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Morphological comparison of early stages of two Japanese species of eight-barbel loaches: *Lefua echigonia* and *Lefua* sp. (Nemacheilidae)

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Abstract. The larval and juvenile development was compared between *Lefua echigonia* and *Lefua* sp., both endemic and endangered species in Japan. *L. echigonia* larvae collected in sunny wetlands were planktonic and swam in the middle to upper layers in lentic waters, whereas *L.* sp. larvae swam with their abdomen facing toward the substrate along the river bottom in well shaded waters of mountain streams. Larvae and juveniles of both species have a distinct dark band on the lateral side of snout. *L. echigonia* larvae have melanophores on the dorsal body, gut region, and around the lateral midline, whereas melanophores distribute broadly on the body in *L.* sp. larvae. Eyes are located more dorsally in larvae of *L.* sp.: in the ventral view of the head, the eyes of *L. echigonia* larvae could be seen, but those of *L.* sp. larvae could not be seen. *L. echigonia* larvae and juveniles have relatively large eyes and eye diameters were larger than the snout lengths. *Lefua* sp. larvae and juveniles have relatively small eyes and eye diameters were smaller than the snout lengths. These characters of melanophore distribution, eye size, and eye location are concluded to show adaptation for each habitat.

Key words: larval development, morphology, habitat

Introduction

The Japanese eight-barbel loach, *Lefua echigonia* (Japanese: hotoke-dojo) is distributed from Tohoku to Kinki districts of Honshu Island, Japan (Hosoya 2002). It occurs in streams and springs flowing through wetlands, irrigation canals of rice fields, and small ponds on flood plains. The fluvial eight-barbel loach *L.* sp. (Japanese: nagare-hotoke-dojo, sensu Hosoya 2002) is an undescribed species separated from *L. echigonia*, based on morphological, ecological (Hosoya 2002), and genetic (Sakai et al. 2003) studies. *Lefua* sp. is distributed in the coastal region of the Inland Sea of Seto and the Sea of Japan sides of Fukui and Kyoto prefectures, and inhabits the upper reaches of mountain streams with gravely bottoms (Hosoya 2003). *Lefua* sp. coexists with *L. echigonia* in Kyoto and Hyogo prefectures (Hosoya 2002).

Morphological differences between adults of the two species were described by Hosoya (2002). The snout of *L. echigonia* does not bear a dark oblique band; if a band is present, it is light grey and indistinct (Fig. 1A). Their eyes locate dorsolaterally on the head. Dark spots are scattered on the dorsal and caudal fins. Whereas *L.* sp. possess a snout with a distinct dark oblique band (Fig. 1B). Eyes locate on the dorsal side of head. No dark spots on dorsal and caudal fins, or sparse if present. Larval and juvenile development of *L. echigonia* has been described by Okada & Seiishi (1938), Miyadgi et al. (1976) and Hosoya (1988). However, no study has been made on the development of *L.* sp. Furthermore, larval and juvenile development in nemacheilid loaches has been scarcely reported. Therefore, the aim of the present study is to describe and compare the larval and juvenile development of *L.* sp. with that of *L. echigonia*.

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Fig. 1. *Photos of* L. echigonia *(A),* L. *sp. (B).*

