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Ontogenetic Changes in Pigmentation Pattern in *Mesanthura miyakoensis* (Crustacea: Isopoda: Anthuridae)

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Species in the anthurid isopod genus *Mesanthura* have specific, dorsal dark pigmentation patterning on the body. Though *Mesanthura* species have traditionally been distinguished mainly by differences in the dorsal pigmentation pattern in females, the stability of the pigmentation pattern within species had not been investigated, and information was lacking on ontogenetic variation in the pattern. Our study showed the following for *M. miyakoensis*. (1) Mancae begin to show dorsal pigmentation in the marsupium roughly 9 days before their release. (2) The pigmentation pattern in the first-instar mancae (first free-living stage) differs from that in later instars. (3) The pigmentation pattern in females is discrete and stable from putative second-instar mancae through females lacking oostegites, and distorted but recognizable in ovigerious females. (4) The pattern in males is different from and less discrete than that in females; it remains similar through the molt from subadult to adult male but changes markedly with age, leading to heavy pigmentation of the body. (5) The pigmentation pattern in mancae and females remains stable and observable after storage in ethanol for at least 13.7 months. Our results suggest that comparisons of pigmentation pattern across species in *Mesanthura* taxonomy should be restricted to females in the post-manca or later stages.

Key words: Anthuroidea, color, Crustacea, manca, sexual dimorphism

INTRODUCTION

The moderately diverse anthurid genus *Mesanthura* Barnard, 1914 is common at shallow depths in tropical and temperate seas (Müller, 1993) and currently contains 51 valid species (Boyko et al., 2024). Species in *Mesanthura* have a species-specific dark pigmentation pattern on the body (Poore, 2001) and are distinguished mainly by differences in the dorsal pattern in females (Wägele, 1984a) (Fig. 1). Other interspecific morphological differences are minimal (Müller, 1993).

This taxonomic focus on pigmentation has potentially led to ambiguity or misidentification. Anthurids undergo direct development and are generally protogynous (Poore and Bruce, 2012), meaning that after release from the mother's marsupium (brood pouch), young *Mesanthura* isopods likely first develop as females and then change sex to become males. In some species where only males were known, differences in pigmentation pattern were used to distinguish those males from the females of putative congeners (e.g., Wägele, 1984b). However, this approach assumed that no ontogenetic changes in pigmentation pattern occurred (cf. Poore and Bruce, 2012) as the females developed, or after they developed into males.

To our knowledge, no previous study has systematically investigated ontogenetic changes in pigmentation pattern in *Mesanthura*. Some studies (e.g., Müller, 1993) have reported the sexes to have different patterns, whereas others (e.g., Pires, 1981) have reported the same pattern. Only in *Mesanthura protei* Kensley, 1980, polymorphism of female pigmentation patterns has been reported (Kensley and Poore, 1982; Chew et al., 2015). None of these previous studies molecularly confirmed whether their studied animals were conspecific; it is possible that ones they identified as the same species (i.e., males and females; polymorphic females) were not, in fact, conspecific.

In this study, we examined ontogenetic changes in the pigmentation pattern in *Mesanthura miyakoensis* Nunomura, 1979 to address (1) how the pattern differs between males and females; (2) in what sex and at what ontogenetic stages the color pattern is taxonomically informative; and (3) whether storage in ethanol affects the color pattern. Through rearing observations, we were able to examine the time at which pigmentation first appears in brooded mancae, and to obtain data for the first free-living instar stage and two stages of males. Finally, we used DNA sequences to assess conspecificity for a male and female with markedly different pigmentation patterns.

