

Nest structure, incubation and hatching in the Trinidadian leaf-frog *Phyllomedusa trinitatis* (Anura: Hylidae)

J. Roger Downie¹, Mohsen Nokhbatolfoghahai², Duncan Bruce¹, Joanna M. Smith³, Nina Orthmann-Brask¹, and Innes MacDonald-Allan¹

¹School of Life Sciences, University of Glasgow, UK. E-mail: roger.downie@glasgow.ac.uk.

²Biology Department, Faculty of Sciences, University of Shiraz, Iran. E-mail: nokhbeh@hotmail.com.

³Institute of Science and the Environment, University of Worcester, UK. E-mail: frojo2525@hotmail.com

Abstract

Nest structure, incubation and hatching in the Trinidadian leaf-frog *Phyllomedusa trinitatis* (Anura: Hylidae). New findings on nesting and hatching are reported for the leaf-nesting tree frog *Phyllomedusa trinitatis*. Nest height and leaf cover (one or many leaves; well or poorly covered) were very variable. Contrary to a previous report, eggless jelly capsules were scattered amongst the eggs, with substantial jelly plugs located above and below the egg clutch. Plugs were composed of capsules embedded in a matrix of somewhat different composition. The upper plug acted as a barrier to water entry. Contrary to previous reports, isolated eggs could develop in aquatic media, the later stage on entry the better: aeration and salt composition of the water influenced this ability. Egg clutches developed equally well whether the covering leaves were alive or dead. Single eggs incubated in water hatched prematurely; when placed in water, eggs near hatching stage hatched within a short time; such eggs on a dry or damp substrate did not hatch till much later. The hatching of any embryo in a group appeared to act as a stimulus to neighbours to hatch. Observations on nests with the covering leaves replaced by clingfilm showed that emergence is preceded by a lengthy period of hatching behavior, with individuals hatching, wriggling and stimulating larger and larger groups to hatch, and releasing a frothy fluid which leads to the dissolution of the lower jelly plug and emergence of the hatchlings. Contrary to a previous report, hatching gland cells were observable on the laterodorsal surface of the head. When hatchlings emerged from the nest, they were mostly at Gosner stage 25 with gills fully resorbed. When earlier embryos were induced to hatch, they rapidly shortened their external gills. These findings are discussed in comparison with observations on the related genus *Agalychnis*.

Keywords: *Agalychnis*, hatching, leafnests, Phyllomedusinae.

Received 4 April 2012.
Accepted 4 March 2013.
Distributed June 2013.

Resumo

Estrutura do ninho, incubação e eclosão de *Phyllomedusa trinitatis* (Anura: Hyllidae). Relatamos aqui novas descobertas sobre a nidificação e a eclosão de *Phyllomedusa trinitatis*, uma perereca que constrói ninhos em folhas. A altura do ninho e a cobertura das folhas (uma ou muitas folhas, muito ou pouco cobertas) foram muito variáveis. Contrariamente a um relato anterior, cápsulas gelatinosas sem ovos estavam espalhadas em meio aos ovos, com tampões gelatinosos substanciais localizados acima e abaixo da massa de ovos. Os tampões eram compostos de cápsulas envoltas em matriz de composição levemente diferente. O tampão superior funcionava como uma barreira à entrada de água. Diferentemente de relatos anteriores, ovos isolados poderiam se desenvolver em meio aquático, desenvolvendo-se melhor quando em estágios mais avançados: aeração e composição salina da água influenciaram essa capacidade. As desovas desenvolveram-se igualmente bem estando as folhas de cobertura vivas ou mortas. Ovos isolados incubados na água eclodiram prematuramente; quando imersos em água, ovos perto do estágio de eclosão eclodiram em um curto período; em substrato seco ou úmido esses ovos eclodiram muito mais tarde. A eclosão de qualquer embrião em um grupo pareceu agir como um estímulo para que os embriões vizinhos eclodissem. Observações de ninhos com as folhas de cobertura substituídas por uma película plástica revelaram que a emergência é precedida por um longo período de comportamento de eclosão, com indivíduos eclodindo, se contorcendo, e estimulando grupos cada vez maiores a eclodir, e liberando um fluido espumoso que leva à dissolução do tampão gelatinoso inferior e à eclosão. Contrariamente a um relato anterior, células glandulares de eclosão foram observadas nas superfícies laterodorsais da cabeça. Quando girinos emergiram do ninho, encontravam-se principalmente no estágio 25 de Gosner, com as brânquias completamente reabsorvidas. Quando embriões precoces eram induzidos a eclodir, suas brânquias externas encurtaram-se. Essas descobertas são discutidas em comparação a observações feitas no gênero aparentado *Agalychnis*.

Palavras-chave: *Agalychnis*, eclosão, ninhos em folhas, Phyllomedusinae.

Introduction

The neotropical hyllid subfamily Phyllomedusinae includes several genera and a total of 58 species (Frost 2011). Of these 30 are assigned to the genus *Phyllomedusa*. *Phyllomedusa trinitatis* Mertens, 1926 occurs throughout Trinidad (but not Tobago) and also in northern Venezuela. Faivovich *et al.*'s (2010) phylogenetic analysis of the Phyllomedusinae confirms Duellman's (1974) and Barrio-Amorós's (2006) suggestion that *P. trinitatis* belongs to the *P. tarsius* species group. Budgett (1899) seems to have written the first report on nesting and embryonic development in *Phyllomedusa*, followed by Agar (1910). More recent work (Kenny 1966, 1968, Pyburn and Glidewell 1971, Pyburn 1980, Lescure *et al.* 1995, Vaira 2001, Rodrigues *et al.* 2007) has confirmed and extended their early findings.

Phyllomedusa mate in trees and bushes above ponds and ditches. The eggs (clutch size: mean 395 – see Results) are deposited as a mass on to a leaf or leaves which the adults fold, enclosing the eggs more or less completely. In addition to eggs, the female deposits eggless jelly capsules as solid plugs at the top and bottom of the egg mass, and individually amongst the eggs. The eggs are large (2.3 mm diameter in *Phyllomedusa trinitatis*: Nokhbatolfigoghahai *et al.* 2005) and develop over several days to an advanced stage at hatching when the lower jelly plug liquefies, allowing the larvae to drop into the water below, all tadpoles exiting the nest together, or over a short period (Agar 1910, Kenny 1966, 1968).

Both Budgett (1899) and Agar (1910) noticed that *Phyllomedusa* eggs swell considerably during development and Agar deduced that the source of the fluid must be the eggless capsules,

which decreased in size during incubation. This suggestion was later confirmed experimentally by Pyburn (1980). A curious feature noticed by Budgett (1899), in comparison to other amphibians, is that *Phyllomedusa* eggs do not develop well in water. Budgett reported that newly-laid eggs died “immediately” when placed in water, whereas others incubated in moist conditions developed well for some days. Pyburn (1980) reported that eggs did not die immediately in water, but that development proceeded only for a short time.

The timing of hatching and emergence from nests in amphibians which breed out of water is of considerable interest. A series of reports (e.g. Warkentin 1995, Gomez-Mestre *et al.* 2008) has shown that in the phyllomedusine frog *Agalychnis callidryas* (Cope 1862), where eggs are deposited on leaves and left open, egg predation by snakes stimulates early hatching (allowing many to escape predation), whereas in unpredated nests, hatching occurs at a later stage: more fully developed hatchlings are better equipped to evade aquatic predators. Flooding can also act as a stimulus for premature hatching (Gomez-Mestre *et al.* 2008).

The enclosure of the egg clutch within a leaf makes it possible for hatching of individual eggs in *Phyllomedusa* to be separate in time from larval emergence from the nest: this factor makes the whole process potentially quite different from *Agalychnis*, where larvae enter water as soon as they hatch. Kenny (1968) noted that emergence from the nest sometimes occurred over a considerable period, but suggested that simultaneous hatching of a complete clutch could have an aquatic predator satiation effect allowing survival of the majority. Another possible factor in the timing of hatching is the availability of water. As in many amphibians, *Phyllomedusa* breeding is related to wet weather, but development to hatching takes several days and the pool or ditch can dry up in that time: it could therefore be adaptive for hatching to be triggered by rainfall events.

