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Postembryonic development of sexual dimorphism in a skeleton shrimp

（トゲワレカラにおける後胚発生過程での性差発現）

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Department of Biological Sciences, Graduate School of Science,

The University of Tokyo

東京大学大学院理学系研究科生物科学専攻

Yohei Otomo

大友　洋平

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ABSTRACT

Sexual dimorphism is the phenotypic difference between males and females which is universally seen over the animal kingdom. However, because sexes of the same species share most of the genes that control their development and growth, it has been one of the major research subjects to understand mechanisms of evolution and development of sexually dimorphic traits. Generally, sexual differences appear during the course of postembryonic development. Since sexually dimorphic traits, such as male weapons or ornaments, directly affect on the reproductive success, it should be adaptive that the development of these traits completed at the timing of sexual maturation, as observed in most of insects. However, insects are known to stop to grow after sexual maturation, and this mode is called determinate growth. In contrast, because many animals exhibit indeterminate growth in which growth and morphological alterations occur even after sexual maturation, sexually dimorphic traits are also modified. Therefore, it is crucial to reveal growth patterns of each species and each sex during the whole lives of animals with indeterminate growth, for understanding the evolution and developmental mechanisms of sexual dimorphism.

Thus, how sexual dimorphism in indeterminate growth appears and how growth pattern in each sex affects on its development would be important questions to answer. Nevertheless, little is known about the growth pattern, and the developmental bases underlying the expressions of sexually dimorphic traits, especially in animals with indeterminate growth, due to the difficulty to select the appropriate study model. Among crustaceans which usually show the indeterminate growth, caprellids (Caprellidae: Amphipoda), also known as skeleton shrimps, often show apparent sexual dimorphism during relatively short generation period. Due to these biological features, I thought that caprellids could be an ideal study model. In this study, therefore, I focused on the species of caprellids

Caprella scaura Templeton, 1836 as an experimental model in which a rearing and breeding system were successfully established.

The aim of this study is to examine how sexually dimorphic traits appear and become conspicuous during the whole life of an individual, and how that pattern differs between sexes in animals with indeterminate growth. To answer these questions, this study focused on the expression pattern of the sexual dimorphism in a skeleton shrimp, *C. scaura*.

Firstly, to elucidate the patterns of morphological changes during postembryonic development in both sexes, extensive observations were carried out on fixed samples of *C. scaura*. The results showed that sexually dimorphic traits appeared at the timing of sexual maturation in both sexes. However, they also suggested that there was a sexual difference in the developmental course of sexually dimorphic traits after sexual maturation, in which the size of male weapon increased through their lives, whereas the growth of female brood pouch, in which adult females incubate eggs until hatching, stopped after the appearance at the adult stage.

In crustaceans, the expression of sexually dimorphic traits is regulated by various hormones secreted from gonads and the male-specific gland, called androgenic gland. Therefore, in the next, the relationship between the development of these organs and sexual dimorphism was investigated. As a result, although the formation of female brood pouch was occurred simultaneously with ovarian maturation, spermatogenesis in males seemed to have started even in juveniles without conspicuous sexually dimorphic traits. This suggested the possibility of the presence of polymorphism among sexually matured males, and even alternative reproductive strategies in males of *C. scaura*.

To investigate the possibility of sexual differences in the developmental process of sexually dimorphic traits and the timing of sexual maturation, knowledge on the relationship between molting and morphological alteration in *C. scaura* is essential, because growth, sexual maturation and morphological alteration in arthropods must be associated with

molting. But, as the instar of each individual could not be determined from fixed samples, breeding experiment of newly hatched larvae were performed to track the growth via molts during the whole lives of them.

The results showed that sexually dimorphic traits appeared at the approximately seventh instar in both sexes, and males rapidly increase their body size after sexual maturation. Furthermore, unexpectedly, the number of molts clearly differed between sexes, in which males stopped to grow up to tenth instar, whereas females continue to molt to nineteenth instar until their death. These suggested that it was important that the sharp increase of growth rate and cessation of molting which occurred only in adult males were the key on the development of sexually dimorphic traits in *C. scaura*. Thus, it is possible that the genes differentially expressed only in adult males produce the sexual difference in the developmental trajectories, leading to the expression of sexual dimorphism.

Therefore, lastly, to search genes responsible for sexual differences in growth pattern in *C. scaura*, the expression patterns were compared between sexes at each postembryonic stage. These results showed that in adult males, *doublesex* (*dsx*) was highly expressed, while *shade* (*shd*) which involved in the synthesis of molting hormone, was down-regulated. *dsx* is known not only to promote sex determination and differentiation to males in crustaceans, but also to suppress expressions of genes related to molting. Thus, it is suggested that sex-specific expression and also temporal dynamics of expression of *dsx* may play a central role to produce the differences in the developmental trajectory of sexually dimorphic traits in *C. scaura*.

In summary, this study revealed the morphological alteration and the developmental trajectories of sexually dimorphic traits during the whole life of an individual in each sex of *C. scaura*. Although sexual dimorphism of *C. scaura* appeared at approximately same timing in both sexes, male weapon traits became conspicuous especially after sexual maturation, and the development of sexual dimorphism was affected by different growth rate and the

number of molts. Furthermore, these sexual differences might be regulated by the sex-specific expression, pluripotent function and temporal dynamics of *dsx* gene. This sexual difference in postembryonic development of *C. scaura* would be originated from differences in reproductive strategies between sexes. Given that examples of polymorphism and alternative reproductive strategies in males of many species of crustaceans, and also in other animals with indeterminate growth, it is possible that changes in the regulation of sex-specific genes may be one of the sources of life history evolution.

GENERAL INTRODUCTION

Sexual dimorphism and postembryonic developmental trajectories

Sexual dimorphism is the phenotypic difference between males and females. Because sexual dimorphism is widespread among the animal kingdom, it has attracted considerable interest from biologists to understand how the morphological diversity found in animals had evolved (**Figure 1**; Emlen, 2008). Since sexes of the same species share most of the genes that control development and growth, it could be assumed that the sex-biased expressions and regulations of those shared genes accomplish adult sexual dimorphism during development (Badyaev, 2002).

Sexual dimorphism generally appear during the course of postembryonic development, and the pattern of morphological difference between sexes varies depend on the ecology and/or reproductive biology of each species (Shine, 1989; Temeles *et al*., 2000; Badyaev, 2002).

Molecular and developmental mechanisms underlying the development of sexual dimorphism have been investigated, mainly focusing on adult sexual-size dimorphism (SSD), and male weapons for male-male competition and/or ornaments for attraction to females (Fairbairn, 1997; Badyaev, 2002). Especially in insects, coordinated regulations among genes related to sex determination and differentiation, growth and molting, and positional information, onto the development of male weapons or ornaments, such as beetle horns, has been elucidated (Cotton *et al*., 2004; Bonduriansky and Rowe, 2005; Emlen, 2008; Emlen *et al.*, 2012).

However, given that sexual dimorphism appear during the course of postembryonic development, it is also crucial to understand the growth patterns of each species and/or each sex. For instance, it is known that SSD is produced by differences in growth patterns between the sexes (Temeles *et al.*, 2000; Badyaev, 2002). Although molecular developmental

mechanisms underlying the development of sexually dimorphic traits have so far been studied extensively in insects, it is known that almost all insects exhibit determinate growth in which they stop to molt and grow, after sexual maturation (Richards and Davies, 1977; Ernsting *et al*., 1993; Hariharan *et al*., 2016). In contrast, many animals exhibit indeterminate growth in which their growth and morphological changes continue even after sexual maturation (Karkach, 2006; Hariharan *et al.,* 2016). Therefore, in such animals with indeterminate growth, it is possible that the sexual dimorphism could newly arise or could be exaggerated by sex-specific developmental regulations.

Furthermore, it is suggested that, although determinate growth can be seen in various animal taxa, indeterminate growth is an ancestral state because species with determinate growth are always found in the phylogenetically derivative positions (Hariharan *et al*., 2016). Therefore, it is assumed that determinate growth is evolved from indeterminate growth by changing the timing of sexual maturation and those of the growth pattern. Given this evolutionary trajectory, knowledge on the developmental trajectories of sexually dimorphic traits in indeterminate growth would contribute to understand the evolution of morphological diversity and life history. However, little is known about these patterns and regulations, due to the difficulty in selecting the appropriate model organism.

Crustaceans: with indeterminate growth and apparent sexual dimorphism

The crustacean species (Crustacea, Arthropoda) exhibit both determinate and indeterminate growth and sexual dimorphisms (Hartnoll, 1978, 1983; Hariharan *et al*., 2016). Although the phylogenetic position of crustaceans has long been discussed, the current consensus is that they constitute the group of Pancrustacea, together with Hexapoda (Richter, 2002; Schwentner *et al*., 2017; Giribet and Edgecombe, 2019). Sexual dimorphism in crustaceans is often found in overall body size and/or appendage sizes, such as chelipeds, that are exaggerated in males and used in reproductive behaviors. Generally, in crustaceans,

since females can mate and spawn during brief time just after molting, males engage in pre-copulatory guarding of females (Conlan, 1991; Jormalainen, 1998; Wada *et al.,* 1999) and male-male competitions over females frequently occur (Barki *et al*., 1998; Emlen, 2008; Yoshino *et al*., 2011; Lord *et al.*, 2021).

Growth patterns and molecular developmental mechanisms in relation to sex determination/differentiation and molting, have been studied in many decapod species including shrimps or crabs (order Decapoda) (Hyde *et al*., 2019; Farhadi *et al*., 2021). However, investigations on postembryonic development in decapods have some difficulties, due to their large body size, complicated life cycles with planktonic larval stages, and long generation time. Therefore, it is necessary to choose appropriate species to study postembryonic development throughout their lifetime.

Study materials

For the appropriate model species to be used for studies on postembryonic development, caprellids (Amphipoda: Malacostraca), commonly referred to as skeleton shrimps, belonging to the order Amphipoda, Malacostraca are focused in this study.

The order Amphipoda is one of the largest taxa in the phylum Arthropoda, containing more than 12,000 species (Arfianti *et al*., 2018). Among them, caprellids belong to the family Caprellidae (Lowry and Myers, 2013). Although the peculiar body plan of caprellids has been focused on in some studies (e.g., Ito *et al*., 2011; Copilaş-Ciocianu *et al*., 2020), it is also known that they exhibit a particularly apparent sexual dimorphism among amphipods (**Figure 2A**; Conlan, 1991). Reproductive biology of caprellids is almost similar to that of other crustaceans, i.e., males show pre-copulatory behaviors and frequently engage in male-male competitions (**Figure 2C**; Conlan, 1991). Males of caprellids develop relatively longer body segments on the first and second pereonites (thoracic segment) and larger appendages on the first antenna and the second gnathopod, used as weapon traits (Arimoto, 1976; Lim

and Alexander, 1986; Takeshita and Henmi, 2010). On the other hand, females develop the brood pouch on the ventral side of the third and fourth pereonites, in which eggs are incubated until hatching.

Although caprellids are found in various marine environments, including deep sea floor, species inhabiting in shallow waters are very easy to be collected, because they can be found attaching on the seaweed, bryozoans, or even artifacts, such as ropes and buoys (**Figure 2B**; Arimoto, 1976; Thiel *et al*., 2003).

Moreover, amphipods are direct developers, and thus, there is no planktonic stage in their life cycles (Poore, 2005; Wilson, 2009) and newly hatched larvae look like a miniature of adults. Although generation time is variable among amphipods, it is known that most of species distributing in temperate zone have relatively short generation time among crustaceans, and reproduce year-round (Wildish, 1982; Paris *et al*., 2021). Caprellids are known to become sexually matured in less than one month (Woods, 2009; Ito *et al.*, 2011; Baeza-Rojano *et al*., 2013).

Thus, these characteristics of caprellids could make them the appropriate animal to study postembryonic development, particularly focused on the sexual dimorphism. Although molecular developmental studies on caprellids are still very limited, it is expected that knowledge on insects and crustaceans can be applied for studies in caprellids. Therefore, as the next step, it was needed to select the species of caprellids which can be easily collected and bred in the lab.

As a result, it was found that *Caprella scaura* Templeton, 1836 can easily be collected in waters near the Misaki Marine Biological Station. A number of individuals of *C. scaura* are often found, attaching on bryozoans, especially *Bugula neritina* (Linnaeus, 1758), and is considered to feed mainly on detritus (Guerra-García *et al*., 2003, 2011). Then, it is also revealed that *C. scaura* can be bred, and even reproduced in tanks in the lab, by feeding

artificial pellets for fishes, diatoms and brine shrimps. Therefore, *C. scaura* was used as the study animal in this study.

In addition, it is known that *C. scaura* contains several subspecies which can be distinguished by external morphological features (Arimoto, 1976). However, the validity of each subspecies has been suspected because they are not distinguished in molecular phylogenetic analysis, and the status of distinguishable traits varied among individuals (Aoki and Kikuchi, 1990; Krapp *et al*., 2006). So, in this study, *C. scaura* is treated as a single valid species.

Study purpose

The aim of this study is to examine whether alteration on sexually dimorphic traits continues after sexual maturation, for investigating the developmental course of sexual dimorphism in animals with indeterminate growth. To test this hypothesis, this study focused on the development of sexually dimorphic traits in a skeleton shrimp, *C. scaura*. Moreover, genes differentially expressed between sexes in different postembryonic stages were searched in order to give an insight into the molecular developmental mechanisms underlying the formation of sexual dimorphism in *C. scaura*.

In Chapter 1, observations on fixed samples of *C. scaura* were performed in order to elucidate the postembryonic development and associated morphological changes in both sexes. In this study, it was revealed that the postembryonic development of *C. scaura* can be classified into three stages, i.e., larva, juvenile, and adult, and distinguishable characters for each stage were also described.

Next, in Chapter 2, to reveal the relationships between the development of external sexually dimorphic characters and the gonadal development in order to imply effects of

endocrine factors, observations on internal structures and investigation for the status of reproductive organs for juveniles and adults were performed.

Lastly, in Chapter 3, rearing experiments were performed in order to track the growth and morphological alterations of newly hatched larvae, and examine whether there are sexual differences in growth pattern and in the development of sexually dimorphic traits. Also, by comparing the gene expression pattern among each sex and each postembryonic developmental stage, candidate mechanisms underlying the formation of sexual differences, observed in growth pattern and development of sexually dimorphic traits, were investigated.

CHAPTER 1

Ontogenetic expression of sexually dimorphic traits in the skeleton shrimp *Caprella scaura* (Crustacea: Amphipoda)

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Abstract

In sexual dimorphism, males often exhibit exaggerated characters as weapons or ornaments. Among the numerous amphipod species (Amphipoda, Crustacea) showing sexual dimorphism, caprellids (Caprellidae) are characterized by considerably larger males that possess weapons, although the developmental processes underlying these sex-related differences are largely unknown. Therefore, to clarify the process of sexual differentiation during postembryonic development in caprellids, morphometric analyses of *Caprella scaura* were conducted. Principal component analysis using 31 morphometric traits showed drastic allometric changes occurring at two ontogenetic body length (BL) points (i.e., 3.8 and 8.8 mm). In individuals larger than 3 mm, head spines appeared in both sexes, and penises did only in males, allowing the discrimination of juveniles from larvae. Moreover, in larger males (BL > 8.8 mm), traits used in reproductive behavior, i.e., the first antenna, second gnathopod, and first to fifth pereonites, were extremely exaggerated. Observations of pre-copulatory behavior along with morphological assays revealed that sexually mature males could be identified by the size ratio between the triangular projection and palmar spine on the propodus of the second gnathopod. In contrast, female maturation could be determined by the marginal setae of oostegites forming a brood pouch. The body size distribution of sexually

mature females was concentrated within a narrow range of BLs (6–9 mm), whereas that of sexually mature males showed a broader range (BL 9–18 mm), suggesting that, in *C. scaura*, males continue to molt and grow even after sexual maturation via indeterminate growth, to increase their lifetime reproductive success.

