Bonn zoological Bulletin 67 (2): 109–116
2018 · Behr N. & Rödder D.
https://doi.org/10.20363/BZB-2018.67.2.109

ISSN 2190–7307
http://www.zoologicalbulletin.de

Research article

urn:lsid:zoobank.org:pub:939B24A9-ED39-4CF6-82D7-EB7E5933343C

Larval development stages and husbandry of the Rice Frog *Microhyla mukhlesuri* Hasan et al., 2014 (Anura: Microhylidae)

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Abstract. We describe captive management and larval development of *Microhyla mukhlesuri*, a recently described microhylid frog from Bangladesh, southern Yunnan, Thailand, Laos, and Vietnam, at the scientific animal keeping facility of the Zoological Research Museum Alexander Koenig (ZFMK). Beginning at Gosner stage 25, for each larval stage detailed characteristics are provided and additionally developmental time is compared to other members of the genus *Microhyla*. Herein, we present first observations on captive reproduction of the species.

Key words. Conservation breeding, larval staging, tadpole morphology.

INTRODUCTION

The genus *Microhyla* Tschudi, 1838 currently comprises 41 species of small ground-dwelling frogs which are commonly referred to as rice frogs (Frost 2018). The group is widely distributed across Asia, occurring from the Japanese Ryukyu Islands and China to the north, across India, Sri Lanka and South-east Asia to the islands Sumatra, Borneo, Java, and Bali in the southeast (Frost 2018). Morphological characteristics comprise a generally small body size and a narrow mouth, a brown to reddish dorsal coloration with variable dark markings, smooth skin on dorsum, absence of vomerine teeth and paratoid glands, fingers without webbing and a hidden tympanum covered with skin (Poyarkov et al. 2014; Seshradi et al. 2016).

Microhyla mukhlesuri Hasan, Islam, Kuramoto, Kurabayashi & Sumida, 2014 was recently separated from its sister taxon *M. fissipes* Boulenger, 1884 and is not yet listed by the IUCN Red List of Threatened Species (Hasan et al. 2014; IUCN 2018). Yuan et al. (2016) suggested that *M. mukhlesuri* is distributed in Bangladesh, southern Yunnan, Thailand, Laos, and Vietnam and thus suppose that it also occurs in Myanmar and Cambodia, resulting in a wide distribution in Southeast Asia. For a few microhylid species of the genus *Microhyla* information on breeding ecology, captive management, and also larval staging tables are available (e.g., Shimizu & Ota 2003; Narzary & Bordoloi 2013; Wang et al. 2017). There is still a lack of information on the ecology and life history, including larval development, of *M. mukhlesuri*. Although this species might currently not be threatened by extinction, there are at least three species in the genus *Microhyla* that are currently listed as Endangered (i.e., *M. pulchella* Poyarkov, Vassilieva, Orlov, Galoyan, Tran, Le, Kretova & Geissler, 2014, *M. sholigari* Dutta & Ray, 2000 and *M. zeylanica* Parker & Osman-Hill, 1949) and one even as Critically Endangered (i.e., *M. karunaratnei* Fernando & Siriwardhane, 1996) by the respective IUCN Red List accounts (Biju et al. 2004; Manamendra-Arachchi & de Silva 2004a; Manamendra-Arachchi & de Silva 2004b; IUCN SSC Amphibian Specialist Group 2017). Therefore, captive management and information on larval development of *M. mukhlesuri* presented in this paper might be used analogously for more threatened closely related species.

Herein, we describe different tadpole stages of *Microhyla mukhlesuri* for the first time and present our captive management methods for this species in the scientific animal keeping facility of the Zoological Research Museum Alexander Koenig (ZFMK), Bonn, Germany. Furthermore, we documented the tadpoles' body surface every two to three days to examine general growth within the larval stage.