The work reported here focuses on several aspects of *Phyllomedusa trinitatis* nests and hatching. We provide a quantitative analysis of eggs, jelly capsules and jelly plugs, and a histochemical comparison of capsules and plugs. Enclosure of the egg clutch by a folded leaf or leaves separates the eggs from the environment, protecting them from dehydration and possibly from predation, but likely restricting respiratory gas exchange. Respiratory activity of the living leaf might mitigate this problem, and we tested this possibility by comparing incubation in living and dead leaves. We also assessed nest predation occurrence. The high mortality of *Phyllomedusa* eggs in water is somewhat surprising, given that incubation in water is the norm for amphibian eggs and we assessed whether aquatic development could be improved in balanced salt solutions. Finally, we investigated stimuli for hatching and emergence from the nest: are *Phyllomedusa* embryos responsive to external stimuli, like *Agalychnis*, or does enclosure in a leaf insulate the hatching process from external influences?

Materials and Methods

General Methods

Nest collection and maintenance.— *Phyllomedusa trinitatis* nests were collected from locations in northern Trinidad, principally the William Beebe Research Station (Simla), Verdant Vale, Arima Valley, a roadside ditch on the Lopinot Road north of Surrey Village and a large flooded area at Sunset Drive off the Lopinot Road. At Simla, nests were found overhanging three sets of artificial ponds designated as upper front (in the front garden of the research station); upper back (by the kitchen); lower (a set of four concrete ponds in the forest below). The lower pond site is heavily shaded compared to the others. Nests were observed and collected June–August over the years 1994, 1996, 2008, 2010, and 2011. Depending on the observations to be made, nests were marked and left in position, or

collected by cutting the stems of the leaves enclosing the nests and transferring them to the laboratory in a 2 L polyethylene container containing a little water. Nests were then incubated outside or in the laboratory (at Simla or at the University of the West Indies, St Augustine) at ambient temperature (approximately 28°C) either in 2 L containers with moist absorbent tissue on the bottom, or suspended just above such a container containing water to catch the eventual hatchlings. In a few cases, nests were suspended upside down i.e. with the top jelly plug at the bottom in order to test whether the jelly plugs differed in their properties relative to hatching. Any hatchlings not required for further observations were returned to their original locations. Nest collection was permitted by the Trinidad Government's Wildlife Section, Forestry Division.

Developmental staging and fixation.— Embryos and larvae required for assessment of developmental stages were fixed in Bouin's fluid or 10% formol-saline, then later staged using Gosner (1960). Kenny (1968) described the developmental stages of *Phyllomedusa trinitatis* and we found this helpful for stage recognition. However, we report the stages according to Gosner for ease of comparability.

Tissue analysis.— Bouin and formol-saline fixed tissues were processed for paraffin wax histology using standard methods. Sections cut at 7 µm were stained using a combined Alcian blue–PAS procedure (Bancroft and Gamble 2008) with Meyers haematoxylin as counterstain. Sections were examined using a Leitz microscope over a range of magnifications. Glutaraldehyde fixed specimens were processed for scanning electron microscopy (SEM) by standard methods, as described in Nokhbatolfoghahai and Downie (2007). Images were edited using Adobe Photoshop V7 software.

Data analysis.— Statistical analyses including student's t tests, Mann-Whitney U tests and ANOVA were performed using Minitab version 15 or SPSS version 16.0.

Experiments and Observations

Nest descriptions, dissections and fixation.— A sample of collected nests ($N = 18$) was examined for extent of coverage of eggs by leaves and other features. Another sample of nests ($N = 5$) collected 1–2 days (Gosner stage 16 after about two days development) after oviposition (assessed by checking that eggs were still at early stages of development) were dissected to measure their different components. Each nest was removed from its surrounding leaves and weighed intact (on an electronic balance to 0.01 g). Top and bottom jelly plugs were removed and weighed separately. Eggs and single jelly capsules were separated using watchmakers forceps and the total mass of eggs and capsules weighed separately. During the separation of eggs and capsules, any obvious patterns of capsule distribution were noted. Samples of top and bottom jelly plugs, and single capsules both from such early stage nests and nests closer to hatching were fixed in Bouin's fluid or formol-saline for histology.

Effect of leaves on incubation, hatching and emergence; assessment of predation.— To test whether living leaves were important to the incubation process, we needed a location where we could regularly monitor incubation and hatching in intact nests. This was possible in 2008 at Simla, where adequate numbers of nests were produced at the upper front and back, and lower ponds. After deposition, nests were left intact, but a container was placed below each nest with enough water to collect the hatchlings. Nests were checked morning and evening and sometimes more frequently. Nest height, number/type of leaves and approximate coverage of the eggs were all noted. These were the control nests: the sample was accumulated over about five weeks.

Experimental nests were collected from several sites, including Simla. The stems of all leaves were cut and each nest suspended from a wooden platform constructed at Simla lower pond i.e. all experimental nests were incubated

at the same location, close to many of the control nests. A collecting water container was located below each nest. The experimental nest sample was collected over the same time period as the controls.

For both controls ($N = 11$) and experimentals ($N = 12$) the pattern of hatchling emergence was recorded. Samples ($N = 10$ – 16) of early and later emerging hatchlings were fixed in formol-saline to assess their Gosner (1960) stage and size (total length to 0.1 mm using a binocular microscope and eyepiece scale). Once emergence was complete, the number of live hatchlings was counted plus the number of unhatched eggs/embryos, both in the water and remaining in the leaf nest. Air temperature at the incubation sites was recorded using a digital thermometer accurate to 0.1°C morning and afternoon. Control nests from this experiment plus other marked nests left *in situ* at Lopinot Road and Sunset Drive (2011) were checked for evidence of nest predation. We looked for signs of opening the nests and substantial loss of eggs/embryos: we would not have detected loss of single eggs.

Incubation of eggs in different aquatic media.—Incubation was carried out in the laboratory in 2 L polyethylene containers each holding 500 ml of medium at a depth of 1.5 cm. The medium was continuously aerated via an airstone. The media were: (1) de-chlorinated tapwater; (2) frog Ringer; (3) 100% Holtfreter's solution; (4) 10% Holtfreter's solution (New 1966). This range of media was chosen to assess whether incubation could be more successful in balanced salt solutions than in water alone. Each container held 10 *P. trinitatis* eggs removed singly from newly deposited nests ($N = 5$) or from nests incubated for four days ($N = 2$): for each nest, we set up one container for each of the four media: one newly deposited nest was incubated in the laboratory and used as a source of four day eggs. Development was assessed after two, three, four, five and six days. As controls, clumps of eggs from each nest were incubated on the surface of damp tissue in closed 2 L polyethylene containers. Some specimens

were fixed in formol-saline for microscopical examination and Gosner stage determination.

Incubation of eggs in air on substrates of different levels of hydration.—Incubation was carried out in the laboratory in 9 cm diameter plastic petri dishes each with a layer of absorbent tissue on the bottom. Each dish received 4, 6 or 10 ml de-chlorinated tap-water and five individual eggs from day 1 *P. trinitatis* nests ($N = 3$). For each nest, five dishes were set up for each treatment. Development was assessed after six, seven, eight and nine days. In some cases, embryos and hatchlings were fixed in formol-saline for morphological examination. In one case, egg diameter was measured using callipers accurate to 0.1 mm to assess any diameter changes. Eggs remaining in the parent nests were incubated in the laboratory on damp tissue in 2 L polythene containers with loose fitting lids, and acted as controls.

Water as a stimulus for hatching.—To test whether rainwater was absorbed by nests, and whether it can stimulate hatching, de-chlorinated tap water was dripped down the centre of the main leaf on to the top jelly plug of eleven suspended nests. We used two successive batches of 20 ml water, taking about 10 seconds to pour, five minutes apart; five nests were at days 2–3 incubation, six were close to hatching, about day 6. Nests were suspended such that the top jelly plug upper surface was horizontal. A beaker collected any water emerging after this procedure. The amount of water collected was used to test whether nests absorbed water, and whether absorption ability differed between early and late nests. The timing of hatching was noted in the late stage nests.

