Study on the Growth Process and Behavior of Polyclad Flatworms

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SUMMARY

Polyclad flatworm is almost exclusively marine-living invertebrate belonging to the class Rhabditophora (phylum Platyhelminthes). Among the class, polyclads are characteristic in having a planktonic larval stage in the growth process. Also, they are known to associate with various species of marine invertebrates through relationships such as predatory-prey interference and symbiosis. However, studies addressing such characteristic features of this group have not been popular. Details on the polyclad growth process from the planktonic stage to the benthic form, behaviors seen in the different growth stages, and the affect they have ecologically are yet to be observed and evaluated. This is presumably because their delicate body and unknown behavioral patterns made it difficult to handle them in the laboratory. As a result, most of the polyclad research concerns identification, taxonomy, and embryonic development. One possible solution present in overcoming the technical difficulties in studying polyclads is establishing a laboratory culture system. In this study, I established a laboratory culture system for four polyclad species, *Notocomplana koreana*, *Pseudostylochus*

obscurus, *Comoplana pusilla*, and *Stylochoplana paraistica*, the former two species being free-living and the latter two species symbiotic. These species hatch out as lobe-less larvae with four eyespots, but the number of eyespots increases in later development. Cross-like and triangularly shaped larvae are observed in *N. koreana* and *P. obscurus*, respectively. After settlement, a pale area appears on the body of juveniles and then develops into the copulatory complexes. All four species could be successfully reared on brine shrimp, but only *C. pusilla* and *N. koreana* achieved reproductive maturation by this method. In *P. obscurus*, switching the food to the gastropod *Monodonta labio* induced sexual maturation. In *S. parasitica*, culturing the flatworms with the host chiton, *Liolophura japonica*, was necessary for growth and sexual maturation and suggested a strong dependency of the flatworm to the host chiton.

Taking advantages of the culture system established in this study, behavioral and ecological study was also attempted. I first assessed the phototactic behavior of *P. obscurus* larvae. Most of the larvae react negatively to light in the early stages, and gradually switch to positive. In addition, initial swimming direction of the larvae to the light stimulus was altered by the lighting condition before the stimulus was given.

Second, I examined the growth and reproductive maturity of the polyclad *Stylochoplana parasitica*, a species commensal to chitons. From the field observations, they were found to reproduce during the summer months from June to August, and the juveniles become symbiotic in the fall of the same year, which was supported by the growth process observed in the laboratory culture. These results show that the successful culturing system not only allowed the close observation of the growth process, but to evaluate behavioral changes that take place during this time and how the polyclads relate to other invertebrates in more detail.

INTRODUCTION

Members of the order Polycladida (polyclads) are dorsoventrally flattened worms belonging to the class Rhabditophora, the non-parasitic group of the phylum Platyhelminthes. Relatively large in size among the rhabditophorans, they are easily observed with the naked eye, and approximately 800 species are reported (Martín-Durán and Egger, 2012), most of which are exclusively marine (Prudhoe, 1985). The majority of them are free-living, but some are reported to be symbiotic with hermit crabs (Prudhoe, 1968; Lytwyn and McDermott, 1976), gastropods (Kato, 1933) and other marine invertebrates (Prudhoe, 1985). Although some platyhelminthes such as planarians have been known for high regeneration capacity (Duran et al., 2016), polyclads are said to have low regenerative capacity, and reproduce only by oviposition. Polyclads are hermaphrodites, yet are not known to self-fertilize, and thus they do not reproduce asexually (Prudhoe, 1985). With an exception of one species, all polyclads have a planktonic larval stage, which is a characteristic only seen in this group and the parasitic Platyhelminthes subgroup, the Neodermata (Rawlinson, 2014). A single

species in another free-living platyhelminth group, a catenulid *Rhynchoscolex simplex*, was once reported to have a larval stage called Luther's larva (Reisinger, 1924), but this larval form is now recognized as a direct developing form (Martín-Durán and Egger, 2012).

The larvae of polyclads are ciliated over the entire body, which has been considered the evidence for a phylogenetic link to other groups that develop through similarly shaped larvae, such as trochophore larvae (Ballarin and Galleni, 1987) and pilidium larvae (Prudhoe, 1985). However, current consensus considers the polyclad larval stage to be an independently acquired trait from the larval forms observed in the other taxonomical groups (Martín-Durán and Egger, 2012). Some variation is seen in the number of lobes they possess, wherein the typically observed four- and eight-lobed types are called Götte's or Müller's larvae, respectively (cf. Prudhoe, 1985; Shinn, 1987; Rawlinson, 2010). Five-, six-, seven-, and ten-lobed types have also been reported, but are not given individual names; instead, typically the five-lobed type is included in Götte's larva, while larva with more lobes are referred as Müller's larvae (Rawlinson, 2014). Another rare type of lobed larval stage, usually called Kato's larva but also

called intracapsular Müller's larva or intermediate developers, are seen to complete metamorphosis within the eggshell. In this case, the larva lacks a planktonic stage, and the young animal hatches out, resembling a miniature version of the adult (Kato, 1940; Rawlinson et al., 2011). Only two species are known to have this type of reproduction, but *Planocera reticulata* was reported to usually hatch as a lobed, pelagic larvae (Teshirogi et al., 1981; Martín-Durán and Egger, 2012). The other species, *Amakusaplana acroporae*, is the only species to exclusively reproduce in this manner (Rawlinson et al., 2011). Besides these larval forms, where the morphology of the larva differs greatly from that of the mature adult, there is a third type of development through a planktonic stage which lacks lobes at any time in the growth process, both before and after hatching (Kato, 1940; Ballarin and Galleni, 1984; Prudhoe, 1985; Shinn, 1987; Smith et al., 2002). This type of hatchlings have been referred to in various names in previous reports, such as 'directly developing juveniles,' 'direct developers' (Martín-Durán and Egger, 2012), 'miniature adults,' a 'form resembling an adult' (Kato, 1940; Prudhoe, 1985; Smith et al., 2002; Bolaños and Litvaitis, 2009), a 'juvenile worm' (Ballarin and Galleni, 1984), and 'pelagic larva lacking arms' (Ruppert,

1978). The evolutionary relationship of these different larval types is still under question. Götte's larvae and direct developers are only found in suborder Acotylea (species without suckers), while Müller's and intracapsular Müller's larvae are found both in suborder Acotylea and Cotylea (species with ventral suckers) (Rawlinson, 2010; Rawlinson et al, 2011; Martín-Durán and Egger, 2012). Even two to three different types of larvae have been reported from the same genera within the Acotylea (Martín-Durán and Egger, 2012).

Observations reveal that the planktonic larval stage of polyclads can last for a few days to a few months (Prudhoe, 1985; Smith et al., 2002; Johnson and Forward Jr., 2003). A few reports have been made on metamorphosis in species with the Müller's or Götte's larval stage, noting changes such as resorption of the lobes, dorsoventral flattening of the body, multiplication of the eyespots, change in phototaxis from positive to negative, and progressive branching in the intestines (Anderson, 1977; Ruppert, 1978; Shinn, 1987; Smith et al., 2002). A recent paper describes the requirement of algal food for development of Götte's larva (Allen et al., 2017). However, details on post-embryonic development, such as larval behavior, settlement, growth, and the

process of reproductive maturation, are still lacking for most of the larval types (Ruppert, 1978; Shinn, 1987; Chen et al., 1990).

Descriptions on the late development, behavior, and ecological strategy are generally lacking in free-living platyhelminthes groups (Rawlinson, 2008; Rawlinson and Litvaitism 2008; Rieger, 1998). Polyclads are carnivorous and prey on various marine invertebrates, but the amount of ecological impact given by the polyclads is little studied, (Prudhoe, 1985; Janiak et al., 2016). Also, some polyclads have been recorded to be symbiotic to other marine invertebrates, but how strongly they associate with the host species are not clear (Prudhoe, 1985). Most of the reports on polyclads have focused on taxonomical descriptions (Rawlinson, 2014). Embryonic development is relatively well studied since the eggs are easy to acquire by keeping mature specimens if they can be captured (e.g. Younossi-Hartenstein and Hartenstein, 2000; Rawlinson et al., 2008; Lapraz et al., 2013). The eggs are also a good sample for observation since they are usually transparent (Deguchi et al., 2009). Overall, polyclad studies were focused on limited areas, and this is presumably because of difficulty in collecting specimens due to their delicate body and cryptic behavior (Rawlinson, 2008). In

addition, long-term laboratory experiments have been difficult due to little available knowledge in food preferences for specific species of polyclads (Rawlinson et al., 2011). Polyclads can survive an extensive period without food, as reported in the case of *Martigrella crozieri*, where the flatworm lived up to 143 days without being fed. The capability of oviposition was also observed under starved condition but considerable decrease in body size could not be avoided (Lapraz et al., 2013). One possible solution to approach the difficulties in studying polyclads is establishing a laboratory culturing system.

If captured without damage, benthic juveniles and adults of some species such as *P. obscurus* (Teshirogi et al., 1981) and *M. crozieri* (Lapraz et al., 2013) are known to be maintainable in the laboratory. However, not many attempts have been made on culturing the larvae, and most of them have been unsuccessful (Shinn, 1987). Deguchi et al. (2009) reported one of the successful cases, where the embryonic development of naturally spawned eggs and fertilized eggs obtained from egg receptacles in the sexually mature specimens were observed in a symbiotic polyclad species, *Comoplana pusilla*. They also collected the larvae from the host gastropods as well as larvae that hatched in

the laboratory and cultured until they reached sexual maturity. However, only little observation has been made on the growth process after hatching to sexual maturation.

In this study, I have attempted to establish a laboratory culture method from hatching to sexual maturation for four species of direct-developing polyclads: *Comoplana pusilla*, *Notocomplana koreana*, *Pseudostylochus obscurus*, and *Stylochoplana parasitica*. The different conditional needs for each species were found, and the growth processes were observed. Using the culturing system, behavioral and ecological studies were also undertaken. Phototaxis is suggested to take a large part in invertebrate larval behavior and have previously been studied for the lobed, Müller's larva (Johnson and Forward Jr., 2003). In the present study, the phototactic behavior during the planktonic period of a direct developer, *P. obscurus*, was observed for the first time. Also, field observations were made on *S. parasitica*, which is symbiotic to chitons. Together with the growth process observed in the laboratory, I illustrated the lifecycle of this species in the field population.

MATERIALS AND METHODS

Sample Collection

Comoplana pusilla, *Notocomplana koreana*, and *Pseudostylochus obscurus* were all collected at Nabeta Bay, Shimoda City, Shizuoka Prefecture, Japan, while *Stylochoplana parasitica* was captured in two places, Nabeta Bay and Tatado Beach, Shimoda City. All sampling was done during low tide at an intertidal area for the first three species and above the tideline for *S. parasitica*.

Specimens of the symbiotic *C. pusilla* were collected from the gastropod *Monodonta labio* (Linnaeus, 1758), in which *C. pusilla* is reported to most commonly occur among several habitable gastropod species (Kato, 1933; Deguchi et al., 2009; Fujiwara et al., 2014). The gastropods were collected, taken back to the laboratory, and dissected to search for internal flatworms. In total, five individuals of *C. pusilla* were collected, three in February and two in July 2012, with only a single flatworm found per gastropod.

The free-living species *N. koreana* and *P. obscurus* were collected at the bottom or underneath rocks around the tideline. Specimens were gently removed using a soft-tipped brush and placed into a plastic container filled with seawater. Several specimens with morphological similarities were placed together in the same container. Five *Notocomplana*-like specimens were collected in January 2011, and four specimens of *P. obscurus* were collected in February and March 2012 for establishing the laboratory culture system. For phototaxis experiments, three mature specimens of *P. obscurus* were collected in December 2016 in Nabeta Bay.

Specimens of *S. parasitica* were collected once a month from February 2015 to January, 2016. Thirty samples of possible host specimens were removed from the rock surfaces by wedging tweezers. The removed animals were individually placed in vinyl bags with seawater to be brought back to the laboratory. The flatworms live in the pallial groove of the host, thereby the chitons were placed with the ventral side turned upwards in a glass container filled with seawater and observed under the stereoscopic microscope (Olympus SZX7; Olympus Co., Tokyo, Japan). The muscular foot was moved out of the view and the specimen was kept stretched using tweezers. When

polyclads were present, they were washed out from the host by creating a current with a pipette. The collected specimens were then sucked with the pipette and placed in a 16 ml plastic, seawater-filled container with a lid. The specimens from the same host were placed in the same container.

Species identification

Some external characteristics were helpful in identification, but I made histological sections for *Comoplana pusilla*, *Notocomplana koreana*, and *Pseudostylochus obscurus* to examine the internal structure of the copulatory apparatus to confirm the species identification according to the traditional method for polyclads. Reproductively mature specimens were taken from the cultured batches and used for the identification of *N. koreana* and *C. pusilla*. For *P. obscurus*, the structures of the genital organs were observed in the parent specimens, which were cultured individually at the time of oviposition. The procedures for making sections are written in the next section. Preparing the sections for all of the sampled specimens of *Stylochoplana parasitica* were unrealistic. Therefore, they were identified according to the morphological

description by Kato (1935). Regarding the species name, we employ the nomenclature by Faubel (1983) supplemented with that by Prudhoe (1985) (indicated in parentheses for reference) when they differ. The diagnostic features of the species are as follows.

Comoplana pusilla (Bock, 1924) (*Stylochoplana pusilla* Bock, 1924); a polyclad species found from inside gastropods, most commonly from *M. labio*. The body size was smaller than previously reported, measuring 2–3 mm in length, 1–1.5 mm in width (Fig. 1A) (cf. Kato, 1933, 1934). The body shape was oval with brown colored dorsal side marked by darker lines branching around the pharynx. Ventral surface was pale without any patterns or evident adhesive organ. A pair of long tentacles were positioned, one on each side of the brain (Fig. 1B). At the base of the tentacles were 10–20 tentacular eyespots (positioned at the base of tentacles); together with the cerebral eyespots (distributed around the brain), 40–55 total eyespots were counted (Fig. 1B). The genital complexes were seen as pale, oval area close to the hind end in sexually mature individuals. In sectioned samples, the seminal vesicle opens into the prostatic organ (Fig. 1C). The vagina forms a large anteriorly directed curve and then turns posteriorly, connecting to the uterine canal, and finally opens into Lang's vesicle (a

bulbous structure of the vagina, which is believed to function as a storage space for sperm after copulation). Lang's vesicle of *C. pusilla* is observed as a clover-shaped structure when observed from dorsal or ventral side. Although the male and female genital pores were expected to open into the common genital atrium, they were often observed to end at different positions (Fig. 1C), but this was possibly an artefact of contraction during fixation of the specimen.

Notocomplana koreana (Kato, 1937) (*Notoplana koreana* Kato, 1937); the coloration was greatly altered by the gut content (e.g., it appeared orange after feeding on brine shrimp) (Fig. 2A), but no evident patterns could be seen on the dorsal or ventral side. A pair of short tentacles were seen, one on each side of the brain with 150–200 eyespots in total (Fig. 2B). The appearance was quite similar to *Notocomplana humilis*, except for the bluntly pointed posterior end in *N. koreana* and uniform body width in *N. humilis*, but deciphering them from external physical characteristics were difficult. In reproductively mature individuals, the genital apparatus was seen just behind the pharynx (Fig. 2A). In the internal structure, *N. koreana* could be distinguished from *N. humilis* by proportionally smaller prostatic vesicle compared to

the seminal vesicle and by a male atrium (Fig. 2C) larger than that in *N. humilis* (Hagiya, 1993). Lang's vesicle were relatively larger than previously reported (Hagiya and Gamo, 1992; Hagiya 1993) (Fig. 2D).

Pseudostylochus obscurus (Yeri and Kaburaki, 1918); oval body with a characteristic small median notch at the hind end (Fig. 3A). A pair of short tentacles were seen, one on either side of the brain with up to 150 eyespots around the area shortly after sexual maturation. Dorsal surface was brownish green and covered with numerous brown specks. Ventral surface was pale colored without notable patterns or evident adhesive organ. In mature specimens, the genital apparatus could be recognized just in front of the notch at the tail end. Seminal vesicle was muscular, nearly equivalent in size to the prostatic vesicle, which was positioned dorsally and slightly posteriorly to the seminal vesicle (Fig. 3C). Penis cone-shaped, positioned in the male atrium. The relative size of Lang's vesicle differed between specimens (Fig. 3C), likely a variation between individuals (Hagiya, personal communication).

Stylochoplana parasitica Kato, 1935; This species was identified by the following characteristics; 1) living in the chiton, commonly found in the pallial groove,

2) pale in color without any evident patterns, 3) a pair of fairly small tentacles at about one-sixth the body length from the anterior end, and 4) eyespots only around the cerebral and tentacular area, with the maximum total number of about 50.

Histological Observations

To examine the reproductive organs, serial histological sections were made according to the method of Newman and Cannon (1995). For fixation, individual specimens were laid on filter paper and placed on 10% formalin seawater for at least a day, which was frozen in advance and gradually melted after the samples were placed. Using the frozen solution prevented the specimens from curling during fixation, which made it easier to distinguish and examine the internal organs in the histological sections. Fixed samples were stored in 70% ethanol. Next, the samples were dehydrated in ethanol-xylene series and embedded in paraplast (Sherwood Medical Co., St Louis, MO, USA). The samples were processed into 8-μm-thick sections, which were stained with Carrazzi's hematoxylin and eosin Y. Histological sections were observed under an inverted microscope (Olympus CKX41).

Rearing adult animals

The specimens were reared in differently sized columnar glass containers with glass lids to avoid evaporation of water and alteration in salinity. Seawater pumped from the sampling site to the laboratory was used after filtered twice using 10-μm and 1-μm membrane filters (Advantec Co., Ltd, Tokyo, Japan). Different food organisms were chosen for each polyclad species, thus the seawater of their rearing containers was changed according to different schedules based on the food type. At that time, the containers were cleaned with a sponge to avoid algal accumulation on the container's surface as well as to remove leftover food and undigested excrement from the specimens. Some exceptions to the above procedures were taken in the case of *Stylochoplana parasitica*, since they had to be cultured with their host chiton.

Comoplana pusilla specimens were kept without the host gastropods in containers sized 3 cm in diameter and 1.5 cm in depth. February and July samples were kept in separate groups, and they were reared at 22–24°C with no lighting except when handled for culture treatment. Hatched, swimming brine shrimp larvae was used for food. They were microwaved until they were no longer swimming (about 30 seconds

with a 700 W microwave for a container with a volume of about 80 ml). This was to make the brine shrimp sink and accessible for the polyclads to feed. Whole pieces of the shrimp were placed in the containers for food. The water, along with the food, was exchanged once every one or two days. Eggs were laid 90 days after collection for the February sample and one month later for the July sample, although they are reported to lay within the week of collection when fully mature specimens are collected (Deguchi et al., 2009).

Notocomplana koreana specimens were kept in containers 9.5 cm in diameter and 4.5 cm in depth, at room temperature, without any specific lighting conditions. Various types of small invertebrates collected at the sampling site, such as crustaceans and gastropods, were given as food once every two days; food remaining one to two hours after feeding was removed to avoid water deterioration. Water was changed every other day. The first eggs were laid 2 weeks after field collection.

Pseudostylochus obscurus specimens were reared similar to the method used for *N. koreana* but were kept in a larger container, 17.2 cm in diameter and 4.5 cm in depth, with diced portions of *M. labio* supplied as a food. When maintained individually,

they were kept in containers the same size as those used for *N. koreana*. The collected specimens were found copulating upon encounter in the field, and the eggs were laid three weeks after capture.

Chitons with *S. parasitica* living inside were individually kept in a glass container similar in size to those used for *N. koreana* using natural seawater, which was changed daily. Food was given to neither the flatworm nor the chiton, but the container was exposed to the laboratory light to grow algae on the surfaces as a possible food source for the host specimens.

Collection of embryos and culture of larvae

In this thesis, the planktonic and the immature benthic stages will be called larva and juvenile, respectively, to distinguish their behavioral and morphological differences as well as to avoid using multiple naming terms.

Although there are some exceptions, polyclads basically lay their eggs from late winter to early autumn in the field (Teshirogi et al., 1981; Prudhoe 1985; Chen et al., 1990). The reproductive period differs between species, but they tend to be prolonged in

laboratory environment (Teshirogi et al., 1981). The eggs are laid in a capsule, and those capsules are laid in a mass of gelatinous sheet-like structures, called egg plates; these are laid on the hard surfaces such as the container wall, but are sometimes seen to be laid on the underside of the water surface in the laboratory (Lapraz et al., 2013). *Stylochoplana parasitica* was an exception, where most of the egg plates were found on the underside of the chitons, and more rarely on the sides of the container. For all four species ,the number of eggs in a capsule is usually one, in rare occasions two. The egg plate was in a form of zig-zag chain closely aligned beside each other in a random length and pattern, as it has been reported for *Comoplana pusilla*, *Pseudostylochus obscurus*, and *S. parasitica* (Kato, 1940; Teshirogi et al., 1981). The egg plates of *Notocomplana koreana* were also in a similar shape. Cleavage begins immediately after oviposition if the eggs are fertilized. After confirmation of this process, the egg plates were left alone for several days until the gelatinous layer became hardened. Then, they were carefully removed with a needle and placed in separate containers filled with filtered seawater. The seawater was changed daily, and maintained at 22–24°C for *C. pusilla*, *N. koreana*, and *P. obscurus*, and 20°C for *S. parasitica*. The embryonic

developmental process for all four species resembled that of *Notocomplana humilis* reported by Teshirogi et al. (1981) except in *S. parasitica*, where the body color began to darken around the developmental stage when the eyespots began to appear. The larvae hatched in about 2 weeks after the egg plate being laid.

