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Description of a Novel Procedure to Aid in Emergence of Larval La Palma Glass Frogs (*Hyalinobatrachium valerioi*) to Improve Survival Rate with Implications for Captive Management and Conservation

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Abstract: New, more efficient ways to accomplish objectives are key to improving the ability of zoological and conservation organizations to protect the animals they study and care for. Here, we describe an ovocesarean procedure, a novel task to assist the hatching of larval anurans in cases when allowing for hatching to occur naturally presents a risk to the survival of the progeny. This study focuses on two clutches of La Palma Glass Frogs (*Hyalinobatrachium valerioi*) where the eggs were not laid over a water body for the tadpoles to drop into upon emergence from the egg. A simple, three-step procedure that can be performed in just a few minutes resulted in a 95% success rate in emergence and led to increased survivability in the tadpoles that were assisted. Procedures that assist hatching of embryos and neonates are discussed in many circles of animal care but have not been described in detail to provide assistance to those that are not in a situation where they can learn it from a professional. This description of the ovocesarean procedure assigns a definitive, technical term to assisted hatching and can easily be extrapolated to other oviparous animals. Although the focal species here is of a Least Concern conservation status, this procedure can be key in improving reproductive success in other, more threatened species of anurans.

Keywords: ovocesarean; *Hyalinobatrachium*; assisted hatching; captive reproduction; conservation

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1. Introduction

In ex situ wildlife conservation, breeding programs are used to create captive populations to safeguard species threatened with extinction in the wild. It is thus an essential function for captive breeding programs to innovate tasks that can improve the effectiveness of their work. The purpose of this report is to provide a description of a procedure which can be applied to help with the emergence of larval anurans by releasing them from the egg membrane in cases where deposition occurs in an area where the survivability of the offspring is in jeopardy: the ovocesarean procedure. The term ovocesarean refers to the cleaving of the shell in amniotes and the vitelline membrane in amphibians, which contain the individual embryos of oviparous animals. This title has been used before in discussing the cutting of bird and reptilian eggs in various publications in Latin America [1,2]. This procedure can be directly applied to oviparous amphibians, and it could maximize the efficiency of propagation when working with species of anurans that may deposit eggs in areas which may lead to offspring mortality. An ovocesarean procedure is simple but may be crucial when working to conserve captive species threatened with extinction. Although this demonstration is performed on a species with stable populations in the wild, it is

directly applicable to other anuran species with similar reproductive dilemmas and/or with a more severe survival status.

Species within the family Centrolenidae are distributed throughout the riparian habitats created by mountain streams in the neotropics from Southern Mexico through Central America and the Andes, and through Venezuela to the Guiana Shield and south to Argentina [3]. These species are generally identifiable by their transparent skin on the ventral surface which varies between genera, with species within the genus *Hyalinobatrachium* having complete transparency and the intestines and liver are covered by a white peritoneum, while this peritoneum in other genera can cover nearly all of the ventral surface [4,5]. Of the 156 species within Centrolenidae, 50 are listed as either endangered or critically endangered [6], indicating that ex situ conservation strategies are necessary to ensure that these unique frogs continue to survive in the areas in which they occur. Although there has been plenty of research performed on the Centrolenid species, much of this research has focused on the important in situ investigations into natural history, ecology, and reproductive biology in nature.

Species within the genus *Hyalinobatrachium* are widely distributed, ranging north to south from Southern Mexico to Bolivia and west to east from Ecuador to the Guiana Shield. These species are generally smaller and nearly all reproductive behaviors, from calling to courting to egg deposition, are performed on the underside of large-leaf, riparian plant species such as Heliconia and Philodendron [5]. By comparison, the Hyalinobatrachium species are of less concern regarding their conservation status, with only 6 of the 41 representative species being endangered, although there are several listed as vulnerable or threatened [6]. Here we use *H. valerioi* as a sample species.

The La Palma Glass Frog (*Hyalinobatrachium valerioi*) is a small arboreal anuran species of the *Centrolenidae* family native to Colombia, Ecuador, Panama, and Costa Rica [3]. It is classified as Least Concern by the IUCN [7], but is a rare species to be kept in zoo collections and although they are becoming more popular in private collections, they are still very uncommon. Only attaining a length of 20–25 mm and weighing little more than 1 g, this species presents difficulties in its diet and husbandry regimens. *Hyalinobatrachium* species naturally deposit 10–80 eggs measuring ~2.0 mm in diameter on the undersides of large leaves or palms above a water source where the male has attracted the females by producing a mating call consisting of intermittent high-pitch chirps. This courting behavior is generally triggered by a seasonal increase in humidity and precipitation during the rainy season [8]. Thus, captive reproduction of the *H. valerioi*, like in most frogs of the Centrolenid species, involves cycling between rainy and dry seasons.