Observations on embryos around the time of hatching; hatching and emergence in whole nests.—At five and again at six days of incubation, 20 eggs from one clutch were carefully removed into a petri dish and observed using a dissecting microscope to assess movements of embryos and positions of the external gills. At seven days, eggs were removed from the same nest for observation; eggs were

also removed from a different nest and placed in 9 cm diameter plastic containers on three different substrates: dry plastic, plastic covered by damp tissue (8 ml de-chlorinated tap water), plastic immersed to a depth of 1.5 cm in water. Each container held 10 eggs; there were three containers with each substrate. Containers were covered and eggs observed after 30, 60, 90, 120 and 150 min to assess the progress of hatching. Hatching times were also noted in the nest from which these eggs were taken. A sample of 20 eggs from this clutch was also artificially hatched, by gently puncturing the outer capsule of each egg under water. These hatchlings were fixed in glutaraldehyde after 20 ($N = 10$) and 50 ($N = 10$) minutes. At the same time as the 50 minutes group, 10 unhatched eggs from the parent clutch were also fixed. Fixed embryos were later observed using a dissecting microscope (after removing outer capsules from unhatched eggs) to assess the morphology of the external gills. External gills were cut from the body, and using an eyepiece graticule, measurements were made of ramus length and three randomly chosen filaments for each gill. Measurements were made only on a small sample since the remaining specimens were needed for scanning electron microscopy where it was better for the gills to remain attached.

In order to follow the process of hatching and emergence in complete clutches, leaves were carefully removed either partially or completely from nests at different stages of development. When leaves were very firmly stuck to the clutch, complete removal would damage some eggs, so some leaf area was retained. Each clutch was then wrapped in clingfilm with the top and bottom remaining open. A plug of damp tissue was placed on the top jelly plug to prevent complete closure of the clingfilm. Each wrapped nest was suspended by means of a clip from a tripod of sticks above a tub of dechlorinated tap water (Figure 1). Initially, we attempted to follow hatching and emergence by time lapse filming. However, picture resolution was poor and it proved preferable simply to watch nests around the time of hatching.



Figure 1. Three *Phyllomedusa trinitatis* nests with complete clutches transferred to clingfilm covers and suspended above water.

Results

Nest Parameters, Positions, Leaf Arrangement, Predation, Sizes, Components

We found nests 3–300 cm above water (mean 102.2 ± 77.1 SD, $N = 24$). The Lopinot Road ditch had little overhanging vegetation and the 3 cm height nest was constructed there from many narrow blades of grass at the edge of the ditch. Most nests were made in low shrubs or vines, but the highest was in a tree overhanging Simla lower pond. There was no consistency in leaf type or number per nest: frogs appeared to make nests from whatever leaves were available at their chosen location. In a sample of 17 nests where we counted the number of leaves used, 35% used 2, 41%–3, 12%–4 and 12% more than four (these used grass or bamboo blades). The most completely enclosed nests were made by folding large single leaves. When more, smaller leaves were used, nests tended to be more exposed to the air, occasionally up to 50% of the nest surface area. In a sample of 17 nests where we estimated the percentage coverage of the egg clutch, the mean coverage was $82.6\% \pm 9.9$ SD. Generally, the top jelly plug was open to the atmosphere

while the bottom plug was usually well enclosed. Although most nests were made from leaves directly above water, this was not always the case, with nests located as far as 1 m away from the water's edge. In the 23 nests marked and left *in situ* at three different locations, we saw no evidence of predation.

All nests had top and bottom jelly plugs, and clear eggless capsules scattered amongst the creamy-white eggs: there were no obvious accumulations of capsules close to the leaf surface. Mean ($N = 5$; \pm SD) nest components were: total wet mass (g) 19.4 ± 2.5 ; top plug 2.5 ± 1.1 ; bottom plug 3.5 ± 0.6 ; eggs 8.2 ± 1.8 ; eggless capsules 4.5 ± 1.0 . Component proportions were quite consistent across this sample, with bottom plugs heavier than top plugs in all but one case. In the larger sample transferred to clingfilm parcels, we noticed more variability, especially in the bottom plugs, sometimes small with eggs close to the base of the nest (Figure 2). Dried samples of jelly plugs and yolkless capsules contained 3–4% dry matter i.e. are 96–97% water in the fresh state. The number of eggs per clutch ranged from 109–629 (mean 395.6 ± 130.7 SD, $N = 23$).



Figure 2. Three *Phyllomedusa trinitatis* nests with part of leaf covering removed, to show variation in clutch size and jelly plugs.

We followed changes in egg diameter in fixed specimens during days 1–6 of incubation (Table 1). Data are for the outermost diameter, including the thin jelly coat, since this size is easily measurable. Jelly coats were about 0.15 mm thick at day 1 i.e. up to 24 hours after deposition), so eggs early in development had a diameter of about 2.5 mm.

We stained wax sections of eggs and jelly plugs with a combined periodic acid-Schiffs (PAS) and alcian blue (AB) technique capable of discriminating between different types of mucopolysaccharide. Jelly plugs were clearly composed of capsules embedded in a continuous matrix (Figure 3). Capsules each had a dense centre formed of skeins of tangled fibrous material, with the periphery formed of less dense, more granular or amorphous material. The surrounding matrix did not have a fibrous appearance. The capsules were AB positive with the matrix PAS positive. There was no obvious difference between the top and bottom plugs, or between plugs near the start and towards the end of incubation. Eggs had a thin outer covering of fairly fibrous mainly PAS positive material.

Does it matter to hatching success whether the leaves are alive or dead, or which may up the nest is?

Control nests at the well shaded Simla lower pond experienced temperatures ranging from 24–32°C; the remaining control nests at Simla upper ponds were also mostly in the shade with daytime temperatures somewhat higher than at the lower pond. Temperature differences may therefore have influenced incubation durations but we did not measure temperatures continuously. At the end of the incubation period, leaves of control nests were still alive and healthy; leaves of experimental nests were generally dry and withered, clearly dead.

Control nests ($N = 11$) had mean (\pm SD) clutch sizes of 419.5 ± 130.0 and mean proportions of unhatched eggs $12.9 \pm 12.7\%$. Experimental nests ($N = 12$) had clutches of

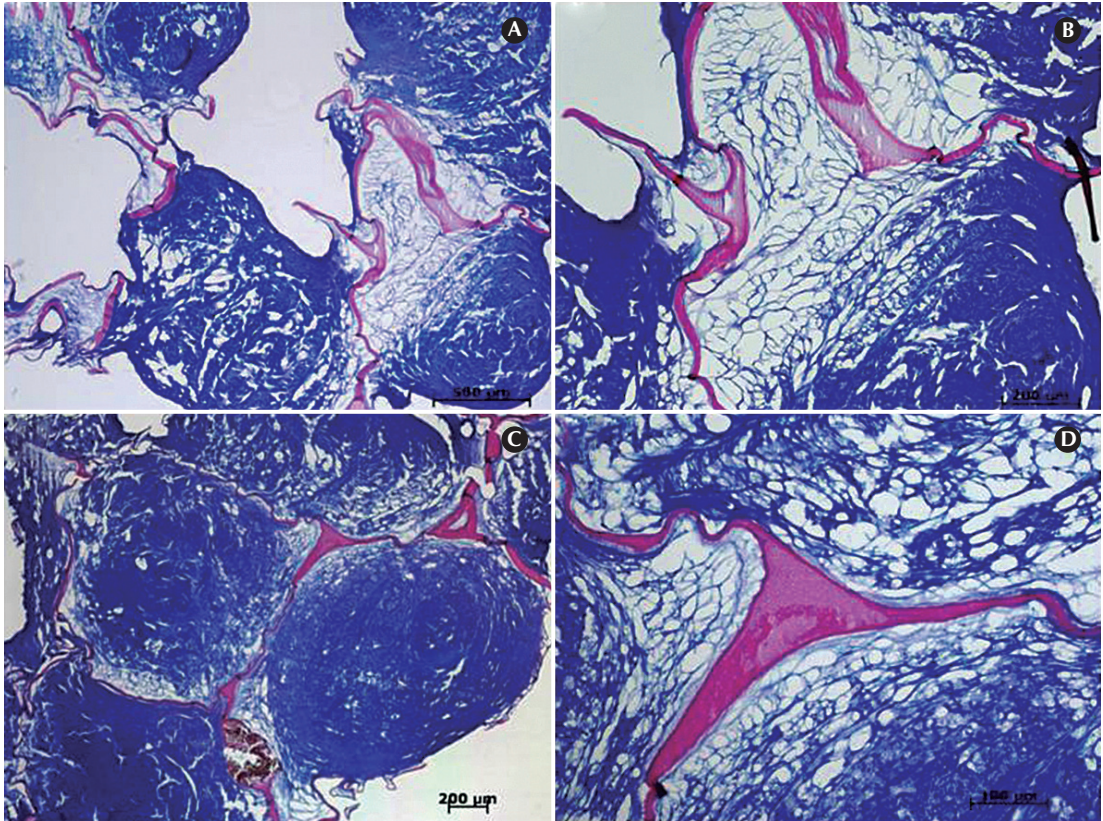


Figure 3. Alcian blue–PAS stained sections of eggless capsules of *Phyllomedusa trinitatis* within jelly plugs at low (A, C) and higher (B, D) magnifications, to show the embedding matrix and the fibrous nature of the capsular material at early (A, B) and late (C, D) stages of incubation. Embedding matrix stained magenta; capsules blue.

