

EMBRYONIC EXTERNAL NARES IN THE MICROHYLID *ELACHISTOCLEIS OVALIS*, WITH A REVIEW OF NARIAL DEVELOPMENT IN MICROHYLID TADPOLES

M. NOKHBATOLFOGHAHA^{1,2} AND J. R. DOWNIE¹

¹*Institute of Biomedical and Life Sciences, Division of Environmental and Evolutionary Biology, University of Glasgow, Glasgow, UK*

²*Biology Department, Faculty of Sciences, University of Shiraz, Iran*

Microhylids have the nares closed during most of tadpole life. In the microhylid *Elachistocleis ovalis* the external nares initially form in late embryonic stages but the nares then close when independent feeding begins and re-open at the start of metamorphosis. Examination of other microhylid species suggests that this may be a general feature. Given the uses of the olfactory system in tadpoles (e.g. kin recognition, predator detection), closure of the system in microhylids is a curious feature which needs further investigation.

Key words: anuran, development, nostrils, olfaction, Microhylidae

INTRODUCTION

Paired ectodermal placodes that form near the tip of the snout of anuran embryos at Gosner (1960) stage 17 are the earliest signs of the olfactory organs. The placodes invaginate to form olfactory pits (presumptive external nares), and soon after hatching each pit begins to divide into the anteromedial vomeronasal organ and the posterolateral principal cavity. The lumen of the principal cavity extends towards the roof of the buccal cavity and breaks through to form the choana (internal naris) by Gosner stage 28 in *Xenopus laevis* (Hansen *et al.* 1998; staged using the approximation table in McDiarmid & Altig, 1999). Klein & Graziadei (1983) showed that the olfactory placode is composed of a superficial cell layer which forms the supporting cells of the olfactory epithelium, with microvillate apical surfaces, and a deep layer that becomes sensory cells with ciliated apical surfaces.

It has been known for some time that the olfactory development of larval microhylids is unusual. Parker (1934) noted that the external nares of free-living microhylid tadpoles “do not appear until shortly before metamorphosis.” Wassersug (1980) examined the internal oral anatomy of three species of microhylid tadpole (stages 32-37) and noted that the internal narial depressions were not perforated in any case. Haas (2003) concurred with this conclusion from examination of six more microhylid species, noting that the nostril passages became open only during metamorphosis, and that closed nostrils are one of a set of derived characters that distinguish microhylid tadpoles from those of other groups.

Altig & McDiarmid (1999) briefly reported that embryos of the tadpoles of *Gastrophryne carolinensis* and *Microhyla heymonsi* had external narial openings; they found that the external openings closed at later stages, and remained closed during most of larval development.

During a study of the external features of embryos and larvae from a wide range of anuran taxa, we made similar observations in the microhylid *Elachistocheis ovalis* (Schneider). Here we present our observations on this species and re-examine the specimens reported by Parker (1934).

MATERIAL AND METHODS.

Eggs and tadpoles of *E. ovalis* were collected from temporary pools in Trinidad over a number of years. Embryos were incubated in well-aerated dechlorinated tapwater at 28°C in the laboratory and fixed in 2.5% glutaraldehyde in phosphate buffer. Tadpoles were grown in particle-rich pond water and fixed in Bouin's fluid at stages before and around metamorphosis (Gosner 37-44). Specimens were prepared by conventional methods for both scanning electron microscopy (SEM) and light microscopy using semi-thin resin embedded sections stained with Toluidine Blue or wax sections stained with PAS. For comparison, larvae of two species with primitive olfactory organ development (*Bufo bufo*, collected in the UK; *Hyla crepitans*, collected in Trinidad) were also fixed for light microscopy. Microhylid embryos and larvae held in the collections of the Natural History Museum, London (BMNH) were examined with a dissecting microscope.

RESULTS

As viewed by SEM, the positions of the nasal placodes of *E. ovalis* were first detectable as flattened areas dorsal to the oral invagination at stage 18 but became more obvious as invaginations at stage 19 when the embryos hatched. By stage 20, each external naris was a circular pit with a flattened base composed of densely packed cells, most of which bore short apical microvilli, but some of which were ciliated. The pits were deeper by stages 21-22 and remained distinct through stages 23-24, but by stage 25, they had disappeared; their previous positions were faintly visible as a circular arrangement of the cells (Fig. 1a-e). Although

