



Restricting living space: Development and larval morphology in sticky frogs (Microhylidae: *Kalophrynus*) with different reproductive modes

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Abstract

We compare reproductive features, development, and larval morphology in three closely related species of sticky frogs (*Kalophrynus* Tschudi, 1838) inhabiting the lowland and mountain forests of Vietnam and displaying a variety of reproductive modes. While *K. interlineatus* breeds in open temporary ponds, *K. honbaensis* and *K. cryptophonus* are phytotelm-breeders using tree hollows and bamboo stems for reproduction. Their tadpoles also differ in trophic specialization: larval *K. interlineatus* are typical suspension-feeders, whereas *K. honbaensis* and *K. cryptophonus* are obligatorily oophagous. All three species differ in egg and clutch sizes, duration of embryonal period and hatching stage, and the structure of the larval digestive tract and skeleton. Based on external and internal morphology, we conclude that tadpoles of *K. interlineatus* and *K. cryptophonus* represent two “extremes” of the adaptive spectrum of microhylid larvae, while *K. honbaensis* displays a set of transitory traits. Relying on these new findings in anuran biology, we discuss reproductive, ontogenetic, and morphological rearrangements during the transition from pond breeding to phytotelm breeding and from microphagy to macrophagy as well as the significance of the revealed adaptations to different habitats and larval life modes.

Keywords

chondrocranium, embryonization, oophagy, phytotelm breeding, tadpole, trophic specialization

Introduction

Anuran amphibians display an extreme diversity of reproductive and developmental modes that allow them to occupy a rich variety of aquatic, terrestrial and arboreal habitats (Haddad and Prado 2005; Elinson and del Pino 2012; Nunes-de-Almeida et al. 2021). In particular, many species, instead of spawning in open ponds, breed in micro waterbodies, including phytotelmata (Lannoo et al. 1987; Malkmus and Dehling 2008).

Breeding in small and usually secluded spaces offers a number of benefits, such as protection against predators and avoidance of interspecific competition, among others (Crump 2015). At the same time, restricting the vital space imposes certain limitations on developing larvae, mainly due to the shortage of food resources (Caldwell and Araújo 2004). The need to overcome these limitations naturally leads to changes in the tro-

phic specialization of tadpoles, their morphology and their development.

Phytotelm-breeding anuran species can adopt different strategies allowing the raising of a relatively numerous progeny in small volumes of water. One of them is the production of endotrophic larvae, which do not need food uptake and develop until metamorphosis solely at the expense of their large internal yolk deposits. Another strategy is larval oophagy, i.e., feeding on conspecific eggs. Both strategies are similar in the energetic source (yolk) but obviously require different larval adaptations.

Endotrophic tadpoles, as they are not actively feeding, do not exhibit any specific features in their oral morphology that differentiate them from their exotrophic relatives or show some reduction in nonfunctional trophic structures (Blommers-Schlösser 1975; Vera Candioti et al. 2011; Formas 2013). However, they can differ in some degree of embryonization (sensu Matsuda 1987), which implies the incorporation of the early larval stages into intraovular development: much data show that species with endotrophic tadpoles tend to have larger eggs, longer embryonic periods and hatch at more advanced stages than exotrophic tadpoles (Blommers-Schlösser 1975; Krügel and Richter 1995). In contrast, obligate oophagy, i.e., feeding exclusively on eggs, is usually associated with specific transformations of the larval oral apparatus, digestive tract and chondrocranium. Thus, oophagous tadpoles display reduced or highly specialized keratinous mouthparts and a set of features characteristic of macrophagous anuran larvae, including a shortened gut and reduced branchial baskets in the hyobranchium (Rowley et al. 2012; Vassilieva et al. 2013; Vera Candioti et al. 2021).

Oophagy is widespread among mainly tropical anurans, especially within families whose members are prone to predation at their larval stages, e.g., Hylidae, Leptodactylidae, Rhacophoridae, and Dendrobatidae (Lannoo et al. 1987; Brust 1993; Kam et al. 1996; Lehtinen and Nussbaum 2003; Gibson and Buley 2004). Nevertheless, in a rather unexpected way this trophic specialization was also found in the family Microhylidae, although the tadpoles of these frogs almost always lack keratinous mouthparts and are mostly microphagous.

Larvae of Asian microhylids are in their vast majority pond-dwelling suspension or neustonic filter feeders (Altig and Johnston 1989; Khan 2000; Vassilieva et al. 2017a). However, the obligatory oophagous tadpole of *Nanohyla arboricola* (formerly assigned to *Microhyla*) has recently been described from montane tropical forests of Vietnam (Poyarkov et al. 2014; Vassilieva et al. 2017b). Compared with other known *Microhyla* and *Nanohyla*, this species displays a combination of both embryonization traits and deep transformation of its external and internal larval morphology due to phytotelm-dwelling and oophagy. The profound differences between larval *N. arboricola* and other closely related species, however, make it difficult to understand the evolution of its most striking features.

Another group of microhylid frogs, the genus *Kalophrynus* Tschudi, 1838, can help to shed some light on the

morphological evolution associated with the transition from typical pond-dwelling larvae to highly specialized tadpoles adapted to life in phytotelmata.

Sticky frogs *Kalophrynus* represent a separate subfamily Kalophryninae and number currently 27 species distributed from India throughout Indochina and Southern China to Indonesia and the Philippines (Frost, 2023). The reproductive biology of most species remains unstudied; however, some of them are known to spawn in open ponds, some are known or suspected to breed in micro waterbodies (in phytotelmata or holes in the ground), and some species have endotrophic tadpoles (Lim and Ng 1991; Manthey and Grossmann 1997; Matsui 2009; Dehling 2011; Zug 2015; Haas et al. 2022).

Three species of sticky frogs are encountered in southern Vietnam (Fig. 1), all differing in their reproductive biology: the striped sticky frog *Kalophrynus interlineatus* (Blyth, 1855) spawns in temporary open ponds, the Hon Ba sticky frog *Kalophrynus honbaensis* Vassilieva, Galoyan, Gogoleva & Poyarkov, 2014 reproduces in large tree hollows, and the bamboo sticky frog *Kalophrynus cryptophonus* Vassilieva, Galoyan, Gogoleva & Poyarkov, 2014 breeds inside thin bamboo internodes. Thus, these species represent a good model for studying the impact of reproductive modes on the developmental and morphological features of their progeny. Among these features are the possible tendency toward embryonization as well as the differences in the external morphology of larvae living in open waterbodies and limited water volumes, varieties of trophic specialization of tadpoles developing with and without food limitations, and related morphological characters in the chondrocranium, hyobranchium and digestive tract.

Methods

Field observations as well as egg, larva and adult specimen collections occurred in May–July 2018 in three nature conservation areas in southern Vietnam: Cat Tien National Park (Tan Phu District, Dong Nai Province; approximate coordinates 11°26.45'N, 107°24.77'E, elevation: 140 m a. s. l.); Hon Ba Nature Reserve (Cam Lam District, Khanh Hoa Province; approximate coordinates 12°07.26'N, 108°56.9'E, elevation: 1500 m a.s.l.); and Loc Bao Forest (Lam Dong Province, Bao Lam District, approximate coordinates 11°44.31'N, 107°42.15'E, elevation: 800 m a.s.l.).

Spawning and development of the tadpoles of *K. interlineatus* in natural forest ponds were observed during stationary fieldwork at the forest station of the Tropical Centre in Kat Tien National Park. To count the number of eggs in a clutch, three couples in amplexus were caught and placed in plastic aquaria for spawning. Breeding sites of *K. cryptophonus* and *K. honbaensis* were located during short-term field expeditions by thoroughly searching for accessible tree hollows or bamboo stems during



Figure 1. Species of the sticky frogs, *Kalophrynus*, inhabiting lowland and mountain forests in the south of Vietnam: **A** *K. interlineatus*, Cat Tien National Park (adult male, SVL 42.1 mm); **B** *K. honbaensis*, Hon Ba Nature Reserve (adult male, SVL 38.7 mm); **C** *K. cryptophonus*, Loc Bao forest (adult male, SVL 28.8 mm, photo by E. Galoyan).

the day and night. The locations of all of the breeding sites found were marked with GPS coordinates. For each of the phytotelmata, the tree/bamboo stem diameter and the height of the hollow/internode above the ground were measured. The volume of liquid contained in the hollow/internode was evaluated after scooping it out together with the eggs or larvae. The number of eggs within each clutch and the number of tadpoles in each hollow/internode were counted.

