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Morphology of developing larvae and a dichotomous key for five bubble-nesting *Betta* species (Teleostei: Osphronemidae)

SANTI POUNGCHAREAN[1,2] & DOMES LIMPIVADHANA[2]
[1]*Department of Fishery Biology, Faculty of Fisheries, Kasetsart University, Chatuchak, Bangkok, Thailand.*
ffisstpr@ku.ac.th; https://orcid.org/0000-0002-8521-4897
[2]*Kasetsart University Natural Histories Museum of Fisheries (KUMF), Faculty of Fisheries, Kasetsart University, Chatuchak, Bangkok, Thailand. ffidml@ku.ac.th https://orcid.org/0000-0001-7815-274X*

Abstract

The morphology of larvae of five bubble-nesting *Betta* species (*B. imbellis*, *B. mahachaiensis*, *B. siamorientalis*, *B. smaragdina*, and *B. splendens*) from Thailand was described from specimens reared in the laboratory from wild-caught parents. For all species, the mean notochord length (NL) of the larvae ranged from 2.37 to 2.58 mm. Larvae reached the flexion stage within 9–15 days (NL = 3.22–5.21 mm) and the juvenile stage within 40–45 days (standard length = 10.45–12.18 mm). The main characteristics of hatched larvae for all species were an oblong and slightly compressed body, a small and rounded head, an oval to rounded eye, and a yolk sac without oil droplets. Pre-flexion larvae for all species have an oblique, terminal mouth, and pectoral buds begin to develop after day 1. During the post-flexion larval stage, rays for the caudal, dorsal, anal, ventral, and pectoral fins begin to develop. Juveniles have a fully developed ventral fin with 2–3 dorsal, central, and ventral stripes, and a caudal spot. The minimal and maximal myomere number and fin rays differed between species. For all species, there were 1–2 dorsal spines, 7–9 dorsal rays, 9–12 pectoral rays, 1 ventral spine, 4–5 ventral rays, 2–4 anal spines, 22–28 anal rays, and 10–12 caudal rays. The different larval stages also differed in pigmentation patterns between species. Pigmentation patterns on the head (pre-orbital, sub-orbital, post-orbital, and sub-opercular bars) and longitudinal stripes on the side of the body could be used to distinguish between different larval stages as well as different species.

Key words: betta, fish larvae, morphological development, ontogeny, Osphronemidae

Introduction

Fishes in the genus *Betta* (Osphronemidae), commonly known as bettas, are popular aquarium fish and well known for their colourful bodies and fighting behaviours. Many species of this genus are highly valued in ornamental fish markets in Thailand and abroad, particularly in countries such as the USA, Japan, Singapore, and France. The commercial distribution of *Betta* species for the aquarium fish trade comprises a significant component of the Thai economy. Between 2000 and 2017, exports were valued at 15–25 million Thai Baht annually (Department of Fisheries, 2018; Monvises , 2009). Thailand also provides the highest diversity of *Betta* species for the aquarium market. Species of the genus *Betta* are categorised into two groups based on whether they mouthbrood their eggs and offsprings or build bubble-nests for their eggs. There are seven species of mouthbrooders and five species of bubble-nesters that are native to the waters of Thailand. The bubble nesters are the crescent betta *B. imbellis* Ladiges (1975), Mahachai betta *B. mahachaiensis* Kowasupat *et al*. (2012), *B. siamorientalis* Kowasupat *et al*. (2012), blue betta *B. smaragdina* Ladiges (1972), and Siamese fighting fish *B. splendens* Regan (1910) (Kowasupat *et al*., 2012; Sriwattanarothai *et al*., 2010). In the aquarium trade, bubble-nesters are typically the most popular due to their colourful bodies and fighting behaviours. Broodstocks of this group are therefore subject to a substantial amount of selective breeding to satisfy market demands. Characteristics that are targeted typically relate to fin morphology, colouration, strength, and fighting manner.

Currently, wild populations of *Betta* species are still used to source broodstock for crossbreeding and selective breeding. The resulting hybrid offspring are sorted based on suitability for the ornamental fish trade or the sport of

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fish fighting, and lower quality offspring are used for fish bait or sold at low prices (Department of Fisheries, 2018; Monvises *et al*., 2009). However, in some cases, unwanted offspring are released into natural waters (for example, to reduce the costs of continued care), likely leading to the genetic contamination of existing wild populations and representing an ecological threat to aquatic ecosystems (Chaichana *et al*., 2011; Welcomme & Vidthayanon, 2003). Consequently, disagreements regarding the original morphology of wild *Betta* species have begun to emerge in the scientific community (e.g., Panijpan *et al*., 2017; Rüber *et al*., 2004). To resolve this problem, genetic analyses and re-examination of the relevant type specimens and original descriptions are necessary. Additionally, biological descriptions of immature stages can help to discern the original morphology of species versus morphology resulting from selective breeding. However, to date there have only been descriptions of the immature stages for two bubble-nesting species which occur in Thailand, *B. smaragdina* and *B. splendens* (Termvidchakorn, 2005; Termvidchakorn & Hortle, 2013).

This study aimed to describe morphology and development of immature stages for the five, wild, bubble-nesting *Betta* species native to Thailand. For each immature stage, we measured notochord and body sizes, took measurements of head, body, and appendage features, and illustrated features and pigmentation in detail. We then generated a dichotomous key that can identify all five species at the newly hatched, pre-flexion, post-flexion, and juvenile stages and deposited all larval specimens in museum collections for reference. The information presented in this study further develops knowledge on the early-life histories and phylogenetic relationships of the *Betta* genus and can be used to inform the conservation and management of wild populations in the future.

Materials and methods

Broodstock collection and breeding

Broodstocks of the five bubble-nesting *Betta* species were sourced either as close as possible to the type specimen's locality or possessed characteristics that most closely matched the description of the type specimen. Species, range of approximate size in mm of standard length (SL), and collection locations (Figure 1) were as follows: *B. imbellis* (28.00-30.00 mm SL)—a marshland in Takua Pa Sub-district, Takua Pa District, Phang Nga Province (8°48′47″ N, 98°23′45″ E); *B. mahachaiensis* (32.00-35.00 mm SL)—a Nypa swamp beside Mahachai canal, Khok Kham Sub-district, Mueang District, Samut Sakhon Province (13°34′02″ N, 100°19′33″ E, type locality); *B. siamorientalis* (30.00-32.00 mm SL)—a paddy field in Samed Nuea Sub-district, Bang Khla District, Chachoengsao Province (13°43′46″ N, 101°13′23″ E, type locality); *B. smaragdina* (28.00-31.00 mm SL) a small canal in Non Ko Sub-district, Sirindhorn District, Ubon Ratchathani Province (14°59′08″ N, 105°30′18″ E); and *B. splendens* (33.00-36.00 mm SL)—an agricultural area connected to Thawi Wattana canal in Sala Thammasop Sub-district, Thawi Watthana District, Bangkok Province (13°45′55″ N, 100°22′06″ E, type locality).

Rearing was done at the Laboratory of Kasetsart University Museum of Fisheries (Natural History; KUMF), Faculty of Fisheries, Kasetsart University, Bangkok, Thailand. Broodstocks comprised a male-female mating pair kept in a 20 × 25 × 30 cm glass container and separated by a clear plastic panel. Emergent plants (*Ipomoea aquatica*) were provided as a substrate for males to build a bubble-nest. Brood pairs were deemed as being ready to spawn when males displayed nest-making behaviour and changed body colouration and when females developed genital papillae. These phenomena usually occurred within 1–2 weeks of release into glass containers. The clear plastic panel was then removed, and the pair usually spawned within 1–3 days. After spawning, the female was immediately removed to prevent her from eating the eggs, and the male was allowed to provide continuous care for the fertilized eggs and young.