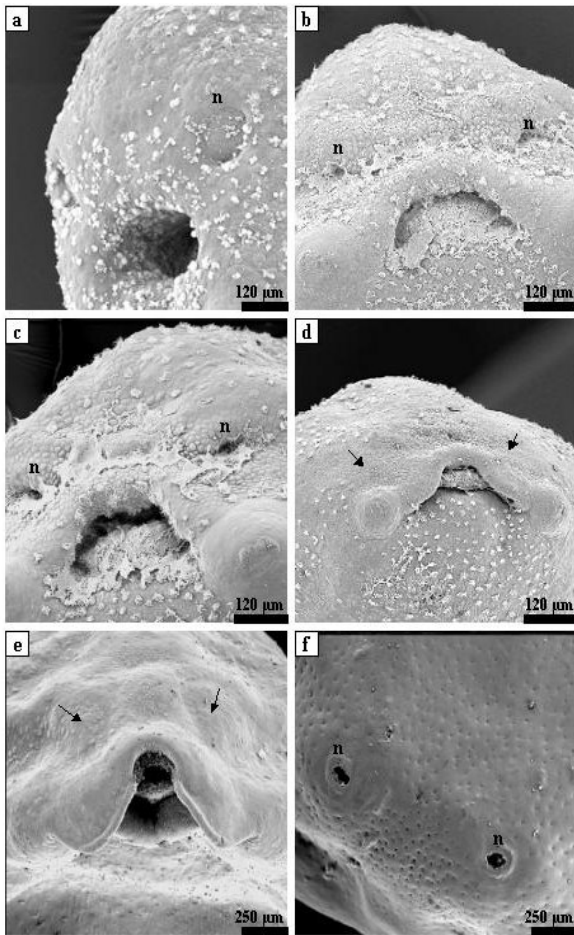


FIG. 1. Scanning electron micrographs of *E. ovalis* at embryonic and early metamorphic stages showing external nares. a: stage 20, b: stage 23, c: stage 24; d: stage 25; e: stage 25-26; f: stage 42. Labels: n - external nares; arrow - positions of former external nares. Structures below the nares in a-e are the central mouth and lateral adhesive glands.

they had a few ciliated cells in their proximity, these late embryonic/early larval external nares were not densely surrounded by ciliated cells, as Nokhbatolfooghahi & Downie (in press) report for the external nares of many other tadpole species. At stage 37, there were no signs of external nares. At stages 38/39, a pair of surface elevations was visible at the expected positions of the external nares but openings were absent. By stage 42, the external nares were clearly open (Fig. 1f).

In *Bufo bufo* and *Hyla crepitans* (Fig. 2 a-c), the external narial openings connect via an olfactory canal with a thickened olfactory epithelium to the internal nares in stage 26 tadpoles. In *E. ovalis*, the olfactory pits at stage 23 have a basal thickened olfactory epithelium; by stages 25 and 27, the external opening has closed but there is an internal opening and an elaborately thickened olfactory epithelium.

Table 1 shows data from specimens in the BMNH together with our data from *E. ovalis*. Early larval stages were available only for *Kalophrynus pleurostigma*, and these confirmed the presence of nasal pits at stages 19-

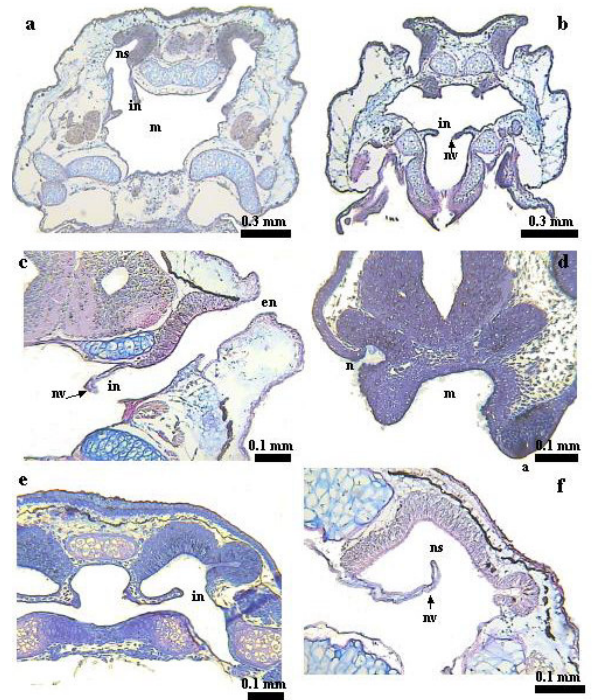


FIG. 2. Sections through the nostril region of three species of early larvae. a,b: *B. bufo*, stage 26, two sections through the same tadpole to show internal nares (a) and external nares (b). c: *H. crepitans*, stage 26, section showing both internal and external nares. d: *E. ovalis*, stage 23 showing external naris with thick olfactory placode at its base. e: *E. ovalis*, stage 25 showing internal naris and olfactory epithelium: external naris now closed. f: *E. ovalis*, stage 27 showing further development of the olfactory epithelium. Labels: a - adhesive gland; en - external naris; in - internal naris; m - mouth cavity; n - nostril; ns - nasal sac; nv - nasal valve. All figures photographed from PAS-stained wax sections, except d, semi-thin Toluidine Blue-stained resin section.

