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Ontogenetic variation in *Chironomus flaviplumus* (Diptera, Chironomidae) larvae

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Chironomus is a large genus of Diptera comprising about 400 species and occurs worldwide except for Antarctica. The strong morphological cross-taxon similarity of chironomid larvae renders identification at the species level difficult. Here, we analyzed the morphology of larvae of *Chironomus flaviplumus*, an easily cultured species employed as a bioindicator in polluted environments, to determine identifying morphological characteristics at the first through fourth instar. Observed differences appearing at each instar include the presence or absence of setae on the body and tubules on the 10th and 11th body segment, the number of seta interna in the mandible, and the presence or absence of ring organs in the antennae. Some specific morphological characteristics did not change after hatching. Our findings provide a reliable method for identifying *C. flaviplumus* larvae.

Keywords: Chironomidae, *Chironomus*, larvae, morphology, ontogeny

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Introduction

Chironomidae is a family of non-biting midges in the order Diptera that are superficially similar to mosquitoes. Chironomid larvae can be found worldwide in locations ranging from deep lake waters (e.g., at >1,000 m depth in Baikal Lake) to the Arctic, and show high diversity in aquatic ecosystems. The life cycle of a chironomid involves complete metamorphosis in four stages: egg - larva (first to fourth instar) - pupa - adult. The larval stage is aquatic (water column or sediment), while the adult is terrestrial and winged (Kwak, 2015; Silva *et al*., 2021). Chironomid larvae have important ecosystem functions as food sources for predators (Ferrington, 2008; Orel *et al*., 2014; Schaller, 2014; Allgeier *et al*., 2019; Baranov *et al*., 2019; Brovini *et al*., 2023) and often appear dominant in polluted freshwater environments (Prolux *et al*., 2013).

In chironomid larvae, identification at the species level using only morphological characteristics tends to be uncertain, and identification at the species level using published classification keys is difficult. For this reason, most previous studies have attempted to provide identification at the genus level (Hamerik *et al*., 2018; Cranston, 2019). Some studies have also classified species using a reverse identification method, where genetic analysis techniques are for species identification and then connected to morphological characteristics (Prolux *et al*., 2013). However, in many cases the gene sequence has been registered in the NCBI or BOLD systems databases without a clear species identification, with further clarification still outstanding (Han *et al*., 2023). A previous study confirmed the morphological characteristics of instar larvae of the genus *Chironomus*, but focused on distinguishing instar larvae collected in the field for toxicity analyses rather than on a detailed analysis of morphological characteristics for species-level identification (Rebechi and Navarro-Silva, 2012).

In this study, *Chironomus flaviplumus* was selected as the model organism because this species is easy to culture and is often used in toxicity tests due to its high resistance to contamination (Kawai *et al*., 1989; Kwak *et al*., 2002; Tang *et al*., 2010). We analyzed the morphological development process of *C. flaviplumus* larvae in the first to fourth instar stages and compiled the morphological characteristics into a primary identification key usable at the larval stage.

Materials and Methods

The samples were obtained from a breeding colony

Table 1. Number of body, head, and dissection slides for each larval instar of *Chironomus flaviplumus*.

Type of prepared slide	Instars				Total
	I	II	III	IV	
Head capsule	2	2	1	1	6
Whole body	6	2	3	3	14
Dissected	9	7	4	5	25
Total	17	11	8	9	45

maintained in a culture room of the Environmental Ecotoxicology Laboratory, Chonnam University. This colony's cultivation started about four years ago (more than 50 generations in the culture room). The breeding tank used for culture was a square insect collection container of 22.5 × 15 × 16 cm. Sediment in the tank was sand with a grain size of 0.2–0.5 mm (about the grain size of sugar, naturally sourced), and dust was removed before use. M4 medium was prepared and used as a breeding medium following OECD (2010). An aerator was installed in the tank to continuously supply oxygen, and LED lighting was attached to adjust the photoperiod. Larvae were fed with Tetra Min (Tetra, Germany), Tetra Bits (Tetra, Germany), or similar tropical fish feed crushed and mixed with the breeding water at a ratio of 1 : 10, at a volume complying with US-EPA 2000 standards. The temperature inside the laboratory was maintained at 25 ± 1°C under a light-dark cycle of 16 : 8 hours.