For all three species, part of the eggs from each clutch were placed in plastic containers filled with clear rainwater, incubated at 26°C, and then fixed in 10% formalin at the hatching stage. Several tadpoles of all three species at developmental stages 35–38 were fixed in 10% formalin for measurements and morphological examination; five to seven advanced tadpoles of each species were placed in plastic containers filled with water from their native microhabitat, and their development until metamorphosis completion was observed. The rest of the clutches and tadpoles were returned to their native microhabitat.

Species identification of the tadpoles was based on morphological and natural history criteria. Tadpoles of *K. interlineatus* were reared from eggs obtained from amplexing pairs and grown to mid-larval stages in the aquarium; in parallel, the full developmental cycle of tadpoles was observed in natural waterbodies. Males of *K. honbaensis* were observed calling near breeding hollows and, once, an adult male was found inside a hollow with egg clutch; tadpoles were observed at various developmental stages and always differed clearly from the tadpoles of *Nanohyla arboreicola*, the only other microhylid phytotelm-breeder in Hon Ba forest, and other syntopic microhylid species. The tadpoles of *K. cryptophonus* were found inside bamboo stems with calling males and were first described by Vassilieva et al. (2014) at early-larval stages. The young metamorphs of all three species displayed characteristic genus-specific traits (head shape, body and limbs shape, some features of coloration – see Results) and clearly differed from metamorphs of all known coexistent microhylid species.

Hatching larvae and advanced tadpoles were photographed with a LEICA EZ4 dissecting stereo microscope (Germany) with a digital photo attachment and staged according to Gosner’s simplified table (1960) and the de-

scription of normal development in a microhylid species (Shimizu and Ota 2003). Measurements were taken to the nearest 0.1 mm with an ocular micrometer for eggs and hatching larvae and with digital calipers for advanced larvae. Tadpole morphometric parameters mostly followed Altig (2007); the snout-vent length (SVL) of newly metamorphosed juveniles (stage 46) was also measured.

For all three species, larval chondrocranium and hyobranchium morphology was examined in three tadpoles (stages 37–39) that were stained as whole mounts for cartilaginous tissue with Alcian blue and cleared with 1% KOH. Designations of skeletal elements mostly followed Haas (2003) and Trueb et al. (2011).

For all three species, the digestive tract length (DTL) was measured by removing the entire digestive tract from three formalin-fixed tadpoles (stages 36–38) and extending it on a piece of filter paper.

The voucher series of tadpoles were deposited in the herpetological collection of the Zoological Museum of the Lomonosov Moscow State University (*K. interlineatus*: ZMMU A-7891; *K. honbaensis*: ZMMU A-7892; *K. cryptophonus*: ZMMU A-7893).

Taxonomy followed the constantly updated database “Amphibian Species of the World” by Frost (2023).

Results

Reproduction and development

Breeding sites

The intensive mass breeding of *K. interlineatus* was observed periodically during May, June, and July in its natural habitat represented by plain monsoon, semideciduous tropical forest, including secondary and highly disturbed areas at elevations ca. 100–150 m a.s.l., with air temperature variation of 26–31°C and humidity level of approximately 92–96%. Spawning occurred in various kinds of still waterbodies: temporary ponds, small rainy pools and flooded areas of the forest. Males (up to several tens) formed noisy choruses on the ground near the breeding

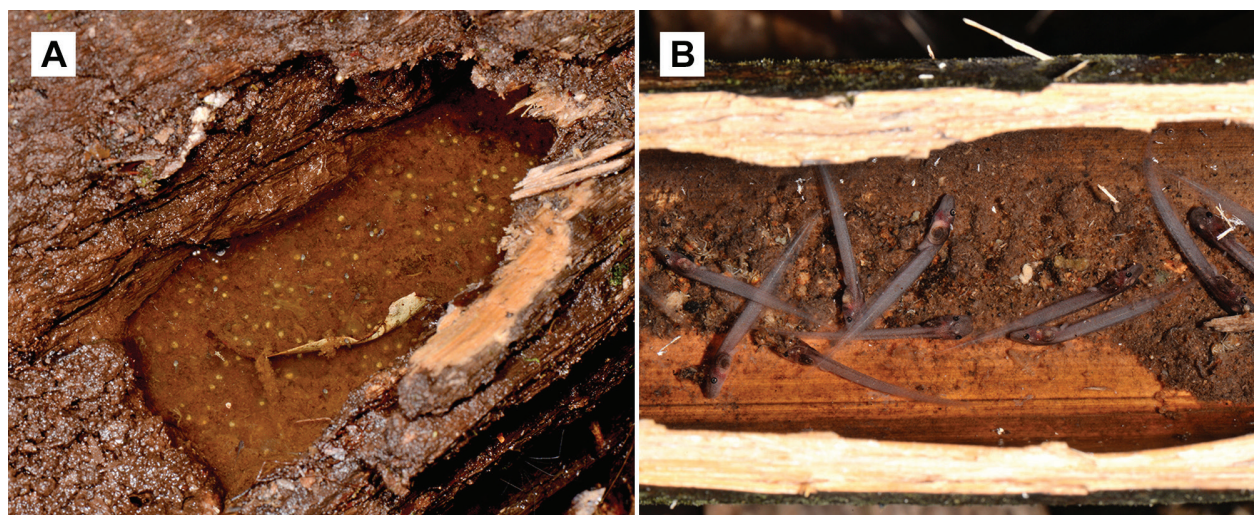


Figure 2. Breeding sites of phytotelm-breeding *Kalophrynus* species in Vietnam: **A** hollow in a log containing the developing egg clutch of *K. honbaensis*; **B** bamboo internode containing advanced tadpoles of *K. cryptophonus*.

ponds mostly at night after heavy rains; massive spawning was often observed simultaneously with other microhylid species (*Kaloula indochinensis*, *Kaloula pulchra*, *Microhyla heymonsi*, *Microhyla mukhlesuri*, and *Micryletta erythropoda*) and other frog species, such as *Chironomantis nongkhorensis*, *Rohanixalus vittatus*, *Polypedates megacephalus* (Rhacophoridae), *Fejervarya limnocharis*, and *Occidozyga martensii* (Dicroglossidae).

The breeding of *K. honbaensis* in the Hon Ba Nature Reserve was observed for five days in June at the height of the rainy season. Breeding habitat was represented by an 1800×200 m area of primary montane polydominant evergreen forest on the top and steep slopes of a narrow mountain ridge at elevation ca. 1500 m a.s.l., with air temperature variation of 18–22°C and humidity level of approximately 91–92%. Egg clutches and tadpoles were found in water-filled cavities, mostly at ground level or slightly elevated (up to 20–30 cm), distant from each other by 350–900 m; of six breeding sites, two were represented by holes in tree butts, three by hollowed logs (Fig. 2A), and one by a plastic container (200 mL) left by humans. The diameter of natural cavities varied from 10–12 to 28 cm, and their capacity varied from 150 to 300 mL. Males (from one to three at once) were observed calling on the ground close to breeding hollows. In one case, several egg clutches of Truong Son moss frog, *Theloderma truongsoneense* (Rhacophoridae), were observed on the inner wall of a tree hollow containing eggs of *K. honbaensis*.

The breeding of *K. cryptophonus* in Loc Bao Forest was observed for three days in May, at the beginning of the rainy season, on a limited parcel, ca. 120×30 m, of secondary, disturbed mountain high polydominant evergreen tropical forest with an abundance of bamboo (*Phyllostachys* sp.) at an elevation of 800 m a.s.l., with air temperature variation of 26–28°C and humidity level of approximately 89–92%. Egg clutches and tadpoles were found inside water-filled internodes of dead bamboo stems (Fig. 2B): vertical or fallen, distant from each other by 3–60 m, with diameter 3–3.6 cm, partially filled with

20–40 mL of liquid, sometimes highly viscous because of egg mucus. Males were observed to call individually from inside the breeding bamboo stems. In three cases, developing eggs of the bamboo moss frog *Theloderma laeve* (Rhacophoridae) were observed in the same internodes with *K. cryptophonus* clutches or larvae.

