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Source: Zoological Science, 35(1) : 28-38

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zs170114

ZOOLOGICAL SCIENCE **35**: 28–38 (2018)

Post-Embryonic Development and Genital-Complex Formation in Three Species of Polyclad Flatworms

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Without the establishment of effective culturing systems, little can be known about the late developmental stages of polyclad flatworms. Here, we report a laboratory culturing system for three polyclad species: *Comoplana pusilla*, *Notocomplana koreana*, and *Pseudostylochus obscurus*, and we describe changes in their morphology from hatching to reproductive maturity. 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 three species could be successfully reared on brine shrimp, but only *C. pusilla* and *N. koreana* achieved reproductive maturation in such a culturing system. In *P. obscurus*, switching the food to the gastropod *Monodonta labio* induced sexual maturation.

Key words: growth process, laboratory culture, marine invertebrates, Platyhelminthes, Polycladida

INTRODUCTION

Members of the order Polycladida (polyclads) are relatively large dorsoventrally flattened worms within the class Rhabditophora of the phylum Platyhelminthes. There are approximately 800 recognized polyclad species (Martín-Durán and Egger, 2012) that are almost exclusively marine (Prudhoe, 1985) and the majority of these are free-living, though some are reported to be symbiotic with hermit crabs (Lytwyn and McDermott, 1976), gastropods (Kato, 1933) and other marine invertebrates (Prudhoe, 1985). Whereas other platyhelminthes such as planarians have been known for high regeneration capacities (Duran et al., 2016), polyclads are said to have low regenerative capacity, and reproduce only by oviposition. They are hermaphrodites, yet are not known to self-fertilize, and thus they do not reproduce asexually (Prudhoe, 1985). A planktonic larval stage is seen after hatching but it is limited to this free-living flatworms. A single species of the 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 Neodermata subgroup is the only other platyhelminth group to produce planktonic larvae (Rawlinson, 2014).

The larvae of polyclads are ciliated over the entire body, and this has been considered evidence of 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 larval stage to be an independently acquired trait (Martín-Durán and Egger, 2012). Some variation is seen with 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), although *Planocera reticulata* was reported to usually hatch as 8-lobed, pelagic Kato's larvae (Teshirogi et al., 1981; Martin-Duran and Egger, 2012). Besides 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 lacking lobes before and after hatching (Kato, 1940; Ballarin and Galleni, 1984; Prudhoe, 1985; Shinn, 1987; Smith et al., 2002). This type of hatchling have been referred to in various names in past 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).

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, the shift from a positive to negative phototaxis, and progressive branching in the intestines (Anderson, 1977; Ruppert, 1978; Shinn, 1987;

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doi:10.2108/zs170114

Smith et al., 2002). A recent paper describes the requirement of algal food for development of Götte's larva (Allen et al., 2017). However, detailed information on post-embryonic development—such as larval behavior, settlement, growth, and the process of reproductive maturation—is still lacking for many of the larval types (Ruppert, 1978; Shinn, 1987; Chen et al., 1990).

Here, we report the establishment of a culturing method from hatching to sexual maturation in the laboratory and describe the growth of three species of direct-developing polyclad flatworms: *Comoplana pusilla*, *Notocomplana koreana*, and *Pseudostylochus obscurus*. *Comoplana pusilla* is a symbiotic species that has been successfully cultured previously (Deguchi et al., 2009), and *P. obscurus* has been reported maintainable in the laboratory (Teshirogi et al., 1981). However, only little or no observation has been made on the growth process after hatching to sexual maturation for all three species. The planktonic stage and the immature benthic stage will be called larva and juvenile, respectively, to distinguish their behavioral and morphological differences as well as to avoid using multiple terms to address the same growth stage, as mentioned above.

MATERIALS AND METHODS

Collection of samples

All specimens used in this study were collected during low tide from an intertidal area at Nabeta Bay, Shimoda City, Shizuoka Prefecture, Japan.

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, 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 were placed together in the same container if they appeared morphologically similar. Four specimens of *P. obscurus* were collected in February and March 2012. *Notocomplana koreana* lacks characteristics in its external features compared to the other two species, so five *Notocomplana*-like specimens were collected in January 2011, and then cultured together.