We used 45 larvae in this ontogenetic study. Numbers by instar and details are listed in Table 1. First instar larvae had hatched 1–12 hours previously, and second to fourth instar larvae were respectively collected 3–7 days, 9–10 days, and 15 days after hatching. For examination of samples from first to third instar larvae, slides were created (sandwich method) by fixing samples with CMC-10 mounting medium (Masters Company, USA) in a living state, and semi-permanent slides were made by dissecting the heads of some fixed samples. For the observation of fourth instar larvae, slides were created using a mount solution; glycerin-ethanol blend at the ratio 1 : 1. The head and tail segments were dissected for detailed observation, and semi-permanent slides were produced as above. Detailed morphological observations were conducted in the order of overall body shape and head structure, antenna, mandible, maxilla, mentum, and tail segment. We used the terminology of Cranston (2019) to describe and discuss the structures (Fig. 1).

Results

In the first to fourth instar larvae samples, several morphological characteristics did not change after hatching: eyespot and seta distribution on the dorsal part of the head, mentum, and ventromental plate (Figs. 1, 2). The two pairs of eyespots on the head were vertically aligned from hatching onward, and the dorsal head setae were distributed symmetrically in the same positions in all instars (Fig. 1). The size of the teeth of the mentum increased from the first to fourth instar larvae and teeth were arranged neatly, showing a slight difference in distribution shape but maintaining a tooth structure of 6-3-6. The same shape of the ventromental plate was maintained in all larvae stages, and striae always started from a similar position (Fig. 2).

The differences in morphological characteristics from the first to fourth instar larvae were as follows.

Body (Fig. 3): The first instar larva body had long bare setae between segments, fully developed antennae, and anal tubules. Two long setae existed near the anal tubules. However, no lateral and ventral tubules (a principal characteristic of the genus *Chironomus*) were observed (Fig. 3A, 3F). In second instar larva, ventral tubules appeared, and body setae declined in number and/or size. The long setae near the anal tubules were strongly degenerated and reduced (Fig. 3B). In third instar larva, lateral tubules began to appear (Fig. 3D) while most body setae disappeared; setae near the anal tubules almost disappeared completely. The only difference observed in fourth instar larva was an increased length of the lateral and ventral tubules (Fig. 3C, 3E).

Antenna (Fig. 4): In first instar larva, only the ring organ was undeveloped, while other characteristics, such as blades, accessory blades, and segments, were well developed (Fig. 4A). The only difference in antenna between first and fourth instar larvae was size, except for the ring organ, which developed from the second instar larva onward (Fig. 4B).

Pre-mandible (Fig. 5A–D): The pecten epipharyngis on the premandible began to develop from the second instar larva; only surrounding structures were slightly developed before that (Fig. 5A). There were 10 teeth on the pecten epipharyngis in the second instar larva (Fig. 5B), gradually increasing to 12–13 in the third instar larva and 14–16 in the fourth instar larva together with a size increase (Fig. 5C, 5D).

Mandible (Fig. 5E–H): The number and shape of inner teeth did not change from the first to fourth instar larvae, and the seta-subdentalis and posterior distal setae were well developed from hatching. However, seta-interna started to develop from the second instar larva (Fig. 5F). The seta-shaped organ on the dorsal tooth was observed from the third instar larva onward (Fig. 5G).

Maxilla (Fig. 5I–K): In first instar larva, only the lacina and maxillary palps were distinguishable, while the other projection-type structures only presented as trace shapes.

