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Notes on lung development in South African ghost frogs (Anura: Heleophrynidae)

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ABSTRACT

Lungs are a prototypical trait of most tetrapods, but some amphibians have become secondarily lungless over evolutionary time. Anuran (frog) tadpoles offer an opportunity to examine lung loss from an evolutionary perspective, because there are many independent instances where lungs are not inflated until adulthood, and so are functionally lost. Lung loss is typically associated with living in fast-flowing streams, and so we examined larval lung development in the stream specialist family Heleophrynidae. We find that one genus, *Hadromophryne* Van Dijk, 2008, has large lungs as a tadpole, while the other genus, *Heleophryne* Sclater, 1898, has much smaller, stunted lung buds. We further speculate how this information changes our understanding of how the specialised torrent form has evolved in this specialised group.

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The ghost frogs of South Africa (Anura: Heleophrynidae) are a particularly odd and historically mysterious family of frogs. The phylogenetic placement of the group among frogs has been historically controversial, with previous authors aligning the heleophrynids with bufonid toads, Australian myobatrachids, South American leptodactylids, or pelobatoids (Noble 1931; Ford and Cannatella 1993; Ruvinsky and Maxson 1996; Haas 2003). Modern molecular phylogenetics appear to have finally resolved this issue, with several studies independently placing the Heleophrynidae as sister to the rest of suborder Neobatrachia, which includes the majority of extant anurans (Frost et al. 2006; Pyron and Wiens 2011; Feng et al. 2017). Within Heleophrynidae, there are two sister genera, the type genus *Heleophryne* Sclater, 1898 consists of five species spanning different ranges of the Cape Fold Mountains in South Africa and the monotypic *Hadromophryne*

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natalensis Van Dijk, 2008 has populations spread across the more northern Drakensberg escarpment of South Africa, Lesotho, and Eswatini (Van Dijk 2008; Channing et al. 2012).

The life history of heleophrynid tadpoles is not well known, except that they inhabit cold, fast-flowing streams (Channing et al. 2012). Heleophrynid tadpoles are torrent-adapted, with large, sucker-like mouths they use to adhere to rocks in stream rapids and torrents (Noble 1929; Channing et al. 1988). This derived phenotype is not unique to ghost frog larvae: the tadpoles of North American *Ascaphus*, some Australian pelodyadines, the West African *Odontobatrachus*, and others all live in torrents and have converged on similar internal and external morphology (Gradwell 1971; Gradwell 1975; Haas and Richards 1998; Haas 2003; Doumbia et al. 2018).

Common adaptations of stream-adapted tadpoles are a ventral suction apparatus (an oral sucker in heleophrynid tadpoles and others), dorso-ventral flattening of the body, and lung reduction or loss of function (Altig and Johnston 1989; Haas 2003). More generally, lung or swim-bladder loss are associated with a stream ecology across salamanders and fish (Gee et al. 1974; Wilkinson and Nussbaum 1997). Lung loss, in particular, has received significant attention in the context of the evolution of the lungless plethodontid salamanders, which Wilder and Dunn (1920) attributed to a historical association with streams (but see Ruben and Boucot 1989). The evolution of lung loss in fast-flowing water is often attributed to the increasing difficulty of rheotaxis (i.e., moving in a current) when an animal is positively buoyant (Beachy and Bruce 1992; Bruce et al. 1994). Additionally, respiratory lungs are less likely to be necessary in the presumably well-oxygenated environment of cold, fast-flowing streams (Noble 1931). In accordance with this, some torrent-adapted tadpoles, including *Ascaphus truei* and *Litoria dayi*, delay lung inflation until metamorphosis (Haas 2003). This contrasts with non-stream dwelling tadpoles, which almost exclusively breathe air to fill the lungs from an early age, with the notable exception of bufonid toad tadpoles (Wassersug and Seibert 1975; Ultsch et al. 1999; Wells 2010; Schwenk and Phillips 2020). Additionally, it has been shown that functional larval lung loss can be experimentally induced in *Xenopus laevis* tadpoles by preventing air-breathing, showing the possibility for behavioural plasticity to play a role in mediating lung evolution (Rose and James 2013; Rose 2014). However, no study has yet demonstrated the presence of environmentally driven lung variation in natural populations.