MATERIAL AND METHODS

Species identification. Since *Microhyla* species are often cryptic and hence hard to distinguish from each other, species identification was confirmed by DNA barcoding

Received: 27.04.2018
Accepted: 25.10.2018

Corresponding editor: W. Böhme
Published: 01.11.2018

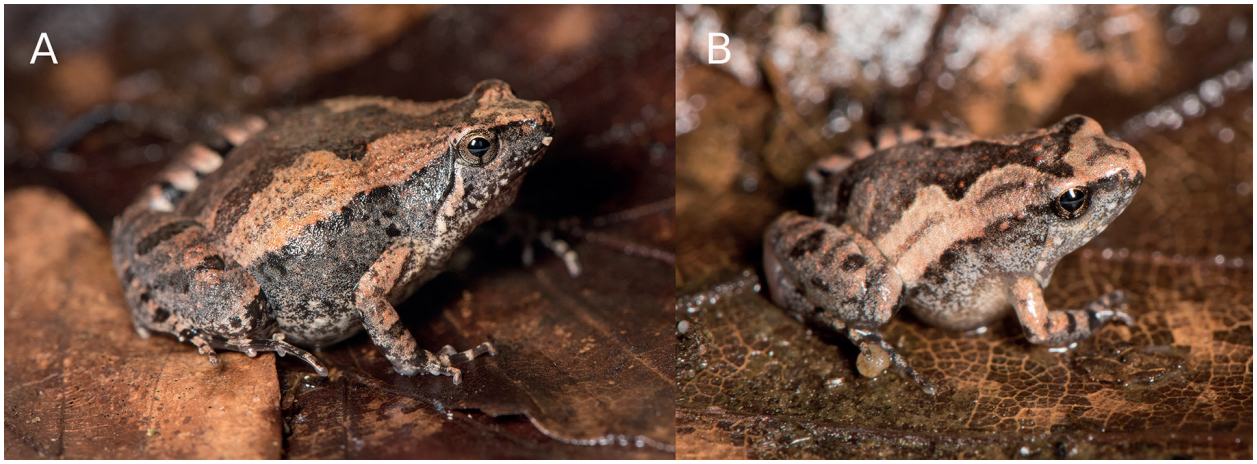

Fig. 1. Live specimens of *Microhyla mukhlesuri* at the ZFMK. A: adult frog; B: juvenile frog one week after metamorphosis.

Table 1. Developmental data of the fastest developing tadpoles. Stage: developmental stage according to Gosner (1960); Age: number of days after hatching; Diagnostic features: characteristic features of the respective stage; L: length, D: diameter.

Stage	Age [d]	Diagnostic features
25	1	Body nearly transparent, single pigment cells visible on whole body, wide mouth with obvious mouthparts, well developed eyes, closed operculum covers gills, spiracle forms ventrally on left side
26	10	Hind limb buds start to develop (L < ½ D)
27	12	Further development of hind limb buds (L ≥ ½ D)
28	14	Further development of hind limb buds (L ≥ D)
29	–	Further development of hind limb buds (L ≥ 1.5 D)
30	16	Further development of hind limb buds (L ≥ 2 D), limbs become slightly bent
31	17	Foot paddle starts to develop
32	19	First slight indentation on foot paddle visible (between toes 4 and 5)
33	19	Second indentation on foot paddle visible (between toes 3 and 4)
34	21	Third indentation on foot paddle visible (between toes 2 and 3)
35–36	24	Fourth indentation on foot paddle visible (between toes 1 and 2)
37	27	All toes completely separated
38–39	–	Metatarsal tubercle and subarticular tubercle appear
40	30	Foot tubercles, toe pads completely developed
41	32	Forelimbs visible under the transparent skin, atrophy of the mouthparts begins
42	33	Forelimbs emerge, mouth positioned anterior to nostril
43	35	Mouth angle between nostril and eye, tail begins to atrophy
44	35	Mouth angle beneath eye, tail already strongly reduced
45	36	Mouth angle posterior to eye, only a tail stub is left; coloration and pattern slightly developed
46	37	Tail completely resorbed, metamorphosis completed; development of coloration and pattern completed

using a fragment of the mitochondrial 16S rRNA. Sequences were obtained as described in Koch et al. (2013). The final sequence (GenBank Accession MH232034) was compared with sequences of *Microhyla* species available in GenBank and following the definition of *M. mukhlesuri* as proposed by Yuan et al. (2016).

