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SHORT COMMUNICATION

Embryonic development and mortality in *Hyalinobatrachium pulveratum* (Anura: Centrolenidae) of south-western Costa Rica

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The population biology and ecology of most members of the neotropical family Centrolenidae, or glass frogs, are unknown. Glass frogs deposit their eggs in a gelatinous mass on vegetation overhanging streams, the eggs hatch, and the tadpoles drop into the water, where they complete development (Savage 2002). This study will contribute to our limited understanding of centrolenid reproductive ecology by quantifying variation in clutch size, embryonic development and embryonic mortality in a population of *Hyalinobatrachium pulveratum*.

Hyalinobatrachium pulveratum egg masses were observed along a 175-m section of the Quebrada Bilingual in the Osa Peninsula, Puntarenas Province, Costa Rica (8°26′N, 83°22′W). The stream is bordered on the east by continuous lowland wet forest (Hartshorn 1983) and on the west by a forest buffer and cattle pasture. The Osa Peninsula is characterized by distinct rainy (May to December) and dry seasons (January to April) and precipitation averages 4000–6000 mm y^{-1} (L. Gilbert, unpubl. data).

Egg masses were found on overhanging vegetation on both sides of the stream up to 3 m in height. Each clutch was digitally photographed and individually marked. Subsequent daytime monitoring events of each clutch, or egg checks, included taking digital photographs and recording the dates hatching commenced and concluded and mortality events. Each clutch was monitored until the last egg hatched or the embryo died. Monitoring took place on 70 d of the 102-d study period. Clutches were monitored every other day from 19 July to 22 August and almost every day, except during heavy flooding, from 23 August to 29 October.

The number of eggs was quantified to determine clutch size using the first photograph, because it is easiest to count eggs at an early embryonic stage before the jelly swells with uptake of water (*pers. obs.*). Photographs were enlarged and each egg was individually marked to ensure that it was counted only once. If clutch size was uncertain, two or more photographs were examined to reach a consensus or the clutch was excluded.

Developmental rate and variation in embryo colour were assessed for a subset of clutches. The subset included clutches with low embryo mortality (<10%) and consistent monitoring from oviposition to hatching (maximum of 2 d missed). Developmental stages (Gosner 1960) were determined from photographs, thus only gross morphological features, not fine features or movement, were visible. Stages were collapsed into five categories, stages 1–14, 15–17, 18–19, 20–22 and 23–25. All visible embryos in each photograph were examined, the clutch was assigned to a category, and values for each day of development were averaged to estimate developmental rate. Variation in embryo colour (indicating vascularization) was assessed throughout development by categorizing clutches as yellow-green, pink and red from photographs. Comparisons were made among clutches within each colour category to ensure consistency.

Embryonic and hatching durations were estimated using photographs and field notes. The first photograph of each clutch was used to estimate clutch age (in days) by comparing with clutches of known ages. Minimum and maximum embryonic duration are defined as the number of days from egg deposition until the first and last embryos hatched, respectively. Several clutches were excluded from these calculations because monitoring began late in development. If hatching was not observed in entirety (i.e. observations were not daily), it was

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assumed to have concluded during the day or night prior to the egg check when the clutch was observed with empty egg capsules. Thus, the maximum embryonic duration may be overestimated for these clutches. The hatching duration of each clutch was defined as the number of days from the first indication of hatching to the date when hatching was estimated to have concluded.

The extent and causes of mortality were determined from photographs and field notes. Descriptions of embryonic mortality by Villa (1977), McDiarmid (1978), Hayes (1991), Warkentin (1995, 2000), and Lips (2001) aided in identifying the types of *H. pulveratum* mortality. I recognized four types of mortality: (1) Failure to develop or unfertilized eggs, characterized by embryos that developed abnormally and turned white. (2) Predation by arthropods, characterized by an irregular pattern of egg removal and an opaque appearance to the jelly, or by snakes, assigned to clutches with all or a large proportion of the embryos and jelly removed without damage to remaining embryos. (3) Desiccation, eggs or late-stage embryos that turned yellowish to white in clutches with little or no jelly during periods of low precipitation. (4) Flooding, characterized by the disappearance of whole clutches or leaves with clutches during periods of heavy precipitation. Fungi were observed but are not considered a separate cause of mortality because in each case they represented post-mortem infection. The number of living embryos present before and after mortality events was quantified. Embryos were recorded as hatching successfully if they were in a late stage of development during one egg check and empty egg capsules were observed at the next egg check. Time did not permit the use of catch cups, a method to assess embryonic survival developed by Hayes (1983); therefore, hatched embryos cannot be distinguished from those predated during the hatching period.