For feeding, the broodstocks were fed in the morning and afternoon with enough adult artemia and grindal worm, while the larvae were fed with enough larval and adult moina. Throughout this study, the betta larvae and juveniles were reared under water temperatures ranging between 26-28 °C in laboratory room condition.

Collection of specimens for different immature stages

We attempted to sample larval fish according to the recommendations of Termvidchakorn (2005), which required the collection of at least 20 specimens from a series of 18 different age classes (newly hatched, and at 1, 2, 3, 4, 5, 7, 9, 12, 15, 19, 23, 27, 31, 35, 40, 45, and 50 days). However, we could not obtain the recommended number of specimens as our broodstocks had low fecundity of less than 200 offspring per pair, and a portion of each pair always died during the nursing period. Therefore, we used only 3–5 specimens per size class to represent each stage.

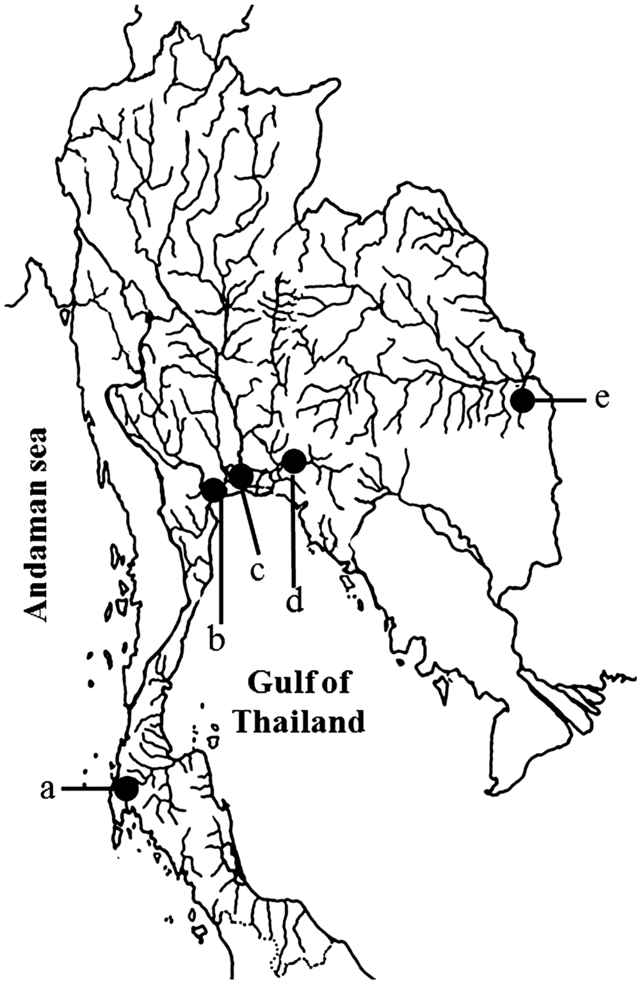

FIGURE 1. Collection localities of broodstocks for the five *Betta* species from Thailand: *B. imbellis* (a), *B. mahachaiensis* (b), *B. splendens* (c), *B. siamorientalis* (d), and *B. smaragdina* (e).

For ethical statement, this study was performed under the approval for animal care and use for scientific research. The authors S. Poungcharean. and D. Limpivadhana receiving a respective license number U1-02081-2558, and U1-09253-2663. The specimens were anesthetized by using 200 ppm eugenol solution before preserved in 4% neutralized formalin condition and deposited in the laboratory at KUMF.

Collection and analysis of morphological data

The developmental stages were classified as the yolk-sac, pre-flexion, flexion, post-flexion, and juvenile stages, according to Balon (1985). Morphological measurements followed Leis and Carson-Ewart (2000) and were taken with a digital Vernier calliper to the nearest 0.01 mm (Mitutoyo, 150 mm range ± 0.01 mm). They included notochord length (NL), standard length (SL), head length (HL), head depth (HD), eye diameter (ED), pre-anal length (PreAL), and body depth (BD) (Figure 2). Notochord length was used to record growth rates at different ages from newly hatched to pre-flexion stages, and SL was used from post-flexion to juvenile stages. Relationships between age and size were analysed using the correlation coefficient (r^2) and the simulated equation.

For each stage, three body dimensions were examined (head length, body depth, and pre-anal length) by photographing specimens with a scale bar under a stereomicroscope. For yolk-sac to pre-flexion stages, the body dimensions were expressed as a percentage of notochord length (%NL), and for post-flexion to juvenile stages as a percentage of standard length (%SL). Additionally, two head dimensions (head depth and eye diameter) were also examined and expressed as a percentage of the head length (%HL). Myomeres were counted in the pre-flexion stage when the bodies of specimens were transparent, and fin rays were counted in juvenile specimens when fins were completely developed. Patterns for melanophores (pigmentation patterns) were recorded and comprised ‘punctate spots' and 'stellate spots' (Neira *et al.*, 1998), 'bars' for dark pigments positioned on head regions, 'stripes' for longitudinal dark lines on the sides of the body, and 'caudal spot' for the dark band at the base of caudal fin (Schindler & Schmidt, 2006) (Figure 2). In this study, eighteen size series per betta species were examined. Each species comprised 54 specimens for *B. mahachaiensis*, *B. smaragdina*, *B. siamorientalis*, and *B. splendens,* and 80 specimens for *B. imbellis*.

Results

Comparative growth rates and morphologies of immature stages for all species

Age and body size (smallest and mean) of reared larval fish at the newly hatched, flexion, and juvenile stages, and the KUMF catalogue number for reference specimens are shown in Table 1. The largest species at hatching were *B. mahachaiensis* (NL = 2.58 ± 0.02 mm [n=3]) and *B. siamorientalis* (NL = 2.57 ± 0.00 mm [n=3]), followed by *B. smaragdina* (NL = 2.47 ± 0.02 mm [n=3]), *B. splendens* (NL = 2.40 ± 0.03 mm [n=3]), and *B. imbellis* (NL = 2.37 ± 0.02 mm [n=5]). After hatching, the yolk sac was completely absorbed within 3–4 days. At the beginning of the flexion stage, the notochord tip was bent dorsally at an angle of more than 30°. The fastest species to develop to the flexion stage were *B. splendens* (NL = 3.68 ± 0.05 mm at day 9 [n=3]) and *B. smaragdina* (NL = 4.90 ± 0.11 mm at day 9 [n=3]), followed by *B. siamorientalis* (NL = 5.21 ± 0.01 mm at day 12 [n=3]), *B. imbellis* (NL = 3.68 ± 0.03 mm at day 12 [n=4]), and *B. mahachaiensis* (NL = 3.22 ± 0.05 mm at day 15 [n=3]). Three species had completely developed ventral fins and were classified at the juvenile stage after 40 days: *B. siamorientalis* (SL = 12.18 ± 0.55 mm [n=3]), *B. smaragdina* (SL = 12.11 ± 0.66 mm [n=3]), and *B. splendens* (SL = 11.89 ± 0.38 mm [n=3]). The other two species exhibited a slower development of 45 days to reach this stage: *B. imbellis* (SL = 10.75 ± 1.03 mm [n=4]) and *B. mahachaiensis* (SL = 10.45 ± 0.11 mm [n=3]).