Development

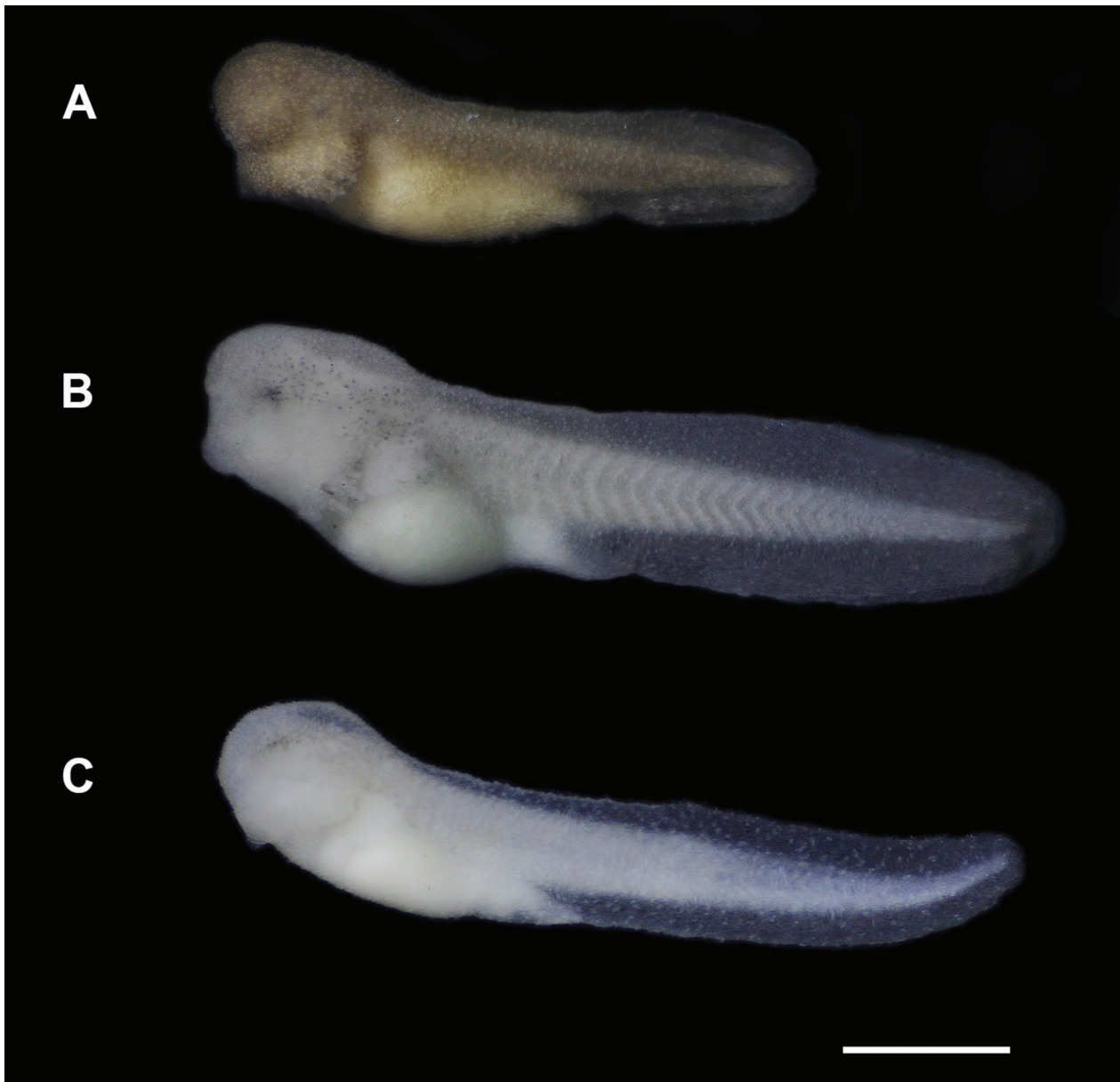
Data on egg and clutch sizes and embryonal and larval development in all studied species are summarized in Table 1.

In *K. interlineatus*, egg clutches were deposited in portions, while the couple in amplexus floated in water, making periodic dives. Single-layered egg clusters containing hundreds of pigmented eggs with dark brown animal poles and creamy-white vegetative poles floated on the water surface. The embryonal period was very short: the eggs deposited at night usually hatched by the upcoming evening. Hatching larvae were at early developmental stages, with vestigial gills, a closed mouth and an unpigmented iris (Fig. 3A). In natural ponds, the larval period was approximately two weeks, and metamorphosis was completed rapidly: usually, it took approximately 10–12 hours from stage 42 (first forelimb emergence) to stage 46 (total tail stub resorption).

In *K. honbaensis*, egg clutches floated or were partially immersed in water and mucus inside the breeding hollows. Of the six observed breeding sites, two contained eggs at the beginning of cleavage, three contained eggs at various stages of embryonal development, and one contained 423 tadpoles at stages 28–39. This number greatly exceeded the maximum number of eggs in found clutches, and it is unknown whether all of these tadpoles hatched from a single clutch. Freshly laid eggs were weakly pigmented, with pale gray animal poles and greenish-yellow vegetative poles. Larvae hatched for several hours at variable stages, mostly with slightly branching gills and partially pigmented irises (Fig. 3B). The duration of the larval period is not known precisely: the observed development

Table 1. Main reproductive and developmental characteristics of three *Kalophrynus* species.

Species	Clutch size (number of eggs)	Egg diameter (mm)	Embryonal period (days)	TL at hatching (mm)	Hatching stage	Larval period (days)	SVL at stage 46 (mm)
<i>K. interlineatus</i>	2900±574.2 (2494–3306)	1.1±0.1	<1	3.6±0.1	20–21	13–15	4.5±0.3
<i>K. honbaensis</i>	178.8±71.7 (123–283)	1.5±0.1	2.5	5.1±0.7	20–22	>15	6.8±0.2
<i>K. cryptophonus</i>	43±11.0 (32–56)	1.4±0.1	3	5.3±0.4	23–24	>15	5.5±0.2

**Figure 3.** Early larvae of three *Kalophrynus* species at the hatching stage: **A** *K. interlineatus*; **B** *K. honbaensis*; **C** *K. cryptophonus*. Scale bar: 1 mm.

from midlarval stage 38 to metamorphic stage 46 took 13 days; the development from stage 42 to the completion of metamorphosis took two days.

In *K. cryptophonus*, oocytes were unpigmented, yellowish-cream. Their development occurred in very small quantities of water, rather in liquid mucus. Of the six in-

ternodes used for breeding, four contained egg clutches (two freshly laid, with oocytes at the early stages of cleavage, one at the neurula stage, and one at the beginning of hatching), and two more contained larvae at stages 29–33 and 34–38 (32 and 95 tadpoles, respectively). The last number greatly exceeded the maximum egg number in

Table 2. Main morphometric characters and body proportions (average \pm SD) of the tadpoles of three *Kalophrynus* species. Abbreviations: TL, total length; BL, body length; TaL, tail length; BW, maximal body width; BH, maximal body height; TH, maximal tail height; TBW, tail base width; SVL, snout-vent length; SSp, snout-spiracle length; DF, maximal dorsal fin height; VF, maximal ventral fin height; IP, interpupilar distance; RP, rostrum-pupilar distance; ED, horizontal eye diameter; MW, horizontal mouth width.