373.7 ± 133.1 and unhatched eggs $20.4 \pm 16.1\%$. Clutch sizes in the two groups were not significantly different (two-sample t-test: $t = 0.83$, $df = 20$, $p = 0.414$ NS). Although the proportion of unhatched eggs was higher in experimentals than in controls, this difference was not significantly different (arcsine transformed percentages; two-sample t-test: $t = 1.36$, $df = 20$, $p = 0.188$ NS).

Table 2 shows size (total length) and Gosner stage at emergence in controls and experimentals. In some nests in both groups, small numbers of hatchlings emerged some time before the others:

for example, in one experimental nest, four emerged by 21:00 h another 12 by 10:00 h the next morning; no more had emerged by 21:00 h that night but the remainder, over 200, had emerged by 09:00 h the next morning; in other nests, the whole clutch emerged over less than one hour. The longest period between first and last emergence was about two days (three out of 23 nests); four out of 23 took about one day; the remainder less than one day. In both control and experimental groups, early emergers were significantly smaller than late emergers (student's t tests: controls $t = 2.13$, $df = 14$, $p = 0.052$;

Table 1. *Phyllomedusa trinitatis* egg diameters after different incubation durations.

	Mean egg diameter \pm SD in mm (N) after different incubation durations			
	1 day	3 days	5 days	6 days
Nest 1	2.7 \pm 0.1 (4)	2.8 \pm 0.1 (6)	–	3.4 \pm 0.1 (5)
Nest 2	2.9 \pm 0.1 (6)	3.0 \pm 0.1 (4)	3.3 \pm 0.1 (7)	3.7 \pm 0.1 (8)
Nest 3	2.9 \pm 0.1 (14)	2.9 \pm 0.1 (4)	3.1 \pm 0.1 (6)	3.3 \pm 0.0 (2)

Table 2. Stage and total length of hatchlings of *Phyllomedusa trinitatis* from control and experimental nests. N = number of nests. In each nest, 6–10 hatchlings measured and mean stage/ length calculated.

Category	N	Mean Gosner stage \pm SD	Mean total length \pm SD (mm)
Control: early emergers	5	24.4 \pm 0.8	12.4 \pm 0.8
Control: final emergers	11	24.9 \pm 0.2	13.4 \pm 0.8
Experimental: early emergers	5	24.2 \pm 0.6	12.9 \pm 0.4
Experimental: final emergers	9	25.0 \pm 0.1	13.8 \pm 0.5

experimentals $t = 3.0$, $df = 12$, $p = 0.009$). A significant difference in Gosner stage at emergence was observed in experimental clutches (Mann-Whitney U test: $U_{5,9} = 1.0$, $df = 14$, $p = 0.002$) but not in control clutches ($U_{5,11} = 16.5$, $df = 16$, $p > 0.05$). In neither early nor late emergers were there any significant differences in stage or size between controls and experimentals (two-sample t-tests: early stage controls vs. experimentals, $p = 0.725$; late stage controls vs. experimentals, $p = 0.432$; early length controls vs. experimentals, $p = 0.310$; late length controls vs. experimentals, $p = 0.232$). Note that the vast majority of late emergers and many early emergers were already at stage 25 (complete resorption of external gills).

A possible difference between controls and experimentals could be in the synchronicity of emergence. We did not know the precise time

span between first and last emergence in all nests, but it was possible to classify these times into short (a few hours), medium (more than a few hours, but less than a day) and long (more than one day) for five control nests and five experimentals. There was no significant difference ($\chi^2 = 2.0$; $p > 0.1$), with short, medium and long spans occurring in both groups, but the sample size was small.

When a nest was suspended upside down i.e. with the top jelly plug below, emergence occurred normally. This may not be surprising, given that examination of a hatched nest shows that both jelly plugs are liquefied during the emergence process, indicating that they are structurally identical. Jelly isolated from a nest at an early stage and incubated on damp tissue in a petri dish did not liquefy even after two weeks.

Do nests absorb rainwater, and does additional water stimulate hatching?

When water was dripped slowly on to the horizontal top jelly plugs of early stage nests ($N = 5$) it spilled over the sides and was collected below: there was no evidence of any water absorption. When the same experiment was done on late nests ($N = 6$, all at stage 23, when hatching of individual eggs can be stimulated by water: see below) again negligible water was absorbed. There was no evidence for emergence being brought forward in this experiment, since it occurred either several hours afterwards, or the next day. It is worth noting here that the folding of the leaf often shields the upper jelly plug from direct exposure to rainfall.

How well do isolated eggs develop in water with different ionic balances?

In eggs incubated in different media, although there was some variation between eggs from different nests, a general pattern emerged (Table 3). When eggs were placed in medium about half-way through development, their probability of continuing to hatching was high, but eggs placed in medium at earlier stages developed poorly, with very few reaching hatching and generally abnormal in various ways if they did hatch. Hatching generally occurred prematurely, sometimes as early as Gosner stage 20, but mostly around stage 22 and never as late as stage 25. Of the four media used 10% Holtfreter supported development of both early and late eggs slightly better than the others. Dead eggs were often attacked by fungus, but it was not clear if the fungus caused death, or only attacked eggs already dead. Control clumps of eggs developed normally and hatched about one day earlier than successfully developing eggs in water.

How well do isolated eggs develop in air, and does substrate hydration matter?

Isolated eggs from three early stage nests were incubated on substrates of three hydration levels. Survival rates were high (Table 4). The clearest result is that hatching in isolated eggs was delayed compared to the nest they were taken from, generally by about a day. For nest 1, we fixed a sample of hatched and unhatched embryos at day 8, to check if there was any clear difference in developmental stage. In both groups, most were at Gosner stage 25 (hatched 94%; unhatched 90%) with the remainder at stage 24: there was no difference between hatched and unhatched individuals. There was no consistent difference in hatching pattern between the three hydration levels: for example, in nest 1, more hatched relatively early on the 10 ml substrate than on 4 ml, but the reverse was the case in nests 2 and 3. We measured egg diameters only for nest 3. Eggs absorbed more water and therefore swelled to a larger diameter on the 10 ml substrate than on 6 ml, with the 4 ml batch least swollen. For the different substrates, egg diameters were significantly different both at 4 and 6 days (day 4: $F_{2,59} = 72.5$, $p < 0.001$; day 6: $F_{2,55} = 102.7$, $p < 0.001$. Post-hoc Tukey tests confirmed that all three treatments differ from one another). For the 4 ml substrate, there was no increase in egg diameter between days 4 and 6; but for both the 6 and 10 ml substrates, egg diameters increased significantly over that time (6 ml: $t = 4.14$, $p < 0.001$, $df = 36$; 10 ml: $t = 5.09$, $p < 0.001$, $df = 43$).

What is the proximate stimulus for hatching, and how do the embryos change around the time of hatching?

By five days of incubation, external gills were long and extensive, held close to the inner wall of the capsule. Embryos were quite active: we counted the number of twitches (rapid flexing

Table 3. Development of isolated eggs of *Phyllomedusa trinitatis* in different aquatic media (d = days; st = Gosner stage). All percentages based on starting number.