Noble (1929) examined the lungs of the now critically endangered Table Mountain Ghost Frog, *Heleophryne rosei*, and determined that much like the Pacific Tailed Frog, *Ascaphus montanus*, *H. rosei* tadpoles lacked inflated lungs. However, more than 50 years later, Wassersug and Heyer (1988) described the closely related (and, at the time, congeneric) *Hadromophryne natalensis* as being lunged. Wassersug and Heyer (1988, p 2) describe the lungs as “small, less than 25% of the buccal floor; uninflated”. More than a decade later, Haas (2003) also scored *H. natalensis* as lunged in a study examining anuran phylogeny based on larval characters.

It is somewhat strange that *H. rosei* would have uninflated lungs, while *H. natalensis* would not, given their close phylogenetic relationship and ecological affinity. Common sense and parsimony would suggest that a torrent-adapted lifestyle is ancestral for the group, and lunglessness is often a component of this condition in tadpoles. While never specifically remarked on previously in the literature, the apparent discrepancy between *Hadromophryne* and *Heleophryne* could make some functional sense, as

Hadromophryne natalensis tadpoles have been known to leave the water and climb up on wet, vertical rock-faces with its sucker, while *Heleophryne* are not known to leave the water (Channing et al. 2012). Perhaps lungs might be useful during climbing when the gills are not being supplied with fresh water for aquatic breathing, as seen in some semi-terrestrial cyclorhampids (Dias et al. 2021). This could be interpreted as either a non-parasimonious re-evolution of larval lung use in *Hadromophryne*, or retention of lungs in *Hadromophryne* due to selection for semiterrestriality.

If *Heleophryne* and *Hadromophryne* differ with regard to their lung status, then lunglessness is not fixed in the Heleophrynidae, while torrent-dwelling is. This finding could have implications for how larval lunglessness and torrent-dwelling evolved in tadpoles of this group and amphibians in general. If torrent-adaptation is ancestral for heleophrynids, but lunglessness is not, then ghost frogs would provide the best example of a putative step-wise evolution of larval lunglessness following the move into streams and evolution of other torrent-derived features. To resolve this question, given the independent examiners for both genera, we examined the lung morphology of four species of heleophrynid tadpole and here describe those findings.

We conducted tadpole dissections at the Port Elizabeth Museum (Bayworld) in Gqeberha, South Africa and the Kansas University Biodiversity Institute in Lawrence, Kansas with full permission (Table 1) to ascertain the lung status of heleophrynid tadpoles. We examined *Hadromophryne natalensis* ($n = 9$), *Heleophryne hewitti* ($n = 2$), *Heleophryne purcelli* ($n = 3$), and *Heleophryne regis* ($n = 1$) tadpoles. Museum specimens had all been fixed in formalin and were dissected manually under a dissection scope while submerged in 70% ethanol or water, depending on museum protocols. We selected tadpoles between Gosner stages 25 and 44 (Gosner 1960) for dissection. Tadpoles were dissected to find evidence of lungs or lung buds by placing each tadpole ventral side up in an ethanol-filled plastic container with a thin layer of cured silicon at the bottom. We then made a sagittal incision from the heart to the vent as well as transverse incisions on either side at these same points. The ventral skin flaps were then pulled back and

Table 1. Museum specimen voucher numbers.