Once the species has spent a few weeks feeding and the females begin to produce eggs, which are readily visible via the transparent ventral surface, they can be moved into a rain chamber to deposit those eggs. This species can be difficult when it comes to harvesting and rearing eggs because they may be laid on the sides or doors of the tank, or in an area that is not over a body of water. While tadpoles from species in this genus have been documented to launch themselves up to 40 cm from the egg mass [9] in the clutches presented in this article, the odds of survival were zero due to the lack of water in the enclosures. Therefore, the egg mass must be moved onto a petri dish, or the leaf on which the eggs are laid can be clipped and placed into a container to allow the clutch to finish development. Phenotypic plasticity in this genus, resulting in the rushed development and early emergence of larvae, does not affect larval mortality [10], but it does affect the size at emergence which is likely to have an effect on the fitness of the offspring [11,12]. Placing the egg clutch into a container to monitor development is an excellent way to ensure developmental success and greatly reduces the threats of parasitism, destruction, and desiccation.

While some species can readily adjust their conditions for emergence in response to threats or cues via hatching plasticity [13], in instances where the threat is post-emergence from the egg, the clutch must be safely harvested. The practice of cutting the leaf on which the eggs have been deposited to be placed in a container for safety removes this threat, but assistance for safe, timely emergence is beneficial to the probability of survival. This

practice has led to a hatching success rate of ~95% within our Centrolenid frogs in instances where natural emergence and development would be impossible.

Although parental clutch attendance is well documented in *Hyalinobatrachium* species, it is vital only to defend and hydrate egg clutches instead of providing a biotic shield against detrimental bacteria or fungi [14]. Because the protection and hydration of the clutch is ensured when it is placed in a sterile container, the act of removing the clutch from the adult tank presents no threat to developmental issues of the larvae.

2. Materials and Methods

The subjects of these procedures were two *Hyalinobatrachium valerioi* egg clutches that were deposited in a holding tank on leaves that were not overhanging a suitable deposit area in a housing enclosure. The clutch of 30 (Clutch 1, Figure 1)) eggs was laid the night of 23 July 2019 and removed four days after discovery. The second clutch of 45 eggs was deposited on 3 August 2019 and was removed five days after discovery (Figure 2). Both clutches were deposited on a large-leaf Philodendron (*P. burle marxii*) by females in a group of animals in a sex ratio of 1.4 (1 male and 4 females). Because these clutches were laid in an enclosure not constructed to accommodate breeding, the larvae would not survive without being pulled due to the lack of a receptive pool in which the tadpoles could develop.



Figure 1. Male *Hyalinobatrachium valerioi* displaying clutch guarding of two egg masses that were deposited four nights apart; notice the embryonic development in the anterior clutch which was harvested but not documented so is not covered in this report. This behavior is common in the genus *Hyalinobatrachium*. Clutch 1 in our study is indicated by the arrow.

Determination of the incubation time that the species in question spends in the egg is an important variable to consider. For *Hyalinobatrachium valerioi*, this time period can be between 9 and 16 days [15]. The ovocesarean procedure in this genus can generally be performed at Gosner stage 25 [16] (~day 14) to allow optimum development while reducing the risk of fungal infection due to stagnation that may occur. Allowing the larvae to reach this stage of development allows for the full development of the respiratory machinery

that will allow the tadpoles to draw oxygen from the water in the rearing pool upon release from the egg. Fungal infection leading to embryo death did occur in both clutches used here and can be seen in Figure 3. Close attention to differential development rates in the embryos may require discretion when performing the ovocesarean procedure. Both clutches in this study were harvested by clipping the stem of the individual leaf and placing the leaf in a fully sealed plastic container (8 oz. Karat to-go container) which, as stated in the introduction, minimizes the risk of contamination and desiccation. While in the container, the development of the embryos was readily monitored by slightly opening the lid. Because species within the genus *Hyalinobatrachium* deposit eggs on the underside of the leaf, they are generally sheltered from harsh lighting in the wild. Thus, the artificial lighting of the clutch as it develops should not be direct and should be kept to a minimum to reduce stress on the developing tadpoles. There were no specific lights used for the developing clutches.



Figure 2. Clutch 2, consisting of ~40 developing eggs from a group of *Hyalinobatrachium valerioi.* Eggs were deposited 12 days earlier on the underside of a *Philodendron burke marxii* and in this figure are at stage 22 [16], putting the date of the needed ovocesarean procedure approximately two days away.

