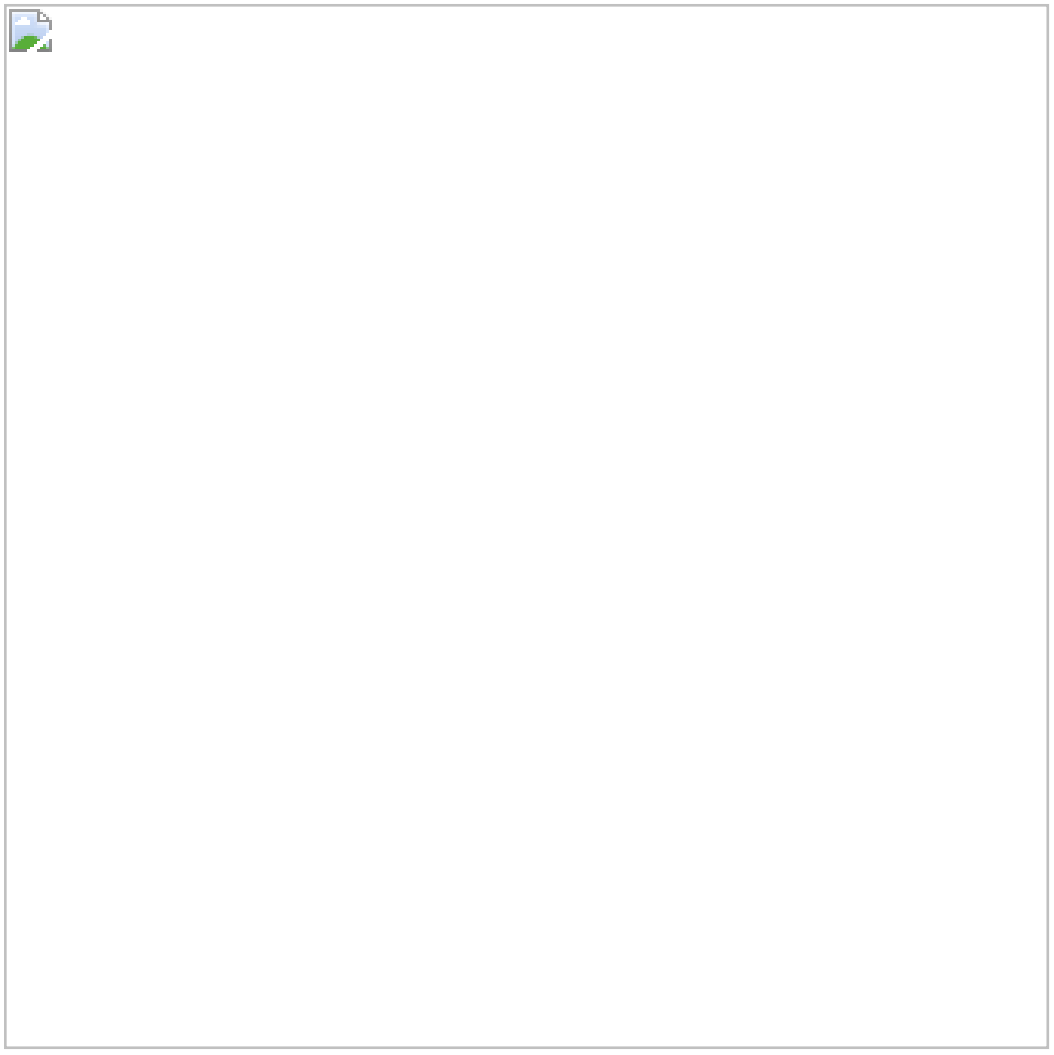

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Life Cycle of the Japanese Green Syllid, *Megasyllis nipponica* (Annelida: Syllidae): Field Collection and Establishment of Rearing System