MATERIALS AND METHODS

Animals, staging, and measurement

Twenty-six isopods were collected from intertidal coral rubble at Tatsukushi (32°47′09.3″N 132°51′22.2″E), Kochi, Shikoku,

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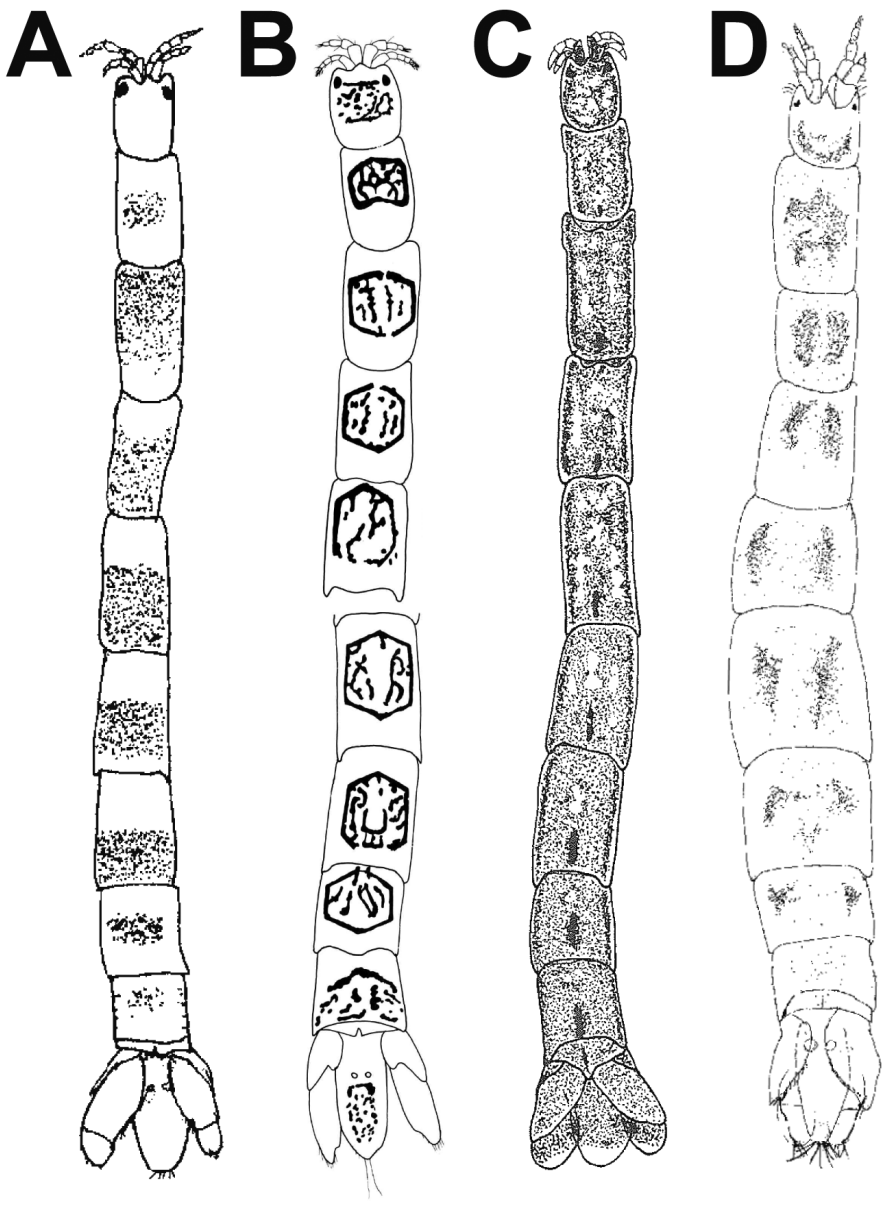

Fig. 1. Examples of pigmentation patterns in *Mesanthura* females. **(A)** *M. cinctula* Nunomura, 2006. **(B)** *M. miyakoensis* Nunomura, 1979. **(C)** *M. nigrodorsalis* Nunomura, 1977. **(D)** *M. saikaiensis* Nunomura, 2016. Each illustration is reproduced with permission from Nunomura (2006), Nunomura (1979), Nunomura (1977), and Nunomura (2016), respectively.

