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ZOOLOGY

Artificial reproduction in the breeding of bullfrogs on a small frog farm for several years

Marcelo Maia Pereira* and **Carlos Eduardo Marques de Oliveira Filho**

Fundação Instituto de Pesca do Estado do Rio de Janeiro, Rodovia RJ 145, Km 96, 56.660, Zona Rural, 27660-000, Rio das Flores, Rio de Janeiro, Brazil.
*Author for correspondence. E-mail: mmaiap2001@yahoo.com.br

ABSTRACT. Reproduction is a critical factor for the success of animal production. This study aimed to diagnose and evaluate the technique of using hormones to induce reproduction and artificial fertilization of bullfrogs on a small frog farm. The artificial reproduction of bullfrogs was evaluated over six years: 2016/2017, 2017/2018, 2018/2019, 2019/2020, 2020/2021, and 2021/2022. Growth and reproductive aspects were evaluated, totaling 16 different metrics. Data obtained from biometric measurements and reproductive assessments were analyzed using descriptive statistics. The main parameters investigated were the number of eggs (units, un), the production of tadpoles at 14 days (units, un), and the effective reproduction rate (%). Results by year were as follows: 2016/2017 (8,500.0 un; 6,000.0 un; 70.59%); 2017/2018 (22,750.0 un; 8,600.0 un; 37.80%); 2018/2019 (53,700.0 un; 5,850.0 un; 32.04%); 2019/2020 (18,258 un; 7,170.0 un; 13.35%); 2020/2021 (67,500.0 un; 70 un; 0.09%) and 2021/2022 (7,000.0 un; 200 un; 1.24%). The results indicate that artificial fertilization for bullfrog production was most effective during the initial years of the study. However, reproduction, even with this technique, did not result in spawning outside the spring and summer periods. Overall, the use of artificial fertilization technology on a small frog farm demonstrated good results.

Keywords: aquaculture; gametes; production.

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Introduction

The estimated production of frogs in Brazil in 2019 was 400 t in live weight, with 88% coming from the exotic bullfrog species (*Aquarana catesbeiana*) (Ribeiro & Toledo, 2022). In 2022, world production of frogs, considering both farmed and extracted animals, reached 229 thousand tons (FAO, 2024). The state of Rio de Janeiro was a pioneer in frog production in Brazil and is currently one of the largest frog producers. Its production is concentrated among small producers, with dynamics linked to low-production enterprises and reproduction based on natural techniques (Esteves et al., 2023).

In any agricultural activity, it is essential to have detailed records to minimize errors and optimize quality. Rural bookkeeping is rarely practiced among frog farmers (Esteves et al., 2023). In the reproductive sector, the need for information is even greater to ensure production efficiency (Rezende et al., 2020). Frog farming in Brazil faces several challenges, including the continuity of reproduction (Leal & Pereira, 2021).

The artificial reproduction technique has several applications: addressing reproductive failure through *in vitro* fertilization (Kouba & Vance, 2009); providing greater safety against amphibian diseases and minimizing the use of space for live animals (Kouba et al., 2009); increasing gene flow, maximizing genetic diversity, minimizing inbreeding, managing effective population size, and performing detailed pedigree analysis to reduce genetic drift (Clulow et al., 2014); and increasing genetic diversity in small groups of amphibians (Silla & Byrne, 2019).

Despite not being novel technologies, hormonal induction and artificial fertilization techniques (Agostinho et al., 2000) for bullfrogs in Brazil are not widely used among frog farmers. Among the hormones adopted, buserelin acetate has been the most prevalent in recent studies (Leal & Pereira, 2021).

The aim of this study was to analyze the reproductive and productive aspects of bullfrogs using various zootechnical evaluation parameters. This research is justified by the need to provide greater support to frog farmers who lack basic information in this area and to emphasize the importance of zootechnical records for decision-making.

Material and methods

The procedures adopted were approved by the Ethics Committee on the Use of Animals (CEUA) of the Rio de Janeiro State Fishing Institute Foundation (CEUA/FIPERJ; case no. 010/2021). The research was conducted on a small-scale frog farm classified as having a minimum intervention area of less than 200 m^2 (INEA, 2014). The experimental period spanned six consecutive years, starting in July of one year and ending in June of the following year.