Voucher No.	Specimen data			
	Species	Locality*	Gosner Stage	Inflated lungs
KU196020	<i>Hadromophryne natalensis</i>	KZN	25	yes
KU196020	<i>Hadromophryne natalensis</i>	KZN	25	yes
KU196020	<i>Hadromophryne natalensis</i>	KZN	25	yes
NA**	<i>Hadromophryne natalensis</i>	KZN	25–30	yes
NA**	<i>Hadromophryne natalensis</i>	KZN	25–30	yes
NA**	<i>Hadromophryne natalensis</i>	KZN	25–30	yes
NA**	<i>Hadromophryne natalensis</i>	LIMP	25–30	yes
NA**	<i>Hadromophryne natalensis</i>	LIMP	25–30	yes
NA**	<i>Hadromophryne natalensis</i>	LIMP	25–30	yes
PE_T419b	<i>Heleophryne hewitti</i>	EC	38	no
PE_T419a	<i>Heleophryne hewitti</i>	EC	41	no
KU196021	<i>Heleophryne purcelli</i>	WC	25	no
KU196021	<i>Heleophryne purcelli</i>	WC	25	no
PE_T278	<i>Heleophryne purcelli</i>	WC	38	no
PE_T034	<i>Heleophryne regis</i>	EC	39	no
PE_A1489	<i>Heleophryne regis</i>	WC	adult	yes

*Abbreviations of provinces of South Africa: KZN = KwaZulu-Natal, LIMP = Limpopo, EC = Eastern Cape, WC = Western Cape.

**Tadpoles recently collected and not yet accessioned. Contact authors for details.

pinned to reveal the spiral intestine and liver, which were carefully removed with forceps and stored. After removing the intestine, liver, and any fatty tissues, the lungs were scored as either functional and large or small, non-functional lung buds. In tadpoles, the lungs sit along the dorsal wall of the visceral cavity and connect to the glottis where they lie alongside the most anterior portion of the oesophagus. We also dissected one adult *H. regis*. All museum specimen voucher numbers are included in [Table 1](#). Care was taken to preserve specimens as fully as possible, and dissected specimens and all associated material was labelled and returned to museums' collections.

We found that all examined members of *Heleophryne* lack functional lungs in the larval phase, and that *Hadromophryne* tadpoles have small, but apparently functional, inflated lungs. *Heleophryne* tadpoles have small, non-functional lung buds that are very difficult to see, even at magnification ([Figure 2](#)). We dissected one metamorphic individual of *H. hewitti*, which also lacked functional lungs and an adult *H. regis*, which had short, inflated lungs that extended ~ 25% of the way down the body cavity and were unpigmented. *Hadromophryne* tadpoles have very thin, unpigmented lungs that extend ~ 25% of the way down the visceral cavity ([Figure 2](#)).

To confirm that *Hadromophryne* lungs are indeed functional, we collected an additional three individuals from two populations near Hillcrest, KwaZulu-Natal, South Africa and Haenertsberg, Limpopo, South Africa. Tadpoles were euthanized and dissected before fixation. These dissections revealed fully air-filled lungs in three individuals ([Figure 3](#)), lungs with small bubbles trapped within in two individuals, and one individual had similar lungs without any air present. There were no obvious differences between the populations. It is typical for fresh dissections of tadpole lungs to vary in the degree of inflation, as air can often be displaced from the lungs by the dissection process itself, and so we concluded that *Hadromophryne* lungs are indeed functional and filled with air.