Japan, on 13 June 2021 and 26 July 2021, and from 1 m depth at the same locality on 10 October 2022. All individuals were photographed alive to record pigmentation patterns, 15 of which (seven mancae, two post-mancae, five females lacking oostegites, and one subadult male) were then fixed and preserved in 70–99% ethanol; the others were used for rearing experiments (see below). We categorized individuals into one of six ontogenetic stages: (1) **manca**, without pereopod 7; (2) **post-manca**, with incomplete pereopod 7; (3) **female lacking oostegites** (components of the marsupium), with complete pereopod 7 and slender antennula but without oostegites; (4) **ovigerous female**, with complete pereopod 7, slender antennula, and oostegites; (5) **subadult male**, with enlarged eyes and swollen antennula lacking aesthetascs; and (6) **adult male**, with enlarged eyes and swollen antennula covered with numerous aesthetascs. Ovigerous females brood eggs and hatchlings in a marsupium and release mancae. Head length (from tip of rostrum to posterior end of head) was measured from digital images by using ImageJ (http://rsb.info.nih.gov/ij).

Verification of male–female conspecificity with 16S rRNA sequences

To pair a conspecific male and female molecularly, we determined partial mitochondrial 16S rRNA (16S) sequences from three specimens, namely, one subadult male (similar pigmentation pattern as Fig. 5A) collected on 13 June 2021, and one post-manca (similar pigmentation pattern as Fig. 3C) and one ovigerous (Fig. 3E) female collected on 10 October 2022. DNA was extracted from a pereopod of each specimen by using a NucleoSpin Tissue XS Kit (Macherey-Nagel, Germany). Primers Copepod16S_F and Copepod16S_R (Kakui et al., 2023) were used for PCR amplification and cycle sequencing; amplification conditions with KOD One (Toyobo, Japan) were as described by Kakui et al. (2023). Nucleotide sequences were determined with a BigDye Terminator Kit ver. 3.1 and a 3730 DNA Analyzer (Life Technologies, USA). The sequences obtained were deposited in the International Nucleotide Sequence Database (INSD) through the DNA Data Bank of Japan, under accession numbers LC787697 (subadult male), LC787698 (post-manca), and LC795631 (ovigerous female).

Rearing

Eleven individuals (four mancae, three post-mancae, two females lacking oostegites, one ovigerous female, and one subadult male) collected on 10 October 2022 were maintained in several small aquaria filled with seawater from the sampling locality at room temperature and under ambient light. The ovigerous female released seven mancae during the study period, and these were maintained individually, as above. Reared individuals were intermittently observed and photographed to record dorsal pigmentation patterns, and were fixed and preserved in 70–99% ethanol when they died. Potential food organisms (several polychaetes, several tanaidaceans, a nematode, and pieces of algae) collected with the *Mesanthura* individuals on 10 October 2022 were offered occasionally, but we did not observe the *Mesanthura* individuals feeding on any of them.

RESULTS AND DISCUSSION

Verification of male–female conspecificity with 16S rRNA sequences

The genetic distances among the 16S sequences of three specimens were 0.2–0.9% (p-distance), much lower than the 3–10% “gray zone” (Riehl et al., 2018) in which distances can represent either interspecific or intraspecific variation in various isopods. This confirmed that our females and males were conspecific, and we treated the individuals used in this study as all the same species.

Early ontogeny and pigmentation of mancae in the marsupium

Ten eggs were observed in the marsupium of a female on 10 October 2022 (Figs. 2A, 3E). The female released seven mancae: two on 6 November 2022 (this release may have been induced through pipetting the female), one between 6 and 11 November, and four on 11 November. This ovigerous female lost the marsupium through one molt; the anterior and posterior halves of her exuvia were retrieved on 22 November.

The embryos on 10 October were oblong and enclosed with chorion, suggesting they had been laid into the marsupium a few days earlier (cf. Mercer et al., 2008); if this was correct, then the brooding period (from a few days before 10 October to 6 November) of this individual was about 1 month. Three embryos may not have hatched, though we do not know their fate.