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Some polychaete species in the family Syllidae exhibit distinctive life cycles, in which a posterior part of the body of an individual detaches as a reproductive individual called a "stolon". This type of reproductive mode is known as stolonization or schizogamy. Although a number of observations have been reported, and techniques using molecular markers have recently been applied to characterize this phenomenon, little is known about the developmental and physiological mechanisms underlying stolonization. In the present study, *Megasyllis nipponica*, a common syllid species distributed throughout Japan, is proposed as a model to reveal the developmental and physiological mechanism of stolonization, and the rearing system to maintain it in laboratory conditions is described. This species was repeatedly sampled around Hokkaido, where more dense populations were found from August to October. The animals were maintained in the laboratory under stable long-day condition (20°C, 16L:8D), and fed mainly with spinach powder. Stolonization processes, spawning, embryonic and postembryonic development were observed and documented, and the required period of time for each developmental stage was recorded. The complete generation time was around two months under the rearing condition. The information provided is valuable to maintain this and other syllid species in the laboratory, and hence contributes to the establishment of new evolutionary and developmental research lines in this group of annelids.

Key words: stolonization, schizogamy, reproductive mode change, spawning, annelid, regeneration, development

INTRODUCTION

Syllids are polychaetes that belong to the family Syllidae, a member of Phyllodocida (Annelida). There are about 700 described species in the family, and they show distinctive life cycles with diverse reproductive modes (Franke, 1999; Aguado et al., 2015a). Most of the species are benthic and produce epitokes, which swim and spawn in the reproductive season (epitoky) (Malaquin, 1893). Epitoky is classified into two major types: epigamy and schizogamy. In epigamy, the entire body of a benthic individual becomes an epitoke, by altering its morphology and behavior when it becomes reproductively mature (Daly, 1975; Garwood, 1991; Franke, 1999). In contrast, in schizogamy, only posterior segments of an individual undergo transformation into a reproductive individual (called a "stolon") filled with eggs or sperm. The process producing stolons is also called "stolonization". During stolonization, gonads (testes or ovaries) develop in the posterior segments of a parental benthic individual. Then, a stolon head is newly formed with functional eyes and anterior appendages and the posterior segments produce swimming chaetae, suitable for their spawning behavior. Stolonization, or schizogamy, is further classified into two types, i.e., scissiparity, in which a single stolon develops in a reproductive cycle, and gemmiparity, in which multiple stolons develop at the same time (Franke, 1999; Aguado et al., 2015a).

As described above, stolonization is a postembryonic process during which sexual units are produced from an

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original individual, also known as a "stock". Stolonization includes the process of regeneration that occurs after stolonization or even before, when the stolon is still linked to the stock (Ribeiro et al., 2018). Recent molecular phylogenetic studies suggested an evolutionary scenario in which stolonization has evolved twice within Syllidae (Aguado and Bleidorn, 2010; Aguado and San Martin, 2009; Aguado et al., 2012, 2015a). However, little is known about the developmental and physiological mechanisms underlying this intriguing biological phenomenon.

To clarify the developmental and physiological mechanisms of stolonization, a rearing system for a model syllid species is required. In this sense, in the syllid species *Syllis prolifera*, ecological and developmental features have been studied extensively (Franke, 1999; Massa Gallucci and Gambi, 2014). Recently, *Typosyllis anotoni* has been proposed as a model species (Aguado et al., 2015b) and used to investigate the regenerative abilities (Weidhase et al., 2016, 2017) and the gene expression of stem cell markers during reproduction (Ponz et al., 2018). Furthermore, an RNA-seq study was recently carried out in *Syllis magdalena*, suggesting that several neurohormones may be involved in the stolon formation (Álvarez-Campos et al., 2018).

As noted above, stolonization has recently been studied in three different species of Syllidae. However, the developmental mechanisms underlying this phenomenon is still far from fully understood. In the present report, we describe a study of the rearing conditions of *Megasyllis nipponica* (Imajima, 1966), an abundant species in Japan. The information provided about this fourth species available for developmental experimental approaches is valuable for comparative studies and researches into the diversity and evolution of the fascinating reproductive modes and regeneration capacities in Annelida.