25, and their later absence. Absence of nostrils as early as stage 25 was confirmed for three species. The precise stage at which nostrils opened in later tadpoles varied from stage 39-41, based on a few available species.

DISCUSSION

In *E. ovalis*, the nasal pits develop at the same stage as in other anurans, but they close externally around the start of independent feeding and re-open at the start of metamorphosis. Internally, *E. ovalis* has an olfactory epithelium similar to that in other anurans, at least in early tadpole stages, and open internal nares; we did not examine the internal nares after stage 27.

Parker (1934) wrote that the "external nares do not appear until shortly before metamorphosis" although he examined the late embryonic stages only of *Kalophrynus pleurostigma* and *Microhyla borneensis*; in the former, he noted shallow nasal pits in recently hatched larvae, which we confirmed as stages 19-25. He made no mention of nasal pits in *M. borneensis* hatchlings, and these specimens are no longer available.

TABLE 1. Nostril appearance in developmental stages of different species of Microhylidae.

Species	Stages nostrils visible	Stages nostrils not visible	Stages nostrils not clear
<i>Calluella guttulata</i>	44, 46	37, 38, 42	43
<i>Glyphoglossus molossus</i>	42, 44	25, 26, 30, 35, 36, 37, 37/38	40, 41
<i>Kaloula pulchra</i>	39, 42/43, 45	38	38/39
<i>Kalophrynus pleurostigma</i>	19/20, 24, 25	29/30	-
<i>Chaperina fusca</i>	42	29, 32	25, 26
<i>Microhyla berdmorei</i>	41, 42, 42/43, 44	25, 34, 35, 37, 37/38	39/40, 40
<i>Microhyla butleri</i>	-	36, 36/37, 38	40
<i>Microhyla heymonsi</i>	-	36, 36/37, 37, 38	-
<i>Microhyla achatina</i>	-	37, 39	-
<i>Microhyla pulchra</i>	40, 43, 46	34	-
<i>Microhyla ornata</i>	-	25, 36, 37, 38, 42	43/44
<i>Microhyla rubra</i>	43, 44	37	-
<i>Elachistocleis ovalis</i>	19-24, 42	25, 26, 27, 37, 38/39	-

Altig & McDiarmid (1999; personal communication) reported open nares in embryos of *Gastrophryne carolinensis* and *Microhyla heymonsi* with later closure that persisted through most larval stages. They also stated that the external nares of nidicolous species of the sub-family Cophylinae are open at least at the surface throughout development, but it is not known if this applies to other nidicolous microhylids. Blommers-Schlösser (1975) reported that nostrils appeared late in the development of the swimming tadpoles of the cophylina *Platyhyla grandis*, though still before metamorphosis.

Open external nares have been reported from swimming tadpole stages in a few microhylid species. Berry (1972) showed open external nares from stage 26 onwards in *Phrynella pollicaris*; Mohanty-Hejmadi *et al.* (1979) described nostrils, without comment, in all stages of *Uperodon systoma* from late embryos onwards. Kirtisinghe (1958) described nostrils in tadpoles of *Ramanella palmata* and *Kaloula pulchra taprobanica*, but did not give stages. Parker (1934) observed late stage *Ramanella* tadpoles, but found nostrils opened only in *K. pulchra* tadpoles with well-developed hindlimbs (later than stage 38; our Table 1). Wassersug (1980) examined the internal narial depressions of three species of microhylid tadpoles from stages 32-37 and found that they were not open in any case.

It would clearly be helpful to examine embryonic and early larval stages in more microhylid species but, if it is possible to generalise from the present data, external nares develop normally in microhylid embryos. These apertures then close about the time of independent feeding in nearly all species with free-swimming tadpoles but remain open in species with larger eggs developing on land. Internal nares also form normally, then close in at least some tadpole species and re-open at metamorphosis.