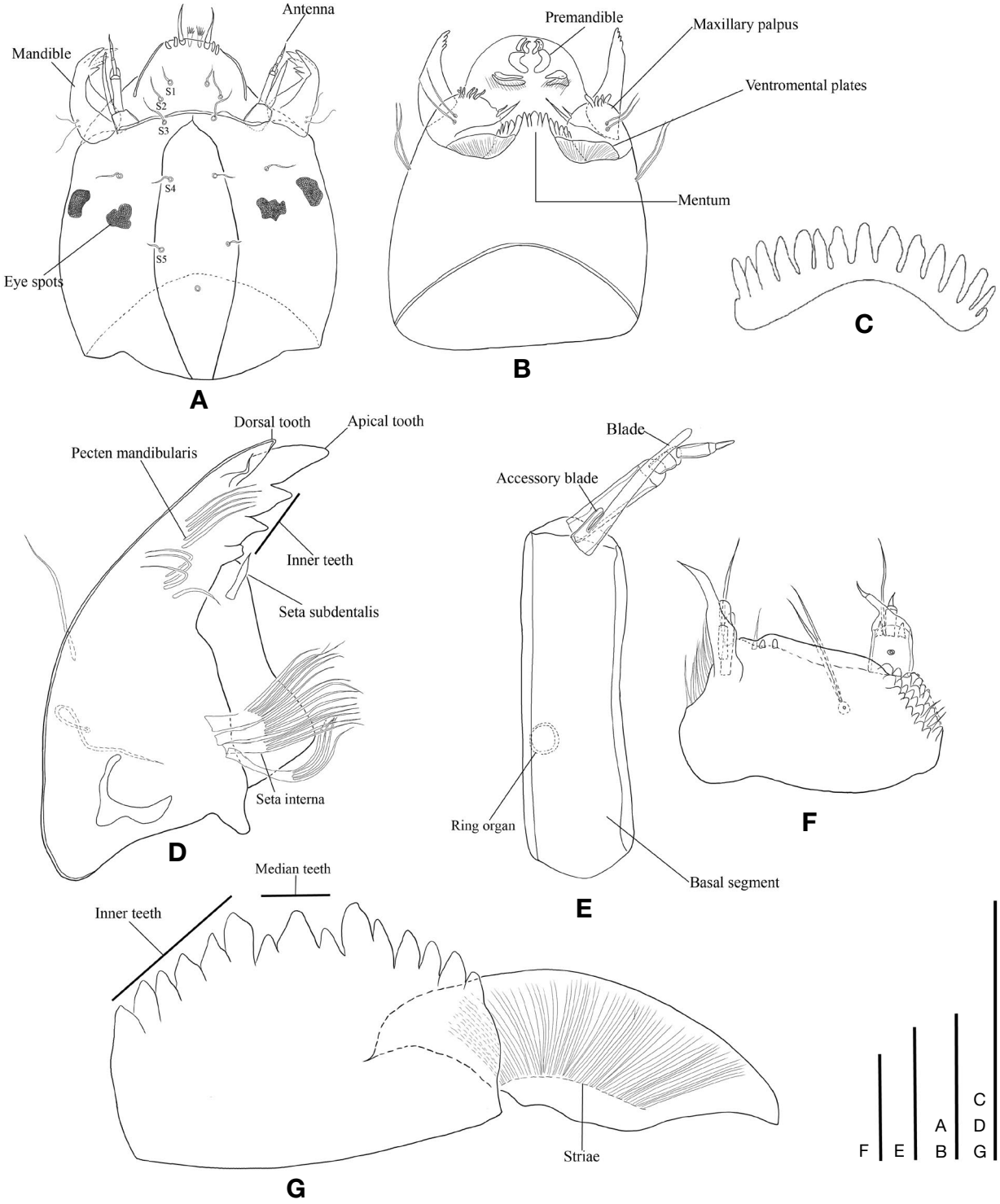

Fig. 1. Morphological designation of *Chironomus flaviplumus* fourth instar larva. A: Dorsal view of head capsule; B: Ventral view of head capsule; C: Pecten epipharyngis; D: Mandible; E: Antenna; F: Maxilla; G: Mentum & Ventromental plate. All scale bars: 50 μm.

