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Normal Development of an Aquatic Spawning Tree Frog, *Buergeria japonica* (Amphibia: Rhacophoridae)

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Abstract: The family Rhacophoridae, including *Buergeria japonica*, shows a great diversity of reproductive patterns, but the knowledge of developmental processes is still limited. The genus *Buergeria* is a sister group to all other rhacophorids and shows a conservative, probably primitive, reproductive mode for this family. Thus, it is valuable to clarify the developmental process in this genus for understanding the evolution and diversification history of reproductive modes and developmental processes across the broader family members. In this study, we describe the normal development of *B. japonica* by rearing eggs and larvae under ambient temperatures of 27±1°C. The developmental speed of *B. japonica* from fertilization to gill elongation (stage 20), corresponding to their hatching period, was faster than most of other anuran species, when comparison was made using relative age, which is independent of temperature. The rapid embryonic development may be advantageous in their highly fluctuating breeding environment.

Key words: Aquatic breeder; *Buergeria*; Normal development; Rhacophoridae

Introduction

The Anuran family Rhacophoridae shows a wide variety of egg-laying strategies, which are classified into four categories: aquatic spawning, gel nesting, foam nesting, and direct development (Meegaskumbura et al., 2015). It is believed that the rhacophorid species have evolved from aquatic spawning to terrestrial spawning in the above order (Meegaskumbura et al., 2015). Molecular phylogenetic analyses indicate that the genus *Buergeria* is sister to all other rhacophorids (Yu et al., 2009; Pyron and Wiens, 2011). Despite the presence of variation in reproductive patterns, there is a lack of knowledge on the corresponding variation in developmental processes of the rhacophorid frogs. Currently, complete developmental stages have been reported for *Zhangixalus arboreus* (as *Rhacophorus*; Iwasawa and Kawasaki, 1979), *Kurixalus eiffingeri* (Kishimoto and Hayashi, 2017), and *Polypedates teraiensis* (Chakravarty et al., 2011). For *Buergeria japonica* only an incomplete developmental table has been reported (Taba et al., 2013). Gosner's (1960) developmental stage is the most famous developmental staging system for Anura and has been used in various studies of this animal group (e.g., Wassersug and Duellman, 1984; Dayton and Fitzgerald, 2001), but there is considerable interspecific variation in developmental

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patterns and larval morphological change during development (Sayim and Kaya, 2008). Thus, it is desirable to describe developmental stage for each species and genus of rhacophorids.

The six known species of *Buergeria* occur in the Ryukyu Islands, the Japanese Islands, Taiwan, and Hainan Island (Matsui and Tominaga, 2020; Frost, 2021). Unlike other genera of Rhacophoridae, all species in *Buergeria* spawn in running water environments (Matsui and Maeda, 2018). *Buergeria japonica* is the smallest species of this genus (Matsui and Maeda, 2018) and is endemic to Amami and Okinawa Islands, Ryukyu Archipelago, Japan (Matsui and Tominaga, 2020). This species is widely distributed from lowlands near the coast to mountainous areas, human settlements, and around paddy fields (Matsui and Maeda, 2018). The breeding occurs from March to November, in a variety of shallow, slow-flowing water bodies, ranging from mountain streams, ditches in urban area, and to waterways in paddy fields.

The clarification of the developmental process of *Buergeria*, which is considered to have a non-specialized breeding mode, will help us to understand the evolution of breeding mode and developmental processes in other rhacophorid species. Therefore, the purpose of this study is to describe the normal developmental stages of *B. japonica* and provide baseline data for understanding embryonic and larval development in the family Rhacophoridae.

Materials and Methods

Frogs were caught in ditches around University of the Ryukyus, Nishihara, Okinawa Prefecture and brought back to the laboratory at 21:00–22:00 h from April to September, 2020. A single gravid female and single male were paired in plastic containers (45×30×15 cm), filled with water to a depth of 2–3 cm, in a thermostatic chamber set at 27±1°C overnight. The eggs thus laid in the laboratory were used for observation. Eggs and larvae were reared under constant air temperature conditions of 27±1°C which corresponds to the mean air temperature of Okinawa in June. The larvae of this species are often found in pools of slow or almost still water, and thus we used an air pump to create a gentle current. Well-aerated tap water was used for rearing, and the water was changed every second day. Larvae of each clutch were kept in two or three plastic containers (45×30×15 cm) filled with water to a depth of 5–6 cm to avoid a crowding effect. The maximum number of embryos/larvae at the early developmental stages (stage 1–23) in each cage was less than 60. The larvae were evenly divided into each container. Five to ten individuals were fixed every day, and then, at the middle and late of the developmental stage (stages 24–44), the number of individuals in each container became less than 20. Commercial rabbit food (Rabbit Food Soft from Yeaster Company Limited) was used as food for larvae, and approximately one small spoonful of foods was given once a day.

Embryos or larvae were fixed in 5% formalin every 1–2 h during early development, and twice daily after hatching. As far as possible, tadpoles were preserved in the morning and evening, and more than four specimens in each larval stage were preserved. A total of 284 eggs/embryos and178 larvae from a total of five pairs were used for this study.