Because the container was completely sealed, the humidity remained high (~100%) and thus the risk of desiccation was extremely low. These cups were maintained at a temperature of 24–28 °C, which was the ambient temperature of the room in which they were maintained and lines up with conditions in the wild [8]. In the wild, male *H. valerioi* will tend to the clutch primarily to defend it from predators but also to ensure that the clutch remains hydrated by direct water release (urination) or less direct water transfer (osmotic transfer) [10,17,18]. Oxygen consumption of the larvae in the egg mass is extremely low [18], so the routine inspection of the clutch provides sufficient oxygen for the clutch within the container.

During the ovocesarean procedure, adding spring water to create a water level of approximately 4 mm in the base of the cup allows the newly released tadpoles a place to retreat after their emergence and helps to stabilize the leaf. Use of a spray bottle also containing spring water is recommended to clear out the empty membrane and remove loose tadpoles from the cutting area. The tools used in this procedure were all from the 30pc dissection kit from Dexsur [19]. Lysing of the vitelline membrane was performed using a scalpel with a No. 11 blade, which has a point allowing highly precise cuts. A curved teasing probe was also used to maneuver and manipulate the delicate embryos while cutting. Placing the point of the teasing needle up against the embryo helps stabilize

the egg (Figure 3A,D,F). Placing the scalpel at the base of the teasing needle and dragging the blade through the vitelline jelly surrounding the embryo causes the vitelline membrane to rupture (Figure 3B,E,G). The teasing needle and scalpel were then used to widen the hole in the egg, thus allowing the larvae to escape. Once freed, the tadpoles were coerced into the water and out of the way of the procedure area (freed tadpoles indicated by arrows in Figure 3C,E,H).

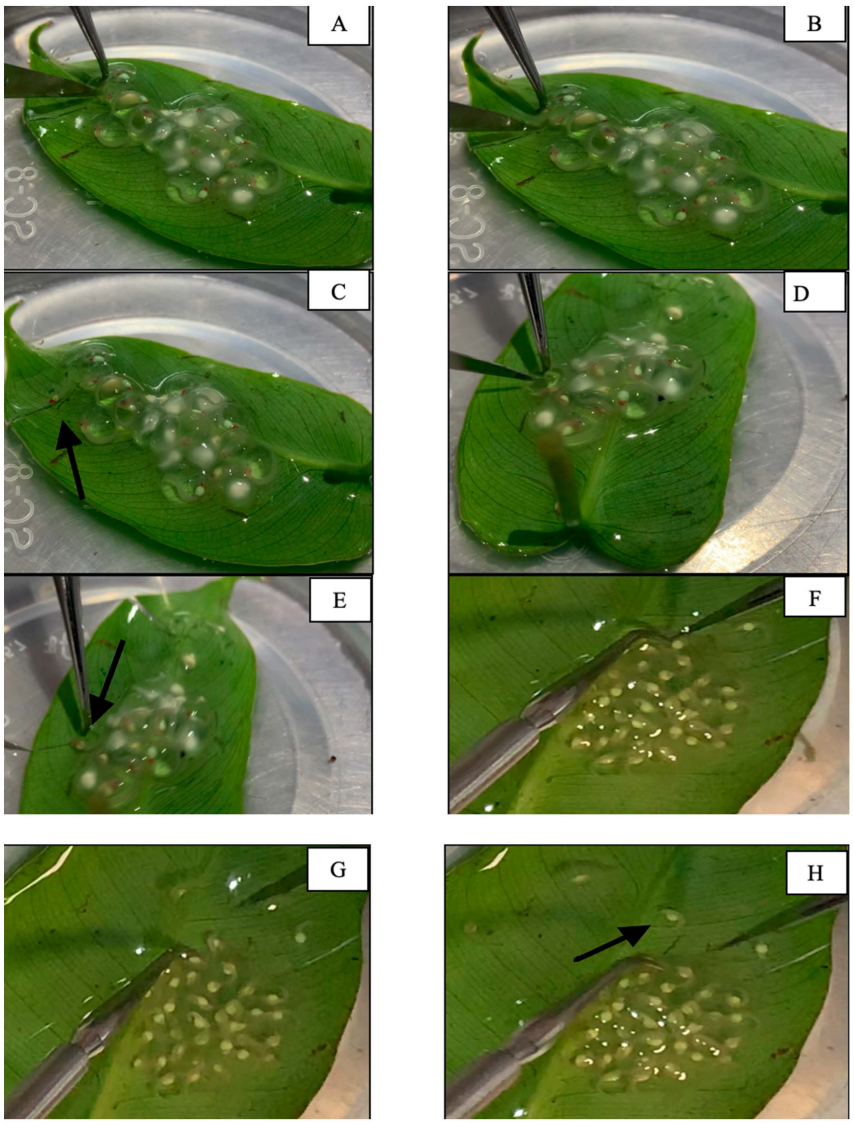

Figure 3. Step-by-step shots of three individual ovocesarean procedures. (**A**,**D**,**F**) Placement of the tip of the teasing needle near the embryo to stabilize the egg. (**B**,**E**,**G**) Dragging of the scalpel blade through the vitelline membrane. (**C**,**E**,**H**) Arrows indicate the newly released tadpoles.