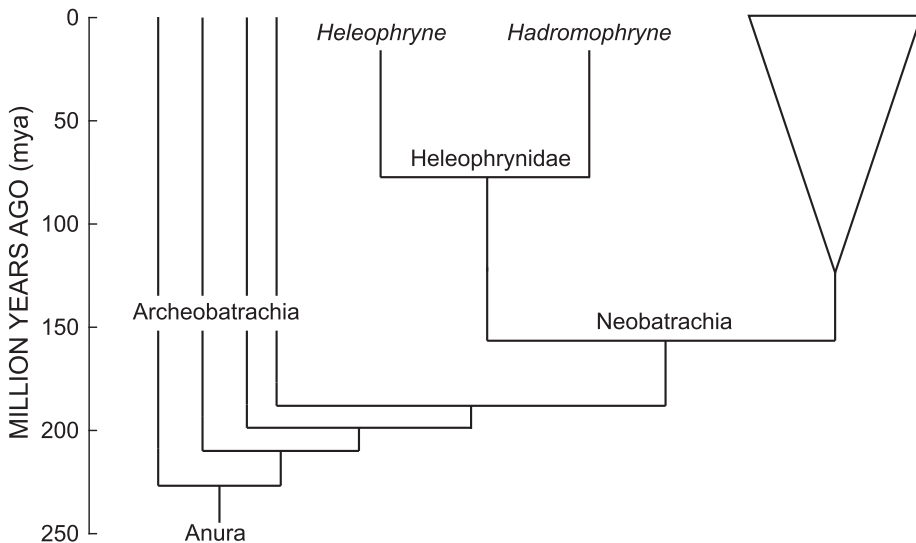


Figure 1. Evolutionary relationships of the Heleophrynidae. An abbreviated ultrametric tree based on [Feng et al. \(2017\)](#) and [Jetz and Pyron \(2018\)](#).