Relationships between age and body size (NL for pre-flexion stage and SL for post-flexion stage) for all five *Betta* species are shown in Figure 3. The trend lines and simulated equations represent only the hatching to flexion stages. Although *B. imbellis* had the smallest size at hatching (NL = 2.37 ± 0.02 mm) and slow growth in the pre-flexion period (NL = 0.0820Age + 2.7282, r^2 = 0.8694), it showed the fastest growth in the post-flexion stage (SL = 0.3855Age –2.9679, r^2 = 0.9873). In contrast, *B. mahachaiensis* had the largest size at hatching (NL = 2.58 ± 0.02 mm) but was the slowest growing in the post-flexion period (SL = 0.1880 Age + 0.3882, r^2 = 0.9134). The other three species (*B. siamorientalis*, *B. smaragdina*, and *B. splendens*) showed steadier growth in the pre- and post-flexion periods.

TABLE 1. Age (days) and body size (mm) at three immature developmental stages for five *Betta* species.

Species (catalogue number)	Newly hatched stage		Flexion stage			Juvenile stage		
	Smallest size	Mean ± SD	Age	Smallest size	Mean ± SD	Age	Smallest size	Mean ± SD
	(mm NL)	(mm NL)	(Days)	(mm NL)	(mm NL)	(Days)	(mm SL)	(mm SL)
B. imbellis (KUMF 7761)	2.36	2.37 ± 0.02	12	3.66	3.68 ± 0.03	45	10.72	10.75 ± 1.03
B. mahachaiensis (KUMF 7762)	2.57	2.58 ± 0.02	15	3.18	3.22 ± 0.05	45	10.34	10.45 ± 0.11
B. siamorientalis (KUMF 7763)	2.57	2.57 ± 0.00	12	5.20	5.21 ± 0.01	40	11.63	12.18 ± 0.55
B. smaragdina (KUMF 7764)	2.45	2.47 ± 0.02	9	4.82	4.90 ± 0.11	40	11.58	12.11 ± 0.66
B. splendens (KUMF 7765)	2.40	2.44 ± 0.03	9	3.64	3.68 ± 0.05	40	11.51	11.89 ± 0.38

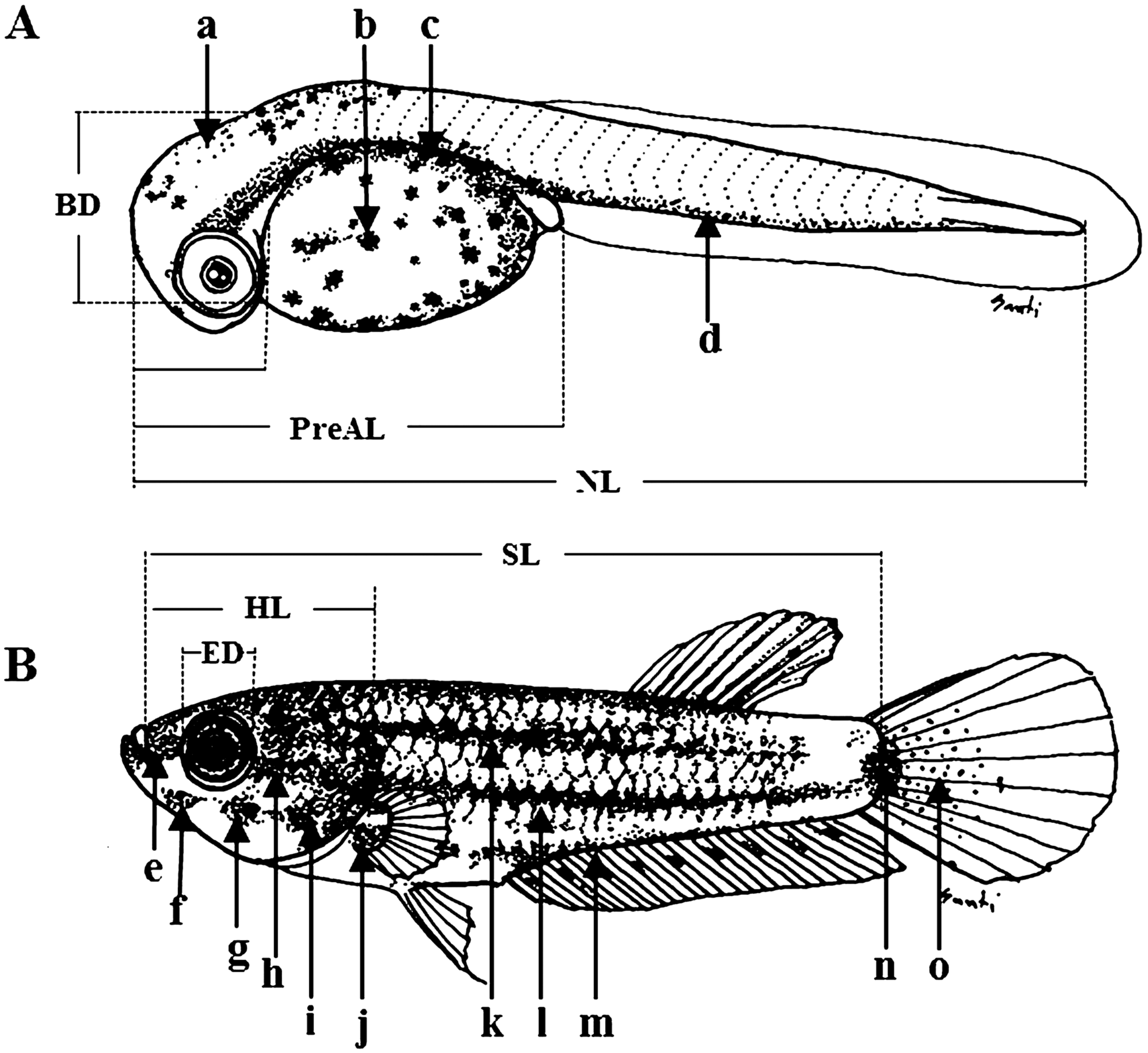

FIGURE 2. General illustrations of *Betta* species' yolk sac larvae (A) and juvenile (B) showing morphometric characters and dark pigmentation patterns. Abbreviations of body dimensions: NL = notochord length, SL = standard length, BD = body depth, HL = head length, PreAL = pre-anal length, ED = eye diameter. Abbreviations for pigmentation patterns: a = punctate spot, b = stellate spot, c = longitudinal stripe along dorsal edge of yolk sac, d = faded stripe along ventral edge of tail, e = pre-orbital bar, f = first sub-orbital bar, g = second sub-orbital bar, h = post-orbital bar, I = post-orbital bar, j = pectoral fin base bar, k = dorsal stripe, l = central stripe, m = ventral stripe, n = caudal spot, o = small punctate spots on caudal fin.

Morphology of immature stages for five *Betta* species

For each of the five species, we described morphology and produced illustrations of the newly hatched, pre-flexion, flexion, post-flexion, and juvenile stages. For all species, specimens at each stage shared some general morphological features. All newly hatched larvae had an oblong and slightly compressed body, a small and rounded head, an oval to rounded eye without pigmentation, no oil droplet in the yolk sac, and the anus was at the anterior half of the body. Pre-flexion larvae had complete eye pigmentation, a developed and oblique terminal mouth, and the pectoral buds were beginning to develop after day 1. Post-flexion larvae were beginning to generate rays for the caudal, dorsal, anal, pectoral, and ventral fins. In juveniles, the ventral fin was fully developed, there were 2–3 dorsal, lateral, and ventral stripes, and a caudal spot was beginning to appear. The number of meristic characters (myomeres and fin rays) was variable across and within the five species (Table 2).

