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Developmental Stages of Lotic-breeding Toad, *Bufo torrenticola*, with a Comparison to Lentic-breeding *B. japonicus formosus* (Amphibia: Anura: Bufonidae)

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Abstract: The development of lotic-breeding *Bufo torrenticola* is described from zygote to completion of metamorphosis in captivity at 13±1°C. We delimit 56 developmental stages based on external features. We describe developmental stages so as to be comparable to common stages that are widely used for anurans. We also compare the larval development between *B. torrenticola* in lotic water and lentic water, and with lentic-breeding *B. japonicus formosus* in lentic water. Our results suggest tadpole mouth size in *B. torrenticola* is determined by genetic factors, but the tail muscle volume is determined by both genetic and environmental factors.

Key words: External gill; Japanese stream toad; Mouth width; Tadpole morphology; Tail height

Introduction

In amphibians, morphological adaptation to a new habitat often involves speciation (Noble, 1931). In addition, the larval characters can show extensive environmental adaptation (Orton, 1953). Although such novel and adaptive larval characteristics have been described, their developmental bases are not demonstrated well in many cases. The Japanese stream toad, *Bufo torrenticola* Matsui, 1976a was described as distinct species, separating from the Japanese toad, *B. japonicus*. The Japanese stream toad lays eggs in running water, and the tadpoles inhabit streams in central Honshu, mainland Japan (Matsui, 1975). The larvae of *B. torrenticola* have wide mouth (especially when compared to lentic-breeding *B. japonicus*) which functions like a sucker and allows them to attach to stones in running water (Matsui, 1975). This wide mouth is also used for grazing algae on the stones in running water (Matsui, 1976b). The tail is muscular with a low fin, a likely adaptation to produce strong swimming force needed to counteract fast-flowing water (Matsui, 1975). These morphological adaptations to streams enable

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the separation of breeding habitat between sympatric populations of lotic-breeding *B. torrenticola* and lentic-breeding *B. japonicus* (Matsui, 1976b). However, the development of the adaptive morphology of *B. torrenticola* is not completely understood. Iwasawa and Saito (1989) compared the shape of mouth and body size at several larval stages between *B. torrenticola* and *B. japonicus*. However, Iwasawa and Saito (1989) made univariate comparisons between the two species, rather than employing bivariate comparisons which could take possible shape change into account. Further, the developmental stages of *B. japonicus* used by Ichikawa and Tahara (1966) that Iwasawa and Saito (1989) used were not standardized for comparison to the stages system developed by Gosner (1960), which is the commonly used developmental stages for frogs. Gosner (1960) provided generalized developmental stages in frogs, which enable comparison between species (McDiarmid and Altig, 2000). Gosner (1960) has accordingly been referred to by many anuran developmental studies (e.g. Iwasawa and Futagami, 1992; Shimizu and Ota, 2003; Wang et al., 2017). However, previous studies of developmental stages of Japanese toads did not refer to Gosner (1960), impeding comparisons between Japanese *Bufo* and other anurans. Here, we describe the complete development of lotic-breeding *B. torrenticola* for the first time so as to be comparable to the common anuran staging systems that are widely used. We also compare shape changes through development of *B. torrenticola* with related lentic-breeding species *B. japonicus*.

Materials and Methods

Breeding

We collected three pairs of adult *Bufo torrenticola* on 3 May 2020 from Kutsuki, Takashima City, Shiga Prefecture, Japan. The air temperature was 14.3°C and the water temperature was 12.3°C when we collected the pairs at 20:00 (flow velocity 0.48 m/sec). We brought these pairs into a laboratory (located in central Kyoto City 32.7 kilometers away from Kutsuki), and placed each pair into a separate plastic containers (62.4×43.2 cm in area, 31.6 cm in height, three containers in total), filled with enough tap water (23±1°C) as to cover the dorsal surface of the pairs' bodies. The water was Chlorine-free, made by holding tap water in a large tank for a few days prior to collection of amplectant pairs. After egg deposition on 4 May, we cut egg strings into ca. 10 cm lengths for allowing aspiration (Iwasawa, 1987b). Strings were kept in small plastic containers (25.7×17.3 cm in area, 4.3 cm in height) and petri dishes (8.8 cm in diameter) until they developed into tadpoles with spiracles. We then divided tadpoles into two groups, lotic water and lentic water, with group each consisting of 100 or fewer individuals. We kept each group in a container of the same size (29.2×19.0 cm in area, 17 cm in height) filled two-thirds with water. All containers were kept in an incubator (air temperature 14±1°C, water temperature 13±1°C). In one container, we attached a small electric pump (3.5×4.7 cm in area, 5.8 cm in height) to produce the lotic water like the natural habitat of the tadpole of *B. torrenticola*. In the other container without a pump there was no water current. After forelimbs appeared we reduced the water level and tilted the containers so that the metamorphs could move out of water (land : water=1 : 1 in space). We replaced the water in the containers every second day (replacement water was dechlorinated as described above). We fed goldfish food (Hikari Co., Ltd.) to the tadpoles. We scattered one gram of food over the water surface in each container after every water change.

Developmental Stages

We distinguished and combined developmental stages following Gosner (1960) and Iwasawa (1987a), because the former is commonly used developmental staging system for frogs (McDiarmid and Altig, 2000), and the latter is the only previously available data on developmental stages of Japanese toads (*Bufo*).

For the description of each stage we sampled eggs, embryos or tadpoles (usually five) at random. We chose Gosner (1960) or Iwasawa (1987a) for each stage of development and made complete developmental stages. Details of numbers of specimens examined are given in Table 1. Our new stages are italicized in this article, as "*Stage*". We followed Iwasawa and Futagami (1992) and separated these stages into eight categories as follows: Cleavage-blastula stage (*Stages* 1–11); Gastrula (12–17); Neurula (18–23); Tail bud (24 and 25); External gill (26–34); Hindlimb bud (33–41); Hindlimb formation (42–50); and Metamorphosis (51–56).

Measurements

All measurements were taken to nearest 0.01 mm with a digital caliper under the stereoscopic microscope (character dimensions are shown in Fig. 1). After fixation in 5% formalin, we measured total length (TL) and observed the embryos and tadpoles at each stage with a stereomicroscope. From *Stage* 24, we measured body length (BL) at each stage. In addition, we used 211 specimens from *Stage* 40 for comparing across *B. torrenticola* in still water (hereafter, lentic *B. torrenticola*), *B. torrenticola* in flowing water (hereafter, lotic *B. torrenticola*), and *B. japonicus formosus* in still water. Details of the sample sizes of each stage per species are given in Table 3. From *Stage* 40 we measured oral width (OW), oral height (OH), tail length (TAL), maximum tail height (MTH), tail muscle height (TMH) and tail muscle width (TMW) as described by McDiarmid and Altig (2000), and maximum head width (MHW) and mid-tail muscle height (mTMH). We measured gill length (GL) and MHW at the stage with maximum gill size. We did not use BL but MHW for relative gill length comparison, because in the stage, ventral figure was not drawn in Iwasawa (1987a) and the border between body and tail was not shown in Matsui (1987). Drawings were prepared using camera-lucida. Voucher specimens are deposited at the Graduate School of Human and Environmental Studies,