Frogs (*Aquarana catesbeiana*) were kept in 3-m^2 stalls with a maximum density of 10 animals m^{-2}, a photoperiod of 14 hours of light and 10h of darkness (using a digital timer, MANPLEX®), and humidity above 60% (measured using a digital thermo-hygrometer with max/min function, INCOTERM®). Temperatures were measured daily (water and ambient temperatures at 8:00 am and 3:00 pm, using a digital maximum-minimum thermo-hygrometer with an external probe, INCOTERM®). Males were separated from females in adjacent stalls (Figure 1).

The breeding location, referred to as 'flooded stall', housed the bullfrog breeders throughou the study period. The frogs were kept submerged in water at a constant depth of 2 cm. The animals were fed *ad libitum* with commercial feed containing 40% crude protein designed for carnivorous tropical fish, administered directly into the water. The water for the farm was sourced from a spring stream located within the property and in a legal reserve, ensuring it was free from contamination from rural residences or livestock farms. Water pH was monitored weekly using a digital pH meter (AKSO®).

Figure 1. Male (a) and female (b) bullfrog (*Aquarana catesbeiana*) breeders used for artificial reproduction on a small frog farm.

The number of available breeders varied each year, with the number of males and females, respectively, as follows: 2016/2017 (3 and 3), 2017/2018 (10 and 10), 2018/2019 (25 and 15), 2019/2020 (15 and 15), 2020/2021 (10 and 20), and 2021/2022 (20 and 10). This variation was due to several factors: the initial year had fewer breeders, and in subsequent years, the number increased through frog reproduction and the availability of breeders on the frog farm.

Biometric measurements were performed monthly, with animals weighed on a precision scale accurate to 0.01 g (MARTE®). Health and reproductive status were assessed as follows: animals housed in the holding stall were evaluated for mating suitability based on characteristics indicative of sexual maturity (Lima & Agostinho, 1992).

Males were identified by a throat region displaying intense yellow coloration and dilation at the base forming a crop, thumbs with well-developed sponges or calluses, and a response to the amplexus reflex (Costa et al., 1998a). Females were assessed visually for belly volume and abdominal circumference (Costa et al., 1998b), with both sexes qualifying for selection if they weighed over 200 g. Suitable animals were selected for artificial fertilization using the methodology described by Agostinho et al. (2000), which involves administering two hormone doses of 1 mL each to females-one initially and another eight hours later. Males received a single dose of 0.2 mL one hour prior to oocyte extraction from the females. The hormone used was buserelin acetate, a GnRH (gonadotropin-releasing hormone) analogue that stimulates the anterior pituitary gland to secrete LH (luteinizing hormone) and FSH (follicle-stimulating hormone) (Sincroforte®).

Upon positive response to the hormone treatment, females underwent oocyte extraction by gentle abdominal compression using the thumb. Oocytes were then collected into a clean container and weighed on a digital scale with 0.0001 g precision (SHIMADZU®). This extraction process typically took about one minute.

Females that did not respond to the treatment were paired for 24 hours with a male that also received hormonal treatment at the appropriate dosage for induced mating in a 3 m^2 stall containing a 5 to 7 cm water column, a protocol employed in the years 2020/2021 and 2021/2022.

Males (3 to 10 specimens, the highest frequency being 6) were induced to spermatize, and semen was collected using a 2.0 mL pipette inserted into the male's cloaca. The collected sperm or semen (Figure 2) was then continuously poured over the spawn, which was in an appropriate container, followed by an additional 50 mL of water to aid fertilization. The container was then gently and continuously shaken for about two minutes to mix the gametes (Figure 2).

Post-fertilization, the eggs were transferred to a graduated bucket (L) and any clumps removed. The spawn was stirred by hand for 20 min. to hydrate the eggs (Figure 2). Five samples were subsequently taken and transferred to a beaker, with the total volume and number of eggs recorded and the average calculated.

Over the years, modifications were made to the egg hydration process. In the years 2016/2017 through 2019/2020, eggs were hydrated using water at ambient temperature. Starting in 2020/2021 and continuing in 2021/2022, the water was heated to a minimum of 23°C using heaters designed for domestic aquariums.

After hydration, the eggs were placed in 3-m^2 brick tanks filled to a height of 10 cm for incubation (Figure 2), maintaining a maximum density of 80 eggs per liter. These eggs were monitored until the 14th day post-fertilization to count the tadpoles produced.