Measurements (mm) and proportions	<i>K. interlineatus</i>		<i>K. honbaensis</i>		<i>K. cryptophonus</i>	
	n = 9	St. 35–38	n = 10	St. 35–38	n = 10	St. 35–38
TL	12.6 \pm 0.6		19.6 \pm 0.7		20.3 \pm 1.2	
BL	4.7 \pm 0.3		5.5 \pm 0.2		4.6 \pm 0.1	
TaL	7.9 \pm 0.4		14.0 \pm 0.6		15.7 \pm 1.1	
BW	3.2 \pm 0.3		3.4 \pm 0.3		2.9 \pm 0.3	
BH	2.2 \pm 0.1		2.4 \pm 0.2		2.1 \pm 0.1	
TH	2.4 \pm 0.2		3.1 \pm 0.2		2.5 \pm 0.1	
TBW	0.9 \pm 0.1		1.3 \pm 0.1		1.1 \pm 0.1	
SVL	5.1 \pm 0.4		7.1 \pm 0.4		5.2 \pm 0.2	
SSp	4.3 \pm 0.4		4.5 \pm 0.2		3.7 \pm 0.1	
DF	1.0 \pm 0.1		0.8 \pm 0.1		0.6 \pm 0.1	
VF	1.0 \pm 0.1		1.0 \pm 0.1		0.7 \pm 0.1	
IP	3.1 \pm 0.2		2.9 \pm 0.2		2.3 \pm 0.1	
RP	1.8 \pm 0.1		1.7 \pm 0.2		1.6 \pm 0.1	
ED	0.8 \pm 0.1		0.9 \pm 0.1		0.8 \pm 0.1	
MW	1.1 \pm 0.1		1.7 \pm 0.1		1.1 \pm 0.1	
TaL/BL	1.71 \pm 0.11		2.53 \pm 0.13		3.44 \pm 0.18	
BW/BL	0.70 \pm 0.04		0.61 \pm 0.05		0.64 \pm 0.07	
BH/BL	0.48 \pm 0.03		0.43 \pm 0.02		0.47 \pm 0.03	
RP/BL	0.31 \pm 0.02		0.26 \pm 0.03		0.33 \pm 0.03	
SSp/SVL	0.83 \pm 0.06		0.63 \pm 0.02		0.71 \pm 0.04	
TH/BH	1.08 \pm 0.08		1.29 \pm 0.07		1.15 \pm 0.06	
TBW/BW	0.27 \pm 0.02		0.37 \pm 0.02		0.38 \pm 0.03	
DF/VF	1.02 \pm 0.08		0.79 \pm 0.08		0.76 \pm 0.10	
ED/BL	0.18 \pm 0.02		0.16 \pm 0.01		0.18 \pm 0.02	
MW/BW	0.33 \pm 0.03		0.49 \pm 0.04		0.39 \pm 0.07	

the observed clutches, and it is unknown whether all 95 tadpoles hatched from a single clutch. Larvae hatched at a relatively advanced state, with gills partially covered with developing opercular fold, opened mouth, and slightly pigmented iris (Fig. 3C). The duration of the larval period in this species is also not known precisely, but the development from midlarval stage 37 to metamorphic stage 46 took 11 days. The development from stage 42 to the completion of metamorphosis took two days.

External larval morphology

Morphological description is based on 9–10 larvae of medium larval stages. The external appearance of the tadpoles is shown in Fig. 4. The main morphometric parameters are summarized in Table 2.

***Kalophrynus interlineatus*.** In dorsal view (Fig. 4A), body broadly elliptical, with maximum width at the gill level; snout bluntly rounded. In lateral view (Fig. 4B), body ovoid and slightly compressed dorsoventrally, with a flat dorsum and convex belly. Tail moderately long, broadly lanceolate, and slightly more than one and half times longer than the body; tail musculature rather weak-

ly developed (tail base less than a third of the maximal body width); tail ending with terminal filament (approximately one-fourth of the tail length). Tail fins rather tall, reaching their maximum height at the middle third of the tail length; dorsal fin not extending on the trunk and ventral and dorsal fins nearly equal in height. Spiracle medial, transversal, narrow, and opening at the rear of the body; covering membrane rather long, with a serrated free margin. Vent tube medial and oblique. Eyes strictly lateral and moderately large. Nostrils sealed at larval stages. Nasolacrimal grooves and lateral line not visible. Mouth terminal, transversal, and relatively narrow. Lower labium not visible in dorsal view; upper labium short and rounded, with two lateral flaps hanging over narrow spoon-shaped lower labium. External papillae and keratinized mouthparts absent.

In life, at all larval stages, dorsal coloration dull brown with pink or reddish marbled pattern; belly paler, with a pinkish tint; tail fins densely pigmented, grayish-brown with irregular pink or orange spots and yellow or orange speckling; terminal, tapering parts of tail fins unpigmented, stem of the terminal filament gray; spiracle flap unpigmented. Iris black with golden speckling and wide golden ring around the pupil. In preservative, brown and reddish tints fading to pale gray, tail with a gray marbled pattern.

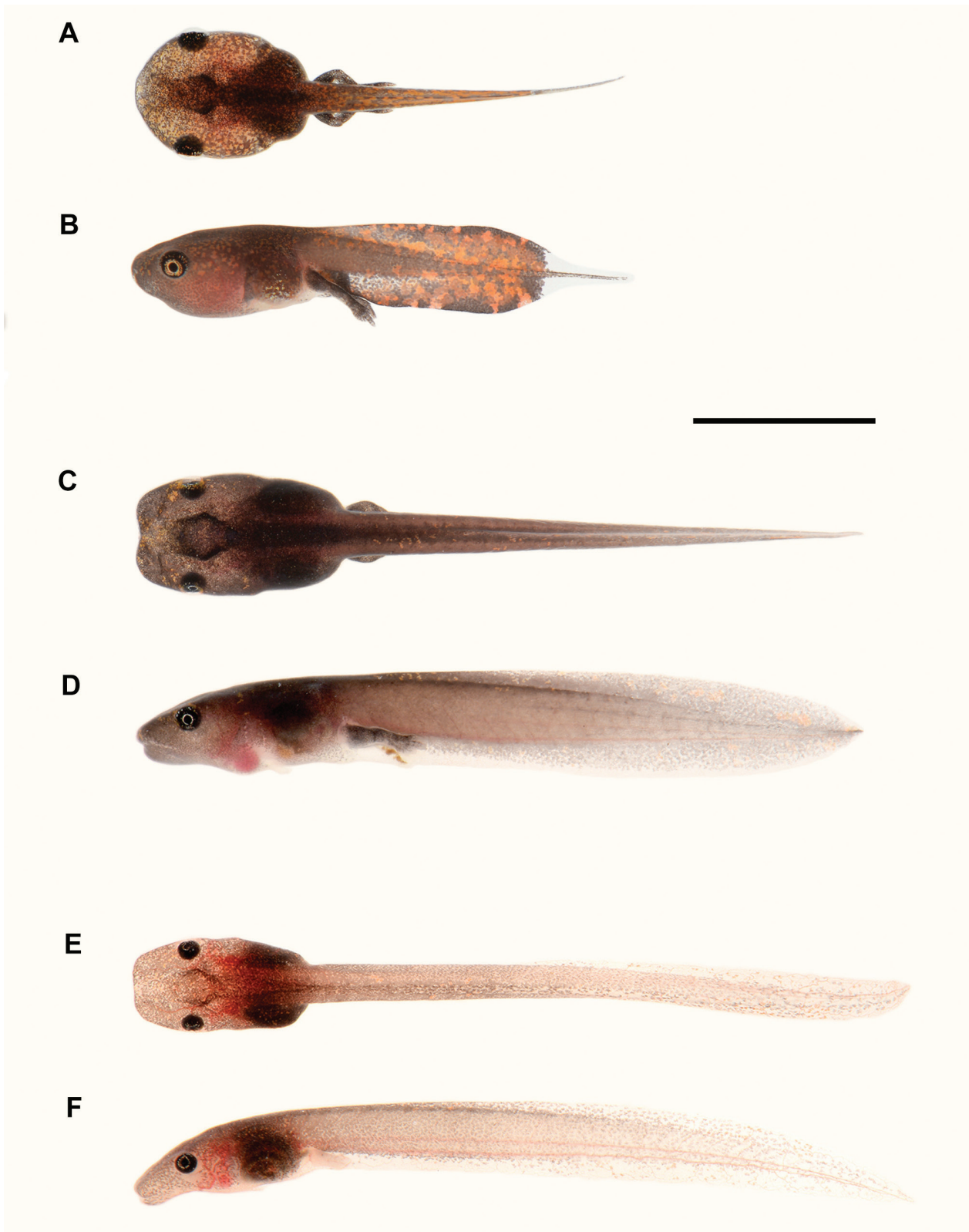


Figure 4. External morphology and coloration in life of the tadpoles of three *Kalophrynus* species at stages 36–37: *K. interlineatus* (ZMMU A-7891), **A** dorsal view, **B** lateral view; *K. honbaensis* (ZMMU A-7892), **C** dorsal view, **D** lateral view; *K. cryptophonus* (ZMMU A-7893), **E** dorsal view, **F** lateral view. Scale bar: 5 mm.