Observations and photographing of early development were conducted using a binocular stereoscopic microscope (Nikon SMZ 800N with Nikon DS-Fi1). The external features of the middle to late developmental stages were recorded together with photographs (Olympus TG-4). Measurements of egg diameter, size of yolk plug, tail length, and total length for embryos and larvae were made using software ImageJ-1.53e based on photographic data. The names and characteristics of each developmental stage and the time required to reach each stage were recorded using Gosner's (1960) developmental stage (hereafter referred to as G-stage) as a standard. The diagnostic characteristics of

several G-stages (G-stages 15, 18–20; rotation, muscular response, heart beat, and gill circulation) could not be observed in *Buergeria*, and some details provided by Gosner (1960) (G-stages 26–37, 42–46; limb bud I–V, toe development VI–XIII, and metamorphosis complete XXI–XXV) were insufficient for description of their characteristics in each stage. Stagings for *Zhangixalus arboreus* (original by Iwasawa and Kawasaki, 1979 and modified by Maeda and Matsui, 1989) and *Kurixalus eiffingeri* (Kishimoto and Hayashi, 2017) were also used as references.

The developmental speed of amphibians is strongly negatively correlated with temperature (Duellman and Trueb, 1986), and is also the case in *B. japonica* at least partially (Taba et al., 2013). Thus, indices little affected by temperatures are necessary for comparing intra- and interspecific temporal variation in development. We thus calculated relative age of each stage as a ratio of the time reaching to the stage to the time at the final stage (i.e., metamorphosis) for comparison among species. Relative age at hatching was calculated for three rhacophorid species (*Zhangixalus arboreus* [Iwasawa and Kawasaki, 1979, modified by Maeda and Matsui, 1989], *Kurixalus eiffingeri* [Kishimoto and Hayashi, 2017], and *Polypedates teraiensis* [Chakravarty et al., 2011]) and for 12 species of other families. We converted the specific stages in each species to G-stage and compared the G-stage at hatching among species. Because developmental time has been reported to vary inversely with ovum size (Kaplan, 1980), we investigated the correlation between egg diameter and relative age at hatching with Spearman's rank correlation using the software R version 4.0.5. (R Core Team, 2014).

Results

Developmental speed and characteristics of each developmental stage

The developmental process from fertilization to the completion of metamorphosis was divided into the eight phases following Iwasawa and Kawasaki (1979). Based on changes in external appearance, we recognized 44 stages. Each of the eight phases was comprised of between 3 to 8 stages. The photographs in each stage are shown in Figs. 1–4.

Phase I: Fertilization, cleavage, and blastula formation: Stages 1–8 (Fig. 1)

Stage 1: Fertilization. The egg size is 1.2±0.1 mm (Table 1). Animal hemisphere is blackish brown and vegetal hemisphere is creamy brown. The shape of animal hemisphere is rounded; the stereotaxic rotation stage and the appearance of a gray crescent stage (G-stages 1 and 2) could not be divided in this study (see Table 1).

Stage 2: 2-cell. First division observed and two divided spheres are formed at 0.96± 0.11 h after fertilization (Table 1, Fig. 5).

Stage 3: 4-cell. Second division begins and four divided spheres are formed.

Stage 4: 8-cell. Third division begins with four small and four large divided spheres.

Stage 5: 16-cell. Fourth division begins. Regular egg cleavage occurred up to this stage.

Stage 6: 32-cell. Fifth division begins. Irregular egg cleavage starts at this stage.

Stage 7: Mid-cleavage (morula). Irregular cleavages continue. In addition a pale area is observed in the cells.

Stage 8: Late-cleavage (blastula). Blastomeres are smaller in the animal hemisphere than in the vegetal hemisphere.

Phase II: Gastrula formation: Stages 9–11 (Fig. 1)

Stage 9: Dorsal lip. Gastrulation begins and dorsal lip of protostome is seen as a slit at 7.69±0.79 after fertilization (Table 1, Fig. 5).

Stage 10: Mid-gastrula (large yolk plug). The yolk plug is formed after 10.4±1.0 h (Table 1, Fig. 5). The diameter of the yolk plug is 0.8 mm, which is approximately 2/3 of the egg diameter.

Stage 11: Late-gastrula (small yolk plug).

Table 1. Duration of embryonic development and size at each stage in *Buergeria japonica* with corresponding stage numbers of *Incilius valliceps* (Gosner, 1960), *Zhangixalus arboreus* (Iwasawa and Kawasaki, 1979), and *Kurixalus eiffingeri* (Kishimoto and Hayashi, 2017). Asterisks indciate hatching stage. Mean±SD with range in parentheses are shown for duraiton and size.