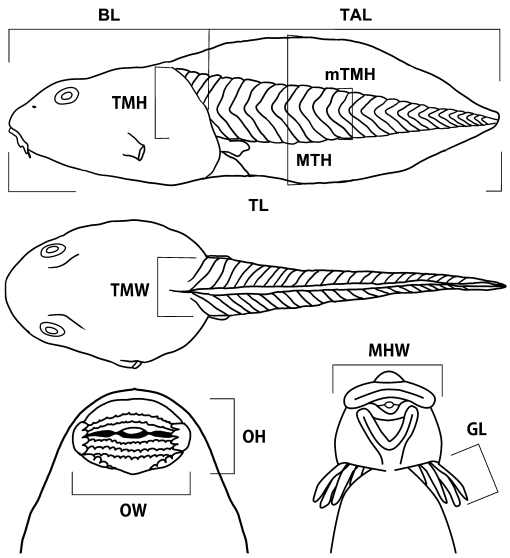

FIG. 1. Character legend for tadpole morphology.

Kyoto University (KUHE). All procedures followed the Animal Experiment Guideline of Kyoto University and were approved by the institutional review committee of the Graduate School of Human and Environmental Studies of Kyoto University (approval nos. 30-A-7 and 20-A-5).

Comparison

To compare with a close relative (see Igawa et al. [2006] for their phylogenetic relationships), we grew eggs of *Bufo japonicus formosus* from Minamiechizen-cho, Fukui Prefecture (about 100 individuals) using the lentic water husbandary protocol as described above. We compared tadpoles across species and treatments after they reached *Stage* 40.

We compared ratios of OH/BL, OW/BL, TMW/TAL, TMW/TMH, TAL/TOL, MTH/TAL, TMH/MTH, mTMH/MTH and mTMH/TMH among the lentic *B. torrenticola*, the lotic *B. torrenticola*, and *B. japonicus formosus*, by Kruskal-Wallis test and Steel-Dwass test ($p<0.05$ as significant) using R software (R Development Core Team, 2021).

In order to test for developmental changes in

TABLE 1. Stages of normal development of *Bufo torrenticola*. The initial number represents the frog's stage as defined by us, while the numbers in parentheses (G1–G46) and brackets (I1–I45) represent the corresponding developmental stages as defined by Gosner (1960) and Iwasawa (1987a), respectively. Total length (in mm) is given as $\bar{x}$±SD, followed by ranges and sample sizes in parenthesis. Age (h:m) indicates the minimum age.

Stage number	Stage	Age (h:m)	Total length (in mm)	Diagnostic features
1 (G1, G2)[I1]	Early uncleaved	0:00	3.16±0.21 (2.87–3.40, n=5)	The embryo rotates for orientation completed with upper location of animal hemisphere. Animal hemisphere is dark brown and vegetal one is cleamy in color.
2 (G2)[I1]	Late uncleaved	0:34	3.13±0.19 (2.85–3.40, n=7)	The upper part of the embryo becomes flat.
3 (G3)[I2]	2-cell	3:34	3.20±0.13 (3.01–3.44, n=10)	The 1st cleavage (meridional) begins. 2 blastomeres.
4 (G4)[I3]	4-cell	5:22	3.08±0.21 (2.87–3.34, n=6)	The 2nd cleavage (meridional) begins. 4 blastomeres.
5 (G5)[I4]	8-cell	7:01	3.23±0.31 (2.87–3.8, n=6)	The 3rd cleavage (latitudinal) begins. 4 small blastomeres and 4 large ones.
6 (G6)[I5]	16-cell	7:59	3.03±0.16 (2.79–3.41, n=10)	The 4th cleavage (meridional) begins. 16 blastomeres.
7 (G7)[I6]	32-cell	8:57	3.07±0.24 (2.69–3.46, n=8)	The 5th cleavage (latitudinal) begins. 32 blastomeres. The clevages are more or less delayed in vegetal hemisphere.
8 (G8)[I7]	Morula	18:40	3.22±0.22 (2.94–3.51, n=7)	The 6th cleavage begins.
9 (G8)[I8]	Blastula I	20:38	3.12±0.21 (2.83–3.34, n=3)	The division of blastomeres advances. The blastomeres become quite small.
10 (G9)[I9]	Blastula II	22:39	2.97±0.16 (2.77–3.25, n=7)	The division of blastomeres advances more. The cells of the upper hemisphere are very small.
11 (G9)[I10]	Blastula III	25:56	3.02±0.24 (2.52–3.23, n=6)	The surface of the embryo looks smooth.
12 (G10)[I11]	Appearance of blastoporic lip	43:25	3.21±0.15 (3.03–3.19, n=3)	The invagination begins.
13 (G10)[I12a]	Semicircular blastoporic lip	44:05	3.27±0.06 (3.19–3.34, n=3)	The blastoporic lip extends laterally and becomes semicircular.
14 (G10)[I12b]	Horseshoe-shaped blastoporic lip	44:48	3.19±0.06 (3.10–3.26, n=5)	The blastoporic lip becomes horseshoe-shaped.
15 (G11)[I13]	Large yolk plug	46:32	3.22±0.18 (2.80–3.41, n=8)	The lateral tips of blastoporic lip fuses ventrally and make blastopore circular.
16 (G12)[I14]	Middle yolk plug	52:51	3.32±0.26 (2.98–3.73, n=5)	The yolk plug becomes slightly smaller.
17 (G12)[I15]	Small yolk plug	67:50	3.50±0.13 (3.39–3.69, n=3)	The yolk plug becomes smaller. The neural plate and groove appear faintly.
18 (G13)[I16]	Neural plate with primary neural fold	71:51	3.33±0.09 (3.20–3.45, n=5)	The yolk plug almost disappears. Primary neural fold surrounds the neural plate.
19 (G14)[I17]	Secondary neural fold	86:46	3.62±0.14 (3.45–3.8, n=3)	Secondary neural fold appears.
20 (G14)[I18]	Late secondary neural fold	87:41	3.48±0.34 (3.14–3.82, n=2)	Secondary neural fold becomes clearly visible. The bulge of the jaw group and the hyoid group be distinguished in the anterior half of the primary neural fold.
21 (G15)[I19]	Neural tube I	88:41	3.74±0.18 (3.49–3.89, n=3)	The neural folds approach each other.
22 (G16)[I20]	Neural tube II	96:55	3.27±0.05 (3.21–3.34, n=3)	The neural folds make contact in the trunk region. Primary gill bulge becomes obvious. Left and right primordia of adhesive organs adhere on the median surface.