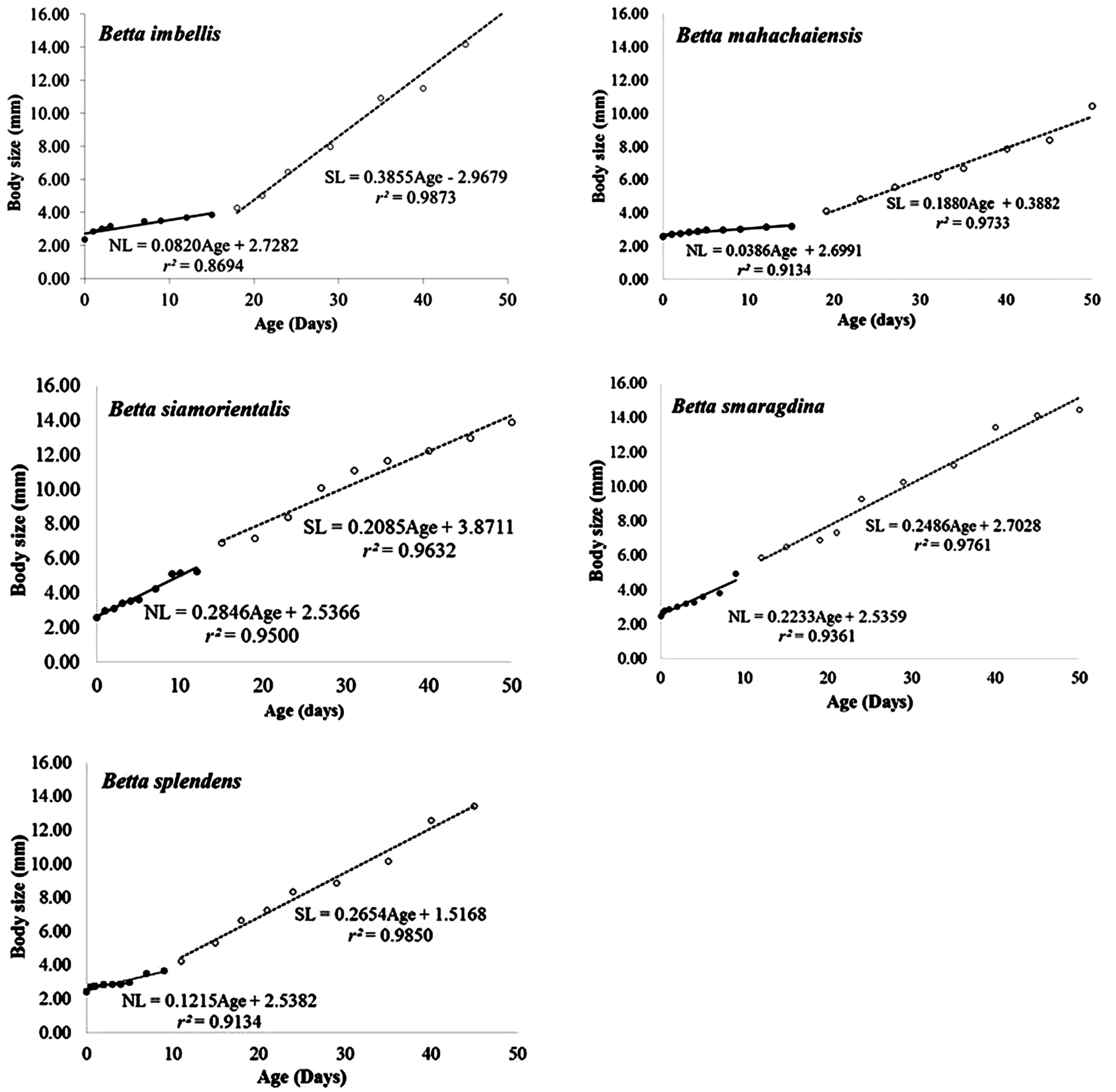

FIGURE 3. Relationship between age (days) and body size (mm) from hatching to 50 days for five *Betta* species. Solid lines with filled circles represent.

1. Morphological development of *B. imbellis*

At the newly hatched stage, larvae (NL = 2.37 mm; Figure 4a) had an oblong and slightly compressed body (BD = 18.66% of NL), a small and rounded head (HL = 13.43% of NL), and large, oval-shaped eyes (ED = 66.67% of HL). The PreAL = 47.76% of NL. Stellate spots were scattered throughout the posterior ¾ of the yolk sac. Melanophore pigments were not present on the head and anterodorsal region of the body, and there was no longitudinal stripe above the yolk sac. **At the pre-flexion stage**, day 1 larvae (NL = 2.82 mm; Figure 4b) had a rounded eye that became proportionately reduced to the length of the head (ED = 52% of HL). Slight pigmentation was beginning to appear on the dorsal region of the head and anterodorsal region of the body. A post-orbital stripe was present which was discontinuous with an upper longitudinal stripe on the yolk sac. **At the flexion stage**, day 3 (NL = 2.98 mm; Figure 4c), day 9 (NL = 3.39 mm; Figure 4d), and day 12 larvae (NL = 3.68 mm; Figure 4e) had stripes on the upper and ventral edges of the gut, which were with a fading pigmentation in the middle of the gut, and then disappeared

TABLE 2. Number of myomeres and fin rays for five *Betta* species. In parenthesis is a number of the examined specimen (n) for myomere count in the pre-flexion stage when the bodies of specimens were transparent, followed by (+) a number of examined specimens for fin ray count in the juvenile stage when fins were completely developed.

Meristic character	*B. imbellis* (*n* = 20 + 18)	*B. mahachaiensis* (*n* = 22 + 21)	*B. siamorientalis* (*n* = 22 + 20)	*B. smaragdina* (*n* = 20 + 21)	*B. splendens* (*n* = 20 + 22)
Myomeres					
Pre-anal	8–10 (mode 9)	9–10 (mode 10)	9–10 (mode 9)	8–9 (mode 8)	9–10 (mode 9)
Post-anal	20–22 (mode 21)	19–20 (mode 19)	19–20 (mode 20)	19–20 (mode 20)	19–21 (mode 21)
Total	29–30 (mode 30)	28–29 (mode 29)	28–29 (mode 29)	28–29 (mode 28)	29–31(mode 30)
Fin rays					
Dorsal spines	2	1	1	1	1–2 (mode 1)
Dorsal rays	7–8 (mode 8)	7–8 (mode 7)	7–8 (mode 7)	9	8–9 (mode 9)
Pectoral rays	11–12 (mode 12)	9–10 (mode 9)	10–11 (mode 11)	10–12 (mode 11)	11–12 (mode 12)
Ventral spines	1	1	1	1	1
Ventral rays	4–5 (mode 5)	5	5	6	5
Anal spines	4	2	2	2	3
Anal rays	22–24 (mode 23)	21–24 (mode 24)	22–25 (mode 25)	26–28 (mode 27)	24–26 (mode 26)
Caudal rays	11–12 (mode 11)	11–12 (mode 12)	10–11 (mode 10)	11–13 (mode 11)	10–12 (mode 12)

by late pre-flexion stage. **At the post-flexion stage**, day 18 larvae (SL = 3.86 mm; Figure 4f) had pigmentation on the air bladder and ventral edges of the gut. Day 31 larvae (SL = 10.20 mm; Figure 4g) had dorsal, lateral, and ventral stripes, and a caudal spot. **At the juvenile stage**, day 40 larvae (SL = 12.88 mm; Figure 4h) had a lateral stripe extending to the caudal spot, and a small spot or region of light pigmentation appeared around the second post-orbital region and at the base of the pectoral fins.