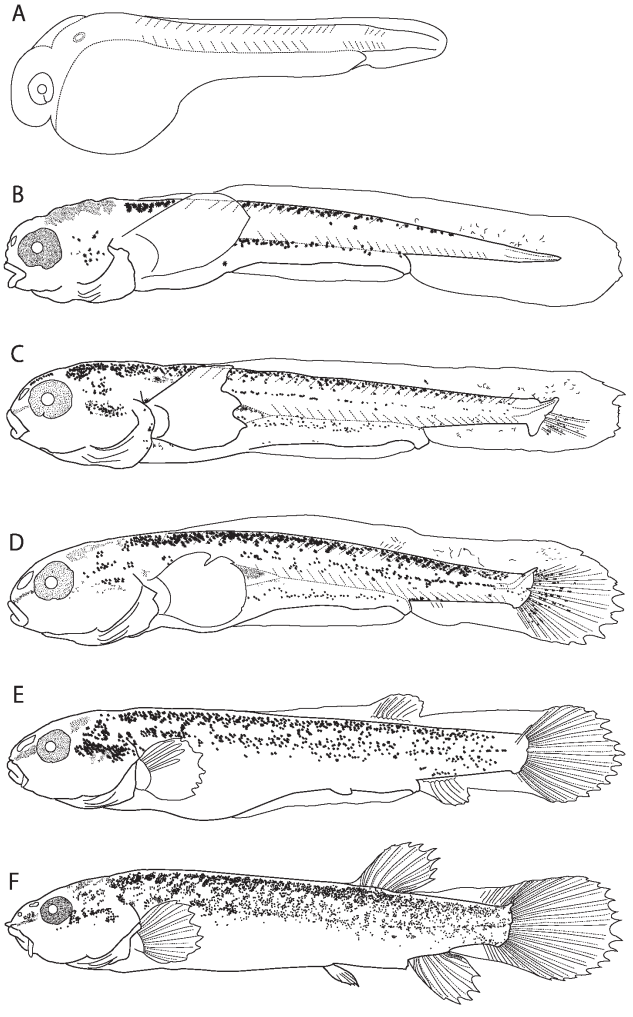

Fig. 2. *Developmental stages of* L. echigonia*. A) 2.6 mm TL newly hatched larva; B) 4.2 mm BL preflexion larva; C) 6.1 mm BL flexion larva; D) 7.6 mm BL flexion larva; E) 10.7 mm BL postflexion larva; F) 13.1 mm BL juvenile.*

Material and Methods

Samples of larvae and juveniles of *L. echigonia* were collected at a sunny wetland in a fallow rice field, connected to the Kako River system, in Hyogo prefecture, Japan, from late April to mid July, 1997. Samples of larvae and juveniles of *L.* sp. were collected at a tributary of the River Kako, in Hyogo prefecture, Japan, from July to August, 1996. But earlier stages of both species were described by incubating eggs which were spawned in rearing tanks.

Specimens were preserved in 5 % formalin. The definition of each developmental stage followed Kendall et al. (1984). Body length (BL) of 37 specimens of *L. echigonia* and 41 specimens of *L.* sp. was measured to the tip of the notochord (newly hatched, preflexion and flexion larvae) and to the posterior edge of hypural plates in postflexion larvae and juveniles using an ocular micrometer for examining ranges of developmental stages. Furthermore, 34 out of the above *L. echigonia* specimens and 28 out of the above *L.* sp. specimens were also measured for pre-anal length (PAL), head length (HL), eye diameter (ED) and snout length (SNL) for examining relative growth. Sketches of larval development of six specimens of *L. echigonia* and seven specimens of *L.* sp. were made for examining morphometric and meristic characters using a stereoscopic microscope with a drawing tube within a year from preservation. Some specimens were stained with cyanine 5-R for observations (Kinoshita 1987).

Results

Larval development

General morphology – body lengths of larvae and juveniles at each developmental stage are shown in Table 1. In *L. echigonia*, newly hatched larvae (2.56-2.60 mm TL, n = 3), two days after spawning, had an unpigmented body with colourless eyes, and were characterized by a large yolk sac (Fig. 2A). Dorsal and ventral finfolds were slightly developed. Pectoral fin buds were scarcely apparent. Notochord end did not elongate and was still indistinct. At 4.2 mm BL (Fig. 2B), notochord end scarcely flexed. Mouth opened and food items were observed in the gut. The yolk was not observed. Notochord end had begun to flex at 5.5 mm BL, was flexing at 6.1 mm BL (Fig. 2C) and had finished to flex at 7.6 mm BL (Table 1). The 4.2 mm BL, 6.1 mm BL and 7.6 mm BL larvae had 24 + 12 = 36 myomeres. One pair of outer rostral barbels had elongated in post-flexion larvae (10.7 mm BL) (Fig. 2E), although these were not seen in flexion larvae (7.6 mm BL, Fig. 2D). At 13.1 mm BL, nostrils divided into two portions (Fig. 2F). Eyes were relatively large and the snout was short in all larval and juvenile stages (Fig. 2).