Stage No.	Stage	n	Time (h) from fertilization	Egg diametor or total length (mm)	Stage of Gosner (1960)	Stage of *Z. arboreus*	Stage of *K. eiffingeri*
1	Fertilization	9	0	1.2±0.1 (1.1–1.2)	1, 2	1	1
2	2-cell	9	0.96±0.11 (0.83–1.06)	1.2±0.1 (1.1–1.2)	3	2	2
3	4-cell	11	1.48±0.23 (1–1.71)	1.2±0.1 (1.1–1.2)	4	3	3
4	8-cell	7	1.65±0.32 (1–1.78)	1.2±0.1 (1.2–1.3)	5	4	4
5	16-cell	7	2.00±0.11 (1.91–2.11)	1.2±0 (1.2–1.2)	6	5	5
6	32-cell	29	2.27±0.27 (1.88–3.0)	1.2±0.1 (1.2–1.3)	7	6	6
7	Mid-cleavage	26	3.70±0.67 (3.0–5.51)	1.2±0.1 (1.1–1.2)	8	7	7
8	Late-cleavage	27	5.35±0.81 (4.0–7.0)	1.2±0.1 (1.1–1.2)	9	8–10	8–9
9	Dorsal lip	22	7.69±0.79 (6.13–8.5)	1.2±0 (1.2–1.2)	10	11	10
10	Mid-gastrula	15	10.40±0.95 (8.91–11.5)	1.2±0.1 (1.2–1.3)	11	12–13	11
11	Late-gastrula	15	12.55±1.43 (10.96–14.91)	1.2±0.1 (1.1–1.2)	12	14–15	12
12	Neural plate	24	14.68±1.97 (12.96–18.0)	1.2±0 (1.2–1.2)	13	16	13
13	Neural folds	6	16.09±2.12 (14.18–18.5)	1.3±0.1 (1.2–1.3)	14	17–18	14–15
14	Closure of neural folds	10	16.85±1.98 (15.01–20.5)	1.3±0.1 (1.2–1.3)	15	19	16
15	Neural tube	4	19.73±2.07 (16.91–21.91)	1.5±0 (1.5–1.5)	16	20	17
16	Tail bud I	8	20.42±3.33 (16.91–23.91)	2.0±0 (2.0–2.0)	17	21–25	18
17	Tail bud II	9	25.46±5.01 (18.96–31.65)	2.0±0 (2.0–2.0)	18	26	19
18	Tail bud III	13	30.59±3.82 (26.85–37.75)	2.4±0.1 (2.4–2.5)	19	27	19
19	Gill bud appearance	15	35.83±4.26 (28.93–47.91)	3.1±0 (3.1–3.1)	19	27	19
20*	Gill elongation	17	46.81±12.31 (22.93–68.2)	4.9±0.1 (4.9–5.0)	20*	26–30*	20
21	Cornea transparent	10	69.86±12.61 (62.2–90.71)	6.1±0.3 (6.0–6.5)	21–22	31	21–22
22	Early opercular development	8	93.13±16.61 (84.06–132.2)	6.9±0.1 (6.8–7.0)	23	31–32	23*
23	Late opercular development	7	132.43±25.48 (96.91–198.95)	7.5±0.5 (7.1–8.0)	24–25	33–34	24–25*
24	Limb bud development I	8	152.52±41.80 (120.58–255.2)	9.4±0.6 (9.0–10.1)	26		26
25	Limb bud development II	7	164.76±34.79 (144.86–246.2)	12.7±0.5 (12.1–13.0)	27	35	27
26	Conical limb bud I	9	207.83±34.94 (174.88–294.2)	15.0±0 (15.0–15.0)	28	36	28
27	Conical limb bud II	7	256.89±33.48 (193.18–328.18)	16.4±0.5 (16.0–17.0)	29	37	29
28	Knee joint appearance	8	289.14±37.30 (217.43–351.68)	20.8±0.8 (19.9–21.5)	30		30
29	Oar-shaped limb bud	6	308.15±42.42 (260.56–373.71)	22.6±0.6 (22.0–23.0)	31	38	31
30	Fourth and fifth toes appearance	10	366.03±56.80 (275.13–436.81)	24.7±0.3 (24.5–25.0)	32	39	32
31	Third and fourth toes appearance	8	384.30±70.20 (299.63–491.01)	24.8±0.3 (24.5–25.0)	33	39	33
32	Second toe appearance	6	414.75±76.74 (312.63–514.5)	26.6±1.2 (26.0–28.0)	34	40	34
33	First toe appearance I	7	436.01±95.93 (344.45–586.65)	27.5±0.5 (27.0–28.0)	35	41	35
34	First toe appearance II	6	512.50±107.40 (369.63–630.1)	29.6±1.2 (29.0–31.0)	36	41	36
35	Hindlimb development I	5	533.86±111.59 (410.5–653.31)	29.5±0.5 (29.0–30.0)	37	41	37
36	Hindlimb development II	8	556.54±131.22 (390.81–726.51)	29.6±0.6 (29.0–30.0)	38	42	37
37	Emergence of subarticular tubercles of toes	6	614.40±142.08 (469.13–820.95)	33.5±1.3 (32.5–35.0)	39		38
38	Completion of subarticular tubercles of toes	9	649.69±179.47 (415.6–896.4)	34.3±3.1 (31.0–37.0)	39		39
39	Degeneration of cloaca	8	730.02±189.29 (533.9–988.5)	36.6±1.5 (35.0–38.0)	40–41	43	40–41
40	Forelimb appearance	6	782.96±204.31 (580.31–1,037.98)	35.6±3.2 (32.0–38.0)	42	44	42
41	Degeneration of tail I	7	853.64±221.54 (615.95–1,092.38)	23.3±3.5 (20.0–27.0)	43	45	43
42	Degeneration of tail II	7	932.16±196.30 (656.81–1,132.33)	15.6±1.2 (15.0–17.0)	44		44
43	Degeneration of tail III	9	975.26±200.14 (698.08–1,158.73)	12.3±0.6 (12.0–13.0)	45		45
44	Completion of metamorphosis	6	982.81±189.22 (731.36–1,158.73)	10.3±0.6 (10.0–11.0)	46	46	46