Hatched larvae of *C. pusilla*, *N. koreana*, and *P. obscurus* were transferred to a new columnar glass container, sized 3.0 cm in diameter and 1.5 cm in depth. To avoid overcrowding, 20 to 30 individuals were kept in each container. For *N. koreana* and *P. obscurus*, enough number of specimens were obtained from a single batch hatched in one day, but several days were needed to recover 20 to 30 individuals of *C. pusilla* larvae because of the small size of their egg plates. For *S. parasitica*, 20 to 30 larvae were collected in a single glass bowl with a volume of about 0.5 L. Since culturing the hatched larvae with chitons resulted in death in large portions of the larvae, they were grown independently of the host for at least 30 days.

Larvae of *C. pusilla*, *N. koreana*, and *P. obscurus* were fed microwaved brine shrimp larvae, prepared in a similar way to the brine shrimp given to adults of *C. pusilla*. The polyclad larvae could feed on the swimming brine shrimp larvae, but microwaved

food was given for easier observation of the feeding behavior. The food was given and the water was exchanged twice per day (for the phototaxis experiment, the frequency was reduced to once a day). Larvae were temporarily transferred to a separate container at times when the original container was washed. No specific lighting conditions were imposed. For *S. parasitica*, hatched brine shrimp larvae were similarly provided but previously fed with Chlorella based powdered feed (Super Capsule Powder, Chlorella Industry Co., Ltd, Tokyo, Japan). Also, the larvae were moved to a transparent, plastic container with a diameter of about 5.5 cm with filtered seawater filled at a depth of about 0.5 to 1.0 cm. After the larvae and brine shrimp were placed in the container, the container was placed on top of a light box to provide light from beneath, since this treatment made the larvae approach the food efficiently. The samples were left for about 2 hours until most of the larvae have moved off from the brine shrimp, puffed up and become orangish in color from feeding. The food was provided once to twice a day.

Settled specimens of *C. parasitica*, *N. koreana*, and *P. obscurus* were reared according to the same procedure as in the larval state, but *N. koreana* specimens were transferred to larger containers (9.5 cm in diameter and 4.5 cm in depth) when they

reached ~5 mm in average length to avoid overcrowding. To induce reproductive maturation of *P. obscurus* specimens, the food was changed to the foot of *M. labio* from day 80–90 after hatching, the same food item offered to mature specimens. The snail was diced before given, since this species were only seen to feed on items that were small enough to be fully engulfed. On feeding, the leftover food and excrement of the flatworms caused deterioration of the water quality. Therefore I checked the water conditions regularly and cleaned the containers as necessary. Once the specimens grew to a body size of approximately 3–4 mm length, they were moved to a larger container, similar in size to those used for *N. koreana*, while the remaining portions of the replicates was still cultured with brine shrimp for comparison. For *S. parasitica*, a group of 10 larvae was placed together with chitons after individual culture of at least 30 days. The chitons and larvae were placed in a columnar glass container similar to those used for *N. koreana*, with natural seawater. The container was closed with a glass lid and left in room light, so that the chitons could feed on algae that start to grow on the surfaces of the container.

Morphological observations of polyclads

For all growth stages, the largest specimen available in each replicate was selected for observation. The descriptions mainly relied on photographs. During the planktonic stage, the free-swimming larvae were recorded using a digital camera (Nikon D5000; Nikon Co., Tokyo, Japan) mounted on a stereoscopic microscope Olympus SZX7. When recording the images, I mainly targeted feeding times as well as occasions when the animals were resting at the surface of the water, since in most other times the larvae were actively swimming and were difficult to be recorded. Selected larvae were also gently compressed on a glass slide with a coverslip and observed under an inverted microscope that was likewise equipped with a digital camera (Nikon D5000); the amount of seawater on the slide could be adjusted using a pipette and paper towel so that the larvae did not easily crawl around and would remain undamaged for further culturing. Body measurements were made mainly from stereoscopic microscope images, but, when clear images could not be obtained, they were made based on images acquired from the inverted microscope. Detailed structures, such as the eyespots, were observed from the inverted microscopic images.

After settlement, the external features were observed with or without the aid of a stereoscopic microscope, depending on the individual size. For immobilization of the specimens, polyclads were either anesthetized with menthol in some cases, or the seawater on a glass slide was reduced to immobilize the animal as much as possible, so that it could be photographed in a petri dish or on a glass slide with 1-mm grid paper placed underneath. Anesthetic was used only for *N. koreana* and *P. obscurus*; all *C. pusilla* and *S. parasitica* specimens were observed under a microscope without the use of anesthetic, since their body seemed especially delicate and vulnerable to the use of anesthetics. The internal structures after settlement were observed under the inverted microscope, similar to the procedure used for viewing animals in the planktonic stage, except that the eyespots were observed from the dorsal side and the genital complexes from the ventral side to obtain clear views.

When anesthetizing specimens, saturated menthol was used. The crystals of menthol were dissolved into filtered seawater in a bottle of about 50 ml in volume. This was added gradually on to the body of the animal, while the curled or folded edges of the specimen were gently flattened out. Excess mucus was removed with tweezers, so

that the body would not be torn or damaged in the process. The same care was taken when helping the animal to recover from the anesthetic. For some *P. obscurus* specimens that outgrew the size of the glass slide, they were placed on a clear rectangular plastic lid (11.7 × 7.8 cm). To add enough pressure to flatten out the organism, a smaller clear plastic lid (8.8 × 5.7 cm) was used instead of a coverslip. The frequency of the morphological observations for *C. pusilla, N. koreana*, and *P obscurus* were as follows: once per day for the first 30 days after hatching; once every two days from day 31–60 post-hatching; every 5 days from day 61–100 post-hatching; every 10 days from day 101 and onward after hatching. However, the intervals between observations were sometimes shortened, for example when the genital organs started to develop. For the symbiotic polyclad *S. parasitica*, the morphology was recorded every 2 days when the larvae were individually cultured from the chitons, and every 5 days after adding the chitons. Data lack for some of the sampling scheduled dates, since the flatworms could not be found hiding inside the chitons.

Analysis of phototaxis

The swimming chamber for *P. obscurus* larvae was made using a silicon sheet, 2 mm thick. Cutting this in a rectangular shape 6 × 4 cm in size, a smaller rectangular space of 2 × 5 cm was cut, leaving an open end at the longitudinal direction. This sheet was placed on a glass slide and covered with a coverglass (2.4 × 6.0 cm) to make it water-tight (Fig. 4A).

Violet (395 nm), blue (440 nm), cyan (470 nm), teal (510 nm), green (550 nm), and red (640 nm) light on the Lumencor® SPECTRA X light engine (Lumencor Inc., Beaverton, OR, USA) was simultaneously lit to create the white light stimulus through a light cable. The stimulus was placed about 5 cm apart and 0.5 cm above the open end of the chamber at about 30° angle to produce a gradient within the swimming area. Also, the lighting cable was aligned with the midline of the chamber to provide the light stimulus thoroughly in the chamber. All data were recorded using a video camera (Panasonic HC-W870M, Panasonic Co., Osaka, Japan) placed over the chamber (Fig. 4B). Two different light intensities were tested, adjusted by the computer connected to the lighting unit. Using a quantum sensor (MQ-200 Quantum Separate Sensor, Apogee

Instruments Inc., Logan, UT, USA) the brighter area in the weaker lighting condition was measured as 1.8 to 2.4 × 10^{19} photons m^{-2} s^{-1} and the darker area measured as 1.8 to 3.0 × 10^{18} photons m^{-2} s^{-1}, while in the stronger lighting condition, the brighter and darker areas were 4.2 to 4.8 × 10^{20} photons m^{-2} s^{-1} and 3.0 to 3.6 × 10^{19} photons m^{-2} s^{-1}, respectively.

All experiments were conducted in a curtained off compartment to cut off any light from the surrounding environment. About 50 specimens (minimum: 35, maximum: 83) were placed in the chamber, and were either dark or light adapted for 15 minutes before experimentation. For light adaptation treatment, two LED lights were lit from the opposing sides of the chamber to minimize the affect of directional light. For the dark adaptation treatment, the chamber was placed in a box with a lid. At the end of each adaptation treatment, the movement of the specimens was recorded as a control. Infrared flashlight (UniqueFire T-20, Shenzhen Homesafety Electronic Col, Ltd, Guangdong, China) was used to provide a light source for recording under darkened condition without disturbing the adapted conditions. The specimens were then provided with light stimulation for 90 seconds. The distribution of flatworms in the chamber at

90 seconds and the swimming behavior at 3 seconds after light illumination was recorded by the video camera.

Larval responses to lighting conditions were further assessed in the following experiment. After the larvae was exposed to the light stimulus for more than 90 seconds, a break of 5, 10, 30, 60, 90, or 120 seconds darkness was given, and the light stimulus was resumed. The swimming direction of the larvae 3 seconds after resuming the light stimulus was recorded in the samples cultured for 0, 15, and 33 days after hatching.

The numbers of specimens at the proximal or distal 1 cm ends of the chamber were counted after 90 seconds for positive or negatively phototactic, respectively (Fig. 5A). Some specimens were immobile throughout the experiments, and they were not counted for the assessment. The video recorded data were analyzed by a tracking software Bohboh (Bohbohsoft, Tokyo, Japan) to evaluate the movement of the larvae. Also, the direction of movement 3 seconds after the light stimulus was determined to evaluate the reaction of larvae after different adaptation conditions. The tracked data from Bohboh was processed by ImageJ (https://imagej.nih.gov/ij/) for assessment. Defining the direction of light stimulus as 0°, specimens moving to the range of 330° to

360° and 0° to 30° were counted as those showing a positive phototaxis, while those moving to 150° to 210° were counted as showing a negative phototaxis (Fig. 5B). Overlapping tracks or specimens moving adjacent to the sides of the chamber were excluded.

Field study of *S. parasitica* and the host chitons

The chitons were transferred to a glass container filled with seawater, which was placed over a 1mm grid paper underneath. After the body was stretched, the specimens were photographed using a digital camera (Pentax Optio WG-1 GPS, Ricoh Imaging Co., Ltd, Tokyo, Japan). The specimens that were curled up due to damage during collection were pressed down with tweezers to stretch the body as much as possible, as well as taking a photograph from the lateral direction. The ventral sides were checked and also photographed when the specimens were found with polyclad eggs.

The flatworms were placed on a plastic petridish filled with seawater and photographed using a digital camera Nikon D5000, the procedure similar to that of their

hosts. The photographed data were used to measure the specimen size using the software ImageJ. For the chitons, the length was measured to represent the specimen size. For the flatworms, the body area was measured instead of length or width, since the body outline could be altered due to their soft bodies..

Statistical analysis of *Stylochoplana parasitica* field study

To evaluate if the proportion of chitons inhabited by flatworms differed between the two sites at each month, Fisher's exact test was performed. For the evaluation of relationship among flatworm size, the host chiton size, and number of symbiotic flatworms per host, flatworms and chitons were grouped according to the number of symbiotic specimens. Tukey's HSD test was performed between those groups if the size of flatworms and chitons were affected. All statistical calculations were preformed on R ver. 3.4.2.

RESULTS

Growth from hatching to settlement for *Comoplana pusilla*, *Notocomplana koreana*, and *Pseudostylochus obscurus*

Some similarities were observed for the larvae of *Comoplana pusilla*, *Notocomplana koreana*, and *Pseudostylochus obscurus*, but the larvae of *Stylochoplana parasitica* showed characteristic patterns only seen in this species. Therefore, the growth process of *C. pusilla*, *N. koreana*, and *P. obscurus* will be discussed here, and the growth process of *S. parasitica* will be presented individually in the later section.

Larvae of *C. pusilla*, *N. koreana*, and *P. obscurus* swam actively in the water column and gathered near the brightened side of the container, suggesting a positive phototactic response. When provided with brine shrimp, the larvae swam in circles while gradually approaching the food item, and then they crawled over the shrimp before finally settling to consume it: the posterior half of the polyclad's body pressed against the shrimp while the anterior half was sometimes raised, and the inner contents of the brine shrimp was sucked out with the muscular pharynx. After taking in enough

food, the larva began to swim freely again. In some instances, an individual puffed up its body into an ovoid shape (much like a balloon) and the body color changed according to the gut contents. Feeding was observed within the day of hatching. The larva cultured without food became smaller in size and died several days after hatching. The swimming behavior continued for about 15 days. Approximately 2 to 3 weeks after hatching, the duration of time gliding along the surface of the container gradually lengthened until the animals settled and became completely benthic. No change in culture method was taken nor specific cues were provided to induce settlement.

C. pusilla hatched out as lobe-less, direct-developing larvae with a total of four eyespots (Fig. 6A, Table 1). During the planktonic period, small black spots appeared on the hind end at about day 7 post-hatching (Fig 6B), but these eventually disappeared after settlement. The species occasionally clung lightly to the sides of the container in the free-swimming period but readily swam off when a mechanical shock was given. From about day 11–13 post-hatching, 4–8 new eyespots began to appear (Table 1). At first, at least one eyespot was added on each side behind the four eyespots originally present at hatching; after settlement, these spots became grouped as cerebral

eyespots. Posterior to the cerebral eyespots, two eyespots appeared alongside each other on each side, positioned closer to the lateral edges of the body than the eyespots formed previously. These new pairs of eyespots eventually became grouped as the tentacular eyespots (Fig. 6C). Out of 212 cultured specimens, 70 survived until settlement (33.02%).

N. koreana hatched out as lobe-less larvae, with a total of four eyespots (Fig. 7A, Table 2), similar to *C. pusilla*. They developed into cross-like body shape from one week after hatching until settlement (Fig. 7B). This body form was seen for all the specimens and only when the animal was swimming. This shape could be converted into an oval shape by mechanical stimulus, such as compression by a coverslip. Once they began to assume the cross-like shape, two new eyespots appeared behind the initial four eyespots. At approximately day 10 post-hatching, an additional eyespot appeared on each side behind those previously formed; during the next 4–5 days, paired eyespots appeared further behind the existing eyespots (Fig. 7C, Table 2). The paired eyespots became the tentacular eyespots, while all the others grouped as the cerebral eyespots, as

seen in *C. pusilla* (Fig. 6C). 82 specimens out of 106 cultured survived until settlement (77.36%).

P. obscurus hatched out as lobe-less larvae with four eyespots (Fig. 8A, Table 3), similar to both *C. pusilla* and *N. koreana*. Between day 5 and 10 post-hatching, two additional cerebral eyespots and a pair of tentacular eyespots appeared on each side (Table 3). These eyespots were positioned similarly to the other two species, but the second set of cerebral eyespots and the tentacular eyespots appeared almost simultaneously. From approximately day 10 post-hatching, all the larvae took on a triangular shape, with one apex seen at the posterior end and the other two positioned at lateral sides of the anterior end of the body (Fig. 8B). Similar to the larvae of *N. koreana*, this triangular body shape changed to oval under the compression of a coverslip, and it persisted until settlement. By that time, some specimens exhibited an additional cerebral or tentacular eyespot on either side, resulting in as many as 14 eyespots in total (Fig. 8C, Table 3). 155 specimens out of 184 cultured survived until settlement (84.24%).

Growth from settlement to reproductive maturity for *Comoplana pusilla*, *Notocomplana koreana*, and *Pseudostylochus obscurus*

The larvae of all three species took on an elongated spheroid or likewise three-dimensionally rounded forms. As the planktonic period reached the end, the larvae gradually became dorsoventrally flattened in proportion, and the pharynx changed from tubular to plicated. After settlement, the eyespots appeared at different rates and in different positions, depending on the species (Tables 1-4). The patterns of eyespot formation became unclear as compared to their appearance in the larval period. With growth, the distribution of eyespots was laterally asymmetric regarding number and placement.

Comoplana pusilla did not dramatically change in size or shape in post-settlement stage, as compared with the other two species. The tentacles first appeared as a pair of small humps in the area of the tentacular eyespots and gradually grew in length. Multiple eyespots were visible as the tentacles developed, since the latter seemed to lack pigmentation (Fig. 9A). Process of maturation was evident at about day 35 post-hatching with the formation of a pale area near the tip of the tail, where the

genital complexes began to develop (Fig. 9A, B). Within the following 10 days, the distinctive clover-shaped Lang's vesicle could be observed. Within another two weeks, the vagina became evident and the genital pore (a common genital atrium in this species) opened (Fig. 9C). Finally, eggs accumulated in the uterine canal at about 20–30 days after onset of the genital-complex formation (Fig. 9D, E). Eggs are reported to be fertilized in the uterine canal (Prudhoe, 1985), and oviposition is observed shortly after eggs are accumulated (Teshirogi et al., 1981). Thus, the time of egg accumulation in the uterine canal could be used to approximate the time needed for sexual maturation, and *C. pusilla* is estimated to reach reproductive maturity in about 70 days post hatching. However, an exceptional case was observed where a single specimen was confirmed to carry eggs as early as 54 days after hatching. The body size and the number of eyespots continued to increase during the maturation process (Table 1) and seemed to cease by approximately 60 days post-hatching, which coincided with the commencement of reproductive maturity. Forty two specimens out of the originally collected 212 survived until 70 days post hatching (19.81%).

The body of *Notocomplana koreana* gradually elongated after settlement (Table 2). A pale area appeared behind the posterior end of the pharynx at around day 40 post-hatching (Fig. 10A, B), signaling the process of sexual maturation, similar to *C. pusilla*. During the next 5–10 days, the areas of the male and female genital complexes became distinguishable as different clusters and the gonopores were opened (Fig. 10C). The gonopores became more distinct during the following 10 days, especially the male gonopore which attained a slightly jagged outline. At around 10–15 days after the formation of the gonopores, a set of male organs, namely the prostatic vesicle, seminal vesicle, seminal canal and penis, started to be formed. In the female complex, Lang's vesicle gradually became distinguishable; during the next 5–10 days, the other female organs, such as the vagina and the oviducts, became evident, and the previously recognized structures were more defined. Later, sperm was observed in both the seminal canal and seminal vesicle, followed by eggs being stored in the oviducts (Fig. 10D, E). It is predicted to take approximately 70 days from hatching to sexual maturation, and the period between the first confirmation of the formation of the genital organs to an accumulation of eggs was approximately 30 days. At 70 days post hatching, 82 out of

106 specimens survived (77.36%), which is 100% survival rate after settlement. The size of the body and the number of eyespots continued to increase during and after reproductive maturation, reaching a maximum body size at around 150 days, and the maximum number of eyespots established at around 200 days post-hatching (Table 2).

***Pseudostylochus obscurus* fed with brine shrimp**

Juveniles of this species were oval in shape, but with a somewhat blunt anterior end and a slightly narrower and rounder hind end (Fig. 11A). The dorsal surface was scattered with small brown spots that gradually darkened and became more distinct with growth. Although these juveniles survived on brine shrimp, their external features showed no signs of growth or maturation other than the multiplication of eyespots (Table 3). The formation of genital organs was not evident even 140 days post-hatching (Fig. 11B). Out of 57 specimens allocated for continuous brine shrimp culture, 45 specimens survived to this period (78.95%).

Pseudostylochus obscurus* fed with *Monodonta labio

After changing the food to *Monodonta labio* at 80 to 90 days after hatching, some specimens were observed to shake and twitch, even disintegrate, by unknown causes, whereas the dorsal coloration gradually darkened and the body size became considerably larger and progressively circular in shape among healthy individuals over the course of 5–10 days (Tables 3 and 4). The characteristic median notch on the hind end was seen within 20 days after the food was changed, and the external appearance came to fit the descriptions of this species (Fig. 12A).

The first signs of sexual maturation could also be observed around this time, as early as 14 days after changing the food, as a pale spot located anterior to the tail notch, which was visible only from the ventral side (Fig. 12B). Within the next 5 days, the male and female gonopores became noticeable, but the male gonopore was typically more distinguishable. During the following 5–10 days, the area of the male and female genital complexes became evident and separated, with structures like the seminal vesicle, prostatic vesicle and the vagina being recognizable. During another 5 days, the structures of other female organs, such as the oviducts, Lang's vesicle and its duct,

became evident, and the other structures grew more defined (Fig. 12C). Then sperm started to fill up the seminal canal and seminal vesicle. Finally, stored eggs were apparent in the oviducts after the next 5–10 days (Fig. 12D, E). Thus, about 30 days were required from the onset of genital-organ formation to the storage of eggs. In total, about 50 to 60 days from the time of food change were required for this species to sexually maturate, and 38 of the 73 treated specimens survived until this time (52.05%).

Growth and reproduction of *Stylochoplana parasitica*

The eggplates of *S. parasitica* are yellow in color in the early stages of development but gradually turned darker in color from around the time when eyespots start to appear on the embryos (Fig. 13A). The larvae hatched out as lobeless, direct developing larvae that are similar in morphology to the other cultured species except for the body color (Fig. 13B). This species also differed from the previously cultured flatworms in that the larvae floated at the water's surface or frequently cling to the sides of the container. The eyespot multiplication pattern was also slightly different. *S. parasitica* hatch with 4 eyespots (Fig. 13B, C), but no additional eyespots were seen at

7 days after hatching (Fig. 13D), although new eyespots were usually found for the other cultured species at the same stage. Additional eyespots were found around 2 weeks after hatching, and the eyespot type (cerebral or tentacular) is not clear compared with the larvae of other three species that were cultured (Table 5, Fig. 13E).

When cultured with only brine shrimp, no maturation process or considerable growth was seen in *S. parasitica* (Table 5), similar to what was observed in *P. obscurus*. Brine shrimp cultured *S. parasitica* seemed closer to the larval stage rather than being a sexually immature benthic juveniles as in the case of *P. obscurus*; morphology of *S. parasitica* remained black, oval, and not dorso-ventrally flattened.