TABLE 1. (continued)

Stage number	Stage	Age (h:m)	Total length (in mm)	Diagnostic features
23 (G16)[I21]	Neural tube III	100:38	3.52±0.20 (3.24–3.85, n=7)	The neural tube is completed. Secondary gill bulge becomes obvious. A dent appears in the primordium of adhesive organ.
24 (G16)[I22]	Early tail bud	117:29	3.91±0.24 (3.29–4.33, n=15)	The bulge of anterior kidney becomes clearly visible. The adhesive organ secretes mucus. Oral fossa formation takes place. There are some individuals that hatch.
25 (G17)[I23]	Late tail bud	142:09	TL 4.70±0.21 (4.41–4.97, n=5) : BL 4.19±0.20	The nasal and oral fossa, optic vesicle and fin primordium appear.
26 (G18)[I24, I25]	Gill bud I	5 (days)	5.91±0.09 (5.82–5.99, n=2) : 5.36±0.04	Muscular response to mechanical stimulation. The gill buds appear.
27 (G18)[I26]	Gill bud II	7	7.32±0.32 (6.70–7.58, n=5) : 4.89±0.27	Primary gill bud begins to ramify.
28 (G19)[I27, I28]	Gill bud III	9	7.48±0.04 (7.42–7.51, n=3) : 5.24±0.22	Secondary gill bud begins to ramify. A visible pulsation below and behind the gills appears by heart beat.
29 (G20)[I28]	External gills I	9	7.83±0.27 (7.42–8.34, n=7) : 5.09±0.50	The gills elongates. The optic vesicle becomes deep.
30 (G20)[I29]	External gills II	10	8.52±0.58 (7.64–9.53, n=6) : 5.51±0.34	The gill circulation becomes seen as a movement of corpuscles through the external gill filaments.
31 (G20)[I30]	External gills III	11	10.43±0.36 (9.99–10.88, n=3) : 6.36±0.30	The gills elongates most. Anlage of the labial teeth appears and becomes divided into two.
32 (G21, G22, G23)[I30, I31]	Opercular development I	13	12.35±0.65 (11.4–13.06, n=4) : 6.44±0.51	The cornea becomes transparent. The base of gills is covered with operculum
33 (G24, G26)[I31, I32, I33]	Opercular development II	14	12.11±0.05 (12.07–12.19, n=3) : 5.99±0.33	The shape of hind limb bud can be seen. Papillas appears. Degeneration of adhesive organ begins.
34 (G25, G26)[I32, I34]	Opercular development III	15	12.97±0.10 (12.85–13.09, n=3) : 6.93±0.34	Hind limb bud: length <1/2 basal width. Larbal teeth grow on the upper jaw. The fin becomes transparent. The gills completely disappear.
35 (G27)[I33, I35]	Limb bud I	16	14.44±0.73 (13.53–15.32, n=3) : 7.36±0.79	Hind limb bud: length ≥1/2 basal width. Beginning of feeding. Maturation of labial teeth. Labial teeth formula=2/3. Anus opens and excretes.
36 (G27)[I34]	Limb bud II	17	16.33±1.77 (13.1–19.7, n=21) : 7.77±0.72	Hind limb bud: length ≥1/2 basal width. Spiracle appears from left side.
37 (G28)[I35]	Limb bud III	24	19.12±1.42 (15.79–21.48, n=18) : 8.39±0.52	Hind limb bud: length ≥1 basal width.
38 (G21, G28)[I35]	Limb bud IV	30	19.97±2.12 (16.16–23.74, n=12) : 8.78±0.45	Hind limb bud: length ≥1 basal width. The adhesive organ disappears.
39 (G29)[I35]	Limb bud V	35	19.37±0.75 (18.31–19.91, n=3) : 8.10±0.27	Hind limb bud: length ≥1.5 basal width.
40 (G30)[I35]	Limb bud VI	37	19.64±1.68 (18.00–21.97, n=3) : 7.99±0.29	Hind limb bud: length ≒2 basal width.
41 (G31)[I36]	Appearance of knee junction	43	23.52±1.67 (21.23–25.13, n=3) : 9.71±0.71	Hind limb bud is slightly bent at the anlage of the knee junction.
42 (G32)[I37]	Individual toes I	45	28.86±0.60 (28.13–29.61, n=3) : 11.76±0.34	A shallow indentation between the primordia of the 4th and 5th toes appears.
43 (G33)[I38]	Individual toes II	51	29.58±0.61 (28.82–30.21, n=4) : 12.31±0.19	A shallow indentation between the 3th and 4th toes appears.

TABLE 1. (continued)

Stage number	Stage	Age (h:m)	Total length (in mm)	Diagnostic features
44 (G34)[I39]	Individual toes III	54	30.26±1.78 (27.55–32.87, n=7) : 12.90±0.76	A shallow indentation between the 2th and 3th toes appears.
45 (G35)[I39]	Individual toes IV	57	32.97±0.76 (32.33–34.28, n=5) : 13.34±0.45	The primordium of the 1st toe is faintly recognizable.
46 (G36)[I40]	Individual toes V	59	35.86±2.31 (31.61–39.76, n=15) : 14.97±1.18	Hind limb bud is slightly bent at the anlage of the ankle junction. Webbing slightly appears between the toes.
47 (G37)[I41]	Individual toes VI	77	37.99±0.44 (37.55–38.42, n=2) : 17.69±0.91	The primordium of the 1st toe is clearly recognizable. Hind limbs move.
48 (G38, G41)[I41]	Tubercles I	80	40.14±1.29 (38.85–41.43, n=2) : 15.54±0.71	The metatarsal tubercles slightly appear. The cloacal tail piece disappears. Well-developed forelimbs are seen through the pectoral skin.
49 (G39)[I41]	Tubercles II	84	40.52±0.92 (39.6–41.44, n=2) : 16.55±0.31	The subarticular tubercles slightly appear.
50 (G40)[I41]	Tubercles III	86	40.01±0.06 (40.04–40.16, n=2) : 16.60±0.18	The total length of the larva is the maximum. The metatarsal and subarticular tubercles are clearly recognizable.
51 (G42)[I42]	Early appearance of forelimbs	90	37.42±1.02 (36.5–38.84, n=3) : 14.44±0.72	Right forelimb appears. The eyeball begins to protrude. Mouth fissure begins to spread from side to side.
52 (G43)[I43]	Late appearance of forelimbs	92	37.57±1.22 (36.35–38.79, n=2) : 15.02±1.34	Left forelimb appears from spiracle. Mouth fissure does not reach half of the eyeball.
53 (G44)[I44a]	Early degeneration of tail	105	23.16±3.77 (25.11–32.64, n=2) : 13.76±0.25	Tail length ≅body length. The angles of the mouth reach the level of the posterior half of the eyeball.
54 (G44)[I44b]	Late degeneration of tail	109	19.02±2.53 (16.62–22.52, n=3) : 13.56±1.24	Tail length ≅2/1 body length. Landing.
55 (G45)[I44c]	Stub-like tail	115	13.24±1.71 (11.53–14.94, n=2) : 12.43±1.71	The tail are like a stub. The angles of the mouth reach the level of the posterior margin of the eyeball.
56 (G46)[I45]	Completion of metamorphosis	128	11.72±0.98 (10.74–12.69, n=2) : 11.50±0.76	The tail disappears. The skin adhesions where the forelimbs came out.

mouth shape we calculate OH/BL and OW/BL ratios in the lentic *B. torrenticola* at *Stages* 32 to 52 (equivalent to stages 31 to 42 in Ichikawa and Tahara [1966]), at which point the extensive change in the size of the mouth in the lentic *B. torrenticola* was observed (Iwasawa and Saito, 1989).