***Kalophrynus honbaensis*.** In dorsal view (Fig. 4C), body subrectangular and slightly elongated, with a broad, concave snout. In lateral view (Fig. 4D), body subtriangular and compressed dorsoventrally, especially in the rostral part of the head. Tail rather long, nearly two and half

times longer than the body or more in some specimens. Tail musculature rather well developed (tail base more than a third of the maximal body width); tail tip broadly acuminate. Tail fins moderately tall, reaching their maximum height at the distal third of the tail length; dorsal



Figure 5. Young metamorphs of three species of *Kalophrynus*: **A** *K. interlineatus* (SVL 4.9 mm); **B** *K. honbaensis* (SVL 6.7 mm); **C** *K. cryptophonus* (SVL 5.5 mm).

fin not extending on the trunk, ventral fin slightly higher than the dorsal fin. Spiracle medial, narrow, and opening at the middle of the belly; covering membrane rather short, with a finely serrated free margin. Vent tube medial and oblique. Eyes lateral and moderately large. Nostrils sealed at larval stages, narial openings developing only at premetamorphic stages 41–42. Nasolacrimal grooves faintly visible before stage 38, at more advanced stages visible from narial protuberances to the anteroventral margin of the orbit. Mouth terminal, transversal, and relatively wide (approximately half of the body width or even slightly more during earlier midlarval stages 35–36). In dorsal view, lower labium slightly visible, semicircular. Upper labium short and concave, with an even edge. Lateral lobes and keratinized mouthparts absent.

In life, at stages 35–41, body and tail uniformly dark brown with scarce irregular pinkish or orange speckles, belly and tail underside paler; tail fins pigmented, grayish with blurred orange spots; spiracle flap unpigmented. Iris black with goldish-silver speckling and narrow metallic ring around the pupil. In preservative, brown color fading to dull gray.

Kalophrynus cryptophonus. In dorsal view (Fig. 4E), body pyriform or subrectangular (depending on gut fullness), rather elongated, with a broad, blunt, and even or slightly concave snout. In lateral view (Fig. 4F), body subtriangular and markedly compressed dorsoventrally, especially in the rostral part of the head; rostral part rather long, nearly one third of the body length. Tail very long, nearly three and half times longer than the body or even more in some specimens. Tail musculature well developed (tail base more than a third of the maximal body width); tail tip tapering and acuminate. Tail fins low, reaching their maximum height at the distal half of the tail length; dorsal fin not extending on the trunk, ventral fin higher than the dorsal fin. Spiracle medial, transversal, narrow, and opening at the middle of the belly; covering membrane short, with an even free margin. Vent tube medial and oblique. Eyes dorsolateral and moderately large. Nostrils sealed at larval stages. Nasolacrimal grooves and lateral line not visible. Mouth terminal, transversal, slit-like, and relatively wide (more than a third of the body width at stage 38 and almost half of the body width at earlier stages). In dorsal view, lower labium not visible;

upper labium short, straight or slightly concave, with an even edge. Lateral lobes and keratinized mouthparts absent.

In life, at early stages (26–31), larvae almost unpigmented, pinkish-white; at midlarval and late larval stages (35–39), skin semitransparent, body and tail uniformly grayish-brown with scarce irregular orange speckles; belly and tail paler than the dorsum; tail fins slightly pigmented, spiracle flap unpigmented. Iris black with scarce golden speckling and narrow, dotted ring around the pupil. In preservative, color fading to pale yellowish-gray.

Metamorphs

The newly metamorphosed froglets of all three species were extremely small and fragile and rather similar externally (Fig. 5). All had a stocky appearance, with bluntly triangular snout, lateral bulging eyes, slender limbs with thin, short, and tapering digits, and smooth skin. Brownish or yellowish dorsal coloration contrasted with darker coloration on flanks. Unlike adults, all froglets displayed whitish or bluish dots on the dorsum and hind limb uppersides. In *K. interlineatus* (Fig. 5A), a darker triangular spot marked the dorsum from the nape of the head to the groins.

Digestive tract

Kalophrynus interlineatus. Tadpole digestive tract of roughly equal thickness, without distinct larval stomach; intestine arranged in a dense round six-looped spiral (Fig. 6A). Length of the digestive tract varying in fixed specimens (BL 4.2–4.7 mm) from 21.5 to 23.5 mm, depending on the tadpole size and gut fullness; DTL/BL 5.01 ± 0.08 ($n = 4$). In all tadpoles, the intestine evenly filled with a thin, brown or dark brownish-green detritus-like mass with fine particles of inorganic material.

Kalophrynus honbaensis. Digestive tract including a well-defined large larval stomach with thin, extensible walls. Fully extended, filled stomach in satiated larvae occupying the entire ventral cavity; intestine arranged in three loops (Fig. 6B). Length of the digestive tract

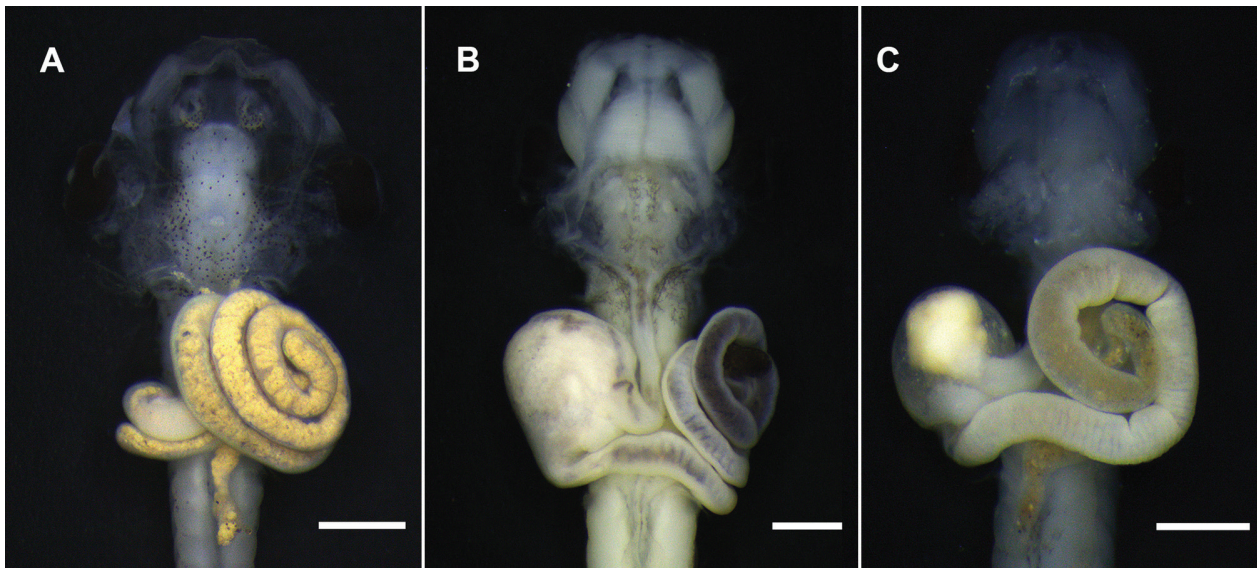


Figure 6. Larval digestive tract of three *Kalophrynus* species, stages 37–38 (in preservative). **A** *K. interlineatus* with gut filled with fine detritus; **B** *K. honbaensis* with extended larval stomach and gut filled with ingested yolk; **C** *K. cryptophonus* with a whole partially digested egg in the larval stomach. Scale bar: 1 mm.

varying in fixed specimens (BL 5.3–6.3 mm) from 17.6 to 22.9 mm, depending on the tadpole size and stomach fullness; DTL/BL 3.39 ± 0.25 ($n = 4$). In most advanced tadpoles (stages 35–41), stomachs containing various amounts of amorphous whitish mass resembling the yolk of frog eggs; intestine filled with loose, amorphous brownish mass.

Kalophrynus cryptophonus. Tadpole digestive tract including a well-defined large larval stomach with thin, extensible walls. Fully extended, filled stomach in satiated larvae occupying the entire ventral cavity; intestine arranged in two loops (Fig. 6C). Length of the digestive tract varying in two loops (Fig. 6C). Length of the digestive tract varying in fixed specimens (BL 4.9–5.2 mm) from 12.3 to 14.9 mm, depending on the tadpole size and stomach fullness; DTL/BL 2.70 ± 0.11 ($n = 4$). In most advanced (stages 35–38) tadpoles from two bamboo stems, stomachs containing various amounts of amorphous whitish mass resembling the yolk of frog eggs and, in some cases, whole eggs of the same size as *K. cryptophonus* intact eggs; intestine filled with a loose, amorphous grayish mass.