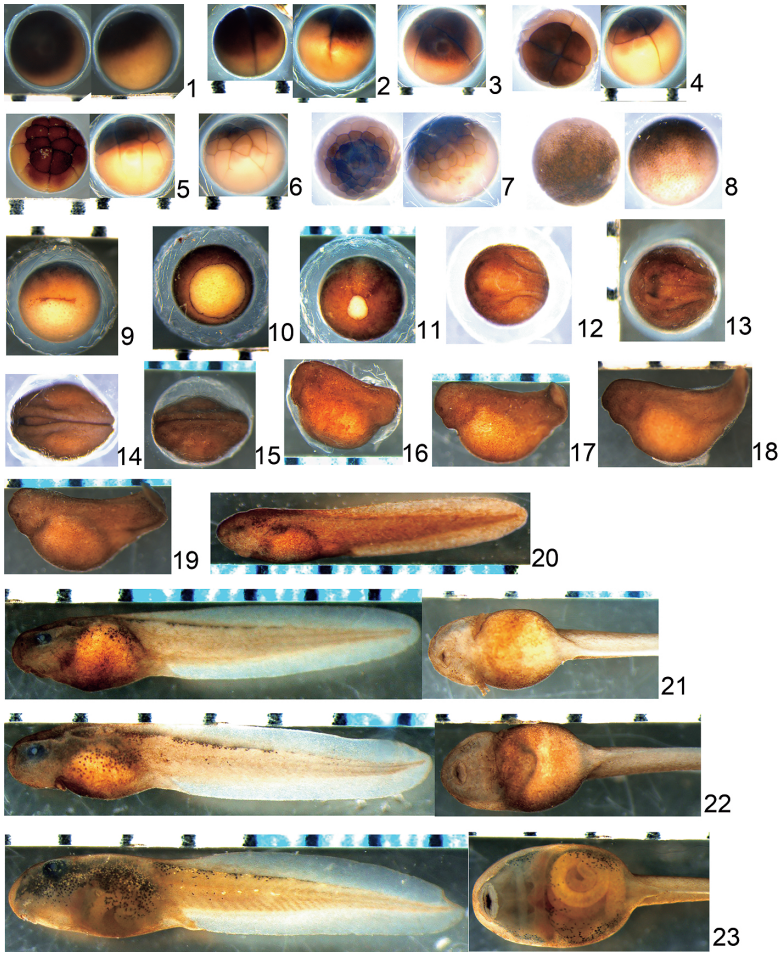

FIG. 1. Fertilization, egg cleavage, blastula formation, gastrula formation, neural embryo, tail bud embryo, and external gill stages (stages 1–23) of *Buergeria japonica*. One scale in each developmental stage corresponds to 1 mm.

Yolk plug becomes smaller than 1/3 of the egg diameter.

Phase III: Neural embryo: Stages 12–15 (Fig. 1)

Stage 12: Neural plate. The yolk plug disappears and neural plate appears at 14.68 ±1.97 h after fertilization (Table 1, Fig. 5).

Stage 13: Neural folds. Neural folds appear as ridges.

Stage 14: Closure of neural folds. The raised neural folds begin to close. The embryo begins to elongate along the anterior-posterior axis. Rotation of neural embryos during this stage (Gosner, 1960) is not observed.

Stage 15: Neural tube. Neural folds close completely.

Phase IV: Tail bud: Stages 16–18 (Fig. 1)

Stage 16: Tail bud I. Tail bud begins to elongate at 20.4±3.3 h after fertilization (Table 1, Fig. 5).

Stage 17: Tail bud II. Tail length becomes 1/3 of head-body length. Nostrils are opened.