Why does the olfactory system open up in the first place, and why does it later close off? It is likely that the formation of the nasal placodes and their subsequent invagination to form nasal pits is an essential early stage in

the chain of developmental interactions that forms the olfactory organs. Therefore, even if the olfactory system is to remain unused during most of tadpole life, it has to undergo these initial stages. Wassersug (1980) suggested that the closure of the internal nares in microhylid tadpoles was related to the efficiency of their buccal pumps; a reduction in the number of openings removes sources of leaks. This may be so, but it is surprising that this is a big enough advantage to overcome the loss of a sensory modality and it has yet to be established in how many microhylid species internal narial closure occurs. The sensitivity and uses of the olfactory system in tadpoles has not been fully investigated (review by Dawley, 1998) but it is responsible for chemical mediated kin recognition in some species and predator detection in others. *E. ovalis* tadpoles do not form shoals (Downie, personal observations), so it is unlikely that they show kin recognition, but the ability to detect predators would seem of importance. The stages at which the anuran olfactory system becomes functional has not been fully established, but in *X. laevis*, there is evidence for functionality just before the start of independent feeding (Hansen *et al.*, 1998). Assheton (1896) reported a powerful cilia-generated current sweeping into the olfactory pits of recently-hatched *Rana temporaria* larvae. If the olfactory cells are functional at this stage, the current may bring useful sensory information which could then be available to microhylid larvae in the early post-hatching stages when their external nares are still open.

ACKNOWLEDGEMENTS

We thank Margaret Mullin and Ian Montgomery for technical assistance and advice, the late Professor Peter Bacon and colleagues at the University of the West Indies for laboratory space, Barry Clarke and Mark Wilkinson for their help in accessing and studying specimens at BMNH and Ronn Altig for sharing unpublished results and commenting on a draft of this paper. We are also grateful for a comment by Alex Haas on the development of the internal nares in microhylids. JRD acknowledges financial assistance from the Carnegie

Trust and the University of Glasgow; MN carried out this work while on a Government of the Islamic Republic of Iran postgraduate scholarship.

REFERENCES

- Altig, R. & McDiarmid, R. W. (1999). Body plan development and morphology. In *Tadpoles: the biology of anuran larvae*, 24-51. McDiarmid, R.W. and Altig, R. (Eds). Chicago: University of Chicago Press.
- Assheton, H. A. (1896). Notes on the ciliation of the ectoderm of the amphibian embryo. *Quarterly Journal of the Microscopical Society* **38**, 465-484.
- Berry, P. Y. (1972). Undescribed and little known tadpoles from West Malaysia. *Herpetologica* **28**, 338-46.
- Blommers-Schlösser, R. M. A. (1975). Observations on the larval development of some Malagasy frogs, with notes on their ecology and biology (Anura: Discophinae, Scaphiophryninae and Cophylinae). *Beaufortia* **24**, 7-26.
- Dawley, E. M. (1998). Olfaction. In *Amphibian Biology, 3: Sensory Reception*, 711-742. Heatwole, H. (Ed). Australia: Surrey Beatty & Sons.
- Gosner, K. L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* **16**, 183-190.
- Haas, A. (2003). Phylogeny of frogs as inferred from primarily larval characters (Amphibia, Anura). *Cladistics* **19**, 23-89.
- Hansen, A., Reiss, J. O., Gentry, C. L. & Burd, G. D. (1998). Ultrastructure of the olfactory organ in the clawed frog, *Xenopus laevis*, during larval development and metamorphosis. *Journal of Comparative Neurology* **398**, 273-288.
- Kirtisinghe, P. (1958). Some hitherto undescribed anuran tadpoles. *Ceylon Journal of Science (Biological Sciences)* **1**, 171-6.
- Klein, S. L. & Graziadei, P. P. C. (1983). The differentiation of the olfactory placode in *Xenopus laevis*: a light and electron microscope study. *Journal of Comparative Neurology* **217**, 17-30.
- McDiarmid, R. W. & Altig, R. (1999). Research. In: *Tadpoles: the biology of anuran larvae*, 7-23. McDiarmid, R. W. and Altig, R. (Eds). Chicago: University of Chicago Press.
- Mohanty-Hejmadi, P., Dutton, S. K. & Mallick, S. C. (1979). Life history of Indian frogs 2: the marbled balloon frog *Uperodon systoma* Schneider. *Journal of the Zoological Society of India* **31**, 65-72.
- Nokhbatolfoghahai, M., Downie, J. R., Clelland, A. K. & Rennison, K. (2005). The surface ciliation of anuran amphibian embryos and early larvae: patterns, timing differences and functions. *Journal of Natural History* **39**, 887-929.
- Parker, H. W. (1934). *A monograph of the frogs of the family Microhylidae*. London: British Museum (Natural History).
- Wassersug, R. (1980). Internal oral features of larvae from eight anuran families: functional, systematic, evolutionary and ecological considerations. *University of Kansas Museum of Natural History, Miscellaneous Publication* **68**, 1-146.

Accepted: 18.10.04