In *L.* sp., newly hatched larvae (3.5-4.5 mm TL, 3.3-4.3 mm BL, n = 7), five days after spawning, had

Table 1. *Body length (mm) of each developmental stage of* L. echigonia *and* L. *sp.*

Stage	*L. echigonia*	*n*	*L.* sp.	*n*
Newly hatched larva	2.56-2.60*	3	3.28-4.28	7
Yolk sac larva	-		5.16-5.56	4
Preflexion larva	4.21-4.92	4	5.78-6.19	5
Flexion larva	5.46-7.64	11	6.17-8.75	14
Postflexion larva	7.23-11.36	13	8.74-11.08	6
Juvenile	12.37-13.09	6	11.40-12.90	5

** Total length: notochord end did not elongate.*

unpigmented body with colourless eyes, characterized by large yolk sac (Fig. 3A). One pair of small hollows, representing the nostril buds, was observed in front of the eyes, but small hollows were not observed at 2.6 mm TL of newly hatched larvae of *L. echigonia*. Dorsal and ventral finfolds were developed and pectoral fin buds were apparent. At 5.6 mm BL of yolk sack larva (Fig. 3B), four days after hatching, a yolk sack was large and mouth opened but did not function. At 6.2 mm BL (Fig. 3C), six days after hatching, notochord end scarcely flexed. The larva had a functional mouth and started feeding. The yolk was still observed. Notochord end begun to flex at 6.2 mm BL (other individual; Table 1), was flexing at 6.6 mm BL (Fig. 3D), at 8.1 mm BL (Fig. 3E) and finished to flex at 8.8 mm BL (Table 1). The 6.6 mm BL and 8.8 mm BL larvae had 24 + 11 = 35 myomeres. One pair of outer rostral barbels begun to form at 6.6 mm BL (flexion larva; Fig. 3D) and elongated at 8.1 mm BL (flexion larva; Fig. 3E) but rostral barbels were not observed at 7.6 mm BL in flexion larva of *L. echigonia*. At 9.8 mm BL (postflexion larva; Fig. 3F), two pairs of inner rostral barbels began to appear. At 12.1 mm BL (juvenile; Fig. 3G), nostrils had divided into two portions and a pair of nasal barbels had already started to develop at the anterior nostril, but this character was not observed at 13.1 mm BL of *L. echigonia*.

Pigmentation – in *L. echigonia*, eyes were pigmented at 4.2 mm BL (Fig. 2B). Melanophores were mainly distributed on the dorsal head, dorsal trunk, dorsal aspect of the gut region, and dorsal gas bladder in preflexion larvae (Fig. 2B). Melanophores on the trunk in preflexion larvae of *L. echigonia* were punctate or stellate, and were darker than those of *L.* sp. At 6.1 mm BL (flexion larva; Fig. 2C), a dark oblique band had started to form internally at snout. Melanophores added along the lateral midline and the caudal fin rays in flexion larvae (Fig. 2C, D). Melanophores on the abdominal side from the base of pectoral to the anus showed a pattern of lower intensity from the dorsal gut. Melanophores on the opercular region were distinct in *L. echigonia* larvae but scarcely present in