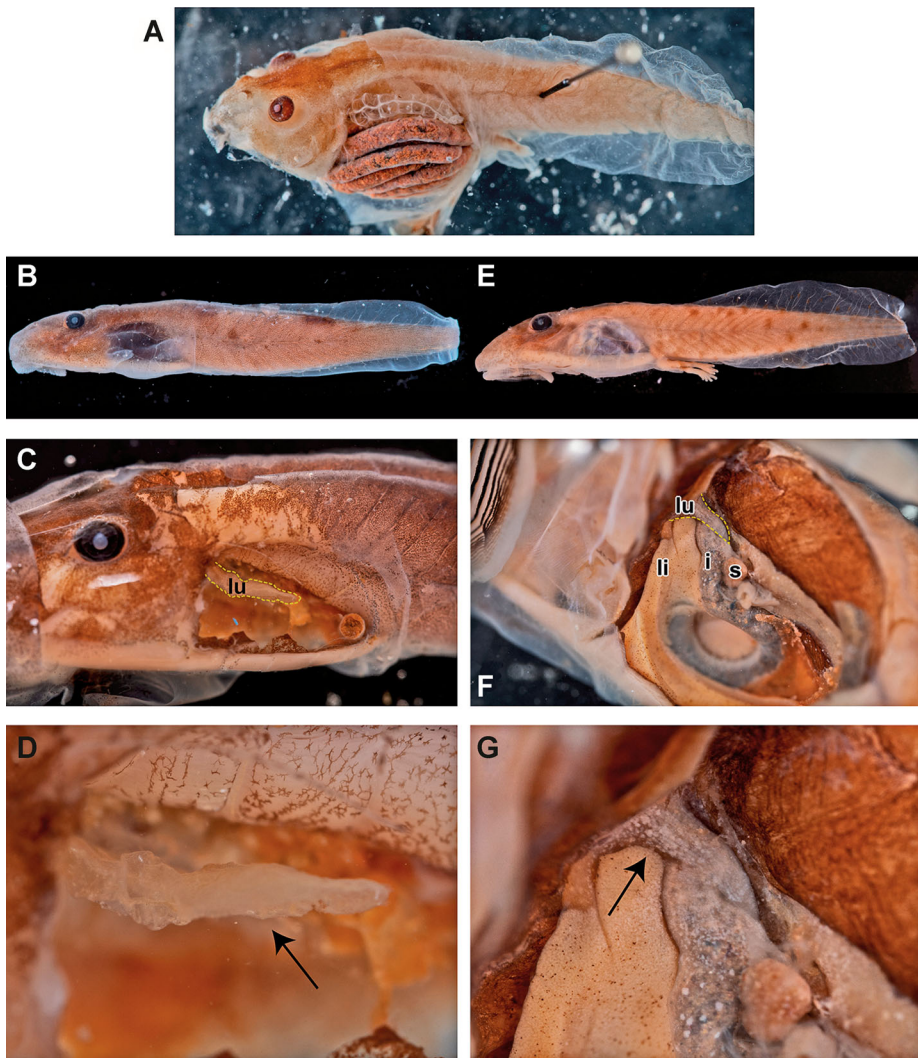


Figure 2. Dissections of tadpole lungs. **A:** large, typical tadpole lungs in *Notaden nicholli* (KU204195). **B:** whole body view of *Hadromophryne natalensis* (KU196020). **C:** dissected lateral view of *H. natalensis* tadpole, showing lung with yellow outline. **D:** close-up of *H. natalensis* lung. **E:** whole body view of *Heleophryne purcelli* (KU196021). **F:** dissected latero-ventral view of *H. purcelli* tadpole, with lung bud outlined in yellow. **G:** close-up of *H. purcelli* lung bud. Abbreviations: i = intestine; li = liver; lu = lung; s = spleen.

Lungs are an essential aspect of the tetrapod condition and are observed across nearly all terrestrial and aquatic tetrapods. Examples of adult lunglessness are extremely rare, including only a single caecilian (*Atretochoana eiselti*), a single frog (*Barbourula kalimantanensis*) and several instances within salamanders (most famously in the family Plethodontidae) (Wake 1966; Nussbaum and Wilkinson 1995; Bickford et al. 2008). However, even in the most well-known cases, some lung tissue usually remains. *Barbourula kalimantanensis* frogs have non-functional, vestigial lung buds (DB Blackburn, *in lit.*) and plethodontid salamanders retain a transient lung bud that is resorbed later in



Figure 3. Fresh dissection of *Hadromophryne* tadpole. A *Hadromophryne natalensis* tadpole dissected before fixation, photographed with an Andonstar AD409 HDMI Digital Microscope. The lungs (outlined in yellow) are full of air. Tadpole was collected in Haenertsberg, Limpopo province, South Africa.

development (Mekeel 1930; Lewis 2016). Lungless tadpoles (whether natural or experimental as in Rose and James (2013)) also retain lung tissue, but have lost lung function, presumably due to similar evolutionary pressures to the adults mentioned above. Tadpoles then present a unique opportunity, because there are many species from independent lineages that lack functional and inflated lungs until metamorphosis. In many cases, tadpoles can be viewed as transitional forms for the evolution of lunglessness because aside from the apparently unique case of *B. kalimantensis*, all known adult frogs possess inflated lungs.

The African ghost frogs are a prototypical example of the torrent-adapted tadpole form, with large, dorso-ventrally flattened bodies and large sucker mouths with many tooth rows (Channing et al. 2012). Both heleophrynid genera share these adaptations, but differ in the state of their lungs. Compared to typical pond species, i.e., *Rana* or *Xenopus*, both genera have reduced lungs, but that reduction is far more extreme in *Heleophryne*. This finding confirms the findings of Noble (1929), Wassersug and Heyer (1988), and Haas (2003).

It is worth considering the evolution of the heleophrynid condition in the context of this information. The common ancestor of heleophrynids and their sister group (the sub-order Neobatrachia) likely had a fairly generalist tadpole with large, inflated lungs, given the spread of larval lungs across the anuran tree of life (Wells 2010). Some descendants of that generalist likely moved into higher elevations and became adapted to living in mountain streams, eventually evolving into the modern heleophrynid form. From this

