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Direct development in African squeaker frogs (Anura: Arthroleptidae: *Arthroleptis*) reveals a mosaic of derived and plesiomorphic characters

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Abstract Direct development has evolved independently several times in anurans and direct-developing species are characterized by large-scale developmental repatterning and a complete, or near complete, absence of most tadpole-specific structures. Earlier studies stressed the similarities among different direct-developing species, but more recent studies have indicated differences in the reduction of tadpole-specific structures among different taxa. Here, we describe egg deposition, clutch characteristics and embryonic development of the direct-developing squeaker frogs of the genus *Arthroleptis*, providing the first detailed description of direct development in Arthroleptidae. Embryonic development in *Arthroleptis* is characterized by the presence of an opercular fold that still encloses the developing forelimbs, the absence of external gills and an only moderately extended tail. A comparison with published information on other direct-developing anurans reveals broad dissimilarities in the formation of an opercular fold and very different tail morphology among different taxa. An egg tooth, often considered characteristic of direct-developing anurans, seems to be restricted to New World Terrarana. The embryonic diversity seen in direct-developing anuran taxa argues against simplistic assumptions about the evolution of direct development.

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Introduction

Direct development has evolved independently as a derived reproductive mode in many lineages of animals including molluscs, crustaceans, echinoderms and amphibians (e.g. Duellman and Trueb 1986; Raff 1992; Scholtz 2000; Collin 2004). In amphibians in particular, direct development is a surprisingly widespread reproductive strategy, as is witnessed by the large number of direct-developing species and its repeated, independent evolution in anurans, salamanders and caecilians (Wake and Hanken 1996; Thibaudeau and Altig 1999; San Mauro et al. 2014). In contrast to the biphasic ancestral state, the absence of an aquatic larval stage makes it possible to reproduce without any direct access to bodies of water and direct development might be favoured where environmental conditions do not provide any suitable aquatic larval habitats (Goin and Goin 1962; Müller et al. 2013; Liedtke et al. 2017). Biphasic aquatic reproduction has been lost in a number of other ways in various amphibians, including terrestrial, non-feeding tadpoles (e.g. Warren 1922; Wake 1980), viviparity (e.g. San Mauro et al. 2014; Sandberger-Loua et al. 2017) or incubating eggs in, e.g. dermal pouches or the male vocal sacs (see Wells 2007 for a comprehensive summary of amphibian reproduction), but direct development is by far the most common terrestrial reproductive strategy.

In all amphibians, direct-developing species are characterized by large scale developmental repatterning and in anurans in particular most tadpole-specific structures, such as the lateral line system, larval mouth parts, the cement gland or the

coiled intestine, are greatly reduced or entirely absent during development (Hanken et al. 1997a). In addition to the profound reduction of larval structures, embryos of most direct-developing frogs show similar modifications, such as an enlarged membranous tail with a highly vascularized fin, a reduction of external gills, an early and nearly simultaneous development of front- and hind limbs and a large yolk reserve (Townsend and Stewart 1985; Bahir et al. 2005; Anstis et al. 2007). The apparent similarity in embryonic development of the few studied direct-developing species has led to the idea that the anuran tadpole might represent a distinct developmental module that has been deleted from the ontogeny in direct-developing species (Callery and Elinson 2000a; Callery et al. 2001; Ziermann and Diogo 2014).

In anurans, direct development, in a strict sense in which development is completed within the egg, has been reported to occur in members of the Arthroleptidae, Bufonidae, Microhylidae, Myobatrachidae, Ranidae, Rhacophoridae, Sooglossidae and Terrarana (Brachycephaloidea sensu Frost 2016). In some of these taxa, all species are direct-developing (e.g. Terrarana), whereas in others, only some are (e.g. Myobatrachidae) and direct development may have even evolved several times independently within the group (e.g. Bufonidae). Although many species of frogs develop directly, developmental data are sparse or absent for many taxa that are known or presumed to develop directly (Thibaudeau and Altig 1999), a fact attributable to the often well-concealed breeding sites (Bahir et al. 2005). Direct development has been studied in detail for only a few species (Townsend and Stewart 1985; Hanken et al. 1992; Bahir et al. 2005; Kerney et al. 2007; Anstis et al. 2007), but short descriptions or cursory notes reporting direct development are available for a greater number of species (e.g. Alcala and Brown 1982; Bourne 1997; Jameson 1950; Krishnamurthy et al. 2002). The most well-known are those of the neotropical genus *Eleutherodactylus* and species formerly assigned to it (Terrarana; Hedges et al. 2008; Heinicke et al. 2009). Although direct development has been confirmed for a number of the ca. 1063 species of Terrarana (Frost 2016), detailed information on various aspects of development is only available for *Eleutherodactylus coqui*, a Puerto Rican species that is characterized by a nearly complete loss of larval characteristics (Townsend and Sewart 1985; Elinson 1990; Hanken et al. 1992). Primarily because of the paucity of information on other direct-developing frogs, especially non-terraranan anurans, *E. coqui* has come to be seen as representative of direct development in anurans in general. However, a recent study of development in *Oreobates barituensis*, a terraranan from northern Argentina, together with older reports (e.g. Lynn 1942; Gitlin 1944; Jameson 1950) indicates that development is not as uniform in Terrarana as previously thought (Goldberg et al. 2012). This, together with the limited amount of data available on other direct-developing species (e.g. Patil and Kanamadi 1997; Bahir et al. 2005; Anstis 2008; Anstis et al. 2007; Narayan et al. 2011), suggests that direct development is not as stereotypical as generally assumed.

Among African frogs, only species of the genus *Arthroleptis* appear to be true direct developers, although a number of related taxa show some degree of terrestrial reproduction (Müller et al. 2007). *Arthroleptis* currently comprises 47 moderately diverse species that are distributed throughout most of sub-Saharan Africa (Blackburn 2008; Frost 2016). All *Arthroleptis* species are assumed to be direct-developing, although only very limited developmental information is available for just two species (Lamotte and Perret 1963; Wager 1965). We investigated the embryonic development of the South African endemic *Arthroleptis wahlbergii* Smith 1849 and the East African dwarf squeaker *Arthroleptis xenodactyloides* Hewitt 1933 based on material collected in the field in South Africa and Tanzania. In this paper, we provide a detailed description of external development, clutch characteristics and egg deposition site. A comparison with published information on other direct-developing species shows that although both *Arthroleptis* species exhibit some general characteristics of direct-developing frogs, they also differ in a number of characters. This highlights the degree of developmental diversity that characterizes the different direct-developing anuran taxa and argues against simplistic assumptions about the evolution of direct development as being merely an excision of a 'tadpole developmental cassette' (Elinson 1990) from the ontogeny of direct-developing taxa.

Material and methods

Developmental series of two species of *Arthroleptis*, *A. wahlbergi* and *A. xenodactyloides* were available for investigation. Of *A. xenodactyloides*, a total of 47 embryos and hatchlings as well as two subadults and three adults were collected in December 2009 and March 2010 at Kigogo and Luhota Forests, Mufindi District, Iringa Region, Tanzania. The embryos were sampled from three field-collected clutches. The clutches were collected by systematically sifting through leaf litter on the forest floor (Fig. 1a), whereas adults and subadult frogs were collected opportunistically in the same general area where the clutches were found.