Captive management of adult frogs. A group of 20 adult *Microhyla mukhlesuri* (Fig. 1A) originating from Vietnam was purchased from a commercial importer in 2017 and housed in a terrarium measuring 60 x 60 x 40 cm (l x w x h) in the scientific animal keeping facility of the ZFMK. The terrarium was filled with remineralized osmosis water up to a depth of about 5 cm. One half of the bottom was covered with a filter pad measuring 40 x 20 x 6 cm as land part. Leaf litter (mainly *Fagus sylvatica* and *Quercus robur*) was scattered on the filter pad and in the water part to provide hiding places. Additionally, some live plants (i.e., *Elodea* sp. and *Microsorum pteropus*) were placed in the water. Both air and water temperatures ranged from 20 °C to 26 °C and humidity varied between 60% and 80%. For illumination LED light strips (Solar Stinger 1100mm Sunstrip Dimmable Driver) were used and the photoperiod was set between 8:00 and 20:00 h. The frogs were fed with young crickets (*Acheta domesticus* and *Gryllus assimilis*) and flightless fruit flies (*Drosophila melanogaster* and *D. hydei)* every two to three days. All prey items were dusted with different vitamin and mineral powders (i.e., herpetal Amphib, herpetal Mineral + Vitamin D3 and herpetal Complete Terrarium) and furthermore crickets were gut loaded with fresh vegetables.

Rearing setup for tadpoles. Tadpoles were transferred into a rearing tank measuring 30 x 30 x 30 cm. This tank was filled with remineralized osmosis water to a depth of about 25 cm and an aquarium heater (SERA SE008710, 50W) was used to keep the water temperature at 24 °C to 25 °C. A few aquatic plants (i.e., *Elodea* sp. and *Microsorum pteropus*) and some dried leaves of *Terminalia catappa* and *Fagus sylvatica* were added to the water as natural bacteriostatics and fungistatics (Chitmanat et al. 2005). Tadpoles were fed with a mixture of crushed fish food (Sera® vipan and Tetra Tablets TabiMin) and *Spirulina* flakes every two to three days. In addition, one stone that was overgrown with algae was placed in the tank and was replaced every other day when most of the algae were eaten. Furthermore, *Daphnia pulex* and different aquatic snails (i.e., *Physella* sp. and *Planorbella* sp.) were added to eat possible food remains. As no filtration was used, half of the water was changed once per week.

The following water parameters were measured in the rearing tank: NO_2 < 0.05 mg/l, NO_3 7.5 mg/l, NH_4 < 0.05 mg/l, Cu < 0.1 mg/l, KH 3°dH, GH 5°dH, pH 6.5–

