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Induced ovulation and egg deposition in the direct developing anuran *Eleutherodactylus coqui*

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Abstract

This study investigates ovulation and egg deposition behaviors in the anuran *Eleutherodactylus coqui* from Puerto Rico in response to stimulation with gonadotropin and gonadotropin releasing hormones. Five hormones were tested by injection over a range of doses, including mammalian LHRH, avian LHRH, fish LHRH, D-Ala6, des-Gly10 ethylamide LHRH and hCG. We report a low level of ovulation and egg deposition in response to all hormones, with the most complete and consistent results from the non-natural D-Ala6, des-Gly10 ethylamide LHRH derivative. To confirm the viability of eggs produced in this manner we performed *in vitro* fertilization experiments that resulted in the development of normal frogs. Reproductive behaviors in *E. coqui* are apparently not controlled by a mammalian form of LHRH as reported in other common laboratory anuran species. D-Ala6, des-Gly10 ethylamide LHRH induces ovulation and deposition of mature and fertilizable eggs in *E. coqui*.

Background

Several amphibian species have been commonly used in studies of reproductive biology. Reasons for this include external fertilization and development in large, easily manipulated eggs. Despite the large amount of information known regarding a few laboratory species (most notably *Rana pipiens* and *Xenopus laevis*), the reproductive biology of the majority of amphibian species remains poorly understood. This is unfortunate as amphibians, and especially anurans, show the greatest diversity in reproductive strategies among all of the terrestrial vertebrates, including internal and external fertilization, terrestrial and aquatic breeding, development with a larval stage, direct external development, ovoviviparity, mass seasonal breeding, continuous breeding, and presence or absence of parental care. This diversity in reproductive strategies can be expected to be a result of differences in the physiological control of reproduction, including hormonal control of sexual behaviors.

Our interest has centered on frogs in the neotropical genus *Eleutherodactylus*. With over 700 described species, this is the largest vertebrate genus and as such is an excellent system for studies of comparative biology [1]. As far as it is known, these species undergo direct development in terrestrial eggs (one species is known to be ovoviviparous [2]), and often exhibit parental care [3]. Many species of these frogs are territorial and continuous or nearly

continuous breeders. It has been shown for one species (*Eleutherodactylus coqui*) that population sizes are limited by the availability of terrestrial retreat and nesting sites as opposed to food availability [4]. These developmental and behavioral adaptations make *Eleutherodactylus* species quite distinct from other commonly used laboratory frog species.

Eleutherodactylus coqui, the common Puerto Rican coqui, has received attention as a model for acoustic communication and developmental biology (For example: [5-9]). We are interested in understanding the hormonal control of ovulation and egg deposition in this and other *Eleutherodactylus* species. Reproductive behavior, including ovulation, can often be induced artificially in other species by injection of the pituitary glands of the same or closely related species [10]. This can be difficult for routine applications due to the need for large numbers of animals that must be sacrificed to harvest the pituitaries. Since this would be problematic for *E. coqui* and most other *Eleutherodactylus* species because they are difficult to collect and keep in captivity, we have here investigated the ovulatory effect of stimulation with peptide hormones.

Several reproductive hormones have been previously shown to induce ovulation in other anuran species [11-15]. In *Xenopus laevis*, human chorionic gonadotropin (hCG) is routinely used for this purpose [11,12]. However, *Xenopus* appears to be unusual in this aspect because it is one of a minority of species of anurans that responds to hCG [10]. In addition to direct stimulation of the gonads with gonadotropins, stimulation with leutinizing hormone releasing hormones (LHRHs) has also been successful in inducing ovulation in some other anurans [14,15]. LHRHs are fairly well conserved among vertebrates and often show considerable cross reactivity between even distantly related species [16]. However, amphibians have several forms of LHRH present in their brains [17]. In *Xenopus laevis*, the mammalian form of LHRH appears to be the functional form controlling the reproductive pathway and leutinizing hormone (LH) and follicle stimulating hormone (FSH) release [18]. However, it is unknown if this form plays the same role in *Eleutherodactylus*. We therefore compared the effect of hCG and several different commercially available varieties of LHRH, including a modified form with improved pharmacological stability and enhanced activity in other species [19]. The purpose of this study was to determine which, if any, vertebrate peptide reproductive hormone was able to induce ovulation and egg deposition in the Puerto Rican frog *E. coqui* as a first step towards elucidating the details of this pathway in *Eleutherodactylus* frogs. Here we describe the results of trials using several hormones and report that all hormones tested produced ovulation and egg deposition in at least one animal and that D-Ala6, desGly10, ethylamide LHRH most reproducibly induced ovulation and egg deposition in this species.