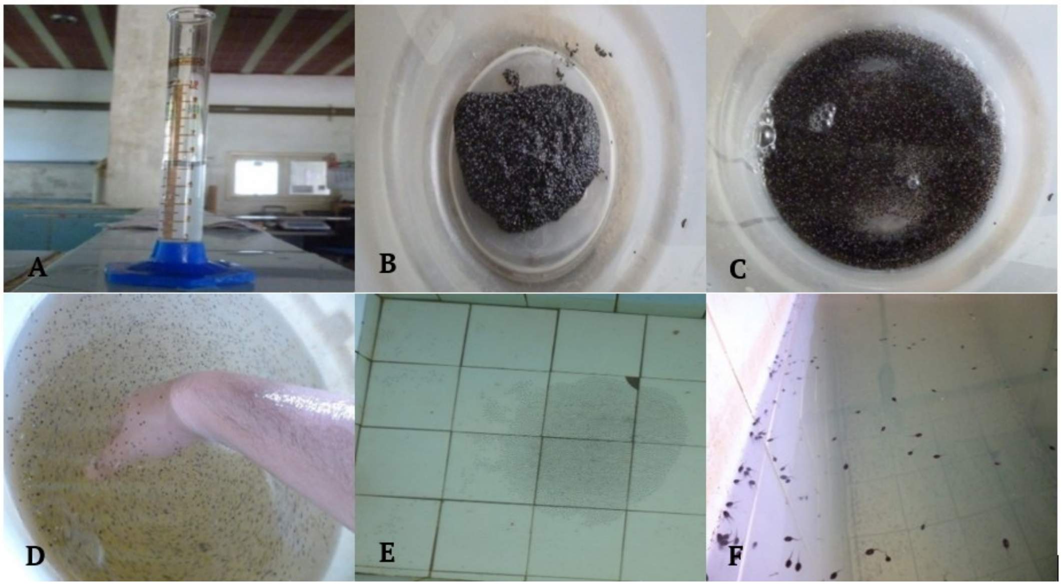

Figure 2. Semen (a), spawning (b), egg fertilization (c), egg hydration (d), egg incubation (e), and tadpoles (f) at 14 days after artificial fertilization of bullfrogs (*Aquarana catesbeiana*) on a small frog farm.

The research assessed the following reproductive and developmental parameters: Live weight of females (g); Spawn weight (g); Eggs (units); Weight of eggs (mg); Semen volume (mL); Tadpole production at 14 days (units); Number of spawning attempts (units); Number of spawnings (units); Spawnings resulting in tadpoles (units); Effective reproduction rate (ERR, %) (Leal & Pereira, 2021),

$$ERR\ (\%) = (Number\ of\ tadpoles\ at\ 14\ days * 100)\ /\ (Number\ of\ oocytes)$$

Ratio between spawn weight and live weight of females,

$$SW{:}FW\ (\%) = (Spawning\ weight\ (g) * 100)\ /\ (Live\ weight\ of\ females\ (g)$$

Ratio between the number of spawnings and the number of spawning attempts (unit),

$$\text{S: A}\ (\%) = (Number\ of\ spawnings\ * 100)\ /\ (Number\ of\ spawning\ attempts)$$

Ratio between the number of spawnings resulting in tadpoles and the number of spawning attempts,

$$\text{T: A}\ (\%) = (Number\ of\ spawnings\ with\ tadpoles * 100)\ /\ (Number\ of\ spawning\ attempts), \text{and}$$

Ratio between the number of spawnings resulting in tadpoles and number of successful spawnings,

$$T:S\ (\%) = (Number\ of\ spawnings\ with\ tadpoles * 100)\ /\ (Number\ of\ spawnings\ obtained)$$

Statistical analysis

The experimental unit in this study was defined as each attempt to spawn bullfrogs through artificial reproduction. Biometric and reproductive parameters recorded annually for the periods of 2016/2017, 2017/2018, 2018/2019, 2019/2020, 2020/2021, and 2021/2022 were analyzed using descriptive (quantitative) statistics via Excel software (MICROSOFT®).

Results

The frequency of female reproductive aptitude did not manifest consistently on a monthly basis. Despite some artificial climatic controls, reproductive responses predominantly occurred during months typical of natural reproduction in the tropical region (spring-summer). Over the six-year study period, artificial reproduction was most successful from October to April, with peak occurrences in December and February.