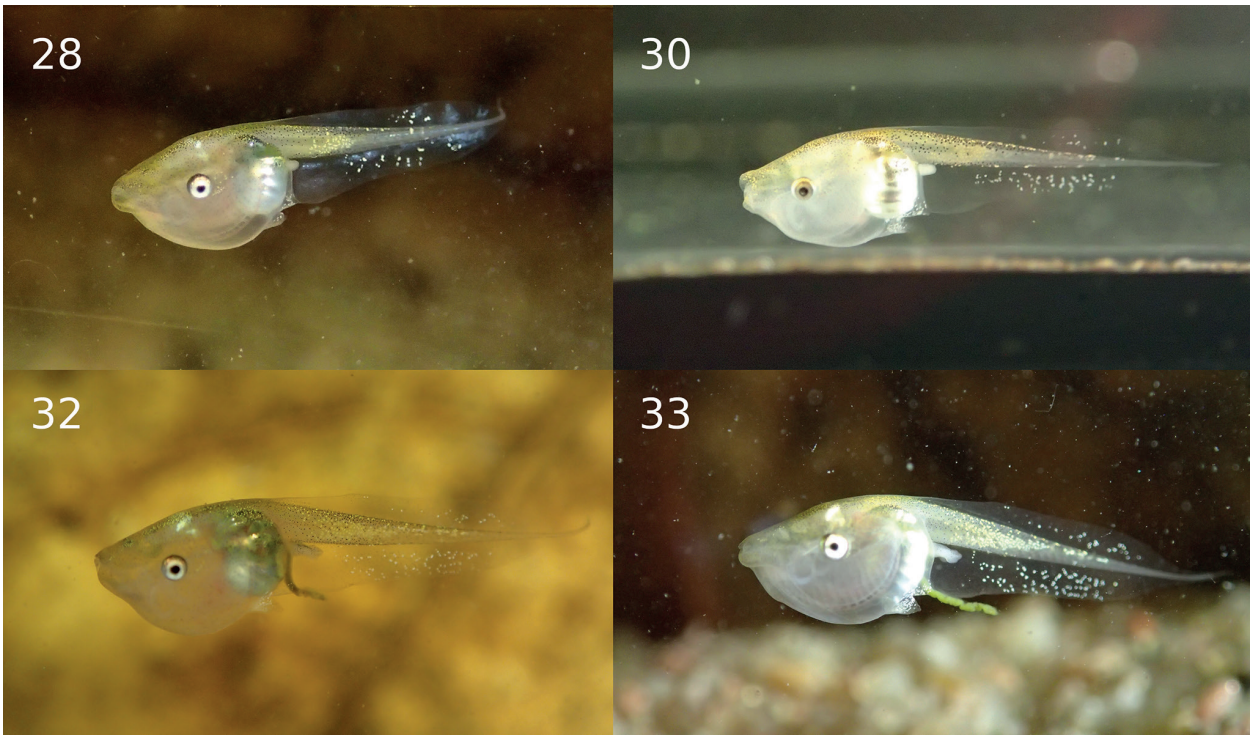

Fig. 2. Tadpoles of *Microhyla mukhlesuri* in Gosner stages 28 to 33. These stages are defined by the growth of hind limbs and foot paddles. Stage 28: hind limbs just became longer than wide (diameter); Stage 30: hind limbs are at least two times as long as wide (diameter); Stage 32: a first indentation appears on the foot paddles, which will later separate toes 4 and 5; Stage 33: second indentation appears on the foot paddles between the developing toes 3 and 4.

Table 2. Comparative larval development time of four species in the genus *Microhyla*: *M. mukhlesuri*, *M. fissipes*, *M. ornata*, *M. okinavensis*. We adjusted the staging data of the other species to fit the staging system after Gosner (1960) and started counting from stage 25 onwards. * The species identified as *M. ornata* by Narzary & Bordoloi (2013) might in fact be *M. mukhlesuri* following Yuan et al. (2016), genetic analyses are necessary; ** Specimens from the Ryukyu Archipelago identified as *M. ornata* by Shimizu & Ota (2003) have been assigned to *M. okinavensis* by Matsui et al. (2005).

	Age [d]			
Stage	***Microhyla mukhlesuri*** (Own data)	***Microhyla fissipes*** (modified after Wang et al. 2017)	***Microhyla ornata**** (modified after Narzary & Bordoloi 2013)	***Microhyla okinavensis**** (modified after Shimizu & Ota 2003)
25	1	1	1.0	1.0
26	10	6	2.5	5.5
27	12	9	3.0	7.5
28	14	12	7.5	9.5
29	–	15	12.5	11.5
30	16	15	16.5	11.5
31	17	17	19.5	13.5
32	19	19	22.5	15.5
33	19	19	25.5	15.5
34	21	22	28.5	17.5
35	24	24	31.5	18.5
36	24	24	33.5	18.5
37	27	26	35.5	20.5
38	–	29	38.5	23.5
39	–	33	40.5	29.5
40	30	33	41.5	29.5
41	32	36	42.5	33.5
42	33	38	43.5	35.5
43	35	38	44.0	35.5
44	35	39	44.5	36.5
45	36	40	45.5	37.5
46	37	41	46.5	38.5

7.5. Lighting was equivalent to the adult setup. When the first tadpoles had developed hind legs a piece of cork bark was added to the aquarium to provide a small land area for metamorphosed frogs.

Rearing setup for juvenile frogs. Metamorphosed frogs were transferred into plastic boxes measuring 33 x 21 x 28 cm with fine mesh lids on both narrow sides. One side of this container was heightened to create a gradient and a water part with a depth of about 2 cm on the lower side. One layer of Hygrolon®, an artificial and highly hygroscopic material originally developed for cultivating orchids and ferns, was used as ground layer to keep the air humidity on a high level of about 70% to 80%. Furthermore, a big layer of dry leaves (mainly *Fagus sylvatica* and *Quercus robur*) and moss was added to the box. Temperatures ranged between 21 °C and 26 °C and illumination was the same as in the adult and tadpole setups. In the first few weeks after metamorphosing the juvenile frogs were fed with tropical springtails (*Collembola* sp.), later on they were additionally fed with dusted *Drosophila hydei*.