When the host chiton, *L. japonica*, was placed in the same container about 30 to 71 days after hatching, the size of the flatworm grew considerably larger (Table 6). The color changed paler and the body became flattened as well as elongated within 10 days after the chitons were placed (Fig. 14A). After 30 days with chitons, a light colored area was seen behind the body, which marked the onset of sexual maturation process (Fig. 14B, C). The flatworm was observed to start to bear eggs after culturing

with chitons for more than 137 days (Fig. 14D, E), which was considerably longer time than the other three cultured species to reach sexual maturity.

Phototactic behavior of *Pseudostylochus obscurus* larvae

Taking advantages of the culture system established in this study, I examined two important issues that have not been solved in the polyclads for a long time. First issue is the phototactic behavior of polyclad larvae. Larvae were inserted into a experiment chamber and illuminated from one side (Fig. 4). Phototaxis was assessed both from the distribution of the larvae after illumination (Fig. 5A) and from the initial swimming direction (Fig. 5B). Because feeding frequencies were decreased in this experiment, the larvae took about 30 days to settle. Young larvae showed negative phototaxis, but this reversed to positive phototaxis around 12 to 15 days after hatching, regardless of light adaptation conditions or the strength of the stimulus (Fig. 15). At around 27 to 33 days after hatching, the proportion of positively phototactic larvae tended to decrease (Fig 15). Thus, the phototaxis reversed around halfway through the planktonic period and became less phototactic when it was close to the settlement

period. The transition from negative to positive phototaxis was seemingly correlated with the appearance of tentacular eyespots or with the increase of cerebral eyespots during the planktonic period. However, this correlation was no longer observed after larvae became close to the benthic stage (Fig. 16).

Next, I examined the initial larval response upon light stimulus by recording the direction of movement (Fig. 5B). Just after hatching, larvae showed directional movements in the light adapted specimens (Fig. 17A) but not in dark adapted groups (Fig. 17B). The directional swimming pattern was observed only when weak light was illuminated (Fig. 17, Fig. 18A); it became less clear with strong light illumination (Fig. 18C). The phototaxis changed from negative to positive with weak light stimulus from 9 to 24 days after hatching in light adapted larvae (Fig. 18A). This response was observed also to strong light in the similar larval stage under light adapted condition (Fig. 18C). However, the directional movement was not evident at any larval stages in dark adapted groups (Fig. 18B, D).

To know more about the mechanism of the phototaxis and light adaptation, a break of darkness was given to the specimen that had been illuminated for 90 seconds. I

found that the initial direction of swimming was reversed when a darkness break was inserted between the light stimuli (Fig. 19). Regardless of stimulus strength, the reversion of swimming direction was significant after shorter darkened breaks. In addition, the reversion was clearly observed in younger specimens.

Field study on symbiotic polyclad species *Stylochoplana parasitica*

Second issue that I examined to take advantage of the culture system was to precisely describe the growth and reproduction of a symbiotic polyclad species, of which ecological life cycle has not been completely understood. *Stylochoplana parasitica* is a white colored polyclad without any distinctive morphological features (Fig. 20A). In the two locations for sampleing, two potential host species were found above the tideline, *Liolophura japonica* and *Acanthochitona achates*. The former was previously reported to be the only host species for this species of flatworm (Kato, 1935; Fig. 20B). A small number of the latter species was collected, but no flatworms were found from this species at either sites (Fig. 21).

Between the two sites, *L. japonica* inhabited by flatworms were more easily found throughout the year in Tatado compared to Nabeta. The prevalence ranged from 5.26% to 38.46% with an average of 21.81% in Tatado, and between 0.00% and 14.81% with an average of 5.85% in Nabeta. Eggs were laid at the ventral side of *L. japonica* (Fig. 20C) and found for a longer period in Tatado compared to Nabeta (Fig. 22).

S. parasitica was found with as much as 5 specimens per chiton with varying sizes (Fig. 23). The size of the flatworms could be affected by the number of individuals living together and the size of the host. To evaluate if such a relation exists, flatworm and chiton sizes were grouped according to the number of symbionts per host for comparison (Fig. 23). No significant differences were found in the size of the chitons at either sites (Tukey HSD test, Tatado $p>0.75$, Nabeta $p>0.15$), but flatworm size differed by the number of worms per chiton. In Tatado, significant difference was found in flatworm size between the cases of one worm per chiton and three worms per chiton, and also between the case of two worms per chiton and three worms per chiton (Tukey HSD test, $p<0.05$). In Nabeta, the flatworm size showed significant difference only

between the case of one worm per chiton and two worms per chiton (Tukey HSD test, $p<0.05$).

Next, I examined the seasonal variation in flatworm body size. Larger size of flatworms were found from June to August in both Tatado and Nabeta, although the size difference was more evident in Tatado samples. From September, smaller sized flatworms were seen and the average size gradually increased towards the summer (Fig. 24, 25).

To confirm the field investigation regarding the growth and reproduction of *S. parasitica*, I observed the process using a laboratory culture method. As described above, *S. parasitica* fed by brine shrimp gradually shrunk from around 60 to 70 days after hatching. The size only reached about 0.21 mm^2 around 30 days after hatching, but did not get any larger (Fig 26). The larvae were capable of living in the chiton as early as 30 days after hatching, and morphological change and sexual maturation process was induced by the presence of the hosts (Fig. 27). *S. parasitica* larvae cultured with the chiton *L. japonica* grew up to 12 mm^2 and became to bear eggs at 4 to 5 months after hatching (Fig. 27). This period for sexual maturation is compatible with the sizes of the

flatworms found in chitons during late spring to early summer (Fig. 24, 25), although it appears to take longer time in the field sample to reach sexual maturity.

DISCUSSION

I successfully achieved to culture four species of polyclad flatworms, including both symbiotic and non-symbiotic species, in the laboratory. To my knowledge, this is the first detailed description of the growth from hatching to reproductive maturity for the lobeless larval type in polyclads. I found several characteristics shared among three of the four species under study (*C. pusilla*, *N.koreana*, and *P. obscurus*) in the planktonic larval stage, such as feeding preference, duration of the planktonic period, the timing of settlement, induction of settlement, and the positions of additional eyespots on the body. These features are possibly shared traits in direct-development larvae characterized by four eyespots at hatching. Observations such as the opening of the mouth in *N. koreana* larvae at around day 5 post-hatching (Kato, 1940) and an unsuccessful attempt in culturing four-eyespot direct-developing larvae with algae (Ballarin and Galleni, 1984) could have resulted from keeping the larvae in conditions that did not meet the food requirements of the larval type. Judging from a well-developed pharynx at hatching, the direct-development larval type is considered carnivorous throughout its lifetime.

In the present study, the culturing conditions needed no specific change for *C. pusilla* or *N. koreana*. Therefore reproductive maturation seems to occur autonomously in these species when they reach adequate body sizes or spend a certain length of time after settlement. In contrast, *P. obscurus* need the change of food from brine shrimp to a gastropod. Therefore, it is considered for this species to require a kind of trigger for reproductive maturation, possibly related to a substance acquired from the gastropod or a metabolic product. The trigger is thought not to be present in the brine shrimp nor a simple body size or time after settlement. It is possible that the *M. labio* could be replaced with another gastropod species found in the same area for alternative food (e.g., *Nerita albicilla* Linnaeus, 1758, and limpets), which may improve the survival rate and condition of cultured *P. obscurus*.

Although the conditions needed for reproductive maturation may differ among the cultured species, all species examined in this study took about 30 days to achieve reproductive maturity, except *S. parasitica* which needed 137 days in a laboratory culture system. Interestingly, the male organs seemed to develop slightly earlier than the female genital complex. This observation agrees with past reports

wherein captured specimens displayed fully developed male organs but not yet matured female structures (Prudhoe, 1985). Additional studies of gonad morphology and detailed observation of gametogenesis will be useful for confirmation and greater understanding of the maturation process.

S. parasitica differed from other three polyclad species in body color, behavior during the larval period, growth process seen under brine shrimp culture, eyespot multiplication pattern during larval period, and the conditions needed for growth and maturation. Considering that chitons are necessary for the lifecycle of this species, the relativity between *S. parasitiac* and chitons seems much stronger than that between another symbiotic species *C. pusilla* and its host *M. labio*, because *C. pusilla* can be reared to reproductive maturation in a free-living state. Also, the fact that *S. parasitica* was able to grow only by the presence of living chitons strongly suggests that the flatworm acquires nutrients from the host in some way. They were not observed to feed on cut pieces of chitons, so that it is a big question how and where they feed. Polyclads are typically known not to be parasitic (Prudhoe, 1985), but the discrimination between parasitism and symbiosis is vague. Association and mutual

affects that two animals have on each other, e.g. parasitism, mutualism, commensalism etc, are often complexed and should be carefully evaluated (Parmentier and Michel, 2013). By using the culturing system that I have established here, the relationship between polyclads and the host animals may be more precisely evaluated.

Field collected sample data throughout the year for *S. parasitica*showed that the largest size specimens were found during the summer when also eggs were laid and that smaller specimens were newly found in September to October. This leads to the prediction that large, mature specimens lay eggs during the summer and die, the eggs hatch during the summer, and the larvae settle and start the symbiotic lifestyle in the chiton in the fall of the same year. In the laboratory cultured specimens, the larvae were able to live inside the chitons as early as 30 days after hatching, and in about a month after living inside the chiton, reached the size of about 1.5 mm^2, which was the smallest-sized worm collected from the field. This suggests that it takes about two months from hatching to reaching the size of about 1.5 mm^2. This span of time fits the time from when the eggs were found to when smaller specimens were sampled. However, it should be noted that the laboratory culturing system does not always reflect

all the conditions of the natural environment. For example, the cultured *S. parasitica* took 137 days to sexually mature, but this could possibly be altered by condition of the chiton, since the flatworms are very dependent on their host. In fact, the chitons were not given any food but the algae that grew on the container wall, which was not confirmed as a valid food source for the chitons. Supposing that the flatworms mature in 137 days, it could be assumed that they become oviporous during winter. However, this was not the case. Therefore, there must be some additional factors in natural condition that was missing in the culturing system.

The successful culture of polyclad species is beneficial not only to the observation of the different growth stages, but also to provide a large number of confirmed species for experimental use. Polyclad identification has so far been based largely on the structure of the genital organs in field collected samples, and thus immature specimens without any distinctive external characters could not be identified. Polyclads do not reproduce asexually and artificial insemination is not practical (Teshigori and Ishida, 1988; Deguchi et al., 2009). The successive process or the mechanism of early and late development is expected to be explored by using cultured

embryos and larvae. Of the four cultured species in this study, *P. obscurus* has some features that are advantageous compared to others. Owing to their relatively large body size, eggs and larvae can be collected in larger quantities, and the maturation timing could be intentionally manipulated with food change. This would be a new strategy in polyclads biology for making molecular basis to study on their reproduction and development experimentally.

One of the first exemplary use of the culturing system of *P. obscurus* in this study was the evaluation of larval phototaxis. This was also the first attempt to evaluate the phototactic behavior of the direct developing larvae. Previously, polyclad larvae were reported to exhibit strong positive phototaxis but predicted to lose this ability at settlement (Prudhoe, 1985; Smith et al., 2002). In the case of Müller's larva, positive phototaxis was found to increase between a week-old and three-weeks old samples (Johnson and Forward Jr., 2003). The phototactic pattern later than 15 days after hatching in *P. obscurus* also agrees with these reports (Fig. 15). This is plausible, since the positive phototaxis first brings the larvae near the surface, increasing the mobility to

carry the animals toward and adequate place to settle, then the animals sink with decrease in phototaxis.

However, observation in *P. obscurus* larvae phototaxis was unusual in that just after hatching out, the larva showed negative phototaxis, then conversed to positive at around 12-15 days after hatching (Fig. 15). This suggests that larva stay low in the water column for a while after they are hatched out from egg mass. Negative phototaxis usually occurs at lower light intensities if any is observed (Forward Jr. and Costlow Jr., 1974; Forward Jr., 1987), and this also applies for Müller's larva (Johnson and Forward Jr., 2003). These are thought as a part of predator avoidance behavior. However, a copepod species is known to display negative phototaxis at higher light intensity according to different growth stages and sexes, suggesting different light environment requirements with ontogeny (Miljeteig et al., 2014). This could also apply to *P. obscurus* larva, where it might be advantageous for the hatched larvae to stay in deeper waters early in their planktonic lives. Other factors such as threshold light intensity and reaction to different wavelength will give more insight to how the light environment affects the behavior of the direct developing larvae. Also, Müller's larva showed

slightly stronger positive phototaxis after dark acclimation (Johnson and Forward Jr., 2003), which is the reaction different from *P. obscurus* and might be also a unique property to discriminate the lobed and unlobed larvae types.

There are several types of photoreceptors in Platyhelminthes. All polyclad eyes are known to be inverted type with pigmented photoreceptors (Founlier, 1984). Electron microscopic studies showed two types of sensory systems exist; the cerebral eye is made up of three cells: one is pigmented cell with ciliary projections and another is rhabdomeric-type photoreceptor cells (Lanfranchi et al., 1981; Lanfranchi and Bedini, 1986). Although few physiological studies have been done in details, it was suggested that the tentacular eyes and cerebral eyespots differ; the former usually receive light from the lateral direction, whereas the latter receive light from above and below (Prudhoe, 1985). In this study, larvae just after hatching showed negative phototaxis, whereas the sign of phototaxis became reversed later (Fig. 15). The positive phototaxis appeared after the tentacular eyespots appeared or during the period when eyespots were increasing, suggesting a correlation between phototactic behavior and tentacular eyes. If this is the case, however, loss of phototaxis prior to settlement could

not be explained. Experimental demonstration, such as surgical removal of eyes, is needed to elucidate the function of both types of eyes.

The position and number of eyespots are considered one of the useful characters for species identification, although I found potentially greater variation in their numbers than was previously recorded. The number of eyespots reached a maximum by the time of reproductive maturity in *C. pusilla*, whereas it continued to increase after reproductive maturity in *N. koreana* and *P. obscurus*. Therefore, the reported number of eyespots could have been underestimated depending on the species and the growth stage. Similar observation has been made on *Martigrella crozieri*, where a larger specimen has a larger number of eyespots than previously reported (Lapraz et al., 2013).

In the present study, I observed that the hatched individuals are planktonic and have morphological characteristic distinct from the benthic specimens, such as number of eyespots, coloration, and body shape. The term ‘direct development’ or ‘juvenile’ has been used to describe the lobe-less type of hatchlings but this might lead to a misunderstanding that hatched individuals are always benthic and their appearance

resembles that of the adults. In polyclads, directly developing juveniles are not generally treated as larvae, since unlike the lobed larvae types, they are considered not to undergo metamorphosis. However, if such planktonic period was seen in nature as I have observed in this study, it is suggested that the direct developers might have an important growth period similar to the larval period in the indirect developers. The cross-like and triangle shaped swimming forms observed for *N. koreana* and *P. obscurus* are only seen at a certain time in the planktonic period and irreversible after settlement. These might be evidences of significance that the planktonic period has for the direct developers. The culturing method provided here encourages further experimental studies and should shed light on the 'direct developing' polyclads and their planktonic periods.

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REFERENCES

Allen JD, Klompen AML, Alpert EJ, Reft AJ (2017) Obligate planktotrophy in the Götte's larva of *Stylochus ellipticus* (Platyhelminthes). Invertebr Reprod Dev 61.2: 110-118

Anderson DT (1977) The embryonic and larval development of the turbellarian *Notoplana australis* (Schmarda, 1859) (Polycladida: Leptoplanidae). Aust J Mar Freshwater Res 28: 303–310

Ballarin L, Galleni L (1984) Larval development in *Echinoplana celerrima* (Turbellaria: Polycladida). T Am Microsc Soc 103: 31–37

Ballarin L, Galleni L (1987) Evidence for planktonic feeding in Götte's larva of *Stylochus mediterraneus* (Turbellaria – Polycladida). Ital J Zool 54: 83–85

Bock S (1924) Eine neue Stylochoplana aus Japan. Ark Zool 16:1–24

Bolaños DM, Litvaitis MK (2009) Embryonic muscle development in direct and indirect developing marine flatworms (Platyhelminthes, Polycladida). Evol Dev 11: 290–301

Chen CP, Tsai WS, Liu CY, Lee QS (1990) Recruitment and induction of settlement of planktonic juveniles of the oyster flatworm, *Stylochus orientalis* Bock (Platyhelminthes, Polycladida). B I Zool Acad Sinica 29: 57–64

Deguchi R, Sasaki H, Iwata K, Echizen M (2009) Reproduction and life cycle of the polyclad flatworm *Stylochoplana pusilla*. Bull Miyagi Uni Edu 44: 53–61

Durant F, Lobo D, Hammelman J, Levin M (2016) Physiological controls of large-scale patterning in planarian regeneration: A molecular and computational perspective on growth and form. Regeneration 3: 78-102

Faubel A (1983) The Polycladida, Turbellaria Proposal and establishment of a new system Part I. The Acotylea. Mitt Hamb. Zool. Mus. Inst 80: 17–121

Forward Jr. RB (1987) Comparative study of crustacean larval photoresponses. Mar Biol 94: 589-595

Forward Jr. RB, Costlow Jr. JD (1974) The ontogeny of phototaxis by larvae of the crab *Rhithropanopeus harrisii*. Mar Biol 26: 27-33

Forward Jr. RB (1976) Light and diurnal vertical migration: photobehavior and photophysiology of plankton. In “Photochemical and photobiological reviews” Ed by KC Smith, Springer, Boston, pp.157-209

Fournier A. (1984) Photoreceptors and Photosensitivity in Platyhelminthes. In “Photoreception and Vision in Invertebrates. NATO ASI Series (Series A: Life Sciences)” Ed by Ali MA, vol 74, Springer, Boston, pp 217-239

Fujiwara Y, Urabe J, Takeda S (2014) Host preference of a symbiotic flatworm in relation to the ecology of littoral snails. Mar Biol 161: 1873–1882

Hagiya M (1993) Notes on some polyclad Turbellarians (Platyhelminthes) from Otsuchi Bay and its vicinity, Iwate Prefecture. Otsuchi Mar Res Cen Rep 19: 31–51 (Japanese)

Hagiya M, Gamo S (1992) Polyclads (Platyhelminthes) collected from the intertidal rocky shore at Manazuru, Sagami Bay. Proc Fac Edu Human Sci, Yokohama Natl Univ 8: 13–24 (Japanese)

Janiak D, Adams J, Rubinoff B, Osman R (2016) Predator-prey interactions of the polyclad, *Euplana gracilis*, and the amphipod, *Apocorophium lacustre*, in the Chesapeake Bay. Estuaries Coasts 40: 513-521

Johnson KB, Forward Jr. RB (2003) Larval photoresponses of the polyclad flatworm *Martigrella crozieri* (Platyhelminthes, Polycladida) (Hyman). J Exp Mar Biol Ecol 282: 103–112

Kato K (1933) On *Stylochoplana pusilla* Bock. Doubutugaku Zasshi 45: 487–490

Kato K (1934) Polycad turbellarians from the neighborhood of the Mitsui Institute of Marine Biology. Jpn J Zool 6: 123–128

Kato K (1935) *Stylochoplana parasitica* sp. nov., a polyclad parasitic in the pallial groove of the chiton Annot Zool Jpn 15.1: 123-129

Kato K (1937) Polyclads from Korea. Japan. Journ. Zool 7: 233-240

Kato K (1940) On the development on some Japanese polyclads. Jpn J Zool 8: 537–573

Lanfranchi A, Bedini C (1986) Electron microscopic study of larval eye development in Turbellarian Polyclad. Hydrobiologia 132: 121-126

Lanfranchi A, Bedini C, Ferrero E (1981) The ultrastructure of the eyes in larval and adult polyclads (Turbellaria). Hydrobiologia 84: 267-275

Lapraz F, Rawlinson KA, Girstmair J, Tomiczek B, Berger J, Jékely G, Telford MJ, Egger B (2013) Put a tiger in your tank: the polyclad flatworm *Maritigrella crozieri* as a proposed model for evo-devo. EvoDevo 4: 29

Linnaeus C (1758) Systema Naturae, per regna tria naturae, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis. Ed. 10, Vol. 1. Salvii, Holmiae 824 pp

Lytwyn MW, McDermott JJ (1976) Incidence, Reproduction and Feeding of *Stylochus zebra*, a polyclad turbellarian symbiont of hermit crabs. Mar Biol 38: 365–372

Martín-Durán JM, Egger B (2012) Developmental diversity in free-living flatworms. EvoDevo 3: 1

Miljeteig C, Olsen AJ, Båtnes AS, Altin D, Nordtug T, Alver MO, Speed JDM, Jenssen BM (2014) Sex and life stage dependent phototactic response of the marine copepod *Calanus finmarchicus* (Copepoda: Calanoida). J Exp Mar Bio Ecol 451: 16-24

Newman LJ, Cannon LRG (1995) The importance of the fixation of colour, pattern and form in tropical Pseudocerotidae (Platyheminthes, Polycladida). Hydrobiologia 305: 141–143

Parmentier E, Michel L (2013) Boundary lines in symbiosis forms. Symbiosis 60: 1-5

Prudhoe S (1968) A new polyclad turbellarian associating with a hermit crab in the Hawaiian islands. Pac Sci 22

Prudhoe S (1985) A monograph on the polyclad turbellaria. British Museum (Natural History) and Oxford University Press

Rawlinson, KA (2008) Biodiversity of coastal polyclad flatworm assemblages in the wider Caribbean. Mar Biol 153:769-778

Rawlinson KA (2010) Embryonic and post-embryonic development of the polyclad flatworm *Martigrella crozieri*; implications for the evolution of spiralian life history traits. Front Zool 7: 1

Rawlinson KA (2014) The diversity, development, and evolution of polyclad flatworm larvae. EvoDevo 5: 9

Rawlinson KA, Litvaitis MK (2008) Cotylea (Polycladida): a cladistic analysis of morphology. Invertebr Biol 127.2: 121-138

Rawlinson KA, Bolaños DM, Liana MK, Litvaitis MK (2008) Reproduction, development and parental care in two direct-developing flatworms (Platyhelminthes: Polycladida: Acotylea). J Nat Hist 42.33-34: 2173-2192

Rawlinson KA, Gillis JA, Billings Jr. RE, Borneman EH (2011) Taxonomy and life history of the *Acropora*-eating flatworm *Amakusaplana acroporae* nov. sp. (Polycladida: Prosthiostomidae). Coral Reefs 30: 693–705

Reisinger E (1924) Die Gattung *Rhynchoscolex.* Z Morphol Ökol Tiere 1.1: 1-37

Rieger, RM (1998) 100 years of research on 'Turbellaria'. Hydrobiologia 383: 1–27

Ruppert EE (1978) A review of metamorphosis of turbellarian larvae. In "Settlement and Metamorphosis of Marine Invertebrate Larvae" Ed by FS Chia, ME Rice, Elsevier, New York, pp 65–81

Shinn GL (1987) Phylum Platyhelminthes with an emphasis on marine turbellaria. In "Reproduction and development of marine invertebrates of the Northern Pacific

Coast" Ed by MF Strathmann, University of Washington Press, Seattle and London, pp 114–128

Smith NF, Johnson KB, Young CM (2002) Phylum Platyhelminthes. In "Atlas of Marine Invertebrate Larvae" Ed by CM Young, MA Swell, ME Rice, Academic Press, San Diego, pp 123–127, 132–137

Teshirogi W, Ishida S, Jatani K (1981) On the early development of some Japanese polyclads. Rep Fukaura Mar Biol Lab 9: 2-31

Teshirogi W and Ishida S (1988) Henkei doubutsu (Uzumushi rui). In "Kaisan musekitsui doubutsu no hassei jikken" Ed by Ishikawa M and Numakunai T, Baihukan, pp 52-69 (Japanese)

Yeri M, Kaburaki T (1918) Description of some Japanese Polyclad Turbellari. J Coll Sci Imp Univ Tokyo 39: 1–54

Younossi-Hartenstein A and Hartenstein V (2000) The embryonic development of the polyclad flatworm *Imogine mcgrathi*. Dev Genes Evol 210:383-398

TABLES AND FIGURES

Table 1. The body size growth and increase in the number of eyespots of *Comoplana pusilla*.