Once all tadpoles were released from their eggs, the leaf was removed and discarded, and the remaining vitelline jelly was suctioned out using a pipet. Tadpoles should be monitored for about 24 h to determine if there are any negative effects from the procedure. Once they have been observed and cleared, they can be moved to a rearing tank to finish their development. Tadpoles that have been released too early in development (<Stage 24) [16] can usually recover if they are maintained in the cup in which the procedure was performed to finish development and are then added to the rearing tank. The tadpoles involved in this study were transported to a 6.5 L clear shoebox container with a small powerhead pump (SunGrow 53 GPH submersible pump, Singapore) to mimic the streams these larvae would inhabit in the wild. While the oxygen consumption of the egg mass is low, this level jumps

significantly upon emergence [18], so be sure to factor in the water level and water movement in this rearing tank. Development time with this setup is normally between three and six months for both naturally emerged and ovocesarean-released larvae, which is in line with other estimates for this species [20,21].

3. Results

Following the ovocesarean procedure, the released tadpoles were inspected for damages in the 8 oz. containers and all but one were found to be healthy and active. The single individual had what appeared to be a hemivertebra developmental malformation with the tail being kinked. This will obviously affect the mobility of this individual in the larval stage, but it is unknown if this might affect the fitness of the offspring post-emergence. Aside from this individual, all of the tadpoles released from these two clutches are feeding and developing naturally. The use of the fine probe allows for direct placement on the vitelline membrane and also for a clean cut of the membrane to release the tadpole, thus nearly eliminating the risk for physical damage. The most prominent exception is in the case of human error in the process. Between these two procedures, there were a total of 58 tadpoles released (Clutch 1: 20; Clutch 2: 38) and thus far they have a 100% survival rate. This indicates that the ovocesarean procedure is safe and effective but it does not speak to the long-term survival of these individuals once they metamorphose into their terrestrial form.

4. Discussion

As zoos and aquariums advance towards being centers of conservation and research, developing and sharing novel ways to improve the efficiency and quality of captive husbandry standards will continue to be a valued task for those that can identify the areas for innovation. The ovocesarean procedure is a simple task that can greatly increase the survivability of larvae deposited in an unfavorable location as long as four simple criteria are met: (1) checking for new clutches is part of the daily routine, (2) developmental aspects of the natural history of the species are known, (3) accurate determination of these stages of development is achievable, and (4) the physical cutting of the membrane can be performed accurately. The idea of tracking embryonic development and cutting the egg when the developmental period has been exceeded has occurred in many genera of ovipositing species with the title "Assisted Hatching", used as a blanket term to refer to this procedure. However, a current search of academic resources for assisted hatching will return many more hits about techniques such as cryopreservation, artificial insemination, and hormone therapy. While these techniques are wildly innovative, when referred to as assisted hatching, they can detract from the ability of researchers to find descriptions of techniques for more simple manipulations of natural reproductive processes. It is for this reason that we introduce the ovocesarean procedure as the lysing of the egg to release an embryo which, as stated above, occurs in many forms across nearly all oviparous clades. Following the methods of a previous publication on the management of glass frog egg masses [22], attempts were made to hang the clipped leaves over a pool of water to allow the tadpoles to emerge naturally into a suitable nursery pond, but were determined to be suboptimal due to the degradation of the host leaf after it was cleaved from the main plant. While there were some successes using this method, the ovocesarean procedure is a response to the methods of maintenance that both allow the host leaf to support the clutch as well as to optimize offspring development when the leaf is laid horizontally in a plastic container. Additionally, the ovocesarean procedure permits the safe release of tadpoles when the host leaf is laid down, which provides a solution for the gravitational drag on the clutch of eggs that naturally occurs when the leaf is oriented vertically.

