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Mass Seed Production Technology of Green Frog, *Rana hexadactyla* Lesson by Captive Breeding

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Abstract: In order to increase the population of green frog (*Rana hexadactyla*) two types of experiment was conducted. The first experiment was designed to expose the frogs in artificial raining system. In the second experiment was conducted to induce the breeding activity by means of pituitary extract. The animals kept in artificial raining system took 26 h for egg deposition. Where as the animals injected with pituitary extract shows only 6.66 h. Fecundity was less (6,600) in animals kept in artificial raining system and it was comparatively high (7,500) in pituitary extract injected animals. Hatching rate, tadpole and frog let survival was much higher in animals exposed to artificial raining system. These were less in the animals injected with pituitary extract. Total rearing periods (6.4 weeks) were less in the animals injected with pituitary extract and it is higher (7.3 weeks) in the animals exposed to artificial raining system. Though the artificial raining system is ideal system for seed production the technology need to be standardized. Once it is standardized it can be very well utilized for the production of seeds in controlled conditions.

Key words: *Rana hexadactyla*, fecundity, artificial raining system, tadpole, frog let, pituitary extract

INTRODUCTION

Exporting trade of frozen frog legs has increased and brought an important impact on the economy of Indian markets. It is ideal lean meat, together with its high protein content, crisp nature and chicken with lobster flavor. The frog meat is preferred in abroad for its delicacy and rich protein. USA, France, Belgium, Netherlands and West Germany are the major importing countries from India. The frog leg industry in India, as in other countries, is explicitly based on the capture of these frogs from the wild. Due to the heavy demand and killing of juveniles and adults, the population of these species in India has started depleting from the nature (Mondal, 1986). The so-called decline in frog populations is not only by exploitation for export, but also due to the harmful effects of extensive use of pesticides and insecticides, during paddy cultivation season (monsoon, coinciding with the breeding of frogs), which can cause mortalities in all stages of the life history, right from the egg stage to the adults (James, 1986; Dutta and Mohanty-Hejmadi, 1978). The breeding season is discontinuous for all the frogs in India. The two species *Rana tigrina* and *R. crassa* are breed only once in a year. In *R hexadactyla*, the breeding season is completed between ends of April through middle of June, in excepting the extreme south eastern parts of Tamil Nadu state, where it occurs between second half of October through middle of November coinciding with north east monsoon. This shows that the breeding is not continuous and the availability of the seed is also restricted to seasons, unlike that of fish or prawn seed, which are available throughout the year. So continuous seed production and development of culture technology is the need of the hour to fulfill the exporting demands and the shortening of exploitation from the natural resources. So induced breeding and seed production of these frogs in

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captive conditions are a prerequisite. Hence, the present study is aimed to produce the seeds in a new experimental set up called artificial raining system. It is also designed to develop a simple technology for the seed production through pituitary injection.

MATERIALS AND METHODS

Collection

Healthy disease free matured males (65-70 mm) and females (110-121 mm) were collected from the ponds of Agaram and Areakosti near Parangipettai (Lat. 11°29'N and Lon. 79°46'E). They were collected by using cast net, or hand picking.

Transportation

The frogs were transported to the laboratory in plastic jars and acclimatized to the laboratory conditions (Temperature 26-30°C; pH 6.5-7.0). There are two types of experiment was conducted for the production of seeds. The first experiment was tried to expose the matured males and females in natural environment, which was artificially designed in the laboratory. The second experiment was tried by inducing the matured females by means of pituitary extract taken from matured females (Pillai, 1986).

Experiment I: Induced Breeding by Artificial Raining System

Terraria for Frogs

Fiberglass tanks having dimensions of 2.5 ft length, 1.8 ft breadth and 1.2 ft height were preferred for their higher durability and easy maintenance. The terraria were placed in such a way that they should get good natural light and ventilation. The top of the tank was covered and tightly tied with small mesh sized nylon net to prevent the escape of the frogs and for regular monitoring of the rearing frogs.