Days after hatching	Number of measured specimens	Length(mm)	Width(mm)	Number of eyespots		
				Cerebral (one side)	Tentacular (one side)	Total
0	3	0.17±0.01 (0.18)	0.13±0.03 (0.16)	2.00±0.00 (2)	0.00±0.00 (0)	4.00±0.00 (4)
5	3	0.25±0.02 (0.27)	0.15±0.03 (0.18)	2.00±0.00 (2)	0.00±0.00 (0)	4.00±0.00 (4)
10	3	0.34±0.05 (0.40)	0.24±0.04 (0.27)	2.00±0.00 (2)	0.00±0.00 (0)	4.00±0.00 (4)
15	3	0.54±0.10 (0.66)	0.37±0.05 (0.42)	3.67±0.52 (4)	2.33±0.52 (3)	12.33±0.58 (13)
20	3	0.83±0.08 (0.90)	0.48±0.14 (0.64)	3.83±0.75 (5)	3.17±0.41 (4)	14.00±1.00 (15)
30	3	1.32±0.38 (1.63)	0.69±0.18 (0.89)	5.83±0.75 (7)	5.50±1.38 (8)	22.67±3.79 (27)
40	3	2.11±0.35 (2.46)	0.85±0.11 (0.97)	9.5±0.84 (10)	9.17±1.17 (11)	37.33±2.08 (39)
50	3	2.16±0.36 (2.39)	1.00±0.24 (1.20)	10.17±1.72 (12)	10.50±1.05 (12)	41.33±5.13 (47)
60	2	2.12±0.01 (2.14)	1.12±0.07 (1.17)	10.00±2.45 (12)	11.00±1.41 (13)	42.00±8.49 (48)
70	2	2.18±0.29 (2.38)	1.10±0.01 (1.11)	10.50±2.38 (13)	12.00±0.00 (12)	45.00±5.66 (49)

The maximum size measurements and the number of eyespots are recorded in parentheses.

Table 2. The body size growth and increase in the number of eyespots of *Notocomplana koreana.*

Days after hatching	Number of measured specimens	Length(mm)	Width(mm)	Number of eyespots		
				Cerebral (one side)	Tentacular (one side)	Total
0	4	0.25±0.02 (0.27)*	0.20±0.04 (0.25)*	2.00±0.00 (2)	0.00±0.00 (0)	4.00±0.00 (4)
5	5	0.41±0.06 (0.49)*	0.31±0.05 (0.38)*	2.13±0.35 (3)	0.00±0.00 (0)	4.25±0.50 (5)
10	6	0.67±0.14 (0.93)*	0.54±0.16 (0.78)*	3.25±0.89 (5)	1.00±0.85 (2)	8.67±2.73 (11)
15	6	1.03±0.32 (1.34)*	0.90±0.11 (1.06)*	4.00±0.29 (5)	2.25±0.62 (4)	12.00±0.00 (12)
20	6	1.79±0.36 (2.01)*	1.09±0.23 (1.41)*	4.58±0.90 (7)	2.92±1.00 (4)	12.67±1.21 (15)
30	6	2.08±0.52 (2.52)	1.06±0.15 (1.25)	7.42±2.02 (10)	4.25±0.62 (6)	23.33±3.50 (29)
40	6	3.04±0.41 (3.38)	1.51±0.13 (1.68)	11.92±1.16 (14)	6.00±1.21 (9)	36.00±2.00 (39)
50	6	4.69±0.45 (5.15)	2.08±0.53 (2.79)	17.30±1.64 (20)	7.80±1.14 (10)	50.20±3.42 (55)
60	6	5.48±0.41 (6.04)	2.27±0.39 (2.57)	21.83±4.73 (32)	9.83±0.94 (11)	63.33±10.13 (79)
70	6	7.38±1.21(8.60)	2.38±0.31 (2.84)	23.00±6.86 (38)	10.70±2.02 (14)	67.33±15.88 (96)
80	6	8.20±1.29 (9.86)	2.54±0.42 (3.06)	27.33±8.51 (47)	13.80±1.29 (17)	82.17±18.52 (116)
90	4	7.81±1.00 (8.70)	2.43±0.25 (2.62)	32.63±10.10 (45)	13.90±1.96 (18)	93.00±24.24 (118)
100	6	9.39±1.93 (12.09)	2.78±0.22 (3.00)	31.38±6.63 (42)	14.50±2.15 (19)	92.17±16.45 (114)
150	2	13.01±0.91 (13.65)	3.55±1.04 (4.29)	52.25±2.87 (54)	23.00±2.45 (25)	150.50±9.19 (157)
200	2	13.96±2.01 (15.38)	4.05±1.06 (4.80)	62.25±5.12 (69)	29.30±4.99 (33)	183.00±15.56 (194)

The maximum size measurements and the number of eyespots are recorded in parentheses. The asterisks mark the measurements made from images under the inverted microscope.

Table 3. The body size growth and increase in the number of eyespots of *Pseudostylochus obscurus* with brine shrimp culture.

Days after hatching	Number of measured specimens	Length(mm)	Width(mm)	Number of eyespots		
				Cerebral (one side)	Tentacular (one side)	Total
0	4	0.36±0.05 (0.43)*	0.22±0.02 (0.25)*	2.00±0.00 (2)	0.00±0.00 (0)	4.00±0.00 (4)
5	6	0.42±0.03 (0.44)	0.35±0.01 (0.35)	2.08±0.29 (3)	0.00±0.00 (0)	4.17±0.41 (5)
10	6	0.56±0.08 (0.62)	0.48±0.08 (0.54)	3.83±0.58 (5)	2.00±0.43 (3)	11.67±1.03 (13)
15	6	0.65±0.15 (0.75)	0.59±0.03 (0.61)	4.08±0.51 (5)	2.67±0.49 (3)	13.50±0.84 (14)
20	6	1.33±0.29 (1.59)	0.75±0.11 (0.90)	6.75±1.42 (9)	4.25±0.45 (5)	22.00±2.83 (26)
30	6	1.80±0.40 (2.12)	1.12±0.18 (1.33)	9.25±2.30 (15)	6.75±1.96 (11)	31.67±7.03 (40)
40	6	2.46±0.35 (2.91)	1.43±0.13 (1.58)	11.83±1.95 (15)	10.20±2.08 (13)	44.00±5.87 (54)
50	6	2.73±0.20 (2.94)	1.56±0.11 (1.73)	13.25±1.60 (16)	13.30±1.14 (15)	53.00±4.20 (59)
60	6	3.01±0.30 (3.53)	1.84±0.12 (2.01)	14.50±1.73 (18)	15.40±2.47 (21)	59.83±5.91 (69)
70	6	3.56±0.26 (3.99)	1.95±0.19 (2.19)	17.75±2.60 (23)	20.00±2.41 (25)	75.50±6.86 (84)
80	6	4.15±0.22 (4.40)	2.23±0.09 (2.38)	20.33±3.06 (25)	23.20±3.07 (27)	87.67±9.00 (100)
90	3	4.37±0.17 (4.55)	2.47±0.03 (2.50)	23.00±2.90 (27)	29.00±2.28 (33)	104.00±3.61 (107)
100	2	5.08±0.06 (5.50)	2.60±0.06 (2.64)	23.74±2.50 (27)	29.30±2.50 (32)	106.00±8.49 (112)
110	2	6.32±0.49 (6.67)	2.93±0.17 (3.05)	23.50±2.52 (27)	32.50±4.20 (37)	112.00±1.41 (113)
120	2	5.99±0.03 (6.01)	2.79±0.34 (3.03)	25.25±1.89 (28)	33.50±2.08 (36)	117.50±3.54 (120)
130	2	6.38±0.36 (6.64)	2.63±0.38 (2.90)	25.25±1.26 (27)	34.50±4.20 (39)	119.50±7.79 (125)
140	2	4.88±0.56 (5.28)	2.16±0.15 (2.27)	23.00±2.94 (27)	29.30±4.79 (35)	104.50±3.54 (107)

The maximum size measurements and the number of eyespots are recorded in parentheses. The asterisks mark the measurements made from images under the inverted microscope.

Table 4. The body size growth and increase in the number of eyespots of *Pseudostylochus obscurus* with *Monodonta labio* culture.

Days after feed type change	Number of measured specimens	Length(mm)	Width(mm)	Number of eyespots		
				Cerebral (one side)	Tentacular (one side)	Total
10	4	6.35±1.29 (8.18)	3.50±0.37 (3.91)	21.00±3.07 (25)	29.60±4.17 (36)	101.25±13.50 (120)
20	4	9.71±1.56 (10.98)	5.74±0.73 (6.59)	25.38±4.03 (31)	33.00±2.93 (37)	116. 75±11.90 (131)
30	4	11.72±1.61 (13.13)	6.63±1.23 (8.44)	25.13±1.89 (29)	32.90±4.36 (40)	116.00±9.20 (127)
40	4	14.27±1.68 (16.67)	7.97±1.79 (9.67)	26.88±3.64 (35)	39.30±6.04 (48)	132.25±19.10 (158)
50	4	18.06±3.37 (22.34)	9.39±2.35 (12.50)	30.50±4.04 (38)	43.60±6.21 (51)	148.25±17.60 (164)
60	2	18.43±5.76 (22.50)	12.18±0.72 (12.69)	34.00±4.16 (38)	47.30±2.06 (49)	162.50±9.19 (169)

The maximum size measurements and the number of eyespots are recorded in parentheses. The feed was changed between 80 to 90 days after hatching.

Table 5. The body size growth and increase in the number of eyespots of *Stylochoplana parasitica*.

Days after hatching	Number of measured specimens	Body size (mm^2)	Number of eyespots (total)
0	3	0.028±0.001(0.029)	4.00±0.00 (4)
5	3	0.050±0.012 (0.063)	4.00±0.00 (4)
10	3	0.058±0.021 (0.087)	4.00±0.00 (4)
15	3	0.080±0.022 (0.108)	7.67±3.09 (12)
20	3	0.096±0.020 (0.116)	9.33±2.36 (11)
30	3	0.173±0.042 (0.233)	12.00±0.82 (13)
40	3	0.176±0.036 (0.216)	12.33±0.47 (13)
50	3	0.169±0.037 (0.206)	12.67±0.47 (13)
60	3	0.152±0.035 (0.196)	14.00±0.82 (15)
70	3	0.149±0.027 (0.187)	13.33±1.25 (15)
80	3	0.119±0.019 (0.134)	13.33±0.47 (14)
90	2	0.112±0.007 (0.119)	12.50±0.50 (13)
100	3	0.083±0.014 (0.096)	12.67±0.94 (14)
110	2	0.076±0.009 (0.085)	10.67±2.49 (14)
120	2	0.116±0.067 (0.183)	10.00±1.00 (11)
130	1	0.061±0.000 (0.061)	12.00±0.00 (12)

The maximum size measurements and the number of eyespots are recorded in parentheses.

Table 6. The body size growth and increase in the number of eyespots of *Stylochoplana parasitica* with chiton, *Liolophura japonica* culture.

Days after culture with chitons	Number of measured specimens	Body size (mm^2)	Number of eyespots (total)
0	3	0.180±0.028 (0.210)	12.00±0.82 (13)
10	2	0.360±0.046 (0.406)	12.50±0.50 (13)
20	N.D.	N.D.	N.D.
30	3	1.270±0.620 (2.142)	16.67±2.05 (19)
40	3	2.373±1.161 (4.012)	18.00±1.63 (20)
50	3	3.645±0.925 (4.923)	23.67±1.25 (25)
60	3	4.139±1.909 (6.776)	24.67±1.25 (26)
70	3	4.693±1.442 (6.726)	29.33±2.05 (32)
80	3	4.783±1.487 (6.879)	27.33±0.47 (28)
90	3	4.445±1.312 (6.286)	28.33±3.40 (33)
100	2	5.663±1.279 (6.941)	26.50±0.50 (27)
110	2	7.335±3.559 (10.894)	30.00±5.00 (35)
120	N.D.	N.D.	N.D.
130	1	5.802±0.000 (5.802)	24.00±0.00 (24)

The maximum size measurements and the number of eyespots are recorded in parentheses. Culture with chitons began at about 30, 65, or 71 days after hatching.

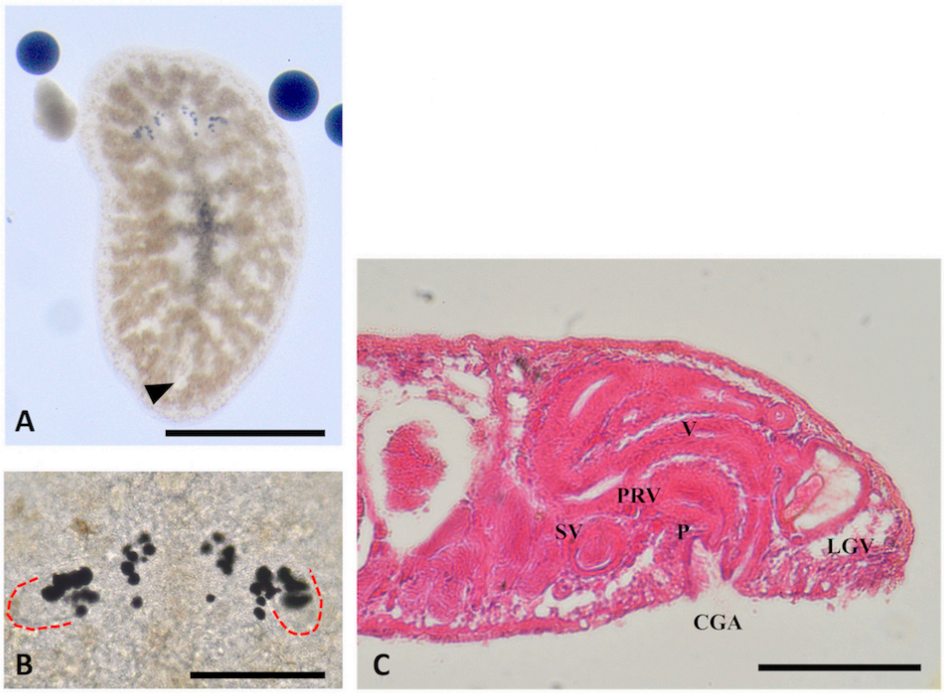

Fig. 1. Key characters for the identification of *Comoplana pusilla*. (**A**) External features; the arrowhead points to the area of the genital organs. (**B**) Eyespots of a sectioned sample; the anterior end is to the top; the dotted lines outline the tentacles on the head. (**C**) Section sample of the genital organs; the posterior end is on the right. Abbreviations: CGA, common genital atrium; LGV, Lang's vesicle; P, penis; PRV, prostatic vesicle; SV, seminal vesicle; V, vagina. Scale = 1 mm (**A**), 200 μm (**B**, **C**).

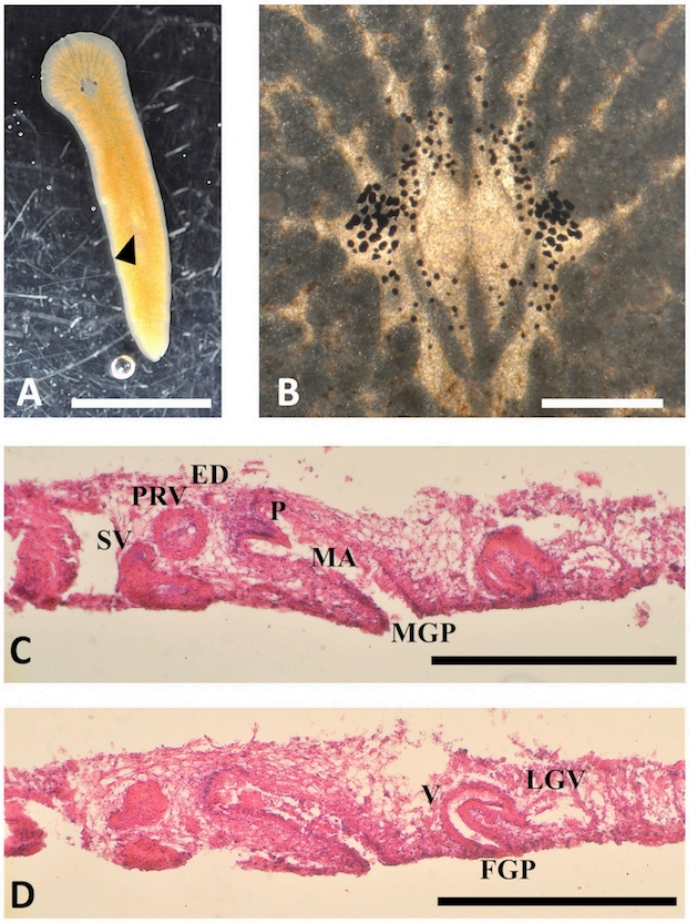

Fig. 2. Key characters for the identification of *Notocomplana koreana*. (**A**) External features; the arrowhead points to the genital complexes. (**B**) Eyespots when fully developed; the anterior end is to the top. (**C**), (**D**) Section samples of the genital organs; the anterior end is to the left. Abbreviations: ED, ejaculatory duct; FGP, female gonopore; LGV, Lang's vesicle; MA, male atrium; MGP, male gonopore; P, penis; PRV, prostatic vesicle; SV, seminal vesicle; V, vagina. Scale = 5 mm (**A**, **C**, **D**), 500 μm (**B**).

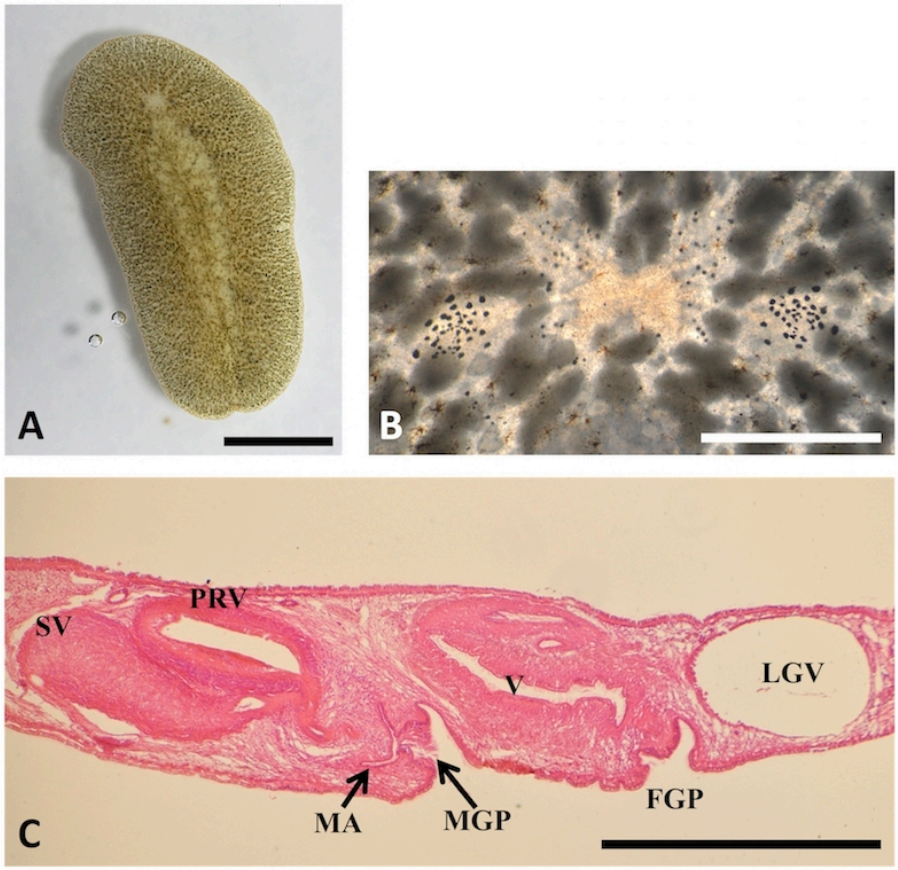

Fig. 3. Key characters for the identification of *Pseudostylochus obscurus*. (**A**) External features. (**B**) Eyespots on a mature specimen selected from the culturing system; the anterior side is to the top. (**C**) Section sample of the genital organs; the posterior side is on the right. Abbreviations: FGP, female gonopore; LGV, Lang's vesicle; MA, male antrum; MGP, male gonopore; PRV, prostatic vesicle; SV, seminal vesicle; V, vagina. Scale = 1 cm (**A**), 1 mm (**B**, **C**).