Chondrocranium and hyobranchium

Kalophrynus interlineatus. Larval chondrocranium (Fig. 7A) widely elliptic, compressed dorsoventrally, delicate, and widely fenestrated. Cranial floor mostly cartilaginous with membranous central part covered ventrally with a thin, laced bony plate of the parasphenoid. Braincase opened dorsally; tectum synoticum narrow, weak, and smoothly arched forward, without a medial process. Otic capsules rounded and rather small (approximately 27% of chondrocranium length). Palatoquadrate cartilage mostly narrow, with a broadened reinforced rostral part, connected to the braincase anteriorly by rather narrow quadra-

to cranial commissures and posteriorly by short, thin ascending processes. Articular process of the palatoquadrate rather narrow. Defined muscular process absent, lateral margins of palatoquadrate with low elevated marginal ridges protruding dorsally. Subocular bar thin, narrow, and fragile. Posterior part of palatoquadrate connected to the otic capsule by the larval otic process. Subotic process on the ventral surface of the palatoquadrate well defined. Lateral posterior process long, narrow, and markedly extending beyond the lateral limits of the otic capsule. Crista parotica forming a bump-like outgrowth. Subocular fenestrae large and elliptically elongated. Choanal openings wide and elliptical. Ethmoid plate short, subquadrate. Trabecular horns rather elongate, with rostral ends fused to the suprarostrals cartilage with faintly visible suture. Suprarostrals cartilage entire, forming a rather wide thin plate convex rostrally. Mandible (Fig. 7B) delicate, formed by short Meckel's cartilages tapering medially, with prominent dorsal processes. Infraostrals cartilage extremely thin, subtle, and filiform. Hyobranchium (Fig. 7C) length approximately 85% of the total head length. Ceratohyals narrow, with faintly formed rounded anterior processes and very prominent, thin, spike-like anterolateral processes, slender posterior processes and lateral processes formed with thin sheet of cartilage. Basihyal (anterior copula) absent. Basibranchial (posterior copula) slender and fused to narrow hypobranchial plates. Ceratobranchials I–IV long, thin, widely expanded, joined with numerous branchial commissures (up to 21–22 between ceratobranchials I and II) and forming a voluminous branchial basket (approximately 75% of the total length of the hyobranchium). Three well-defined, flattened spiculae fused dorsally to each hypobranchial plate.

Kalophrynus honbaensis. Larval chondrocranium (Fig. 7D) subrectangular, slightly longer than wide (maximum length/maximum width 1.1), flattened dorsoventrally,

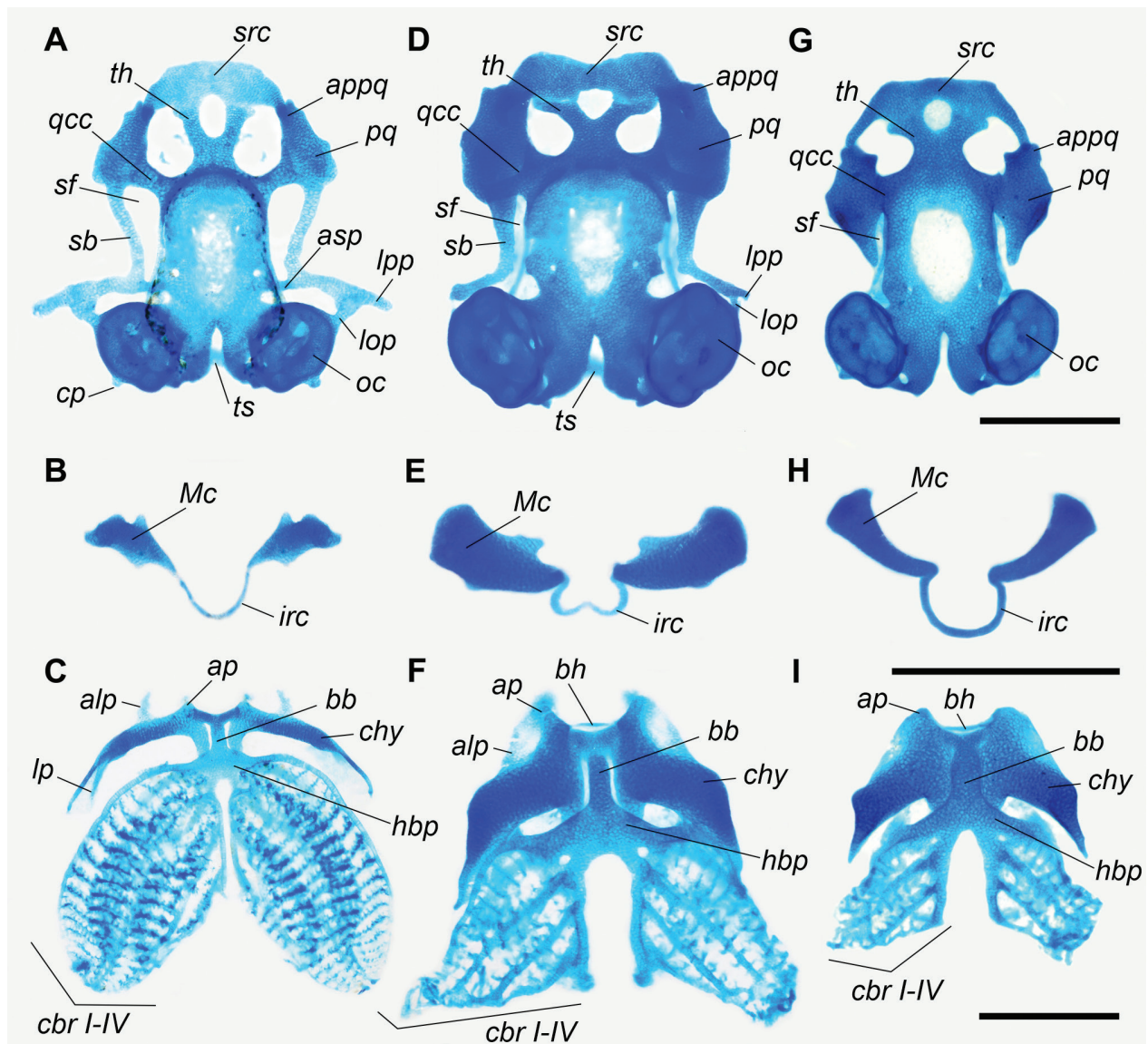


Figure 7. Larval skeleton of three *Kalophrynus* species, stages 37–38: *K. interlineatus*, **A** chondrocranium, **B** mandible, **C** hyobranchium; *K. honbaensis*, **D** chondrocranium, **E** mandible, **F** hyobranchium; *K. cryptophonus*, **G** chondrocranium, **H** mandible, **I** hyobranchium. Scale bar: 1 mm. Abbreviations: *alp* – anterolateral process, *ap* – anterior process, *appq* – articular process of the palatoquadrate, *asp* – ascending process, *bb* – basibranchial, *bh* – basihyal, *cbr* – ceratobranchials, *chy* – ceratohyal, *cp* – crista parotica, *hbp* – hypobranchial plate, *irc* – infracranial cartilage, *lop* – larval otic process, *lp* – lateral process, *lpp* – lateral posterior process of the palatoquadrate, *Mc* – Meckel’s cartilage, *oc* – otic capsule, *pq* – palatoquadrate, *qcc* – quadratocranial commissure, *sb* – subocular bar, *sf* – subocular fenestra, *src* – suprarostril cartilage, *th* – trabecular horns, *ts* – tectum synoticum.