Species identification

Some external characteristics were helpful in identification, but we made thin sections to observe the internal structure of the copulatory apparatus to confirm the species according to the traditional method of polyclad identification. Reproductively mature specimens taken from the cultured batches were used for the identification of *N. koreana* and *C. pusilla*. For *P. obscurus*, the structures of the genital organs were observed in the parent specimen, which was cultured individually at the time of oviposition. The procedures for thin sections are written in the next section. The key characteristics for identification are as follows. Regarding the species name, we employ the nomenclature by Faubel (1983), but that by Prudhoe (1985) also indicated in parentheses for reference.

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 is oval with brown colored dorsal side marked by darker lines branching around the pharynx. Ventral surface is pale without any patterns or evident adhesive organ. A pair of long tentacles are 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 organisms. 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, 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 above or below. Although the male and female genital pores were expected to open into the common genital atrium, they often seemed 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 is 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 are seen, one on each side of the brain with 150–200 eyespots in total (Fig. 2B). The appearance is quite similar to *Notocomplana humilis*, except for the bluntly pointed posterior end for *N. koreana* and uniform body width in *N. humilis*, but deciphering them from physical characteristics are difficult.

In reproductively mature individuals, the genital apparatus was seen just behind the pharynx (Fig. 2A). In the internal structure, *N. koreana* could be identified from *N. humilis* by proportionally smaller prostatic vesicle compared to the seminal vesicle, and larger male atrium (Fig. 2C) than previously observed in *N. humilis* (Hagiya, 1993). Lang's vesicle appeared relatively large than previously reported (Hagiya and Gamo, 1992; Hagiya, 1993) (Fig. 2D).

Pseudostylochus obscurus (Yeri and Kaburaki, 1918); in the oval body, this species has a characteristic small median notch at the hind end (Fig. 3A). A short pair of tentacles is present, one on either side of the brain with up to 150 eyespots around the area shortly after sexual maturation. Dorsal surface brownish green and covered with numerous brown specks. Ventral surface is 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 is muscular, nearly equivalent in size to the prostatic vesicle, which is 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).

Observations using stained thin sections

To examine the reproductive organs, serial thin sections were made according to the method of Newman and Cannon (1995). For fixation, individual specimens were laid on filter paper and placed on a frozen solution of 10% formalin seawater for at least one day. The formalin ice was melted at the surface before laying down the specimen. 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). Finally, 8-μm-thin sections were made and stained with Carrazzi's hematoxylin and eosin Y.

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 labo-

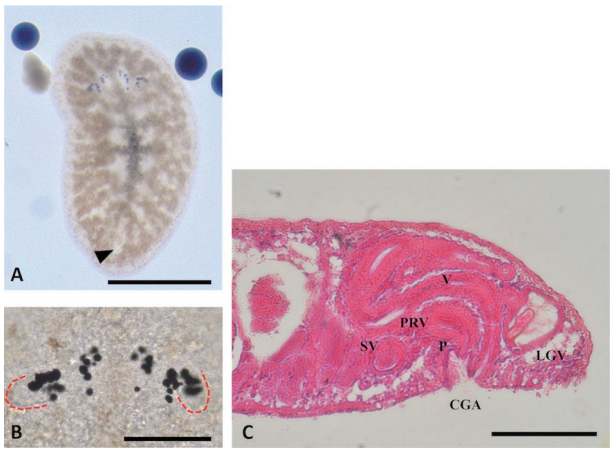

Fig. 1. Key characters for the identification of *Comoplana pusilla*. **(A)** External features; the arrow points to the area of the genital organs. **(B)** Eyespots of a sectioned sample; the anterior end is at the top; the dotted lines outline the tentacles on the head. **(C)** Section sample of the genital organs; the anterior end is on the left. 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)**.

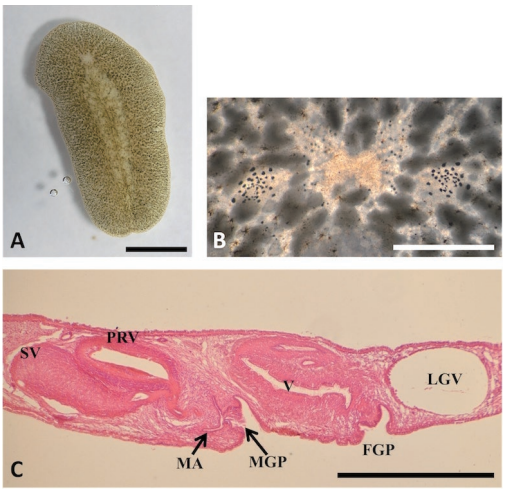

Fig. 3. Key characters for the identification of *Pseudostylochus obscurus*. **(A)** External features. **(B)** Eyespots on a mature specimen selected from the culturing system; the anterior side is at the top. **(C)** Section sample of the genital organs; the anterior side is on the left. Abbreviations: 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)**.