The estimated post-metamorphosis age of bullfrogs used for artificial reproduction ranged from 6 to 48 months. The effective reproductive lifespan was observed to be longer in males than in females.

Over the six-year period, the average weight of female bullfrogs on the day of reproduction ranged between 524 and 825 g. This parameter is crucial as it is indicative of the animal's age and its readiness for reproduction, exceeding the minimum weight required by the reproductive aptitude standard. The average weight of the spawns produced by these females varied from 31.3 to 111.6 g over the years. Notably, the methodology varied across different years; some years exclusively utilized artificial fertilization, while others also implemented mating induction. The latter typically yields better results. Specific years when artificial induction was employed are detailed in the Materials and Methods section. The proportion of spawn weight to female weight fluctuated between 5.8 and 12.9% throughout the study. This variation is influenced by the age of the females, with older females generally producing heavier spawns (Table 1).

The total number of eggs produced by the set of bullfrog females ranged from 8,500 to 78,300 units across the study period, influenced by various factors, particularly the number of reproductive attempts and successful spawnings. The weight of individual bullfrog eggs varied from 7.0 to 12.6 mg, correlating with the age of the females, as older and larger females typically produce larger eggs.

Regarding male reproductive contributions, the only parameter assessed in this study was the volume of semen collected for artificial fertilization, which varied between 9.3 and 14.8 mL over the evaluated years.

Table 1. Average live weight (g) of females, spawn weight (g), ratio of spawn weight to live weight of females (SW:FW, %), estimated number of bullfrog eggs (units), estimated egg weight (mg), and average semen volume per spawn (mL) obtained per year on a small frog farm.

Year	Live weight (g)*	Spawn weight (g)*	SW:FW (%)*
2016/2017	716.7±55.0	37.5±3.5	4.8±0.4
2017/2018	524.1±78.0	31.3±11.7	5.8±2.1
2018/2019	533.7±138.5	38.5±13.3	6.8±2.0
2019/2020	616.7±169.9	86.8±97.7	11.1±9.9
2020/2021	804.8±105.4	111.6±67.0	12.9±7.5
2021/2022	554.7±179.9	31.4±27.6	5.8±5.2
Year	Egg weight (mg)*	Eggs (un)*	Tadpoles (units)*
2016/2017	9.0±1.4	4,250.0±1,060.7	3,000.0±1,816.6
2017/2018	8.4±2.0	3,791.7±1,452.7	1,720.0±1,425.6
2018/2019	9.5±3.8	4,564.5±677.7	1,950.0±1,126.4
2019/2020	8.7±2.6	8,950.0±8,438.2	1,434.0±1,676.4
2020/2021	12.6±3.0	8,700.0±5,140.3	5.8±20.2
2021/2022	7.0±0.8	5,250.0±3,201.5	130.1±61.2
Year	Total eggs (units)	Total tadpoles (units)	Semen volume (mL)
2016/2017	8,500.0	6,000.0	11.5±2.8
2017/2018	22,750.0	8,600.0	14.8±4.8
2018/2019	18,258.0	5,850.0	9.6±0.5
2019/2020	53,700.0	7,170.0	13.0±7.5
2020/2021	67,500.0	70.0	11.0±1.0
2021/2022	7,000.0	200.0	9.3±2.9

*Mean ± Standard deviation.

The number of bullfrog tadpoles produced 14 days post-artificial fertilization showed significant annual variability, ranging from 70 to 8,600 units. This fluctuation can be attributed to several factors, including ambient and water temperature changes and the Covid-19 pandemic, which affected the work schedule and management practices of the animals.

The effective reproduction rate, which measures the yield of tadpoles 14 days post-fertilization relative to the initial number of eggs in the spawn, also demonstrated substantial variability, ranging from 0.09% to 70.59% over the study period (Figure 3).

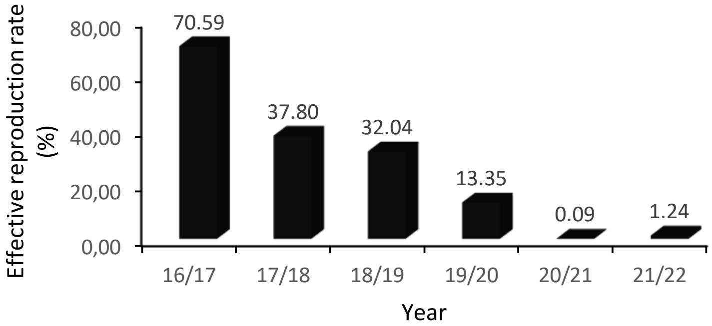

Figure 3. Effective reproduction rates (%) after spawning per year on a small bullfrog (*Aquarana catesbeiana*) farm.