Recognizable shapes were formed from the second instar larva onward, and the wart-like structures observable in the fourth instar larva began to appear from the third instar larva onward (Fig. 5J).

The morphological characteristics from the first to fourth instar larvae are summarized in Table 2.

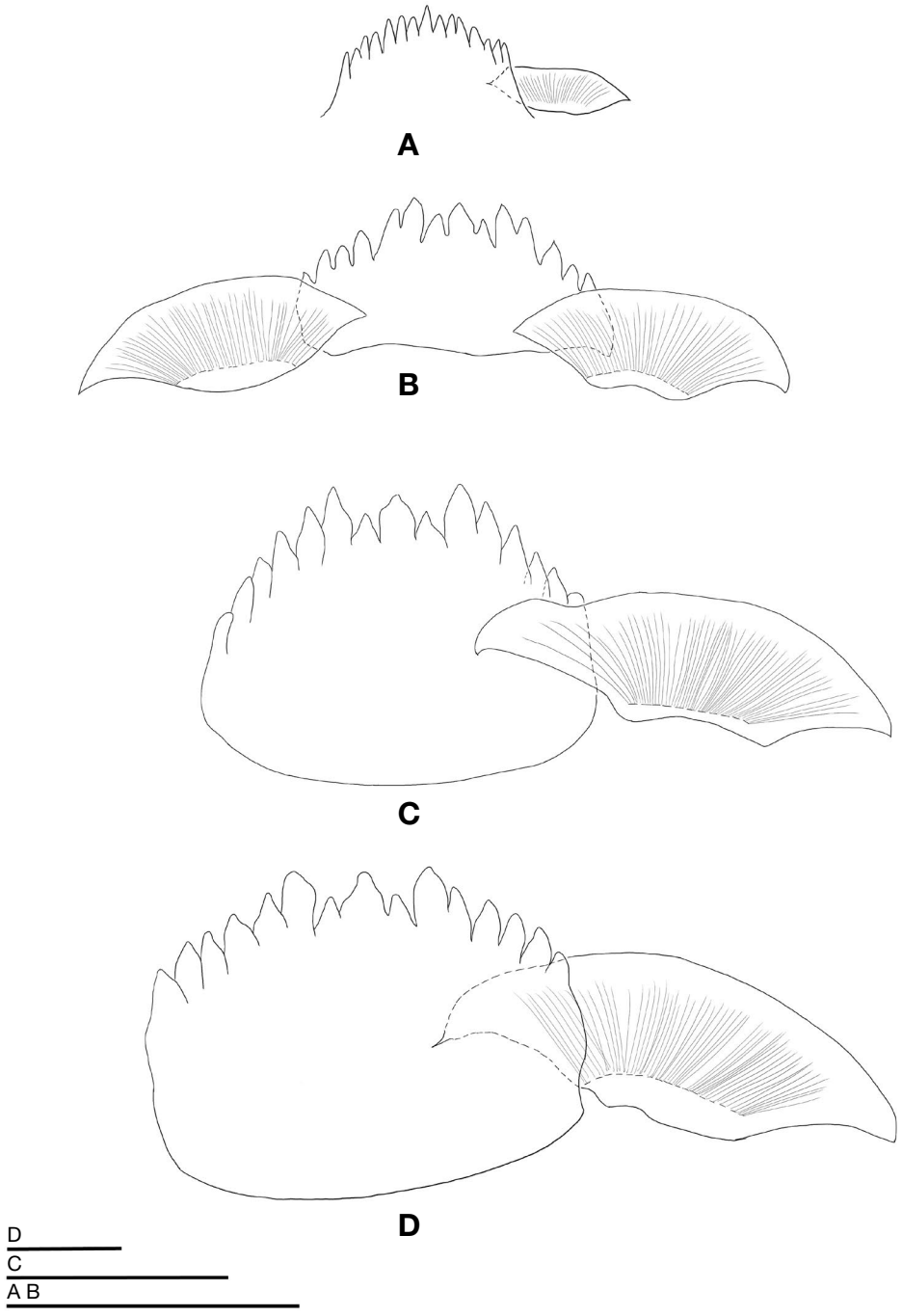

Fig. 2. Mentum and ventomental plate shape from first to fourth instar larvae of *Chironomus flaviplumus*. A: first instar; B: second instar; C: third instar; D: fourth instar. All scale bars: 50 μm.