Data acquisition and evaluation. To document growth and development of the tadpoles every two to four days photos of 10 randomly chosen tadpoles were taken from 10th of June to 1st of September 2017. On the last day of growth documentation only the five remaining larvae, which had not completed metamorphosis at that time, were photographed. For this the tadpoles were transferred into a Petri dish, which was lightened from below to increase the contrast between tadpole and background in the recorded photos. All photos were taken with a digital camera (Olympus TG-2). Additionally, morphological data of the fastest developing tadpoles were recorded to document the developmental stages. The photos were

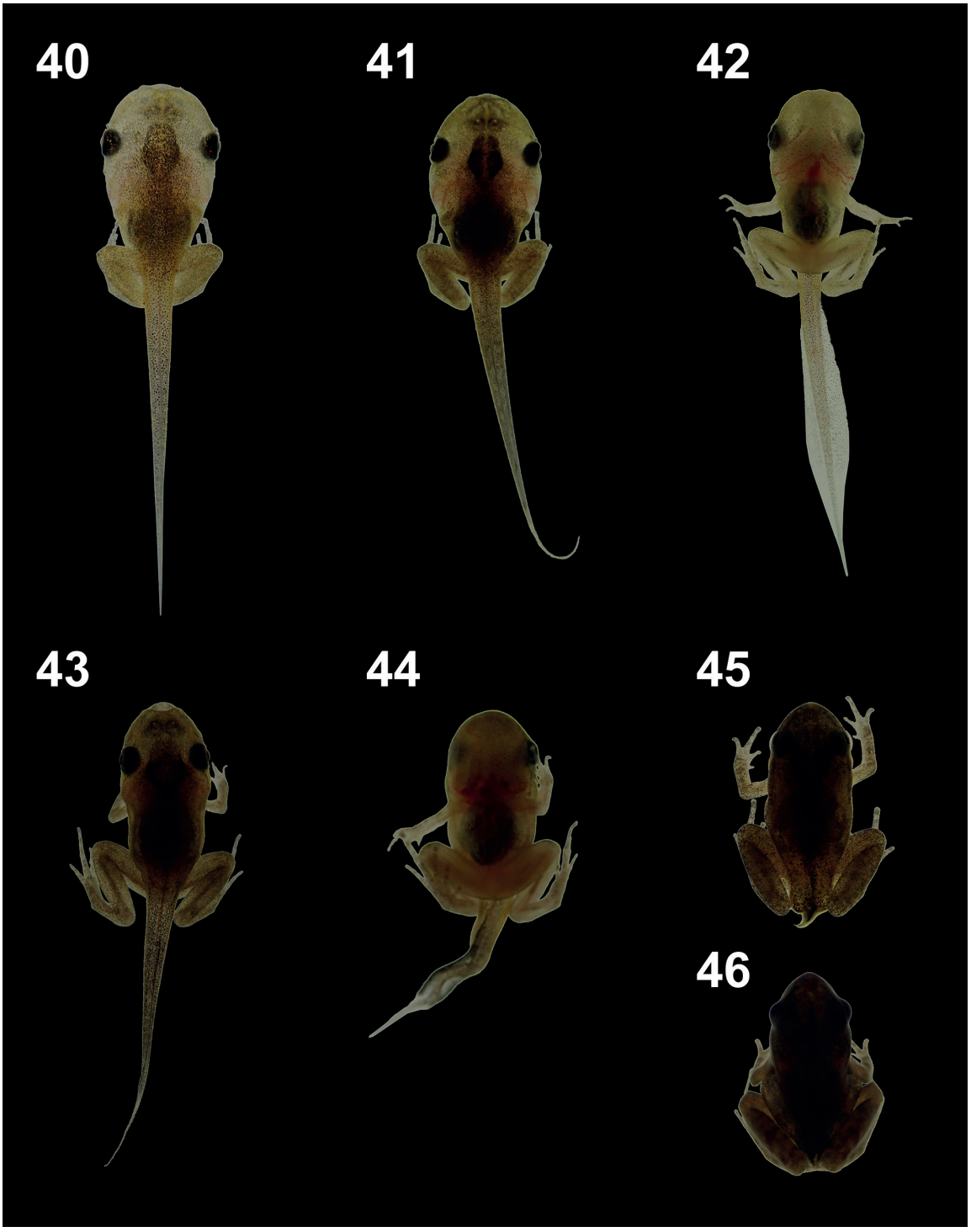

Fig. 3. Gosner stages 40 to 46 of *Microhyla mukhlesuri* tadpoles, representing the last steps to metamorphosis. Stage 40 (dorsal view): development of hind limbs and toe pads is completed and foot tubercles have developed; Stage 41 (dorsal view): the forelimbs have become well visible under the tadpoles' transparent skin, the mouthparts begin to atrophy; Stage 42 (ventral view): both fully developed forelimbs have emerged, mouth angles are positioned anterior to the nostrils; Stage 43 (dorsal view): tail degeneration begins, mouth angle is positioned between nostril and eye; Stage 44 (ventral view): the tail is already strongly reduced and the mouth angles are now positioned beneath the eyes; Stage 45 (dorsal view): the tail is greatly reduced and the mouth angles are positioned posterior to the eyes, additionally, the coloration has already slightly developed; Stage 46 (dorsal view): with the completed reduction of the tail and further development of the coloration, metamorphosis is finished.

analyzed with the tool SAISAQ (Kurth et al. 2014) on the open source statistics platform R (R Developmental Core Team 2016). This software package semiautomatically processes image files and computes the surface area of a tadpole, which is highly correlated with its body mass. This method is non-invasive and therefore suitable for repeated measurements on (small) live animals without causing much handling stress. Larval stages were examined following the universal anuran larvae staging table developed by Gosner (1960). We identified tadpole stages between Gosner stage 25 and 46, starting with the stage in which the tadpoles were found and finishing with completion of metamorphosis. Voucher specimens were deposited in the herpetological collection of the Zoological Research Museum Alexander Koenig, Bonn (ZFMK 101119−101122 [adults], ZFMK 101528 [juvenile], ZFMK 101529 [metamorph]), and ZFMK 101530 − 101531 [larvae]).