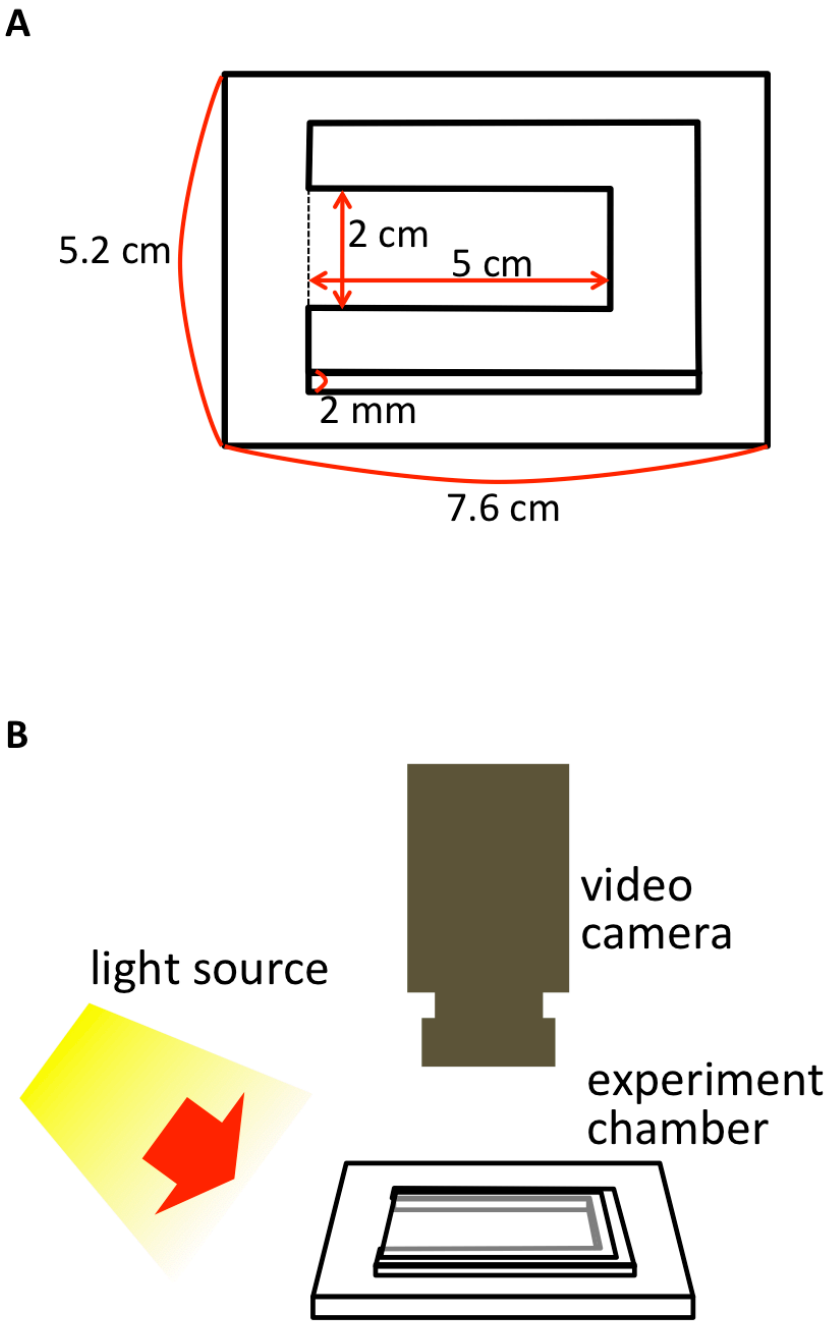

Fig. 4. **(A)** Structure of the chamber used for the phototaxis experiment. **(B)** Experiment setup for the phototaxis experiment.

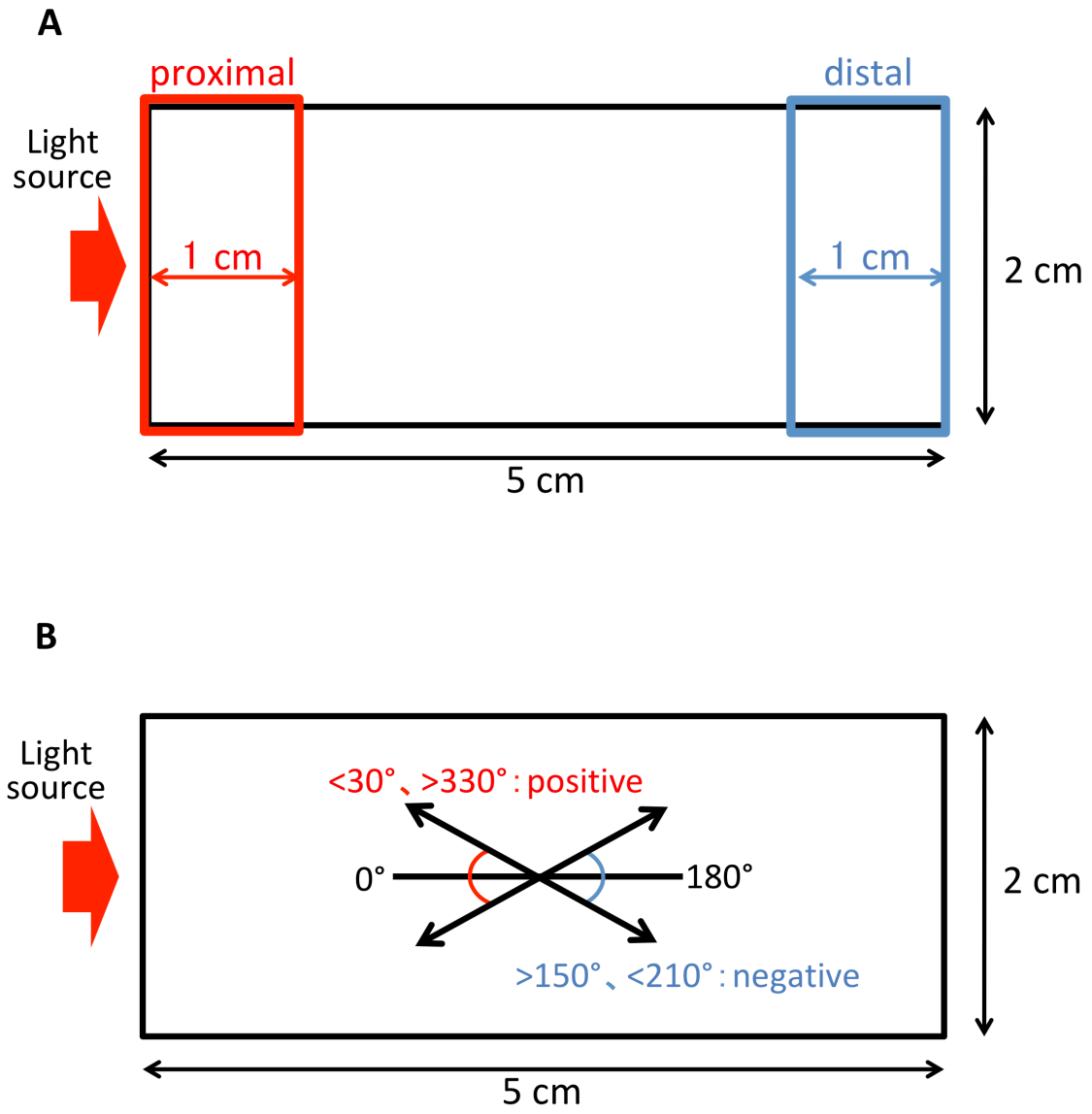

Fig. 5. The standards set to measure phototaxis according to **(A)** distribution of larvae after 90 seconds of light stimulus, and **(B)** swimming direction at 3 seconds after light stimulus.

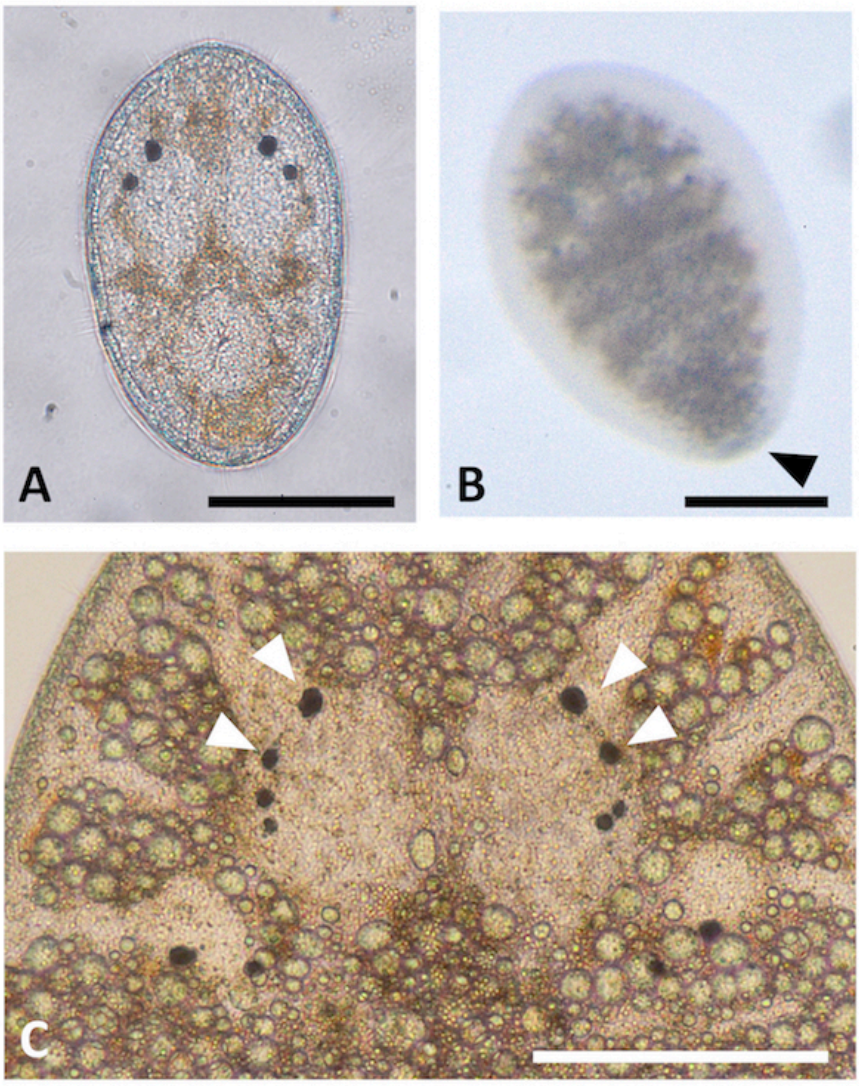

Fig. 6. Larva of *Comoplana pusilla*. **(A)** Newly hatched larva. (**B**) Larva 14 days post-hatching; the arrowhead marks the black spots at the posterior end. (**C**) Eyespots of larva14 days post-hatching; the original four eyespots are marked by arrowheads. Scale = 100 μm (**A**), 200 μm (**B**, **C**).

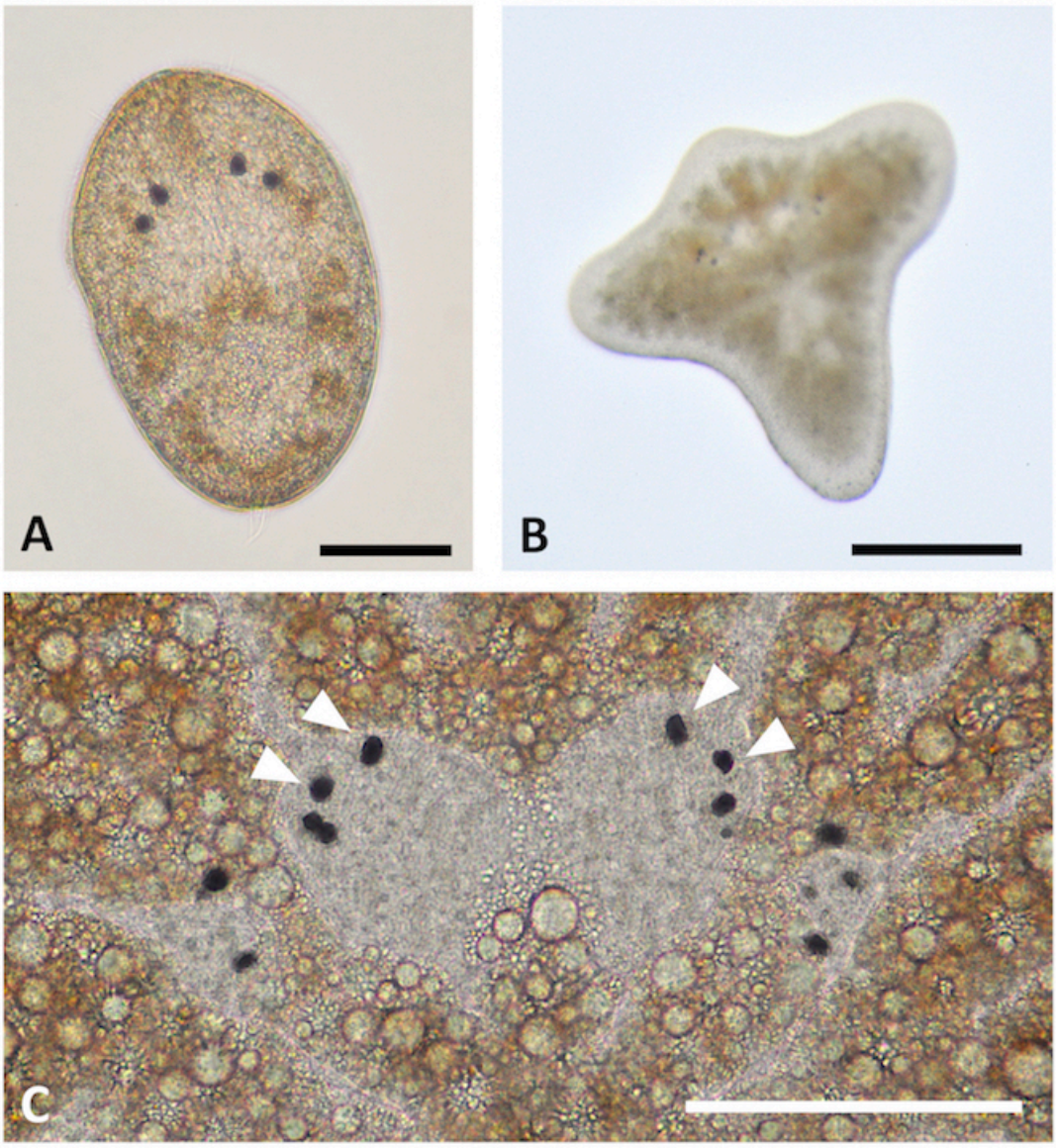

Fig. 7. Larva of *Notocomplana koreana*. (**A**) Newly hatched larva. (**B**) Larva 13 days post-hatching, showing the characteristic cross-like body shape. (**C**) Eyespots of larva 14 days post-hatching; the original four eyespots are marked by arrowheads; the anterior side is at the top. Scale = 100 μm (**A**), 500 μm (**B**), 200 μm (**C**).

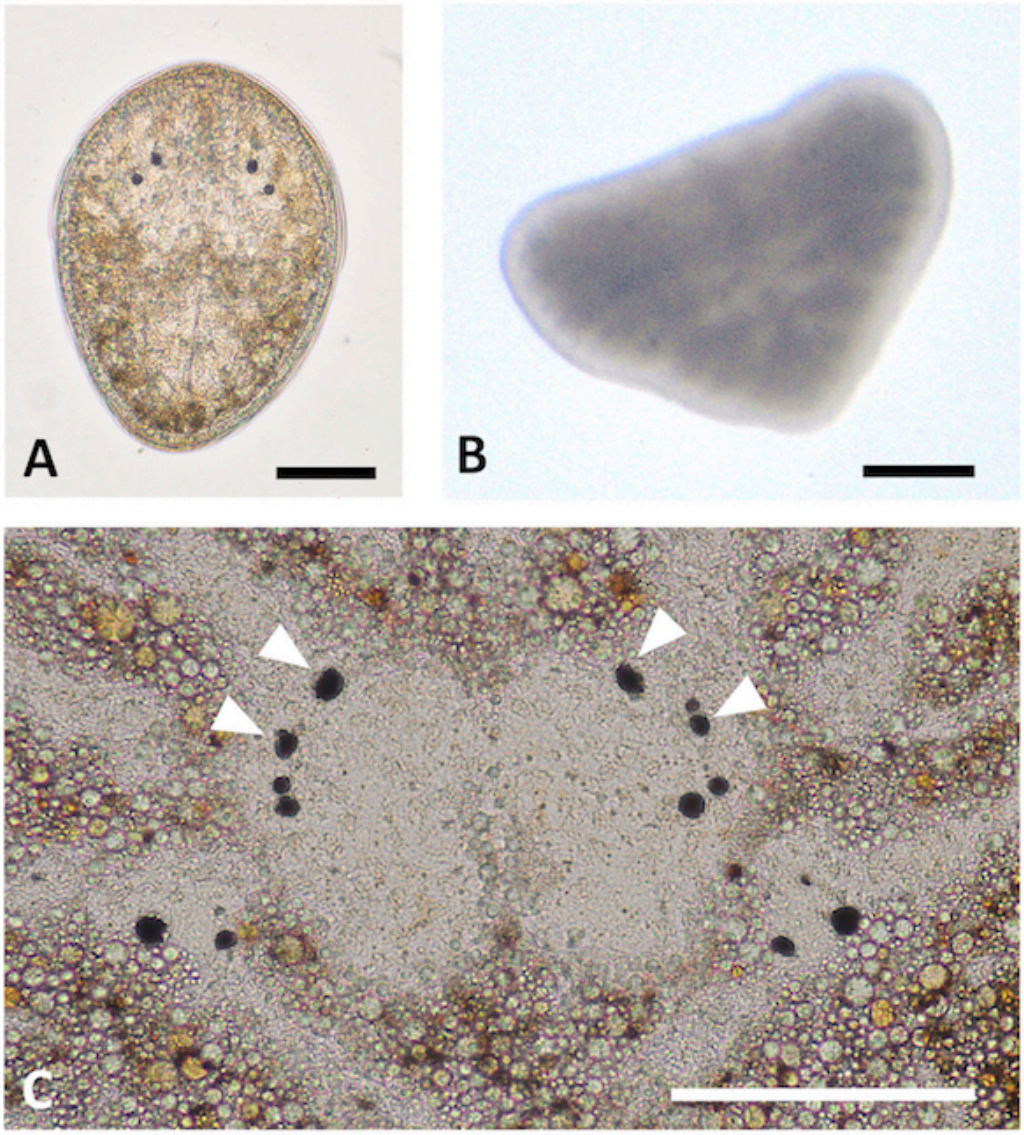

Fig. 8. Larva of *Pseudostylochus obscurus*. (**A**) Newly hatched larva. (**B**) Larva 13 days post-hatching, showing the characteristic triangular body shape. (**C**) Eyespots of larva 14 days post-hatching; the original four eyespots are marked by arrowheads; the anterior side is at the top. Scale = 100 μm (**A**), 200 μm (**B**, **C**).