Spearman's rho correlation coefficients were calculated to examine relationships between (1) maximum embryonic duration and mean daily precipitation, and (2) the number of clutches that initiated or concluded hatching and the quantity of precipitation during the night (measured from 18h00 to 06h00) prior to the egg check. One-tailed tests were used with the expectation that the relationship between precipitation and embryonic duration would be negative and the relationship between the number of clutches hatching and precipitation would be positive. Clutches that were not monitored from the first day after egg deposition or not observed daily while hatching were excluded. Because recent studies of some species with arboreal clutches have found that embryos hatch early in response to substantial clutch mortality (Vonesh 2005, Warkentin 1995), I tested whether *H. pulveratum* embryos respond similarly. Variation in maximum and minimum embryonic duration was compared among three mortality groups using one-way ANOVAs. The mortality groups included clutches with more than 10% mortality caused by either developmental failure followed by fungal infection ($n = 3$) or predation ($n = 4$) and clutches with less than 10% mortality ($n = 47$). I chose $\pm 10\%$ because predation resulted in at least 10% mortality, while mortality from typical developmental failures accounted for less mortality. All statistical tests were performed using SPSS 10.1 software and data presented are the mean $\pm$ SE.

Sixty-six clutches were monitored during the study period. The bright yellowish-green eggs are deposited in a single layer on the upper leaf surface. Unlike other centrolenid egg masses, the jelly surface is crenulated (R. McDiarmid, *pers. comm.*) and usually remains so until the embryos hatch. Clutch size was 42.3 ± 0.8 and ranged from 23 to 57 eggs ($n = 62$). Maximum embryonic duration per clutch, from oviposition until the last egg hatched, was 9–24 d (16.0 ± 0.4, $n = 55$), while the minimum duration was 6–19 d (12.5 ± 0.4, $n = 55$). Hatchling duration was 1–10 d (4.0 ± 0.3, $n = 56$). The majority of eggs in a clutch typically hatched in the time between two egg checks, but often a few embryos remained in the clutch for several additional days. The number of clutches initiating hatching and nocturnal rainfall were positively correlated (Spearman's rho $= 0.34$, $n = 40$, $P = 0.02$), but there was no relationship between the number of clutches concluding hatching and nocturnal rainfall (Spearman's rho $= 0.09$, $n = 40$, $P = 0.30$). Maximum embryonic duration and mean daily rainfall were negatively correlated (Spearman's rho $= -0.34$, $n = 28$, $P = 0.04$).

Developmental rate and embryo colour were assessed in 12 clutches (Figure 1). The transitions between stage categories were variable among clutches, but all reached

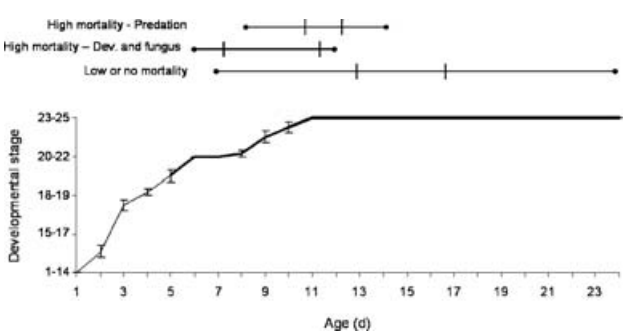

Figure 1. *Hyalinobatrachium pulveratum* clutch development over time (mean $\pm$ SE, $n = 12$ clutches). Developmental stages (Gosner 1960) are grouped. Line thickness shows increasing redness of embryos, with colour changing gradually from yellow-green (thin line) to pink, then red (thick line). Embryonic duration in different mortality groups ('dev.' signifies development) is bounded by the ranges (circles), with inner vertical lines representing the minimum and maximum durations, or start and end of hatching, averaged across clutches.

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Table 1. Fate of *Hyalinobatrachium pulveratum* egg clutches and individual eggs in the Osa Peninsula; percentages are of the total monitored. Embryos in two clutches suffered from more than one type of mortality.

	Clutches		Eggs	
	Number	%	Number	%
Total monitored	59		2513	
Total with an estimated 0% mortality	25	42	2140	85
Total with some type of mortality	34	58	373	15
Failed to develop or unfertilized	22	37	117	5
Predation	5	9	103	4
Desiccation	6	10	25	1
Flooding	3	5	128	5

stages 23–25 within 11 d. At 2 d, embryos in most clutches had tail buds (stage 17) and over the following 2–3 d embryos attained greater head definition and body elongation (stages 18 and 19). During days 5–8, embryos were in stages 20–22, as the tail elongated and the cornea became transparent. Half of the clutches reached stages 23–25 within 9 d. Early-hatching embryos, in clutches with both high and low mortality, were estimated to be in stages 20–22. The yellowish-green egg colour persisted for at least the first 5 d of development, then changed from light to dark pink over the following 5 d, and finally to darker shades of red during the remainder of development (Figure 1). Embryos began to hatch during the pink stages, but most hatched when red.