Of *A. wahlbergii*, a total of 80 embryos and hatchlings were sampled from four clutches of eggs collected in December 2015 at Eshowe and Entumeni Forest in KwaZulu-Natal, South Africa. In addition, several juvenile and adult specimens (Fig. 2) were available for comparison. One clutch was field-collected and the other three clutches were collected

Fig. 1 Habitat (**a**) and clutch (**b**) of *Arthroleptis xenodactyloides* at Luhota Forest, Mufindi, Southern Udzungwas, Tanzania. **c** Clutch of *A. wahlbergii* at Entumeni Forest, KwaZulu-Natal, South Africa. Clutches were photographed in situ after the removal of covering leaves and soil

from a temporary field enclosure. We constructed this using shade cloth to build a fence of ca. 40-cm height to enclose a space of approximately 1.5 m^2 in an area with thick, moist leaf litter and closed canopy where several adult *A. wahlbergii* had been found. We collected four gravid females and four males from the immediate surrounding area and maintained them in the enclosure for 2 weeks. At least once every 48 h we inspected the enclosure and moistened the leaf litter if no rain had occurred. Because no spontaneous egg deposition had occurred after 12 days, we gave all males and females a single injection of Amphiplex, consisting of des-Gly^{10}, D-Ala^{6}, Pro-LHRH (Bachem; 0.4 μg/g body weight; GnRH-A) and metoclopramide (Sigma; 10 μg/g body weight; MET), to induce oviposition (for details, see Trudeau et al. 2010). Two days after the hormonal stimulation, we dismantled the enclosure and found three clutches buried beneath the leaf litter in the relatively loose, uppermost layer of soil. No adults were found in attendance and the general clutch characteristics did not differ from the field-collected clutch.

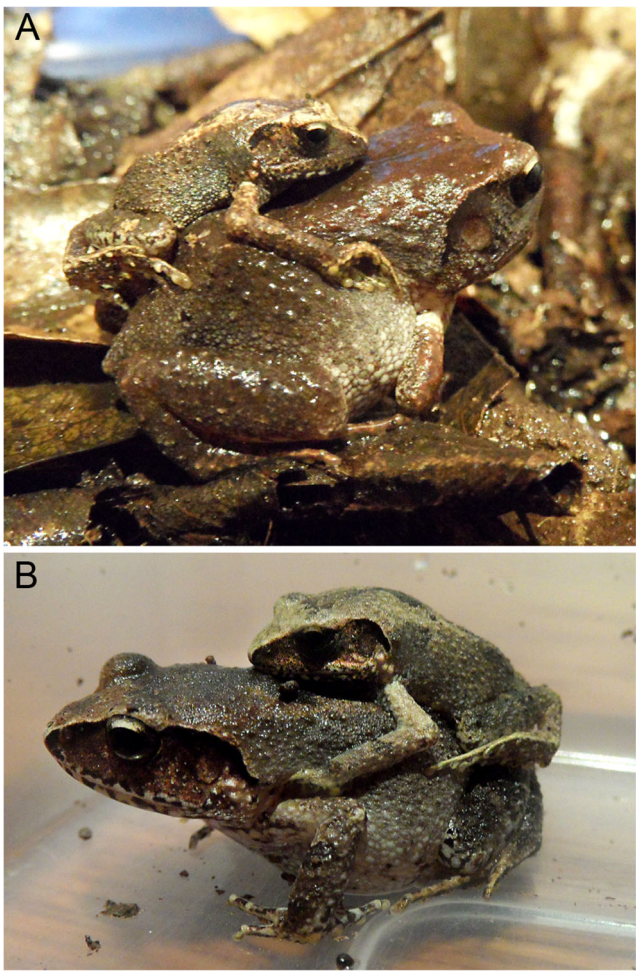


Fig. 2 Pair of *Arthroleptis wahlbergii* in amplexus. The male on top is slightly displaced to the side in **a**, showing the outward facing palm and elongated third finger. **b** Pair in full amplexus; note the disproportionately long forearm of the male

We transferred clutches to Petri dishes and maintained them under field conditions at ambient temperatures, sampling embryos at regular intervals (every 1–2 days). Specimens were euthanized using tricaine methanesulfonate (MS222; Fluka), fixed in 4% neutral-buffered formalin and subsequently stored in 70% ethanol. Voucher specimens of *A. xenodactyloides* have been deposited in the collections of the Museum of Comparative Zoology (MCZ A-149005-10). Conspecificity of the egg clutches was confirmed through raising individual eggs to hatching.

Descriptions of embryo and egg morphology are based on microscopic observations of live and preserved material. Drawings and photos were made using a Zeiss Discovery V12 stereomicroscope (Carl Zeiss) with an attached drawing mirror and an AxioCam digital camera. The description of embryonic development in *Arthroleptis* is based on the staging table for *E. coqui* (Townsend and Stewart 1985). Although different direct-developing species show considerable variation in external morphology and developmental timing (see 'Discussion'), we decided to follow the table by Townsend and Stewart (1985) because it has been widely used for the

description of direct-developing anurans (e.g. Bahir et al. 2005; Anstis et al. 2007; Anstis 2008; Goldberg et al. 2012) and is therefore the most suitable framework for comparing interspecific differences in development. Unless stated otherwise, reported observations refer to both *A. wahlbergii* and *A. xenodactyloides*.

Results

All three clutches of *A. xenodactyloides* were found hidden within moist leaf litter on the forest floor and consisted of 12, 16 and 19 eggs, respectively. The eggs were laid about 3 to 5 cm below the surface of the leaf litter, directly between leaves or inside curled leaves. A clutch consisted of single eggs (sensu Altig and McDiarmid 2007) that were more or less clustered, with some scattering of individual eggs or small groups of eggs (Fig. 1b). In the areas of the forest where the clutches were found, the leaf litter seemed considerably thicker, several layers deep, than in other parts of the forest and the ground had a gentle slope (Fig. 1a). The single clutch of *A. wahlbergii* found in a natural setting contained 23 eggs and was collected in a little *Macadamia* woodland on the outskirts of Eshowe. We collected it from a shallow mound of relatively compact earth, where it was buried about 3 cm deep inside the soil, underneath deep leaf litter. The three clutches obtained from the enclosure contained 19, 19 and 31 eggs. The two smaller clutches contained a number (four and nine) of unfertilized eggs. All clutches were found buried about 1–2 cm deep in the soil underneath leaf litter (Fig. 1c). In both *A. wahlbergii* and *A. xenodactyloides*, no adults were found attending the clutches.

The eggs of both species were non-pigmented and encased in a clear egg capsule. In *A. wahlbergii*, we observed five different layers of the egg capsule (Fig. 3a; no data available for *A. xenodactyloides*). The two outer layers were extremely thin, with the jelly of the first layer being more robust than in the second layer. Soil and other particles adhered to the outer surface of the eggs, but increasing wetness reduced the stickiness of the outer layer. The third and fourth layers were thicker and of a more rubbery consistency (Fig. 3a). The fifth layer was relatively thin again and surrounded the embryo (Fig. 3b, c). It tightly enveloped the embryo and was difficult to remove in preserved specimens. Apart from the unfertilized eggs of *A. wahlbergii*, all eggs developed normally and no fungal infections were observed. All clutches of *A. xenodactyloides* had already commenced development at the time of collection and the youngest embryos represented in our material are at Townsend and Stewart stage 3 (TS 3). We also could not observe a single clutch throughout the entire development, but from the material available, it took 30 days between TS 3 and hatching, and we therefore estimate the entire embryonic development to take around 32 to 33 days. For *A. wahlbergii*, almost the entire embryonic development could be observed in a single clutch and it took 30 days from gastrulation (TS 1) to hatching. Based on these data, we estimate the entire embryonic development to take not more than 31 to 32 days. Hatching in *A. xenodactyloides* occurred at an average size of SVL 3.7 mm (±0.2; $n = 3$) and hatchlings of *A. wahlbergii* had an average size of SVL 5.5 mm (±0.2; $n = 5$).