Stage 18: Tail bud III. Tail length≦1/2 of

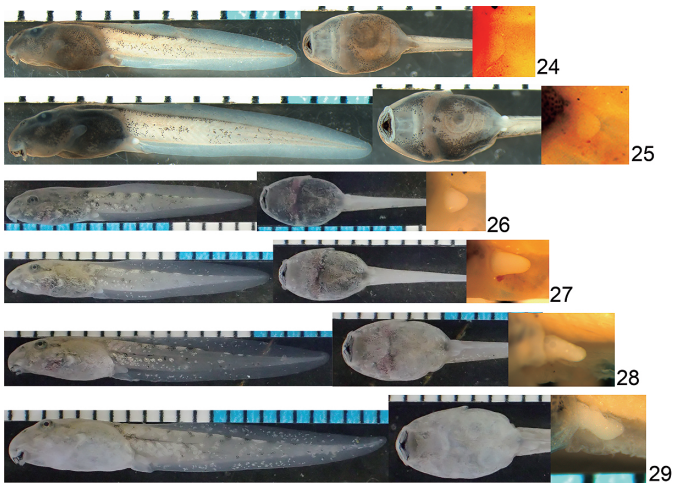

FIG. 2. Hindlimb bud stages (stages 24–29) of *Buergeria japonica*. One scale in each developmental stage corresponds to 1 mm.

head-body length. Muscular responses are observed from this stage.

Phase V: External gill: Stages 19–23 (Fig. 1)

Stage 19: Gill bud appearance. Tail length ≧1/2 of head-body length. Gill buds appear.

Stage 20: Gill elongation. Tail length >head-body length. Gill elongation. Melanin deposition observed in the dorsal retina. Blood circulation is observed in the gills. Hatching begins in this stage at 46.8±12.3 hours after fertilization (Table 1, Fig. 5).

Stage 21: Cornea transparent (completion of external gill). Mouth begins to open. The early opercular development is observed. Cornea becomes transparent. Melanin deposition is evident in the retina.

Stage 22: Early opercular development. The opercular fold completely covers the right side of the gills. The primordial labial teeth are observed.

Stage 23: Late opercular development. The left side of the gills is also covered by the opercular fold during the closure of operculum stage. The heartbeat is observed at the closure of operculum stage when the pigmentation starts to fade. Blood circulation in the tail fin is not observed at this stage. Labial teeth are formed simultaneously in the upper and lower lips. Larvae begin to feed.

Phase VI: Hindlimb bud: Stages 24–29 (Fig. 2)

Stage 24: Limb bud development I. Length of hindlimb bud<1/2 diameter at 152.5± 41.8 h after fertilization (Table 1, Fig. 5).

Stage 25: Limb bud development II. Length of hindlimb bud≧1/2 of diameter.

Stage 26: Conical limb bud I. Length of hindlimb bud is equal to the diameter.

Stage 27: Conical limb bud II. Length of a hindlimb bud becomes 1.5 times its diameter. The labial teeth are completed (1:3+3/1+1:2 or 1:4+4/1+1:2).

Stage 28: Knee joint appearance. The hindlimb buds reach their maximum length. The hindlimb buds slightly bend at the base of the knee junction.

Stage 29: Paddle shaped limb bud. The terminal half of the limb bud becomes paddle shaped.

Phase VII: Hindlimb formation: Stages 30–38 (Fig. 3)

Stage 30: Fourth and fifth toes appear. The appearance of fourth and fifth toes stage is

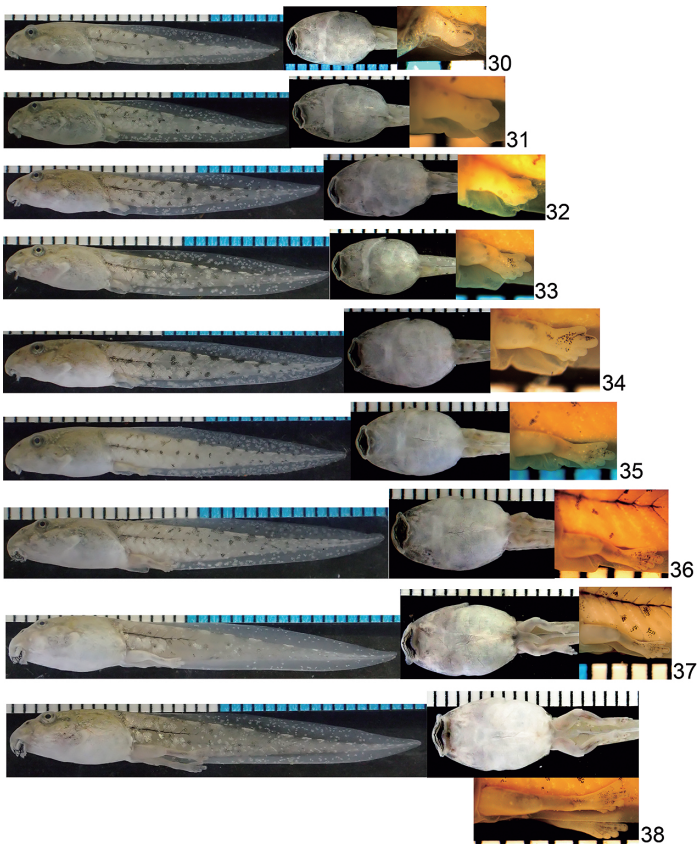

FIG. 3. Hindlimb formation stages (stage 30–38) of *Buergeria japonica*. One scale in each developmental stage corresponds to 1 mm.

reached at 366.03±56.80 h after fertilization (Table 1, Fig. 5).

Stage 31: Third and fourth toes appear. Slight depression is formed between the third and fourth toes.