Methods

Eleutherodactylus coqui were collected near El Verde Field Station in El Yunque National Forest, Puerto Rico. The frogs were housed in the laboratory in 38 l glass aquaria separately or in pairs as described [20]. The aquaria each had approximately four cm of moist peat moss as a substrate with ten-centimeter long, 2.5 cm diameter poly vinyl chloride pipe sections as retreat sites. A shifted, 12 hour day photoperiod was maintained so that night began at 12:00 PM (noon). Twice a week the frogs were fed three-week old crickets (Fluker's Cricket Farm, Port Allen, LA) dusted with vitamin powder (Blair's Super Preen Nutritional Supplement, Neon Pet Products, La Mirada, CA). After a period of about two weeks the frogs typically became gravid, which was determined by gently applying pressure to the abdomen to examine for the presence of large, white egg masses.

Snout vent length and weight of gravid frogs was 43.5 +/- 5.0 mm (sd) and 8.9 +/- 1.0 g, respectively. Hormones were purchased from Sigma-Aldrich company and prepared by dilution in phosphate buffered saline solution (PBS) (138 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.2) to a concentration of 1 mg/ml for LHRHs and 5 mg/ml for hCG. Stock solutions were stored at -80°C until use. For injections, the stock solutions were further diluted with PBS to a total volume of 100 μl. Gravid females were placed into the corner of a plastic bag to restrain the frog and a 1 ml tuberculin syringe was used to deliver a sub-cutaneous injection into the anterior dorsum. The frogs were then returned to the aquarium in the plastic bag for observation. Injections were done at roughly 12:00 PM so that females would ovulate during the dark phase of the photoperiod. The effect of the hormone administration was assessed the following morning at approximately 8:00 AM. Except as described below, all frogs were injected only once. *In vitro* fertilization experiments were carried out by mincing the testes from a single frog in sperm dilution buffer (10 mM NaCl, 0.2 mM KCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.5 mM Hepes pH 7.5) and adding this solution dropwise over the tops of the eggs. All animals were handled and experiments performed in accordance with the standards outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Results and Discussion

Five different peptide hormones were tested for their ability to induce ovulation and egg deposition in *E. coqui*: mammalian LHRH (Glu1, His2, Trp3, Ser4, Tyr5, Gly6, Leu7, Arg8, Pro9, Gly10), avian LHRH (Gln8), fish LHRH (Trp7, Leu8), D-Ala6, desGly10, ethylamide LHRH, and

Table 1: Summary of hormones tested, hormone doses, ovulation results, and numbers of eggs deposited by animals that deposited eggs.

Hormone and dose used(ug)	Number of animals tested	Number of animals that ovulated	Number of eggs deposited
PBS control	6	0	0
Mammalian LHRH			
3	3	0	0
7	3	1	4
20	3	0	0
28	3	0	0
33	3	1	3
Avian LHRH			
3	2	0	0
7	2	0	0
20	3	0	0
28	3	1	6
33	3	0	0
Fish LHRH			
3	2	0	0
7	3	1	1
20	3	1	5
28	4	0	0
33	4	0	0
D-Ala, des-Gly, eth LHRH			
5	2	1	4
10	3	0	0
15	3	1	1
20	15	10	23, 36, 36, 36, 36, 35, 29, 1, 2, 38
hCG			
25	1	0	0
35	1	0	0
100	2	0	0
140	3	0	0
165	3	2	18, 23
200	3	0	0

hCG. Increasing doses of each hormone were used to establish a dose response curve (see table 1). Ovulation was observed in at least one trial with each of the hormones. No ovulation was observed following injection with PBS alone in six gravid animals. Ovulation was observed in two cases using 7 and 33 μg of mammalian LHRH, in which case the frogs deposited four and three eggs, respectively. No ovulation was observed in thirteen other trials using from 3 to 33 μg of mammalian LHRH. Ovulation was observed on one occasion using 28 μg of avian LHRH, in which case the frog deposited six eggs. No ovulation was observed in twelve other trials using from 3 to 33 μg of avian LHRH. Ovulation was also observed in two cases using 7 and 20 μg of fish LHRH, in which case the frogs deposited one and five eggs, respectively. No ovulation was observed in fourteen other trials using from 3 to 33 μg of fish LHRH.