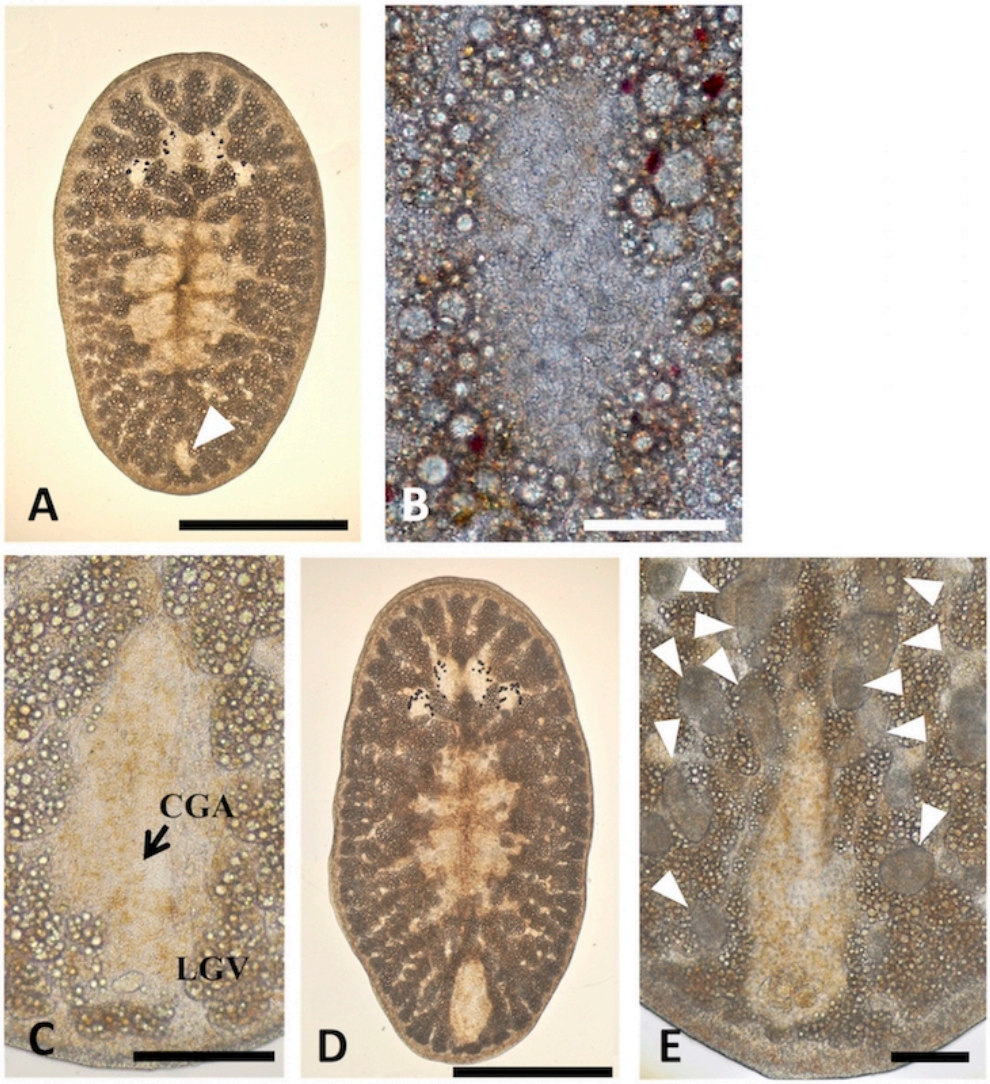
A
B
CGA
LGV
C
D
E

Fig. 9. (**A**) Genital organs of *C. pusilla* as they started to develop approximately 35 days after hatching. The specimen was compressed for observation. The area of the genital organs is marked by an arrowhead. (**B**) Enlarged image of the area of the genital organs, observed from the ventral side; the anterior side is at the top. (**C**) Genital complexes about 20 days after onset of the genital organ formation. (**D**) Grown specimen at 70 days after hatching. (**E**) Area of the genital organs in a fully matured specimen, with arrowheads pointing to eggs. Abbreviations: CGA, common genital atrium; LGV, Lang's vesicle. Scale = 1 mm (**A**, **C**), 100 μm (**B**, **D**), 200 μm (**E**).

A
B
C
SV+PRV
P
MGP
FGP
LGV
D
E
SV
PRV
SC
P
MGP
FGP
OD
V,
LVD
LGV

Fig. 10. (**A**) Specimen of *N. koreana* 50 days after hatching. Formation of the genital organs (arrowhead) are visible from the dorsal side at the posterior end of the pharynx. (**B**) Enlarged image of the area where genital organs are starting to develop, at 50 days post-hatching; the anterior side is at the top. (**C**) Specimen 60 days after hatching, and about 20 days after the sexual organs began to develop. The area where the seminal vesicle and the prostatic vesicle are formed begins to appear as a pale area, but the two structures are still indistinguishable. (**D**) Specimen 100 days after hatching. Eggs are stored in the uterine canals, evident as a white line encircling the pharynx. (**E**) Fully mature state of the genital organs. Some of the eggs are indicated by arrowheads. Abbreviations: FGP, female gonopore; LGV, Lang's vesicle; LVD, duct of Lang's vesicle; MGP, male gonopore; OD, oviduct; P, penis; PRV, prostatic vesicle; SC, seminal canal; SV, seminal vesicle; V, vagina. Scale = 1 mm (**A**, **C**), 200 μm (**B**, **D**), 500 μm (**E**).

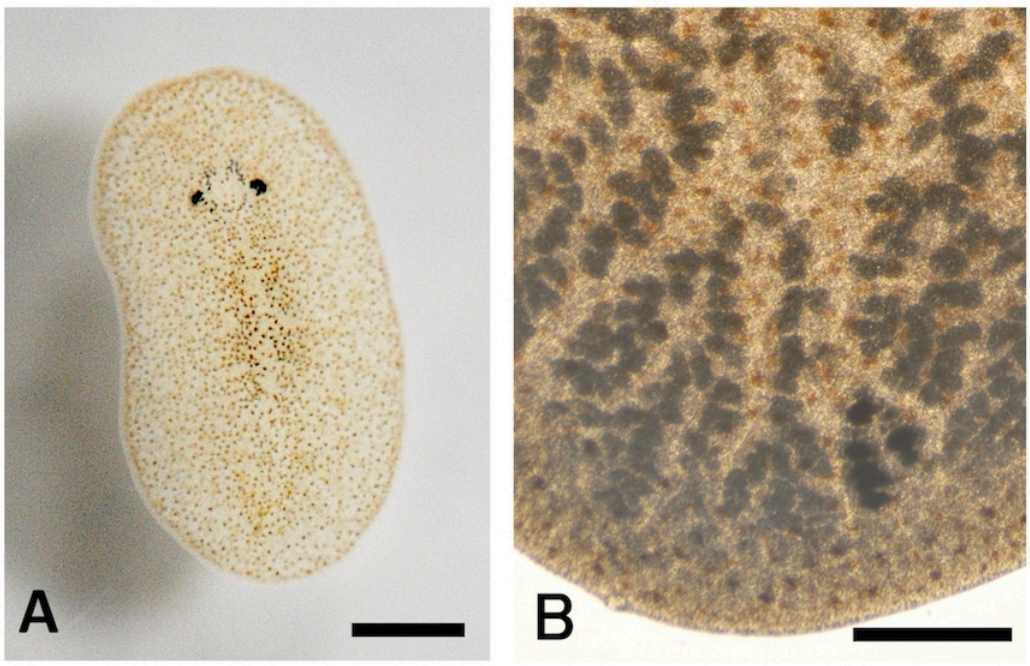

Fig. 11. (**A**) A specimen of *P. obscurus* 140 days after hatching, and which was continuously fed with brine shrimp. (**B**) Area behind the pharynx, viewed from the ventral side; no indication of formation of the genital organs can be seen. Scale = 1 mm (**A**), 500 µm (**B**)

A
B
C
D
E
F
SV+PRV
MGP
FGP
OD
V,
LVD
SC
SV
PRV
MGP
FGP
OD
V,
LVD
LGV
SV

Fig. 12. **(A)** Specimen of *P. obscurus* 20 days after changing the food to the gastropod *Monodonta labio*. (**B**) Area in front of the median tail notch, 20 days after the food was changed, viewed from the ventral side, showing the genital organs beginning to form (arrowhead); the anterior end is at the top. (**C**) Specimen about 30 days after the food was changed, viewed from the ventral side; the anterior end is at the top. The area where the seminal vesicle and prostatic vesicle are formed appears as a pale area, but the two structures are still indistinguishable. (**D**) Fully mature specimen. Eggs are evident as a pale line encircling the pharynx. (**E**) Specimen about 45 days after the food was changed. Most of the reproductive structures are well developed and easily distinguishable; the seminal canal and seminal vesicle are filled with sperm. (**F**) Eggs accumulating (circles) in the area slightly anterior to the seminal vesicle. Abbreviations: FGP, female gonopore; LGV, Lang's vesicle; LVD, duct of Lang's vesicle; MGP, male gonopore; OD, oviduct; P, penis; PRV, prostatic vesicle; SC, seminal canal; SV, seminal vesicle; V, vagina. Scale = 5 mm (**A**, **D**), 500 μm (**B**, **C**, **E**, **F**).

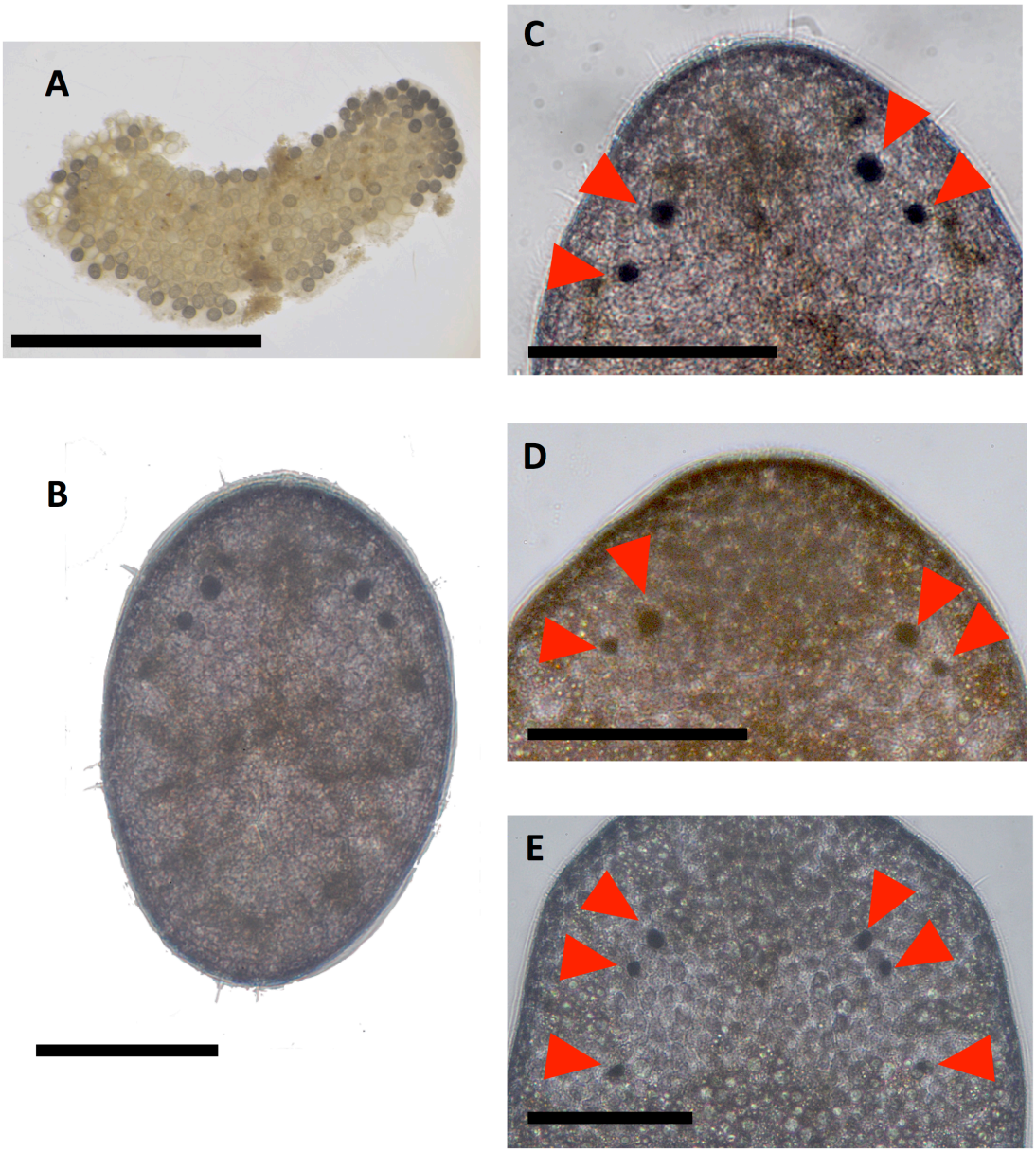

Fig. 13. (**A**) Egg mass (egg plate) of *S. parasitica*. (**B**) Hatched larva of *S. parasitica*. (**C-D**) Change in number of eyespots in (**C**) 0 days after hatching, (**D**) 7 days after hatching, and (**E**) 14 days after hatching in *S. parasitica* larva. Arrowheads indicate the positions of the eyespots. Scale = 3 mm (**A**), 100 μm (**B-E**).

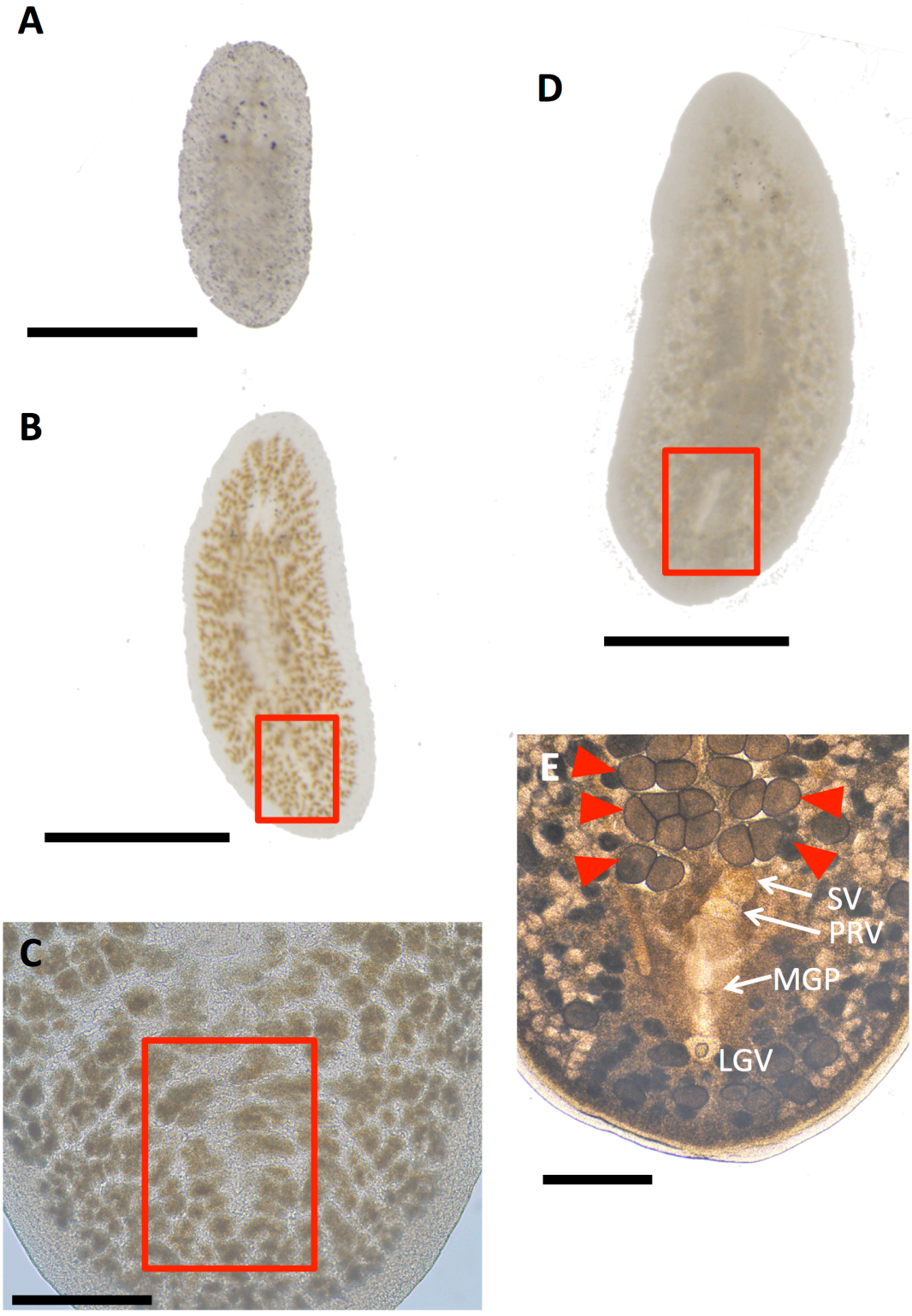
A
B
C
D
E
SV
PRV
MGP
LGV

Fig. 14. Morphological changes seen in *S. parasitica* after culturing with chitons. (**A**) *S. parasitica* cultured with chitons for 10 days. (**B**) *S. parasitica* cultured with chitons for 30 days. Development of genital organs could be confirmed as a light colored area at the hind end (red square). (**C**) Close up view of (**B**). (**D**) *S. parasitica* 137 days after culturing with chitons. This was the earliest timing when fully developed genital organs with egg storage were confirmed. (**E**) Close up of the genital organs seen as a red square in (**D**). Eggs are indicated by arrowheads. Abbreviations: LGV, Lang's vesicle; MGP, male gonopore; PRV, prostatic vesicle; SV, seminal vesicle. Scales = 500 μm (**A, E**), 1 mm (**B**), 300 μm (**C**), 2 mm (**D**).

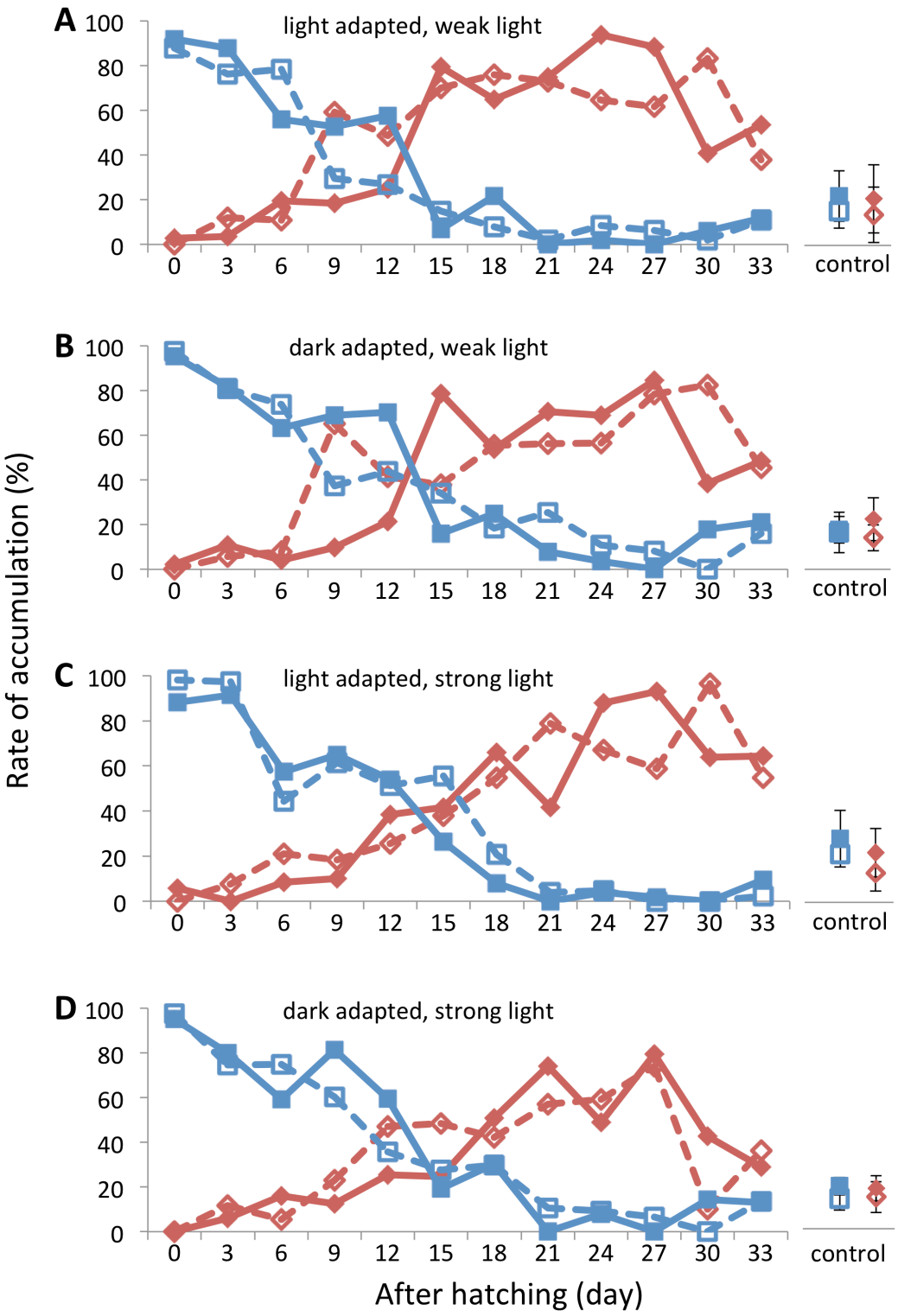
A
light adapted, weak light
B
dark adapted, weak light
C
light adapted, strong light
D
dark adapted, strong light
Rate of accumulation (%)
After hatching (day)
control
0
20
40
60
80
100
0 3 6 9 12 15 18 21 24 27 30 33

Fig. 15. Change of larval phototactic behavior after hatching in *P. obscurus*. Percentages of larvae distributed at the proximal (red, positive phototaxis) or distal (blue, negative phototaxis) region in the experimental chamber (Fig. 5A) are plotted against time after hatching. Data from two different batches (open and closed symbols) are plotted. (**A**) light adaptation, weak light-illuminated; (**B**) dark adaptation, weak light-illuminated; (**C**) light adaptation, strong light-illuminated; (**D**) dark adaptation, strong light-illuminated. Control shows the percentage of larvae distributed at proximal or distal section of the chamber prior to the light stimulus was given. Data from all sampled dates were averaged and plotted with standard error.