2. Morphological development of *B. mahachaiensis*

At the newly hatched stage, larvae (NL = 2.58 mm; Figure 5a) had an oblong and slightly compressed body (BD = 17.91% of NL), a small, rounded head (HL = 14.93% of NL), and large, slightly rounded eyes (ED = 6.50% of HL). The PreAL was 47.01% of NL. Stellate and small punctate spots were scattered on the dorsal region of the head, the anterodorsal region of the body, and the upper and lower edges of the posterior ¾ of the yolk sac. A longitudinal stripe was lacking above the yolk sac. **At the pre-flexion stage**, day 1 larvae (NL = 2.70 mm Figure 5b) possessed a pre- and post-orbital bar which was continuous with the upper longitudinal stripe of the yolk sac and a faded stripe along the ventral edge of the tail. **At the flexion stage**, day 3 (NL = 2.78 mm; Figure 5c), day 9 (NL = 3.20 mm; Figure 5d), and day 15 larvae (NL = 3.22 mm; Figure 5e) had clusters of melanophores on the upper and lower edges of the gut which faded with age. **At the post-flexion stage**, day 18 larvae (SL = 3.82 mm; Figure 5f) had dorsal and lateral stripes and a caudal spot. For day 31 larvae (SL = 6.24 mm; Figure 5g), the dorsal and lateral stripes merged in the posterior region of the body. **At the juvenile stage**, day 40 larvae (SL = 7.57 mm; Figure 5h) had pigmented bars on the lower maxilla, sub-orbital and opercular regions, and base of the pectoral fins that lined up with ventral stripes along the base of the anal fin. The anterior region of the caudal fin possessed small, scattered, punctate spots.

3. Morphological development of *B. siamorientalis*

At the newly hatched stage, larvae (NL = 2.57 mm; Figure 6a) had an oblong and slightly compressed body (BD = 20% of NL), a small and rounded head (HL = 12.89% of NL), and large, rounded eyes (ED = 66.67% of HL). The PreAL was 44.29% of NL. A continuous longitudinal stripe began posterior to the eye and extended along the dorsal edge of the yolk sac to the gut opening. Stellate and small punctate spots were present on the anterodorsal region of the body and around the dorsal and ventral edges of the posterior ¾ of the yolk sac. The ventral edge of the yolk sac possessed 5–6 stellate spots. **At the pre-flexion stage,** day 1 larvae (NL = 2.85 mm; Figure 6b) had pre-orbital and post-orbital bars that were continuous with the upper longitudinal stripe of the yolk sac. **At the flexion stage,** day 3 (NL = 3.12 mm; Figure 6c), day 9 (NL = 5.10 mm; Figure 6d), and day 12 larvae (NL = 5.21 mm; Figure 4e)

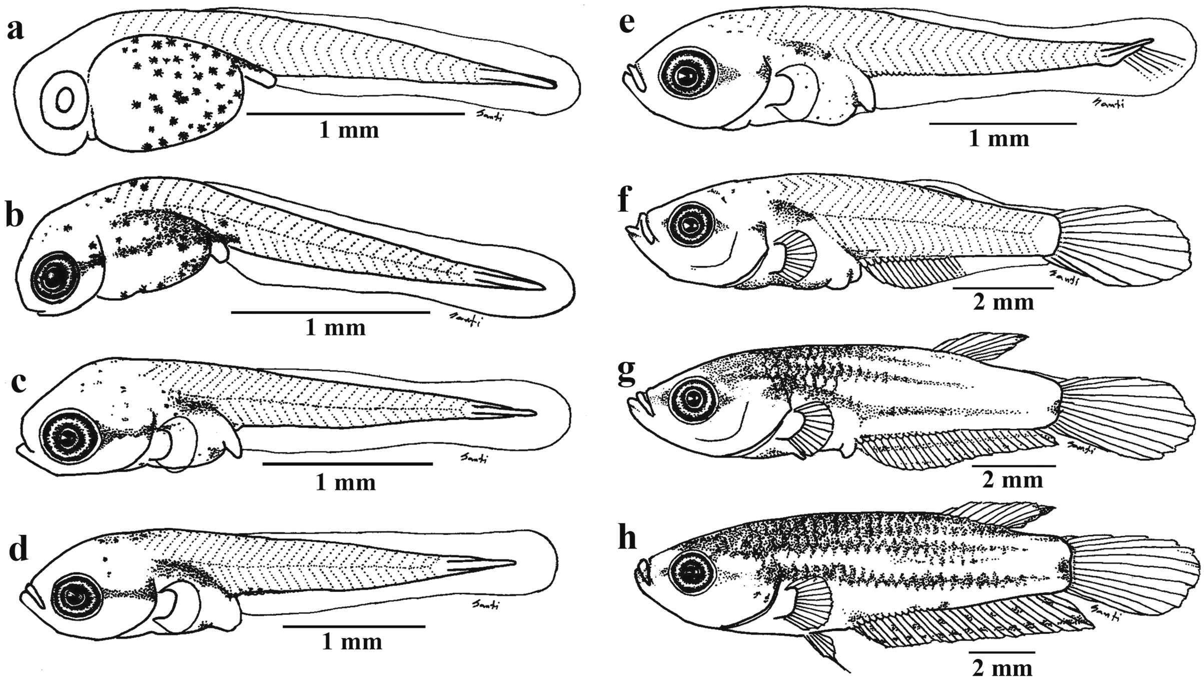

FIGURE 4. Morphological and pigmentation features of immature stages of *Betta imbellis* (KUMF 7066). (a) Newly hatched larva (NL = 2.37 mm); (b) day 1 yolk sac larva (NL = 2.82 mm); (c) day 3 pre-flexion larva (NL = 2.98 mm); (d) day 9 pre-flexion larva (NL = 3.39 mm); (e) day 12 flexion larva (NL = 3.68 mm); (f) day 18 post-flexion larva (SL = 3.86 mm); (g) day 31 post-flexion larva (SL = 10.20 mm); (h) day 40 juvenile (SL = 12.88 mm).