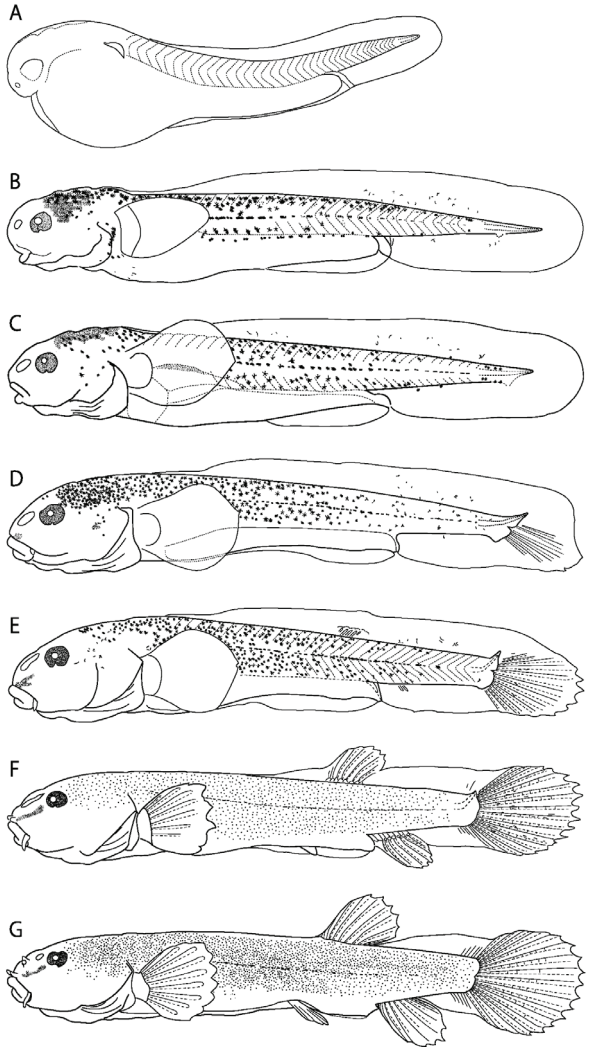

Fig. 3. *Developmental stages of* L. *sp. A) newly hatched larva; B) 5.6 mm BL yolk-sack larva, four days after hatching (laboratory-reared specimen); C) 6.2 mm BL preflexion larva, six days after hatching (laboratory-reared specimen); D) 6.6 mm BL flexion larva; E) 8.1 mm BL flexion larva; F) 9.8 mm BL postflexion larva; G) 12.1 mm BL juvenile.*

L. sp. larvae. Melanophores locating in cleithral area below the pectoral fin were observed in *L. echigonia* larvae but they were not observed in *L.* sp. larvae. A dark oblique band on the snout became distinct in 7.4 mm BL larvae. At 10.7 mm BL (postflexion larva;

Fig. 2E), melanophores gradually increased on the dorsolateral body and around the lateral midline. At 13.1 mm BL (juvenile; Fig. 2F), melanophores on the body were distributed with higher density patches.
In *L.* sp., at 5.6 mm BL (Fig. 3B), eye was pigmented. Melanophores appeared on the dorsal head, dorsal and lateral of the body, and lateral midline. Melanophores seemed to be present on the dorsal gas bladder but they were not distinct. At 6.2 mm BL (Fig. 3C), 2-4 melanophores were present on myomeres and were more sparse on the caudal region. In preflexion larvae of *L.* sp., melanophores on the trunk were distributed closer to the lateral midline. But in preflexion larvae of *L. echigonia* melanophores were not distributed along the lateral midline and were remarkably distributed on the dorsal-side and ventral-side. Melanophores on the trunk in preflexion larvae of *L.* sp. were branched

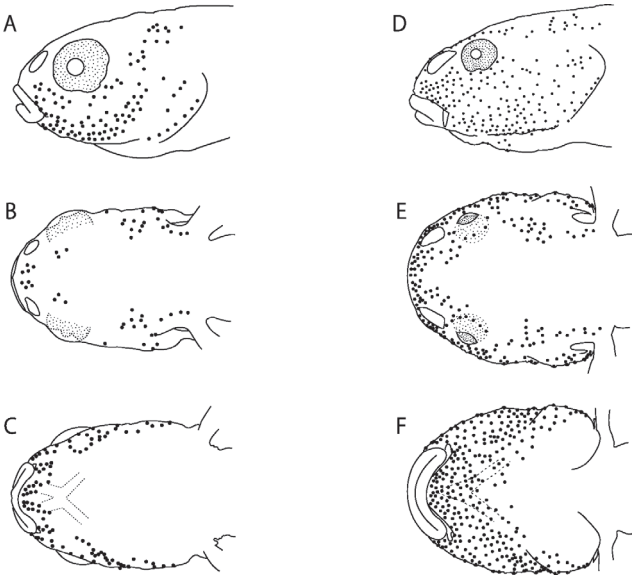

Fig. 4. *Morphology of the head of* L. echigonia *(A-C) and* L. *sp. (D-F). A dot indicates a free neuromast.*