It should be mentioned here that the intention of this procedure is only to assist in the hatching of egg masses that would not survive in their original deposition site. In cases where a clutch is laid above a rearing pool, the eggs are left to develop naturally. With the ovocesarean procedure, the survivability of tadpoles from previous clutches post-emergence is 95%, which we believe is an indication of the low level of stress on the tadpoles

and the relative ease of this procedure. In the clutches presented here, survivability post-emergence is at 100%, which is near the survival rate of previous clutches post-emergence, although the long-term survival and fitness of the single individual with the hemivertebra malformation is something we will observe and hopefully report on in the future. Future studies on this procedure will focus on identifying differences of survival and fitness between naturally emerging *H. valerioi* vs. those released via ovocesarean. While we did have mortality due to fungi (22.6%), we were able to release 58 tadpoles combined, which represented both clutches involved. In cases where the eggs are left to develop naturally, fungal invasion generally envelops the entire clutch and can be linked to the spreading of spores by the male during clutch hydration [6], whereas in isolation it is able to be managed. This procedure was developed to salvage a reproductive event from a species that is rare in captivity but is doing well in the wild. However, we do recognize that this procedure can be utilized in Centrolenidae species that are of a more dire conservation status. With approximately one-third (50/156) of the species within this family being either endangered or critically endangered [6], and the viability of ex situ captive reproduction programs as forms of active conservation [23], the possible application of innovative methods of assisted hatching will continue to become more of a staple in the conservation role of zoos and aquariums.

With the introduction of the ovocesarean procedure, we hope that familiarity with the process of aiding emergence for developed egg-laying animals can spread to become a more utilized conservation tool. Oviparous animals such as crocodilians, squamates, and chelonians can be assisted with emergence if the process takes longer than anticipated to complete or if the eggs are believed to be at risk of infections. Within anurans, a clear, gelatinous egg albumen and jelly make identifying developmental issues and fungal infections easy even for novice keepers. Therefore, if a clutch of anuran eggs is pulled from an enclosure because of a perceived or potential threat to the well-being of the offspring, it is likely that the survivability of that clutch will be higher than if it was left at the original site. While species within Centrolenidae are capable of adjusting their developmental rates through developmental plasticity in response to unfavorable conditions [24], this assumes that the emerging larvae will survive after emergence and that the removal of the deposition site from its original location will not affect the development of these larvae.

In the future, we plan on doing our best to ensure that there are no clutches deposited in unsuitable enclosures where they are unable to develop naturally; however, it is difficult to predict exactly when deposition will occur. In cases where this mistake occurs, we will continue to perform the ovocesarean procedure to allow clutches to survive in instances where they would otherwise perish. When possible, we plan to take the opportunity to run statistical analyses on other forms of assisted hatching versus the ovocesarean procedure, including those cared for naturally by the adults. We value the natural processes of reproduction for the overall health and well-being of amphibians, including the post-deposition care displayed by the *Hyalinobatrachium* species. This runs contrary to our intentions to innovate reproductive processes by removing clutches and raising them in vitro, which we believe highlights the necessity of this process.

Application of this procedure is possible in the near future as human encroachment into the montane rainforests threatens the water quality, host plant density, and prey species diversity for the delicate Centrolenid frogs. In species that might be endemic to a single quebrada or creek bed, abiotic factors that stem from human activity such as these may affect the survival of these species in general, and it may be necessary to mobilize all efforts, both in situ and ex situ, to salvage these species. In instances such as these, it is the hope of the authors that the ovocesarean procedure might assist in the survival outcomes of species that are threatened, either by direct application or by being a stepping stone to another process or technology that can have a greater impact on the objectives of the effort. Additionally, with some of the *Hyalinobatrachium* species being sympatric [5], the role of assisted hatching procedures such as ovocesarean may be helpful in understanding the natural history differences between two sympatric species in the

event that they hybridize. *H. valerioi* is known to hybridize with a sympatric congener *H. aureoguttatum* in captivity (Buttermore, unpublished data; Figure 4) and there are pertinent questions about embryonic development and fitness that may be better explained by affecting the emergence times of these progeny. This adds an additional consideration into the application of novel procedures within care regimens that have the ability to not only allow for better understanding of the natural history of a species, but also to allow for better decisions regarding the conservation practices of such species.



Figure 4. Hybridization event between *H. valerioi* (female) and *H. aureoguttatum* (male) occurring in a large communal breeding enclosure. These offspring were viable and did develop and emerge into a rearing pool.

With global anuran species populations decreasing, the need for captive propagation initiatives at zoos, aquariums, private collections, and museums has increased. With more eyes focusing on their downfall, public institutions such as these can be key in bringing the plight of the world's amphibians to an even larger audience. We hope to see the ovocesarean procedure evolve to fulfill the conservation needs of oviparous species in need. Innovation and the buildout of inventive practices will be key in preserving focal species in the future.

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