Mean survivorship per clutch was estimated to be $85\% \pm 4\%$ ($n = 59$); nearly half of the clutches (42%) hatched without embryonic mortality (Table 1). Mortality occurred in 34 clutches and failure to develop affected the most clutches (37%). In the majority of those clutches, one to three embryos died; however, in one clutch about 25% of embryos died and in two clutches over 80% died. Desiccation was the cause of mortality in six clutches (10%); typically three or fewer embryos died per clutch, with one exception in which 31% of embryos died. In these few cases of high mortality from developmental failure or desiccation, fungal infection followed 2–3 d after embryo death. Five clutches (9%) were predated, with two clutches partially predated by arthropods, likely katydids (M. Hayes, *pers. comm.*) or wasps (K. Warkentin, *pers. comm.*), and one completely and two partially predated probably by snakes. Flooding events caused the mortality of the largest number of embryos (5% or 128 eggs), because usually the entire clutch was affected due to loss of the clutch substrate. Mortality caused by abiotic factors varied in a temporal manner, with desiccation causing mortality in July and August and flooding-related mortality occurring in September.

Embryos in clutches with more than 10% mortality (high mortality) started hatching an average of 2–5 d earlier and finished hatching an average of 4–5 d earlier than clutches with less than 10% mortality (low mortality; Figure 1). Predated clutches suffered mortality before day 3 ($n = 3$) or on day 7 ($n = 1$) of development and the remaining embryos began hatching several days later. Clutches with developmental failures suffered mortality on day 2 ($n = 2$) or day 5 ($n = 1$), fungal infection followed 2–3 d later, and hatching of surviving embryos started 3–6 d later. There were significant differences in average maximum embryonic duration (ANOVA; $F = 9.20$, $df = 53$, $P < 0.0001$) and average minimum embryonic duration (ANOVA; $F = 7.16$, $df = 53$, $P = 0.002$) among clutches in the three mortality groups. Tukey's post-hoc tests revealed that the high mortality groups were different from the low-mortality group but not different from each other for maximum embryonic duration and the group with high mortality from developmental failure was different from the low-mortality group for minimum embryonic duration.

Hyalinobatrachium pulveratum clutch characteristics and embryonic development generally agree with descriptions in previous studies. However, mean clutch size was 8 or 22 embryos smaller than that in Hoffmann (2004) and Villa (1984), respectively, and embryonic duration was up to 12 d longer than reported in Hoffmann (2004). Environmental conditions may be partly responsible for differences in embryonic duration among studies and for variation within and among clutches in the present study. Understorey air temperature is known to vary depending on the amount of canopy cover (Hubbell & Foster 1986) and to influence the developmental rate of anuran eggs (Alford 1999). Hoffmann (2004) raised clutches in an outdoor laboratory, which likely differed in abiotic conditions in comparison to a natural setting. The results suggest that moisture also affects embryonic development, with the quantity of rainfall correlated with embryonic duration and the initiation of hatching.

Hatching of *H. pulveratum* embryos was similar to that of *H. fleishmanni* in that some embryos remain in the egg mass for up to 9 d after the majority of embryos hatch (Hayes 1991). Some late-hatching *H. pulveratum* embryos appeared to be larger-sized and more developed, partially evident by a reduction in yolk reserves (*pers. obs.*), while other late-hatchers appeared to develop at a slower rate than earlier-hatching embryos in the same clutch. Additional study is needed to document embryo developmental stage at hatching and size-dependent risks to hatchlings in a stream environment.

Survivorship of *H. pulveratum* embryos (85%) was high in comparison to other glass frogs. Hayes (1991) estimated that only 21–38% of *H. fleischmanni* embryos hatched successfully. Estimates of whole clutch survival ranged from 61–71% (Greer & Wells 1980) to 32% (Jacobson 1985) for *H. fleischmanni* and 47% for *Centrolenella prosoblepon* (Jacobson 1985). Throughout

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the study period, mortality caused by failures to develop affected a small number of embryos in a large number of clutches, a typical pattern reported in studies of anuran embryonic mortality (Hayes 1991, Lips 2001, Vonesh 2005). Clutch predation was infrequent and larval drosophilids, an important source of embryonic mortality in other centrolenids (Hayes 1991, Villa 1984), were not observed.

The results suggest that *H. pulveratum* embryos in clutches with substantial mortality start and finish hatching earlier than embryos in clutches with low mortality. This response may be an attempt to avoid imminent mortality caused by fungal infection on decaying embryos or related to the negative and potentially lasting experience of predated embryos in the same clutch. The timing of hatching varies with risk of mortality for several species; for instance, *Agalychnis callidryas* embryos hatch early in response to snake and wasp predation and fungal infection (Warkentin 1995, 2000; Warkentin *et al.* 2001). Plasticity in hatching age merits further research on members of the Centrolenidae.

This study will contribute to the small amount of data available on centrolenid population ecology, improving comparative research within and among anuran families. Similar to other centrolenids, embryonic development was long in duration and hatching within and among clutches was temporally variable. In contrast to other centrolenids, embryonic mortality caused by predation and desiccation was low.

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