Landscaping and Vegetation of the Terrarium

Landscaping is very much important for adult frogs, because the adult spend lot of time on land securing their prey and for rest. So, good quality sand was washed 3-4 times and a layer of sand was poured to a height of 4-5 cm in one half of the tank. Live plants were placed on the soil for hiding purpose and to create a natural habitat. For breeding they require water, so the next half was filled with freshwater and smooth bricks were placed to take rest. To create natural situation green floating vegetation like azolla, eichornia, pistia and some hydrophytes were also allowed to grow.

Lighting

Light plays a major role in enhancing the breeding activity of the frogs. So adequate lighting was provided for the experimental tanks. A light source of 1000 lux was provided and photoperiod of 12 h L: 12 h D was maintained to enhance the growth of algae and aquatic plants.

Artificial Raining

Artificial raining system was created to produce drizzling during night to induce the mating of frogs. In this system, a perforated tray having dimensions of 1.25 ft length and 0.75 ft width was allowed to hang over the experimental tank at a height of 2.5 ft. The tray was supplied with water from the water tank through a tube. Controls were kept to regulate water flow in the tank at both inlet and outlet of the tube. Addition to artificial rain, a small fan was placed to face the tank surface and diagoual to the fall of water, which supplies cool breeze to the tank surface. A tape recorder containing a prior recorded voice of thunder was played near the culture tanks.

Stocking

Healthy males and females were stocked in a ratio of 3:2. Optimum environmental conditions were maintained during the experiment (Temperature 26-30°C; pH 6.5-7.0; L: D 12:12). The animals were fed with worms, maggots, flies, cockroaches and insects. The water in the tank was regularly exchanged and excess feed was removed while water exchange.

Mating

When continuous good shower was provided the male with the nuptial coloration moves to breeding site and started croaking. By hearing their chorus females followed the males. Immediately male jnmps on dorsal side of female (Amplexus stage) and was pectoral, took place at the edge of the water.

Egg Deposition

Finally the breeding pair took shelter among the aquatic vegetation and subsequently breeds. The eggs were laid singly and remain attached to the leaves of the aquatic vegetation. The reserve food in the yolk sac was enough to maintain them until hatching.

Hatching of Eggs

Hatchling span was lasted about 48-55 h at the temperature of 26-30°C. The tadpoles were smaller and more slender with narrow and longer tail. Snout was more produced and the mouth was rather small with five rows of teeth in the mouth disc. They were olive green with darker blotches and whitish below with no dark spots on the tail. The lower surface of the head and the body was silvery and there were usually silvery spots on the side of the head, body and tail.

Maintenance of Tadpoles

The tadpoles (150 Nos./tank) were stocked in a separate tank. The water used for rearing was free from toxic chemicals, especially chlorine. Optimnm environmental parameters were maintained for the rearing of tadpoles (Temperature 23-28°C; pH 6.5-7.5; Dissolved oxygen 4-6 ppm; L: D 12: 12 h). The tadpoles were fed with algae, partially decomposed vegetables and boiled/blanched leafy vegetables, pelleted fish and shrimp feeds and also chopped boiled fish. Feeding was done 4 times a day at every 4 h (9.00, 1.00, 17.00 and 19.00 h). Excess feed in the tank was removed regularly while water exchange especially in the morning hours.

Early Frogs

The next step in frog culture system was raising of earlier frogs (16-22 mm snout to vent) from 3 days old hatchling (8-12 mm) which lasted about 5-8 weeks to complete their development. From the 3rd day after hatchling the young tadpoles were start to feed. At this stage well-developed gills were appeared. So vigorous aeration was provided. Since they breath through gills over crowding was avoided. The first visible sign of metamorphosis was the growth of back legs and then front legs. The head has slowly caused it to resemble a frog. During this stage the teeth was shed. When the legs were fully developed the tail begins to shrink and did not fall off the frog. After the front legs are developed the tadpoles were removed from the water and placed in a tank having floating aquatic vegetation. Frogs that still maintain the tail were not feed until the tail was completely absorbed in to the body, after that they were feed with small insects. To avoid escaping nylon net was provided. Once the tadpoles were metamorphosed into frog let stage they were released into the pond.

Experiment II: Induced Breeding by Pituitary Injection

Experimental set up, lighting and feeding for this experiment is similar to the first experiment except artificial raining system.