RESULTS

Developmental stages

The complete normal development of *Bufo torrenticola* was characterized and divided up into a total of 56 developmental stages which we also illustrate (Table 1, Figs. 2, 3). Completion of metamorphosis took a minimum 128 days.

Stages 1 and 2 as we characterised them corresponded to those in Gosner (1960) but are not illustrated in Iwasawa (1987a). For *Stages* 9–25 and 27–31, we followed mainly stages 8–23 and 26–30 in Iwasawa (1987a) as this work was more detailed than Gosner (1960). *Stages* 3–8 corresponded to stages 2–7 in Iwasawa (1987a) and 3–8 in Gosner (1960).

We could not identify stage 24 in Iwasawa (1987a), which was defined by having 13 somites and showing muscular response against stimulation, but we observed a stage showing the muscular response and appearance

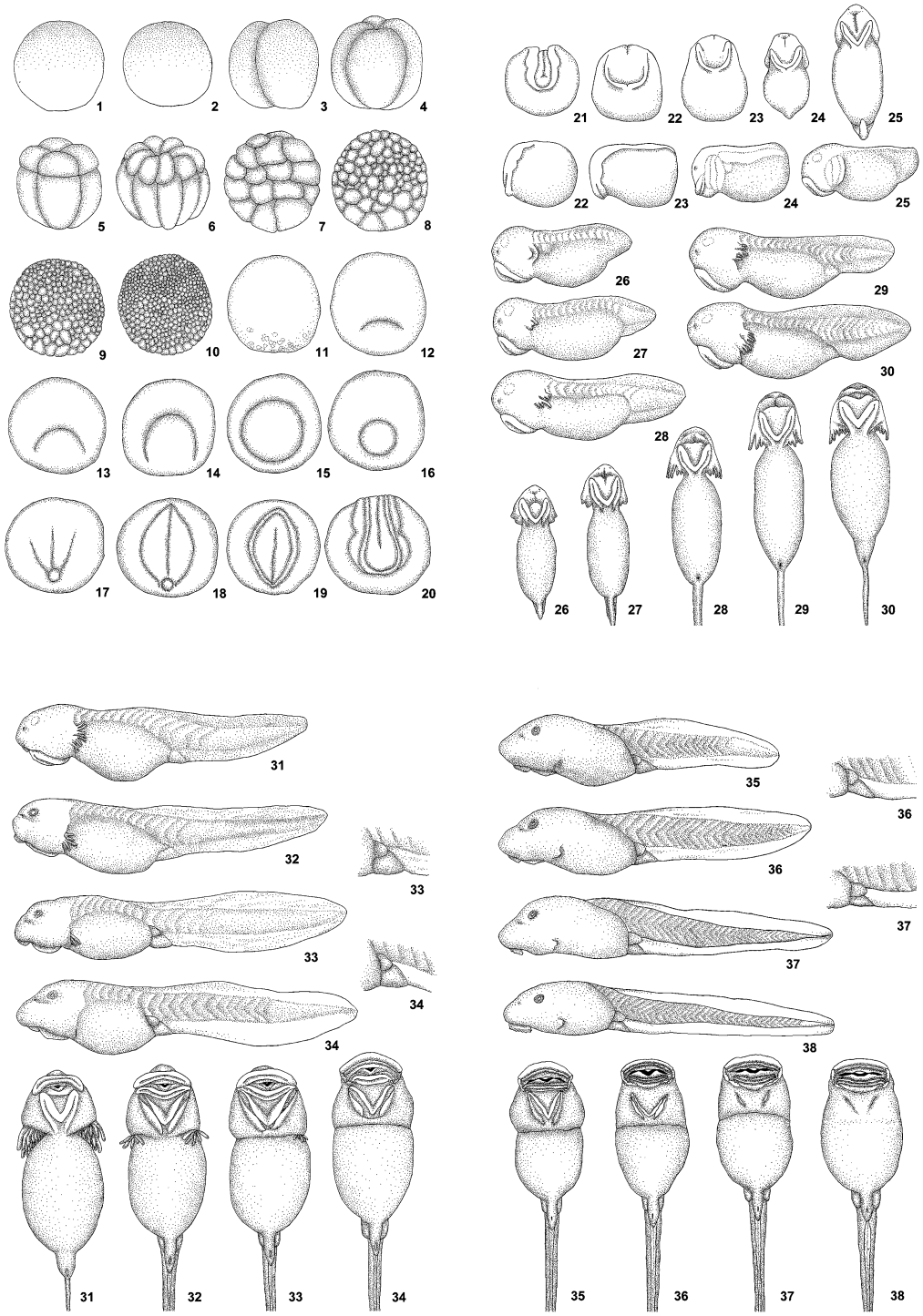

FIG. 2. Illustrations of eggs, embryos and larvae of *Bufo torrenticola* from Shiga Prefecture. Numerals at the bottom right of each drawing correspond to developmental stages defined in Table 1.