Embryonic morphology and development

Both species of *Arthroleptis* were very similar in their development and the following description pertains to both of them, except where stated otherwise.

Head Distinct cephalic areas were delimited by TS 4 and optic and otic regions were clearly differentiated (Fig. 4c). The large

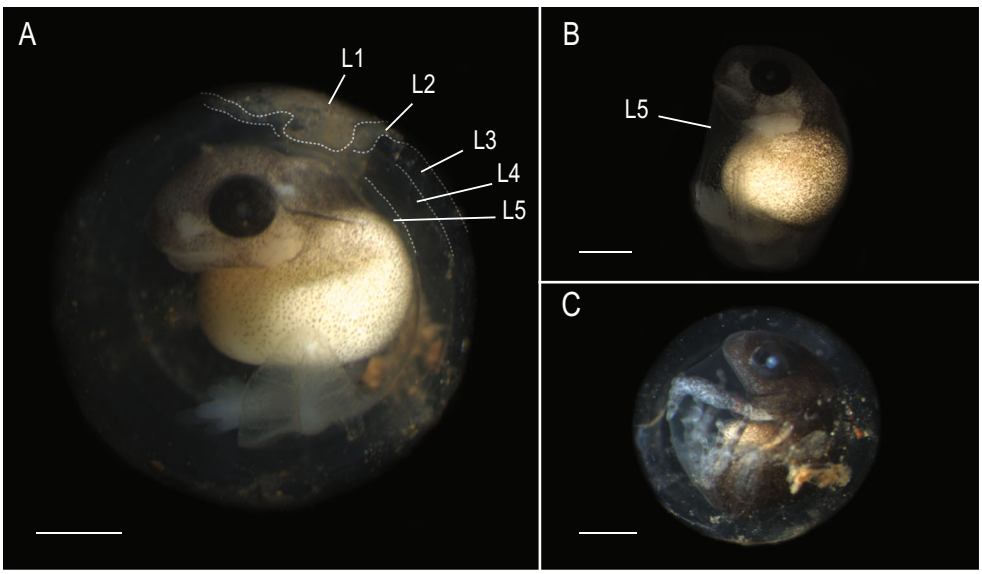


Fig. 3 Different stages of development inside the egg of *Arthroleptis wahlbergii*. **a** TS 9, different layers of the egg capsule are shown. Layers 1 and 2 (*L1* and *L2*) are partly removed. Layers 3 and 4 (*L3* and *L4*) are the thickest of all layers. Layer 5 (*L5*) closely surrounds the embryo. **b** In TS 10, all layers of the egg capsules have been removed except layer 5 (*L5*). **c** In TS 15, last stage inside the egg, hatching occurs at any time

eyes were initially evident as large, unpigmented optic vesicles. Pigmentation of the iris started at TS 6 (Fig. 4e) and the iris became increasingly dark during further embryonic development. The pupil remained clear throughout development. Eyelids were first evident relatively late in embryonic development at TS 15 (Fig. 4l). During TS 6, the mouth opened and the lower and upper jaws were differentiated. The mouth opening widened at TS 8 and the nostrils appeared at the same time (Fig. 4g). By TS 13, the mouth opening extended backwards to below the eye (Fig. 4k). The posterior extent of the mouth opening reached its final position at a level behind the eye during postembryonic development.

Gills At least two gill arch bulges were clearly visible during TS 4 in *A. xenodactyloides* (Fig. 5a). In *A. wahlbergii*, four gill arches were visible during TS 4 (Fig. 4c). The gill arches disappeared from external view at TS 5. External gills were not observed at any stage in the available specimens.

Body pigmentation and body wall Body pigmentation first appeared at TS 6 with the formation of melanophores on the head and in the vertebral region (Fig. 4e). During this stage, a few melanophores also appeared along the paravertebral region of the future body wall overlaying parts of the yolk (Fig. 4e, right). By TS 8, melanophores were widely spread over the head and body and the body wall had expanded laterally to enclose the yolk up to a narrow gap along the ventral side (Fig. 4g). The body wall completely enclosed the yolk ventrally by TS 10–11. In general, dorsal parts of the head and trunk as well as the outer sides of the fore- and hind limbs were more densely pigmented than the lower jaw and the inner sides of the fore- and hind limbs, which only showed slight pigmentation.

Limbs Overall, development was characterized by a very early appearance of the limb buds. Both fore- and hind limb buds first appeared as rounded swellings lateral to the neural tube. Hind limb buds were already distinct at TS 4, whereas the forelimb buds were only faintly indicated and less well defined at this stage (Fig. 4c). The hind limb buds were slightly larger than the forelimb buds and remained so during the differentiation stages. The limb buds increased in size, becoming more elongated and were joined to the trunk by TS 5 (Figs. 4d and 5c). The hind limbs continued to elongate, and by TS 6, slight constrictions of the knee joints appeared, while the forelimb buds were round to ovoid (Fig. 4e). The opercular fold started to overgrow the developing forelimbs at TS 6. At TS 7, the forelimbs were covered to about one third by the opercular fold and were completely covered by TS 9 (Fig. 5d–f). Differentiation of the hand and digits was completed under the opercular fold. Nubs of digits 1–3 and toes 1–4 first appeared at TS 8 (Fig. 5h), and all five toes were visible by TS 9. At TS 10, the forelimbs were fully erupted (Fig. 5g). The subarticular tubercles on hands and feet were first visible at TS 12 (Fig. 5j).

Tail A tail bud first appeared in TS 3 embryos. From the beginning of TS 4, the tail bud elongated and bent on the inside of the vitelline membrane of the embryo (Fig. 5b). There was no consistent orientation of the tail, and some embryos had the tail bent to the left, whereas in others, it was bent to the right. However, tail orientation in individual embryos seemed to remain constant throughout development. A small membranous fin developed at TS 5 (Fig. 5c). At this stage, the tail was about one third of its final length. The tail and its vascularized fin membrane continued to develop and elongate until TS 10, when it reached its full length at a size more or less equal to the body length of the embryo. The tail began to regress at TS 12. By TS 14 (Fig. 4k), the tail was reduced to a small stub and completely resorbed in hatchlings (Fig. 4l). The tail lacked pigmentation throughout development.