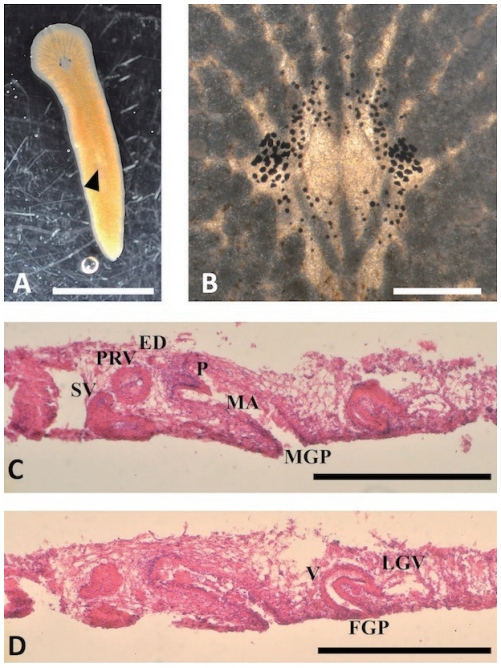

Fig. 2. Key characters for the identification of *Notocomplana koreana*. **(A)** External features; the arrow points to the genital complexes. **(B)** Eyespots when fully developed; the anterior end is at the top. **(C)**, **(D)** Section samples of the genital organs; the anterior end is on the left. 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)**.

ratory was used after filtering 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.

Comoplana pusilla specimens were kept in containers sized 3 cm in diameter and 1.5 cm in depth, in separate groups of February and July samples; they were reared at 22–24°C with no lighting except when handled for culturing treatment. Brine shrimp larvae was used for food, where the hatched, swimming larvae were collected and 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, but 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; any food remaining one to two hours after feeding was removed to avoid water deterioration, and the 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, and offered diced portions of *M. labio* 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.

Collection of embryos and culturing of larvae

Although there are some exceptions, polyclads are generally observed to 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 seem to

extend in laboratory environment (Teshirogi et al., 1981). The eggs, where up to several could be positioned in a single capsule, are laid in a mass of gelatinous sheet-like structures, called an egg plate; these are laid on the hard surfaces of the container, but sometimes seen to be laid on the underside of the water's surface in the laboratory (Lapraz et al., 2013). For all three species, usually one, in rare occasions two, eggs were seen per capsule. The egg plate was in a form of zig-zag chain closely aligned beside each other in no specific pattern, as it has been reported for *C. pusilla* and *P. obscurus*, and similar in form with *N. koreana* (Kato, 1940; Teshirogi et al., 1981). 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 maintained at 22–24°C and changed daily. The embryonic developmental process for all three species resembled that of *Notocomplana humilis* reported by Teshirogi et al. (1981) and the larvae hatched in about 2 weeks from when laid.

Hatched larvae were transferred to a new columnar glass container, sized 3.0 cm in diameter and 1.5 cm in depth. Twenty to 30 individuals were kept in each container, to avoid overcrowding. For *N. koreana* and *P. obscurus*, 20 to 30 larvae per container were obtained from a single batch hatched in one day, but several days were needed to collect the same number of *C. pusilla* larvae because of the small size of their egg plates.

Larvae of all three species were fed microwaved brine shrimp, similarly prepared to those given to *C. pusilla* collected from *M. labio*. The polyclad larvae were able to feed on the swimming brine shrimp larvae, but microwaved food was given to make the feeding behavior easier to observe. The food was given and the water was exchanged twice per day. Larvae were temporarily transferred to a separate container at times when the original container was washed. No specific lighting conditions were imposed.

Settled specimens of all three species were reared according to the same procedure, 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 among a portion 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 the polyclads were only seen to feed on items that were small enough to be fully engulfed. With every feeding, the leftover food and excrement of the flatworms caused deterioration of the water quality, therefore we 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 to those used for *N. koreana*), while the remaining portions of the replicates was still cultured with brine shrimp, for comparison.