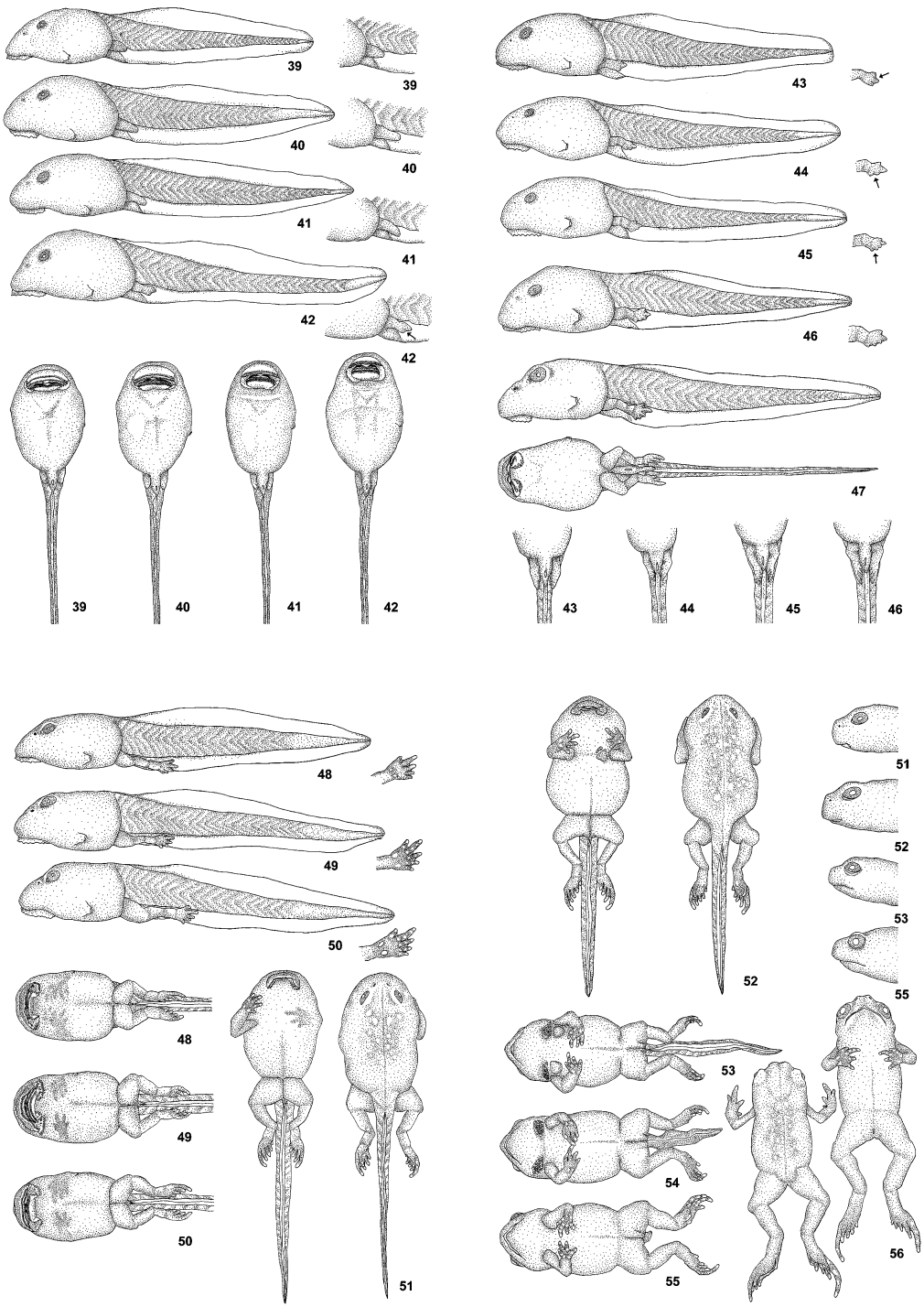

FIG. 3. Illustrations of larvae of *Bufo torrenticola* from Shiga Prefecture. Numerals at the bottom right of each drawing correspond to developmental stages defined in Table 1.

of gill buds (shown in stage 25 in Iwasawa [1987a]) at the same timing. Thus, we could not separate stage 24 from stage 25 in Iwasawa (1987a) and combined these two stages as to be *Stage* 26.

In both this study and Iwasawa (1987a) hindlimb development began during opercular development.

We adopted Iwasawa's (1987a) stages 33 to 35 as *Stages* 33 to 35 because unlike Gosner (1960) but in concordance with Iwasawa (1987a) the papillae appearance and teeth development were observed as oral disc development. However, the timing of oral disc completion in Iwasawa (1987a) was different from our observations; we observed completion of oral disc development at *Stage* 35 before appearance of the spiracle, while Iwasawa (1987a) observed oral disc completion after the appearance of the spiracle.

The cloacal tail piece was lost at appearance of subarticular tubercles and inner and outer metatarsal tubercles in our observations (*Stage* 48), but lost after completion of development of subarticular tubercle and inner and outer metatarsal tubercles in Gosner's (1960) stage 41.

In hindlimb and toe development, we followed Gosner (1960) and defined *Stages* 33–50, because Gosner (1960) mentioned and defined some stages based on the subarticular tubercle and inner and outer metatarsal tubercles of both limbs, while Iwasawa (1987a) did not mention them.

In metamorphosis, we combined Gosner's (1960) and Iwasawa's (1987a) definitions to create *Stages* 51–55, because Gosner (1960) defined stages based on degree of mouth fissure, and Iwasawa (1987a) defined stages based on degeneration of the tail. At *Stages* 53 and 54 the skin covering the forelimbs tore and the external gills (which were drawn inside the body at *Stage* 34) became partly exposed again. However, in *Bufo japonicus formosus* at *Stages* 53 and 54 the external gills were re-exposed in only one out of eight individuals (Table 2, Fig. 4).

The developmental stages of embryos and larvae were identical between *Bufo torrenticola* and *B. japonicus formosus*, except for body color and relative size of some characters. The larvae of *B. torrenticola* had a darker body than *B. j. formosus*. We could not observe internal organs through dark skin in *B. torrenticola*, but could in *B. j. formosus* through its transparent skin.

Comparison

The median of GL/MHW of *Bufo torrenticola* at the *Stage* 31 (n=3) was 0.52. On the other hand, that of *B. japonicus formosus* was 0.71 at the same stage (data were measured from Iwasawa [1987a]), so the former has shorter gills than the latter, although a statistical test could not be applied.

At *Stages* 40, 42, 43, 45, and 46, OH/BL and OW/BL were significantly ($p<0.05$) larger in *Bufo torrenticola* (Table 3) (based on the Kruskal-Wallis test and Steel-Dwass test, implemented in the R software [R Development Core Team, 2021]). However, no significant difference in these ratios were detected between the lentic and lotic tadpoles of *B. torrenticola*. The larva of *B. torrenticola* had a mouth ratio against BL about two times larger than *B. j. formosus* from the *Stage* 40 until initiation of metamorphosis (Fig. 5).

There were no differences in TMW/TAL, TMW/TMH, TAL/TOL and MTH/TAL between species.

For mTMH/MTH the lotic *Bufo torrenticola* was significantly larger than *B. japonicus formosus* at *Stages* 40 and 43. The lentic *B. torrenticola* has larger mTMH/MTH than *B. j. formosus* at *Stage* 45. The lotic *B. torrenticola* has the largest mTMH/MTH, followed by the lentic *B. torrenticola*, and then *B. j. formosus* at *Stage* 46. In TMH/MTH, the lotic *B. torrenticola* was significantly larger than *B. j. formosus* at *Stage* 42. The lotic *B. torrenticola* has the largest TMH/MTH, followed by the lentic *B. torrenticola*, and then *B. j. formosus* at *Stage* 43. The lentic *B. torrenticola* has larger TMH/MTH than *B. j. formosus* at *Stages* 45 and 46. In MTH/TAL, *B. j. formosus* was larger than the lentic *B. torrenticola* at *Stage*

TABLE 2. Major changes to the developmental stages chart.

Stage	Description
1, 2	Uncleaved eggs
	Correspondence to stage 1 and 2 in Gosner (1960)
9–25	Cleavage-blastula stage, Gastrula, Neurula and Tail bud
	Correspondence to stage 8–23 in Iwasawa (1987a)
26	Gill bud appearance
	Correspondence to stage 24 and 25 in Iwasawa (1987a)
27–31	External gill
	Correspondence to stage 27–31 in Iwasawa (1987a)
33	The timing of hind limb appearance
	Correspondence to stage 31 and 32 in Iwasawa (1987a)
33–50	Hind limb and toe development
	Correspondence to stage 26–40 in Gosner (1960)
34–35	Teeth development and papillas appearance
	Correspondence to stage 33–35 in Iwasawa (1987a) (Timing is different between Iwasawa [1987a] and our observation)
48	Cloacal tail piece
	Correspondence to stage 41 in Gosner (1960) (Timing is different between Gosner [1960] and our observation)
51–55	Metamorphosis
	Correspondence of mouth fissure to stage 42–45 in Gosner (1960) Correspondence of degeneration of tail to stage 44a–c in Iwasawa (1987a)
53, 54	Internal gills reappearance

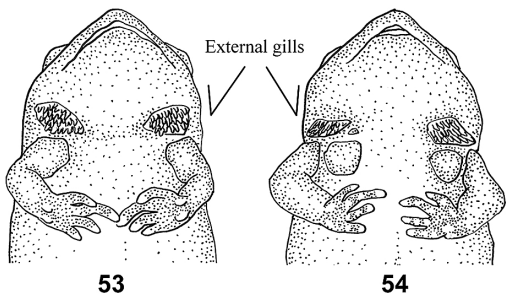

FIG. 4. Exposure of the internal gills at the *Stage* 53 and 54.