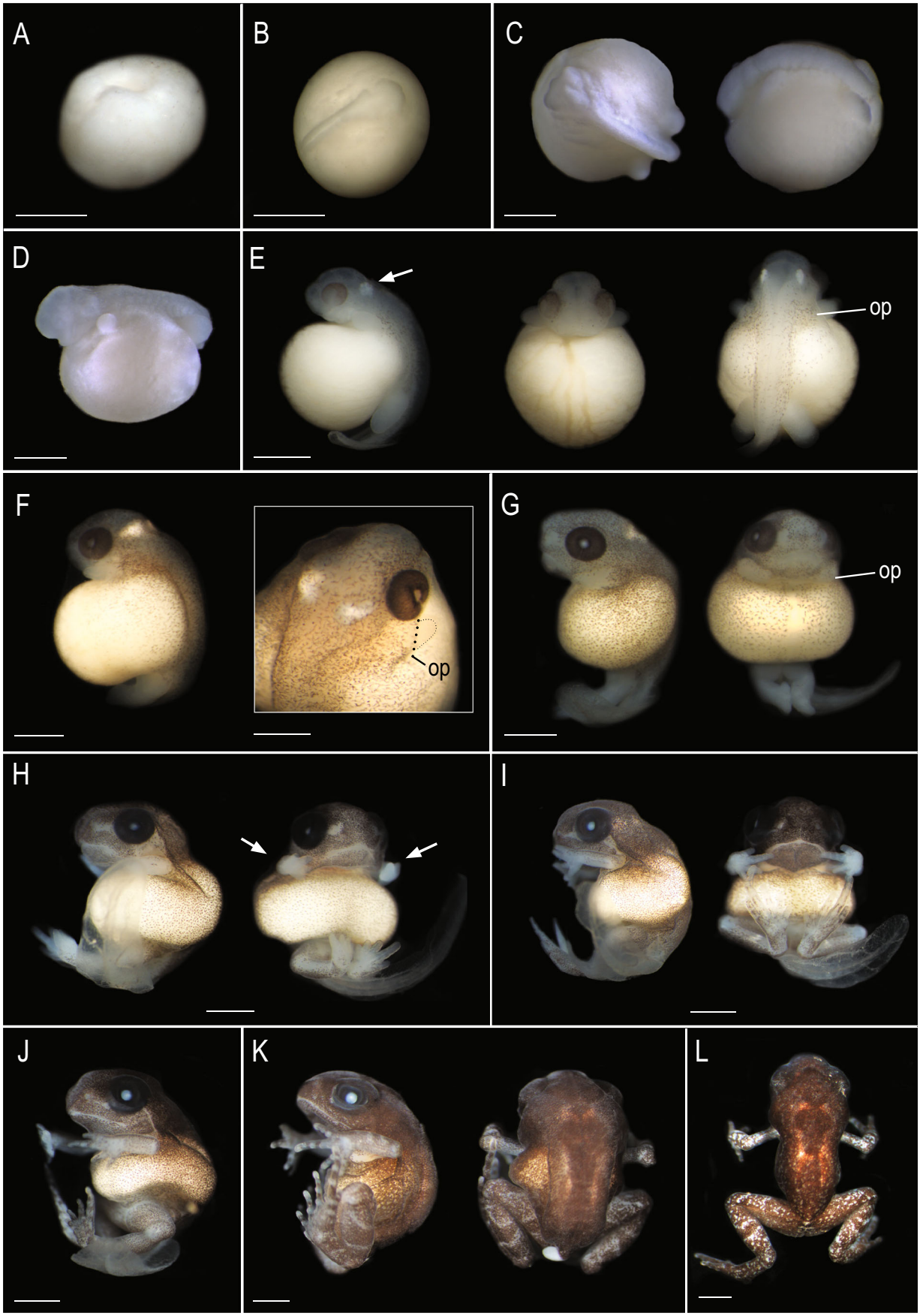
ECD Endolymphatic calcium deposits (ECD) first appeared at TS 6 as two small whitish dots, one on each side of the otic region (Fig. 4e). The ECD expanded during TS 7 (Fig. 4f) and developed extensions in the caudal direction that continued to grow until TS 11. By TS 12, the two ECD antimeres were joined in the midline. At the same stage, the ECD began to be obscured by the increasingly more dense pigmentation and were no longer visible from the beginning of TS 13.

Discussion

Reproduction in *Arthroleptis*

The males of many species of *Arthroleptis* are characterized by moderately to extremely elongated third fingers (Blackburn 2009). The function of this sexually dimorphic trait is unclear but is thought to perhaps be used in male to male combat or during amplexus (see Blackburn 2009 for details). We observed axillary amplexus in *A. wahlbergii* (Fig. 2) and the palms of the hands of the male were facing outward. We did not observe any special motion or posturing of the third finger and it does not seem to play a particular role in amplexus. However, given the large size difference between male and female, the elongated lower arm and third finger might overall improve the hold on the female.

The first observations on reproduction in *Arthroleptis* were made by Barbour and Loveridge (1928), who described three clutches of *A. stenodactylus* eggs (33, 40 and 54 eggs) collected from shallow burrows with some adults, plus an additional clutch of 80 eggs that they suspected to be a mix of at least two clutches. They pointed out the disproportionately large ova in gravid *A. xenodactylus* but did not explicitly infer a direct mode of development. The first explicit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◀ **Fig. 4** External development in *Arthroleptis wahlbergii*. All embryos have been removed from the egg capsules. **a** TS 1. Formation of blastopore. **b** TS 3, dorsal view. Neural folds formed. **c** TS 4, dorsal and frontal view. Early limb bud stage, neural tube formed, gill arches apparent. **d** TS 5. Limb buds attached to trunk. **e** TS 6, *left*: lateral view (*arrow* indicates ECD), *middle*: frontal view, *right*: dorsal view. Opercular fold (*op*) begins to grow over developing forelimb. **f** TS 8, *left*: lateral view, *right*: close-up of head region, with edge of opercular fold and outline of limb bud indicated by *dotted lines*. Opercular fold covers two thirds of the forelimb bud. **g** TS 9, *left*: lateral view, right: ventral view. Opercular fold completely covers forelimbs. **h** TS 10, both forelimbs erupted (*arrow*), tail fin begins to decrease, pigmentation denser. **i** TS 11/12, *left*: lateral view, *right*: ventral view. Toes are much longer, tail begins to decrease in size. **j** TS 13, fingers longer, tubercles visible. **k** TS 14, *left*: lateral view, *right*: dorsal view. Distinct eyelids visible, toes full length, banding patterns on legs evident. **l** TS 15, hatchling. *Scale bars* = 1 mm

statement of direct development in *Arthroleptis* seems to have been made by Loveridge (1953), who reported that *A. stenodactylus* lay their eggs in a burrow and that metamorphosis is completed inside the egg. Guibé and Lamotte (1958) reported direct development for the West African *A. crusculum* and a clutch size of 15 eggs. The eggs were deposited in a spherical chamber of about 1–2 cm in diameter, a few centimetre below the surface of the soil. The wall of the chamber was reported to be rather regular and seemingly lined with mucus (Guibé and Lamotte 1958). Channing (2001) reported a clutch of *A. xenodactyloides* consisting of 20 eggs that he found 20 cm beneath leaf litter accumulated next to a wet rock face on Mt. Zomba, Malawi. Channing and Howell (2006) reported clutches of *A. xenochirus* to consist of about nine eggs that were laid in shallow nests in leaf litter and provided a photograph of a clutch. They also published a photograph of *A. adolfifriederici* eggs at approximately TS 6, and Altig and McDiarmid (2007) published a photograph of a clump of eggs of *A. schubotzi* found in leaf litter, confirming terrestrial reproduction for two additional species.