and were a paler brown than the punctate or stellate melanophores in preflexion larvae of *L. echigonia*. Melanophores internally appeared on the front snout at 6.2 mm BL in flexion larva (other individual; Table 1). At 6.6 mm BL (Fig. 3D), melanophores were distributed more broadly on the body except the caudal region. Internal melanophores extended from the tip of the snout to the posterior at 6.6 mm BL and to 2/3 snout at 8.7 mm BL. At 9.8 mm BL (Fig. 3F), melanophores were distributed more broadly on the body. A dark oblique band on the snout extended to the front of the eye and became distinct. A pattern of small fine melanophores covered the dorsolateral part of the head and body at 12.1 mm BL of juveniles (Fig. 3G).

Fin development – in *L. echigonia*, at 4.2 mm BL (preflexion larva; Fig. 2B), finfold was relatively high, dorsally and ventrally. Especially pectoral fin was large. At 7.6 mm BL (flexion larva; Fig. 2D), buds of dorsal and anal fin rays appeared. At 10.7 mm BL (postflexion larva; Fig. 2E), dorsal and ventral fin rays elongated. Ventral fin-buds had appeared. At 13.1 mm, all fin rays had attained their full counts of D: iii + 7, A: iii + 6, C: 16, P1: 9, P2: 6, and finfold remained in the caudal region (juvenile; Fig. 2F).
In *L.* sp., at 5.6 mm BL (Fig. 3B), finfold was relatively high, almost same depth dorsally and ventrally. At 8.1 mm BL (Fig. 3E), dorsal fin ray buds and anal fin ray buds had appeared. At 9.8 mm BL (Fig. 3F), fin rays of dorsal and anal fins elongated. Pectoral fin rays had appeared. Ventral fin-buds had appeared. At 12.1 mm BL (Fig. 3G), all fin rays had attained their full counts of D: iii + 7, A: iii + 6, C: 16, P1: 9, P2: 6. Finfold still remained in the caudal region.

Comparison of head
In observations of the head in flexion larvae of both species (Fig. 4), free neuromasts of *L.* sp. appeared in larger numbers than those of *L. echigonia*. In the ventral view of the head, eyes of *L. echigonia* could be seen, whereas those of *L.* sp. could not be seen (Fig. 4).

Comparison of relative growth
A large difference was not found in PAL/BL and in HL/BL after the flexion stage of both species (Fig. 5A, B). But there was a distinct difference in SNL/HL and in ED/HL (Fig. 5C, D). Further, in larvae of *L.* sp., ED is smaller than SNL after preflexion stage (Fig. 5E). Whereas, in *L. echigonia* larvae, ED is larger than SNL and about after postflexion stage, ED is smaller than SNL (Fig. 5E).

Ecological note
In the field, larvae and juveniles of *L. echigonia* collected for the present study were planktonic and intermittently swam in the middle to upper layers in shallow water of the wetland.
In tank observations, yolk-sack larvae of *L.* sp. rested on their lateral body on the bottom. When surprised, they showed short distance burst swimming and then rested again. The larvae gradually rested on their abdomen on the bottom with time passed. In two days after the above stage, larvae started swimming intermittently on the bottom and feeding. After swimming, larvae and juveniles moved with their abdomens facing the substrate along the vertical glass