Set up with early stage eggs, days 1–2		
Number of eggs (nests)	Medium	Result
50 (5)	De-chlorinated tapwater	Two nests, 90% eggs dead by d4; 15% developed to st 19. Three nests, 63% developed well to d4, about st 22; 10% hatched prematurely st 21–2.
	Frog Ringer	Two nests, 95% reached st 19 then died; one, all died by st 19; two nests, all died before st 17.
	100% Holtfreter	76% of eggs in all nests developed to st 17–22 and 32% hatched prematurely around st 22. Poor survival after that.
	10% Holtfreter	In all nests, most eggs (74%) developed normally until st 20 and 56% continued to st 22, with 20% hatching prematurely at that stage: a few still alive by day 6.
Set up with well-developed eggs, day 4		
Number of eggs (nests)	Medium	Result
20 (2)	De-chlorinated tapwater	High hatching success 70%, essentially normal, but a little premature
	Frog Ringer	Moderate hatching success, prematurely, 45%, but with most developing fairly well.
	100% Holtfreter	Mixed result: poor development of eggs from one nest, none hatching; but 100% hatching, a little prematurely, from the other.
	10% Holtfreter	High hatching success, a little prematurely, 65%.

of the body) in a group of 20 embryos over 60 seconds: on average there was one per minute per embryo. Each twitch led to a re-arrangement of the external gills. By day 6, twitch frequency had increased to 4.2 per minute per embryo. By day 6 the outer capsules had become very thin and taut, easily burst by touching with forceps.

To test the proximate stimulus for hatching, we carried out two experiments. First, two clumps of eggs were carefully removed from a nest close to hatching (seven days of incubation) and placed on separate leaves. A single egg was punctured in clump A; clump B was left intact.

In clump A, embryos began to wriggle vigorously and nearly synchronously within their capsules at 15 to 25 second intervals. After 4 minutes, 18 had hatched and after 10 minutes, a further three, leaving 13 unhatched in the clump. None of the eggs in clump B had hatched by this time, and wriggling of embryos was not synchronous, nor had hatching occurred in the parent nest. Second, 7 day eggs from a single nest were carefully isolated and placed on one of three substrates (eggs $N = 30$ on each): dry plastic, damp tissue and plastic under water. The numbers hatching over the following 150 minutes were as follows:

Table 4. Survival, size and hatching results for experimental eggs of *Phyllomedusa trinitatis* ($N = 25$ for each treatment and each nest) incubated individually on substrates at different levels of hydration (d = days; ml = millilitres). Hatching times of nests that the experimental eggs were taken from (source) are given in the left-hand column.

Nest	Parameter	Substrate hydration		
		4 ml	6 ml	10 ml
1	% survival to day 8	96	92	96
source	% survivors hatched by d 7 am	0	0	13
hatched by	% survivors hatched by d 7 pm	0	0	17
day 7 am	% survivors hatched by d 8 am	67	57	83
2	% survival to day 9	96	88	88
source	% survivors hatched by d 8 am	8	14	14
hatched by	% survivors hatched by d 8 pm	63	64	27
day 8 am	% survivors hatched by d 9 am	100	100	100
3	% survival to day 9	68	76	92
source	% survivors hatched by d 8 am	76	11	30
hatched by	% survivors hatched by d 8 pm	82	47	70
day 8 am	% survivors hatched by d 9 am	100	84	100
	Mean \pm SD egg diameter (mm) at day 4 (N)	4.2 \pm 0.2 (20)	4.5 \pm 0.2 (19)	4.8 \pm 0.2 (23)
	Mean \pm SD egg diameter (mm) at day 6 (N)	4.2 \pm 0.2 (17)	4.8 \pm 0.2 (19)	5.2 \pm 0.2 (22)

dry substrate, none; damp substrate, one only; immersed—11 eggs had hatched after 30 minutes, a further two after 60 minutes and two more after 150 minutes. The nest from which these eggs were taken hatched the following day, indicating that immersion induced premature hatching in 50% of the eggs tested. A group of these 7 day eggs were hatched by gently puncturing their capsules, then transferred to water. Sub-samples were fixed in glutaraldehyde after 20 and 50 minutes in water; at 50 minutes, a group of embryos from the original clutch were also fixed for comparison. The difference in the external gills between artificially hatched

and unhatched embryos, even as early as 20 minutes was striking (Figure 4 A, B). In unhatched embryos, the main gill ramus was long (mean 1.7 mm \pm 0.5 SD, $N = 3$) with numerous long (mean 1.3 mm \pm 0.6 SD, $N = 3$) thin filaments. In hatched embryos, the gills were short and stubby (ramus lengths 0.8, 0.7 mm, $N = 2$; filaments 0.45, 0.48 mm, $N = 2$).

Examination of the heads of these late stage embryos using SEM showed large numbers of small microvillated cells, fairly evenly dispersed among the common epidermal cells (CEC) of the laterodorsal surface, just anterior to the eyes, ventral to the nostrils, and extending above the

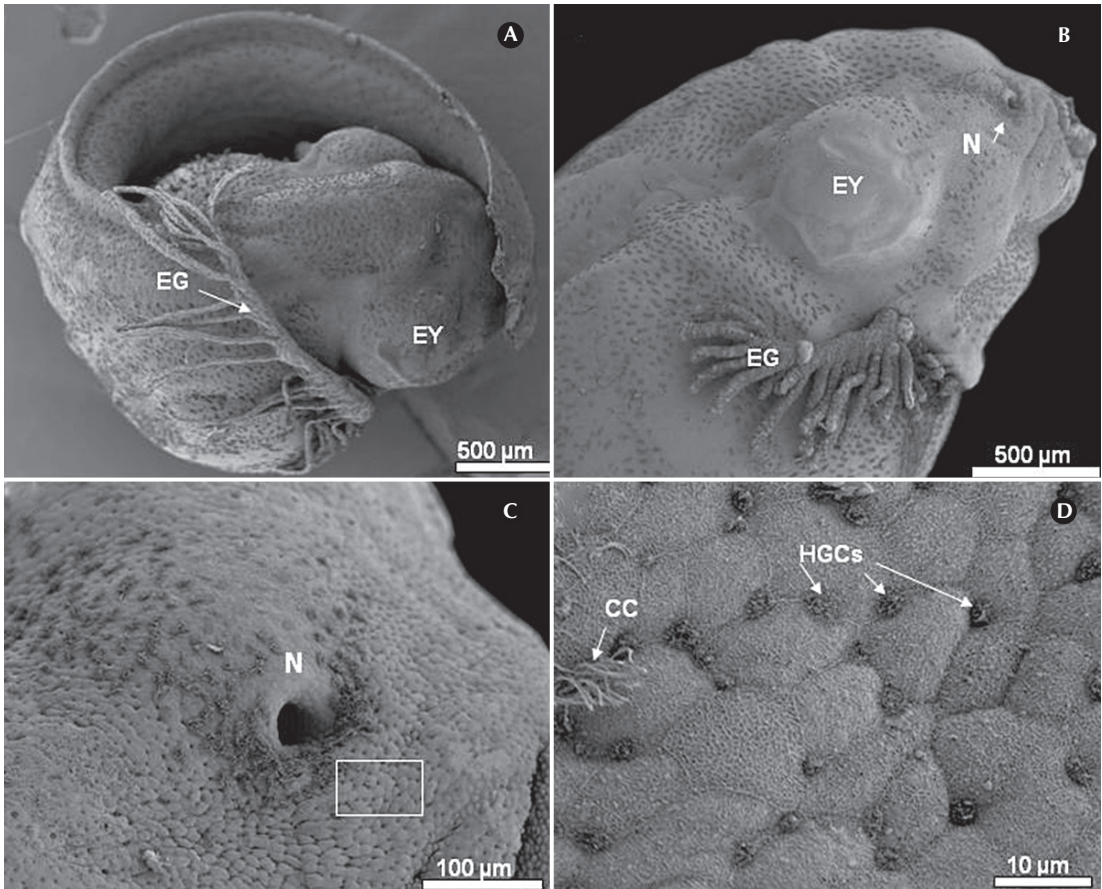


Figure 4. Scanning electron micrographs of *Phyllomedusa trinitatis* embryos: (A) pre-hatching 5–6 days incubation: capsule removed after fixation to show appearance of external gills, (B) fixed 20 minutes after artificial liberation from capsule into water. (C) Nostril region of embryo shown in B; boxed area enlarged in D. (D) high resolution view of boxed area in C. Cell types visible are mainly common epidermal cells (CEC), with one ciliated cell and many hatching gland cells at CEC boundaries. EG, external gill. EY, eye. N, nostril. CC, ciliated cell. HGCs, hatching gland cells.

mouth towards the midline (Figure 4 C, D). These have the morphology characteristic of hatching gland cells (HGC).