Lamotte and Perret (1963) provided a brief description of reproduction and development of the West African *A. poecilonotus* from material collected in Cameroon, where clutches were found in the soil in gardens planted with peanut and cassava. Clutch size averaged 20 to 25 eggs and the large eggs were surrounded by a thick gelatinous capsule that consisted of two to three concentric, spherical layers plus an outer and an inner membrane (Lamotte and Perret 1963). They illustrated five different developmental stages and provided some information on embryonic development, including that the tail was always bent to the left and the forelimbs were hidden under a membrane (presumably the opercular fold) for some unspecified time during development. Interestingly, Lamotte and Perret (1963) reported hatching to occur after 15 to 20 days at a seemingly less advanced stage compared to *A. wahlbergii* and *A. xenodactyloides*. Upon hatching, *A. poecilonotus* still possessed a tail remnant of about half the snout-vent length and eyelids were not yet differentiated. Lamotte and Perret (1963) reported raising their clutches inside a crystallizer at constant temperature (27 °C) and humidity. A constant 27 °C appears to be higher than normal soil temperatures and might explain the comparatively short developmental time reported. Whether hatching generally occurs at an earlier stage in *A. poecilonotus* or might be an artefact of higher-than-average incubation temperatures remains unclear at present. Tapley (2009) reported hatching to occur after about 1 month in captive-breeding *A. stenodactylus*. Some data on reproduction and development in *A. wahlbergii* were provided by Wager (1965), who described discovering eggs in a large pile of leaves, about 3 cm or more below the surface. He reported five clutches that consisted of 11, 18, 20, 26 and 30 eggs, respectively, with the eggs in the individual clutches being somewhat scattered over an area of about 8 cm in diameter. Wager (1965) very briefly summarized embryonic development and also illustrated six stages, but his drawings are very schematic and depict proportions incorrectly. However, he reported that the forelimbs are covered by skin and that hatching occurs at the end of the fourth week of development when the young are fully formed and about 6 mm long, which match our own observations presented here.

Although data on aspects of egg laying or development are only available for nine species of *Arthroleptis* (out of 47), these species do show a similar pattern of reproduction in that eggs are laid terrestrially in a moist, secluded place and develop directly into fully developed young. Egg deposition varies slightly in that eggs are either laid among leaf litter or buried in the soil beneath leaf litter. This is likely a function of the ability to burrow—better in species with a well-developed metatarsal tubercle like *A. stenodactylus* and *A. wahlbergii* and less so in small species with weakly developed metatarsal tubercles like *A. xenodactyloides*—and the local conditions (wetness, shade, thickness of leaf litter, etc.). The spherical breeding chamber described by Guibé and Lamotte (1958) for *A. crusculum* seems unusual and resembles the nesting chamber of *Anhydrophryne rattrayi* as described by Wager (1965). The number of eggs per clutch and size at hatching vary among species and seem to correlate with adult size, with smaller species having fewer eggs and smaller hatchlings and larger species having more eggs and larger hatchlings. The average hatching size of 3.7 mm in *A. xenodactyloides* is among the smallest hatchling sizes reported for any direct-developing anuran, except *Sooglossus* (Callery et al. 2001). Hatching at a somewhat earlier stage in *A. poecilonotus*, as reported by Lamotte and Perret (1963), might possibly be a result of suboptimal rearing and would require confirmation. In captive *A. stenodactylus*, Tapley (2009) observed occasional male egg-guarding behaviour but did not provide further details. In all other investigated species, no adults were found attending the clutches and it seems that dedicated egg-guarding does not occur in *Arthroleptis*. Interestingly, the eggs

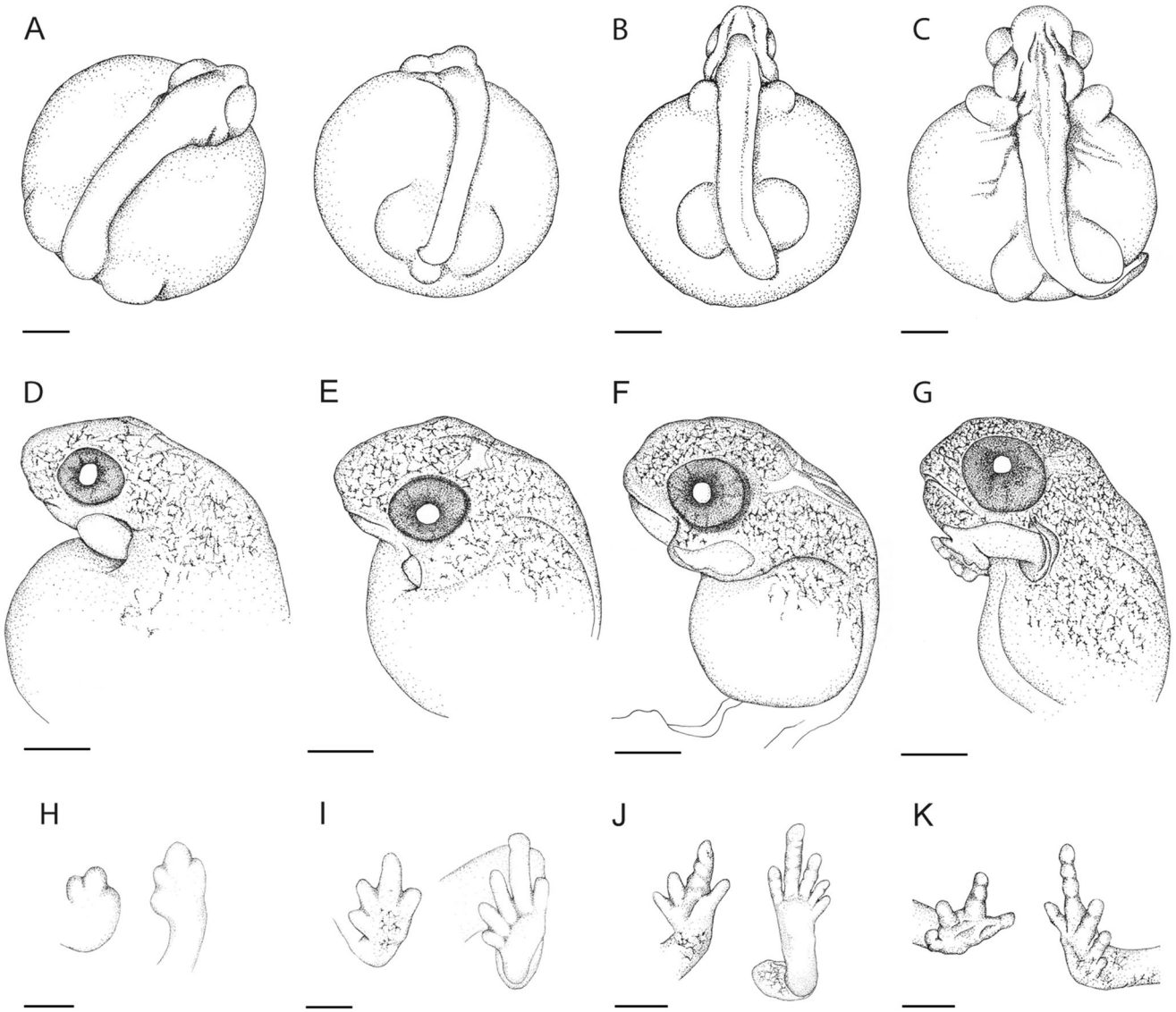

Fig. 5 Details of development of *Arthroleptis xenodactyloides*. **a** TS 3, *left*: dorsal view, *right*: caudal view. **b** TS 4, caudal view. **c** TS 5, dorsal view. **d–g** Development of the opercular fold. **d** TS 7, lateral view. Opercular fold covers one third of the forelimb buds. **e** TS 8, lateral view. Forelimbs almost covered beneath opercular fold. **f** TS 9, lateral view. *Arrow* indicates that forelimbs completely covered by opercular fold. **g** TS 10, lateral view. Forelimbs fully erupted. **h–k** Development of toes and digits. **h** TS 8, dorsal aspect, nubs of three digits and four toes visible. **i** TS 10, dorsal aspect of hand, ventral aspect of foot. Tips of toes and digits enlarged. At this stage, forelimbs are fully erupted and no longer covered by the opercular fold. **j** TS 12. Lateral aspect of hand, ventral aspect of foot. Digits enlarged, tubercles visible. **k** TS 15, ventrolateral aspect. Limbs fully developed. Hatching occurs at any time. *Scale bars*: **a–g** 0.5 mm; **h–k** 0.25 mm

we raised seemed resilient and we lost none to fungal infections. Although little information is available for anurans, in direct-developing salamanders, eggs are more likely to succumb to fungal infections if the attending parent is removed (Wells 2007). *Arthroleptis* eggs might possess some antimycotic properties.