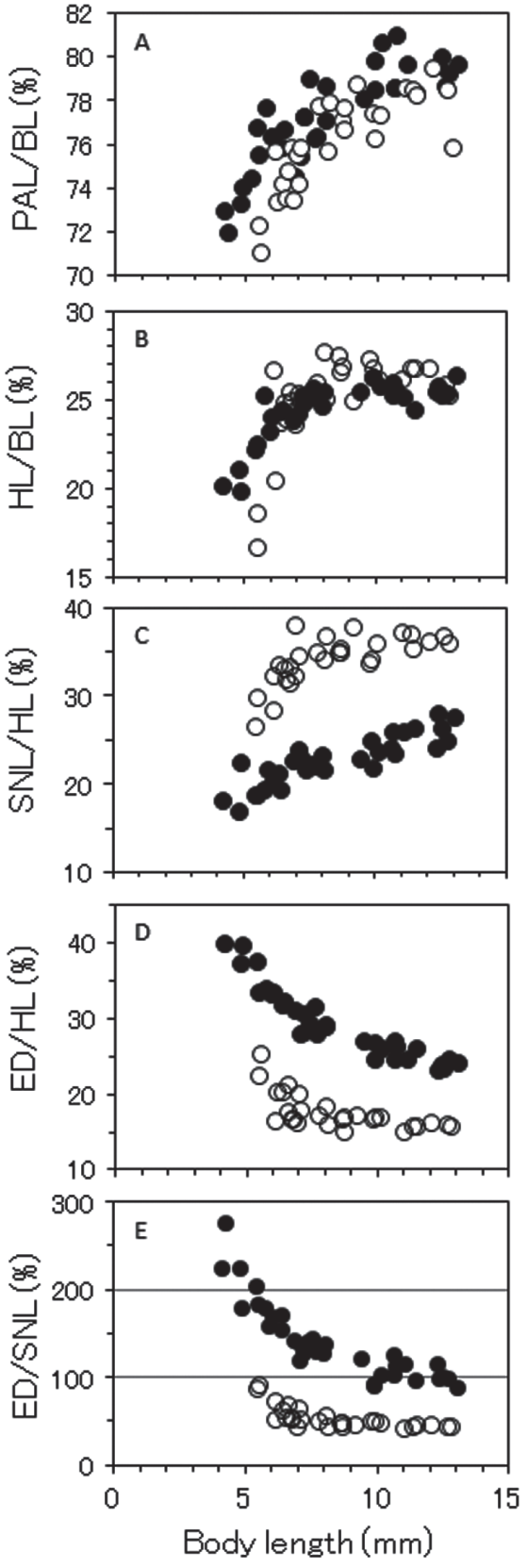

Fig. 5. *Comparisons of relative growth of* L. echigonia *and* L. *sp. BL – body length; PAL – pre-anal length; HL – head length; SNL – snout length; ED – eye diameter. Solid and open circles indicate* L. echigonia *and* L. *sp., respectively.*

walls or the bottom in rearing tanks and along surface of rocks or the bottom in the natural environment.

Digested parts of chironomid larvae were observed in fecal output in specimens of 7.8 mm BL, 11.5 mm BL and 12.7 mm BL of *L.* sp.

Discussion

In the present study, developmental stages of larvae and juveniles of *L.* sp. were described and compared with those of *L. echigonia*. In larval and juvenile development of *L. echigonia*, the observations in previous reports (Okada & Seiishi 1938, Miyadgi et al. 1976, Hosoya 1988) were confirmed again in the present study.

In the present study, newly hatched larvae of *L. echigonia* were 2.6 mm TL and the notochord end did not elongate and the bases of the pectorals were scarcely apparent. In previous reports, TL of *L. echigonia* were 3.7 mm (Okada & Seishi 1938) and 3.4-3.9 mm (Miyadgi et al. 1976) and the notochord end more developmentally elongated and the bases of the pectorals were slightly developed. So newly hatched larvae in the present study were considered to have hatched out in slightly earlier condition than those in previous reports. Still, newly hatched larvae of *L.* sp., being 3.5-4.5 mm TL in the present study, were relatively larger than those of *L. echigonia* in both the present and previous studies. The large size of newly hatched larvae may be yielded from large eggs. In previous reports, diameters of eggs of *L.* sp., being 1.3-1.7 mm (Aoyama et al. 2005) and 1.9-2.3 mm (Aoyama & Doi 2006), were larger than those of *L. echigonia*, being 1.3 mm (Okada & Seishi 1938) and 1.3-1.4 mm (Miyadji et al. 1976). Among relative species, fish inhabiting upper streams tend to yield larger eggs for adaptation to colder mountain streams (Minamori 1960, Shimizu et al. 1998). There might be this trend between both species. More observations on early-food items in larval habitats for both species are needed.