Introduction

Postembryonic allometric changes leading to sexual dimorphism

Sexual dimorphism is one of the important intraspecific phenotypic variations, as it is believed to have evolved through sexual selection (Emlen, 2008). Consequently, sex-specific (mostly male-specific) exaggerated traits, such as weapons or ornaments, have been acquired for mate choice and/or male–male competition. Moreover, it is suggested that these exaggerated traits indicate preferable conditions, such as better nutrition or larger body size (Cotton *et al*., 2004; Bonduriansky and Rowe, 2005; Emlen, 2008; Emlen *et al*., 2012). In addition to exaggerated weapons or ornaments that are specific to either sex, differences in allometry or proportion are also useful in detecting sexual dimorphism (Huxley, 1932; Gould, 1966). Allometry can be used as an index to evaluate the relative size of focal traits, even among individuals with different body sizes. Three types of allometry are defined depending on comparative taxonomic levels: static, ontogenetic, and evolutionary (phylogenetic) (Bonduriansky, 2007). Static allometry describes the relative sizes of body parts, for comparison among conspecific individuals at the same ontogenetic stages (Knell *et al.*, 2004; Voje and Hansen, 2013; Fromhage and Kokko, 2014), and it has often been used to study insects and some crustaceans, in which the total number of molting events in a lifetime is strictly determined. In these animals, it is relatively easy to define comparable postembryonic stages, especially the adult stage, as growth and molting cease after sexual maturation (Hartnoll, 1978, 1983; Emlen *et al*., 2012).

However, sexual dimorphism is also expressed gradually during postembryonic development in species where body growth continues even after sexual maturation, such as arthropod lineages without terminal molt (Hartnoll, 1983; Marochi *et al*., 2019). Therefore, to clarify the proximate mechanisms underlying sexual dimorphism, it is important, especially in such animal species, to examine the appearance of sex-specific differences during

postembryonic development. Morphometric analysis of the ontogenetic allometry occurring during growth can be a useful tool to understand the patterns of postembryonic phenotypic changes (Voje *et al*., 2014; Esquerré *et al*., 2017).

Sex-related differences in caprellids

Among pancrustaceans, sexual dimorphism is often observed in almost all species of Amphipoda (Malacostraca), although they do not exhibit drastic morphological changes such as metamorphosis (Barnard and Karaman, 1991). All amphipod species show direct development, in which newly hatched larvae already have adult-like morphologies (Poore, 2005; Wilson, 2009). Sexual dimorphism in amphipods is often found in body size or appendage morphology, which in most cases has a significant role in reproduction or intrasexual competition (Conlan, 1991).

Among amphipods, sexual dimorphism is especially prominent in caprellids, which belong to the family of Caprellidae, superfamily Caprelloidea (Lowry and Myers, 2003). Compared to other amphipod lineages, caprellids have distinct body plans characterized by elongated thoracic segments (pereonites), while they lack abdominal segments and some appendages (Barnard and Karaman, 1991: Ito *et al.,* 2011). In many caprellid species, intraspecific morphological variations are observed in the relative size of particular pereonites and appendages between sexes and/or developmental stages (Arimoto, 1976). In numerous amphipod species, distinctive sexually dimorphic characteristics are observed in the first and second pereonites and in some segments, such as the basis and propodus of the second gnathopod.

As sexual dimorphism in amphipods is expressed gradually during postembryonic development, and is particularly apparent in caprellids, these species can be regarded as the new optimal model organism to reveal the proximate developmental mechanisms underlying

sexual dimorphism. A number of morphological studies have been conducted on caprellids thus far, but the information is still limited to some specific stages, such as newly hatched larvae or mature adults. Therefore, it is necessary to quantitatively and successively investigate the expression patterns of sexual dimorphism throughout the entire lives of individuals and for single caprellid species, focusing in particular on each trait and defining the postembryonic developmental stages.

Study purpose

The aim of this study was to describe the morphological transitions detected in the postembryonic development of the caprellid *Caprella scaura* Templeton, 1836, with a particular focus on the expression patterns of sexual dimorphism, as apparent in reproductive and weapon traits.

In *C. scaura*, sexual dimorphism occurs during postembryonic development (**Figure 3A–C**; Arimoto, 1976; Sakaguchi, 1989). In addition to presenting male genitalia, which appear in males with a body length (BL) > 3 mm (Sakaguchi, 1989), males become much larger than females and exhibit some male-specific morphological traits, such as the first and second pereonites, as well as the first and second gnathopods (Arimoto, 1976; Guerra-García *et al*., 2011). Furthermore, it was reported that the poison tooth on the propodus of the second gnathopod is developed only in males (Takeshita and Wada, 2012). In contrast to males, mature females are characterized by large pairs of oostegites on the third and fourth pereonites, which form a brood-pouch where eggs are protected and incubated until hatching. Firstly, morphological observations and morphometric analyses were performed to describe the patterns of postembryonic morphological changes. Then, by observing precopulatory behavior in males and oviposition in females, mature adult-specific morphological characters were identified.

Materials and Methods

Animals

Caprella scaura were sampled on 27 April, 26 July, 25 October, and 7 December, 2021, at piers belonging to an aquarium on Hakkeijima Island, Yokohama, Kanagawa, Japan (Hakkeijima Sea Paradise, 35°33′57″N, 139°64′67″E). As most caprellids cling onto various substrates (Barnard and Karaman, 1991), bryozoans, mainly *Bugula neritina* (Linnaeus, 1758), attached to buoys or rope were collected and, among them, numerous *C. scaura* individuals were found. The collected specimens were identified based on the following diagnostic characters defined in Takeuchi (1995): one acute head spine present, pairs of small projections present on the dorsal side of the fourth to seventh pereonites, pairs of grasping spines present on the propodus of the fifth to seventh pereopods (thoracic legs), and only in males, the second pereonite longest among seven pereonites, and the basis of the second gnathopod very long, as long as the second pereonite.

Some of the individuals were fixed and preserved in 70% ethanol or Bouin's solution to observe their external morphology. The others were kept alive in the laboratory for behavioral examinations to identify sexual maturity. The animals were placed in a polyethylene container (L: 21.5 cm × W: 13.0 cm × H: 11.5 cm) filled with 1.5 L of filtered seawater, and a plastic net with a 1-mm mesh (L: 17.0 cm × W: 9.0 cm) was set at the bottom of the container as a substrate to cling on. The water temperature was maintained at 20 ℃ and the water was continuously aerated; the container was exposed to artificial light with a 16/8 h light/dark cycle during the breeding period. The animals were fed daily with artificial feeds for saltwater fish (Hikari Premium Seaweed 70 and Megabyte Red, Kyorin Co., Ltd., Hyogo, Japan) and the diatom *Chaetoceros calcitrans* (Paulsen). Seawater was changed every two to three days.

To conduct observations on newly hatched individuals, eggs were isolated from

ovigerous females and were placed in 12-well plates (Costar 12-well Clear TC-treated Multiple Well Plates, Corning Inc., Corning, NY, USA), which were filled with filtered seawater and were incubated at 20 ℃ until hatching. Filtered seawater was changed once a day. Newly hatched larvae were fixed immediately after hatching in 70% ethanol or 4% PFA/PBS and morphometric measurements were performed.

Morphometric analyses

To comprehensively understand the overall morphological changes occurring during postembryonic development in *C. scaura*, morphometric analyses were performed focusing on 31 traits, i.e., the length of the head and first to seventh pereonite, length of flagellum and the first to third peduncle article of the first antenna, length of flagellum and the first to fourth peduncle article of the second antenna, length of six articles of the first and second gnathopods (basis, ischium, merus, carpus, propodus, and dactylus), and gill length on the third and fourth pereonites (**Figure 3D-G**). The sum of the length of the head and that of the first to seventh pereonite was used as the total body length. Morphological observations on the fixed samples were conducted based on photographs processed using cellSens Standard imaging software (Olympus Corp., Tokyo, Japan), and measurements were taken using ImageJ ver. 1.53e (Schneider *et al*., 2012).

As large differences in BL existed between adult males and females in *C. scaura*, it was necessary to use relative lengths (allometry) against measurements that were less affected by body size for the principal component analysis (PCA). In previous morphometric studies on caprellids, the length of the second pereonite or the total length from the head to the fifth pereonite has been used as an index of individual body size (Bynum, 1978; Lewbel, 1978, Takeshita and Henmi, 2010). However, considering that the second pereonite becomes relatively larger only in mature *C. scaura* males, it seemed inappropriate to use the length

of the second pereonite or the total length from the head to the seventh pereonite as the body size index in this species. In contrast, neither sexual dimorphism nor ontogenetic allometric change is observed in the length of the sixth and seventh pereonites, in which appendages are specialized in grasping substrates for the animals' epibenthic lives. Therefore, it would be reasonable to use the total length of these two pereonites as the body size index of *C. scaura*. Accordingly, body part measurements should be transformed into relative values against the total length of the sixth and seventh pereonites.

Based on the measurements of 31 body parts, PCA was performed. The number of effective principal components (PCs) was determined by scree plot and cumulative proportion. The overall proportional changes were examined by segmented regression analysis applying the least squares method to the obtained PC scores against BLs. The values and numbers of breaking points were determined using the "segmented" function in the package for the R software, "segmented" (Muggeo, 2020). All of the parameters for the analyzed specimens were included into the initial full model, and the best model was determined through the backward stepwise selection based on the Akaike's Information Criterion (AIC) values. The homoscedasticity was examined using the bootstrapped White's test (Jeong and Lee, 1999). The statistical significance of the obtained models was tested via the likelihood-ratio test. In addition, the sex-dependent changes in relative size in the respective appendages and anterior pereonites were examined through a generalized linear model (GLM) with a Gaussian distribution and an identity link function using the inflection points estimated above to detect the BLs that correspond to significant morphological changes occurring during postembryonic development. The best model and statistical significance were determined in the same manner. Furthermore, to evaluate the proportional changes, the appearance of the head spine and external genitalia were specifically examined as the distinctive and quantitative morphological traits that indicate the onset of these changes, based on a binomial logit model. This analysis was performed using a GLM with a binominal distribution with a logit

transformation (R software version 4.1.2; R Core Team, 2021). Then, the best model was determined via the backward stepwise selection based on the AIC with likelihood-ratio test. Similar analyses were conducted on the relative lengths of the head spine (against the head length) for smaller individuals, and those of two propodus spines, poison tooth (PT), and triangular projection (TP) on the second gnathopod (against propodus length) for males. The following growth model was used in this analysis:

$$T = \frac{L_\infty}{1 + \exp\left(-g \cdot L + L_0\right)}$$

where, T is the size of the target body part as the explained variable, L is the body length, L_∞ is the estimated maximum size, g is the parameter changing the growth speed in the target body part, and L_0 is the estimated logical value of the body length when the size of the target body part becomes zero. All of the parameters were estimated using a nonlinear regression function (R software version 4.1.2; R Core Team, 2021).

Scanning electron microscopy (SEM) observation

In caprellids, morphological differences between sexes have been noted particularly in the external genitalia and second gnathopod (Arimoto, 1976). Therefore, to detect sex-related differences, SEM observation was performed on these anatomical traits. The samples, previously fixed in 70% ethanol, were dehydrated for 1 h in increasing ethanol concentrations of 90%, 95% and 100% (20 minutes at each concentration), and were transferred to a hexyamethyldisilazane solution where they were hardened for 1 h, and then into 100% ethanol twice and 100% *t*-butanol thrice for 20 minutes each; finally, they were frozen at -20℃. Subsequently, the fixed samples were freeze-dried using a freeze dryer (model ES-2030, Hitachi Global, Tokyo, Japan) and were coated with silver ions with an ion sputter

coater (model E-1010, Hitachi Global, Tokyo, Japan). The coated samples were observed under a JSM-5510LV scanning electron microscope (JEOL Ltd., Tokyo, Japan).

Evaluation of sexual maturation in males

To identify the developmental stages of sexual maturation in males, behavioral assays were conducted. In many species of crustaceans, males exhibit pre-copulatory behaviors to guard females until spawning (Christy, 1987; Conlan, 1991; Jormalainen *et al*., 1994; Dick and Elwood, 1996). In *C. scaura*, the sequence of reproductive behaviors from pre- to post-mating has already been described (Lim and Alexander, 1986; Schulz and Alexander, 2001). In the present study, a total of 24 pairs of individuals, each consisting of a mature but non-ovigerous female and a male whose BL ranged from 5.2 to 17.8 mm, were placed in a six-well plate (Cell Culture Plate, SPL Life Sciences Co., Ltd., Pocheon, Gyeonggi, Korea), one pair per well, at 21-22℃; the plates were filled with filtered seawater, and then, the pairs' behavior was recorded using a digital video camera (STYLUS TG-3 Tough, Olympus Corp., Tokyo, Japan) for 30 minutes. This behavioral assay was repeated four times. The individuals used in the behavioral assays were fixed in Bouin's solution for morphological observations.

The correlation between pre-copulatory behavior and BL was examined through a binominal logit model. As the relative lengths of three distinctive spines on the lower propodus margin of the second gnathopod seemed to be useful for the identification of sexually mature males, the length of the body and that of these spines were included in the initial full model. In addition, as it has been reported that the difference in body size between sexes affects the occurrence of precopulatory behaviors in other species (Takeshita and Henmi, 2010), the BL ratio between sexes was also considered in this model. Then, the best model was selected using the backward stepwise selection based on the AIC. The analysis

was performed using GLM with a binominal distribution using a logit transformation. The significance of the obtained models was determined via the likelihood-ratio test.

Evaluation of sexual maturation in females

Female maturity was assessed by monitoring oviposition once a day. Ovigerous females were anesthetized with eugenol-based FA100 (DS Pharma Animal Health Co., Ltd., Osaka, Japan) to check eggs in the brood pouch; they were then photographed and their body parts were measured.

In other caprellid species, mature females are known to have conspicuous marginal setae on oostegites, which are the female-specific appendages on the third and fourth pereonites forming the brood pouch (Takeuchi, 1989; Takeuchi and Hirano, 1991). In addition, the female gonopore appears during postembryonic development. To determine the morphological features that indicate female maturity, the correlations between the emergence of ovigerous females and marginal setae of female gonopore were examined through binominal logit models, and the AIC values of each model were compared. The significance of the obtained models was determined via the likelihood-ratio test.