Hatching and Emergence

We wrapped 15 clutches in clingfilm. When we did this very early (day 1–2), development was slowed down and if too late (day 6), liquefaction of the jelly capsules had already

occurred and the egg mass did not adhere well to the clingfilm. However, where the transfer from leaf nest to clingfilm was made about half-way through incubation ($N = 11$), hatching and emergence occurred normally and approximately on schedule. The most clear results came from watching three clutches hatching and timing the events. The pattern was similar in each case. The sequence of events in one clutch was as follows:

22:05 h	Within the egg mass, a few scattered eggs have hatched. A few drops of liquid fall from the base of the nest. Over the next 15 minutes, many more scattered individuals hatch. Intense short bouts (1–2 seconds) of wriggling occur involving most of the hatchlings over intervals of 10, 10, 17, 9, 7, 28, 4, 24, 20, 11, 8 and 18 seconds covering a 3 minute period.
22:25 h	The first hatchlings drop into the water below.
22:30 h	Bottom jelly plug has largely broken down. Four hatchlings have emerged. Within the egg mass, the fluid is frothy as a result of hatchling wriggling.
22:35 h	Wriggling bouts, now involving many more hatchlings occur at 19, 33, 18, 18, 18, 13, 22, 23, 23 second intervals over 3 minutes.
22:38 h	16 now emerged.
22:40 h	40 now emerged. Hatching within the nest is now complete. With each bout of wriggling more hatchlings slide downwards in a mass of frothy liquid.
22:45 h	A large number of hatchlings leave the nest together.
23:30 h	Only scattered hatchlings remain in the nest.
09:00 h	Next morning, only 4–5 live hatchlings remain in the nest.

Discussion

Nest Formation and Structure in Phyllomedusa

Our observations on *Phyllomedusa trinitatis* nests mainly concur with those of Kenny (1966). Nests were found at very variable heights above water and made of many leaf species and sizes, some composed of a single leaf, fully enclosing the eggs, others of several leaves, such as narrow blades of grass, with some of the clutch exposed. Low level nests using grass blades were made only in the absence of more suitable leaves. We found no predation on *P. trinitatis* nests, but other studies have related predation levels to the number of leaves used to construct the nest. Neckel-Oliveira and Wachlevski (2004) found nest predation levels lowest where nests were composed of larger numbers of leaves (*Phyllomedusa tarsius*) and highest in nests of single leaves (*Phyllomedusa tomopterna*), though *Phyllomedusa bicolor* with 1–4 leaves also had high rates. Rodrigues *et al.* (2007) found high predation rates with *Phyllomedusa sauvagii* nests of multiple leaves and low rates with *Phyllomedusa azurea* single-leaf nests. The critical factor may be the proportion of the egg clutch exposed. Neckel-Oliveira and Wachlevski (2004) found staphylinid beetles and phorid flies to be the main causes of complete clutch loss. None of our marked clutches was lost completely, but phorid flies capable of destroying nests are present in Trinidad. In the laboratory, we occasionally left opened nests in containers with small holes in the lids and some of these contained phorid fly maggots after 2–3 days (JRD: laboratory notes).

Kenny (1966) considered *Phyllomedusa trinitatis* a low altitude species, rarely found over 100 m above sea level: however, the populations we studied at Simla and in the Lopinot valley were at about 200 m. Townsend *et al.* (2006) found an adult female at 832 m on Morne Bleu Ridge in the Northern Ridge, but saw no evidence of breeding at this altitude: the frogs may range widely. Our clutch sizes were

similar to Kenny's (1966) who reported a range of 300–600 eggs from three nests. Our larger sample had a mean of 396 eggs per nest. One surprising difference is in the distribution of jelly capsules. Kenny (1966) reported that nests normally had an upper and lower jelly plug, the upper the larger (opposite to our finding), but that in one nest, upper and lower plugs were missing, with jelly capsules scattered amongst the eggs. This implies that Kenny did not generally find jelly capsules among the eggs, quite contrary to our finding, where 43% by mass of the jelly was scattered among the eggs as individual capsules. Pyburn and Glidewell (1971) found that in *Phyllomedusa hypochondrialis* individual capsules were deposited in a definite pattern, mainly along the outer side of the egg mass, forming a protective seal. Agar (1910) described top and bottom jelly plugs and scattered capsules (in no obvious pattern) in *Phyllomedusa sauvagii*. However, Lescure *et al.* (1995) reports top and bottom jelly plugs in *Phyllomedusa vaillantii*, but no individual capsules among the eggs, so this may be a feature that varies between species and, if Kenny's (1966) observations can be confirmed, between individuals.

While we were completing this manuscript, Alcaide *et al.* (2011) published a histological/histochemical study on the oviducts and eggless capsules of *Phyllomedusa sauvagii*: as far as they and we are aware, the capsules have not previously been examined in this way. Our results indicate that the plugs are largely composed of jelly capsules, but are not simply capsules compressed together, as Agar (1910) surmised. They are capsules embedded in a matrix. We used the combined Alcian blue–PAS staining method (Bancroft and Gamble 2008) which can distinguish between acid and neutral polysaccharides. The matrix did stain differently from the capsules and was more amorphous, the capsules having a fibrous appearance. The thin jelly layers surrounding individual eggs stained similarly to the matrix. There was no difference in histological appearance between top and

bottom jelly plugs, nor plugs near the start and end of incubation, and our experiment with inverted nests indicates that both can dissolve to allow emergence of hatchlings. Alcaide *et al.* (2011) also noticed the fibrous nature of the capsular material (they term these “vesicles”), but appear not to have examined the jelly plugs.

Why do Phyllomedusa eggs develop poorly in water?

Budgett (1899) reported that *Phyllomedusa* eggs died “immediately” in water. Pyburn (1980) incubated eggs in pond water from Gosner stage 18 and 20. Stage 18 embryos did not advance beyond stage 19, and all died within 52 hours; stage 20 embryos did not advance beyond late stage 20. At first sight these results are surprising, given that most amphibian species deposit their eggs in freshwater where they develop normally. Two possible factors occurred to us. First, neither Budgett (1899) nor Pyburn (1980) report aerating the water: since *Phyllomedusa* eggs are large and yolky, they may be particularly susceptible to low oxygen levels. Second, associated with terrestrial incubation, the jelly coat and vitelline membrane of *Phyllomedusa* eggs may have altered osmotic and ion balance properties, making aquatic incubation harmful in some way. Our preliminary observations (not reported in detail) suggested that aeration of the water helped egg survival marginally, and we aerated the water in all our experiments. In reviewing methods for the culture of amphibian embryos, New (1966) noted that after the removal of outer jelly coats including the vitelline membrane, most embryos developed poorly in water and required a balanced salt solution to maintain normal development. Holtfreter's solution (100% or 10%) has been widely used for this purpose.

Our results are somewhat different to those of Pyburn (1980). Eggs transferred to any of the media used, at an early stage, developed poorly but those transferred at later stages developed much more successfully, especially in 10% Holtfreter's solution. A notable finding was that

in an aquatic medium, hatching occurred prematurely, as early as stage 20, compared to hatching in intact nests at around stage 25. These results suggest that poor development in water is the result of jelly coat changes, and that it may be possible to devise a culture medium in which *Phyllomedusa trinitatis* eggs could develop normally. Even eggs that developed successfully in water were delayed in comparison with controls in air. This could result from small temperature differences and/or differences in oxygen availability.

Does the living leaf have an active role in Phyllomedusa incubation?