Melanophores on the trunk of *L. echigonia* appeared to be darker and were more remarkably distributed on the dorsal-side in earlier stages than those of *L.* sp. Melanophores heavily distributed on the dorsal surface in early stage of *L. echigonia* have also been described in previous reports (Okada & Seiishi 1938, Hosoya 1988). Hosoya (1988) indicated that in the 5.2 mm TL (5.0 mm BL) of *L. echigonia* larval melanophores were mainly distributed on the dorsal body from the nape to caudal peduncle and on the dorsal aspect of the yolk sack, and the former especially tended to form a vertical row. Whereas, in early stages of *L.* sp.,

paler brown melanophores were distributed more broadly on the body. Habitats of *L. echigonia* catch the sun (Yamashina et al. 1994). Habitats of *L.* sp. are covered by many trees and tend to be shaded even in the daytime (Yamashina et al. 1994, Aoyama & Doi 2006). In general, fish are most vulnerable to ultra violet rays included in sunlight during the egg and larval stages (Olson et al. 2006) and melanophores protect the larval body from sunlight (Dasilao et al. 1998). More observations on difference of distribution of melanophores in dorsal view between early stages of both species are needed in relation to brightness of their environment.

In adult fish, a dark oblique band on the snout is distinct in *L.* sp. but obscure in *L. echigonia* (Hosoya 2002). However, in *L. echigonia* larvae, a dark oblique band on snout was distinctly observed in the present study, which had been the same observation as shown in Hosoya (1988). In *L.* sp. larvae and juveniles, a dark oblique band on the snout was observed in the present study. Dark oblique band on the snout was also observed in *Lefua costata* (see Uchida 1939). Therefore, it might be a common character in early stages of the genus *Lefua*.

Differences between *L. echigonia* and *L.* sp. were also found on eye diameter, snout length and location of eyes. Furthermore, the space in the water column where the larvae and juveniles were active was also different. These differences were considered to be adaptations to their habitat. In the present study, larvae and juveniles of *L. echigonia* were observed showing floating activity in their habitat. In the field, they are floating in water and become benthic at 20 mm in total length (Hosoya 1988). However, *L.* sp. becomes benthic immediately after hatching and does not have floating planktonic stages throughout their life cycle (Hosoya 2003). Larvae and juveniles of *L.* sp. were observed to swim with their abdomen facing toward the substrate along the river bottom in low visibility and well shaded waters of mountain streams or even along the vertical glass walls of rearing tanks. Floating activity in early stages of *L. echigonia* might be an adaptation to a more lentic environment. Meanwhile the breeding season of *L.* sp. is mostly from May to July (Aoyama & Doi 2006, Aoyama 2007) and it is just before or during the rainy season. Moving along substrate without floating in early stages of *L.* sp. might be an adaptation to preventing drifting away even in rapid current.

In the ventral view of the head, the eyes of *L. echigonia* could be observed, but those of *L.* sp. could not be observed. Eyes of *L. echigonia* were larger and those of *L.* sp. were smaller in size. Furthermore, free neuromasts on the head of *L.* sp. have the appearance of being lager in number than those of *L. echigonia*. These observations might show that *L. echigonia* larvae would need to see a wider area including both above and even underneath their body in floating activity and they would mainly depend on a sense of sight. Meanwhile *L.* sp. larvae would need not to see underneath their body in moving along substrate and they would depend more effectively on senses of water flow etc.

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