Results

Allometric changes during postembyonic development

Based on the scree plot (**Supplementary Figure 1**), two effective PCs were determined in the PCA using all of the 31 measured traits. Specifically, in this analysis, 57% of the total variance was explained by PC1 and PC2 (**Table 1**). For PC1, the measurements of each segment of the first antenna and second gnathopod, length of gills on the third and fourth pereonites were heavily loaded and all showed positive scores. In contrast, for PC2, the measurements of the second antenna and first gnathopod were heavily loaded, but almost all of these scores showed negative values. The correlation analysis between PC scores and BL revealed the presence of two significant turning points in terms of allometric changes during postembryonic development. The PC1 scores slowly increased with BL, but an inflexion point was detected at the length of 8.8 mm (**Figure 4**, and **Supplementary Table 1**). This indicates that the relative length of the first antenna, second gnathopod, gills, head, and pereonites, against the total length of the sixth and seventh pereonites (which were heavily loaded traits on PC1) sharply increased at this point. In the case of PC2, the PC scores showed a rapid increase up to a BL of 3.8 mm, but remained almost constant thereafter (**Figure 4B**, and **Supplementary Table 1**). As PC2 was heavily loaded by the second antenna and first gnathopod, this result would indicate that the relative lengths of these traits decreased after reaching this point. Based on the patterns of ontogenetic allometry in *C. scaura*, the BLs during postembryonic development were divided into three size classes: smaller than 3.8 mm, between 3.8 and 8.8 mm, and larger than 8.8 mm, and individuals belonging to them were defined as "small", "middle-sized", and "large", respectively. The biplot using both PC1 and PC2 recognized these three groups; of the three size classes detected, small individuals were considered to reflect the morphology of larvae (**Figure 4C**).

Boundary between larvae and juveniles

To date, no studies have been conducted on the morphology of immature small individuals of *C. scaura*, except one indicating that there are no sex-related differences in newly hatched larvae, but males reaching 3 mm in BL possess an apparent penis on their ventral abdomen (Sakaguchi, 1989). Therefore, it was necessary to identify any distinctive characteristics that define the larval stage for both sexes by investigating morphological traits that appear at the same time as the penis in males.

In this study, it was observed that the head spine appeared at almost the same time as the penis in males (**Figure 5A**). In the analysis using the binominal logit model, there was a clear shift from the stage without a head spine to that with a distinctive head spine (**Figure 5B**; n = 68; likelihood-ratio test, $P < 0.05$; **Supplementary Table 2**), indicating the simultaneous appearance of both the penis and head spine. However, female external genitalia (gonopore) on the ventral surface of the posterior end of the fifth pereonite (**Figure 5C**) appeared at later stages (approximately 6 mm in BL). The shift from females without gonopore to those with it was correlated with BL (**Figure 5B**; n = 118; likelihood-ratio test, $P < 0.05$; **Supplementary Table 2**), which, however, showed a broad range (4.5–7.5 mm); therefore, the appearance of this organ cannot be used to define the larval stage. However, as observed in males, females also showed a clear shift in head spine possession after reaching a BL of 3 mm (**Figure 4B**; n = 118; likelihood-ratio test, $P < 0.05$; **Supplementary Table 2**). Considering the overall PCA results, the appearance of the head spine is suggested to coincide with the allometric change. Thus, as this external trait was proved to be a useful qualitative character to distinguish between larvae and juveniles, immature individuals smaller and larger than 3 mm are hereafter referred to as belonging to the "larval" and "juvenile" stages, respectively.

Ontogenetic allometry and appendage growth

The transition from juvenile to adult is generally defined by sexual maturation, which is reflected in the expression of sexual traits, including reproductive behaviors (Borowsky, 1985; Fahrbach and Robinson, 1996; Minelli *et al*., 2006). Therefore, to identify the adult stage, it is necessary to detect any morphological characteristics that coincide with sexual maturation. In *C. scaura*, males perform a precopulatory guarding behavior when encountering mature females (Conlan, 1991). They guard females in "Type I precopulatory formation" (Aoki, 1996; Schulz and Alexander, 2001), where a male stands just behind a mature female and guards her from other males using the anterior (first to fifth) pereonites. As guarding males cannot escape from other males while guarding females, longer anterior pereonites could be advantageous to keep opponents away. In addition, male–male combats frequently occur over the guarded females during the precopulatory phase (Lewbel, 1978; Takeshita and Henmi, 2010). In male–male combats, both of the competing males extend their bodies to touch the opponent by the first antenna, and then use the second gnathopod to escalate fighting (Lim and Alexander, 1986; Schulz and Alexander, 2001). These traits, which are used during male fighting and reproductive behavior (i.e., the first antenna, the second gnathopod, the head, and the first to fifth pereonites) were heavily loaded on PC1 (**Table 1**). This indicates that the larger individuals shown by PCA (**Figure 4A, C**) may present the morphological characters of sexually mature males.

As different types of appendages have different functions (Takeuchi *et al.*, 2003), ontogenetic allometric changes were investigated based on the functions of specific body parts to detect the morphological characters found only in larger individuals; the first antenna and second gnathopod were chosen as traits used for fighting, the head and first to fifth pereonites for reproductive behavior, the second antenna and first gnathopod for feeding, and the gills on the third and fourth pereonite for respiration (**Figure 6**, and **Supplementary**

Table 3). The results showed that the allometry of the feeding apparatus was larger in larvae and males (**Figure 6A, B**). Respiratory organs showed an almost linear correlation with BL and were slightly larger in large individuals (**Figure 6C, D**), indicating that these organs themselves become larger as BL increases. In contrast, the allometry of traits for fighting and that of anterior pereonites for reproductive behavior were significantly larger in males (**Figure 6E–G**). In particular, these traits were more developed in males with BL > 9 mm, although the second gnathopod and anterior pereonites were also significantly larger in small males than in females.

Moreover, the largest male and female specimens had BL of 17.8 mm and 11.3 mm, respectively, suggesting that females stop growing at a smaller size compared to males. In males larger than 9 mm, there was a marked increase in allometry for weapon traits used during fighting and anterior pereonites used for precopulatory behavior, indicating that there was a significant secondary sexual development.

Morphological characteristics specific to larger males

Subsequently, to easily discriminate sexually mature adults, qualitative morphological characteristics showing the boundary between the juvenile and adult stages were identified for both males and females. In males, the propodus of the second gnathopod was exaggerated as BL increased (**Figure 3G, 6F**); in particular, three projections on the lower margin, i.e., the triangular projection (TP), poison tooth (PT), and palmar spine (PS) from the distal end, were extremely developed (**Figure 7A**; Arimoto, 1976). As it was observed that the PS was apparent in both sexes, but the TP and PT were larger in males, it was hypothesized that the TP/PS and PT/PS ratios could be indicators of sexual maturation in males.

The morphological examinations of these spines showed that individuals of both

sexes that were larger than 5 mm possessed a PT (**Figure 7B, E, H–M**). In larger males, however, this feature was clearly larger in size and sharper in shape compared to that in smaller individuals (**Figure 7C, D, F, G**). Furthermore, SEM observations revealed that only larger males presented a cluster of minute pores at the tip of the PT, which is believed to secrete venom (Schulz and Alexander, 2001; Takeshita and Wada, 2012).

Subsequently, the changes in the relative size of the head spine to head length were also used to identify the boundary between the larval and juvenile stages. It was observed that the head spine continued to elongate after its appearance, and its relative size reached the maximum at a BL of approximately 9 mm (**Figure 8A, B**; and **Supplementary Table 4A**), suggesting that a significant morphological alteration occurs when this body size is reached. The relative sizes of PT, TP, and PS in males increased, but stabilized at a BL of approximately 9 mm (**Figure 8C, E, G**; and **Supplementary Table 4A**), as observed in the head spine. In contrast, in females, although the relative sizes of TP, PT, and PS also increased, they did not reach the maximum point, and especially that of PT was much smaller in females than in males (**Figure 8D, F, H**; and **Supplementary Table 4A**). Furthermore, the analysis of the TP/PS ratio in males showed that it increased with BL and reached the maximum (TP/PS = 1) at a length of ca. 9 mm, when the size of TP became comparable to that of PS. At this developmental point (BL, 9 mm), the relative sizes of the head spine and PT also stabilized (**Figure 8I, J**; and **Supplementary Table 4B**). Thus, significant morphological changes were observed in males as BL reached approximately 9 mm, and at this stage a TP/PS ratio of 1 could be applied to identify the boundary between juveniles and adults.

Evaluation of male sexual maturation

To examine whether the TP/PS = 1 criterion could be used as an indicator for male sexual maturation, it was necessary to identify sexually mature males. Therefore,

reproductive behaviors were observed with a particular focus on males, and the correlation between behavior and the TP/PS ratio was examined.

During the observations, male individuals showed an "assessment behavior", in which they touched the back of females with the first antenna and first gnathopod (Lim and Alexander, 1986; **Figure 9A, B**), and this was regarded as a male reproductive behavior. A binomial logit model was applied to examine the correlation between the presence or absence of this assessment behavior and BL in males, and a gradual shift of the rate occurred at a BL of approximately 9.5 mm (**Figure 9C**; and **Supplementary Table 5**; $n = 22$, $P < 0.05$). Furthermore, a clear correlation was also observed between the occurrence of assessment behavior and the increase of TP size, which reached the PS size, i.e., TP/PS = 1 (**Figure 9D**; and **Supplementary Table 5**; $n = 22$, $P < 0.05$). Additionally, a biplot of the TP/PS ratio versus BL (**Figure 9E**) showed that the smallest male exhibiting assessment behavior had a BL of ca. 9 mm, and only individuals with TP/PS values greater than 1 exhibited this behavior. Overall, the TP/PS ratio can be used to identify sexually mature adult males. Using this criterion, the size range of adult males detected in this study was 9–18 mm.

The results showed that males reached a BL of 9 mm with well-developed fighting traits to perform precopulatory behaviors. In caprellids, sexually mature males are known to perform precopulatory mate guarding and engage in male–male competition over females (Lewbel, 1978; Lim and Alexander, 1986; Conlan, 1991; Takeshita and Henmi, 2010). The exaggeration of traits in larger males reported in this study would be the consequence of sexual selection, as larger males with weapons generally gain advantages during male–male competition (Ward, 1983; Jormalainen *et al*., 1994; Yoshino *et al*., 2011).

Evaluation of female sexual maturation

In other Caprella species, the development of oostegites is divided into immature,

premature, and mature stages (Takeuchi, 1989; Takeuchi and Hirano, 1991). Therefore, this trait can also be used to identify female sexual maturation in *C. scaura*. In the present study, the relationship between oostegite morphology and sexual maturation was investigated in this species via breeding experiments. Like other caprellid species (Takeuchi, 1989; Takeuchi and Hirano, 1991), the *C. scaura* larvae and early juveniles examined did not present any primordial structures for oostegites (**Figure 10A**). When the BL of female juveniles reached 5 mm, the primordia started to develop, although the timing of the developmental onset varied among individuals (**Figure 10B**). According to Takeuchi (1989), premature females are defined by the presence of enlarged oostegites without marginal setae, but only one such individual was investigated in this study (**Figure 10C**). All of the brooding females possessed oostegites with apparent setae (**Figure 10D, E**). The BL of females presenting oostegites with marginal setae ranged from 6 to 11 mm (**Figure 10F, G**; and **Supplementary Table 6**; mean: 8.2 mm, n = 118; likelihood-ratio test, $P < 0.05$), which was slightly larger than the BL associated with the emergence of external genitalia. The BL of brooding females corresponded well to that of females with oostegite setae rather than to that of females showing external genitalia. Thus, the definition of female maturation using oostegite setae was proved to be applicable to *C. scaura*.

Discussion

Indeterminate growth leading to male-specific morphological exaggeration

Overall, the results of this study showed that the process of postembryonic development in *C. scaura* can be classified into three stages, i.e., larval, juvenile, and adult, based on morphological characteristics (**Figure 11**). The BL distribution of mature males showed a wide size range (9–18 mm), whereas that of mature females was concentrated in a narrower range (6–11 mm, but predominantly between 6 and 9 mm) (**Figure 11**). Generally, in arthropods, growth is accomplished by molting, a phase in which the old exoskeletons of previous postembryonic stages are discarded. Almost all insects (except for silverfish and bristle tails; Richards and Davies, 1977; Hariharan *et al*., 2016) and some crustaceans present a determinate growth mode in which sexually mature individuals (adults) cease to further molt and grow (Cheong *et al*., 2015; Hyde *et al*., 2019). Particularly, in insects that undergo determinate growth, adult body size, and traits (including male weapons), which depend on the nutritional conditions during the larval period, cannot be changed after sexual maturity is reached (Cotton *et al*., 2004; Bonduriansky and Rowe, 2005; Emlen *et al*., 2012). In contrast to determinate growth, many crustaceans, including numerous amphipod species, exhibit indeterminate growth, namely, they continue to molt and grow throughout their lives, even after sexual maturation (Hartnoll, 1983; Barnard and Karaman, 1991; Chapin, 2017). In this growth mode, unlike in determinate growth, relatively small but sexually mature males, which are at a disadvantage during male–male competition, can increase their body size and develop weaponry by means of continuous molting and growth. Thus, it is suggested that the wider size range of mature males reflects the male reproductive strategies that could be altered depending on male body size and weapon traits.

To understand the postembryonic development in arthropods, it is important to know the relationship between molting and phenotypic changes, including the reproductive

characteristics. However, only a few studies have been conducted thus far on molting and the associated morphological changes in caprellids; one study in particular suggested that, in *C. scaura*, male individuals with BL reaching approximately 7 mm and 14 mm were seventh and ninth instars, respectively (Sakaguchi, 1990). Furthermore, generally, variations of male phenotypes in animals often correlate with reproductive tactics (Emlen, 2008). For example, larger males compete with other males over mates using their enlarged bodies and weapon traits, while smaller ones avoid fighting and behave as "sneakers" (Laufer *et al*., 1994; Gross, 1996).

Among amphipods, *Jassa falcata* (Montagu, 1808) (Ischyroceridae) is also known to exhibit male dimorphism, especially in the propodus of the second gnathopod, which is used as a weapon during male −male competition (Borowsky, 1983, 1985). The reproductive behavior of this species also varies between the two male phenotypes; specifically, larger males with developed weapons have an advantage in competitions and approach females more vigorously, whereas smaller males with a less developed propodus invest more time and energy in feeding (Borowsky, 1985). Also, in another species (*Jassa marmorata* Holmes, 1905) the smaller males are known to exhibit sneaking behaviors (Kurdziel and Knowles, 2002). Therefore, it is possible that *C. scaura* males too may change reproductive strategies depending on their body size during postembryonic development, where the smaller ones adopt sneaking, while the larger perform precopulatory mate guarding and engage in male−male combats.

Physiological and developmental implications

The molecular and physiological underpinnings of sexual dimorphism have been studied especially in insects and decapod crustaceans, in which some endocrine factors have been identified as responsible for the phenomenon. For example, juvenile hormone (JH) in

insects, and methyl farnesoate (MF) and androgenic gland hormones (AGH) in decapod crustaceans play important roles in the expression of sexual characters (Homola and Chang, 1997; Laufer and Biggers, 2001; Gotoh *et al*., 2011, 2014; Kijimoto *et al*., 2012; Jindra *et al*., 2013). In some gammarid species, MF and AGH contribute to molting and sexual maturation (Hyne, 2011). Furthermore, experimental platforms for amphipods have recently been established, especially for *Parhyale hawaiensis* (Dana, 1853), which is considered an emerging model species in evo-devo studies (Browne et al., 2005; Sun and Patel, 2019; Paris et al., 2022). However, developmental and endocrine mechanisms have yet to be elucidated in caprellids. The present study showed that these species can be good model animals, as they are easy to collect and breed, and exhibit a more apparent sexual dimorphism compared to gammarids. The postembryonic processes revealed here in *C. scaura* will provide the analytical basis for future mechanistic and evolutionary studies.