Both species of *Arthroleptis* investigated here showed a remarkably similar embryonic development. The few details reported in earlier studies (Lamotte and Perret 1963; Wager 1965) are also in agreement with the observations reported here (with the exception of *A. poecilonotus* discussed earlier). *Arthroleptis wahlbergii* and *A. xenodactyloides* are not closely related (Blackburn 2008), and the near identical embryonic development observed in both species and available data on other species suggest that similar patterns of development might likely be characteristic for *Arthroleptis* in general.

Direct development in anurans

Direct development has evolved independently several times in all three orders of amphibians and represents a derived developmental mode in which the aquatic larval stage has

been eliminated as a functionally distinct life-history stage from the ontogeny (Hanken 1989; Wake and Hanken 1996; Hanken et al. 1997a; Wake 2003). The development of direct-developing anurans differs considerably from that of species with a biphasic life cycle in that most adult features, which typically form at metamorphosis in biphasic taxa, already appear during embryogenesis (Wake 1989; Hanken et al. 1997a). Heterochrony is considered a likely reason for these evolutionary changes in the timing of developmental events (Raff 1987; Hanken et al. 1992; Goldberg et al. 2012). Direct-developing species show accelerated growth rates and develop faster in comparison to most tadpoles (Lutz 1948; Callery and Elinson 2000a, b). Less clear is the extent to which larval features are expressed in different direct-developing taxa. Earlier studies focused on the neotropical terraranan *E. coqui*, which shows a near complete loss of larval characteristics (Townsend and Stewart 1985; Callery et al. 2001). This dramatic loss of larval characteristics paired with the scant availability of data on other direct-developing taxa led some authors to suggest that the free-swimming tadpole larva might represent a developmental cassette or module that has been excised from the ontogeny of direct-developing species (Elinson 1990; Callery and Elinson 2000a, b; Ziermann and Diogo 2014). This idea could potentially provide a more mechanistic explanation for the repeated evolution of direct development in anurans. The generality of this assumption however is contingent on the overall similarity of direct-developing anurans. In *E. coqui*, several studies have demonstrated large-scale, ontogenetic repatterning (e.g. Hanken et al. 1992, 1997b). These complex patterns of ontogenetic rearrangements suggest that the evolution of direct development in *E. coqui* might be more than just a simple deletion of the larval phase (Callery et al. 2001). A number of recent investigations have revealed different patterns of reduction of tadpole-specific characters in direct-developing species (e.g. Anstis et al. 2007; Goldberg et al. 2012), which indicates that the effects of direct development on embryogenesis are perhaps not consistent across different lineages that evolved this form of reproduction.

Comparability of TS staging The staging table of Townsend and Stewart (1985) has been used as a basic framework for describing development of terraranan and also non-terraranan species (e.g. Bahir et al. 2005; Anstis 2008; Goldberg et al. 2012). This staging table was originally devised to help assess embryonic age under field conditions and focused on features visible to the naked eye or with the aid of a 10× hand lens (Townsend and Stewart 1985). As such, it is a relatively coarse description of development and condenses developmental events into relatively few stages compared to other staging tables (Gosner 1960; Nieuwkoop and Faber 1967). This is especially the case for the early stages of development; Townsend and Stewart (1985) condensed the early development into three stages, beginning with the fertilized egg to the appearance of the limb and tail buds. As a consequence, embryos at different stages of development can still fall into the same TS stage. This complicates comparison of early development in direct-developing anurans based on stage descriptions derived from Townsend and Stewart's (1985) table. Some authors also employed modified stage definitions, which accounts for a lack of correspondence of the early stages between Townsend and Stewart (1985) and other staging tables (e.g. Bahir et al. 2005; Narayan et al. 2011). To compensate for the broad divisions of early development into stages, Moury and Hanken (1995) subdivided TS 3 into three substages defined by the degree of neural fold closure. In *Arthroleptis*, we also found several discrete stages of development that fit into single TS stages (Table 1). For instance, during the early TS 4, the neural tube is formed, the head is outlined and a tailbud is visible (Fig. 6a). This is followed by the beginning differentiation of the eyes, gill arches and the elongation of the tailbud (Fig. 6b). The late TS 4 comprises distinct eye anlagen and clearly defined forelimb buds (Fig. 6c). To document these, we described all discrete stages in Table 1 but did not alter the numbering of Townsend and Stewart (1985) to retain correspondence with later stages to facilitate comparison of different direct-developing species. We also indicated characters used by Townsend and Stewart (1985) to define stages and employed these to demarcate corresponding stages in *Arthroleptis*.

External gills Small, external gills are present during early embryonic development in some direct-developing species, but their presence seems rather variable, even among closely related species. Although small gill arches occur transitorily in *Arthroleptis*, as in other species, no external gills are present during embryonic development. External gills were also not observed in the myobatrachids *Arenophryne rotunda*, *Myobatrachus gouldii* (Anstis et al. 2007), *Metacrinia nichollsi* (Anstis 2008), the rhacophorids *Philautus viridis* and *Philautus silus* (Bahir et al. 2005) and the craugastorid *Haddadus binotatus* (Goldberg and Candioti 2015). External gills are present for less than one-fourth of the developmental period in *Philautus variabilis* (Patil and Kanamadi 1997). In *Philautus glandulosus*, they appear as lamellae and are also only present for a short period (Krishnamurthy et al. 2002). External gills have further been reported in the ceratobatrachid *Platymantis vitiana* (Narayan et al. 2011). In Terrarana, external gills have been reported in some species but seem to be absent in others. In *E. coqui,* external gills first appear at TS 5 and disappear by TS 9 (Townsend and Stewart 1985). A pair of small, external gills is present as short, stubby ectodermal projections off the third gill arch in *Eleutherodactylus portoricensis* for about 5 days of the 19-day developmental period (Gitlin 1944). No external gills were observed in *Craugastor augusti* (as *Eleutherodactylus latrans*, Jameson

Table 1 Synopsis of external characteristics in developmental stages of *Arthroleptis* embryos. Features in bold indicate diagnostic features of the developmental stages of *Eleutherodactylus coqui* (Townsend and Stewart 1985)