Stage 32: Second toe appears. Slight depression is formed between the second and third toes.

Stage 33: First toe appears I. The base of the first toe becomes slightly visible.

Stage 34: First toe appears II. The base of the second toe becomes clearly visible.

Stage 35: Hindlimb development I. The five toes are completely separated.

Stage 36: Hindlimb development II. The five toes elongate and slight elongation of the hindlimb is observed.

Stage 37: Emergence of subarticular tubercles. The elongation of the hindlimb becomes prominent. Toe joints are clearly recognizable.

Stage 38: Completion of subarticular tubercles. Phalanges develop. The toe disks are completed, the joints of the toes become clearly recognizable, and the hindlimbs begin to function.

Phase VIII: Metamorphosis: Stages 39–44 (Fig. 4)

Stage 39: Degeneration of cloacal tube. The cloacal tube degenerated at 730.0± 189.3 h after fertilization (Table 1, Fig. 5). The cloacal tube disappears and the development of the forelimbs is observed through the

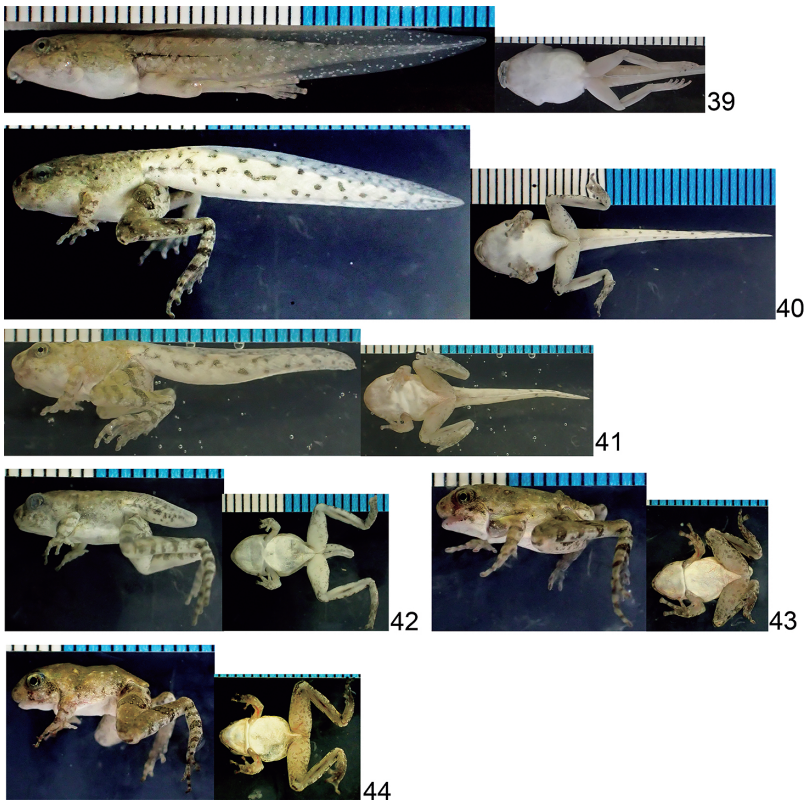

FIG. 4. Metamorphosis stages (stage 39–44) of *Buergeria japonica*. One scale in each developmental stage corresponds to 1 mm.

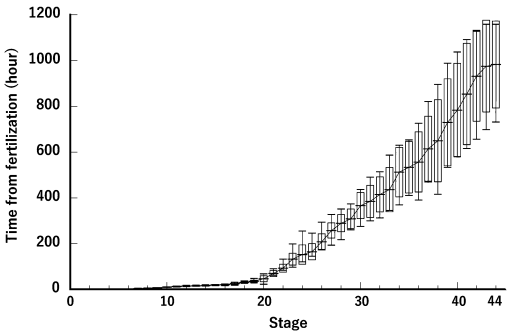

FIG. 5. Developmental speed of *Buergeria japonica*. Box plot shows the range and mean ±SD.

pectoral skin. In addition, the larval mouthparts start degenerating.

Stage 40: Forelimb appearance. Forelimbs emerge on both sides. The labial teeth and horny beaks disappear. The cleft of mouth begins to progress. The angles of the mouth extend to the level of nostril. Hindlimbs have a banded pattern.

Stage 41: Degeneration of tail I. The tail begins to be resorbed. The angles of the mouth reach the level beneath between nostril and eyes. Tongue is formed.

Stage 42: Degeneration of tail II. The angles of the mouth reach the level beneath the center and posterior of eyes. The tail is absorbed and becomes a mass.

Stage 43: Degeneration of tail III. The angles of the mouth are located behind eye and the tail disappears.

Stage 44: Completion of metamorphosis.