Enclosing the eggs in a folded leaf or leaves may have several benefits: most likely, it reduces predation, a major problem for the open nests of *Agalychnis* (Warkentin 1995); as noted above Neckel-Oliveira and Wachlewski (2004) found relationships between leaf cover and predation level in three *Phyllomedusa* species; it also reduces the risk of desiccation, another obvious hazard for amphibian eggs in air, unless humidity is very high and precipitation frequent. However, the enclosed nature of the nest may create a problem for gas exchange. Eggs embedded in masses of jelly can suffer hypoxia, especially when incubated in water with its low oxygen content and slow diffusion rates. Seymour (1999) notes that water uptake into the vitelline space, accompanied by outer capsule thinning, aids oxygen uptake, as does spacing out of embryos, as occurs in *Phyllomedusa* through the presence of scattered jelly capsules. However, Seymour (1999) also notes that when oxygen exchange is restricted by any barriers around the egg mass, this can cause embryo mortality. Burggren (1985) showed that hypoxia is minimised by the convection currents generated by the surface ciliation characteristic of amphibian embryos (Nokhbatolfighahai *et al.* 2005). However, covering the egg mass in a leaf, with thick jelly plugs top and bottom, would seem to minimise the surfaces available for gas exchange, unless

the transpiration and respiration activities of the leaf can be utilised by the eggs. There are examples of amphibian eggs that benefit in this way: the salamander *Ambystoma maculatum* has eggs impregnated with unicellular algae which contribute oxygen during the day, but consume it at night (Valls and Mills 2007).

We expected that if the living leaf made any physiological contribution to egg incubation, cutting the leaf (or leaves) should be detrimental to incubation. However, there were no significant effects on any of the parameters we measured: proportion of unhatched/inviable eggs, size and stage at emergence, or synchronicity of emergence. Some of our sample sizes were small and it might be worth following emergence with continuous recording, but any effects must be minor. The means by which *Phyllomedusa* embryos evade problems of hypoxia need further investigation.

Hatching, Emergence, Water Relations and External Gills

Budgett (1899) and Agar (1910) noticed that as incubation of *Phyllomedusa* nests proceeds, egg diameters increase and jelly capsules diminish. Agar (1910) deduced a transfer of water from capsules to eggs and Pyburn (1980) demonstrated this experimentally, showing that water uptake is essential for successful development. We extended Pyburn's (1980) experiments by incubating individual eggs on substrates with different water availability. Water availability had an effect on water uptake, but survival levels to hatching were high at all the hydration levels tested. In all cases, hatching was delayed by about a day compared to controls. We also incubated eggs close to hatching in three different conditions: dry, damp and immersed. The results were conclusive: many immersed eggs hatched within 30 minutes; no eggs hatched after 2.5 hours in the dry and only one on the damp surface. Finally, we followed the process of hatching and emergence in a complete nest with the leaves replaced by transparent clingfilm, an

approach which appears not to have been attempted before. What do these results tell us about the mechanism of hatching in *Phyllomedusa*?

Agar (1910) noticed that as embryos approached the hatching stages, their outer membranes were so tight that a touch could burst them. When he opened nests around the time of hatching, he found hatched tadpoles in a “mucilaginous fluid”: emergence of the whole nest did not, however, occur until the lower jelly plug had softened, a time of 12–24 hours after tadpole hatching, when most hatchlings left the nest over a short period. Kenny (1968) also noted that if a late stage distended egg was burst artificially, it could set off a chain reaction, with adjacent embryos also hatching over a period of 10–15 minutes, a result similar to ours.

In most amphibians, hatching is the responsibility of the hatching gland cells (HGC), a transient population on the head and part-way along the dorsal surface of the embryo: these cells are generally active around Gosner stages 17–20 and have disappeared by stage 23/4 (Nokhbatolfoghahai and Downie 2007). However, our results make it unlikely that HGC are the immediate mechanism for hatching: rather, osmotic effects with water entering an already thin, taut sac and bursting it, explain the immersion results better. The intense wriggling movements stimulated by the hatching of an individual embryo may also play an instrumental part. Our experiments showed that the hatching of one embryo stimulated its neighbours to hatch. Once enough fluid has been released by the hatching of some embryos, these two effects combine, leading to hatching across the whole clutch. Nokhbatolfoghahai and Downie (2007) could not find HGC in *Phyllomedusa trinitatis* embryos, but here we report a population of surface cells with the morphology of HGC in a somewhat different location to the norm. Our hypothesis is that secretions from these cells are necessary for the breakdown of the jelly capsules and plugs, which we have shown do not break down spontaneously, but have a lesser role in the

hatching of individual embryos, combining with osmotic effects and the intense wriggling activities of the embryos. The delay we recorded in our clingfilm nests between initial hatching and the emergence of most of the larvae was only about 45 minutes, much shorter than the 12–24 hours reported by Agar (1910) for *Phyllomedusa sauvagii*. This may be a species difference, but we expect that there is also considerable individual variation. As we noted, the lower jelly plug can vary considerably both in thickness and in diameter, and this is likely to affect the time the plug takes to break down. The main conclusion, however, is that the mechanism of hatching/emergence ensures that most hatchlings emerge over a short period.

Warkentin (2011) has reviewed evidence that flooding can induce premature hatching in some amphibians, including the phyllomedusine *Agalychnis*. Our results show that immersion in water induces premature hatching in isolated *Phyllomedusa* eggs, but we have not tested the effect of immersion on complete *Phyllomedusa* nests. Because of the relatively high location of most *Phyllomedusa* nests above water, we suspect that flood-induced hatching is not ecologically relevant in this taxon, though it could be a survival mechanism for egg clutches that fall prematurely from their leaf covers.

Budgett (1899) reported that in *Phyllomedusa hypochondrialis* (modern distribution data suggest this was *Phyllomedusa azurea*: Frost 2011), the external gills had disappeared by the stage of hatching, but Pyburn (1980) found hatching in the same species at Gosner stage 23, when external gill regression has just begun. Lescure *et al.* (1995) found hatching in *Phyllomedusa tomopterna* at stage 23, with external gills almost entirely withdrawn. Kenny (1968) reported that hatching in *Phyllomedusa trinitatis* occurred at the stage of maximum external gill development and that gill disappearance occurs over the next 3–4 days. This is quite contrary to our findings, where most hatchlings had already withdrawn their gills and where gill shortening occurred very

quickly after entering water, perhaps as a means of protecting these very delicate structures.

Gomez-Mestre *et al.* (2008) investigated the prevalence of premature hatching in phyllomedusine frogs of the genera *Agalychnis* and *Pachymedusa* in response to two stimuli, vibration (as a cue for snake attack) and flooding. Larvae of these frogs, whose eggs are laid openly on leaves, are able to hatch 30% earlier than normal. Premature hatching in response to flooding is highly conserved in the clade, but the response to snakes varies between species, as expected from a trait with a trade-off: premature hatchlings are more vulnerable to aquatic predators than “normal” hatchlings. In *Agalychnis*, hatchlings possess external gills whether premature or at normal stage, but the gills regress very quickly after entry to water (Warkentin 2000).

Although premature hatching could be induced in *Phyllomedusa trinitatis*, we doubt if this is ecologically relevant. Our experiments suggest that the upper jelly plug acts as a barrier to the entry of water from external sources, and the delay between individual hatching and the softening of the jelly plug that allows the whole clutch to emerge and empty the nest, means that hatching in response to vibration would be unlikely to save the embryos from predation. As it happens, despite the presence in Trinidad of snakes such as *Leptodeira annulata* Garman 1887 (Colubridae), a known frog egg and adult predator (Murphy 1997) none of the 23 nests we observed over their incubation periods in the field showed evidence of predation. In contrast, if we opened nests during early stages and left them with covers allowing access to ants or flies, they were quickly attacked. However, Neckel-Oliveira and Wachlewski's (2004) findings were in sharp contrast: in three Amazonian *Phyllomedusa* species, they found mainly 29%, 59% and 61% of nests predated either partially or totally, mainly by beetles and flies. The reason for this considerable difference between their findings and ours is unclear: perhaps *Phyllomedusa trinitatis* makes better protected nests


than the Brazilian species, or perhaps potential predators are less common in the disturbed habitats in Trinidad.