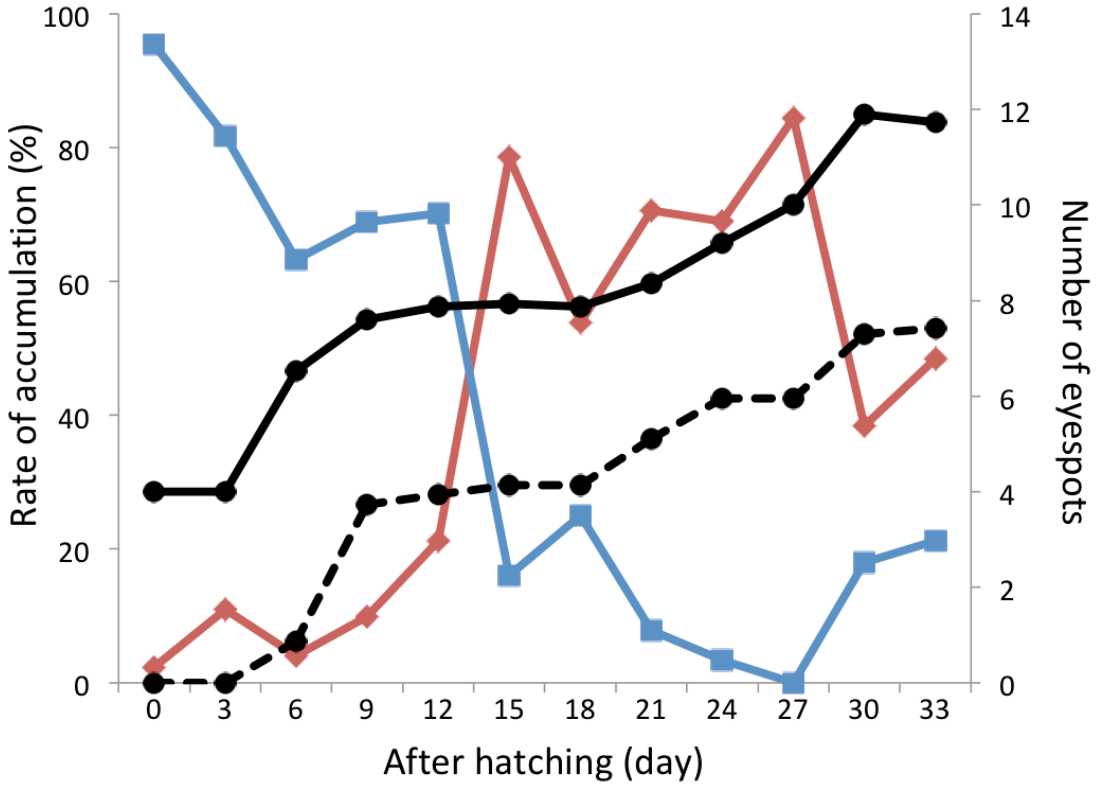

Fig. 16. Relationship between the change of phototactic response and number of eyespots in *P. obscurus*. Exemplary data of phototaxis observed under weak light stimulation after dark adaptation is overlapped with the number of cerebral eyespots (solid line) and tentacular eyes (dotted line). Percentage of larvae distributed at the proximal (positively phototactic) or at the distal (negatively phototactic) region in the experimental chamber are presented in red and blue, respectively.

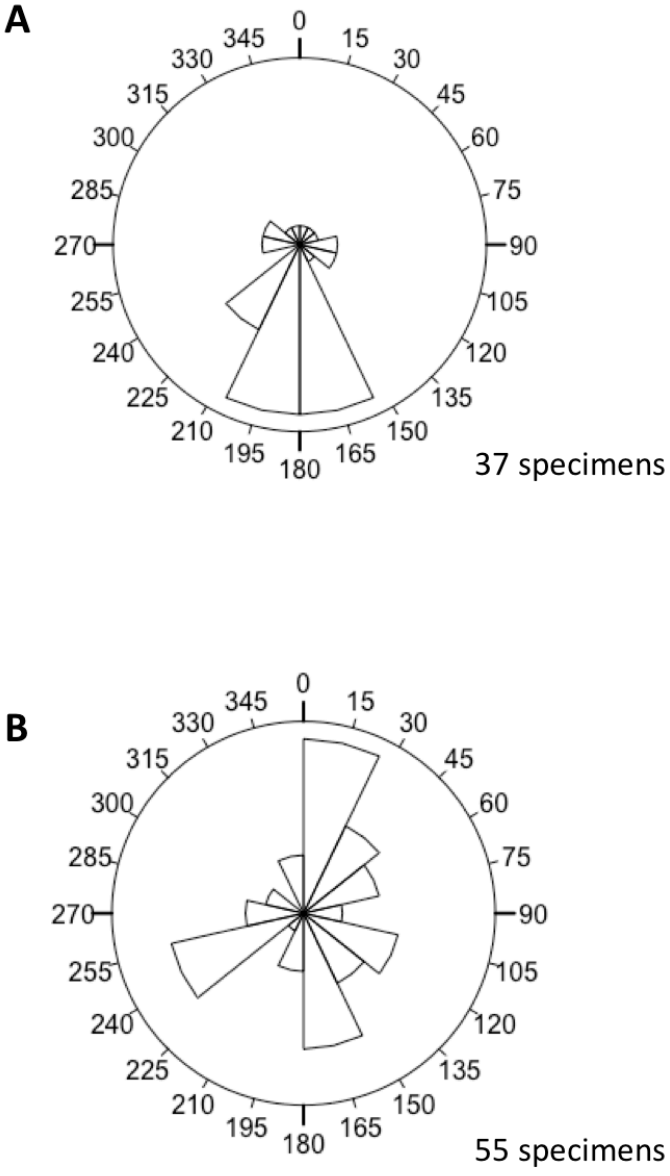

Fig. 17. Distribution of swimming direction after 3 seconds after weak light stimulation in *P. obscurus* larvae at 0 day after hatching. Larvae were adapted to light (**A**) or dark (**B**) before light stimulation.

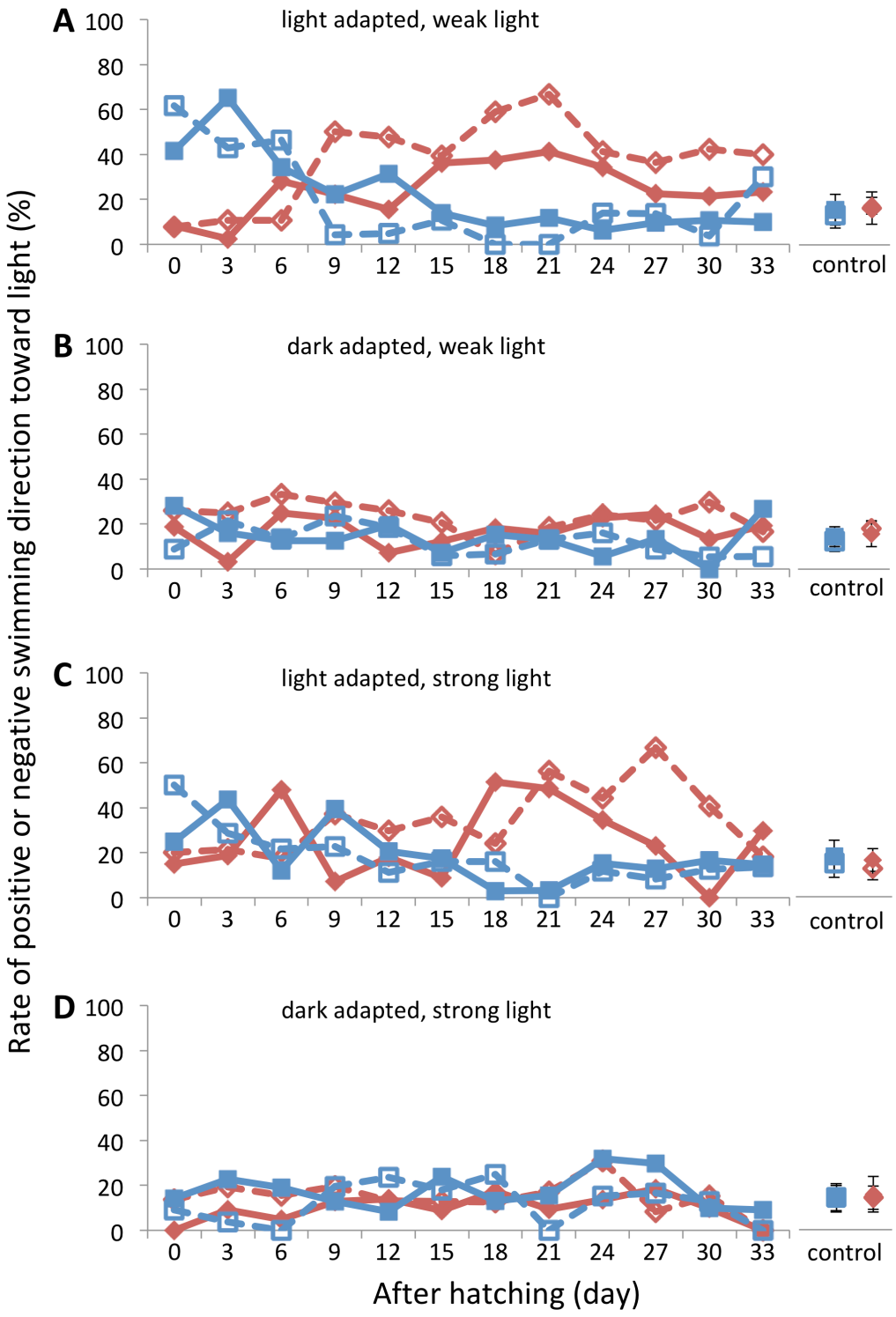
A
light adapted, weak light
B
dark adapted, weak light
C
light adapted, strong light
D
dark adapted, strong light
Rate of positive or negative swimming direction toward light (%)
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24 27 30 33
control
After hatching (day)

Fig. 18. Effect of light or dark adaptation on the initial phototactic response in *P. obscurus*. Percentages of larvae swimming toward the light (red, positive phototaxis) or away from the light (blue, negative phototaxis) in the experimental chamber (Fig. 5A) at 3 seconds after light stimulus are plotted against time after hatching. Data from two different batches (open and closed symbols) are plotted. (**A**) light adaptation, weak light-illuminated; (**B**) dark adaptation, weak light-illuminated; (**C**) light adaptation, strong light-illuminated; (**D**) dark adaptation, strong light-illuminated. Control shows the percentage of larvae swimming towards or away from the light source prior to the light stimulus was given. Data from all sampled dates were averaged and plotted with standard error.

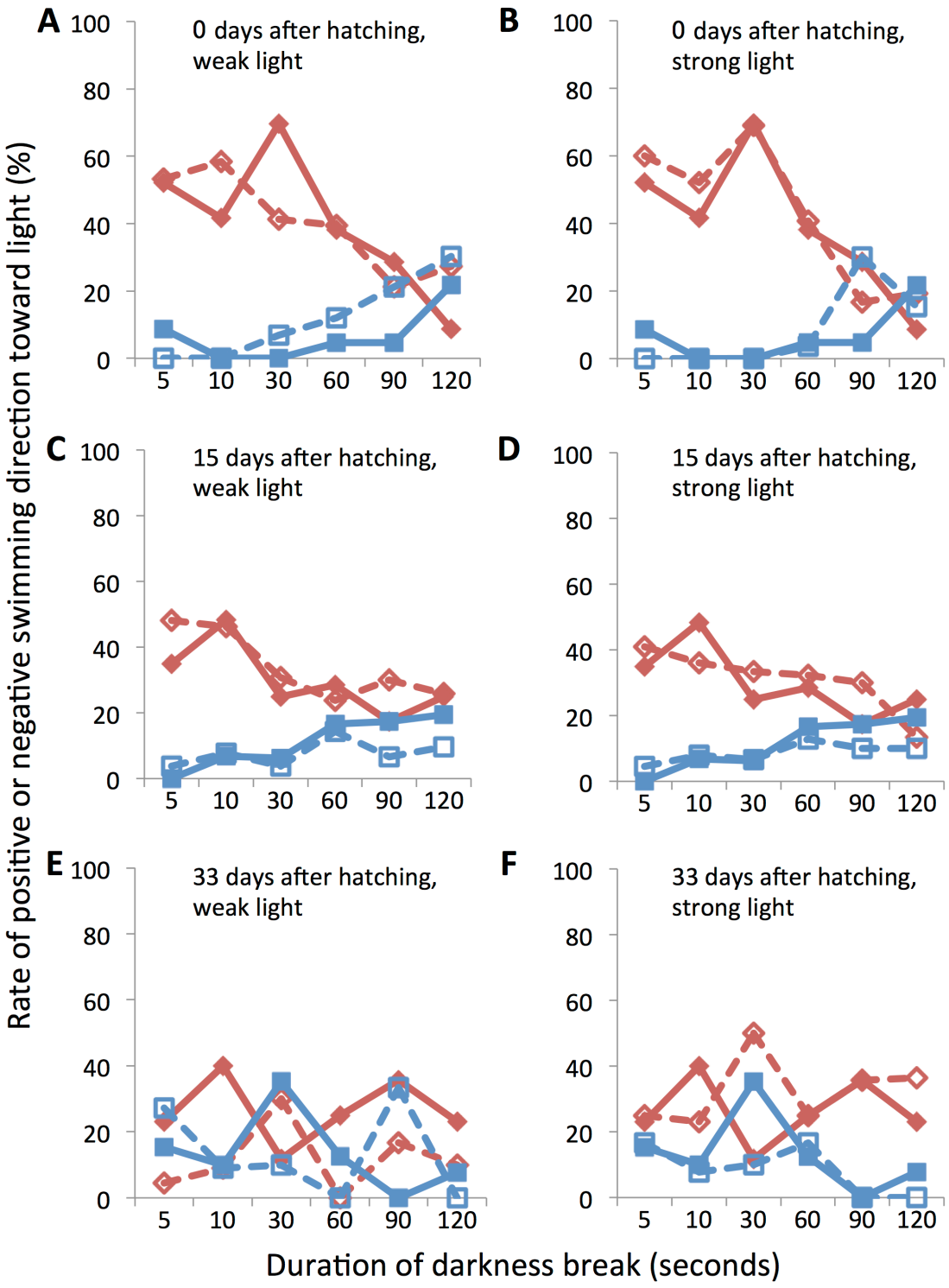
A
0 days after hatching, weak light
B
0 days after hatching, strong light
C
15 days after hatching, weak light
D
15 days after hatching, strong light
E
33 days after hatching, weak light
F
33 days after hatching, strong light
Rate of positive or negative swimming direction toward light (%)
Duration of darkness break (seconds)
0
20
40
60
80
100
5
10
30
60
90
120

Fig. 19. Reversion of phototactic direction by inserting a darkness break in light stimuli in *P. obscurus*. Percentages of larvae swimming toward the light (red, positive phototaxis) or away from the light (blue, negative phototaxis) in the experimental chamber (Fig. 5A) at 3 seconds after dark break are plotted against the length of dark break. Data from two different batches (open and closed symbols) are plotted. (**A**) weak light-illuminated at 0 days after hatching; (**B**) strong light-illuminated at 0 days after hatching; (**C**) weak light-illuminated at 15 days after hatching; (**D**) strong light-illuminated at 15 days after hatching; (**E**) weak light-illuminated at 33 days after hatching; (**F**) strong light-illuminated at 33 days after hatching. Note that positive (red) and negative (blue) phototactic reaction is reversed compared to light adapated samples (Fig. 18A, C).

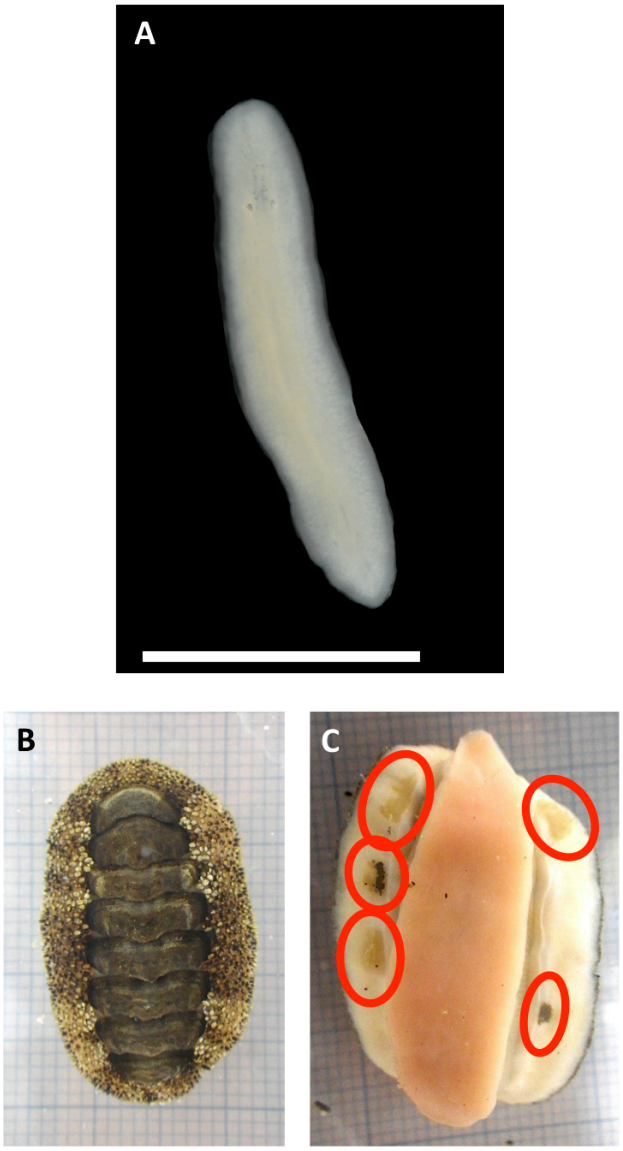

Fig. 20. **(A)** *Stylochoplana parasitica* collected from a chiton. **(B)** The host species of *S. parasitica*, the chiton *Liolophura japonica*. **(C)** The ventral side of *L. japonica* with egg masses of the flatworm (in red circles). Scale = 5 mm (**A**), the smallest grid seen is 1 mm (**B**, **C**).

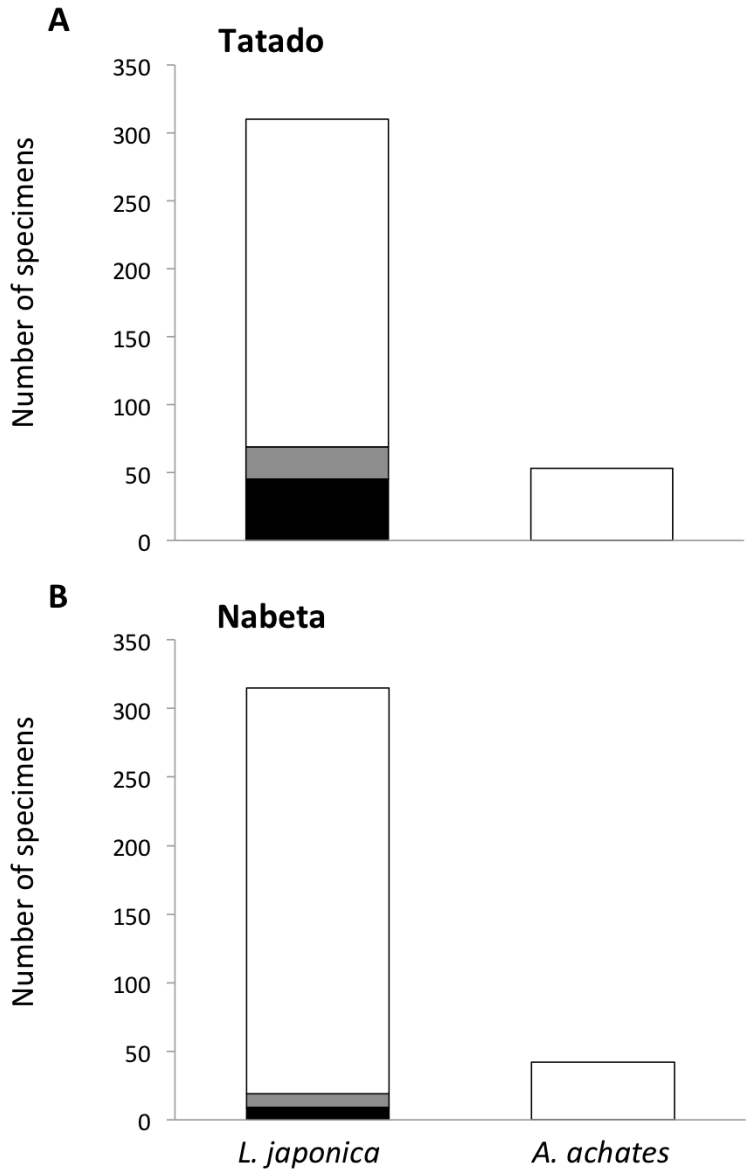

Fig. 21. Total number of chitons with *S. parasitica* inside collected at (**A**) Tatado and (**B**) Nabeta. White bar show chitons without flatworms; grey bars show the number of chiton with a single flatworm inside; and black bars show the number of chitons that had multiple flatworms inside.