Discussion

A previous study suggested that the appendages that could be used for classification are limited to the head capsule, antenna, and mentum (Cranston, 2019); this includes the distribution of setae on the dorsal part of the head, the shape of S1 and S2, the length ratio of the antenna segment, the characteristics of the Lauterborn organ on the antenna, and the shape and number of the central and lateral teeth of the mentum. Other researchers used these features as a pictorial key, as other morphological characteristics for determination at the genus level exist (for example, number of teeth of mentum and the shape of the antenna) (see Park *et al*., 2023, Fig. 3).

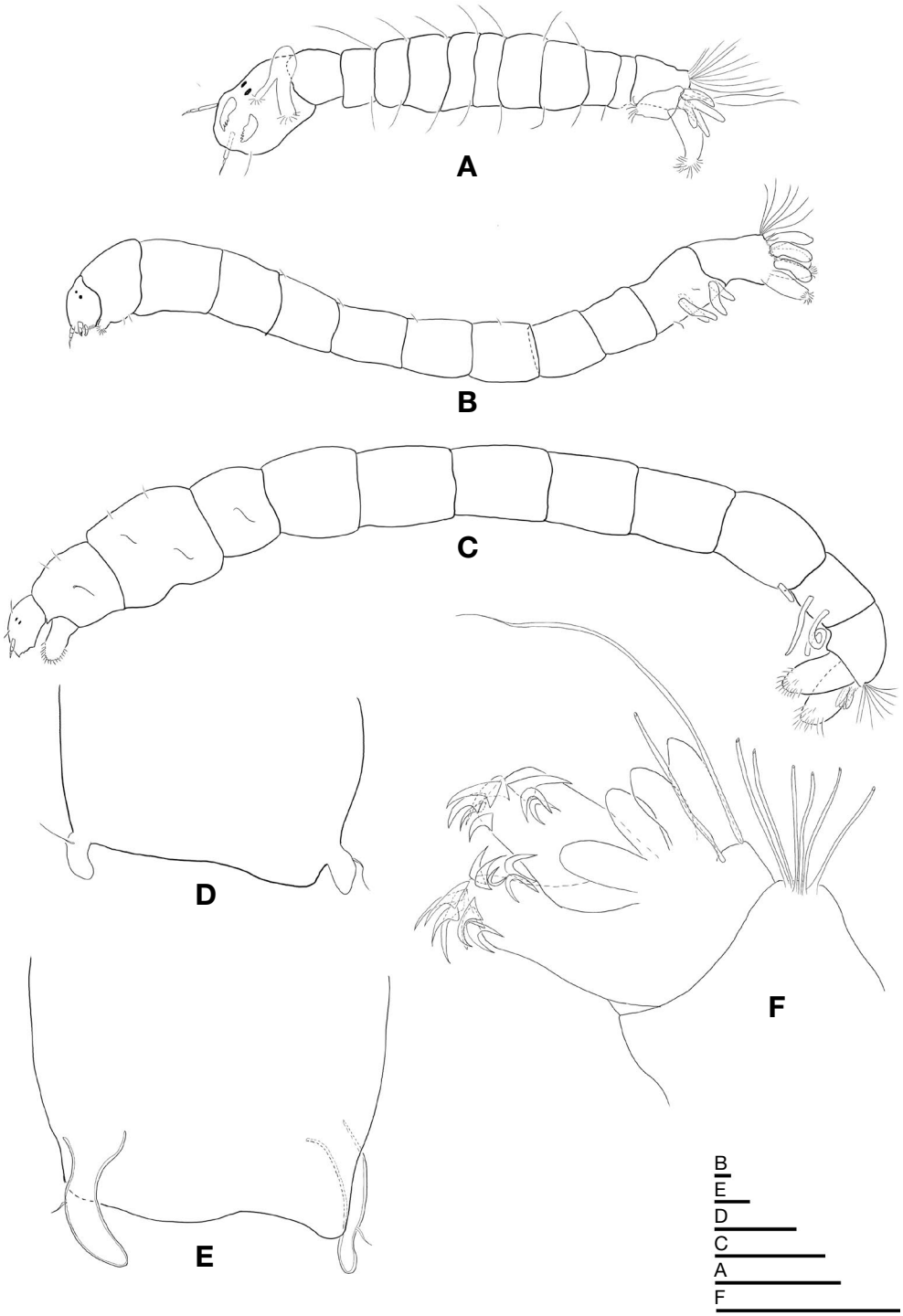

Fig. 3. *Chironomus flaviplumus* body at different instar larvae. A: whole body shape of first instar; B: whole body shape of second instar; C: