis* failed. The only individuals that bred within our laboratories were those that were reared in the laboratory from tadpoles. These animals were originally obtained as the progeny of animals maintained for the purpose of public display at the Melbourne Museum in Victoria. It seems likely that wild-caught animals become imprinted with environmental cues in their location of origin, which we were not able to replicate in the laboratory. Therefore, future attempts at captive breeding may need to rely on captive-bred brood stock. Following the death of one of our wild-caught adults, a pathology report found evidence of myxozoan parasites. At the end of the trials, all remaining wild-caught adults were found to be similarly infected. It remains unclear if the parasite was associated with their failure to breed.

After 2.5 years of growth, all laboratory-reared females produced eggs. All these eggs were produced shortly after a 4-month overwintering period. Two spontaneous matings resulted in fertile eggs (Table 2). In the following year (2009), after an overwintering period of 2 months, these females (now 3.5 years old) failed to breed or even produce infertile eggs, despite the fact that all were heavily gravid with ova. It is unclear if the shortened overwintering period was related to the absence of breeding. There are data indicating that a cold hibernation period is necessary for the maturation of oocytes in *B. asiaticus* [Tchou and Wang, 1963; also see Roth et al., 2009].

Administration of exogenous hormones produced gender-dependant results. Administration of Lucrin® in male frogs did result in an increase in male calling and attempts by males to enter amplexus with females and increased spermiation (approximately 0.85×10^6 spermatozoa per milliliter). Male frogs of other species were also reported to respond well to synthetic LHRH [Obringer et al., 2000; Waggener and Carroll, 1998], resulting in increased ejaculation of spermatozoa. In this study, various hormonal treatments failed to result in ovulation in females, and this is consistent with results obtained in the closely related species, *L. moorei* and *L. aurea* [Browne et al., 2008]. The lack of response in females may limit the use of hormonal induction as a conservation tool in Australian bell frogs (*L. castanea*, *L. raniformis*, *L. aurea*, *L. moorei*). LHRHa (Sigma L4513) has previously been effective for the induction of spawning in *B. marinus* following administration to both males and females [Mann and Bidwell, 2001] and effective in promoting ovulation in *B. fowleri* [Browne et al., 2006b] and *Eleutherodactylus coqui* [Michael et al., 2004]. Administration of hCG, which is universally used for induction of

TABLE 2. Hormonal Induction Trials. All Males Received 20 µg Leuprorelin (Lucrin®)

Trial	Number of males	Number of females	Priming dose	Ovulating dose	Response in females
1	Two wild	Two wild	None	20 µg leuprorelin (Lucrin®)	−ve
2	Two wild	Two wild	10 µg LHRHa (Sigma L4513)+ 500 IU hCG (48 & 96 hr before)	10 µg LHRHa+ 500 IU hCG	−ve
Overwintering at 10–13°C for 4 months					
3	Two domestic (2 years)	One domestic (2 years)	None	None	−ve
4	Two domestic (2 years)	Two domestic (2 years)	None	50 µg LHRHa	Approximately 50 infertile eggs
5	Two domestic (2 years)	Two wild, 55 g and 61 g	8 µg/g progesterone (48 hr prior)	50 µg LHRHa	−ve
6	Two domestic (2 years)	Two wild, 55 g and 61 g	16 µg/g progesterone (48 hr prior)	50 µg LHRHa	−ve
7	Two domestic (2.5 years) and two wild	Two domestic (2.5 years) heavily gravid with eggs	None	None	−ve[a]
Overwintering at 10–13°C for 2 months					
8	Two wild	Two wild	50 µg LHRHa+ 25 µl Ovaprim® 96 hr prior	50 µg LHRHa+ 25 µl Ovaprim®	−ve
9	Two domestic (3 years) and two wild	Two domestic (3 years) heavily gravid with eggs	50 µg LHRHa+ 25 µl Ovaprim® 96 hr prior	50 µg LHRHa+ 25 µl Ovaprim®	−ve

[a]Breeding occurred 19 days after males were injected with Lucrin® and resulted in females producing >1,000 eggs (<10% fertile).

ovulation and spawning in *Xenopus*, was similarly ineffective. Frogs receiving the higher dose of progesterone (16 μg/g) seemed weak shortly after dosing, but recovered within 6 hr. The apparently adverse reaction was surprising considering that an earlier study had administered very high doses of progesterone in *B. fowleri* (~150 μg/g) without reporting any adverse reaction [Browne et al., 2006a], and illustrates the high degrees of variation among anurans with regard to their physiological responses to exogenous hormones.

ACKNOWLEDGMENTS

Funding for this project was provided by the Australian Government's Water for the Future Program and the NSW Government's Rivers Environmental Restoration Program.

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