Megasyllis nipponica is distributed all around Japan from Hokkaido to Shikoku, and is very common in intertidal and subtidal zones (Imajima, 1996). This species reproduces by stolonization, producing stolons one by one (i.e., scissiparity) (Fig. 1). We explored the habitat conditions and seasonal dynamics (i.e., through the number of collected individuals) of this species. Furthermore, we successfully established a rearing method to maintain it under laboratory conditions. Finally, the postembryonic processes and the time course, including the generation time, were also recorded to establish *Megasyllis nipponica* as an experimental model syllid species for future studies.

MATERIALS AND METHODS

Field sampling

Individuals of *M. nipponica* were collected on the intertidal and subtidal zone at a beach in Oshoro Bay, in front of the Oshoro Marine Station, Hokkaido University, which is located in Otaru, Hokkaido (Fig. 2A, B). Especially, at low tide, kelp roots or Rhodophyta (red algae) were collected from shallow rocky reefs (Fig. 2C, D). These algae were washed in plastic trays, where the focal species of syllids was released and collected (Fig. 2E). The species was identified according to previous studies (e.g., San Martín et al., 2014). Collected individuals were kept in small plastic containers (84 × 57 × 44 mm) and brought to the laboratory (Fig. 2F). The number of collected individuals was counted at every sampling date, and the average numbers of sampled syllid individuals were normalized by the number of collectors who participated in each sampling.

Laboratory rearing

In the laboratory, 10–20 individuals were put in the small containers with filtered sea water (FSW) (c.a. 50 ml/case). Six small containers were further put into a larger container with moistened paper towels to prevent desiccation. According to the previous studies of other species such as *Syllis prolifera* (Franke, 1980, 1983; Massa Gallucci and Gambi, 2014), the syllids were maintained under constant rearing conditions (21 ± 1°C, 16L8D) without aeration in an incubator (Fig. 2G). In *S. prolifera*, synchronous stolonization is induced by moonlight (Franke, 1985; Massa Gallucci and Gambi, 2014); we therefore exposed *M. nipponica* individuals to moonlight for one week per month. As food for adult individuals, purchased spinach powder (Mikasa Sangyo, Tokyo) was dissolved in FSW and provided to the syllid cultures (ca. 10 mg/case) every 2 days (Fig. 2H). Fecal pellets were removed with seawater from containers, using a plastic pipette, and clean FSW was added when water level became low. Additionally, fish diet (Hikari Premium Megabite Green, Kyorin Co., Ltd., Himeji), containing fish and shrimp meats, was also supplied to adult specimens (c.a. 5–10 mg/case; Fig. 2I; See also Results). For larvae and juveniles, marine algal plankton, *Nannochloropsis* sp. (ca. 200 µl of Nannochloropsis Green Water, Institute of Environmental Biotechnology, Inc., Yaizu) was mainly supplied (~200 µl/case), since it has been previously considered an optimal food for invertebrate larvae because of its small size and high nutrient content (Antia and Cheng, 1982).

Observations of postembryonic development

After the start of rearing, the number of surviving individuals and the degree of stolonization were recorded daily. When the detachment of stolons occurred, they were transferred to another plastic case. Immediately after encountering stolons of the opposed sex, they started swimming and spawning. Observations of eggs

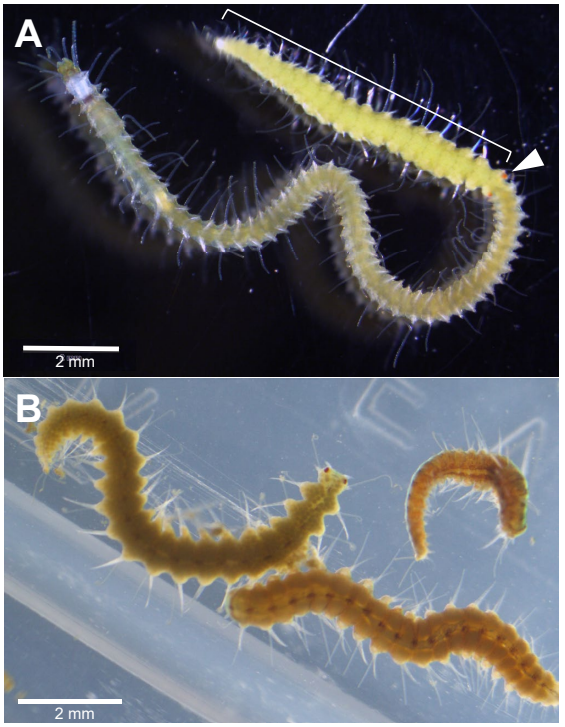

Fig. 1. The focal species *Megasyllis nipponica*. **(A)** Mature adult individual with a well-developed stolon. The stolon is indicated by a bracket, and the eyes of the stolon are indicated by an arrowhead. **(B)** A male and a female stolon, which can be distinguished by their body color.