42. *Bufo j. formosus* has larger MTH/TAL than the lotic *B. torrenticola* at *Stages* 43 and 45. *Bufo j. formosus* has the largest MTH/TAL, followed by the lentic *B. torrenticola*, and then the lotic *B. torrenticola* at *Stage* 46 (Table 4 and Fig. 6). For OW/BL and OH/BL in the lentic *B. torrenticola* we found that these ratios were stable from *Stages* 32 to 50 and decreased from *Stages* 51 to 52 (Table 3 and Fig. 7). In summary, *B. torrenticola* had a more muscular tail and a smaller percentage of fins than *B. j. formosus*. However, the degree of the difference between the species varied between *B. torrenticola* raised with or without water current.

DISCUSSION

This study provides the first complete description and comparisons of the developmental stages of *Bufo torrenticola*. From *Stage* 31 this species has a wide oral disc, which must be adaptation to running water in streams

Table 3. Median of OH/BL and OW/BL. We described the lentic *Bufo torrenticola*, the lotic *B. torrenticola* and *B. japonicus formosus* as A, B and C, respectively. Range is shown in parenthesis and number of samples is shown in bracket.

Stage	Character	A	B	C	Difference
32	OH/BL	0.22 [5] (0.17–0.24)	—	—	—
	OW/BL	0.44 [5] (0.34–0.44)	—	—	—
33	OH/BL	0.22 [6] (0.17–0.27)	—	—	—
	OW/BL	0.37 [6] (0.28–0.42)	—	—	—
34	OH/BL	0.24 [6] (0.19–0.26)	—	—	—
	OW/BL	0.38 [6] (0.36–0.43)	—	—	—
35	OH/BL	0.20 [6] (0.18–0.21)	—	—	—
	OW/BL	0.39 [6] (0.33–0.46)	—	—	—
36	OH/BL	0.22 [6] (0.18–0.24)	—	—	—
	OW/BL	0.41 [6] (0.34–0.52)	—	—	—
37	OH/BL	0.22 [18] (0.14–0.27)	—	—	—
	OW/BL	0.50 [18] (0.41–0.55)	—	—	—
38	OH/BL	0.22 [12] (0.17–0.30)	—	—	—
	OW/BL	0.46 [12] (0.38–0.53)	—	—	—
39	OH/BL	0.23 [6] (0.20–0.26)	—	—	—
	OW/BL	0.44 [6] (0.36–0.48)	—	—	—
40	OH/BL	0.20 [7] (0.14–0.25)	0.22 [12] (0.18–0.26)	0.13 [4] (0.09–0.15)	A,B>C
	OW/BL	0.44 [7] (0.39–0.51)	0.47 [12] (0.41–0.57)	0.25 [4] (0.22–0.31)	A,B>C
41	OH/BL	0.20 [5] (0.18–0.24)	0.23 [3] (0.2–0.3)	0.13[6] (0.1–0.14)	—
	OW/BL	0.40 [5] (0.38–0.43)	0.47 [3] (0.42–0.47)	0.31 [6] (0.24–0.32)	—
42	OH/BL	0.23 [6] (0.19–0.27)	0.23 [4] (0.23–0.26)	0.15 [4] (0.13–0.18)	A,B>C
	OW/BL	0.43 [6] (0.35–0.48)	0.46 [4] (0.33–0.56)	0.28 [4] (0.26–0.32)	A,B>C
43	OH/BL	0.24 [4] (0.17–0.28)	0.26 [6] (0.24–0.28)	0.14 [7] (0.11–0.16)	A,B>C
	OW/BL	0.45 [4] (0.41–0.47)	0.46 [6] (0.42–0.51)	0.26 [7] (0.20–0.27)	A,B>C
44	OH/BL	0.24 [4] (0.24–0.27)	0.30 [3] (0.23–0.32)	0.15 [7] (0.13–0.17)	—
	OW/BL	0.39 [4] (0.32–0.43)	0.47 [3] (0.4–0.47)	0.28 [7] (0.21–0.30)	—
45	OH/BL	0.26 [4] (0.23–0.28)	0.27 [9] (0.22–0.31)	0.15 [11] (0.12–0.17)	A,B>C
	OW/BL	0.45 [4] (0.42–0.54)	0.43 [9] (0.38–0.46)	0.29 [11] (0.27–0.33)	A,B>C
46	OH/BL	0.24 [10] (0.19–0.29)	0.25 [6] (0.23–0.28)	0.15 [12] (0.11–0.18)	A,B>C
	OW/BL	0.46 [10] (0.42–0.51)	0.44 [6] (0.40–0.44)	0.28 [12] (0.23–0.33)	A,B>C
47	OH/BL	0.27 [5] (0.22–0.30)	0.25 [1] 0.25	0.13 [19] (0.10–0.16)	—
	OW/BL	0.46 [5] (0.42–0.50)	0.37 [1] 0.37	0.26 [19] (0.20–0.30)	—
48	OH/BL	0.27 [3] (0.25–0.28)	0.22 [4] (0.20–0.27)	0.13 [12] (0.11–0.18)	—
	OW/BL	0.52 [3] (0.45–0.55)	0.40 [4] (0.38–0.41)	0.25 [12] (0.21–0.32)	—
49	OH/BL	0.25 [1] 0.25	0.25 [3] (0.22–0.3)	0.14 [9] (0.10–0.15)	—
	OW/BL	0.46 [1] 0.46	0.37 [3] (0.36–0.41)	0.26 [9] (0.2–0.29)	—
50	OH/BL	0.25 [2] (0.24–0.26)	0.24 [4] (0.22–0.25)	0.13 [14] (0.1–0.16)	—
	OW/BL	0.49 [2] (0.47–0.5)	0.35 [4] (0.32–0.41)	0.25 [14] (0.22–0.31)	—
51	OH/BL	0.15 [3] (0.12–0.16)	—	—	—
	OW/BL	0.35 [3] (0.31–0.36)	—	—	—
52	OH/BL	0.11 [2] (0.11)	—	—	—
	OW/BL	0.33 [2] (0.31–0.34)	—	—	—

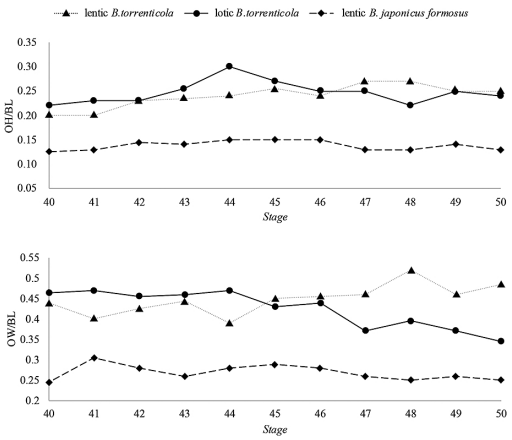

FIG. 5. Developmental difference in values (%) of OH/BL (top) and OW/BL (bottom) in lentic *Bufo torrenticola*, lotic *B. torrenticola* and *B. japonicus formosus* from *Stages* 40 to 50.

and is a unique character for this species. The gills developed at the same stage as lentic-breeding *B. japonicus formosus*, however they do not increase in the length, probably because they live in oxygen rich flowing streams (Matsui, 1976b). The thick and low tail shown in Matsui (1975) was first documented through the detailed developmental stages and is also considered to be advantageous for swimming in streams (McDiarmid and Altig, 2000).