Collection of Pituitary Glands

An incision was made at the posterior part of the cranium with the help of a sharp sterilized scissors and the roof of the skull was removed to expose the brain. The hind brain was disconnected and lifted with a fine forceps to locate the optic chiasma region. The pituitary hangs from the brain in front of optic chiasma was removed and placed in distilled water. Sufficient pituitary glands were removed from the females for injection.

Preparation and Preservation of Pituitary Extracts

Freshly collected pituitary glands were pulverized in a homogenizer with 0.5 mL of distilled water. The extract was centrifuged at 4000 rpm for 10 min and the supernatant was taken for injection. It was immediately preserved in glass Stoppered brown vials containing absolute alcohol and was kept in refrigerator. The preservative solution was changed several times for proper dehydration and defattening of pituitary glands.

Selection of Brooders

Healthy disease free female broods (120 mm) were selected for pituitary extract injection. A female with ripe eggs in the ovary was recognized by keeping the frog in a container having two or three inches depth of water. The brood female had bulging abdomen and the flesh was appear full around the vertebrae of lumbar region.

Injection of Pituitary Extract

A single subcutaneous injection of extract was given to the freshly caught recipient female (110 mm) with the help of a #27 hypodermic needle. The injection was given at the pelvic complex and by the side where the pelvic vein joins the anterior abdominal vein. For effective breeding, an additional dose of 1 mg of cortisone was given after pituitary injection. The injection was given in downward, as the pituitary glands are heavier than water. In order to avoid oozing of extract outside the hole made by the needle was closed by pressing with fingers. The dosage of pituitary varies with the size of the recipient female. More number of pituitary glands has been used for effective breeding.

Stripping of Eggs

The *R. hexadactyla* female was ready to lay their eggs within 3-7 h after injection. Some eggs were released automatically that is the time for stripping. Stripping or squeezing out of eggs from the uteri was done by grasping the legs of the frog in the left hand and place the palm of the right hand over the back of the frog. Then encircling the body just posterior to the fore limbs with the fingers. Finally by gentle closure of the hand in the direction of cloaca forcing the eggs to come out through the vent.

Preparation of Sperm Suspension

Healthy sexually matured males (70 mm) were selected for the preparation of sperm suspension. It was prepared by dissecting out both the testes and chopped them with a scalpel at about 5 mL of pond water. Then the freshly prepared suspension was poured over the eggs for fertilization and placed under 60 volts light. Hatching of eggs, maintenance of tadpoles and appearance of earlier frogs were just like previous experiment Triplicate was maintained for each experiment.

RESULTS

Breeding Period

The results of breeding period in *R. hexadactyla* are presented in Table 1. The amplexus stage was prolonged when the animals were exposed to artificial raining system. They took 26 h for egg deposition. Where as the animals injected with pituitary extract shows only 6.66 h.

Table 1: Fecundity, hatching rate, rearing period and survival of tadpoles and frog let of the *R. hexadactyla* exposed both artificial raining system and pituitary injection

Particulars	Artificial raining system	Pituitary injection
Breeding period (h)	26.00±0.53	6.660±0.48
Fecundity (%)	6.600±0.88	7.500±0.84
Hatching rate (%)	76.66±0.43	71.66±0.43
Tadpole's survival (%)	75.67±0.63	70.00±0.33
Frog let survival (%)	72.33±0.57	68.00±0.43
Rearing period (weeks)	7.300±0.33	6.400±0.56

Fecundity Rate

The number of eggs laid by the animals after brief period of breeding is displayed in Table 1. Fecundity was less (6.600) in animals kept in artificial raining system. It was comparatively higher (7.500) in pituitary extract injected animals.

Hatching Rate

Hatching rate was much higher in animals kept in artificial raining system (76.66%). It was less (71.66%) in the animals injected with pituitary extract (Table 1).

Tadpole Survival

Maximum (75.67%) tadpole survival was recorded in the animals exposed to artificial raining system rather than pituitary injected (70.00%) animals (Table 1).

Froglet Survival

Frog let survival was higher (72.33%) in the animals kept in artificial raining system. However, this was less (68.00%) in the animals injected with pituitary extract (Table 1).