whole body shape of fourth instar; D: lateral tubules of third instar; E: lateral tubules of fourth instar; F: anal tubules and anal setae of first instar. Scale bars: A, B, D, E, 100 μm; F, 50 μm; C, 1 mm.

Using the characteristics of the head capsule and differences in tubules to classify larvae in the genus *Chironomus* at the species level was attempted in the 1930s (Johannsen, 1937). Currently, larvae in the genus are divided into 10 types; salinarius, halophilus, bathophilus, fluviatilis, thummi, reductus, semireductus, melanotus, plumosus, and yama. This classification follows the color of the dorsal and ventral surfaces of the head capsule,

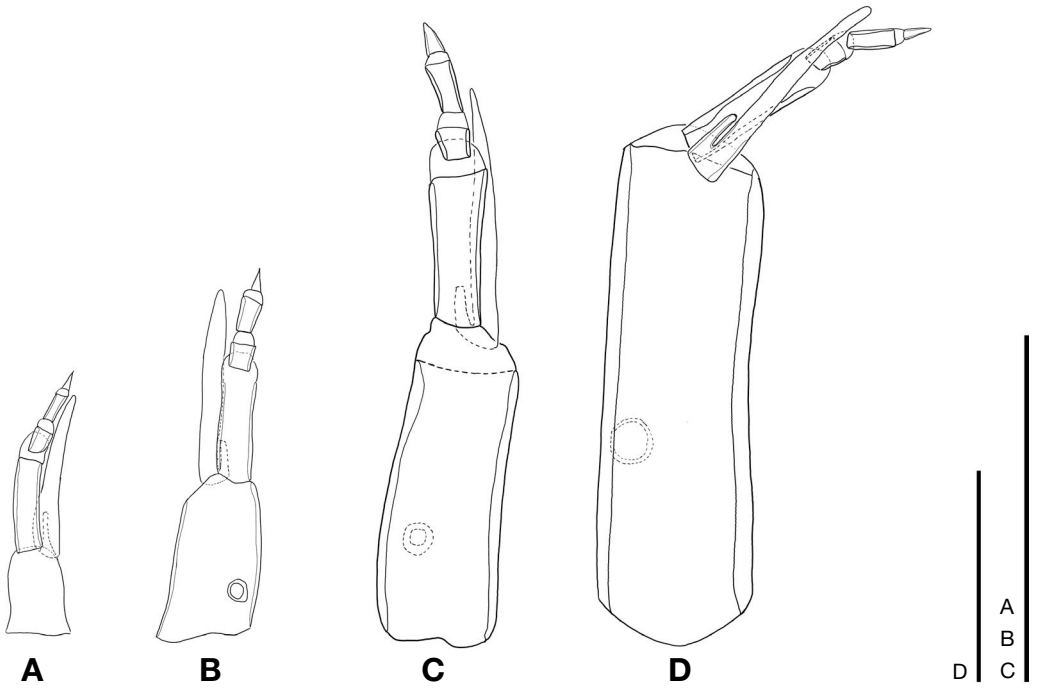

Fig. 4. Antenna shape of *Chironomus flaviplumus* first to fourth instar larvae. A: first instar; B: second instar; C: third instar; D: fourth instar. All scale bars: 50 μm.