CHAPTER 2

Gonadal structure and development in a skeleton shrimp *Caprella scaura* (Crustacea: Amphipoda): Relationship with sexually dimorphic traits and implications for life history

Abstract

In Malacostracan crustaceans, it is known that genes and hormones, expressed or secreted from gonads and endocrine organs play a crucial role in sex determination and differentiation. Thus, to reveal physiological mechanisms underlying the development of sexual dimorphism in *Caprella scaura*, it is important to investigate the developmental course of testis, ovary and the male specific organ, called androgenic gland (AG). However, knowledge on the internal structure of caprellids is still very limited. In this study, to clarify the relationship between the development of gonads and AG, and that of sexually dimorphic traits, comparison on the status of gonads between juveniles and adults was performed, after identification of gonads and AG. As a result, in *C. scaura*, male gonads were consisted of a testis, vas deferens and seminal vesicle, and located on the fifth to seventh pereonite whereas female's ovaries were located on the third and fourth pereonite. Moreover, in males, spermatogenesis seemed to have already started before the emergence and development of sexually dimorphic weaponry traits. These results indicated that, the AG has a central role in the development of sexually dimorphic traits in *C. scaura*. In addition, the discordance between the timing of the onset of spermatogenesis and of the development of sexually dimorphic traits, suggests that morphologically defined juvenile males could engage in mating and reproduction, and such individuals might have alternative reproductive strategies.

CHAPTER 3

Sexual difference in growth pattern and underlying sex-specific gene expressions in a skeleton shrimp *Caprella scaura* (Crustacea: Amphipoda)

Abstract

Because animals with indeterminate growth continue to grow after sexual maturation, it is possible that sexually dimorphic traits can be altered through their live. Thus, to reveal proximate mechanisms underlying the development of sexual dimorphism in such animals, it is important to track growth patterns and morphological changes in each sex. In this study, to examine whether males and females differ in the instar at which sexually dimorphic traits are expressed, and whether growth pattern after sexual maturation differ in *Caprella scaura*, breeding experiments were performed. Moreover, underlying genetic mechanisms were investigated by expression analyses. The result showed that although sexually dimorphic traits were expressed at the approximately same instar in both sexes, there was a clear sexual difference in growth pattern, especially after sexual maturation, i.e., only males rapidly increased in body length and weapon size after sexual maturation, but had the terminal molt. These results suggest that sexually dimorphic regulation in growth pattern could largely affect on the development of sexual dimorphism in *C. scaura*. In addition, expression analyses showed that *doublesex* (*dsx*) was upregulated only in adult males, suggesting that *dsx* might have a central role in controlling not only sex-specific morphological changes, but also growth pattern. These findings suggest that spatio-temporal regulation of sex-specific factors, such as *dsx*, could be the source of various phenotypes in sexual dimorphisms in indeterminate growth animals, and also that of life history evolution.

※第 3 章については，5 年以内に雑誌等で刊行予定のため，非公開.

GENERAL DISCUSSION

Sexual differences in growth pattern and in regulation of development of sexually dimorphic traits

Sexual dimorphism is the phenotypic difference between males and females, and is universally found in the animal kingdom. It has been an important research subject of evolutionary and developmental biology to answer the question how males and females develop different phenotypes, nevertheless sexes of the species should share most of the genes that control development and growth, develop different phenotypes (Badyaev, 2002; Emlen, 2008).

Because sexual dimorphism appears during the course of postembryonic development, it is important to track morphological alteration through a whole life of individuals. For studies on developmental mechanisms of sexual dimorphism, insects have been used as a model animal (Cotton *et al*., 2004; Bonduriansky and Rowe, 2005; Emlen, 2008; Emlen *et al*., 2012; Gotoh *et al.*, 2011, 2014). However, insects are unique to exhibit determinate growth, in which they stop to grow after sexual maturation, whereas most animals have the indeterminate growth mode, in which, in contrast, they can continue to grow even after sexual maturation (Karkach, 2006; Hariharan *et al*., 2016). Therefore, it is likely that sexual difference in growth patterns have a large impact on the development of sexual dimorphism in animals with the indeterminate growth mode. However, due to the difficulty in selecting model animals which can track the whole life, few studies have been conducted on this theme.

This study focused on caprellids, also known as skeleton shrimps, as an appropriate animal to understand the development of sexual dimorphism in indeterminate growth, and I decided to use *Caprella scaura* as a study model, because this species was easily found and collected, and the breeding method was successfully established. The aim of this study was to examine whether sexual dimorphism become prominent by the difference on the growth

pattern between sexes through individual's whole lives. To test this hypothesis, this study focused on the appearance and development of sexually dimorphic traits in *C. scaura*. In addition, to gain insights into the molecular and developmental mechanisms responsible of generating sexual differences, gene expression analyses were also performed.

In Chapter 1, extensive observations on fixed samples revealed the morphological changes and development of sexual dimorphism, and the postembryonic development of *C. scaura* was divided into three morphologically distinguishable stages, that is, larva, juvenile and adult stage. In addition, because the range of body length of adult males were broader than adult females, it was suggested that males would increase their lifetime fitness by continuing to molt and grow, and becoming larger in body and weapon size, even after sexual maturation (**Figure 11**). This may also indicate that there are sexual differences in the development of sexual dimorphism, in which male weapon becomes apparent through their lives, whereas female brood pouch, in which adult individuals incubate eggs until hatching, does not change once it appeared.

This idea was confirmed from the results of breeding experiment in Chapter 3. This experiment confirmed that there was a clear sexual difference in growth pattern between sexes. Interestingly, there was also a sexual difference in the number of molts experienced in individual's life, with females continuing to molt until death, whereas males stop molting after sexual maturation and sexual dimorphism became prominent, suggesting that males of *C. scaura* have the cessation of molting, also called as "terminal molt". As for the life histories of each sex, the presence or absence of the terminal molt could be attributed to the risks associated with molting, constraints on spawning, and differences in reproductive strategies between sexes. On the other hand, it was also suggested that sexual differences on the regulation of genes differentially expressed only in adult males would have large impact to generate the sex-specific growth pattern and development of sexually dimorphic traits in *C. scaura*.

Therefore, it was predicted that the regulations of genes related to sex determination and differentiation, as well as those related to molting, play important roles in such sexual differences in growth patterns. Results of gene expression analysis in Chapter 3 found some differentially expressed genes among each sex of juveniles and adults.

Firstly, *juvenile hormone esterase* (*JHE*) which putatively involved in molting, was highly expressed in juveniles of both sexes. In insects, it is known that *JHE* promotes metamorphosis of larvae to adults by degrading juvenile hormone (JH) which inhibits metamorphosis to adults, and lowering its titer (Share and Rowe, 1988; Riddiford, 1996). On the other hand, in crustaceans, the hormone, methyl farnesoate (MF), has a similar role to JH, and *JHE* also involves in the degradation of MF (Subramoniam, 2001; Sin *et al*., 2015; Toyota *et al*., 2023). Considering that the onset of appearance of sexually dimorphic traits occurs at the transition of juvenile to adult stage in *C.scaura*, it is possible that fluctuation of MF titer has an important effect on it. However, MF is also known to have various roles in crustaceans, such as promotion of settlement of planktonic larvae, involvement of ecdysis, or gonad development in both sexes (Homola and Chang, 1997; Laufer and Biggers, 2001). Therefore, it would be needed to evaluate whether MF promotes the development of sexual dimorphism in *C. scaura*, by MF administration or inhibition of its function.

Moreover, because growth patterns were clearly different after sexual maturation between sexes, where only adult males grow rapidly and finally stop to mole, differentially expressed genes found in adult males would contribute to generate these patterns. Results of expression analyses also showed that in adult males, *doublesex* (*dsx*) was highly expressed, whereas *shade* (*shd*) which involved in the synthesis of molting hormone, was significantly suppressed. *dsx* is well-known gene which involved in sex determination and differentiation, mainly in insects and other arthropods (Kato *et al*., 2011; Geuverink and Beukeboom, 2014; Rohner *et al*., 2021). In crustaceans, *dsx* is expressed highly in testis, and promotes the activity of the androgenic gland (AG), leading to the secretion of

androgenic gland hormone (AGH) which is a key endocrine factor on regulation of masculinization (Yu *et al*., 2014; Li *et al*., 2018; Zong *et al*., 2019). As indicated in Chapter 2, AGH, and also *dsx* are likely to involve in the male-specific growth pattern in *C. scaura*.

In addition, it is suggested that *dsx* suppress the expression of genes related to regulation of molting, such as *phantom* or *shade* (Ledón-Rettig et al., 2017; Jo et al., 2021). Considering these results and knowledge together, sex-specific expression and also temporal expression dynamics and maybe pluripotent function of *dsx* may play a central role to produce the differences in the developmental trajectory of sexually dimorphic traits in *C. scaura*.

Evolutionary implications of life history with indeterminate growth

Polymorphism of weapon traits of adult males is frequently seen in various groups in crustaceans, such as the orders of Decapoda, Isopoda, and Amphipoda (Borowsky, 1985; Kuris *et al.*, 1987; Shuster, 1987; Homola *et al*., 1991; Shuster and Wade, 1991; Laufer *et al.*, 1994; Kurdziel and Knowles, 2002). In addition, it is known that each morph of males often shows different reproductive strategies (Shuster, 1987; Kuris *et al*., 1987). Given these examples, it is possible that males of *C. scaura* may exhibit different reproductive strategies, depending on their size of body or weapon traits, as also indicated from gonadal development of juvenile males of *C. scaura*, in Chapter 2.

Males of *C. scaura* show pre-copulatory behaviors and frequently engage in male-male competitions (Lim and Alexander, 1986). In these male-male competitions, the second gnathopod is mainly used to fight. Furhermore, males sometimes die in these competitions, especially between large males, probably because of lethal venoms produced from the poison-producing glands on the propodus of the second gnathopod (Schulz and Alexander, 2001; Takeshita and Wada, 2012). Based on these observations and results of previous

studies, it seems adaptive to have alternative reproductive strategies for small males of *C. scaura*, such as sneaking. Moreover, it was revealed that even small males can attain large body size and weapons, by subsequent molts after sexual maturation. Therefore, if small males of *C. scaura* adopt some alternative reproductive strategies, it is possible that males change their reproductive strategy with individual lives.

The phenomenon in which same individual changes behavior along with their age or instar during postembryonic developnt, is called "temporal polyethism", or more strictly, "age polyethism" (Franks *et al*., 1997). Temporal polyethism is a concept, firstly proposed for social animals, in which older individuals tend to engage in more risky tasks than younger ones (Tofts and Franks, 1992; Robinson *et al.*, 1994; Goldsby *et al.*, 2012). Although not called as temporal polyethism, sex change can be seen as other well-known examples of the behavioral change over individual age. For example, many fish species can change their sexes during the course of postembryonic development. Fishes showing sex change are known to participate in reproduction as a male or female when their body size is relatively small, and then change their sex to the other, forming harems when they become the largest among the groups (Munday *et al*., 2006; Kuwamura *et al.,* 2020).

Considering that almost all fishes exhibit indeterminate growth (Charnov and Berrigan, 1991), and animals with indeterminate growth would be able to change their phenotype even after sexual maturation, indeterminate mode might be the source to evolve polymorphism among adult individuals, and even various reproductive strategies. Since *C. scaura* can be easily bred and used in behavioral assays, evolution and developmental basis of various life history traits, including temporal polyethism, should be an interesting research topic to be investigated in future.

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FIGURES

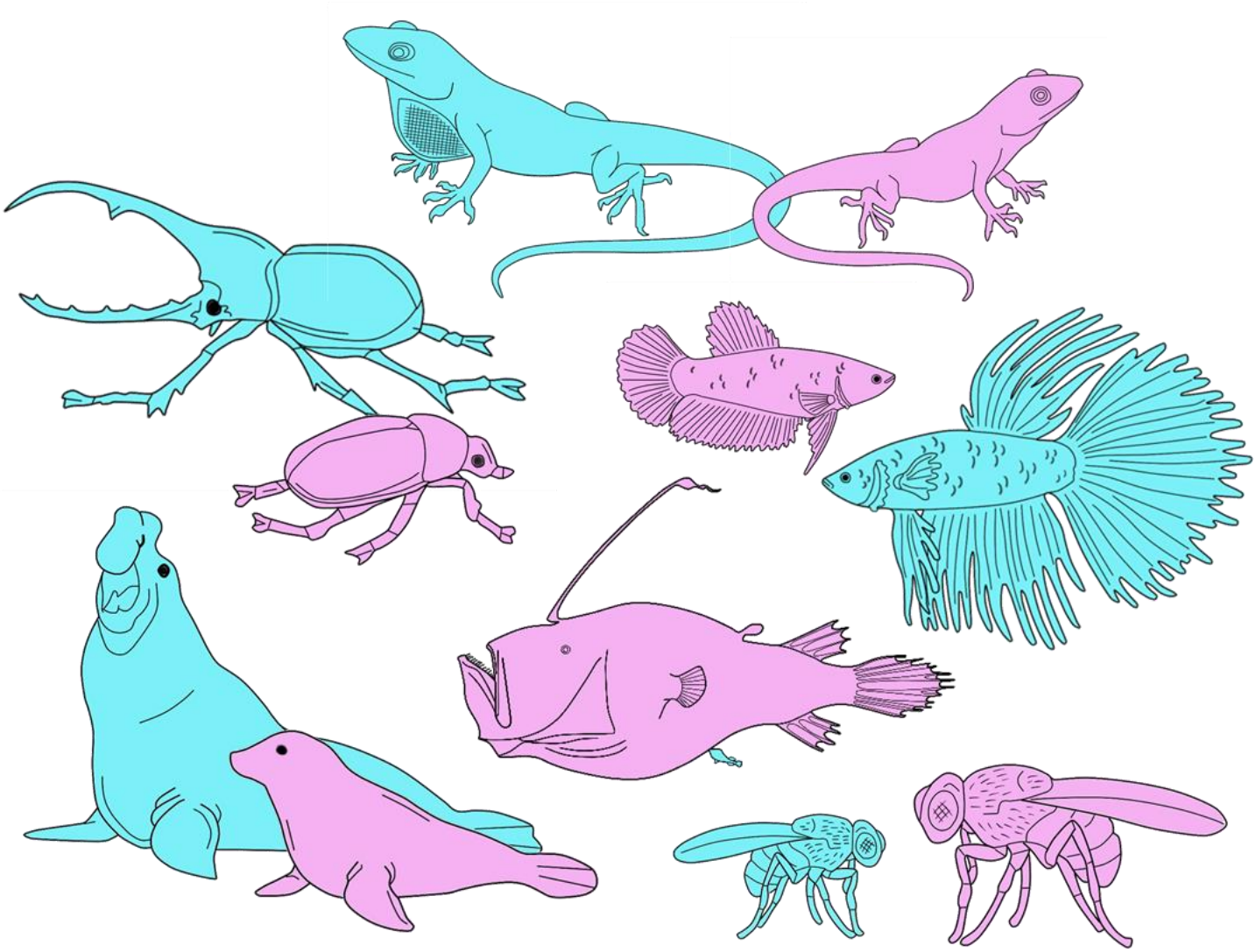


Figure 1

Various modes of sexual dimorphism found in the animal kingdom, modified after Shingleton and Vea (2023). Blue and pink individuals indicate males and females, respectively.