Observations of polyclad morphology

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; Olympus Co., Tokyo, Japan). When recording the images, we mainly targeted feeding times as well as occasions when the animals were resting at the surface of the water, since the otherwise actively swimming larvae tended to remain still at these moments. Selected larvae were also gently compressed on a glass slide with a coverslip and observed under an inverted microscope (Olympus CKX41) 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 animals' external features were observed with or without the aid of a stereoscopic microscope, depending on the individual specimen's size. Animals that did not fit within the microscope's viewing field were either anesthetized with menthol, or the seawater on the slide was reduced to immobilize the animal as much as possible, so that it could be photographed in a petri dish or on a slide glass with 1-mm grid paper placed underneath. Anesthetic was used only for *N. koreana* and *P. obscurus*; all *C. pusilla* 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 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 gain 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 and excess mucus was removed with tweezers, hence 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. Some *P. obscurus* specimens outgrew the size of the glass slide, so they were instead placed on a clear rectangular plastic lid (11.7 × 7.8 cm), and, 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 was 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.

RESULTS

Growth from hatching to settlement

Larvae of all three species swam actively in the water column and gathered near the brightened side of the container, suggesting a positive photo-tactic 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 sometimes raised, and the inner contents of the brine shrimp was sucked out with the muscular pharynx. After taking in enough food, the larva would begin to swim freely again. In some instances, an individual would puff up its body into an ovoid shape (much like a balloon) and the body color changed according to the gut contents (see Supplementary File S1 online). 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 about 15 days. Approximately 2 to 3 weeks after hatching, the time they spent gliding along the surface of the container gradually lengthened until the animals settled and became completely benthic. No change in culturing method was taken nor specific cues

were provided to induce settlement.

Comoplana pusilla hatched out as lobe-less, direct-developing larvae with a total of four eyespots (Fig. 4A, Table 1). During the planktonic period, small black spots appeared on the hind end at about day 7 post-hatching (Fig. 4B), but these eventually disappeared after settlement. The species occasionally clung lightly to the sides of the container in the free-swimming period, which was not commonly seen in the other two species, but these larvae 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 original four eyespots 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. 4C). Out of 212 cultured specimens, 70 survived until settlement (33.02%).

Notocomplana koreana hatched out as lobe-less larvae, with a total of four eyespots (Fig. 5A, Table 2), similar to *C. pusilla*. They were distinguished by a cross-like body shape from one week after hatching until settlement (Fig. 5B). This body form was seen for all the specimens and only when the animal was swimming. This shape could also be coaxed to change into an oval shape by mechanical stimuli, such as compression by a coverslip. Once they began to assume the cross-like shape, two new eyespots appeared behind the

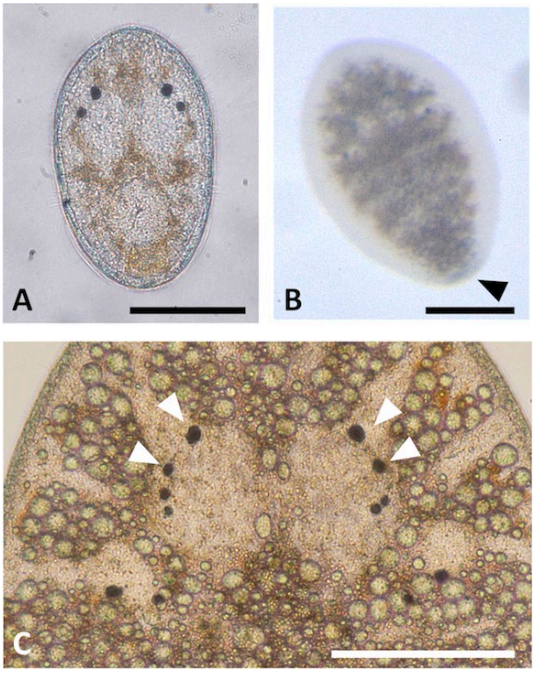

Fig. 4. **(A)** Larva of *Comoplana pusilla*. **(B)** 14 days post-hatching; the arrowhead marks the black spots at the posterior end. **(C)** Eyespots 14 days post-hatching; the original four eyespots are marked by arrowheads. Scale = 100 µm **(A)**, 200 µm **(B, C)**.