The number of spawning attempts through hormonal treatments or artificial fertilization varied annually, ranging from 6 to 12 per year. This variation is explained by several factors, such as the number of available animals and their reproductive condition. The number of successful spawnings achieved through these methods ranged from two to nine per year, while the number of spawnings that resulted in tadpoles 14 days after reproduction varied from one to five. Over the six-year study, there were 18 successful spawnings, averaging three per year.

To more accurately assess the impact of the hormonal reproductive protocol, several ratios were calculated across the study period. The ratio of successful spawnings to total spawning attempts (S:A, %) varied annually from 33.3 to 87.5%. Additionally, the ratio of spawnings that resulted in tadpoles to the total number of attempts (T:A, %) ranged from 8.3 to 71.4% between the evaluated years. The ratio of spawnings that resulted in tadpoles to the total number of successful spawnings (T:S, %) fluctuated between 11.1 and 100%, indicating significant variability in reproductive success across different years.

The temperature of the breeding environment at 8:00 am was a maximum of 25.1°C and a minimum of 12.0°C; at 3:00 pm, the maximum was 33.0°C and the minimum was 21.5°C. The temperature of the water for breeding at 8:00 am was a maximum of 23.3°C and a minimum of 13.1°C; at 3:00 pm, the maximum was 28.2°C and the minimum was 14.8°C. Water and ambient temperatures also exhibited variations in their monthly means, particularly during the fall and winter months, which showed lower average temperatures. Water pH levels throughout the growth and reproduction period ranged from 7.0 to 7.4, averaging 7.2 ± 0.12.

Discussion

The lowest average weight of female bullfrogs recorded at 524 g in this study still exceeds the recommended average of 281.46 g for reproductive capability as suggested by Lima et al. (1998). This discrepancy in weight suggests that other factors may contribute to the variable success rates in response to hormonal application for inducing spawning.

A comparative study by Ribeiro Filho et al. (1998) on the use of different dosages of bullfrog pituitary extract to induce mating demonstrated egg production ranging from 1,280 eggs (1 mg kg^{-1}) to 24,310 eggs (5 mg kg^{-1}). The egg yields in our study aligned with these findings, although they were closer to the minimum rather than the maximum values observed. This outcome warrants consideration of two potential factors: the type of hormonal inducers used and the artificial fertilization techniques employed, which tend to yield lower results compared to hormonal induction.

This study advocates for the use of the effective reproduction rate (%) as a metric (Leal & Pereira, 2021), as it considers the production of tadpoles 14 days post-spawning relative to the initial spawn size. By incorporating both the fertilization rate of eggs and the hatching rate of larvae over an extended period, the ERR offers a more comprehensive and realistic measure of reproductive success. Although typically presenting lower values, this parameter is deemed more reliable for production and practical for frog producers.

The volume of bullfrog semen required for successful fertilization, observed in this study to range from 9.3 to 14.8 mL, aligns well with established literature values (Pereira et al., 2017). Nonetheless, a comprehensive analysis including gamete quality specifically, sperm morphology, motility, vigor, and other characteristics is essential for a more in-depth understanding of fertilization outcomes (Pereira et al., 2012). Future research should incorporate these detailed analyses.

The limited literature on artificial fertilization in bullfrogs shows considerable variability in fertilization rates and other evaluative parameters (Ribeiro Filho et al., 1998; Agostinho et al., 2011; Nascimento et al., 2014). One potential source of this variability could be the quality of male gametes. The *Colégio Brasileiro de Reprodução Animal* [CBRA] (2013) guide for andrological examination of animal semen does not provide specific instructions for amphibians, often necessitating the adoption of protocols intended for fish (Pereira et al., 2013a).

This study proposes the use of relationships between various reproductive parameters, such as the ratio of number of spawnings resulting in tadpoles to spawning attempts (T: A, %). Such zootechnical recordkeeping is paramount for effective management in frog farming. The findings presented can serve as valuable references for technicians and producers working within the reproductive sector.