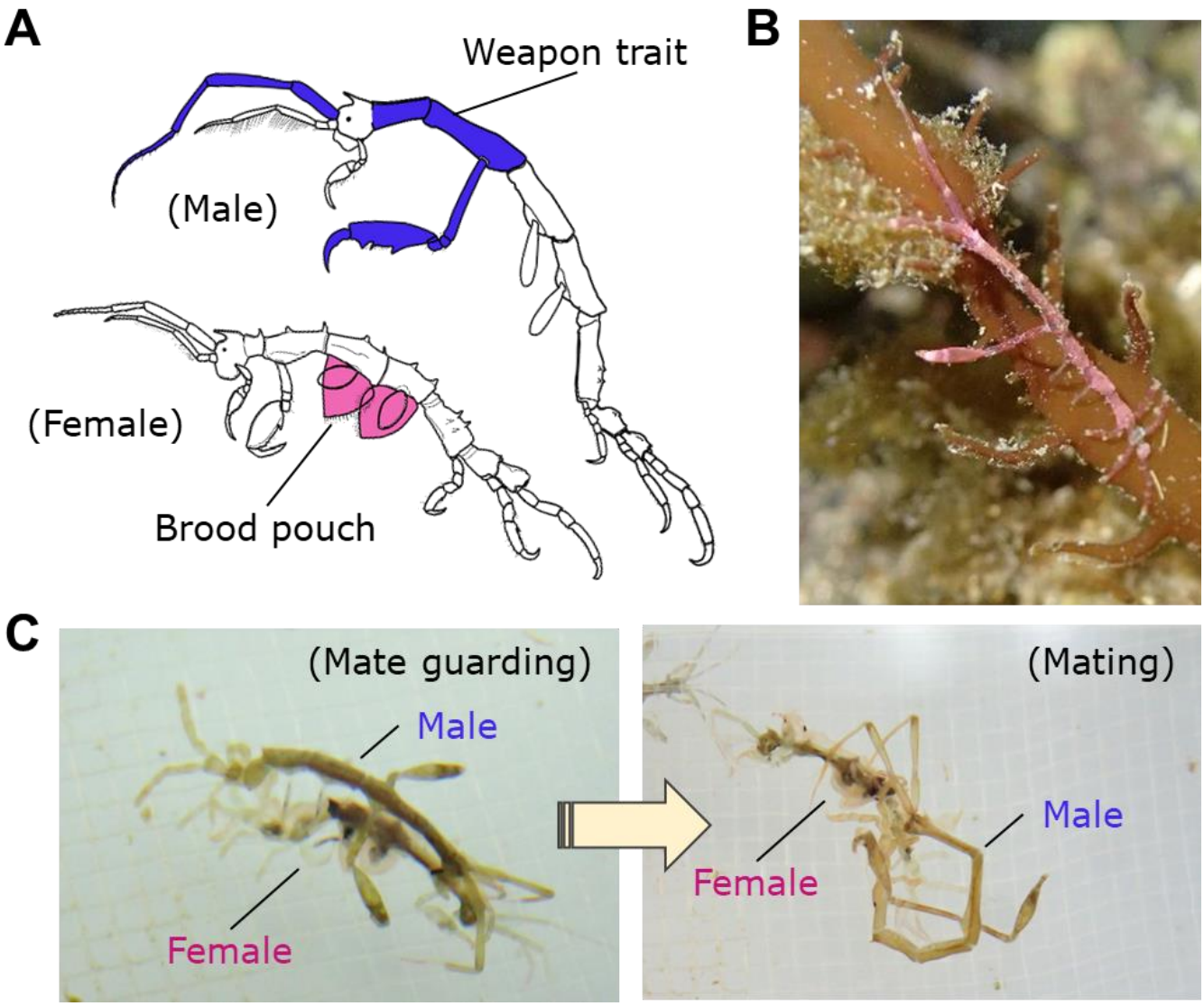


Figure 2

Sexual dimorphism, ecology and reproductive behavior of caprellids. (A) Sexual dimorphism found in caprellids. Males have larger first antenna, second gnathopod and the first and second segments which are used as weapons in male-male competition. Females develop the brood pouch on the ventral side of the third and fourth segments which is used for egg incubating. (B) Caprellids are usually found attaching on such as sea algaes or bryozoans, in wild. (C) Reprodctive behavior of *Caprella scaura*. Males exhibit a mate guarding behavior for mature females, and mate soon after females molted.

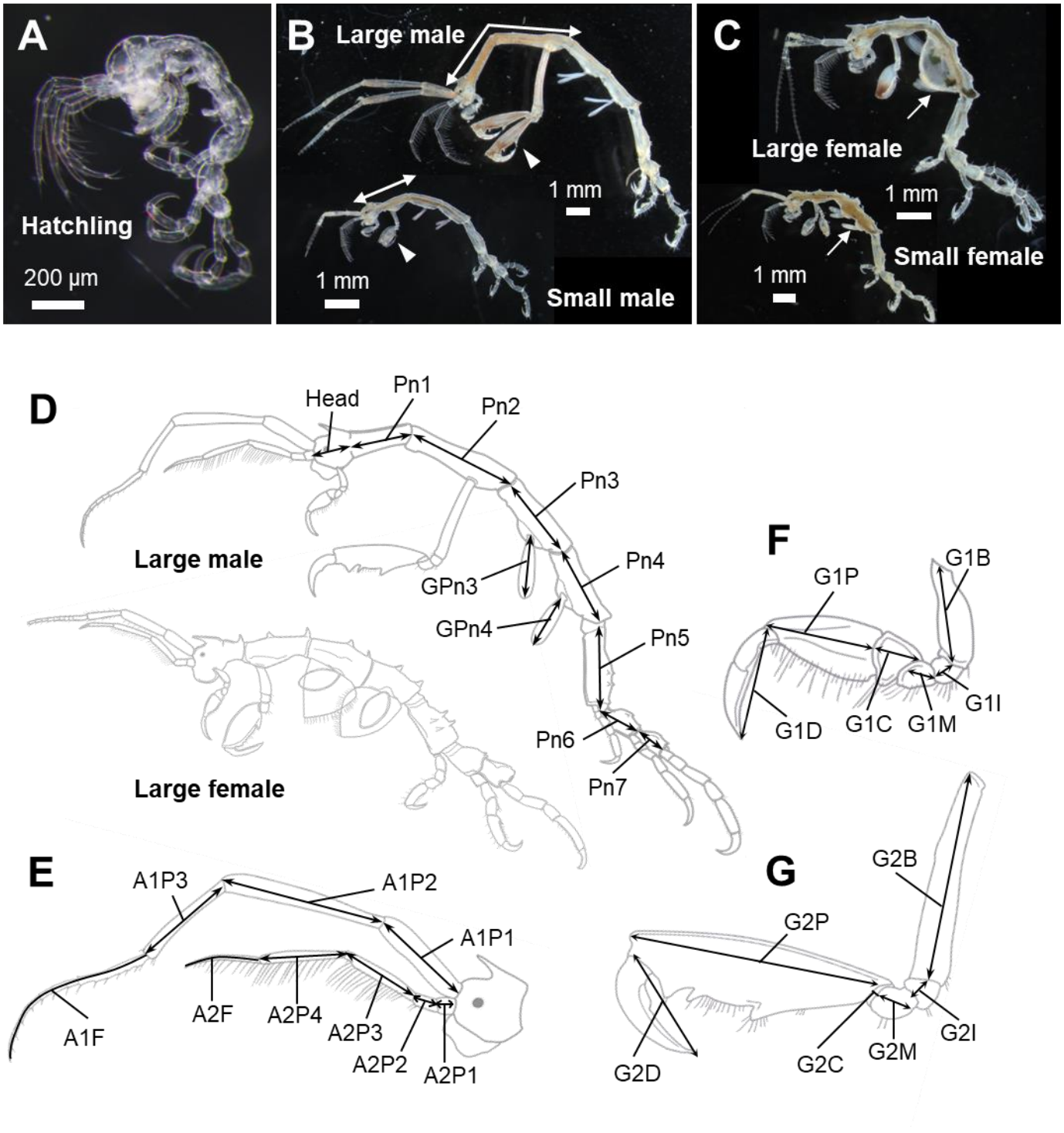


Figure 3 (The caption on the next page)

Figure 3

Photographs of living Caprella scaura individuals and diagrams of the measured traits. (A) Newly hatched larva. (B) Small and large males. Double-headed arrows indicate first and second pereonites, and arrowheads show second gnathopods. (C) Small and large females. Arrows indicate oostegites. (D–G) Diagrams showing the 31 measured traits. Scale bars in A, and B and C, correspond to 200 μm and 1 mm, respectively. (D) Whole body of a large male and female. (E) First and second antennae. (F) First gnathopod. (G) Second gnathopod. Abbreviations: Pn1, first pereonite; Pn2, second pereonite; Pn3, third pereonite; Pn4, fourth pereonite; Pn5, fifth pereonite; Pn6, sixth pereonite; Pn7, seventh pereonite; GPn3, gill on third pereonite; GPn4, gill on fourth pereonite; A1P1, first article of the peduncle of first antenna; A1P2, second article of the peduncle of first antenna; A1P3, third article of the peduncle of first antenna; A1F, flagellum of first antenna; A2P1, first article of the peduncle of second antenna; A2P2, second article of the peduncle of second antenna; A2P3, third article of the peduncle of second antenna; A2P4, fourth article of the peduncle of second antenna; A2F, flagellum of second antenna; G1B, basis of first gnathopod; G1I, ischium of first gnathopod; G1M, merus of first gnathopod; G1C, carpus of first gnathopod; G1P, propodus of first gnathopod; G1D, dactylus of first gnathopod; G2B, basis of second gnathopod; G2I, ischium of second gnathopod; G2M, merus of second gnathopod; G2C, carpus of second gnathopod; G2P, propodus of second gnathopod; G2D, dactylus of second gnathopod.

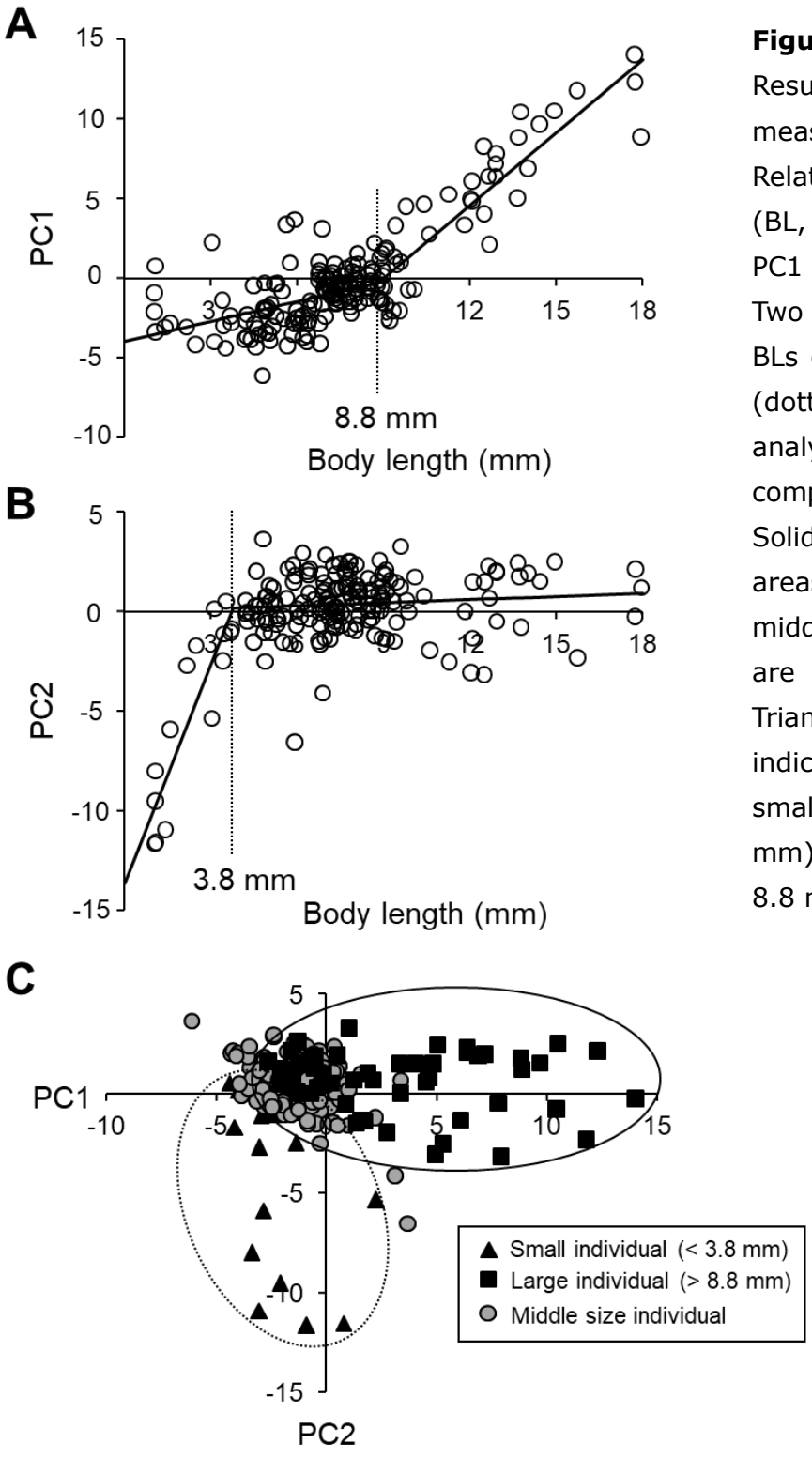


Figure 4

Results of PCA targeting 31 measurements of body parts. (A, B) Relationships between body length (BL, mm) and individual scores of PC1 (A) and PC2 (B), respectively. Two inflexion points were detected at BLs of 8.8 mm (A) and 3.8 mm (B) (dotted line) by segment regression analysis. (C) Biplot of the principal component scores of PC1 and PC2. Solid and dotted circles indicate areas where plots of small and middle-sized, and large individuals are concentrated, respectively. Trianglar, square, and circular plots indicate the three size classes, i.e., small (BL < 3.8 mm), large (BL > 8.8 mm), and middle-sized (3.8 ≤ BL ≤ 8.8 mm), respectively.