Dorsal pigmentation began to be evident in the brooded mancae on 28 October (Fig. 2D), 9 days before the first of them were released, and gradually became more intense (Fig. 2). A pigmentation pattern similar to that of released mancae was observed on 31 October, 6 days before the first mancae were released (Figs. 2E, 6A).

Ontogenetic changes in pigmentation pattern after release of mancae from marsupium

We observed the pigmentation pattern for 26 wild-caught individuals (11 mancae, five post-mancae, seven females lacking oostegites, one ovigerous female, and two subadult males), seven mancae released from the ovigerous female, the same female after molting and becoming a female lacking oostegites, and one adult male derived from one of the two subadult males that molted.

Mancae and females

Two patterns of dark, reddish-brown pigmentation (Fig. 3A, B) were evident in mancae. In Type A (Figs. 3A, 6A) observed in seven wild-caught mancae and all reared mancae in their first instar after release from the marsupium, there was a relatively broad pigmented area in the anterior half of the head, and a narrower band-like pigmented area on each pereonite and fused pleonites 1–5.

The Type B (Fig. 3B) pattern observed in four wild-caught mancae was quite similar to that seen in post-mancae (Fig. 3C) and females lacking oostegites (Fig. 3D). Type B individuals (head length 0.246–0.264 mm; average 0.255 mm; n = 4) were larger than Type A individuals (head length 0.211–0.244 mm; average 0.231 mm; n = 14), suggesting that the manca stage comprised at least two instars (Fig. 4). We did not observe a molt by a first instar manca, and it remains unknown exactly when the pigmentation pattern changes from Type A to Type B. Furthermore, we cannot rule out that the manca stage may contain more than three instars.

The pigmentation pattern from Type B mancae through females lacking oostegites was as follows. (1) In the head, a relatively narrow band of dark brown pigmentation loci (nearly solid, Fig. 3B, C; as rows of dots, Fig. 3D) occupying the anterior half. (2) In pereonite 1, a transverse band of pigmentation (irregular, Fig. 3B; rectangular, Fig. 3C; rounded rectangular, Fig. 3D) occupying less than half the pereonite length, located mid-pereonite. (3) In pereonites 2 and 3, a rounded-square (convex anteriorly, straight posteriorly) zone of pigmentation with dots inside, occupying more than half of each pereonite, located mid-pereonite. (4) In pereonites 4–6, a roughly square (convex anteriorly, straight or convex posteriorly) zone of pigmentation occupying approximately half the segment, located mid-pereonite, with an elliptical, non-pigmented “window” along the midline. (5) In pereonite 7, similarly to pereonite 1, a narrower, transversely subrectangular zone of pigmentation occupying roughly half the pereonite length, more or less mid-pereonite or closer to the anterior end. (6) In fused pleonites 1–5, a dome-shaped zone of pigmentation with a weakly (Fig. 3B) to strongly (Fig. 3C, D) convex anterior margin and straight posterior margin, half or less the segment length, located mid-pleon.