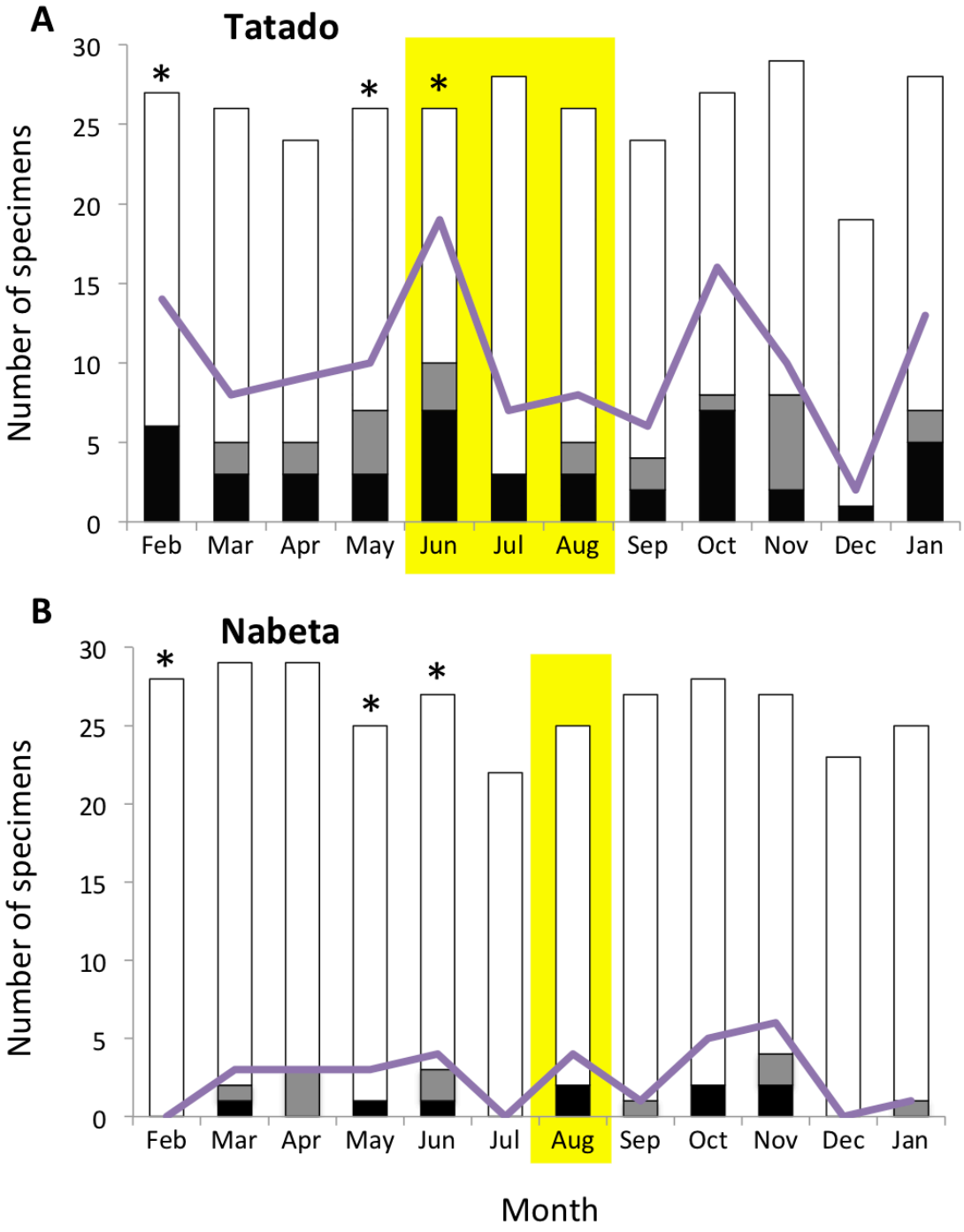
A
Tatado
Number of specimens
30
25
20
15
10
5
0
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
B
Nabeta
Number of specimens
Month

Fig. 22. The number of *L. japonica* and *S. parasitica* collected each month at (**A**) Tatado and (**B**) Nabeta. White bars show chitons without flatworms inside; grey bars show the number of chiton with a single flatworm inside; and black bars show the number of chitons that had multiple flatworms inside. The purple line shows the change in number of *S. parasitica* collected for each month. The yellow colored sections show the months when flatworm eggs were found on chitons. Asterisks show the months when significant difference was seen in the number of chitons with flatworms between the two sampling sites (Fisher's exact test, $p<0.05$).

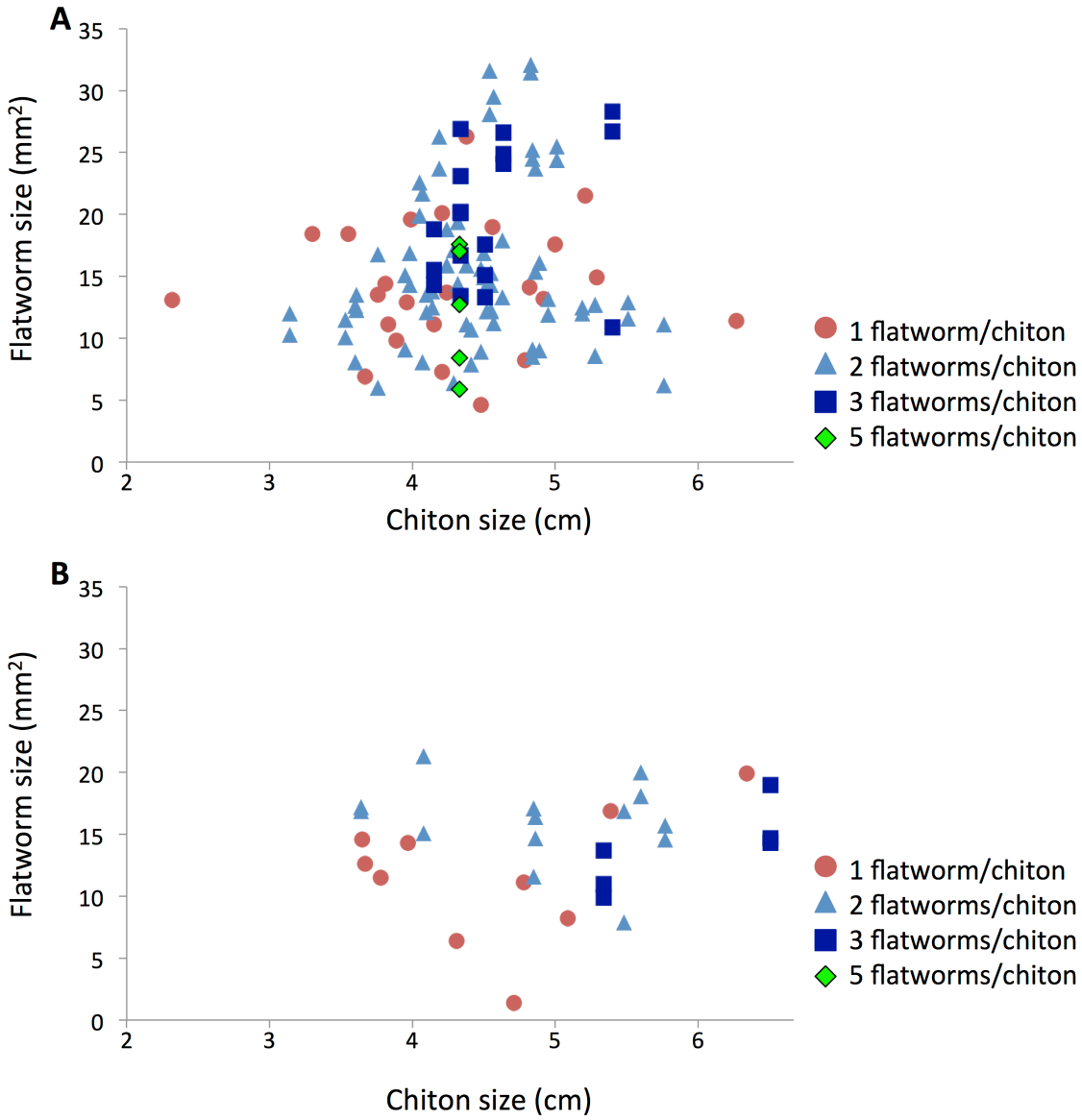

Fig. 23. The size relationship between *S. parasitica* and host chiton at (**A**) Tatado and (**B**) Nabeta. Symbols in red, light blue, dark blue, and green represents one, two, three, and five flatworms per chiton.

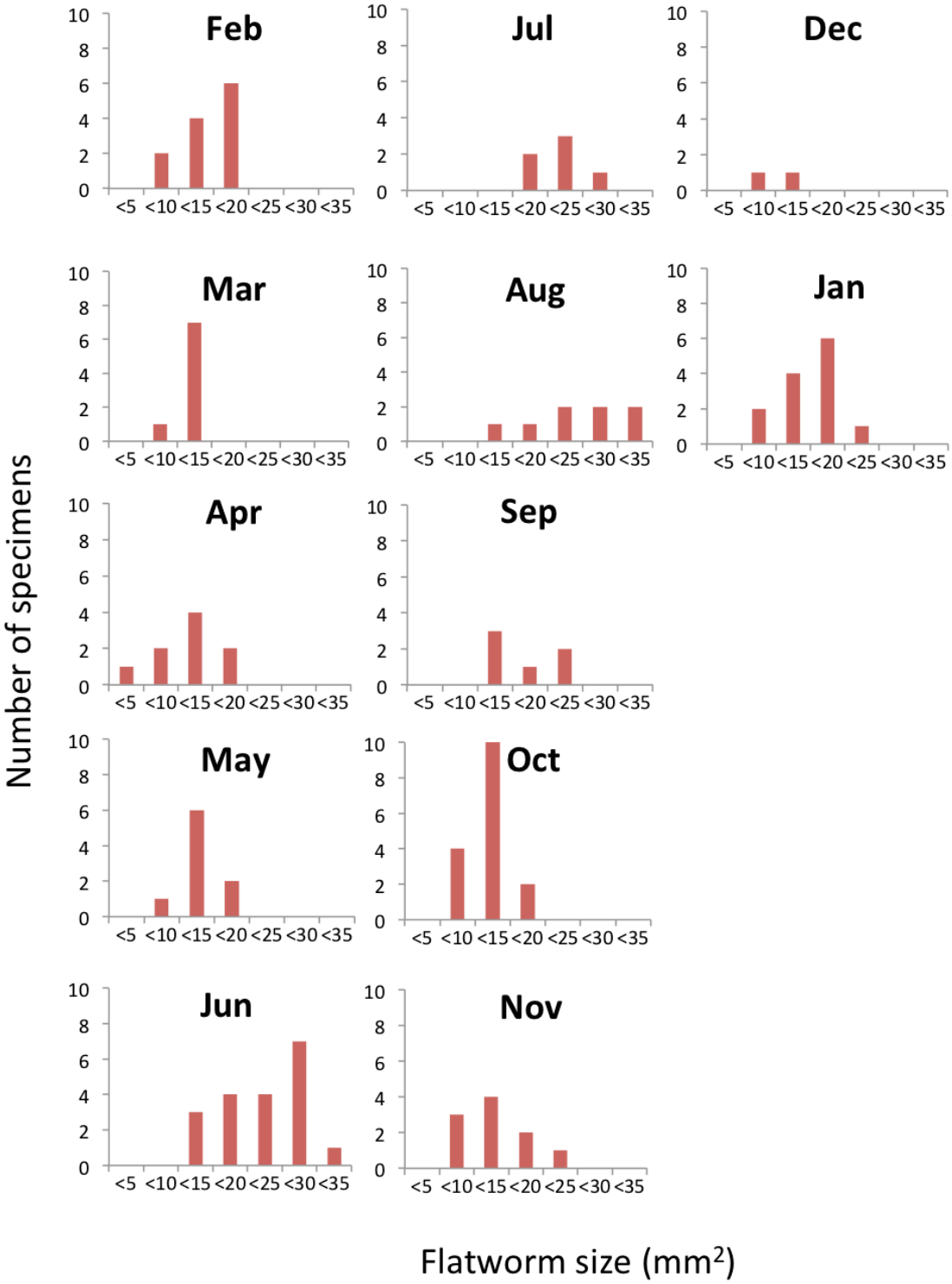

Fig. 24. Size change of *S. parasicita* over the course of the year at Tatado.

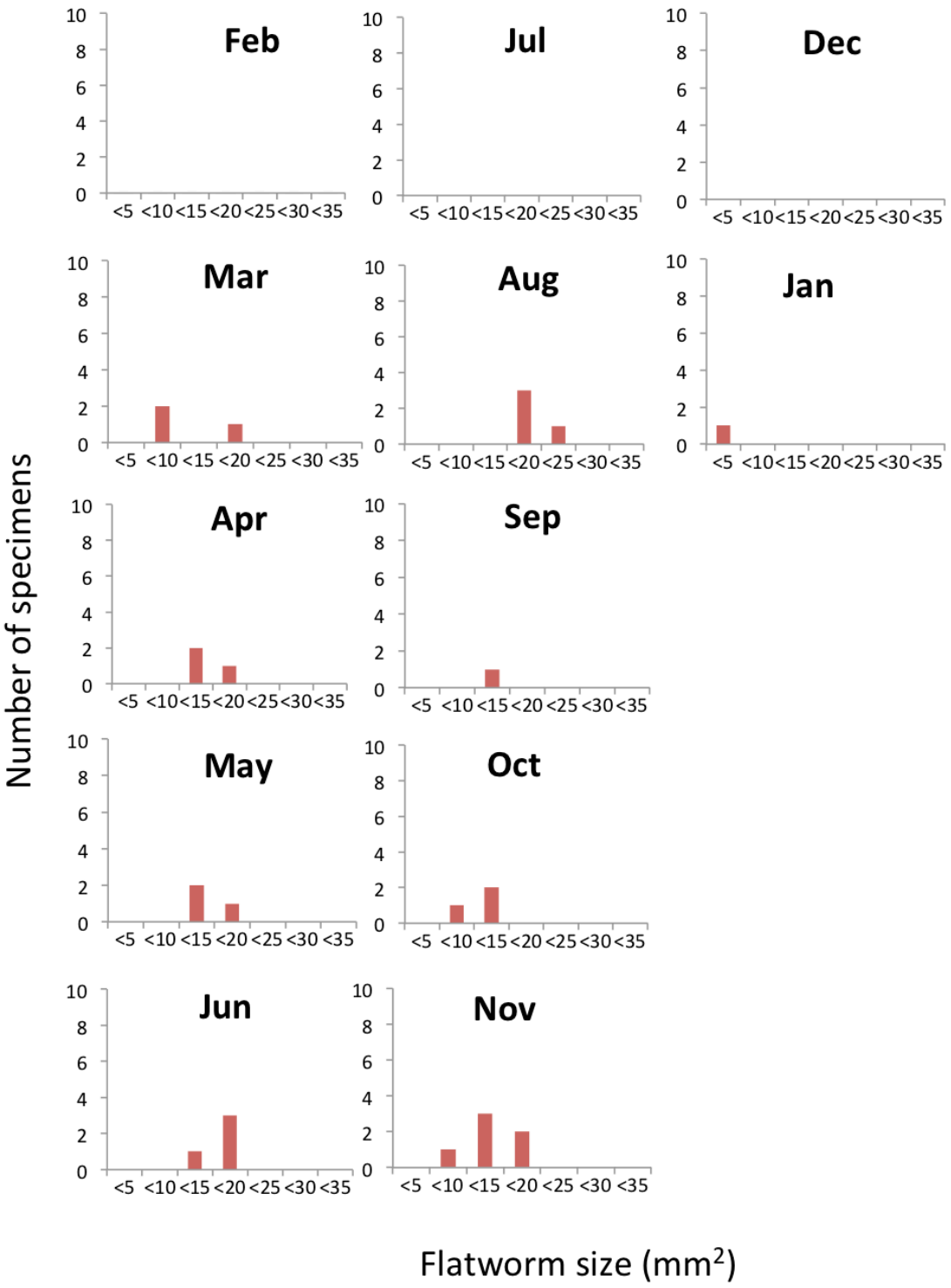

Fig. 25. Size change of *S. parasicita* over the course of the year at Nabeta.

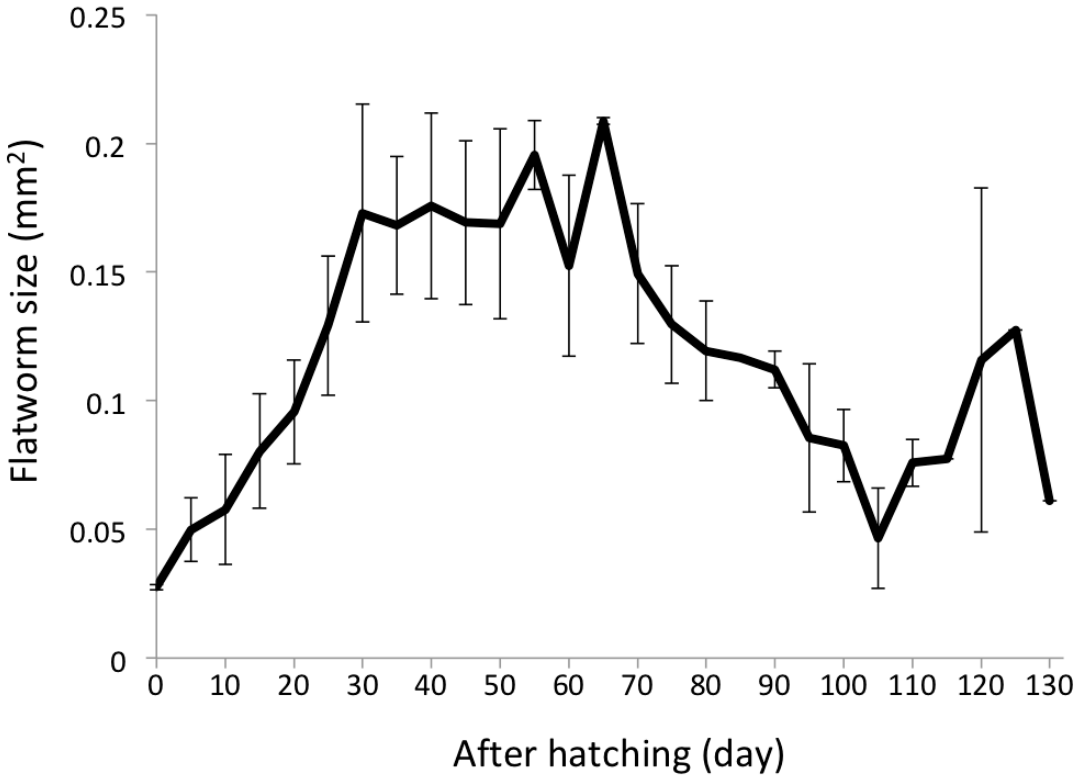

Fig. 26. Growth of *S. parasitica* larva when cultured only with brine shrimp. Bar, standard deviation.

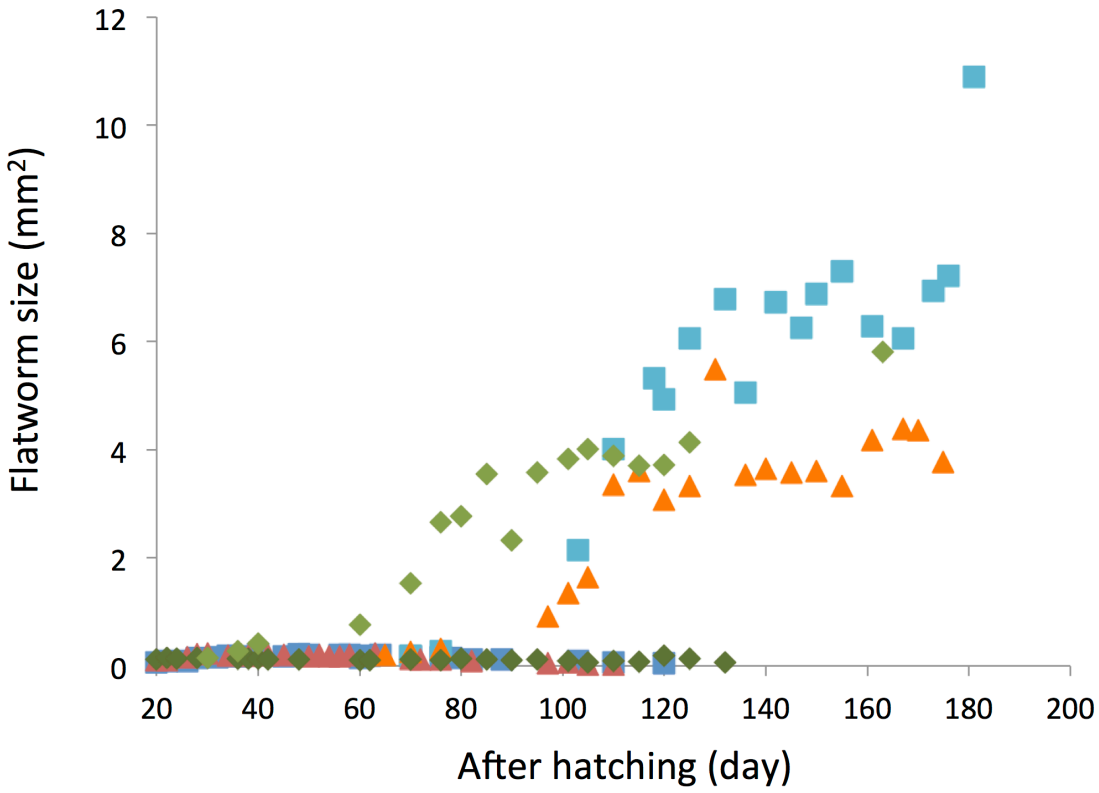

Fig. 27. Growth of *S. parasitica* safter culturing with the chiton *L. japonica*. Chitons were added to the flatworms 30 days after hatching in sample 1 (green diamond), 65 days after hatching in sample 2 (red triangle), 71 days after hatching sample 3 (blue square). A part of the flatworms were continued to be cultured only with brine shrimp (shown in red, dark blue, and dark green with the same symbols as the case of culturing with the chitons).