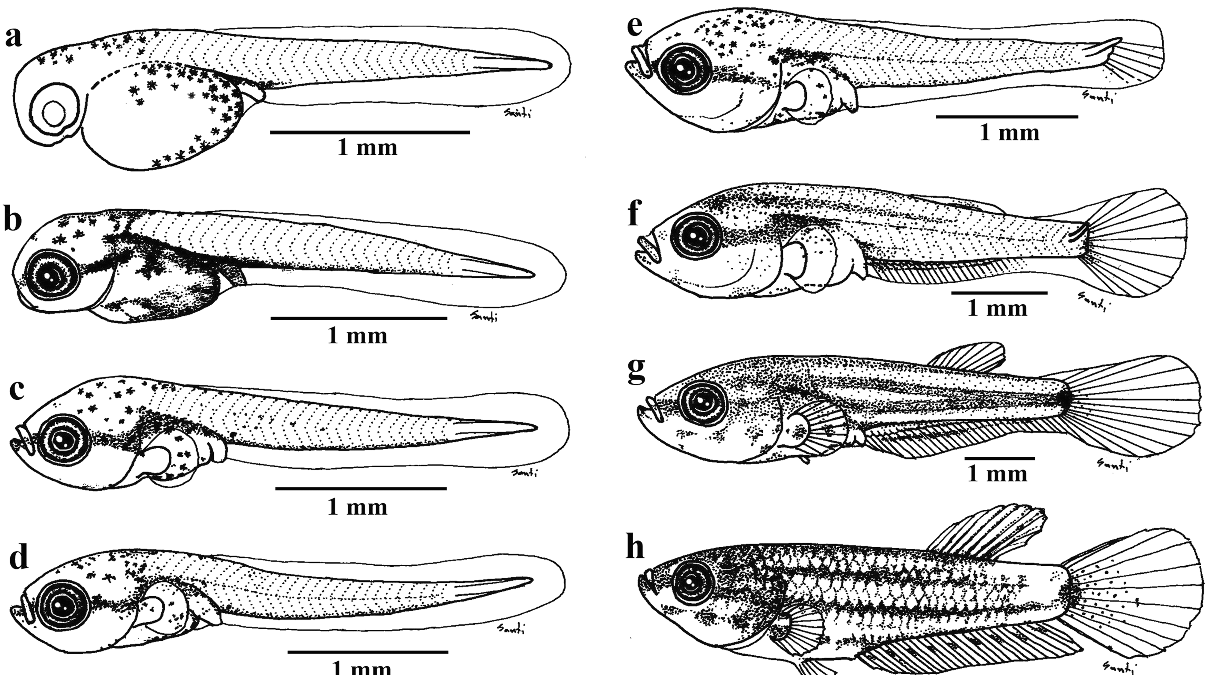

FIGURE 5. Morphological and pigmentation features of immature stages of *Betta mahachaiensis* (KUMF 7067). (a) Newly hatched larva (NL = 2.58 mm); (b) day 1 yolk sac larva (NL = 2.70 mm); (c) day 3 pre-flexion larva (NL = 2.78 mm); (d) day 9 pre-flexion larva (NL = 3.20 mm); (e) day 15 flexion larva (NL = 3.22 mm); (f) day 18 post-flexion larva (SL = 3.82 mm); (g) day 31 post-flexion larva (SL = 6.24 mm); (h) day 40 juvenile (SL = 7.57 mm).

had clustered melanophores on the dorsal region of the head, which increased with age. **At the post-flexion stage,** day 18 larvae (SL = 6.57 mm; Figure 6f) had lateral stripes. The day 23 larvae (SL = 8.68 mm; Figure 6g) possessed dorsal and lateral stripes on the anterior region of the body and a caudal spot. **At the juvenile stage,** day 40 larvae (SL = 11.56 mm; Figure 6h) had dorsal and central stripes which remained separate from each other for their entire length, and bars began to appear around the second post-orbital region and at the base of the pectoral fins.

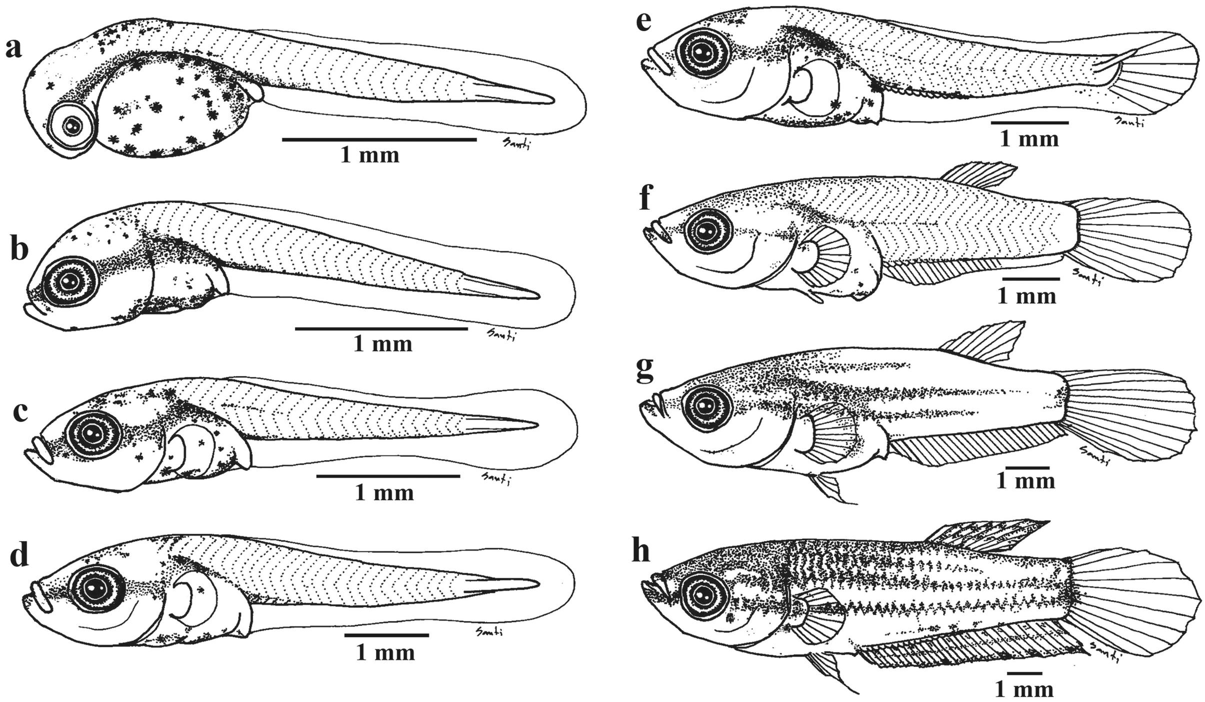

FIGURE 6. Morphological and pigmentation features of immature stages of *Betta siamorientalis* (KUMF 7068); (a) Newly hatched larva (NL = 2.57 mm); (b) day 1 yolk sac larva (NL = 2.85 mm); (c) day 3 pre-flexion larva (NL = 3.12 mm); (d) day 9 pre-flexion larva (NL = 5.10 mm); (e) day 12 flexion larva (NL = 5.21 mm); (f) day 18 post-flexion larva (SL = 6.57 mm); (g) day 23 post-flexion larva (SL = 8.68.40 mm); (h) day 40 juvenile (SL = 11.56 mm).

4. Morphological development of *B. smaragdina*

At the newly hatched stage, larvae (NL = 2.47 mm; Figure 7a) had an oblong and slightly compressed body (BD = 23.60% of NL), a small, rounded head (HL = 14.70% of NL), and large, slightly rounded eyes (ED = 60.00% of HL). The PreAL was 47.06% of NL. There was a longitudinal stripe beginning at the anterodorsal edge of the yolk sac which ended at the gut opening. Stellate and small punctate spots were scattered on the anterodorsal part of the body and around the posterior ¾ of the yolk sac. The ventral edge of the yolk sac had 10–13 stellate spots. **At the pre-flexion stage**, day 1 larvae (NL = 2.79 mm; Figure 7b) possessed a dark bar posterior to the eye that was not continuous with the upper longitudinal stripe of the yolk sac. **At the flexion stage**, day 3 (NL = 3.24 mm; Figure 7c), day 7 (NL = 4.06 mm; Figure 7d), and day 9 larvae (NL = 4.90 mm; Figure 7e) possessed clustered melanophores on the dorsal region of the head which increased with age. **At the post-flexion stage**, day 15 larvae (SL = 6.50 mm; Figure 7f) had begun to accumulate more pigmentation on the anterodorsal surface of the head and air bladder, and post-orbital bars were beginning to form. The day 23 larvae (SL = 7.72 mm; Figure 4g) possessed dorsal and lateral stripes, bars at the first and second sub-orbital, second post-orbital, and at the base of the pectoral fins, and caudal spot. **At the juvenile stage**, day 40 larvae (SL = 10.40 mm; Figure 7h) possessed dorsal and lateral longitudinal stripes that remained separate from each other for their entire length, and small punctate spots were scattered on the anterior part of the caudal fin.