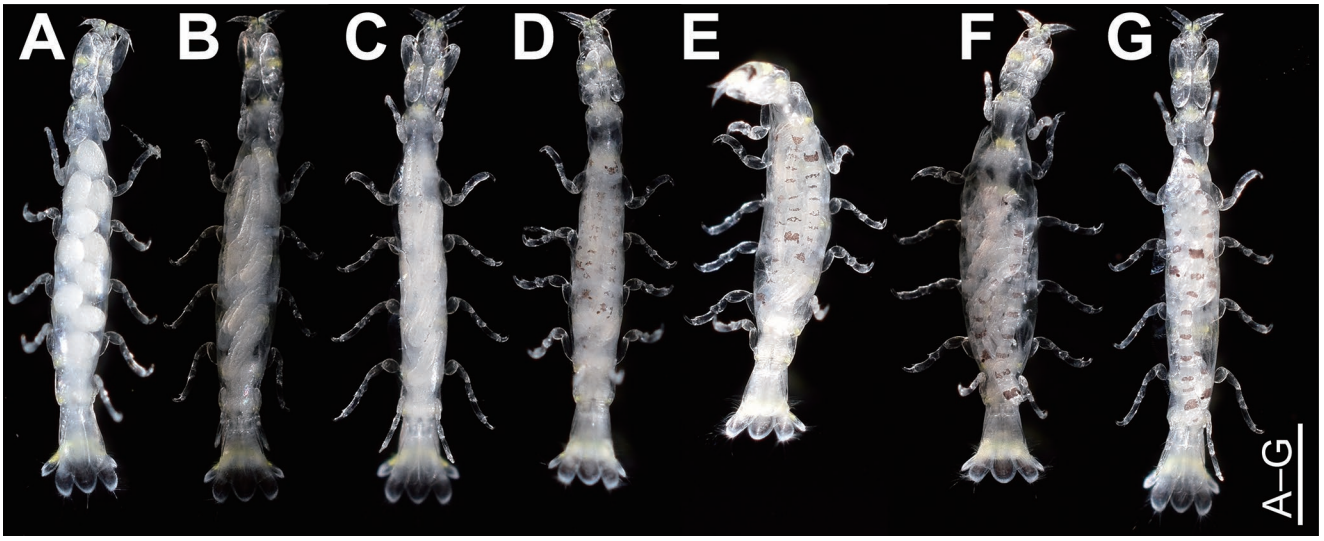

Fig. 2. Development, hatching, and brooding of mancae in *Mesanthura miyakoensis*. **(A–G)** Ventral views of ovigerous female on various dates in 2022. **(A)** 10 October, embryos inside marsupium. **(B)** 20 October, embryos. **(C)** 23 October, embryos. **(D)** 28 October, mancae. **(E)** 31 October, mancae. **(F)** 6 November, mancae. **(G)** 9 November, mancae. Scale bar: 1 mm.

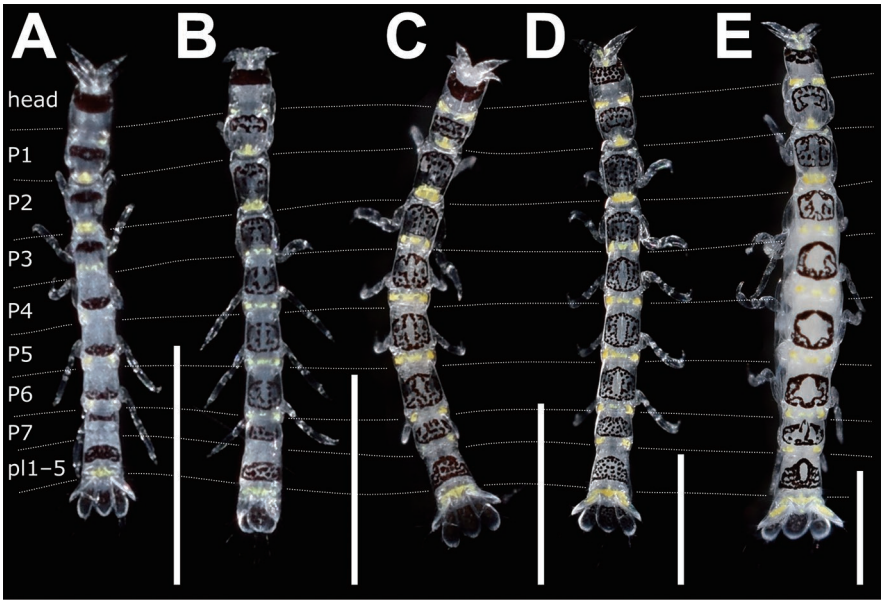

Fig. 3. Pigmentation patterns of female *Mesanthura miyakoensis*, living individuals, dorsal views. **(A)** Manca, first instar, Type-A pigmentation pattern. **(B)** Manca, second (?) instar, Type-B pigmentation pattern. **(C)** Post-manca. **(D)** Female lacking oostegites. **(E)** Ovigerous female. P1–7, pereonites 1–7; pl1–5, fused pleonites 1–5. Scale bars: 1 mm (note that the scale bars are progressively smaller; at the same scale, the individuals illustrated would increase in size from left to right).