Despite being very gravid to begin with, all of the frogs that ovulated and deposited eggs after stimulation with mammalian, avian or fish LHRH deposited a very small number of eggs and remained quite gravid. D-Ala6, desGly10, ethylamide LHRH was the most effective at inducing ovulation and egg deposition. Twelve out of twenty three frogs tested were observed to ovulate and deposit eggs. One of two frogs injected with the lowest dose tested (5 μg) ovulated, depositing four eggs. Three frogs injected with 10 μg failed to ovulate and one frog out of three injected with 15 μg ovulated, depositing a single egg. However, ten of fifteen frogs injected with 20 μg ovulated. Ovulation induced by 20 μg of D-Ala6, desGly10, ethylamide LHRH often appeared to be complete and large numbers of eggs were obtained (23, 36, 36, 36, 36, 35, 29, 1, 2, and 38 eggs (average = 27 +/-14 SD)) in most of the ten clutches deposited. After depositing eggs, the frogs were no longer gravid, except for the frogs that laid only one or two eggs. Using hCG at a dose of 165 μg, ovulation was observed on two occasions. One frog deposited eighteen eggs and the other twenty three eggs. Seven other frogs failed to ovulate using lower doses of hCG between

25 and 140 μg, one frog failed to ovulate at 165 μg and two other frogs failed to ovulate at a higher dose of 200 μg. Severe side effects were observed following injection of higher doses of hCG (165 μg and above). This included hemorrhaging, release of bloody eggs and the death of one of the frogs that ovulated. Other frogs receiving doses of hCG higher than 100 μg showed signs of ataxia and were generally lethargic for several days following treatment.

In two trials using D-Ala6, desGly10, ethylamide LHRH, the viability of deposited eggs was tested by *in vitro* fertilization. In the first trial with thirteen eggs, one embryo developed normally and in the second trial using twenty one eggs, three embryos developed and normal froglets were obtained. This indicates that after hormonal stimulation the ovulated oocytes underwent nuclear maturation and acquired a functional jelly coat after passage through the oviducts.

Conclusions

Although all of the unmodified LHRHs induced some ovulatory activity in *E. coqui*, none was particularly more effective compared to the others either in terms of percentage of animals that laid eggs or the numbers of eggs deposited. Previous work has indicated that a form indistinguishable from mammalian LHRH appears to control reproductive behaviors in *Xenopus* [18], and mammalian LHRH has also been shown to induce ovulation in *Rana catesbeiana* and *Rana temporaria* [14,15]. From the results of this study, it is not obvious that mammalian LHRH is the important form controlling ovulation in *E. coqui*. It is therefore possible that *E. coqui* utilizes a similar, but distinct LHRH to control reproduction, but what this form might be is not clear. Although it is most similar to mammalian LHRH, the D-Ala6, desGly10, ethylamide LHRH derivative possesses several modifications that have been shown to both increase the receptor binding affinity and the pharmacological half-life of the compound [19]. This results in high activity in ovulation assays in mammals and fish that correlates with our observations of high activity in *E. coqui* [19,21]. The number of eggs deposited in response to the D-Ala6, desGly10, ethylamide LHRH derivative is comparable to our previous observations of an average of 23 eggs per clutch laid during natural mating events in this species [20]. The lack of consistent function of hCG in *E. coqui* is not altogether surprising. Although hCG functions well to induce ovulation in *Xenopus*, it does not consistently induce ovulation in many other amphibian species [10] and although hCG did stimulate ovulation in *E. coqui* in a number of cases, it also produced severe hemorrhaging and other side effects at high doses. These results provide a method for induction of ovulation and egg deposition in *E. coqui* that can be used for further studies of the reproductive biology in this species. It will be of interest to see if other *Eleutherodactylus* species respond to these reproductive hormones in a similar fashion.

Author's Contributions

SFM conceived of the study, participated in the design and coordination of the study and drafted the manuscript. CB, ET, ARE, and SV carried out the collection of the frogs, husbandry and hormone injections. All authors read and approved the final manuscript.

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