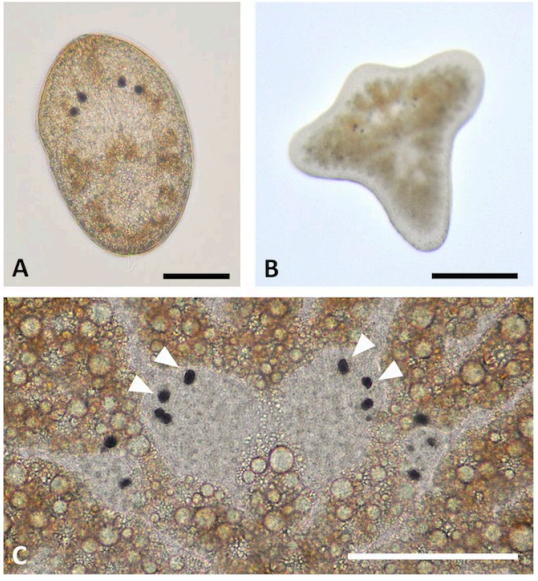

Fig. 5. **(A)** Larva of *Notocomplana koreana*. **(B)** 13 days post-hatching, showing the characteristic cross-like body shape. **(C)** Eyespots 14 days post-hatching; the original four eyespots are marked by arrowheads; the anterior side is at the top. Scale = 100 µm **(A)**, 500 µm **(B)**, 200 µm **(C)**.

Table 1. Body size growth and increase in the number of eyespots of *C. pusilla*.

Days after hatching	Number of replicates	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. Body size growth and increase in the number of eyespots of *N. koreana.*

Days after hatching	Number of replicates	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.

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. 5C, Table 2). The paired eyespots became the tentacular eyespots, while all the others grouped as the cerebral eyespots, as seen in *C. pusilla* (Fig. 4C). 82 specimens out of 106 cultured survived until settlement (77.36%).

Pseudostylochus obscurus hatched out as lobe-less larvae, with a total of four eyespots (Fig. 6A, 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 similarly positioned as in the other two species, but the second set of cerebral eyespots and the tentacular eyespots appeared almost simultaneously. Also 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. 6B; see Supplementary File S2 online). Similar to the larvae of *N. koreana*, this triangular body shape would change 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, creating as many as 14 eyespots in total (Fig. 6C, Table 3). 155 specimens out of 184 cultured survived until settlement (84.24%).

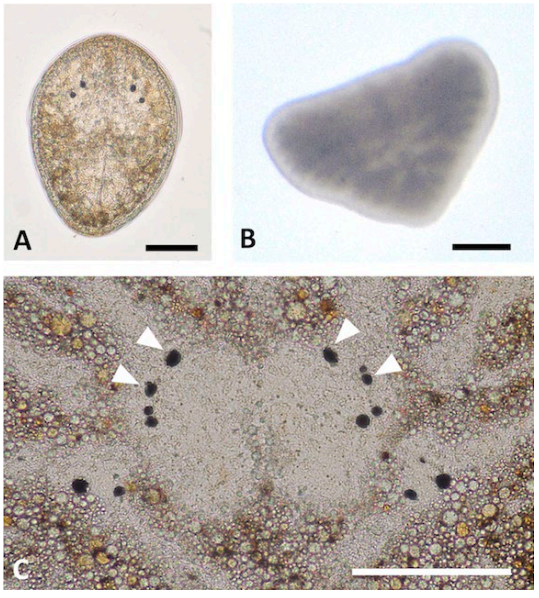

Fig. 6. **(A)** Larva of *Pseudostylochus obscurus*. **(B)** 13 days post-hatching, showing the characteristic triangular body shape. **(C)** Eyespots 14 days post-hatching; the original four eyespots are marked by arrowheads; the anterior side is at the top. Scale = 100 μm **(A)**, 200 μm **(B, C)**.

Growth from settlement to reproductive maturity

Although the larvae were elongated spheroid in shape in all three species, their juveniles gradually became dorsoventrally flattened in proportion, and the pharynx changed from tubular to more plicated. After settlement, the eyespots appeared at different rates and in different positions, depending on the species (Tables 1–4), but the patterns were 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 post-settlement, 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. 7A). Process of maturation was evident at about day 35

Table 3. Body size growth and increase in the number of eyespots of *P. obscurus* with brine shrimp culture.

Days after hatching	Number of replicates	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. Body size growth and increase in the number of eyespots of *P. obscurus* with *M. labio* culture.