study, it is apparent that lung reduction is common to the group, but a total loss of pre-metamorphic lung function is restricted to *Heleophryne*. Either complete larval lung loss occurred in an ancestor of *Heleophryne* after the split with *Hadromophryne*, or larval lung loss evolved earlier in an ancestor of both genera and *Hadromophryne* re-evolved larval lung use. Despite the superficial ecological similarities between *Heleophryne* and *Hadromophryne*, perhaps the lungs serve some adaptive function in *Hadromophryne*, preventing their loss – other similar suctorial tadpoles around the world are known to retain inflated lungs, such as *Hyloscirtus armatus* and *Boophis* spp. (Haas 2003). A possible adaptive explanation is sub-aerial respiration while climbing on wet rock surfaces out of the water, also suggested for semi-terrestrial tadpoles of the genus *Cyclorhamphus* (Channing et al. 2012, Dias et al. 2021). Alternatively, *Hadromophryne* might simply have inherited lungs from a lunged common ancestor, and has not yet evolved a morphological response despite mild selection to lose lungs. It is possible that given enough time, descendants of *Hadromophryne* might also lose lung function during the tadpole phase. Alternatively, some form of developmental bias might constrain adaptive lung evolution in *Hadromophryne* due to genetic or functional pleiotropies.

Hadromophryne also differs from *Heleophryne* in oral anatomy of the larvae. *Hadromophryne* tadpoles retain a posterior keratinised jaw sheath, while *Heleophryne* tadpoles have lost all aspects of the jaw sheath. Once again, *Hadromophryne* retains vestiges of a trait shared by most neobatrachan frogs and which *Heleophryne* has lost completely. Whether these vestiges are maintained by selection, constraint, or chance is unknown, but there is little evidence to support an adaptive explanation in either case. The adults of both genera are very similar, clearly differentiated only by the pronounced crests found on the humeri of *Hadromophryne* (Van Dijk 2008). Our results suggest that the loss of larval lungs is a synapomorphy of *Heleophryne*, and one of the only known diagnostic characters between the two genera.

According to estimates from genetic data, the sister genera *Hadromophryne* and *Heleophryne* diverged ~ 64 million years ago (Jetz and Pyron 2018). The landscape of southern Africa happens to be one of the oldest in the world, and the mountains we see today have been fairly stable since before this divergence (McCarthy and Rubidge 2005). It is noteworthy that the lungless genus, *Heleophryne*, is also the lineage which has diverged and speciated into five extant lineages over the past ~ 64 million years, while *Hadromophryne* apparently has not. Channing (2001) suggested *Hadromophryne* may actually consist of multiple un-recognised species, but subsequent genetic work has not yet revealed evidence for many cryptic species (Grobler et al. 2003). Perhaps the presence of larval lungs gives *Hadromophryne* more flexibility in larval habitat, which in turn leads to greater inter-connectedness of populations, while *Heleophryne* populations are isolated to streams that can support lungless tadpoles reliant on well-oxygenated waters to survive to metamorphosis. This same principle might also help explain why *Heleophryne* includes such a high proportion of species at risk of extinction (Measey 2011). Lungless tadpoles may be extremely reliant on streams with enough oxygen, while lunged tadpoles may be able to survive if the water quality temporarily decreases or temperatures rise. It is also possible that behavioural plasticity in lung use and subsequent impacts on lung development could play a role in these dynamics, but this remains speculative as there is no evidence for natural populations of tadpoles exhibiting plasticity in lung inflation.

The fact that many tadpoles breathe air is an unappreciated aspect of anuran biology. That some heleophrynid tadpoles breathe air while others do not, despite living in the same habitats and sharing an evolutionary history, is a remarkable case study for the evolution of lung loss. *Heleophryne* and *Hadromophryne* are the only known sister genera to share the torrent phenotype and differ in the presence/absence of lungs. This presents a unique opportunity to study the functional ramifications of lungs in torrent-adapted species, as the tadpoles are otherwise extremely similar and have a shared evolutionary history. Future studies could compare the locomotor performance of both genera in a controlled environment to test whether performance of *Hadromophryne* could be improved by the prevention of air-breathing, and whether air-breathing allows *Hadromophryne* tadpoles to tolerate lower oxygen levels than their lungless cousins.

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