TS stage	*Arthroleptis* development
1	**Oviposition**
1	Late-Gastrula, **blastopore** visible
2	Early neurulation stage, **neural folds** forming neural groove
3	- Mid- to late neurulation stage - **Neural groove formed**, not yet closed to a neural tube
4	- **Neural tube formed** - Head outlined - **Forelimb buds** feebly defined, **hind limb buds** evident - Somites slightly visible - **Tail bud** evident
4	- **Eye bulges** visible - **Gill arches** distinct caudal to the eyes - Somites clearly visible - Tail bud elongated
4	- Head more prominent and separated into distinct areas: optic region, mouth and auditory region differentiable - Gill arches disappeared, no gill buds visible
4	- Eye bulges distinct but unpigmented - Forelimb buds round and clearly defined, hindlimb buds round to ovoid - **Limb buds separated from trunk** - Tail bud elongated and slightly curved to one side
5	- **Eyes distinct but unpigmented** - **Limb buds attached to trunk**, forelimb buds clearly defined and smaller than hind limb buds, hind limbs round to ovoid - **Tail elongated with small thin fin**, bends to one side
6	- **Iris** with light brown **pigment** - Forelimbs round to ovoid, **slight knee constrictions** on hind limbs - Opercular fold appears and begins to grow over forelimb buds - **Tail one third of its final length, small fin** - **ECD** (endolymphatic calcium deposits) **first visible** as small white points - **Light pigment over head and trunk**
7	- Iris darker on outer and inner border, colour of iris light brown - Opercular fold covers two thirds of forelimbs - **Knee joints more evident, foot paddles** - **ECD quadrangular patches** - **Tail with vascularized fin** - Pigment denser over head and trunk, spreads over yolk
8	- Opercular fold covers two thirds of forelimbs - **Nubs of digits and toes** - **ECD triangular** with forward **extensions** to eye
9	- Opercular fold closed - **Toes 1–5 demarcated** - Hind limbs have slight pigmentation except on inner sides and on ventral sides of feet - **Tail to two thirds of its final length** with well-vascularized fin - **ECD expand** to one third over trunk
10	- **Iris dark pigment, pupil light grey** - Nares distinct - Forelimbs erupted - Elbows distinct, fingers differentiable, hands ventrally orientated - Knees distinct, Toes 1–5 separated
	- **Tail at full size**, remains unpigmented - Slight pigment over forelimbs except inner sides - Yolk enclosed in body wall
11	- **Iris dark, pupil light grey** - Fingers and toes much longer - Forelimbs with light pigment on outer sides, hind limbs darker pigment on outer sides and light pigment on inner sides - **Tail at full size** - **ECD expanded backwards** toward the trunk - Pigment over dorsum darker
12	- Iris dark, pupil light grey - Fore and hind limbs darker pigment on outer sides, hind limbs light pigment on inner sides and on ventral side of feet - Tubercles slightly visible - **Toes two thirds of their length at hatching** - Tail begins to regress in size
13	- Tubercles distinct on hand and feet, darker pigment on palms of hand and feet - **Tail** further **regressed in size** - **Body heavily pigmented**, light pigment on abdomen - ECD joined in the midline, large longitudinal extensions
14	- Eyelids visible - **Limbs and toes fully developed** - Banding patterns on legs visible - **Tail regressing** - Pigment denser over ventral sides of body
15	- Tail reduced to a small stub - Minimal yolk reserves - **Hatching possible at any time**

1950), *Eleutherodactylus nubicola* (Lynn 1942), *Ischnocnema guentheri* (Lynn and Lutz 1946), *Ischnocnema nasutus* (Lynn and Lutz 1947) and *O. barituensis* (Goldberg et al. 2012).

Opercular fold In free-living tadpoles, the opercular fold covers the external gills and developing forelimbs and forms a gill chamber that opens via a paired or single spiracle (Altig and McDiarmid 1999). In *E. coqui*, epidermal folds briefly develop to cover the gills and the base of the limb buds but disappear rapidly, even before the digits start developing on the forelimbs (Callery and Elinson 2000b). Although present for only a few hours during development in *E. coqui*, these epidermal folds are considered to be homologous with the opercular fold of metamorphosing anurans (Callery and Elinson 2001). As such, the opercular fold is a useful character when comparing direct-developing frogs with respect to their deviation from the ancestral morphology. In *O. barituensis* (Goldberg et al. 2012) and *H. binotatus* (Goldberg and Candioti 2015), the opercular fold encloses the proximal half of the forelimbs and, as in *E coqui*, the opercular fold never completely covers the forelimbs, although it seems to persist

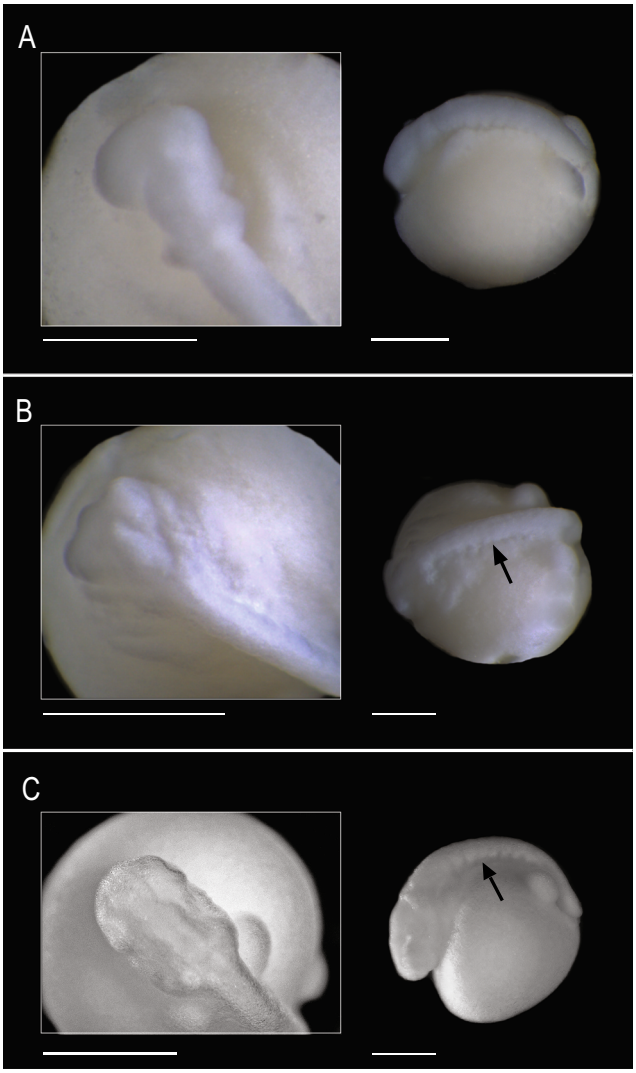

Fig. 6 Different discrete developmental events subsumed within TS 4 in *Arthroleptis*. **a** Early TS 4 of *A. wahlbergii*, *left*: close-up of head region, *right*: lateral view with head facing left. **b** Mid-TS 4 of *A. wahlbergii*, *left*: close-up of head region; *right*: lateral view, *arrow* indicates somites. **c** Late TS 4 of *A. xenodactyloides*, *left*: close-up of head region, forelimb buds clearly defined, *right*: lateral view, *arrow* indicates somites. *Scale bars* = 1 mm

for a somewhat longer period of development. This is substantially different from *Arthroleptis* and all other non-terraranan direct-developing species described so far, in which the developing forelimbs are partially or completely covered by the opercular fold for a comparatively long period of development, although the timing of appearance and disappearance of the opercular fold varies among the different taxa. In *Arthroleptis* and *A. rotunda*, the opercular fold persists for a period of five stages (Anstis et al. 2007), for six stages in *M. gouldii* (Anstis et al. 2007) and *M. nichollsi* (Anstis 2008) and up to eight stages in *P. viridis* (Bahir et al. 2005). Also, the period of complete enclosure of the forelimbs by the opercular folds ranges from a maximum of two stages in *Arthroleptis*, *P. viridis* (Bahir et al. 2005) and *Philautus surdus* (as *Philautus lissobrachius*, Alcala and Brown 1982), to up to six stages of development in myobatrachids (Anstis et al. 2007; Anstis 2008).