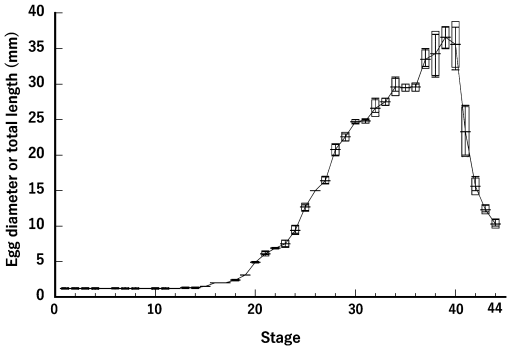

FIG. 6. Egg diameter and total length of *Buergeria japonica* at each developmental stage. Box plot shows the range and mean±SD.

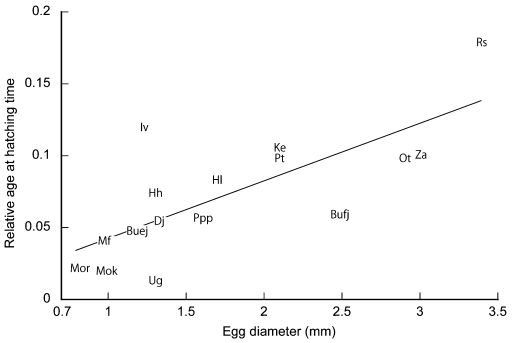

FIG. 7. Relationship between egg diameter and relative age at hatching among 16 anuran species. Abbreviations indicate plots of following 16 species: Buej, *Buergeria japonica*; Ke, *Kurixalus eiffingeri*; Za, *Zhangixalus arboreus*; Pt, *Polypedates teraiensis*; Rs, *Rana sakuraii*; Ppp, *Pelophylax porosus porosus*; Hh, *Hylarana humeralis*; Hl, *Hylarana leptoglossa*; Ot, *Odorrana tormota*; Ug, *Uperodon globulosus*; Iv, *Incilius valliceps*; Bufj, *Bufo japonicus*; Dj, *Dryophytes japonicus*; Mok, *Microhyla okinavensis*; Mf, *Microhyla fissipes*; and Mor, *Microhyla ornata*. The solid line indicates the regression line. Linear regression equation: y=0.040071x+0.00214, R=0.74695.

Under the laboratory rearing conditions provided, the time from fertilization to metamorphosis was 982.8±189.2 h (Table 1, Fig. 5).

Total length

Under the stable rearing conditions provided, the embryos started to elongate from the closure of neural stage (stage 14) and reached their maximum total length at stage 39 (36.6 ±1.5 mm; Table 1, Fig. 6). The snout-vent length (total length) at the completion of metamorphosis stage (stage 44) was 10.3± 0.6 mm (Fig. 6; Table 1).

Relative age at hatching to time for metamorphosis

Relative age of *B. japonica* at stage 20 (G-stage 20; gill circulation), when larvae start hatching, was 0.0476 (Table 2). Relative ages at hatching of 16 frogs significantly correlated with egg diameter (Spearman's rank coefficient Rs=0.702, P<0.01; Fig. 7). The developmental stages at hatching varied from G-stages 17 to 25 among the 15 other frog species for which we had data. The G-stages at hatching of most species were similar to each other and ranged from 19–21, but two bufonid species (G-stage 17), *M. okinavensis* (G-stage 18), and *K. eiffingeri* (G-stages 23–25) (Table 2) were out of this range.

DISCUSSION

Buergeria japonica hatched approximately 47 h after fertilization, at G-stage 20 under rearing condition of 27°C. This developmental duration is shorter than that reported for other rhacophorid species under lower temperatures (106 h from fertilization to hatching at G-stage 20 at 22°C for *Zhangixalus arboreus* [Iwasawa and Kawasaki, 1979], 104 h at G-stage 20 at 26–32°C for *Polypedates teraiensis* [Chakravarty et al., 2011], and 132 h in G-stage 23 for *Kurixalus eiffingeri* at 25±2°C [Kishimoto and Hayashi, 2017]). These variations in the developmental speed among species are mainly affected by temperatures, a phenomena which has already been shown in *B. japonica* (Taba et al., 2013).

In order to directly compare developmental speeds recorded under various temperatures,

TABLE 2. Egg diameter, Gosner's (1960) stage, relative and absolute age at hatching, absolute age at metamorphosis, and references of 16 anuran species.