5. Morphological development of *B. splendens*

At the newly hatched stage, larvae (NL = 2.45 mm; Figure 8a) had an oblong and slightly compressed body (BD = 22.88% of NL), a small, slightly rounded head (HL = 13.28% of NL), and large, slightly rounded eyes (ED = 64.71% of HL). The PreAL was 46.88% of NL. There were stellate spots on the anterodorsal surface of the body and around the dorsal edges and middle of the posterior ¾ of the yolk sac. There were no longitudinal stripes above the yolk sac. **At the pre-flexion stage**, day 1 larvae (NL = 2.69 mm; Figure 8b) possessed a post-orbital bar that was discontinuous with the upper longitudinal stripe of the yolk sac, and there was a faded stripe along the ventral

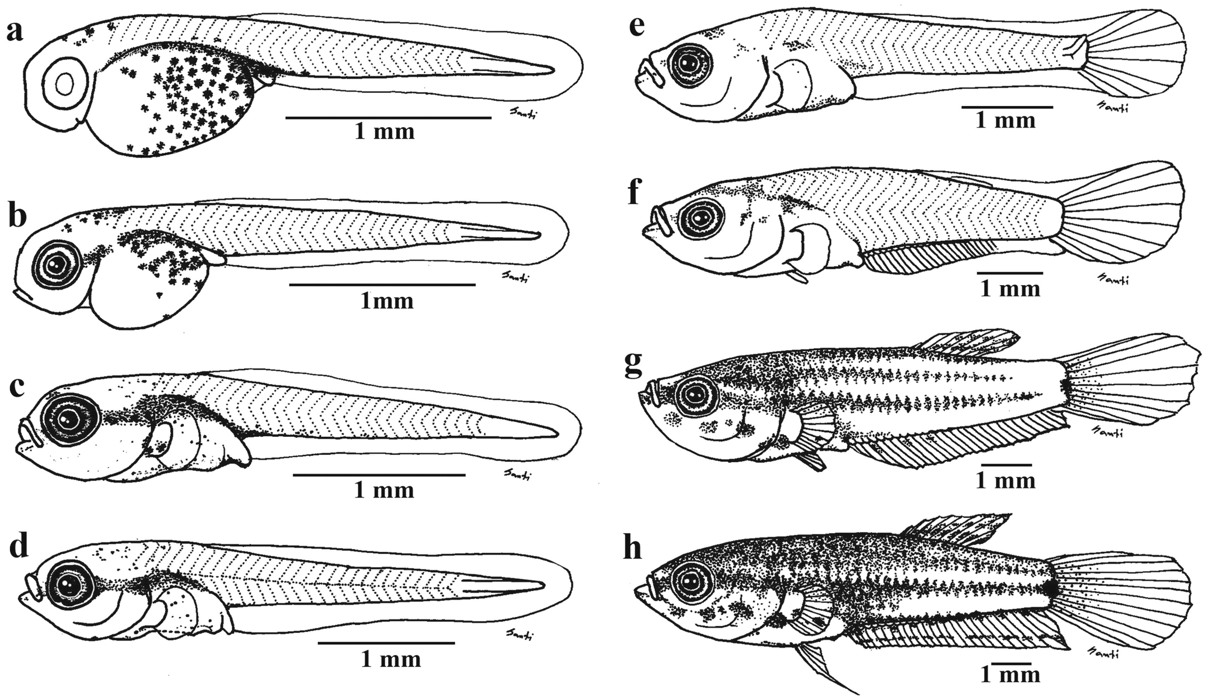

FIGURE 7. Morphological and pigmentation features of immature stages of *Betta smaragdina* (KUMF 7069); (a) Newly hatched larva (NL = 2.47 mm); (b) day 1 yolk sac larva (NL = 2.79 mm); (c) day 3 pre-flexion larva (NL = 3.24 mm); (d) day 7 pre-flexion larva (NL = 4.06 mm); (e) day 9 flexion larva (NL = 4.9 mm); (f) day 15 post-flexion larva (SL = 6.50 mm); (g) day 23 post-flexion larva (SL = 7.72 mm); (h) day 40 juvenile (SL = 10.40 mm).

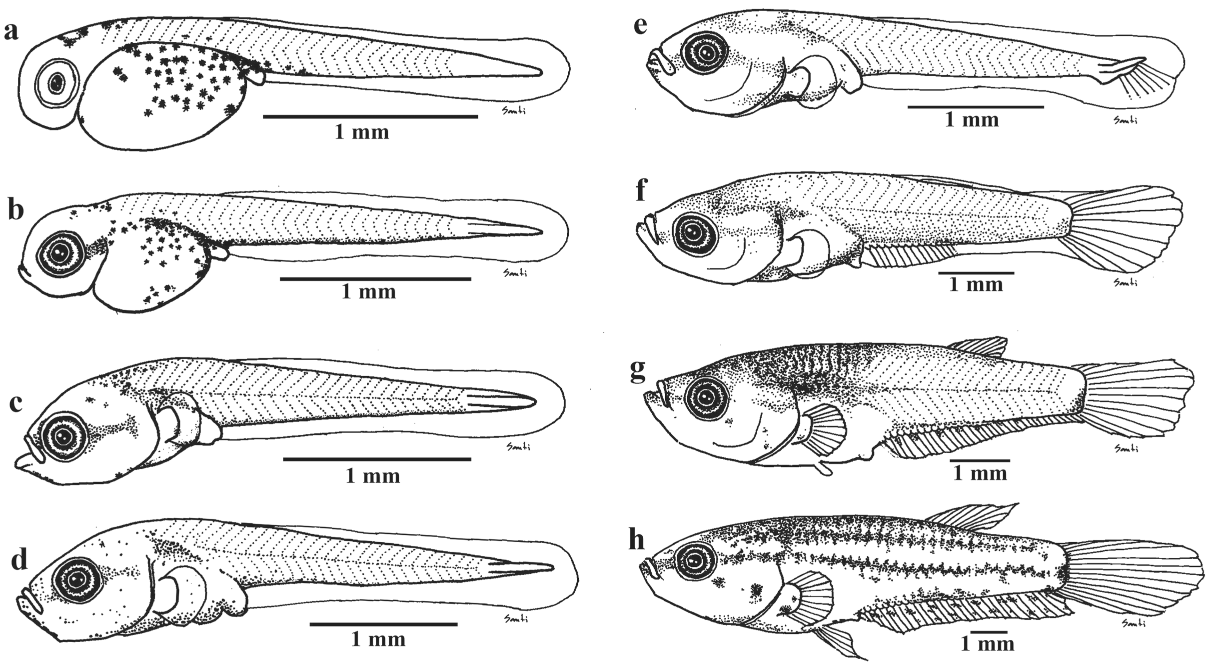

FIGURE 8. Morphological and pigmentation features of immature stages of *Betta splendens* (KUMF 7070); (a) Newly hatched larva (NL = 2.45 mm); (b) day 1 yolk sac larva (NL = 2.69 mm); (c) day 3 pre-flexion larva (NL = 2.86 mm); (d) day 7 pre-flexion larva (NL = 3.42 mm); (e) day 9 flexion larva (NL = 3.59 mm); (f) day 15 post-flexion larva (SL = 5.58 mm); (g) day 18 post-flexion larva (SL = 6.24 mm); (h) day 40 juvenile (SL = 11.78 mm).

edge of the tail. **At the flexion stage**, day 3 (NL = 2.86 mm; Figure 8c), day 7 (NL = 3.42 mm; Figure 8d), and day 9 larvae (NL = 3.59 mm; Figure 8e) had a pre-orbital bar and melanophores on the ventral edge of the gut which increased with age. **At the post-flexion stage**, day 15 (SL = 5.58 mm; Figure 8f) and day 18 larvae (SL = 6.24 mm; Figure 8g) had accumulated more pigmentation on the dorsal part of the head and anterodorsal part of the body. There was a ventral stripe at the base of the anal fin and a caudal spot, both of which increased with age. **At the juvenile stage**, day 40 larvae (SL = 11.78 mm; Figure 8h) had complete dorsal and lateral stripes that remained separate from each other for their entire length, and bars were present on the first and second post-orbital regions and at the base of the pectoral fins.