rather robust, well chondrified, and moderately fenestrated. Cranial floor mostly cartilaginous with membranous central part covered ventrally with a thin, laced bony plate of the parasphenoid. Braincase opened dorsally; tectum synoticum very thin and narrow, weak, and smoothly arched forward, with faintly marked triangular medial process. Otic capsules ovoid, of moderate size (approximately 34% of the chondrocranium length). Palatoquadrate cartilage with massive anterior part forming a large, robust articular process and connected to the braincase by a wide quadratocranial commissure. Low dorsal ridges on lateral margins of the palatoquadrate instead of the muscular process. Subocular bar rather weak, narrowing posteriorly; ascending process absent. Posterior part of the palatoquadrate connected to the otic capsule by a very

short larval otic process. Subotic process absent. Lateral posterior process thin, short, and almost not extending beyond otic capsule. Crista parotica not defined. Subocular fenestrae narrowed and elongated. Choanal openings wide and elliptical. Ethmoid plate short and wide, trapeziform. Trabecular horns short, broadened rostrally and widely divergent with rostral ends fused to the suprarostril cartilage with distinct suture. Suprarostril cartilage entire, forming a rather wide thin plate somewhat narrowed and concave medially. Mandible (Fig. 7E) formed by short, thick Meckel’s cartilages tapering medially, with prominent dorsal processes. Infracranial cartilage thin, delicate, filiform, ω-shaped. Hyobranchium (Fig. 7F) approximately 70% of the total head length. Ceratohyals relatively robust, with prominent, sharp anterior processes and rather

long triangular anterolateral processes connected to anterior processes by a thin membranous sheet. Basihyal thin, horizontal, and rod-like. Basibranchial narrow and fused with widened hypobranchial plates. Ceratobranchials I–IV moderately long and thin, joined with branchial commissures (up to 13–15 between ceratobranchials I and II) and forming a moderately large branchial basket (68% of the total length of the hyobranchium). Three well-defined, flattened spiculae fused to each hypobranchial plate dorsally, the lateralmost being the widest.

***Kalophrynus cryptophonus*.** Larval chondrocranium (Fig. 7G) roughly elliptical, elongated (maximum length/maximum width 1.4), rather robust, well chondrified, and moderately fenestrated. Cranial floor mostly cartilaginous with membranous central part covered ventrally with thin, laced bony plate of the parasphenoid. Braincase opened dorsally; tectum synoticum reduced, only small condensation of chondrified tissue visible medially in the membranous chondrocranium roof between otic capsules. Otic capsules ovoid, rather small (approximately 29% of chondrocranium length). Palatoquadrate cartilage with robust anterior part and large articular process connected to the braincase by relatively wide quadratocranial commissures. Subocular bar reduced posteriorly, ascending and lateral posterior processes absent. Crista parotica not developed. Subocular fenestrae narrow, open-ended posteriorly. Choanal openings wide and elliptical. Ethmoid plate short and wide. Trabecular horns short, broad, and widely divergent with rostral ends fused to suprarostrals cartilage without a visible suture. Suprarostrals cartilage entire, forming a rather broad semilunar plate somewhat narrowed medially. Mandible (Fig. 7H) Ω -shaped, with bar-like Meckel's cartilages tapering medially. Infrarostrals cartilage thin but resilient, round in cross-section. Hyobranchium (Fig. 7I) approximately 65% of the total head length. Ceratohyals robust, with wide rounded anterior processes; anterolateral processes indistinct; posterior processes large, robust, and triangular. Basihyal thin and narrowly spindle-shaped. Basibranchial broad and fused with widened hypobranchial plates. Ceratobranchials I–IV rather shortened, slightly thickened, joined with branchial commissures (up to 9–10 between ceratobranchials I and II) and forming a rather reduced branchial basket (48% of the total length of the hyobranchium). Three well-defined, flattened and slightly widened spiculae fused to each hypobranchial plate dorsally, the lateralmost being the widest.

Discussion

Reproduction and development

The existing knowledge on reproductive modes, development and larval morphology in *Kalophrynus* frogs is very fragmentary and moreover complicated by the taxonomic confusion in older publications. Some cases of erroneous species attributions to *K. pleurostigma* by Berry (1972),

Inger (1985), Lim and Ng (1991), and Leong and Chou (1999) were later resolved by Matsui et al. (2012, 2017) and Zug (2015).

Apparently, many (if not most) *Kalophrynus* species tend to breed in more or less confined waterbodies. These waterbodies can be small, shallow pools as for *K. sinensis* (Maglangit et al. 2020), burrows in the ground as for *K. calciphilus* (Dehling 2011) or diverse kinds of phytotelmata, including pitcher plants *Nepenthes*, hollows in trees and stumps or bamboo stems as for *K. limbooliati* and *K. yongi* and possibly for *K. eok*, *K. barioensis* and *K. puncak* (Lim and Ng 1991; Leong and Chou 1999; Das and Haas 2003; Matsui 2009; Matsui and Nishikawa 2011; Fukuyama et al. 2021). *K. meizon* is rather opportunistic, using small pools, hollows in logs and burrows for reproduction (Inger 1985). In contrast, *K. interlineatus* and *K. palmatissimus* breed in various open waterbodies, including large temporary ponds and flooded areas of the forest (Kiew 1984; Manthey and Grossmann 1997; Vassilieva et al. 2016). Thus, the pond-breeding *K. interlineatus*, on the one hand, and *K. cryptophonus* breeding in very small water volumes inside bamboo internodes, on the other hand, represent the extremes of the known spectrum of reproductive habits within the genus, whereas *K. honbaensis*, which spawns in large tree hollows, displays a transitional state.

It has previously been shown that in Asian microhylid genera *Microhyla* and closely related *Nanohyla* phytotelm-breeding species have larger eggs and smaller clutches than pond-breeding species (Vassilieva et al. 2017b). The same pattern is revealed in *Kalophrynus*: the pond-breeding *K. interlineatus* has smaller egg diameters and much larger clutches than the phytotelm-breeding *K. honbaensis* and *K. cryptophonus*. These findings are in agreement with the extremely scarce published data on egg size in other *Kalophrynus* species provided by Berry (1972) and Matsui (2009): 2.3 mm and 1.88–2.14 mm in phytotelm-breeding *K. limbooliati* and *K. yongi*, respectively. The duration of the embryonal period and degree of embryonization also progress from *K. interlineatus* to *K. honbaensis* and *K. cryptophonus*, leading to the hatching of the larvae at more advanced developmental stages, as also occurs in *N. arboricola*.

No trophic eggs were found in any of the tree hollows or bamboo stems with *K. honbaensis* and *K. cryptophonus* larvae during field observations. Presumably, these tadpoles do not rely on parental provisioning and feed on unfertilized eggs from the clutches from which they themselves hatched and/or eat fertilized eggs and embryos from other conspecific clutches laid in the same phytotelmata. Such feeding mode is characteristic of tadpoles of *N. arboricola*, which were observed to swallow conspecific fertilized eggs and developing embryos during experimental feeding in aquaria (Vassilieva et al. 2017b) or to extract eggs from clutches suspended on the walls of the hollows where they lived (Vassilieva, personal observations 2018). The large number of *K. honbaensis* and *K. cryptophonus* tadpoles of different development stages in some of the hollows or bamboo internodes allows us to assume that different mating pairs may share a common breeding site.

External larval morphology

All studied *Kalophrynus* species develop with the larval stage. More or less detailed descriptions of the external larval morphology exist for very few species: *K. palmatissimus* (Kiew 1984; Manthey and Grossmann 1997), *K. limbooliati* (Berry 1972 – as “*K. pleurostigma* smaller form”; Lim and Ng 1991; Leong and Chou 1999 – as *K. pleurostigma*), *K. kiewi* (Berry 1972 – as “*K. pleurostigma* larger form”), *K. meizon* (Inger 1985 – as *K. pleurostigma*), *K. yongi* (Matsui 2009), and *K. intermedius* (Haas et al. 2022); the previous description of *K. cryptophonus* tadpole by Vassilieva et al. (2014) was based on a very early developmental stage and is revised in the present work. While sharing characteristic features common for most microhylid larvae of Orton’s type II (Orton 1953; Donnelly et al. 1990), such as simplified mouthparts without keratinized elements, a medial spiracle and a medial anal tube, as well as a set of features common for *Kalophrynus* larvae, such as small size and terminal mouth without papillae, the described tadpoles display a variety of body shapes and proportions. The endotrophic larvae of the nepenthophilous species *K. limbooliati* and *K. intermedius* are small, scarcely pigmented, with a rounded body, gut filled with yolk and moderately long tail with blunt tip and medium-tall fins (Lim and Ng 1991; Haas et al. 2022). Among the species studied by us, the tadpole of *K. interlineatus* is externally most similar to another pond-dweller, larval *K. palmatissimus*: both have a rounded body, convex belly, strictly lateral eyes, a rather short and broadly lanceolate tail with a weakly developed muscular portion, tall fins and a thin, fragile terminal filament. This morphological plan is characteristic of many other microhylid tadpoles coexisting with *K. interlineatus* in the same temporary ponds, e.g., for some *Microhyla* or *Micryletta* species (Vassilieva et al. 2016). In contrast, the tadpole of *K. cryptophonus* closely resembles larval *K. yongi* by elongated body, dorsolateral eyes, and extremely long tail with well-developed musculature and low fins. The same features are also characteristic for the larval *N. arboricola* and many nonmicrohylid obligatorily oophagous tadpoles (Lannoo et al. 1987; Lehtinen et al. 2004; Vassilieva et al. 2017b); this allows us to suggest that the tadpole of *K. yongi* may also not be endotrophic, as has been assumed by Matsui (2009), but oophagous, and the yolk in its intestine is not embryonic but rather from eaten eggs. The external larval morphology of *K. honbaensis* displays an intermediate state, with lateral eyes and a moderately long tail with less reduced fins. Possibly, this set of features reflects the evolutionary transition from pond to phytotelm breeding through the use of large cavities for reproduction.