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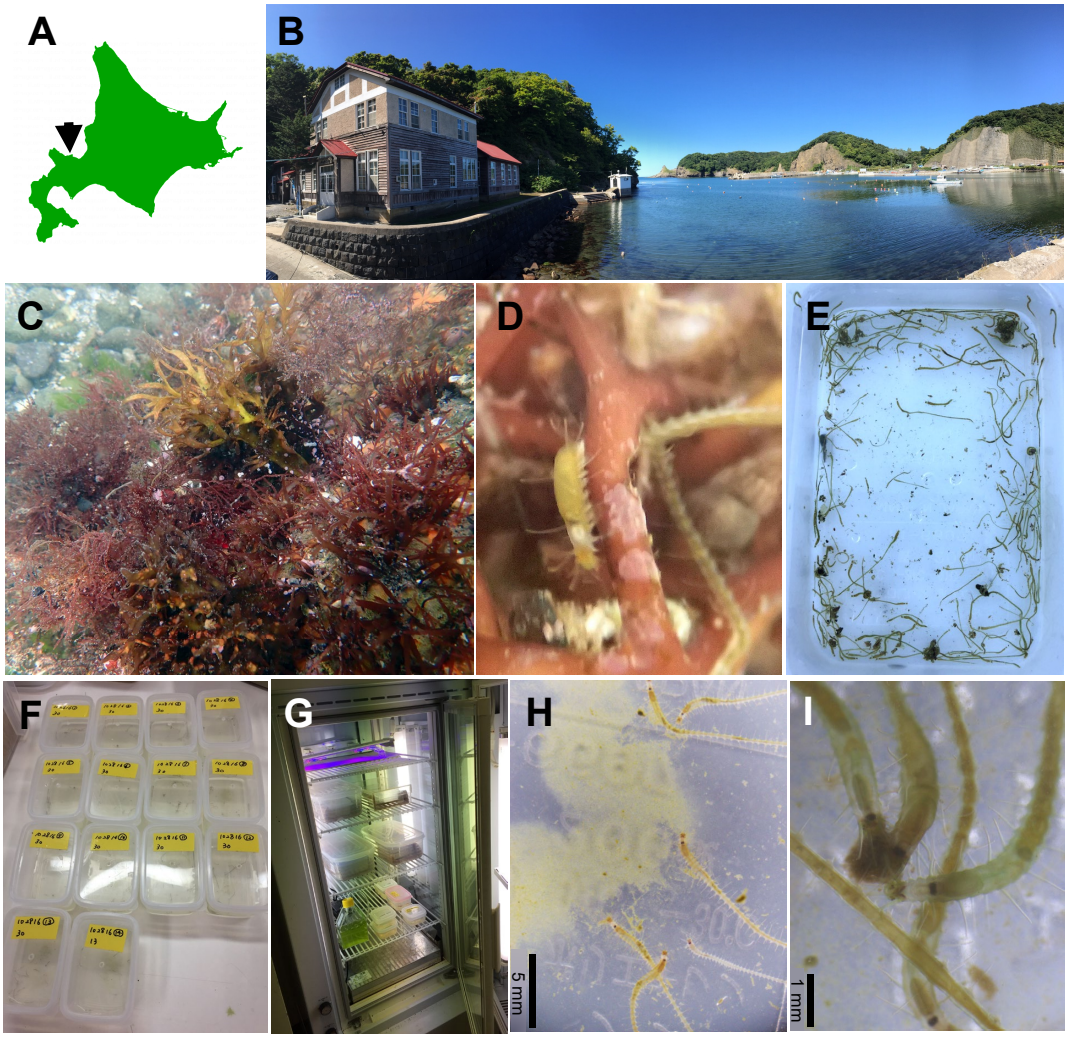