Discussion

The results of this study provide new information on several shared characteristics between the immature stages of the five *Betta* species, such as general body and head shape, and the development of shared features during each stage. This study also revealed some interesting developmental differences between their immature stages. For example, although *B. mahachaiensis* was one of the largest at hatching (NL = 2.58 ± 0.02 mm), it had the longest developmental period and was, therefore, smaller in size than the other species at the flexion stage. *B. mahachaiensis* required 15 days to reach the flexion stage (NL = 3.22 ± 0.05 mm) and 45 days to reach the juvenile stage (SL = 10.45 ± 0.11 mm). In contrast, *B. imbellis* was the smallest at hatching (NL = 2.37 ± 0.02 mm) but grew faster than the other examined *Betta* species, requiring just 12 days to reach the flexion stage (NL = 3.68 ± 0.03 mm) and 45 days to reach the juvenile stage (SL = 10.72 ± 1.03 mm). There are several environmental factors that can influence fish growth. For example, water temperature is the primary factor influencing embryonic and larval development in tropical fishes (Rønnestad & Morais, 2008). Korwin-Kossakowski (2008) has shown that high water temperatures, which are within the optimal range, result in faster yolk absorption and the rapid growth of larvae. Additionally, nutrition is an important factor affecting the growth of late pre-larval and post-larval stages. Adewumi (2018) reviewed proper nutrition such as amino acid, fatty acid, and vitamins are promote normal growth and sustain health of fish, especially fish larvae and juveniles, which sensitive to the dietary nutrition supply. Therefore, the effects of temperature and feeding methods should be considered when interpreting the results of this study which pertain to hatching sizes, the timing of stage transformation periods, and growth rates.

We, therefore, infer that *Betta* species exhibit a linear growth trend, where the Y-intercept of the NL-based equation predicts the ‘size at hatching’, while the slope predicts the ‘growth rate’. There are two alternatives to the equation, which allow it to be optimised to morphology and development phase. The first alternative uses NL as the data input for the estimation of pre-larval age, when the caudal fin has not yet developed. The second alternative uses SL because at the beginning of the flexion stage the larvae lack a measurable notochord due to notochord tip flexion (Viseth *et al*., 2013). Together, the two alternatives allow estimating the age of larvae for *Betta* species when reared in captivity or collected from the wild. However, the equations were limited by the range of the simulated data and the environmental conditions the larvae were reared under, and are, therefore, optimised for larval specimens less than 45 days old.

The morphological descriptions showed that pigmentation patterns of larval or juvenile stages can be used to differentiate between the five examined species, as well as between different larval stages within a species. For example, for yolk-sac larvae, the stellate spots present on the yolk sac, the dorsal region of the head, and the anterodorsal surface of the body, as well as the longitudinal stripe on the dorsal edge of the yolk sac, can be used to identify all five species. For pre- and post-flexion larvae, the pigmentation patterns on the head, such as pre-, sub-, and post-orbital, and sub-opercular bars can be used for identification. For juveniles, the longitudinal stripes on the side of the body, such as dorsal, lateral, and ventral stripes, can be used (Schindler & Schmidt, 2006).

Historically, there have been suggestions to divide the genus *Betta* into two genera based on the different modes of reproduction and parental care. In this case, bubble-nesting species would be classified under the genus *Betta* and mouth-brooding species would be classified under the genus *Pseudobetta* (Kottelat & Ng, 1994; Ritcher, 1981; Tan, 1998). To date, this suggestion has not been accepted due to insufficient evidence supporting the division (Roberts, 1989). Although this uncertainty has prompted extra efforts in classifying and investigating the taxonomy of *Betta* species and their relatives, more information is still required to determine the necessity of a second genus. To advance the identification of members of this group, below, we present dichotomous keys for the five bubble-nesting *Betta* species and their four larval developmental stages, using the results of this study.

Keys to the five bubble-nesting *Betta* species from Thailand based on larval and juvenile stages

Key for newly hatched stage

1a) Longitudinal stripe along dorsal edge of yolk sac present 2
1b) Longitudinal stripe along dorsal edge of yolk sac absent. 3
2a) 5–6 stellate spots on ventral edge of yolk sac; lightly pigmented on dorsal region of head (less than 6 stellate spots). . . . . *B. siamorientalis*
2b) 10–13 stellate spots on ventral edge of yolk sac; heavily pigmented on dorsal region of head (more than 10 stellate spots) . . . . . . *B. smaragdina*
3a) Pigments on head and anterodorsal region of the body absent; stellate spots scattered throughout posterior ¾ of the yolk sac . . . . . . *B. imbellis*
3b) Pigments on head or anterodorsal region of body present; stellate spots scattered on some portions of posterior ¾ of the yolk sac 4
4a) Stellate spots on dorsal and ventral edges of yolk sac present . . . . *B. mahachaiensis*
4b) Stellate spots on ventral edges of yolk sac absent . . . . *B. splendens*

Key for pre-flexion stage (day 3 larvae)

1a) Post-orbital bar continuous with upper longitudinal stripe of yolk sac 2
1b) Post-orbital bar not continuous with upper longitudinal stripe of yolk sac 3
2a) Pre-orbital bar present; heavily pigmented on ventral edge of yolk sac . . . . *B. siamorientalis*
2b) Pre-orbital bar absent; lightly pigmented on ventral edge of yolk sac . . . . *B. smaragdina*
3a) Faded stripe along ventral edge of tail absent . . . . *B. imbellis*
3b) Faded stripe along ventral edge of tail present. 4
4a) Pre-orbital bar present; heavily pigmented on ventral edge of yolk sac . . . . *B. mahachaiensis*
4b) Pre-orbital bar absent; lightly or not pigmented on ventral edge of yolk sac. . . . . *B. splendens*

Key for post-flexion stage (day 15–18 larvae)

1a) Sub-orbital and sub-opercular bars present 2
1b) Sub-orbital and sub-opercular bars absent 4
2a) Post-orbital bar continuous with central stripe. . . . . *B. smaragdina*
2b) Post-orbital bar not continuous with central stripe. 3
3a) Post-orbital bar appears posterior to eye and to the edge of the operculum. . . . . *B siamorientalis*
3b) Post-orbital bar appears only posterior to eye . . . . *B. imbellis*
4a) Heavily pigmented bars present on first sub-orbital, second sub-orbital, first post-orbital, second post-orbital, and at the base of pectoral fins; ventral stripe present. . . . . *B. mahachaiensis*
4b) Lightly pigmented bars present on first sub-orbital, second sub-orbital, and base of the pectoral fins; ventral stripe absent. . *B. splendens*

Key for juvenile stage

1a) Sub-orbital and second post-orbital bars present; small punctate spots scattered on anterior part of caudal fin. 2
1b) Sub-orbital and second post-orbital bars absent; lacking punctate spots on caudal fin 3
2a.) 27–29 anal fin rays (usually 28). . . . . *B. smaragdina*
2b.) 23–26 anal fin rays (usually 26). . . . . *B. mahachaiensis*
3a.) Bars present for first post-orbital, second post-orbital, and base of pectoral fins . . . . *B. splendens*
3b.) Bars present for second post-orbital and base of pectoral fins . . . . *B. siamorientalis*
3c.) Small spot or light pigments appearing on second post-orbital and base of pectoral fins region . . . . *B. imbellis*

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