RESULTS

A total of 79 tadpoles were found on 10th of June 2017 in the water part of the adult breeding group terrarium, no unhatched or unfertilized eggs were left. When detected, all tadpoles were free swimming without yolk sac and showed a strong fleeing reaction when disturbed. They had a mainly transparent body with scattered pigment cells, a wide mouth with already completely developed mouthparts, and the gills were covered by the operculum. Following these features, we determined them as Gosner stage 25 at the day of finding (Table 1). At this stage, tadpoles had a mean body surface of about 0.057 cm² (Fig. 4). Stages 26 to 30 ranged from day 10 to day 16 and were defined by different growth stages of the hind limb buds (Fig. 2). We were not able to differentiate stage 29. At stage 30, the limbs became slightly bent. The following stages 31 to 37 were characterized by the development and indentation of the foot paddle, finishing with the complete separation of all toes, and lasted from day 17 to 27 (Fig. 2). Stages 38 and 39 were not documented. On day 30 the first tadpoles reached stage 40, characterized by developed foot tubercles and the completion of the toe pads. Stages 41 to 46, the metamorphs stages, were characterized by the completion of metamorphosis and ranged from days 32 to 37 (Fig. 3). At stage 41 the developed forelimbs were well visible under the tadpoles' transparent skin. Additionally, the atrophy of the mouthparts began in this stage. Forelimbs emerged at stage 42 at day 33, and the mouth angle was positioned anterior to the nostril. Within the next three stages the mouth angles became translocated more to the distal end of the tadpoles' head, until they were positioned posterior to the eyes at stage 45 at day 36. Furthermore, absorption of the tail began in stage 43 and was finished in stage 46.

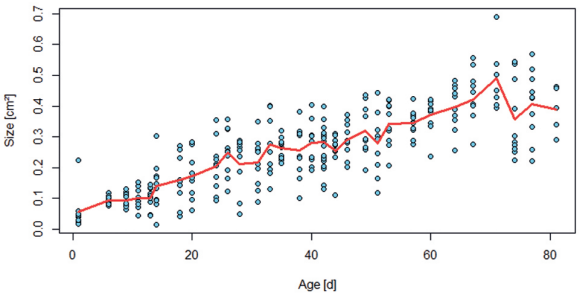

Fig. 4. General growth in tadpoles of *Microhyla mukhlesuri*, based on body surfaces computed with SAISAQ (Kurth et al. 2014). Blue spots represent sizes of individual tadpoles; mean size at the respective day is plotted in the red graph.