Rearing Period

Total rearing periods (6.4 weeks) were less in the animals injected with pituitary extract and it is higher (7.3 weeks) in the animals exposed to artificial raining system (Table 1).

DISCUSSION

The green frog, *R. hexadactyla* plays an important role in the pest control and also acting as a prey for other large animals. Due to heavy collection of frogs for their legs for export, at least in some areas of India, there has been severe decline in frog population. So decline in the frog population will affect the ecosystem (Mohanty-Hejmadi and Dutta, 1981). The most exploited species in India are bullfrog, *R. tigrina* and green frog, *R. hexadactyla*. So in the present study on attempt has been made to enhance the frog population especially green frog, *R. hexadactyla* through captive breeding. Previous report shows that cloudy weather with rains or drizzles favors good breeding in *R. hexadactyla* species (Mondal, 1986). The breeding activities of the frogs are mostly related with monsoon season. So artificial raining system of the present study is highly suitable system for inceptive breeding. In general breeding is normal in ponds and other water bodies filled with aquatic vegetation (Ramasamy, 1962). In the present study also aquatic vegetation was provided to favors the breeding activity. The aquatic vegetation was allowed to grow well in advance in the artificial raining system. This is supported by the study of Ramasamy (1962). They have grown aquatic vegetation one month ahead of breeding season. In such environment the frog thrives and breeds well and large quantity of seeds has been obtained. The vegetation in the water is not only encouraging reproductive behavior but also used to adhere large mass of eggs that laid by the frogs.

In natural condition, *R. hexadactyla* was breed only during monsoon period (October-December). So induced breeding is very much essential to get the seeds in round the year. The *R. hexadactyla* has great potential to responds well to its own pituitary gland. So in the second experiment in the present study is designed to produce seeds by pituitary injection. Hypophysation is carried out in pre-breeding and breeding seasons of frogs. The best time for induced breeding work for *R. trigrina* and *R. crassa* is from end of April through May-June and for *R. hexadactyla* from mid May through June and January-February (Mondal, 1986). Fortunately, in the present study also the induced breeding experiment falls on the above period.

The breeding period is less (6.66 h) in pituitary injected animals rather than the animals in artificial raining system (26 h). Amplexus stage was reported in animals exposed to artificial raining system. So the duration is high in artificial raining system. Where as in pituitary injected animals there was no amplexus stage. So breeding period is comparatively low. In the present study the matured males and females were introduced into the tank in the ratio of 3:2 (Mohanty-Hajmadi and Dutta, 1981) for the purpose of breeding. This ratio varied with different species. Pillai (1986) introduced 1:3 ratios and recommended sex ratio for bullfrog was 1: 5 (Grey Lurz and Avery, 1999).

In some other experiment, *R. hexadactyla* had spawn 3-7 h after injection. In the present study also the animals are ready to spawn about 6.66 h after pituitary injection. So there is no much difference. But in *R. tigrina* and *R. crassa* the time taken for spawning after pituitary extract injection varies from 18-21 and 48-54 h, respectively (Kasinathan and Ramulu, 1989).

Mondal (1975) has been used freshly taken pituitary from live *R. hexadactyla*. Immediately after pituitary extract injection a dose of 1 mg of cortisone was adminisitered for effective breeding. In the present study also fresh pituitary was used and followed by a dose of 1 mg of cortisone.

In general 1-3 pituitary glands are enough for larger species (*R. trigina*, *R. crassa* and *R. hexadactyla*), however, smaller species required more number of pituitary glands. Some female frogs respond well to allied genera pituitary extracts. However, *R. hexadactyla* responded well to its own pituitary. The female pituitary glands are twin potent than males (Mondal, 1986). In the present study also the pituitary was actually taken from female. The pituitary gland in female is bigger in size than the male glands (Rugh, 1941; Mondal, 1975). So it is very easy for plucking and handling than male pituitary. The time lapse between pituitary extract adminisitration and final stripping of eggs varies between 3-4 h for *R. tigrina*, *R. crassa*, *R. cynophylyctis* and *R. limnocharis* and 6-7 h for *R. hexadactyla* (Mondal, 1986).