Internal larval morphology

Differences in the morphology of the larval digestive tract in the three studied species also reflect the evolutionary transition from micro- to macrofeeding. Suspension-feeding tadpoles, such as *K. interlineatus*, have a relatively

long, uniform, spiraled gut evenly filled with thin homogeneous food (Altig and Kelly 1974; Wassersug and Pyburn 1987). In contrast, oophagous tadpoles, such as *K. honbaensis* and *K. cryptophonus*, tend to have a shorter digestive tract and large larval stomach (manicotto glandulare) as an adaptation to food storage and digestion in intermittent macrophagy (Lannoo et al. 1987; Liang et al. 2002). The same differences are observed between other suspension-feeding and oophagous microhylids (Vassilieva et al. 2017b); and, again, *K. honbaensis* displays an intermediate state of gut shortening.

The morphology of the larval chondrocranium and brachial skeleton of the three *Kalophrynus* species also differs markedly. In *K. interlineatus*, these structures are in many aspects similar to those of other pond-dwelling microhylid tadpoles studied to date, in particular, the representatives of the neotropical genera *Hamptophryne*, *Chiasmocleis*, *Gastrophryne*, *Dermatonotus*, and *Elachistocleis* (de Sá and Trueb 1991; Vera Candioti 2007; Trueb et al. 2011; Fabrezi et al. 2012; Ferreira and Weber 2021), as well as the Asian genera *Uperodon*, *Microhyla*, and *Kaloula* (Ramaswami 1940; Haas 2003; Bowatte and Meegaskumbura 2011; Vassilieva 2021; Zhang et al. 2021). All of these tadpoles share features such as a generally broad and depressed shape of the skull, an underdeveloped muscular process of the palatoquadrate, well-developed lateral posterior process, and the presence of larval otic and subotic processes; the wide cristae paroticae are mostly characteristic of neotropical microhylids, but in Asian species they are rather reduced. Other typical features of the microhylid tadpoles are a small mandible with a very thin, delicate infrastralar cartilage and hyobranchium with narrow, widely separated ceratohyals and a voluminous branchial basket. These features are recognized as adaptations for pumping large volumes of water through the buccal cavity and thus for suspension feeding (Wassersug and Hoff 1979). In contrast, the larval chondrocranium and branchial skeleton of *K. cryptophonus* are greatly modified. The complete reduction of the posterior part of the palatoquadrate along with ascending, larval otic and lateral posterior processes is among its most striking features. A similar reduction is found in the rhacophorid oophagous tadpole *Vampyrus vampyrus* (former *Rhacophorus vampyrus*) and is thought to be related to macrophagy (Vera Candioti et al. 2021). The infrastralar cartilage in *K. cryptophonus* seems to be adapted to stronger mechanical stresses than in *K. interlineatus*, which may be related to the need to suck out viscous mucus and swallow larger food. The hyobranchium of *K. cryptophonus* also sharply differs from that of *K. interlineatus* in greatly enlarged, massive ceratohyals giving space for the attachment of potent depressor muscles and, on the contrary, significantly reduced and simplified branchial baskets. This can be attributed to the increasing pumping force during the ingestion of large food and the reduction in the filtering function during the transition to macrophagy (Wassersug and Hoff 1979). The larval skeleton of *K. honbaensis* displays a somewhat intermediate state between these “suspension-feeding” and “macrophagous” extremes: in this spe-

cies, the reduction of the posterior part of the palatoquadrate is not complete, although it affects the ascending and lateral posterior processes; the mandible is reinforced whereas the infrastralar cartilage is still thin and weak; and the hyobranchium is more adapted to macrophagous feeding, although its branchial basket is less reduced than in *K. cryptophonus*.

Conclusions

Our study of the reproductive biology and larval morphology of three closely related species of the genus *Kalophrynus* reveals the exceptional evolutionary plasticity of these microhylids, previously known mostly from fragmentary data. The variability of their breeding modes and larval features reflects their ability to adapt to a wide variety of environmental conditions. Although spawning in open waterbodies is most common among anurans and is considered an ancestral form of breeding, the development of terrestrial forms of reproduction, including the use of phytotelmata, is an important evolutionary trend. Oophagy is an independently emerging strategy in different families to overcome the inevitable food deficit during the development of offspring in confined spaces and is thus an alternative to endotrophic development. In general, the evolution of terrestriality is accompanied by many changes, including the enlargement of eggs and the reduction of clutch size (Duellman and Trueb 1986; Crump 2015). An increase in yolk reserves is natural when it is necessary to ensure larval development for as long as possible under conditions of food deficiency. However, in contrast, many anurans with oophagous larvae display a decrease in egg size compared to related forms without oophagy; this is explained by the replenishment of nutrients by maternal provisioning with trophic eggs (Summers et al. 2007; Furness et al. 2022). In species with the simplest, evolutionarily primitive form of oophagy without parental care, however, tadpoles feed on the unfertilized part of the clutch or on fertilized conspecific eggs (Schiesari et al. 2003; Haddad et al. 2005). This occurs in *N. arboricola* and most likely in *K. honbaensis* and *K. cryptophonus*. In this case, egg enlargement, prolongation of embryonic development, and hatching at later stages, i.e., embryonization, are beneficial under developmental conditions in a protected environment. Terrestriality is associated with evolution via embryonization up to its extreme form, direct development (Callery et al. 2001). Whereas many studies are devoted to the evolution of direct development, the very first steps of embryonization are largely neglected.

In contrast, for *K. interlineatus*, which breeds in open waterbodies, another strategy is advantageous: many small eggs, which develop rapidly in tropical climates, and further tadpoles grow in the food-rich medium of seasonal waterbodies. A striking developmental feature of this species is an unusually short larval period for anurans (only two weeks from hatching to leaving a water) and

rapid metamorphosis. Most likely, such “ephemeral” development is beneficial when breeding in rapidly drying waterbodies, such as rainy puddles or flooded forest areas, and expands the reproductive potential of the species.

Feeding on relatively large eggs requires special morphological adaptations, especially in groups where the basic form of larval feeding is microphagy, as in typical microhylid tadpoles. The features of the external morphology of the oophagous tadpoles *K. honbaensis* and *K. cryptophonus*, as well as the structure of their skeleton and digestive tract, indicate that they do not simply eat eggs concomitantly with other food but are specialized oophages. At the same time, we have the opportunity to trace the morphological rearrangements that accompany the evolution from pond-dwelling suspension feeders to phytotelm-dwelling, highly specialized oophages through an intermediate form such as *K. honbaensis*. Our findings, along with the previously obtained knowledge of *N. arboricola*, suggest that the origin of macrophages based on microphage morphology is not unique among microhylids and can be achieved in different ways. In addition, these findings extend our knowledge of the diversity of Southeast Asian tadpoles and indicate that the distribution of larval oophagy among anurans is broader than was thought until recently.

New data additionally show that plasticity in development and morphology allows anurans to adopt new habitats under challenging conditions. This is especially true for montane conditions, where the absence of suitable spawning ponds and other restrictions impose especially high demands on reproductive and larval adaptations and contribute to reproductive diversification (Haddad and Prado 2005). Indeed, although the adult frogs of the studied *Kalophrynus* species are apparently similar, the diversity of their reproductive modes and larvae is rather impressive.

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