Days after feed type change	Number of replicates	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.

post-hatching with the formation of a pale area near the tip of the tail, where the genital complexes began to develop (Fig. 7A, B). Within the following 10 days, the distinctive clover-shaped Lang's vesicle could be observed. And within another two weeks, the vagina was evident and the genital pore (a common genital atrium in this species) opened (Fig. 7C). Finally, eggs accumulated in the uterine canal at about 20–30 days after onset of the genital-complex formation (Fig. 7D, E). Thus, this species is estimated to reach reproductive maturity in about 70 days post hatching, but 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 *N. 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. 8A, 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. 8C). The gonopores became more distinct during the following 10 days, especially the male gonopore once it attained a slightly jagged outline. Also at around 10–15 days after the formation of the gonopores, the structure of the 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. 8D, E). It is

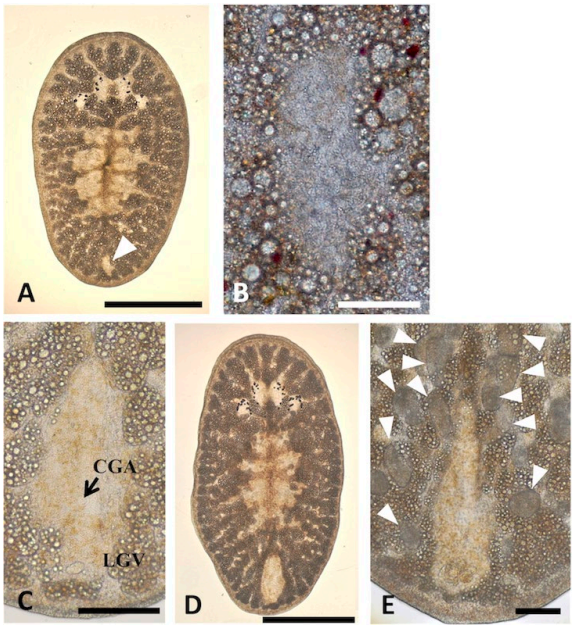

Fig. 7. **(A)** Genital organs of *C. pusilla* as they started to develop approximately 35 days after hatching. The specimen was compressed for observation. The area of the genital organs is marked by an arrowhead. **(B)** Enlarged image of the area of the genital organs, observed from the ventral side; 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)**.

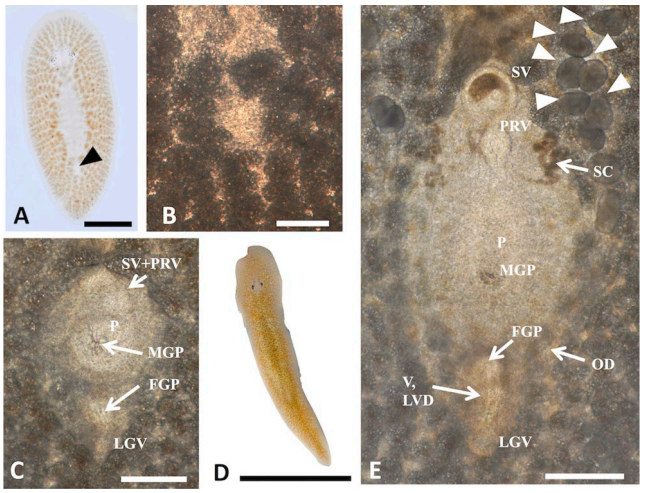

Fig. 8. **(A)** Specimen of *N. koreana* 50 days after hatching. Formation of the genital organs (arrowhead) are visible from the dorsal side at the posterior end of the pharynx. **(B)** Enlarged image of the area where genital organs are starting to develop, at 50 days post-hatching; the anterior side 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; MA, male atrium; 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)**.

predicted that it takes 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. An individual specimen's size and 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. 9A). 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. 9B). 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, some specimens were observed to shake and twitch, even disintegrate, for unknown causes after the food was changed, 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. 10A).

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 anterior to the tail notch, which was visible only from the ventral side (Fig. 10B). 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 clearly recognizable. During another 5 days, the structures of other female organs, such as the oviducts, Lang's vesicle and its duct, became evident, the other structures more defined (Fig. 10C), which was followed by sperm filling up the seminal canal and seminal vesicle. Finally, stored eggs were apparent in the oviducts after the next 5–10 days (Fig. 10D, 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%).