Limbs Simultaneous appearance of the fore- and hind limb buds very early in development is one of the most striking features of direct development (Townsend and Stewart 1985; Elinson 1994; Anstis et al. 2007). In all species investigated so far, fore- and hind limb buds appear more or less simultaneously and develop at a similar rate. At their first appearance during early embryonic development (about TS 4), hind limb buds are usually somewhat more distinct, whereas forelimb buds are smaller and slightly less well defined. The limb buds lengthen during subsequent stages and developing digits are first seen during TS 9 or 10 on the hands and feet. Subarticular tubercles first appear during TS 12, 13, and 14 or during postembryonic development, depending on the species.

Tail Many previous investigators have commented on the highly derived tail morphology of embryos of direct-developing frogs. In general, the tail of direct-developing frogs is usually enlarged with well-vascularized tailfins that are broadly expanded and envelop the embryo to varying degrees (e.g. Townsend and Stewart 1985; Bahir et al. 2005; Goldberg et al. 2012; Goldberg and Candioti 2015). In *O. barituensis* for example, the extensively developed tail envelops nearly the entire embryo (Goldberg et al. 2012). In *H. binotatus*, it forms a sac-like structure wrapped around the hind limbs for a relatively long period (Goldberg and Candioti 2015). This is not seen in either *Arthroleptis* species, where the tail is extended to its full length with a well-vascularized fin but never envelops the embryo. Instead, tail development in *Arthroleptis* is more similar to *E. coqui* (Townsend and Stewart 1985). In most species, including *E. coqui*, the expanded parts of the tail seem homologous to the dorsal and ventral tailfins of tadpole larvae. However, in the terraranans *O. barituensis* (Goldberg et al. 2012), *H. binotatus* (Goldberg and Candioti 2015) and *Pristimantis urichi* (Nokhbatolfoghahai et al. 2010), the expanded part does not correspond to the dorsal and ventral tailfin but represents lateral, fin-like expansions. This particular configuration might be apomorphic for Pristimantinae.

Because of its enlargement and apparently high degree of vascularization, the tail is usually assumed to function mainly as a respiratory organ in embryos of direct-developing anurans (Lutz 1948; Townsend and Stewart 1985; Bahir et al. 2005). A relationship between the presence or absence of gills and tail size has frequently been discussed (Townsend and Stewart 1985; Patil and Kanamadi 1997; Bahir et al. 2005; Goldberg et al. 2012) and it seems plausible that the tail has a potential role as the main respiratory organ, especially when considering that it has lost its locomotory function (Lutz 1948).

However, embryos of direct-developing ceratobatrachids (Alcala 1962; Narayan et al. 2011) and myobatrachids (Anstis et al. 2007; Anstis 2008) show little to no enlargement of the tail or tailfins. In the ceratobatrachid *P. vitiana* (Narayan et al. 2011), the tail is furthermore comparatively short and has only shallow fins. Interestingly, in both ceratobatrachids and myobatrachids, embryonic development is characterized by the reported absence of external gills (Anstis 2008; Narayan et al. 2011). In *P. vitiana*, highly vascularized abdominal sacs are thought to have a respiratory function (Narayan et al. 2011), whereas no specialized respiratory structures have so far been reported for myobatrachids. It seems clear that the tail of ceratobatrachids and myobatrachids has only a limited function as a respiratory organ. The co-option of the larval tail for a respiratory function is therefore not a general characteristic of direct-developing anurans.

The development of the tail in different direct-developing taxa also varies substantially regarding timing and growth rate. For instance, in the myobatrachids *A. rotunda* and *M. nichollsi*, the tail bud forms before the appearance of the limb buds (Anstis et al. 2007; Anstis 2008), whereas in *E. coqui*, it appears after limb buds have formed. In *O. barituensis*, tail development is apparently accelerated and by TS 6, only two stages after the tail bud has appeared, the tail envelops nearly the entire embryo (Goldberg et al. 2012).

Egg tooth An egg tooth has been reported for nearly every investigated species of *Eleutherodactylus* and those formerly referred to this genus (Lynn 1942; Lynn and Lutz 1946; Jameson 1950; Wake 1978; Bourne 1997; Goldberg et al. 2012). Jameson (1950) even considered the development of an egg tooth as a typical feature of terrestrially (i.e. direct-) developing species. Embryos of non-terraranan direct-developing species, however, have all been reported to lack an egg tooth (e.g. this study, Anstis et al. 2007; Bahir et al. 2005; Krishnamurthy et al. 2002). Based on available data, the presence of an egg tooth appears to be restricted to species of the New World direct-developing Terrarana (Hedges et al. 2008) and is presumably apomorphic for this group.

Evolution of direct development

Although direct development evolved independently in several different anuran taxa and generated early interest as an evolutionary phenomenon (e.g. Bavay 1873; Noble 1931), we still have only an incomplete understanding of its exact developmental basis and evolution. At first glance, direct-developing embryos show a high degree of overall similarity in their external development, such as early and nearly simultaneous appearance of limb buds and a more adult-like head morphology. A more detailed comparison however reveals differences in tempo and/or sequence of development of some features, such as an early enlargement of the tail or the presence and extent of an opercular fold (present study; Bahir et al. 2005; Anstis et al. 2007; Anstis 2008; Goldberg et al. 2012) among different direct-developing taxa. There is also variation among direct-developing anurans in the presence or absence of external gills, although no clear pattern is discernible and an often-suspected link between the absence of gills and an elaboration of the tail (Lutz 1948) is not without exceptions. Some taxa like the rhacophorid *Philautus* furthermore express a cement gland and a coiled gut (Bahir et al. 2005), characters more typically associated with tadpole larvae. Several features previously thought to be characteristic for direct-developing anurans, such as an egg tooth, a greatly enlarged tail or only very rudimentary and transitory presence of an opercular fold, seem to be restricted to particular direct-developing taxa and are indeed rather variable among the different direct-developing lineages. The scarce available data on patterns of internal development also present a more heterogeneous picture of the effects of direct development on, e.g. skeletal development (Lynn 1942; Hanken et al. 1992; Kerney et al. 2007).

Even though these differences in developmental morphology and timing suggest that there is no single developmental pattern among direct-developing species, we do not wish to discount the obvious similarities seen in the different direct-developing taxa. However, the idea of some authors (Elinson 1990; Ziermann and Diogo 2014) that the free-swimming tadpole larva represents some form of module that has been deleted from the ontogeny of direct-developing frogs conflicts with the available data and appears too simplistic. While the egg tooth seems to be an evolutionary novelty of Terrarana, most differences seen among direct-developing taxa concern the expression of typical larval features or their lack thereof. At present, it is unclear what accounts for these differences, but several reasons seem possible. The reduction of tadpole-specific features might be idiosyncratic and the similar phenotypes a function of the gradual accumulation of these reductions. Another alternative could be that the trajectory from larval to direct development follows a predictable sequence and the observed differences are the result of the various taxa being at different positions along that trajectory. However, the variability in the developmental timing and pattern of the tail, which seems disconnected from the remaining tadpole characteristics, indicates a more complex pattern of both integration and modularity of larval traits. More detailed studies including external and internal morphology of different direct-developing lineages are needed for a better and more comprehensive picture of the specific, evolutionary changes involved in the larval to direct development transition. More detailed morphological data might further provide valuable clues in the search for the underlying molecular mechanisms leading to the reduction of a free-swimming, feeding tadpole larva and the evolution of direct development.

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