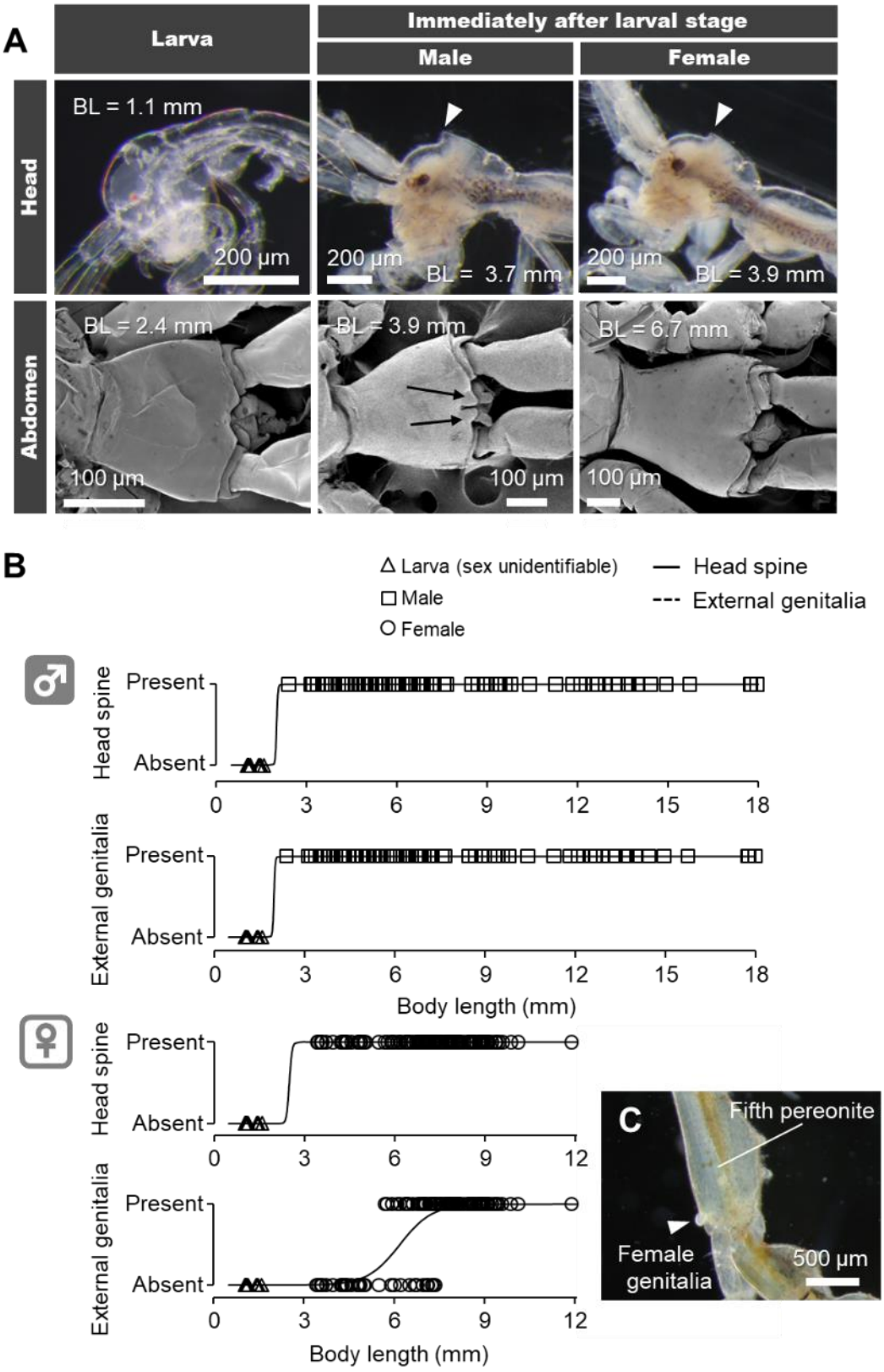


Figure 5

Morphological characteristics used for the identification of larval and juvenile stages. (A) Light microscopic images of head spines (upper row) and scanning electron microscopy (SEM) images of the ventral side of the seventh pereonite and abdomen (lower row) of larvae, and male and female juveniles. White arrowheads show head spines, and black arrows indicate penis pairs. Scale bars in the upper and lower rows correspond to 200 and 100 µm, respectively. (B) Correlation between BL and emergence of the head spine or external genitalia in each sex. Solid lines are regression lines estimated by the logistic model using the parameters estimated in the binominal logit analysis [n = 68 (males), 118 (females); likelihood-ratio test, $P < 0.05$]. (C) Female genitalia on the fifth pereonite (white arrowhead). Scale bar = 500 µm.

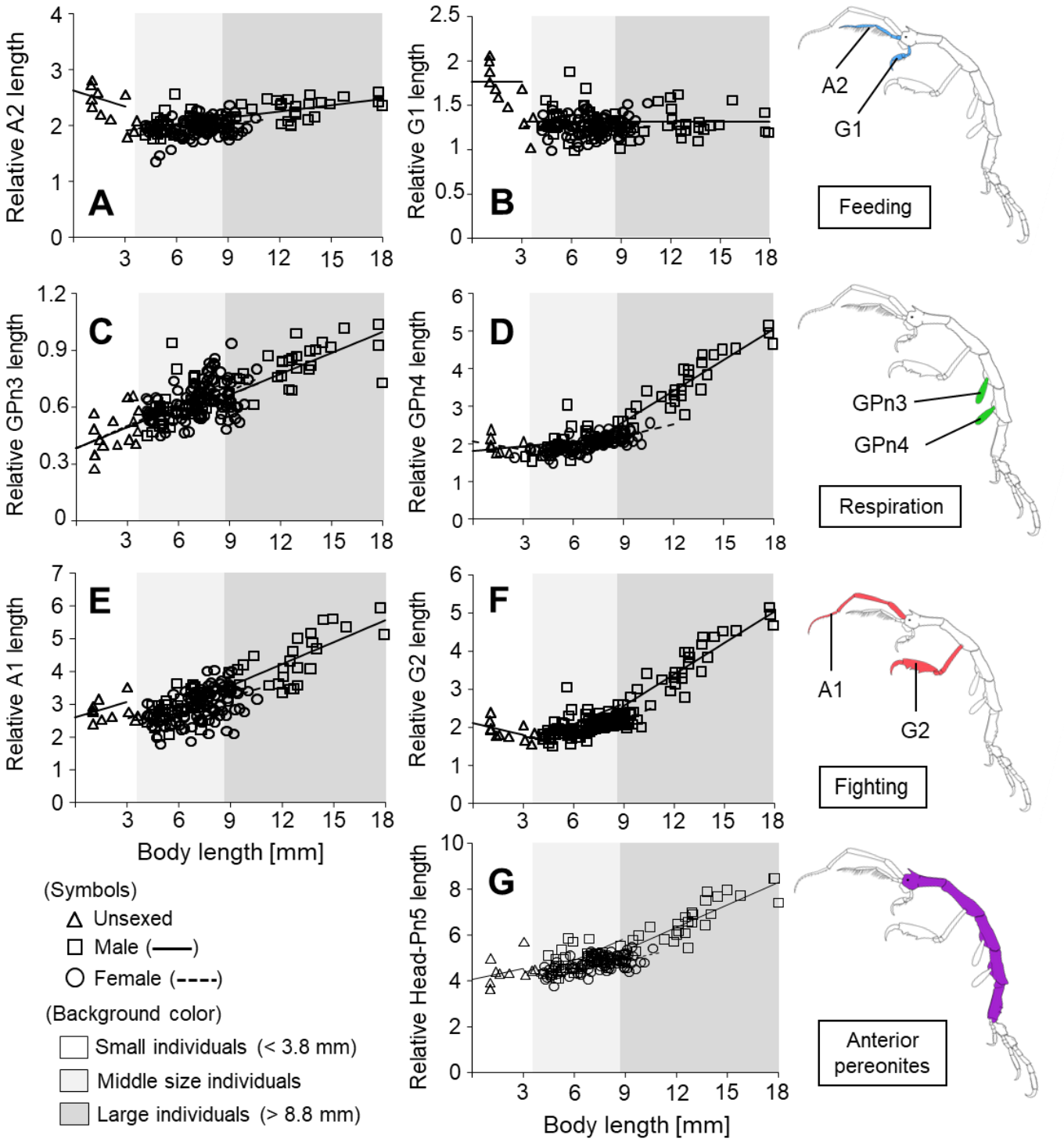


Figure 6

Allometric growth of morphological traits classified by function; second antenna (A) and first gnathopod (B) for feeding; third pereonite gill (C) and fourth pereonite gill (D) for respiration; first antenna (E) and second gnathopod (F) for fighting; anterior pereonites (G, total length of the head and first to fifth pereonites) for precopulatory behavior. The trianglar, square, and circular plots represent unsexed individuals, males, and females, respectively. Background colors indicate the size class defined by PCA: white, small (BL < 3.8 mm); light gray, middle-sized (3.8 ≤ BL ≤ 8.8 mm); dark gray, large (BL > 8.8 mm). Vertical axes represent the relative lengths of the focal traits against the length of the sixth and seventh pereonites, and BL (mm) is used for horizontal axes. The segmented regression lines obtained by the least squares method on principal component scores against BLs are also shown for each sex. The detailed statistical results for these regression lines are summarized in Supplementary Table S3.

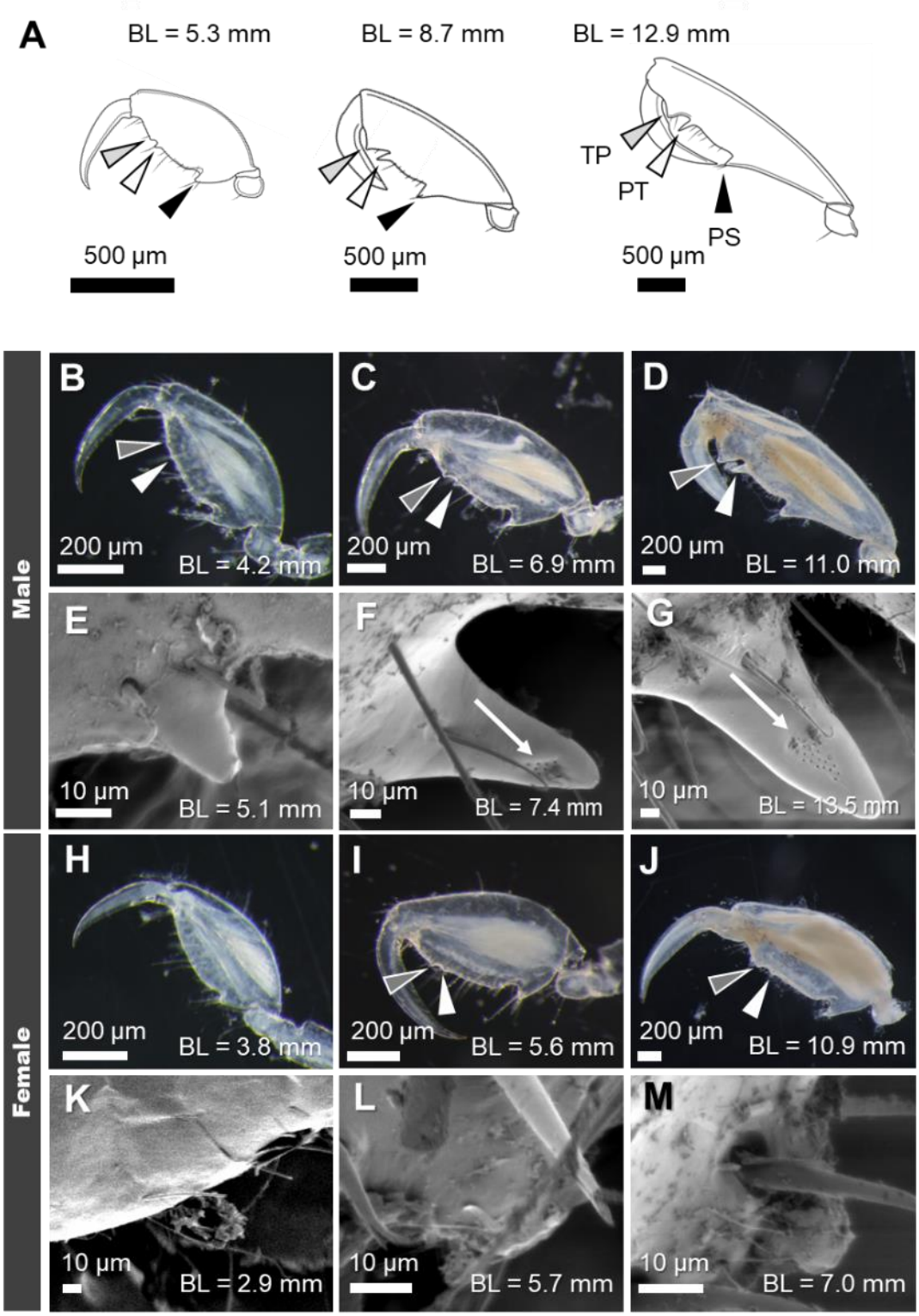


Figure 7 Developmental transition of the propodus of the second gnathopod in both sexes. (A) Diagrams of the propodus of the second gnathopod with three types of spines on the palmar margin, i.e., triangular spine (TP, gray arrowhead), poison tooth (PT, white arrowhead), and palmar spine (PS, black arrowhead) from the distal end near the dactylus. (B–M) Light microscopic images of the propodus of the second gnathopod (B–D, H–J), and SEM observations of the poison tooth (E–G, K–M). (B–G) Male. (H–M) Female. At the distal tip of the PT, multiple small pores were present only in larger males (F, G, white arrow). Scale bars in A, B–D and H–J, and E–G and K–M indicate 500, 200, and 10 µm, respectively.