DISCUSSION

We successfully cultured three species of polyclad flatworms, including both symbiotic and non-symbiotic species, in the laboratory. To our knowledge, we present the first detailed description of the growth from hatching to reproductive maturity for the lobeless larval type. We found several shared characteristics among the three species in the planktonic larval stage, such as feeding preference, duration of the planktonic period, the timing 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 been the result of keeping the larvae in conditions that did not meet the food requirements of that larval type. With a well-developed pharynx at hatching, the direct-development larval type is perhaps carnivorous throughout its lifetime. However, we examined only three of numerous species having this type of development, and further study is needed to confirm these patterns as universal characteristics. For example, we induced larval settlement in these three species of polyclads reared in the laboratory simply by feeding with brine shrimp. Although it is likely that settlement occurs autonomously after individuals attain a certain size, it is possible that additional environmental signals can alter the place and timing of settlement, especially in symbiotic species like *C. pusilla*.

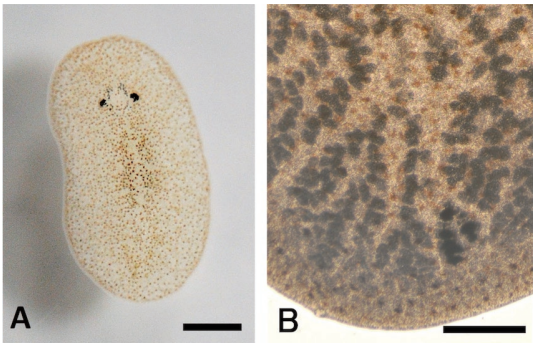

Fig. 9. **(A)** A specimen of *P. obscurus* 140 days after hatching, and which was continuously fed with brine shrimp. **(B)** Area behind the pharynx, viewed from the ventral side; no indication of formation of the genital organs can be seen. Scale = 1 mm **(A)**, 500 μm **(B)**.

Reproductive maturation seems to occur autonomously in *C. pusilla* and *N. koreana*, with possible signals being an adequate body size or length of time after settlement, since the culturing conditions were not dramatically changed for these species in the present study. In contrast, *P. obscurus* seems to require a kind of trigger for reproductive maturation, possibly related to nutrition acquired from the gastropod but not from the brine shrimp, in addition to other probable conditions, such as body size and time after settlement. It seems possible that *M. labio* could be replaced with another gastropod species found in the same area (e.g., *Nerita albicilla* Linnaeus, 1758, and limpets) as food, which may improve the survival rate and condition of cultured *P. obscurus*.

Although the conditions needed for reproductive maturation may differ among these species, all three species examined in this study took about 30 days to achieve reproductive maturity. Interestingly, the male organs seemed to develop slightly sooner than the