Species	Egg diameter (mm)	Gosner's (1960) stage at hatching	Relative age at hatching (A/B)	Age at hatching time (h) (A)	Age at metamorphosis (h) (B)	Rearing temparutre (°C)	References
Buergeria japonica	1.2±0.1	20	0.0476	47	983	27±1	This Study
Kurixalus eiffingeri	2.10±0.14	23–25	0.1038	132	1,272	25±2	Kishimoto and Hayashi (2017)
Zhangixalus arboreus	3	20	0.1004	106	1,056	22	Iwasawa and Kawasaki (1979) modified by Maeda and Matsui (1989)
Polypedates teraiensis	2.0–2.2	20	0.1006	140	1,392	26–32	Chakravarty et al. (2011)
Rana sakuraii	3.4± 0.2	21	0.1786	240	1,344	15±2	Kishimoto and Hayashi (2020)
Pelophylax porosus porosus	1.3–1.9	20	0.0570	108	1,896	19 or 21–29	Iawasawa and Morita (1980)
Hylarana humeralis	1.3	21	0.0737	100	1,350	20.0–25.5	Bortamuli et al. (2010)
Hylarana leptoglossa	1.7	21	0.0832	109	1,313	20.0–25.5	Bortamuli et al. (2010)
Odorrana tormota	2.5–3.32	19	0.0990	152	1,532	18–24	Xiong et al. (2010)
Uperodon globulosus	1.3	20	0.0130	20	1,560	25–27	Narzary and Bordoloi (2013)
Incilius valliceps	1.23	17	0.1190	28	235	25	Limbaugh and Volpe (1957) modified by Gosner (1960)
Bufo japonicus	2.0–3.0	17	0.0593	64	1,080	18	Iwasawa (1987)
Dryophytes japonicus	1.2–1.4	21	0.0547	42	768	24±1	Iwasawa and Futagami (1992)
Microhyla okinavensis	0.97±0.04	18	0.0198	19	960	25±1	Shimizu and Ota (2003)
Microhyla fissipes	0.97±0.02	20	0.0411	42	1,032	22–26.5	Wang et al. (2017)
Microhyla ornata	0.8	20	0.0221	26	1,176	25–27	Narzary and Bordoloi (2013)

relative age at hatching stage was calculated. As a result, the value for *B. japonica* (0.046) is smaller than that for *K. eiffingeri* (0.104), *Z. arboreus* (0.947), and *P. teraiensis* (0.101). Thus, the developmental velocity in *B. japonica* is higher than the others even after removing temperature effects.

It has been reported that developmental time varies inversely with ovum size (e.g., Kaplan, 1980; Williamson and Bull, 1989). Correlation analyses between the relative ages at hatching and the egg diameters among 16 anurans indicate that the smaller relative age (faster development speed) of hatching in *B. japonica* seems to be related to its smaller size of eggs, which contain relatively smaller amounts of egg yolk.

Compared to other rhacophorid frogs, larval *K. eiffingeri* hatches at a more advanced developmental stage (G-stages 23–25). The species is known to deposit eggs above the water surface of pools in tree cavities to prevent fertilized eggs from being eaten by larvae that have already hatched (Kam et al., 1997). Thus delayed hatching must be advantageous in preventing predation of hatching larvae by conspecific larvae.

Rapid development of *B. japonica* in early developmental stage was ascertained at 27°C. This temperature is not especially high, because water temperatures at the spawning site of *B. japonica* used in this study was around 25°C, but it rose to over 30°C in the middle of summer. Because the larvae of this species and its relatives have been reported to be able to live at temperatures of up to around 37°C (Wu and Kam, 2005; Komaki et al., 2016), the actual speed of development in the field would be even faster than that recorded in this study. The larvae of *B. japonica* inhabit various aquatic environments with slow water currents, including artificial ditches, small streams, estuaries near the coast in which the water is affected by salt water (Haramura, 2007), and hot springs (Komaki et al., 2016). The rapid hatching of *B. japonica* seems to be advantageous to avoid death of their embryos at unstable habitats, where heavy rains may change current speed and water volume, and tidal ranges may change salinity concentrations.

The animal hemisphere of egg in *Z. arboreus* is flattened during fertilization to blastula formation stages and its blastocoel cavity becomes visible as a transparent zone during gastrula formation stages (Iwasawa and Kawasaki, 1979). In contrast, the animal hemisphere is circular and no transparent zone is seen at early developmental stage in other species. The egg of *Z. arboreus* is larger (3 mm) in size than that of *B. japonica* (1.2±0.1 mm). Furthermore, in *Z. arboreus* egg yolk remains in the abdomen even at the time of hatching, unlike in *B. japonica*. Iwasawa and Kawasaki (1979) associated the unique developmental morphologies in *Z. arboreus* with the large amount of egg yolk. However, two ranid frogs (*Rana sakuraii* and *Odorrana tormota*), with large eggs similar to *Z. arboreus*, do not have such characteristics (Xiong et al., 2010; Kishimoto and Hayashi, 2020), neither does another foam nesting lineage, *Polypedates* (Chakravarty et al., 2011). Therefore, it is unlikely that the large egg size or the much amount of egg yolk alone is responsible for these characteristics of *Z. arboreus*. It is possible that such developmental characteristics in *Z. arboreus* are unique to the genus *Zhangixalus*.

In conclusion, *B. japonica*, representing a non-specialized, aquatic breeding rhacophorid, exhibits developmental changes in morphology that are common to aquatic spawning species of other families. Other rhacophorid genera differed from *B. japonica* in several points. *Kurixalus eiffingeri*, classified as a gel-nesting species, hatches at a more advanced developmental stage, which is advantageous in preventing predation by conspecific larvae. Foam nesting *Z. arboreus* exhibits blastocoel cavity as a transparent zone during gastrula formation stages, which might be a characteristic specific to the genus. Thus, our results were congruous with the evolutionary process of egg-laying strategies in Rhacophoridae, as suggested by

Meegaskumbura et al. (2015). Further studies employing larger number of species, especially direct-developing species, are necessary.

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