Frog farming in Brazil often suffers from poor administrative management, with many producers lacking basic statistical and reproductive data. The results of the current study also reveal the importance of rural recordkeeping and the adoption of artificial fertilization techniques, which can significantly aid in managing genetic diversity by facilitating crossbreeding between different animal groups and preventing inbreeding.

In a study on the influence of climatic factors on the number of spawnings, Fontanello et al. (1984) noted the pronounced seasonality in bullfrog reproduction, occurring primarily during the spring and summer months in Brazil. This study corroborates those findings, observing spawnings within the same seasonal window, despite employing artificial fertilization techniques and partially acclimatizing the breeding environment to enhance thermal comfort. It was anticipated that such interventions would enable females to reproduce over a more extended period.

The inherent seasonality of bullfrog reproduction significantly impacts farm production schedules, as the breeding sheds and tadpole tanks remain empty for several months each year, limiting production to specific seasons (Leal & Pereira, 2021). However, observations suggest that in regions with a milder climate, if reproduction occurs in early spring and late summer, seasonality has less impact because the breeding period of these animals is longer and thus spans a greater portion of the year.

Tadpole production (units) remained stable during the initial four years of the study but experienced a sharp decline thereafter. The effective reproduction rate (%) per year also showed a continual decrease over the years. These trends are attributed to many factors, particularly water temperature.

Extensive research has been conducted to determine the ideal ambient and water temperatures for bullfrog production (Fontanello et al., 1984; Hoffman et al., 1989; Braga & Lima, 2001; Figueiredo, et al., 2001). The consensus from independent studies for spawning, tadpoles, froglets, and adults suggests that the optimal temperature range lies between 25 and 30°C. Deviations below this optimal range can lead to increased mortality rates among spawn and froglets and hinder their growth.

This study was conducted during the COVID-19 pandemic, which imposed restrictions on operational routines at the frog farm. Movement restrictions led to changes in staffing schedules, resulting in no reproductive attempts during the first half of 2020/2021 and reports of spontaneous abortions in female stalls. Even with artificial induction, the reproduction results during this period were suboptimal, highlighting the extensive impact of the pandemic on agricultural and livestock sectors globally (Cirqueira de Souza & Santana, 2023).

The findings emphasize the importance of meticulous management of reproductive manipulation techniques. Proper timing for hormonal induction, precise dosing, and controlling environmental factors such as temperature and photoperiod are critical for enhancing the production of quality tadpoles and froglets (Pereira et al., 2012; Pereira et al., 2013a and b).

Drawing parallels to advancements in livestock farming, particularly recently in beef cattle within Brazil, this study suggests that frog farming could benefit similarly from technologies linked to the genetic improvement process such as fixed-time artificial insemination, embryo transfer, and *in vitro* embryo production, which maximize productivity (Carvalho et al., 2023).

The outcomes of this research offer valuable insights for strategic planning in animal reproduction, helping frog farmers avoid oversizing breeding facilities and defining the number of female breeders (Ribeiro Filho et al., 1998). This study contributes to the technological knowledge base necessary for sustainable production, emphasizing an integrated approach that does not solely focus on specific components of the system, but instead considers environmental, social, and economic factors (Lamonaca et al., 2023).

The success of artificial reproduction in amphibians is influenced by several factors, including the appropriate choice of hormone for gamete induction, the correct dosages, and the proper techniques for gamete collection, among others (Uteshev et al., 2023). In this study, the hormone buserelin acetate was found to be effective in the reproduction of bullfrogs. Additionally, other amphibian reproduction protocols, such as gamete cryopreservation (Kaurova et al., 2021; Kaurova et al., 2022), could be adopted by national frog farming to improve the reproductive rates observed in this study.

While the results from the last two years were not satisfactory for commercial production aimed at fattening for slaughter, they may still be valuable if the focus shifts towards selecting animals for breeding purposes.

Overall, optimal utilization of available resources such as inputs and techniques in agriculture in general is a necessity for global food security and environmental sustainability (Penuelas et al., 2023).

Conclusion

The application of artificial fertilization technology on a small-scale frog farm demonstrated favorable outcomes, validating its effectiveness. The insights gained from analyzing reproductive techniques and the reproduction rates of bullfrogs in this study will serve as a valuable guide for frog farmers, particularly those operating on a smaller scale.

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