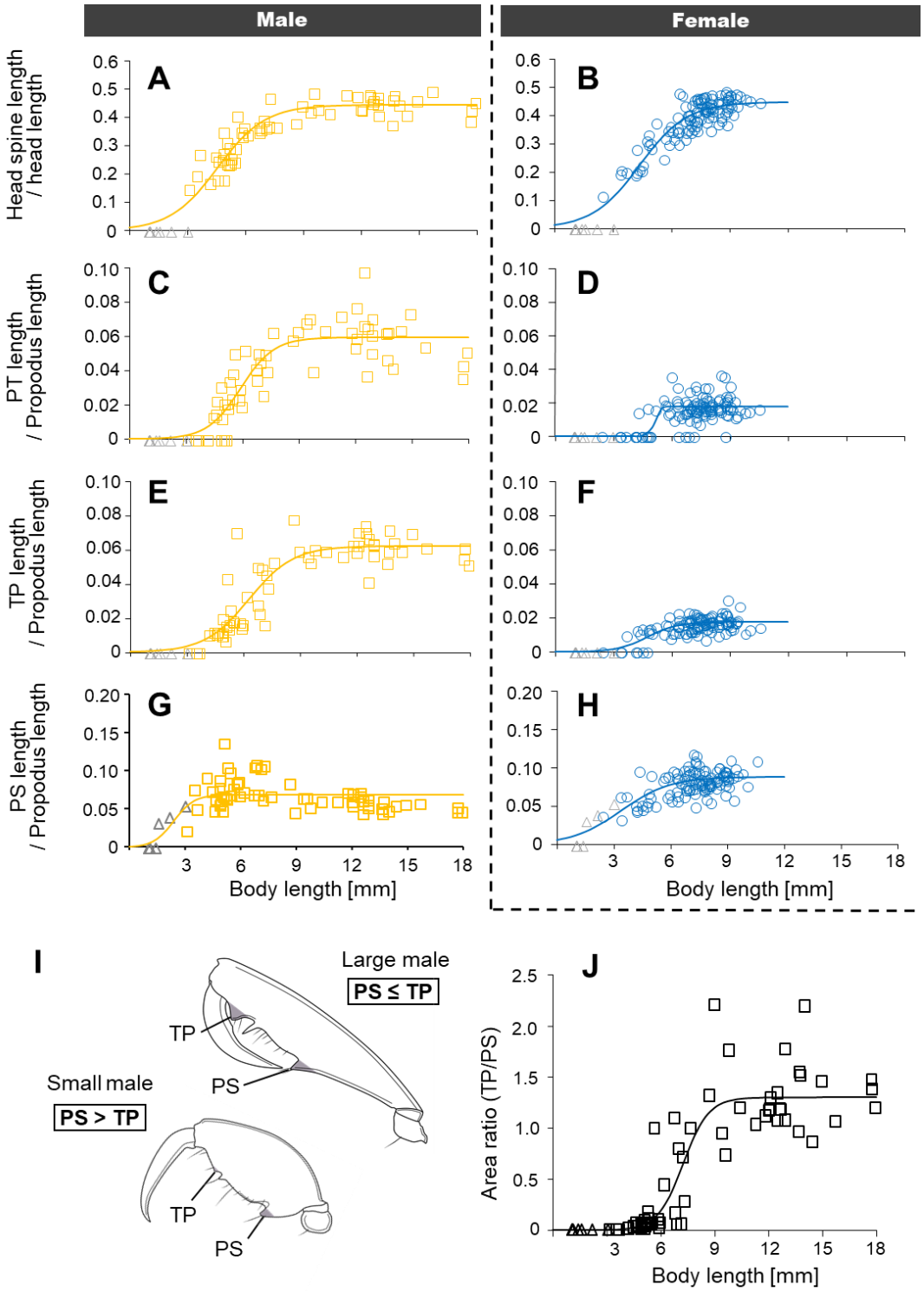


Figure 8

Allometric growth of the head spine and three marginal spines on the propodus of the second gnathopod (TP, PT, and PS) in males (A, C, E, G) and females (B, D, F, H). Square, circular, and triangular symbols in A–H and J indicate male, female, and unsexed larvae, respectively. The relative lengths of spines were plotted against BL (mm) for each sex. The regression curves were obtained using a binominal logit model (see Supplementary Table S4 for the detailed statistics). In males, the relative lengths of the head spine (A), PT (C), TP (E), and PS (G) formed a plateau at a BL of approximately 9 mm. (I) Diagram showing morphological differences between small and large males, in which the relative lengths of TP and PS clearly differ: TP is smaller than PS in small males (PS > TP), while TP is equal to or larger than PS in large males (PS ≤ TP). (J) Area ratios of TP to PS (TP/PS) are plotted against BL (mm), and the regression curve is drawn by nonlinear regression analysis using a logistic model. The TP/PS ratio reached 1 in males with a BL of approximately 9 mm.

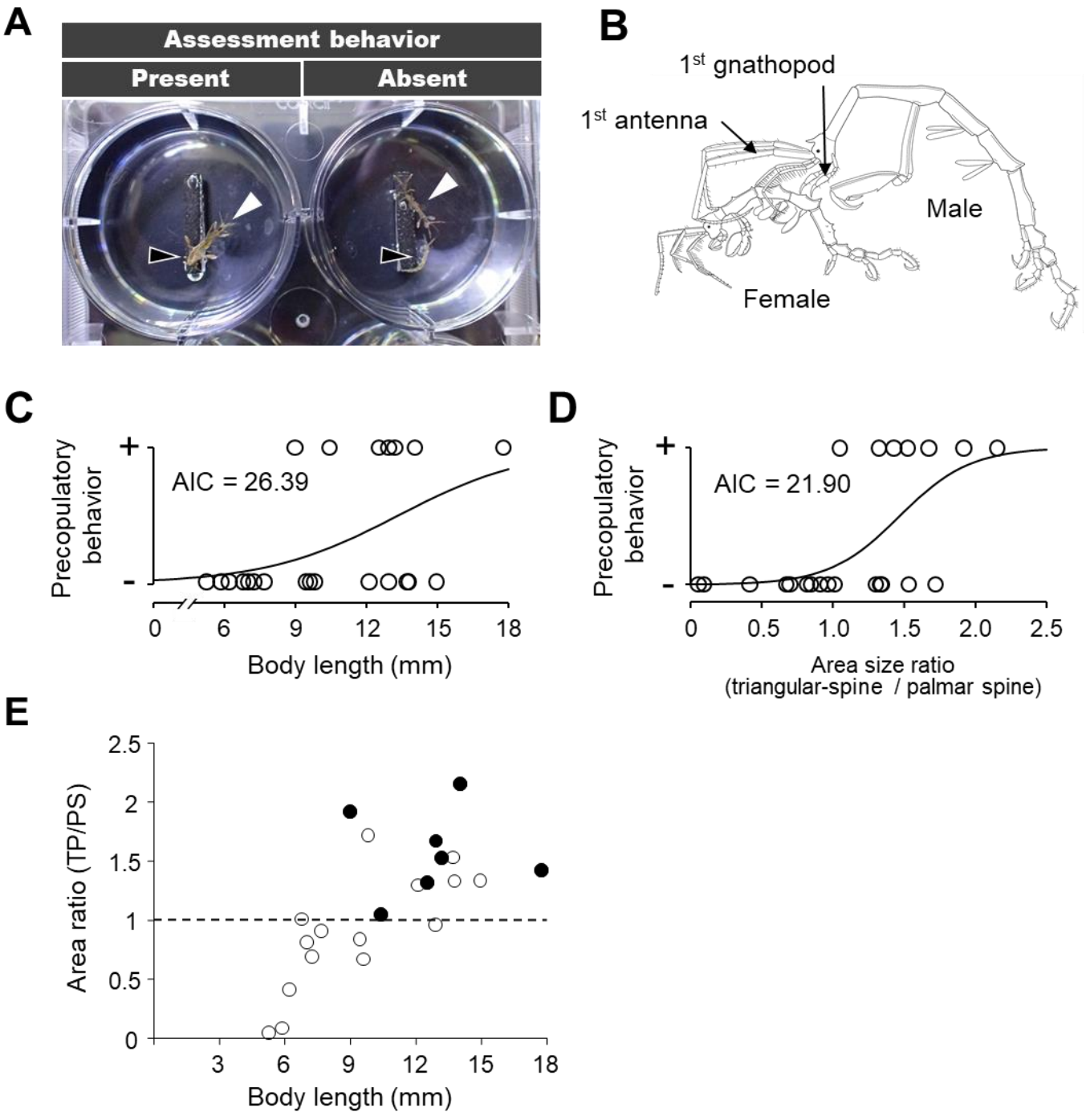


Figure 9

Photographs and diagram of male assessment behavior, and the results of behavioral assays. (A) Photograph depicting the assessment behavior of males. Black and white arrowheads show a male and a female individual, respectively. (B) Diagram of the male assessment behavior in which a male touches the dorsal side of a female with his first antenna and first gnathopod. (C) Occurrence of precopulatory behavior depending on BL. (D) Occurrence of precopulatory behavior depending on the TP/PS ratio. Regression curves obtained through a binominal logit model are shown in (C) and (D) (see Supplementary Table S5 for detailed statistics). (E) Biplot of the TP/PS ratio and BL. Black and white circles indicate males showing or not showing precopulatory behavior, respectively, during the behavioral assays.

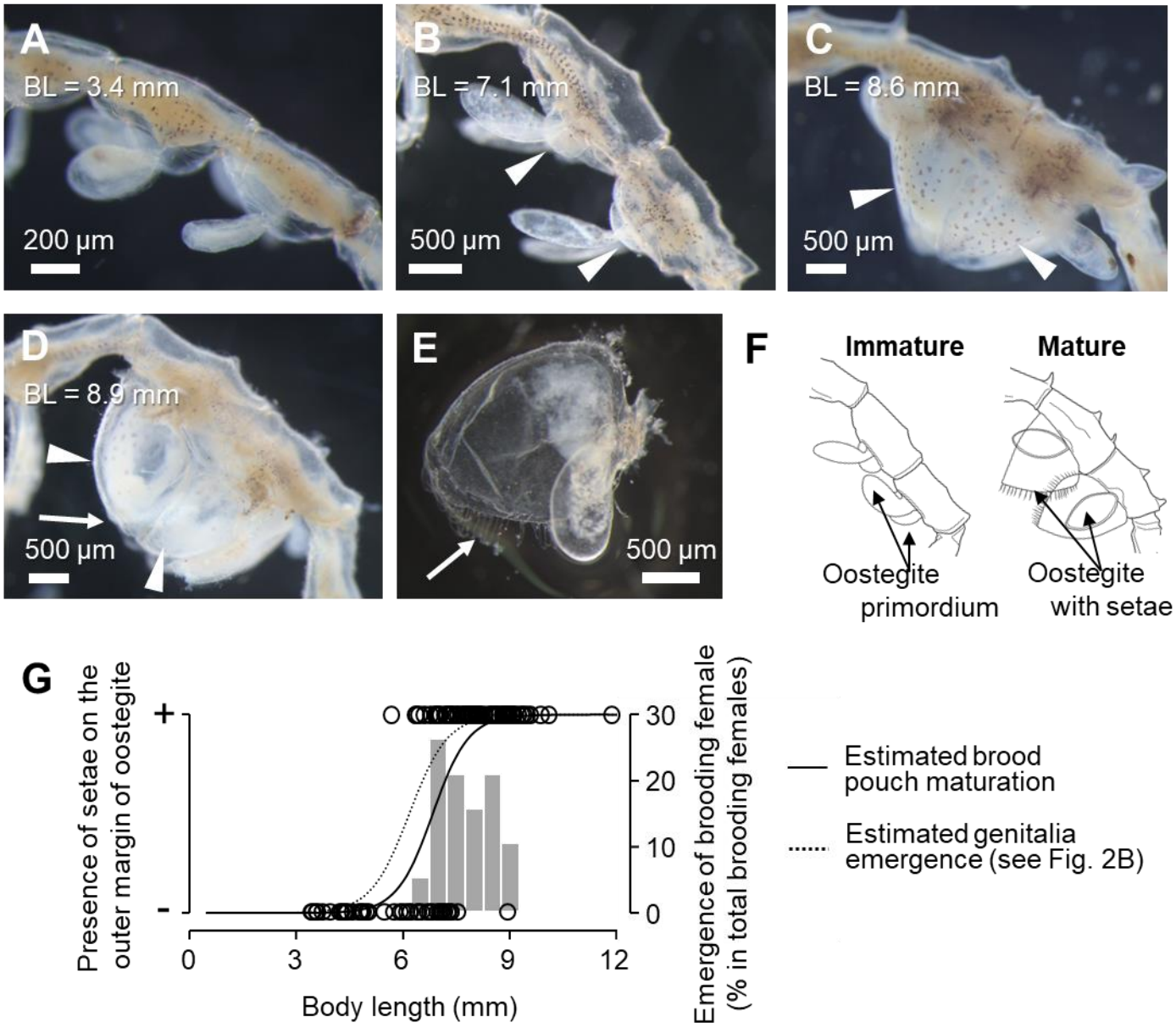


Figure 10

Oostegite development for the identification of female sexual maturation. (A) Only gills are visible on the third and fourth pereonites in young females (BL = 3.4 mm). (B) In relatively developed female juveniles (BL = 7.1 mm), oostegite primordia are visible (arrowheads), although they are much smaller than gills. (C) In larger females reaching sexual maturation, the oostegite primordia are enlarged and form a brood pouch, but marginal setae are still absent. (D) In mature females, the brood pouch is fully developed and setae are visible on it (arrow). (E) Isolated oostegite with marginal setae and a gill on the third pereonite. (F) Diagram of females with immature (left) and mature (right) oostegites. (G) Occurrence of oostegite setae depending on BL, indicating that the completion of brood pouch formation and the emergence of egg-laying females occur simultaneously at approximately the same BL. Solid and dotted lines, respectively show the full formation of the brood pouch and appearance of female external genitalia, depicted based on the analytical results obtained from a binominal logit model (n = 18, 48; likelihood-ratio test, $P < 0.05$; see Supplementary Table S6 for more detailed information). Gray bars show the percentages of egg-laying females at each BL. Scale bars in A and B–E indicate 200 µm and 500 µm, respectively.

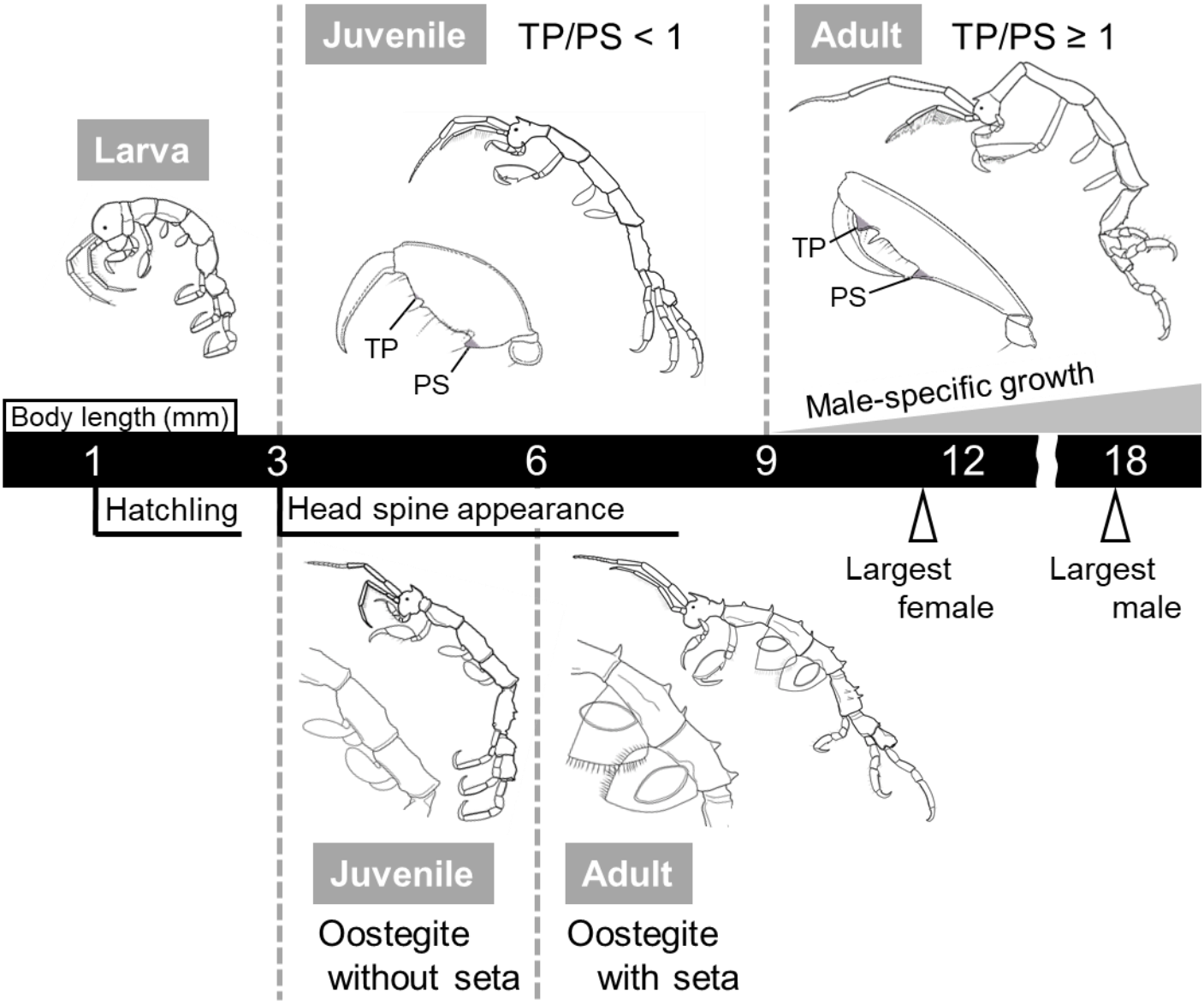


Figure 11

Summary of the postembryonic stages in C. scaura including the diagnostic morphological traits used for the identification of developmental stages (i.e., larval, juvenile, and adult) for both sexes. Newly hatched larvae had a BL of ca. 1 mm without any sex-related differences. Larvae of about 3 mm presented head spines, and the penis appeared in males of the same length, and therefore, this developmental point was regarded as the onset of the juvenile stage. The ratio between areas of TP and PS (TP/PS) in males and oostegite setae in females were used to identify the adult stage at sexual maturation. The body size range of sexually mature individuals differed between sexes: that of adult females was concentrated (BL 6–9 mm), whereas that of adult males was broader (BL 9–18 mm).