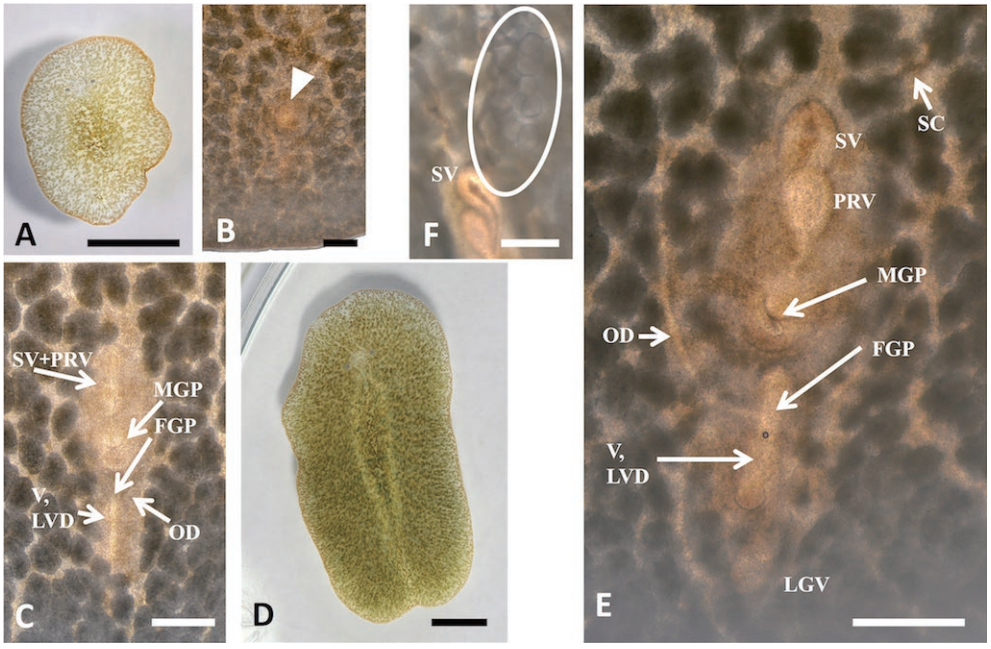

Fig. 10. **(A)** Specimen of *P. obscurus* 20 days after changing the food to the gastropod *Monodonta labio*. **(B)** Area in front of the median tail notch, 20 days after the food was changed, viewed from the ventral side, showing the genital organs beginning to form (arrowhead); the anterior end is 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; MA, male atrium; 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)**.

female genital complex. This assumption agrees with past field observations wherein captured specimens displayed fully developed male organs but not yet mature female structures (Prudhoe, 1985). Additional studies of internal morphology will be useful for confirmation and greater understanding of the maturation process.

The successful culture of polyclad species is beneficial not only for observing the different growth stages, but also for acquiring large numbers of a confirmed species for experimentation. This is due to the fact that polyclad identification is based heavily on the structure of the genital organs, and thus immature specimens and species without distinctive external characters could not be positively identified, especially in field collected samples. The advantage of culturing *P. obscurus*, in particular compared to the other two species, is that the eggs and larvae can be collected in larger quantities, owing to their relatively large size, and that the maturation timing can be intentionally manipulated. Being in control of the maturation timing to some extent is perhaps a new idea for experimentation with polyclads, since they do not reproduce asexually and artificial insemination is not practical (Teshigori and Ishida, 1988; Deguchi et al., 2009).

The position and number of eyespots are considered one of the useful characters for species identification, although we 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 individual *C. pusilla*, whereas it continued to increase after reproductive maturity in *N. koreana* and *P. obscurus*. Therefore, the reported number of eyespots could be an underestimate depending on the species and the extent of the specimens' growth. 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).

Appearance of eyespots with the onset of growth may possibly be an important factor in the animal's behavior. Polyclad larvae are reported to exhibit strong positive phototaxis but may lose this ability at settlement (Prudhoe, 1985; Smith et al., 2002). With a newly established culturing system, sequential study in the laboratory should now be possible, allowing observations on behavioral-change mechanisms after hatching, around the time of settlement, and after reaching reproductive maturity. This could benefit ecological studies in turn, as information on free-living platyhelminths is largely lacking (Rieger, 1998).

From the present study, we have observed that the hatched individuals are planktonic and have morphological characteristic that are distinct from the benthic specimens (such as number of eyespots, coloration, and body shape). The terms 'direct development' or 'juvenile', which have been used to name the lobe-less type of hatchlings, 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 go under metamorphosis. However, if such planktonic period was seen in the wild as we have observed in this study, it is suggested that the direct developers might have an important growth period similar to the larvae 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. We hope that the culturing method provided here encourages further study in the 'direct developing' polyclads and their planktonic periods along with worms providing lobed larval forms, which have been under interest as the unique species to have larval period in the polyclads.

ACKNOWLEDGMENTS

We dedicate this paper to one of the authors, Dr Yasunori Saito, who passed away in December 2015; he offered us generous support and shared his deep interest in marine invertebrates during his many years at the Shimoda Marine Research Center (SMRC), University of Tsukuba. We are also grateful to Dr Morio Hagiya, who passed away in May 2015, for his helpful comments on the taxonomy and identification of polyclads. Thanks are also due to the teaching and technical staff as well as the graduate students at SMRC, for their help with the collection and culturing of animals.

COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

NM designed the study, collected the data, made analysis, and prepared the manuscript. KI and YS contributed in analysis, interpretation of data, and edited the manuscript.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available online (URL: http://www.bioone.org/doi/suppl/10.2108/zs170114.

Supplementary Movie S1. Feeding behavior of *Pseudostylochus obscurus* (24 to 48 hours after hatching) on brine shrimp.

Supplementary Movie S2. A batch of triangular shaped pelagic larvae of *Pseudostylochus obscurus*.

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(Received July 15, 2017 / Accepted October 24, 2017)