The fecundity of the animals kept in artificial raining system was less (6,600) whereas it is higher in pituitary injected animals (7,500). In artificial raining system the spawning is almost natural. But the facility provided for breeding condition in terrarium may be not suitable. No expected calling sites, expected egg deposition sits and adequate territory for calling males. This may be the reason for low fecundity in artificial raining system. The conditions said above are not needed for pituitary injected animals because the pituitary extract was injected directly to the matured female. So high fecundity was reported. The fecundity varies with the species and their age and size, 3,000 to 20,000 eggs can be obtained within a size range of 83-148 mm (*R. trigrina*) 2,000-6,500 eggs in size range of 74-95 mm (*R. crassa*) and 1,100-8,900 eggs (99-124 mm) in *R. hexadactyla* (Mondal, 1986; Kasinathan and Ramulu, 1989). In the present study also the egg number varies 6,600-7,500 (*R. hexadactyla*) for the size groups of 110-121 mm.

In general, about 50-80% hatching rate was reported under normal natural conditions. The hatching of the eggs are mostly depends upon the temperature of the rearing water (Pillai, 1986). Optimum temperature for proper development of eggs ranges between 23 and 27°C. Hatching took place in 18-21 h for *R. trigina* and *R. crassa*. It was 48-54 h in *R. hexadactyla* at above temperature. In the present study hatching rate was maximum (76.66%) in the animals kept in artificial raining

system. It was minimum (71.66%) in the animals injected with pituitary extract. As earlier reports (Mondal, 1986), in the present observation, hatching was took place 48-55 h at the ideal temperature of 26-30°C. The eggs are spawned naturally in artificial raining system. So there is no damage of eggs. However, in pituitary injected animals, the eggs are mechanically brought out. So there is a possibility of damage. This may be the reason for higher hatching rate in the artificial raining system rather than pituitary injected animals.

Tadpole survival in the present study was high (75.66%) in the animals kept in artificial raining system rather than pituitary injected animals (70.00%). In the first case no stress is given and everything is in natural way. But little bit pressure is given during stripping off eggs. So there is less tadpole production in pituitary injected animals. The period of tadpole rearing varies with the species. In *R. trigrina* and *R. crassa* tadpoles complete their metamorphosis within 3-4 weeks of time whereas *R. hexadactyla* takes 5-6 weeks to complete their development. Tadpoles of *R. trigrina* and *R. crassa* are highly carnivorous and have tremendous rate of cannibalism. The latter factor alone accounts for their major mortality in culture operations extending over 90% (Mondal, 1986). But the rearing of *R. hexadactyla* is not a problem, as the tadpoles are herbivorous. The tadpoles showed over 70% survival to early frog stages when they were fed with phytoplanktors. In the present study also reasonable survival was obtained. This is due to the animals are exposed in ideal environmental conditions (Temperature, 23-28°C; pH 6.5-7.5; dissolved oxygen, 4-6 ppm; L: D 12:12 h) as well as provision of vegetable matters as a feed.

Frog let survival was higher (72.33%) in the animals kept in artificial raining system. however; this was less (68%) in the animals injected with pituitary extract. The real problem in the frog culture is on the transformation of tadpoles into early frog stage. From this stage till deaths of all frogs are not only carnivorous but also possess a peculiar mode of feeding in living and moving organisms. In fact how to make provision for sufficient live food for frogs in order to grow them marketable size in confinement was the real problem and for which frog culture met with failures everywhere in the world.

The total rearing periods of the *R. hexadactyla* was 7-3 weeks in the animals kept in artificial raining system and 6.4 weeks in pituitary injected animals. Total rearing period was less in pituitary extract injected animals because in this no amplexus stage. But amplexus stage is there in the animals in artificial raining system.

Comparatively speaking, both systems are ideal for the production of seeds in the laboratory conditions. The induced breeding by pituitary injection technology was already performed in many species of frogs and that technology was almost standardized. For pituitary collection usually the animals are sacrificed and also there is a possibility of egg damage while stripping off eggs. But the above said reasons are not recorded in artificial raining system. Though the artificial raining system is good system but it needs to be standardized. Once it is standardized it can be very well utilized for the production of seeds in controlled conditions.

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