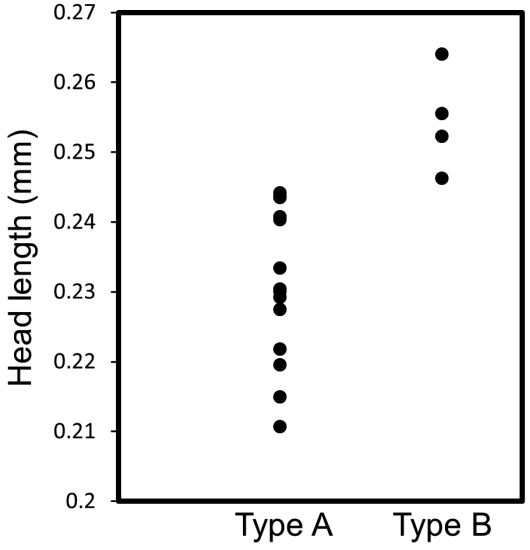

Fig. 4. Distribution of head lengths for mancae of Type A (n = 14) and Type B (n = 4) pigmentation patterns in *Mesanthura miyakoensis*.

The pigmentation pattern in the ovigerous female (Fig. 3E) appeared superficially different from those in Fig. 3B–D, in that many of the pigmentation zones had a moderately (P3, P7, fused pleonites 1–5) to greatly (P4–P6) enlarged non-pigmented "window" along the midline. Nonetheless, the general shapes of the pigmentation zones and their relative sizes and positions persisted until females became ovigerous. Enlargements of the non-pigmented median area may reflect allometric changes within the pigmentation fields as females increase in size and become ovigerous; taking into account differences in the scale bars in Fig. 3, pereonites 4 and 5 in the ovigerous female (Fig. 3E) were roughly twice as wide as in the female lacking oostegites (Fig. 3D) and proportionally even wider than in Type B mancae (Fig. 3B) and post-mancae (Fig. 3C).

In addition to the dark, reddish-brown pigmentation, yellow pigmentation was observed in all individuals except released Type A mancae. Yellow pigmentation occurred mainly at the anterior edge of pereonite 1, the posterior edge of each pereonite, and the base of the tail fan (Fig. 3). Some individuals partially lacked the yellow pigmentation (e.g., an individual without yellow pigmentation at the posterior edge of pereonite 7; Fig. 3B).

Males

Subadult males (Fig. 5A) had medial strands of pigmentation on the head. Small, paired, diagonal or comma-shaped patches of pigmentation were evident anterolaterally on pereonites 1–3 and posterolaterally on pereonites 4–7. On fused pleonites 1–5, three complete, narrow transverse bands of pigmentation were evident posteriorly. Yellow pigmentation was seen in subadult males, as in females, but gradually faded after the molt to adult males and eventually disappeared (Fig. 5B–E). It is unclear whether the pigmentation pattern of subadult males changes gradually within the instar.

Immediately after the molt to adult (Fig. 5B), males retained the subadult pattern, though many of the pigmentation loci were slightly enlarged and more intense. With age following the molt (Fig. 5C–E), and no further molting, the pigmentation loci became increasingly large, extending posteriorly (P1–P3) or anteriorly (P4–P7) along each side of the segment; the posterior half of the head developed an X-shaped pattern (Fig. 5C) and eventually became entirely diffusely pigmented (Fig. 5D, E). The male in Fig. 5E, 27 days after the molt to adult, shows a heavily pigmented head, three nearly complete, narrow transverse pigmented bands in fused pleonites 1–5, and dark pigmentation diffused throughout the whole body. This male died on 3 December 2022, 51 days after the molt to adult.

Impact of ethanol fixation on pigmentation pattern

In various crustacean groups, pigmentation and color are known to fade after long-term fixation in ethanol (Nagasawa and Mizuno, 2022). We thus examined how fixation in ethanol affected the pigmentation pattern in *M. miyakoensis*.