Together with the fully developed coloration at this stage (Fig. 1B), the metamorphosis was completed. The fastest developing tadpole finished metamorphosis as early as day 37 after finding, the slowest developing one at day 98, while most (32) metamorphosed between day 73 and 80. Body surface reached its highest peak at day 71 with a mean surface of 0.490 cm² and the biggest individual measuring 0.689 cm² (Fig. 3). Afterwards, when most tadpoles reabsorbed their tail to finish metamorphosis, general body surface decreased slightly. Directly after the completion of metamorphosis the freshly morphed frogs measured between 0.221 cm² and 0.333 cm², and had a snout-vent length of 6 mm to 7 mm.

DISCUSSION

Our results summarize the first larval staging for free-swimming tadpole stages of *Microhyla mukhlesuri*. As only already hatched tadpoles were found, we were not able to document early embryonic stages of this species. Future captive breeding efforts at the scientific animal keeping facility of the ZFMK will be necessary to complete the staging table.

We assume that the tadpoles had hatched only one or two days before we found them in the breeding group tank. Hence, based on our study tadpoles needed between 38–40 and 98–100 days to complete metamorphosis at water temperatures of 24 °C to 25 °C. This is very similar to the findings of Wang et al. (2017) for the closely related sister taxon *Microhyla fissipes*, which needed 43 days in total and about 41 days after hatching in late stage 23 to complete the metamorphosis at a water temperature of 22.9 °C to 25.4 °C. Furthermore, we found high similarities to other species of the genus *Microhyla*. Measuring from stage 25 onwards, tadpoles of *M. ornata* complete metamorphosis in about 46.5 days at a water temperature of 25 °C to 27 °C (Narzary & Bordoloi 2013), and tad-

poles of *M. okinavensis* in about 38.5 days at 19 °C to 26 °C (Shimizu & Ota 2003). Detailed comparisons of developmental time of different tadpole stages are documented in Table 2. Mohammad Ridzuan (2013) found that tadpoles of *M. nepenthicola* needed 14 days from stage 33 to stage 46, while tadpoles of the aforementioned species needed 18 to 23 days (i.e., *M. mukhlesuri*: 18 d, *M. fissipes*: 22 d, *M. ornata*: 21 d, *M. okinavensis*: 23 d; Table 2). This difference might be due to different rearing setups, especially temperature (information missing for *M. nepenthicola*) and amount of provided food usually have high influences on the rate of larval development (e.g., Duellman & Trueb 1986; Harkey & Semlitsch 1988; Marian & Pandian 1985).

The number of tadpoles we found (n=79) is rather small compared to clutch sizes reported for *M. fissipes* (209 to 564 eggs [Wang et al. 2017]), *M. ornata* (300 to 510 eggs [Narzary & Bordoloi 2013]) and *M. okinavensis* (220 to 910 eggs [Shimizu & Ota 2003]). Hence, we suppose that it was either the first clutch of a recently sexually matured and still not fully grown female, which might produce smaller egg clutches (e.g., Gibbons & McCarthy 1986), or that the majority of either eggs, possibly infertile eggs, or tadpoles had already been eaten by adult frogs. Future breeding efforts might help to collect sufficient data on clutch sizes.

The combined information about larval development, captive breeding and management of these frogs gathered in different studies might become an important factor if measures of conservation necessitate captive breeding programs for the preservation of endangered species and for restocking programs. Currently, the IUCN Red List of Threatened Species (2018) lists four species of the genus *Microhyla* as Endangered or even Critically Endangered (i.e., *M. pulchella*, *M. sholigari*, *M. zeylanica* and *M. karunaratnei*). Furthermore, for seven species of the genus there is not yet enough data available for a classification by the IUCN Red List and ten species are not even listed at all (Frost 2018; IUCN Red List of Threatened Species 2018). The endangered species might benefit from the knowledge gained in our and similar studies as husbandry analogues already today.

Acknowledgements. We want to thank Timo Hartmann and David Hörnes, who helped with the captive management of the frogs, and Ursula Bott, helping with her excellent proof reading skills. Morris Flecks and Claudia Etzbauer helped with the molecular analyses. We are grateful for financial support from the Alexander-Koenig-Gesellschaft, Bonn. Furthermore, we want to thank Anna Rauhaus and Thomas Ziegler, who provided helpful comments to an early version of the manuscript.

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