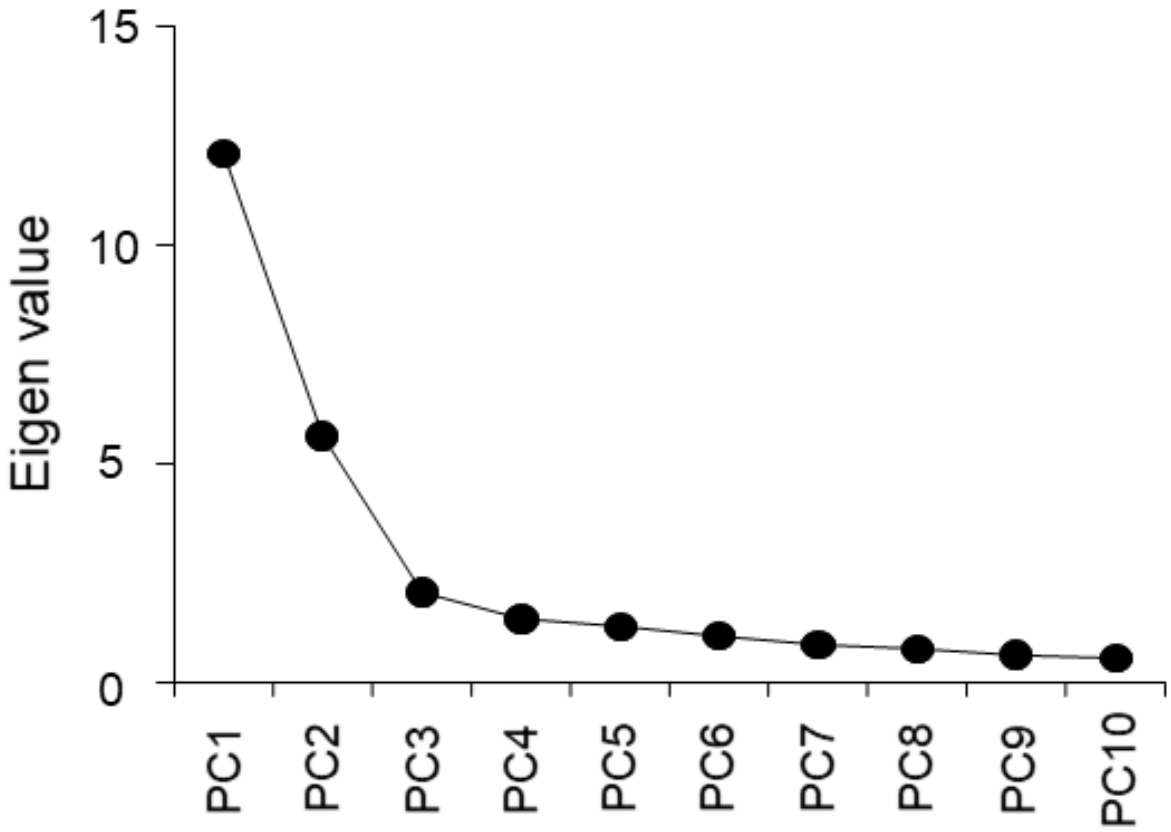
TABLE

Table 1

Principal component loadings and cumulative proportions in the PCA targeting 31 major segments of a caprellid body. The loadings ≥ 0.3 or ≤ −0.3 in the major principal components are shown.

Traits		Segments	PC1	PC2	PC3	PC4	PC5
Principal component loadings	First antenna	A1F					0.8
		A1P1	0.96				
		A1P2	0.95				
		A1P3	0.94				
		A2F		-0.61			
	Second antenna	A2P1		-0.64			
		A2P2		-0.62		0.34	
		A2P3	0.85				
		A2P4	0.84				
	First gnathopod	G1B	0.36	-0.67			
		G1I		-0.57		0.44	
		G1M		-0.53			
		G1C		-0.66			
		G1P		-0.76			
		G1D		-0.76		-0.37	
	Second gnathopod	G2B	0.91				
		G2I	0.63				
		G2M	0.75				
		G2C	0.34	-0.42			-0.33
		G2P	0.96				
		G2D	0.75				
	Gill	GPn3	0.77				
		GPn4	0.73	0.31			
	Head and pereonites	Head		-0.86			
		Pn1	0.92				
		Pn2	0.93				
		Pn3	0.78			0.4	
		Pn4	0.67			-0.4	
		Pn5	0.61		0.33	-0.38	0.3
		Pn6			-0.88		
		Pn7			0.88		
	Cumulative proportion		0.39	0.57	0.64	0.69	0.73

SUPPLEMENTARY FIGURES



Supplementary Figure 1

Scree plot of the PCA in Table 1. The variations in the eigenvalues decreased after PC3, and therefore, only two PCs were considered effective in this analysis.

Supplementary Table 1

Parameters estimated in the segmented regression analyses reported in **Figure 4A and B**. The segmented models were supported by the AIC values when compared to the linear models with no breaking point. The homoscedasticity in this analysis was supported by the bootstrapped White's test (n = 178, P > 0.05).

Explained variable	Number of breaking point	AIC	R^2	Estimated parameter (SE)			
				Intercept	Body length (mm)	Breaking point	Body length ×breaking point
PC1	0	753.2	0.678	-7.032 (0.395)	0.927 (0.048)***		
	1 (best)	684.6	0.786	-3.933 (0.500)	0.418 (0.078)***	-9.756 (1.158)	1.108 (0.119)***
PC2	0	786.3	0.168	-2.386 (0.433)	0.315 (0.053)***		
	1 (best)	656.9	0.607	0.030 (0.364)	0.051 (0.043)*	-13.673 (1.034)***	3.630 (0.372)***

*: $P < 0.05$, ***: $P < 0.001$ (n = 178, likelihood-ratio test)

Supplementary Table 2

Parameters estimated in the analyses conducted using a binominal logit model in **Figure 5B.**

Sex		Male		Female	
Explained variable (presence/absence)		Head spine	External genitalia	Head spine	External genitalia
n		68	68	118	118
Estimated parameter (SE)	Intercept	-1053.5 (78367.5)	-1053.5 (78367.5)	-8.703 (4.531)	-11.604 (2.643)
	Body length (mm)	340.8 (25327.9)***	340.8 (25327.9)***	3.195 (1.559)***	1.902 (0.398)***
Body length at inflection point (mm)		3.09	3.09	2.72	6.10

***: $P < 0.001$ (likelihood-ratio test)

Supplementary Table 3

Parameters estimated in the multiple regression analysis for the regression lines in **Figure 6.**

	Explained variable		Relative A1 length		Relative A2 length		Relative G1 length	
	Model		Best	Full	Best	Full	Best	Full
	AIC		210.0	222.1	-142.0	-131.4	-208.6	-203.3
	R^2		0.685	0.692	0.510	0.520	0.380	0.435
Estimated parameters (SE)	Intercept		1.778 (0.207)	1.594 (0.282)	1.673 (0.038)	1.634 (0.105)	1.258 (0.013)	1.218 (0.085)
	Body length		0.162 (0.027)***	0.19 (0.04)***	0.038 (0.005)***	0.045 (0.015)***		0.005 (0.012)
	Sex	Larval stage (sex is not clear)	0.46 (0.243)	0.47 (1.565)	0.747 (0.164)***	0.691 (0.58)*	0.503 (0.049)***	0.652 (0.474)
		Male	-0.261 (0.236)**	0.001 (0.516)	0.118 (0.027)***	0.108 (0.191)***	0.046 (0.021)*	0.082 (0.156)
	Size class	Small	0.335 (0.182)	0.315 (1.542)	0.201 (0.083)	0.296 (0.571)		0.154 (0.467)
		Large		2.672 (1.909)		0.23 (0.707)		-0.83 (0.578)
	Sex × size class	Male × small		1.75 (4.078)	-0.200 (0.124)	-1.758 (1.511)		-0.122 (1.235)
		Male × Large		-3.037 (2.011)		-0.118 (0.745)		0.685 (0.609)
	Body length × sex	Body length × larval stage		0.061 (0.512)	-0.136 (0.084)	-0.119 (0.19)		-0.166 (0.155)
		Body length × male	0.063 (0.03)*	0.023 (0.083)		0 (0.031)		0.001 (0.025)
	Body length × size class	Body length × small		0.035 (0.46)		-0.024 (0.17)		-0.008 (0.139)
		Body length × large		-0.304 (0.208)		-0.032 (0.077)		0.091 (0.063)
	Body length × sex × size class	Body length × male × small		-0.549 (1.18)		0.455 (0.437)		-0.032 (0.357)
		Body length × male × Large		0.336 (0.223)		0.02 (0.083)		-0.087 (0.068)

	Explained variable		Relative G2 length		Relative GP3n length		Relative GP4n length		Relative Head-P5n length	
	Model		Best	Full	Best	Full	Best	Full	Best	Full
	AIC		-63.6	-58.0	-351.5	-340.2	-333.5	-323.0	147.6	148.5
	R^2		0.921	0.924	0.554	0.580	0.543	0.566	0.834	0.846
Estimated parameters (SE)	Intercept		1.282 (0.118)	1.188 (0.129)	0.379 (0.021)	0.370 (0.058)	0.355 (0.029)	0.313 (0.061)	3.644 (0.186)	3.656 (0.23)
	Body length		0.101 (0.017)***	0.115 (0.018)	0.037 (0.003)***	0.038 (0.008)***	0.042 (0.004)***	0.047 (0.009)***	0.159 (0.027)***	0.157 (0.032)***
	Sex	Larval stage (sex is not clear)		0.859 (0.713)		0.107 (0.323)		0.157 (0.339)		-0.259 (1.273)**
		Male	-0.277 (0.138)***	0.059 (0.235)***		-0.01 (0.106)		0.066 (0.112)	-0.721 (0.221)***	-0.263 (0.419)***
	Size class	Small	0.807 (0.181)**	0.027 (0.702)		-0.198 (0.318)		-0.219 (0.334)	0.408 (0.143)**	-0.187 (1.254)
		Large	-0.933 (0.263)	0.178 (0.869)	-0.053 (0.022)*	-0.166 (0.393)*	0.034 (0.066)*	-0.088 (0.413)*	-0.184 (0.106)**	0.338 (1.552)*
	Sex × size class	Male × small		-0.347 (1.857)		-0.425 (0.84)		-0.8 (0.882)		-0.487 (3.316)
		Male × Large		-1.354 (0.916)		0.293 (0.414)		0.24 (0.435)	-0.336 (0.23)	-1.523 (1.635)
	Body length × sex	Body length × larval stage		-0.244 (0.233)		0.012 (0.106)		-0.017 (0.111)		0.42 (0.417)
		Body length × male	0.076 (0.02)***	0.022 (0.038)		0.004 (0.017)		-0.008 (0.018)	0.170 (0.034)***	0.093 (0.067)
	Body length × size class	Body length × small	-0.198 (0.055)***	0.058 (0.21)		0.064 (0.095)		0.083 (0.1)		0.16 (0.374)
		Body length × large	0.098 (0.03)**	-0.026 (0.095)***		0.01 (0.043)	-0.010 (0.007)	0.001 (0.045)		-0.056 (0.169)
	Body length × sex × size class	Body length × male × small		0.021 (0.537)		0.106 (0.243)		0.204 (0.255)		0.075 (0.960)
		Body length × male × Large		0.165 (0.102)		-0.026 (0.046)		-0.018 (0.048)		0.150 (0.182)

*: P < 0.05, **: P < 0.01, ***: P < 0.001 (n = 178, likelihood-ratio test)

Supplementary Table 4

Parameters estimated in the non-linear regression analysis conducted using a logistic model in **Figure 7 A–H** (A), and **Figure 7J** (B).

A

Explained variable		Head spine length / head length		PT length / propodus length		TP length / propodus length		PS length / propodus length	
Sex		Male	Female	Male	Female	Male	Female	Male	Female
n		68	118	68	118	68	118	68	118
R^2		0.920	0.897	0.808	0.480	0.851	0.638	0.494	0.663
Estimated parameter (SE)	L_∞	0.442 (0.008)***	0.447 (0.009)***	0.059 (0.002)***	0.017 (0.001)***	0.062 (0.002)***	0.017 (0.001)***	0.068 (0.003)***	0.088 (0.003)***
	g	0.813 (0.087)***	0.822 (0.079)***	1.131 (0.235)***	7.277 (6.465)	0.852 (0.148)***	1.286 (0.322)***	1.848 (0.796)*	0.775 (0.133)***
	L_0	3.71 (0.422)***	3.597 (0.347)***	6.677 (1.324)***	37.26 (32.544)	5.358 (0.864)***	6.126 (1.540)***	4.41 (1.926)*	2.623 (0.492)***

*: $P < 0.05$, ***: $P < 0.001$ (t-test for regression coefficient)

B

Explained variable		Area ratio (TP/PS)
n		68
R^2		0.798
Estimated parameter (SE)	L_∞	1.300 (0.059)***
	g	1.413 (0.434)**
	L_0	10.244 (3.000)**

**: $P < 0.01$, ***: $P < 0.001$ (t-test for regression coefficient)

Supplementary Table 5

Parameters estimated in the analysis conducted using a binominal logit model targeting the assessment behavior within the precopulatory mating behavior in **Figure 8C and D**.

Model		Area size ratio (best)	Body length	Full
AIC		21.9	26.4	32.4
Estimated parameter (SE)	Intercept	-5.946 (2.598)	-4.75 (2.214)	74.07 (143.34)
	Body length (mm)		0.356 (0.184)*	-1.90 (12.63)
	Area size ratio (TP/PS)	4.053 (1.857)**		-105.68 (126.27)*
	Body size ratio (female/male)			-153.42 (270.69)
	Body length × area size ratio			4.47 (9.96)***
	Body length × body size ratio			4.95 (27.84)
	Area size ratio × body size ratio			194.50 (240.16)***
	Body length × area size ratio× body size ratio			-8.48 (22.53)

*: P < 0.05, **: P < 0.01, ***: P < 0.001 (n = 22, likelihood-ratio test)

Supplementary Table 6

Parameters estimated in the analysis conducted using a binominal logit model in **Figure 9G.**

Explained variable (presence/absence)		Mature brood pouch
Estimated parameter (SE)	Intercept	-11.726 (2.521)
	Body length (mm)	1.73 (0.352)***
Body length at inflection point (mm)		6.78

***: $P < 0.001$ (n = 118, likelihood-ratio test)