The same dark pigmentation as seen in the living state was maintained in most specimens preserved in ethanol for nearly one year (e.g., compare Fig. 6D with Fig. 6E–G). The only exceptions were two mancae released on 6 November 2022 (Fig. 6A); their pigmentation bands became more

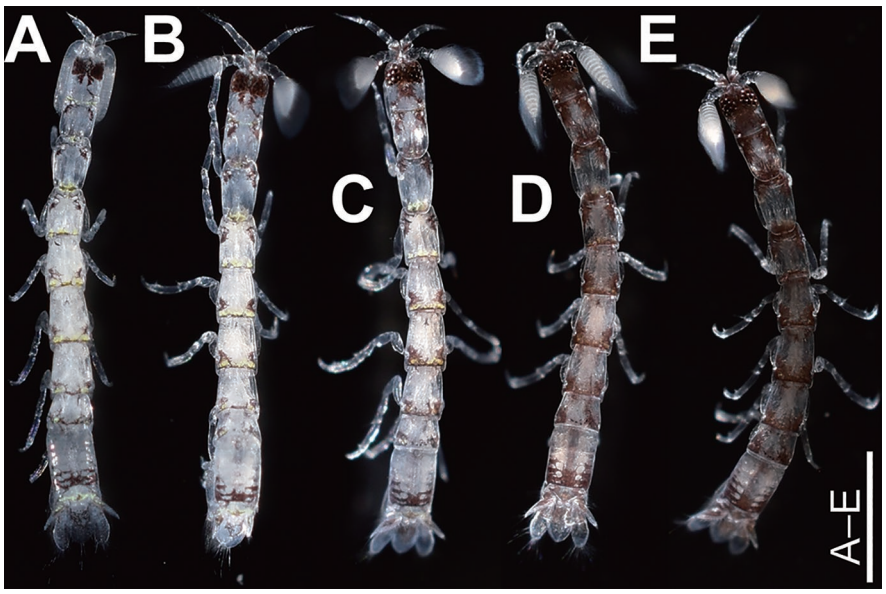

Fig. 5. Changes in pigmentation pattern in a male *Mesanthura miyakoensis*; living individual, dorsal views. **(A)** Subadult male on 10 October 2022. **(B–E)** Same individual as an adult male, 1 day **(B)**, 5 days **(C)**, 19 days **(D)**, and 27 days **(E)** after molting to adult on 13 October. Scale bar: 1 mm.