Completion of metamorphosis took a minimum of 128 days at 13±1°C water temperature in our study. Under natural conditions complete metamorphosis occurs in about 150 days (Matsui and Maeda, 2018). Iwasawa and Saito (1989) reported that *Bufo torrenticola* took 53 days to reach stage 35 of Ichikawa and Tahara (1966) at 8°C water temperatures. In our study, it tooks 24–37 days to reach *Stages* 37–40 (equivalent to stage 35 of Ichikawa and Tahara [1966]) under conditions ca. 6°C warmer than Iwasawa and Saito (1989). The duration to completion of metamorphosis in our results seemed to be very long. However, the studies noted above did not observe until completion of metamorphosis, or kept embryos in very low temperature. In contrast, Iwasawa (1987a) reported that *B. japonicus formosus* from Niigata Prefecture took 58, 44 or 28 days to complete metamorphosis at 15, 18 or 20°C, respectively. Muto et al. (1968) showed that *B. j. formosus* took 37 days to complete of metamorphosis at 20°C. It has previously been documented that *B. torrenticola* has a longer larval life than *B. j. formosus* (Matsui, 1976a; Iwasawa and Saito 1989). *Bufo torrenticola* lays eggs in the stream with year-round stable cool temperature (Matsui and Maeda, 2018) and poor food availability (mainly algae). Conversely, *B. j. formosus* lays eggs in still water with a rich food supply, including some temporary water bodies that dry up relatively rapidly. We hypothesize that *B. torrenticola* does not metamorphose as rapidly as *B. j. formosus* because it occurs in a more stable but nutrient poor environment.

As the forelimbs protruded (*Stages* 53 and 54) we found internal gills were exposed in *Bufo torrenticola*, but not in most individuals of *B. japonicus formosus*, a difference linked to the larger skin tear in the protruding forelimb stages in *B. torrenticola*. We were also not able to find internal organs through the dark skin of *B. torrenticola*, but could do so in *B. j. formosus* through its transparent skin. In our observation, *B. torrenticola* seemed to have thicker skin than *B. j. formosus*, which might be advantageous for protecting the body in fast-flowing streams. Thick skin may be disadvantageous for oxygen intake through skin, the rich supply of dissolved oxygen in streams may compensate for this disadvantage.

Iwasawa and Saito (1989) reported that the mouth of *B. torrenticola* was larger than *B. japonicus formosus* at stages 35–38 as defined by Ichikawa and Tahara (1966). However, they did not consider an interspecific difference in body size into consideration. Here we demonstrate that relative mouth size (OH/BL and OW/BL) decreased through stages in *B. j. formosus*, but was stable in *B. torrenticola*. The larvae of *B. torrenticola* maintain a large mouth size relative to body size even in the stages close to metamorphosis, which could be advantageous in fast-flowing stream environments where the mouth functions as an adhe-

Table 4. Median of mTMH/MTH, TMH/MTH and MTH/TAL. We described the lentic *Bufo torrenticola*, the lotic *B. torrenticola* and *B. japonicus formosus* as A, B and C. Range is shown in parentheis and number of samples is shown in bracket.

Stage	character	A	B	C	Difference
40	mTMH/MTH	0.34 [7] (0.30–0.41)	0.43 [12] (0.27–0.53)	0.31 [4] (0.30–0.35)	B>A, C
	TMH/MTH	0.61 [7] (0.51–0.62)	0.55 [12] (0.40–0.68)	0.54 [4] (0.45–0.71)	A, B, C
	MTH/TAL	0.25 [7] (0.20–0.32)	0.29 [12] (0.21–0.33)	0.30 [4] (0.23–0.32)	A, B, C
41	mTMH/MTH	0.34 [5] (0.32–0.40)	0.35 [3] (0.32–0.38)	0.29 [6] (0.26–0.35)	—
	TMH/MTH	0.55 [5] (0.54–0.63)	0.60 [3] (0.58–0.61)	0.47 [6] (0.44–0.53)	—
	MTH/TAL	0.27 [5] (0.21–0.29)	0.26 [3] (0.25–0.27)	0.27 [6] (0.25–0.29)	—
42	mTMH/MTH	0.34 [6] (0.29–0.38)	0.36 [4] (0.30–0.40)	0.31 [4] (0.25–0.33)	A, B, C
	TMH/MTH	0.50 [6] (0.36–0.59)	0.66 [4] (0.57–0.67)	0.47 [4] (0.40–0.53)	B>A, C
	MTH/TAL	0.26 [6] (0.24–0.29)	0.27 [4] (0.24–0.28)	0.33 [4] (0.31–0.40)	C>A, B
43	mTMH/MTH	0.33 [4] (0.29–0.36)	0.37 [6] (0.35–0.48)	0.32 [7] (0.27–0.37)	B>A, C
	TMH/MTH	0.57 [4] (0.51–0.60)	0.63 [6] (0.56–0.67)	0.48 [7] (0.40–0.56)	B>A>C
	MTH/TAL	0.25 [4] (0.23–0.27)	0.26 [6] (0.21–0.28)	0.29 [7] (0.25–0.31)	C>A, B
44	mTMH/MTH	0.34 [4] (0.31–0.36)	0.33 [3] (0.32–0.39)	0.28 [7] (0.23–0.34)	—
	TMH/MTH	0.59 [4] (0.53–0.64)	0.66 [3] (0.62–0.67)	0.47 [7] (0.39–0.56)	—
	MTH/TAL	0.28 [4] (0.26–0.29)	0.26 [3] (0.20–0.26)	0.32 [7] (0.23–0.36)	—
45	mTMH/MTH	0.40 [4] (0.34–0.41)	0.40 [9] (0.35–0.44)	0.26 [11] (0.19–0.38)	A, B>C
	TMH/MTH	0.65 [4] (0.57–0.76)	0.65 [9] (0.63–0.73)	0.44 [11] (0.43–0.51)	A, B>C
	MTH/TAL	0.23 [4] (0.21–0.24)	0.23 [9] (0.19–0.26)	0.29 [11] (0.26–0.34)	C>A, B
46	mTMH/MTH	0.32 [10] (0.27–0.35)	0.36 [6] (0.32–0.40)	0.28 [12] (0.14–0.32)	B>A>C
	TMH/MTH	0.68 [10] (0.39–0.51)	0.65 [6] (0.60–0.72)	0.46 [12] (0.41–0.52)	A, B>C
	MTH/TAL	0.23 [10] (0.19–0.30)	0.20 [6] (0.18–0.23)	0.26 [12] (0.23–0.29)	C>A>B
47	mTMH/MTH	0.35 [5] (0.24–0.42)	0.45 [1] 0.45	0.22 [19] (0.17–0.30)	—
	TMH/MTH	0.68 [5] (0.57–0.70)	0.67 [1] 0.67	0.49 [19] (0.39–0.59)	—
	MTH/TAL	0.23 [5] (0.21–0.27)	0.20 [1] 0.2	0.27 [19] (0.24–0.35)	—
48	mTMH/MTH	0.28 [3] (0.23–0.32)	0.40 [4] (0.35–0.40)	0.25 [12] (0.20–0.29)	—
	TMH/MTH	0.66 [3] (0.65–0.79)	0.63 [4] (0.61–0.64)	0.53 [12] (0.48–0.61)	—
	MTH/TAL	0.24 [3] (0.22–0.25)	0.21 [4] (0.19–0.22)	0.26 [12] (0.21–0.32)	—
49	mTMH/MTH	0.31 [1] 0.31	0.42 [3] (0.35–0.43)	0.25 [9] (0.17–0.29)	—
	TMH/MTH	0.64 [1] 0.64	0.58 [3] (0.57–0.64)	0.55 [9] (0.49–0.63)	—
	MTH/TAL	0.24 [1] 0.24	0.22 [3] (0.21–0.22)	0.25 [9] (0.23–0.30)	—
50	mTMH/MTH	0.32 [2] (0.30–0.33)	0.35 [4] (0.31–0.46)	0.24 [14] (0.18–0.28)	—
	TMH/MTH	0.62 [2] (0.59–0.65)	0.65 [4] (0.58–0.72)	0.51 [14] (0.37–0.59)	—
	MTH/TAL	0.24 [2] (0.23–0.25)	0.21 [4] (0.19–0.26)	0.25 [14] (0.20–0.29)	—