Table 2. Variation in morphological characteristics of first to fourth instar larvae of *Chironomus flaviplumus*. −: no differences from previous stage.

Morphological types	Instars			
	I	II	III	IV
Body	Well-developed setae on the whole body; strongly developed single pair of setae near anal tubules	Setae length over whole body starts to decrease; ventral tubules begin to develop	Lateral tubules begin to develop; single pair of setae near anal tubules disappears	Setae over whole body disappear almost entirely
Antenna	Ring organ is undeveloped	Ring organ begins to develop	–	–
Mandible	Seta interna is undeveloped	Seta interna begins to develop (two setae)	Three setae interna present; dorsal tooth develops	–
Maxilla	Overall shape unrecognizable; only lacinia and maxillary palp visible	Shape begins to be recognizable	Wart-like organ begins to develop	–

Note. The morphological characteristics of setae distribution of the dorsal head and mentum do not differ between first to fourth instar larvae.

the shape of the mentum, the presence or absence of lateral tubules, the length and shape of ventral tubules, and the shape of anal tubules (Prolux *et al*., 2013; Martin and Chingangbam, 2016). However, the classification of these morphological characteristics is ambiguous enough to be difficult even under a microscope, and there are shortcomings in distinguishing region-specific species not included in this classification.

Chironomus flaviplumus larva was described by Sasa in 1978, who compared it with other species described