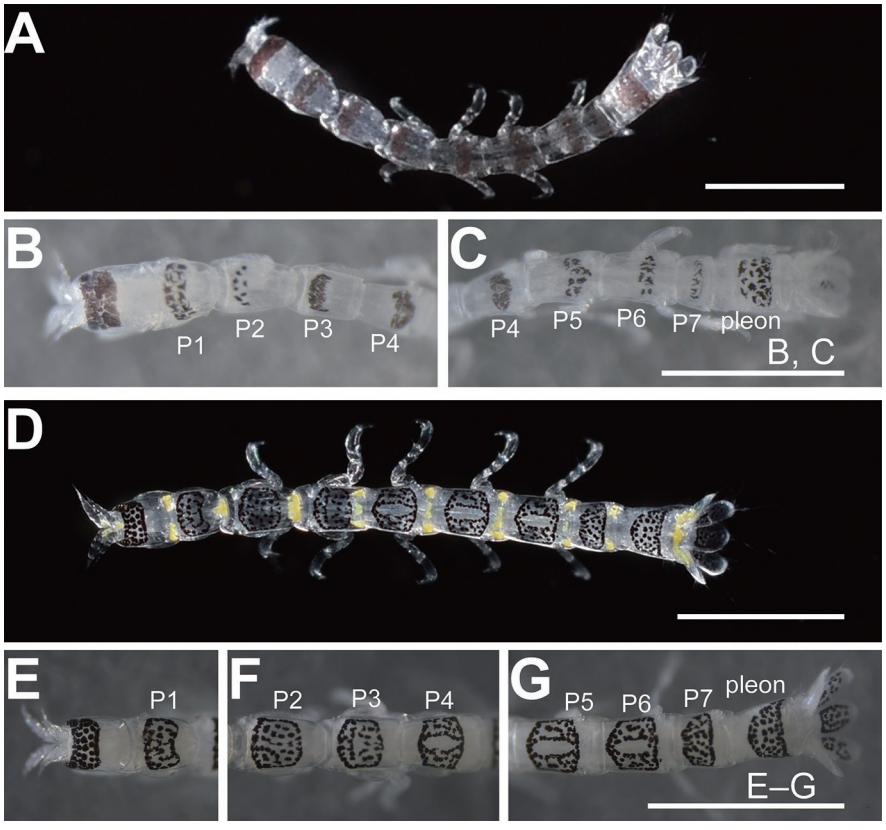

Fig. 6. Pigmentation pattern of living and ethanol-fixed specimens of *Mesanthura miyakoensis*. **(A–C)** Manca released on 6 November 2022. **(A)** Living individual. **(B, C)** Anterior and posterior parts of same individual photographed on 21 November 2023, after ca. 360 days in ethanol. **(D–G)** Female lacking oostegites. **(D)** Living individual. **(E–G)** Anterior, middle, and posterior parts of same individual photographed on 23 November 2023, after ca. 410 days in ethanol. P1–7, pereonites 1–7. Scale bars: 0.5 mm **(A–C)**, 1 mm **(D–G)**.

gappy and dotted after fixation (Fig. 6B, C) though they retained their general sizes and shapes. We interpret this to mean that discrete pigmentation loci maintained their pigmentation, whereas pigment diffusing into tissues surrounding these loci was dissolved by the fixative. Yellow pigmentation disappeared in all ethanol-fixed individuals (Fig. 6E–G vs Fig. 6D).

Our observations on the effects of fixative extended only over about 13.5 months, whereas specimens in museums remain in fixative for decades or even centuries. From our limited observations, we can conclude only that pigmentation patterns (in females) remain relatively intact and taxonomically informative for as long as they remain visible.

Our observations showed that (1) ethanol fixation may cause artifactual pigmentation pattern variation, at least in mancae and (2) some colors disappear after ethanol fixation (yellow in this study).

Taxonomic utility of pigmentation patterns in *Mesanthura* taxonomy

With regard to the taxonomic utility of pigmentation patterns in *Mesanthura* taxonomy, we can summarize the following conclusions from our study of *M. miyakoensis*.

1) The pigmentation pattern in males differs from that in mancae and females. Subadult and adult males (separated by a molt) show a similar pattern. As adult males age, pigmentation loci enlarge until the whole body in living animals appears reddish brown. The pigmentation patterns are not as discrete as in females, and this, coupled with the diffuse pigmentation in older individuals, reduces the taxonomic utility of pigmentation in males.

2) The pigmentation pattern in first-instar mancae differs from that in putative second (and subsequent?) instar mancae. Segmental pigmentation zones are quite similar from putative second-instar mancae to females lacking oostegites, making the overall pigmentation pattern at these stages a useful taxonomic character.

3) The pattern of dark-brown pigmentation seen from putative second-instar mancae to females lacking oostegites was well preserved for up to 410 days (~13.7 months) in ethanol, suggesting that as long as a reason-

able amount of pigment remains, the pigmentation pattern can be compared across species for preserved individuals in these stages.

For taxonomic comparisons among *Mesanthura*, the pigmentation pattern should be observed in living individuals where possible, and additionally in multiple ethanol-fixed female individuals, at the post-manca or later stages.

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COMPETING INTERESTS

We declare no competing interests.

AUTHOR CONTRIBUTIONS

SS conceived and designed the study, performed fieldwork, made observations, and compiled the figures. SS and KK contributed to writing the manuscript and have read and approved the final draft.

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