sive organ (see similar cases in tropical lotic tadpoles: Inger, 1992).

Our results indicated that the larger oral disc in *Bufo torrenticola* than *B. japonicus formosus* is stable and possibly determined by genetic factors, however the tail of *B. torrenticola* became more muscular in individuals kept in water with a current than in still water, suggesting this phenotypic variation is linked to both genetic and environmental factors. The phenotypic plasticity of *B. torrenticola* larvae is possibly an adaptation to differences in water current of breeding streams. Although we did not keep larvae *B. j. formosus* in lotic conditions, we expect they cannot grow up well in such conditions, because they have no adaptive characters for lotic habitats like *B. torrenticola*. Matsui (1972) reported that larvae

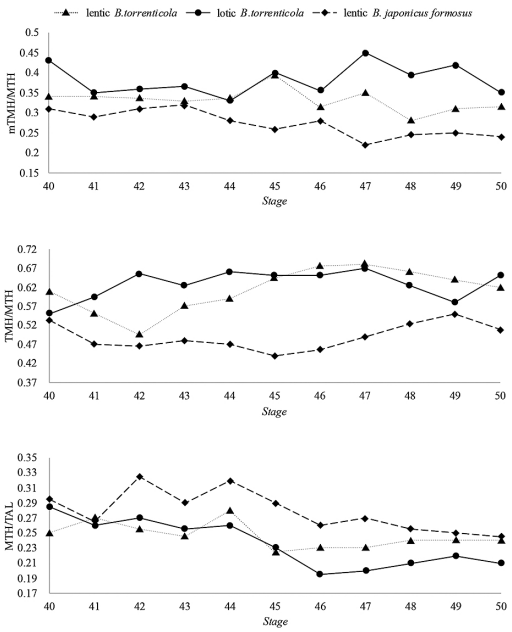

FIG. 6. Developmental difference in values (%) of mTMH/MTH (top), TMH/MTH (middle) and MTH/TAL (bottom) in lentic *Bufo torrenticola*, lotic *B. torrenticola* and *B. japonicus formosus* from *Stages* 40 to 50.

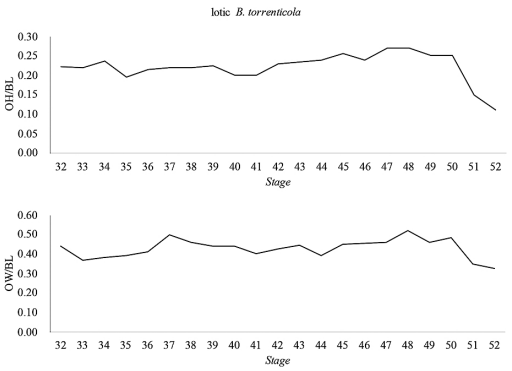

FIG. 7. Developmental difference in values (%) of OH/BL (top) and OW/BL (bottom) in lentic *Bufo torrenticola* from *Stages* 32 to 52.

of *B. bufo japonicus* (presently *B. j. japonicus* or *B. j. formosus*) flushed into streams by flooding from nearby ponds were washed away by water currents.

Asian species of *Bufo* consist of two clades, one contains *B. bankorensis*, *B. japonicus*, *B. gargarizans* (including lotic *B. gargarizans andrewsi* and three lentic subspecies), *B. torrenticola*, *B. tuberculatus*, and *B. stejnegeri*, and the other comprises *B. aspinius*, *B. cryptotympanicus*, and *B. tuberospinius* (Liu et al., 2000; Li et al., 2020). In the first clade, only *B. g. andrewsi*, *B. stejnegeri*, and *B. torrenticola* lay eggs in streams, while the remaining taxa use ponds (Liu, 1950; Matsui, 1986; Matsui and Maeda, 2018; Schmidt, 1931). The stream-breeding species in this clade do not form a monophyletic group (Fong et al., 2020). In the second clade, *B. aspinius* lays eggs in streams, and the breeding habit of *B. cryptotympanicus* and *B. tuberospinius* is unknown. However, tadpoles of *B. tuberospinius* are known to occur in streams and have an abdominal sucker, and metamorphs of *B. tuberospinius* and *B. cryptotympanicus* have indistinct tympanum (Liu and Hu, 1962; Rao and Yang, 1994; Yang et al., 1996). Anurans which live in and along streams have reduced or lost tympanic-columellar system (Duellman and Trueb, 1986). These lines of information suggest that all species of the second clade may be lotic-breeders, which were once treated as an independent genus *Torrentophryne* (Yang et al., 1996). It is obvious that the lotic-breeding and/or lentic-breeding has evolved multiple times in Asian *Bufo*. Fong et al. (2020) show that the lotic ecology of *B. stejnegeri*, *B. torrenticola*, and *B. andrewsi* evolved independently. However, if all toads in the second clade are the lotic-breeding, common ancestor of Asian species of *Bufo* could be assumed as the lotic-breeder. Unfortunately, their phylogenetic relationship has not been resolved with significant supports (Liu et al., 2000; Li et al., 2020). Detailed description of larval development of the other lotic-breeding *Bufo* might contribute to clarify evolution of lotic-breeding and the taxonomy of the genus.

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