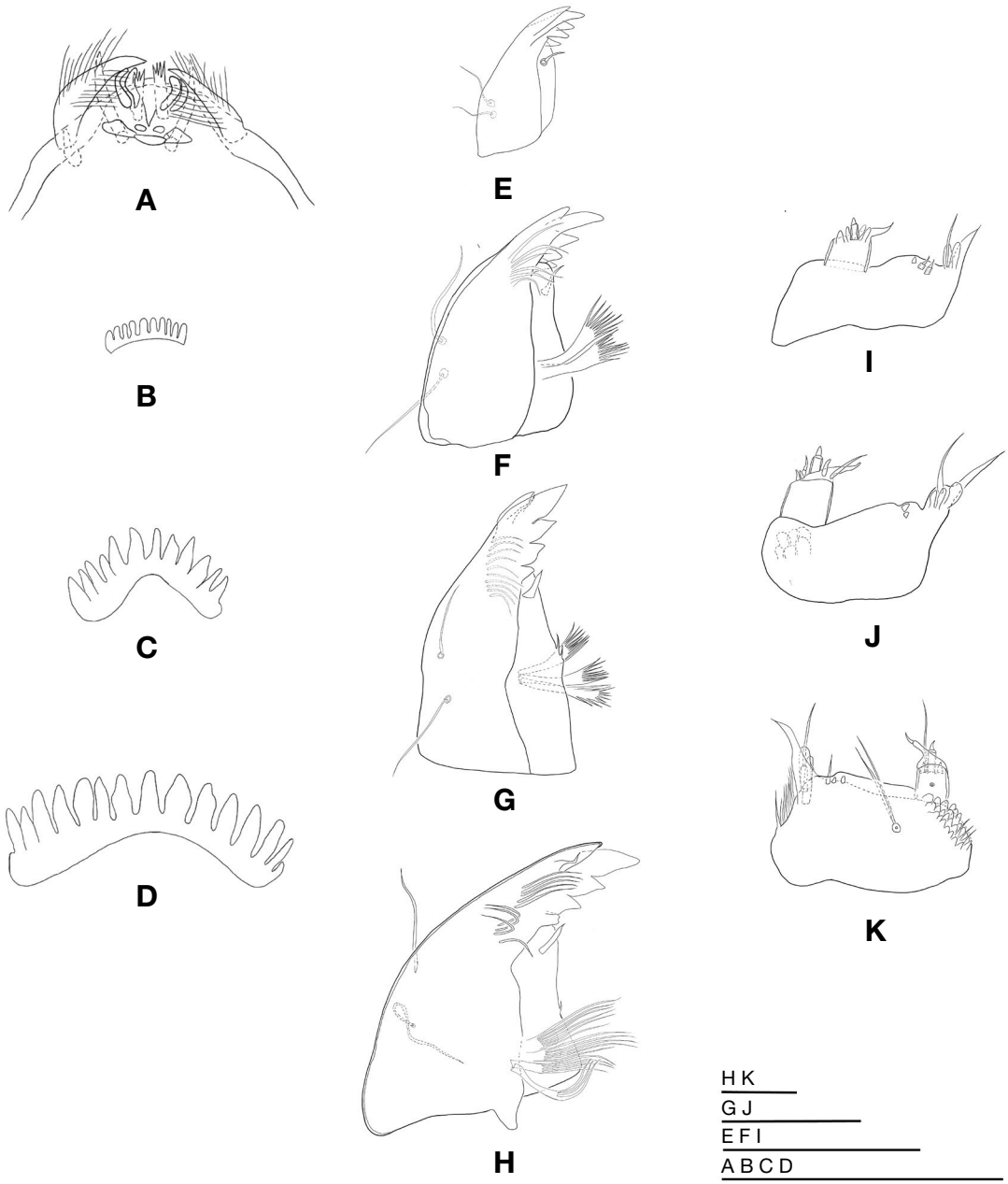

Fig. 5. Premandible, pecten epipharyngis, mandible, maxilla shape of *Chironomus flaviplumus* first to fourth instar larvae. A: premandible of first instar; B: pecten epipharyngis of second instar; C: pecten epipharyngis of third instar; D: pecten epipharyngis of fourth instar; E: mandible of first instar; F: mandible of second instar; G: mandible of third instar; H: mandible of fourth instar; I: maxilla of second instar; J: maxilla of third instar; K: maxilla of fourth instar. All scale bars: 50 μm.

in the literature. The length of the tubules of the 10th segment in *C. flaviplumus* was shorter than in *C. curcumdatus*, but similar to those in *C. kiiensis* and *C. strenzkei*, and the length of anal tubules was described as longer than in other species. However, no detailed comparison of appendages was carried out, since the subjects were categorized based on visible body characteristics. As such, the method of classifying larvae at the species level based on their morphological characteristics has not yet provided a clear solution, and we suggest that the characteristic differences among appendages for each instar shown in the present study will be helpful. We also attempted to determine whether species-level determination could be carried out based on appendages that did not change in their morphological characteristics from the first to fourth instar larvae. However, this proved

infeasible due to the substantial similarity in such characteristics among species within the same genus (e.g., color of inner mandible teeth and characteristics of mentum, ventromental plate, dorsal part of head, antenna).

In order to identify these larvae at the species level, it is necessary to compare the characteristics maintained from the first instar larva onward, but the characteristics that develop from the second or third instar larvae onward may also be helpful. The dorsal tooth of the mandible identified in this study is one example. We suggest it will be necessary to create a classification key at the species level targeting the morphological features that appear prominently in the fourth instar larva, without considering genus characteristics.

Classification of chironomid larvae at the species level based on morphological characteristics remains a problem needing attention. Classification of adults may be straightforward, but in metamorphosing insects, it can be assumed that larval characteristics will change in the fourth instar before the pupa stage. As determining these features is difficult, detailed morphological studies are required to compile usable keys.

Conflicts of Interest

The author of this paper has no affiliation with any interests and is solely responsible for the paper.

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