Conclusion

In discussing the phylogenetic relationships of the phyllomedusine frogs, Faivovich *et al.* (2010) highlighted five key characters relating to reproduction and larval development: bladder filling in advance of oviposition, absent in *Phyllomedusa*; site of oviposition, on leaves in *Phyllomedusa*; leaf folding in *Phyllomedusa* but not in *Agalychnis*; eggless capsules in *Phyllomedusa* but not in *Agalychnis*; site of larval development. Faivovich *et al.* (2010) suggested that there is little ‘plasticity’ in *Phyllomedusa* leaf-folding behavior. This may be a premature conclusion. Some *Phyllomedusa* species seem to be selective in leaf choice, while others are able to make nests from a very wide range of leaf types, including grass blades. This could be regarded as quite “plastic” behavior and further work could reveal what factors influence it, such as clutch size and opportunity, for example. Faivovich *et al.* (2010) mention eggless capsules but not the top and bottom jelly plugs in *Phyllomedusa* nests. We regard these latter as a critical aspect of the *Phyllomedusa* reproductive mode, since they seal the nest from water ingress and protect from various possible predators. At the same time, we contend that the jelly plugs lead to a delay between individual hatching and nest emergence which may limit hatching plasticity though synchronous hatching in itself can be a type of plasticity, as in turtles (Spencer and Janzen 2011). The complexity of structure we report for the eggless jelly capsules suggests that these are worth further investigation, especially in relation to their release of water to assist development, and how this is regulated.

Acknowledgments

This paper is dedicated to the memory of Julian Kenny who sadly died in 2011 while this

paper was in preparation: he pioneered study of *Phyllomedusa trinitatis* and showed the senior author (JRD) how to find their nests. We thank staff at the University of West Indies, especially the late Professor Peter Bacon and more recently Mike Rutherford for the provision of space and general assistance; Ronnie Hernandez at Simla; Nadra Gyan and David Boodoo at the Trinidad Government's Wildlife Section for collection and export permits; students on a series of University of Glasgow Trinidad expeditions for assistance with field and laboratory work; Rebecca Watson for the photographs shown as Figures 1 and 2; JRD acknowledges the Carnegie Trust for the Universities of Scotland and the University of Glasgow for help with fieldwork expenses. MN acknowledges the assistance of the University of Shiraz. We also thank two anonymous reviewers for stimulating comments. 

References

- Agar, W. E. 1910. The nesting habits of the tree-frog *Phyllomedusa sauvagii*. *Proceedings of the Zoological Society of London* 1910: 893–897.
- Alcaide, A. P., M. F. Alcaide, F. J. P. Alcaide, and E. O. Lavilla. 2011. The vesicles of *Phyllomedusa sauvagii* (Anura: Hylidae) nest. *South American Journal of Herpetology* 6: 20–26.
- Bancroft, J. D. and M. Gamble. (eds.). 2008. *Theory and Practice of Histological Techniques*. Philadelphia. Churchill Livingstone/Elsevier. 725 pp.
- Barrio-Amorós, C. L. 2006. A new species of *Phyllomedusa* (Anura: Hylidae: Phyllomedusinae) from northwestern Venezuela. *Zootaxa* 1309: 55–68.
- Budgett, J. S. 1899. Notes on the batrachians of the Paraguayan Chaco, with observations upon their breeding habits and development especially with regard to *Phyllomedusa hypochondrialis*, Cope. Also a description of a new genus. *Quarterly Journal of Microscopical Sciences* 42: 305–333.
- Burggren, W. 1985. Gas exchange, metabolism and ventilation in gelatinous frog egg masses. *Physiological Zoology* 58: 503–514.
- Duellman, W. E. 1974. A reassessment of the taxonomic status of some neotropical hylid frogs. *Occasional Papers of the Museum of Natural History, The University of Kansas* 27: 1–27.
- Faivovich, J., C. F. B. Haddad, D. Baeta, K-H. Jungfer, G. F. R. Alvares, R. A. Brandao, C. Shiel, L. S. Barriento, C. L. Barrio-Amorós, C. A. G. Cruz, and W. C. Wheeler. 2010. The phylogenetic relationships of the charismatic poster frogs, Phyllomedusinae (Anura, Hylidae). *Cladistics* 26: 227–261.
- Frost, D. R. 2011. Amphibian Species of the World: an Online Reference, Version 5.5 (January 2011). Electronic Database accessible at <http://research.annh.org/herpetology/amphibia/index.php>. American Museum of Natural History, New York, USA.
- Gomez-Mestre, I., J. J. Wiens, and K. M. Warkentin. 2008. Evolution of adaptive plasticity: risk-sensitive hatching in neotropical leaf-breeding treefrogs. *Ecological Monographs* 78: 205–224.
- Gosner, K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16: 183–190.
- Kenny, J. S. 1966. Nest building in *Phyllomedusa trinitatis* Mertens. *Caribbean Journal of Science* 6: 15–22.
- Kenny, J. S. 1968. Early development and larval natural history of *Phyllomedusa trinitatis* Mertens. *Caribbean Journal of Science* 8: 35–45.
- Lescure, J., C. Marty, V. Marty, F. Starace, M. Auber-Thomay, and F. Letellier. 1995. Contribution à l'étude des amphibiens de Guyane française. X. Les *Phyllomedusa* (Anura, Hylidae). *Revue Française d'Aquariologie* 22: 35–50.
- Murphy, J. C. 1997. *Amphibians and Reptiles of Trinidad and Tobago*. Malabar. Kreiger Publishing. 245 pp.
- Neckel-Oliveira, S. and M. Wachlevski. 2004. Predation on the arboreal eggs of three species of *Phyllomedusa* in central Amazonia. *Journal of Herpetology* 38: 244–248.
- New, D. A. T. 1966. *The Culture of Vertebrate Embryos*. London. Logos Press. 245 pp.
- Nokhbatolfighahai, M. and J. R. Downie. 2007. Amphibian hatching gland cells: pattern and distribution in anurans. *Tissue and Cell* 39: 225–240.
- Nokhbatolfighahai, M., J. R. Downie, A. K. Clelland, and K. Rennison. 2005. The surface ciliation of anuran amphibian embryos and early larvae: patterns, timing differences and functions. *Journal of Natural History* 39: 887–929.

- Pyburn, W. F. 1980. The function of eggless capsules and leaf in nests of the frog *Phyllomedusa hypochondrialis* (Anura: Hylidae). *Proceedings of the Biological Society of Washington* 93: 153–167.
- Pyburn, W. F. and J. R. Glidewell. 1971. Nests and breeding behavior of *Phyllomedusa hypochondrialis* in Colombia. *Journal of Herpetology* 5: 49–52.
- Rodrigues, D. J., M. Uetanabaro, and F. S. Lopes. 2007. Breeding biology of *Phyllomedusa azurea* Cope, 1862 and *P. sauvagii* Boulenger, 1882 (Anura) from the Cerrado, central Brazil. *Journal of Natural History* 42: 1841–1851.
- Seymour, R. S. 1999. Respiration of aquatic and terrestrial amphibian embryos. *American Zoologist* 39: 261–270.
- Spencer, R. -J. and F. J. Janzen. 2011. Hatching behavior in turtles. *Integrative and Comparative Biology* 51: 100–110.
- Townsend, V. R., D. N. Proud, and M. K. Moore. 2006. *Phyllomedusa trinitatis* (Trinidad Leaf Frog): habitat use. *Herpetological Review* 37: 447.
- Vaira, M. 2001. Breeding biology of the leaf frog, *Phyllomedusa boliviana* (Anura, Hylidae). *Amphibia-Reptilia* 22: 421–429.
- Valls, J. H. and N. E. Mills. 2007. Intermittent hypoxia in eggs of *Ambystoma maculatum*: embryonic development and egg capsule conductance. *Journal of Experimental Biology* 210: 2430–2435.
- Warkentin, K. M. 1999. The development of behavioural defenses: a mechanistic analysis of vulnerability in red-eyed tree frog hatchlings. *Behavioural Ecology* 10: 251–262.
- Warkentin, K. M. 2000. Environmental and developmental effects on external gill loss in the red-eyed treefrog, *Agalychnis callidryas*. *Physiological and Biochemical Zoology* 73: 557–565.
- Warkentin, K. M. 2011. Plasticity of hatching in amphibians: evolution, trade-offs, cues and mechanisms. *Integrative and Comparative Biology* 51: 111–127.

Editor: Franco L. Souza