Fig. 2. Field sampling and rearing system of *M. nipponica*. **(A)** Location of Oshoro Marine Station, which belongs to Hokkaido University. **(B)** Oshoro Marine Station and field sampling site. **(C)** Individuals of *M. nipponica* inhabit in Rhodophyta algae. **(D)** The focal species can easily be distinguished by body color and white and black bands at its phalynx. **(E)** Collected individuals were taken back to the laboratory in plastic containers. In the laboratory, 10–20 individuals were kept in each plastic case **(F)**, and put in an incubator under constant conditions (21 ± 1°C, 16L:8D) **(G)**. Spinach powder **(H)** and fish food **(I)** were supplied as food.

and sperms were carried out. Spawned eggs were maintained, the process of postembryonic development was observed and dates of developmental events were recorded.

Postembryonic stages were classified as follows; EG (egg stage from spawning to hatching), MT (metatrochophore stage from hatchling to juvenile with visible proventricle), JV (juvenile stage, when the proventricle is visible but before the appearance of a black band on the pharynx), BS (black-spotted stage, after the appearance of the black band). The morphology of BS individuals was almost the same as that of adults, so they were treated as adults. The periods required for the different postembryonic stages were recorded daily. It was difficult to track each individual, hence the dates of the first appearance of focal stages in a culture of the syllids were recorded.

RESULTS AND DISCUSSION

Species identification

The selected specimens fully agreed with the available descriptions of *M. nipponica* (Imajima, 1966, 1996; San Martín et al., 2014). Firstly, the species were easily identified as *Megasyllis* because of the presence of segments with several annuli and a strong alternation in cirri length (Fig. 1A). Then, the animals were subsequently identified as *M. nipponica* because of the following features: (1) antennae and dorsal cirri distinctly annulated (Fig. 3A); (2) posterior compound chaetae with distinct subdistal serration on shafts without long filiform subdistal spines (Fig. 3B); (3) tri-annulated segments (Fig. 3C); (4) a pharynx with 10 soft papillae; (5) live specimens with a green body color (sometimes yellowish brown) and a black spot at the posterior part of whitish pharynx (Fig. 1A).

Habitat and seasonality

The specimens were sampled at Oshoro Bay on 13 and 28 October and 2 December 2 2016, 22 February, 28 March, 11 April, 9 May, 26–27 June, and 16–17 September 2017, and 9 June, 14 July, 19 August, 22 September, and 10 October 2018 (Supplementary Table S1, Fig. 4). *Megasyllis nipponica* appears to be one of the most common syllid spe-

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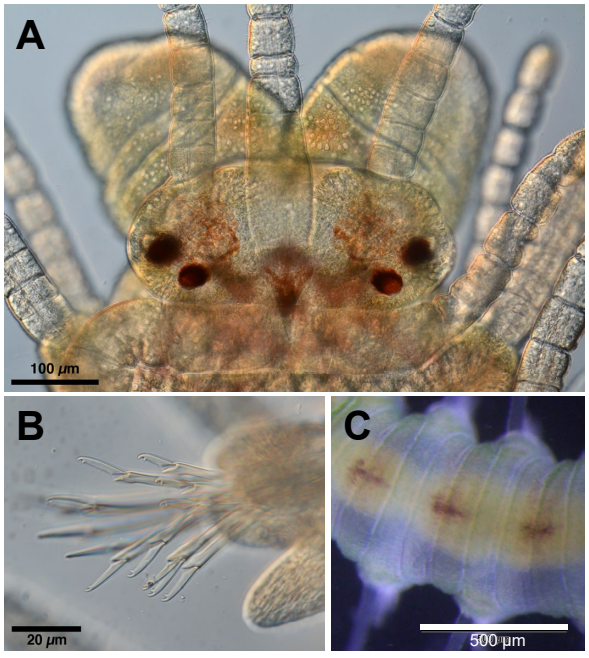

Fig. 3. Key characters for the identification of *M. nipponica*. **(A)** Head morphologies show the characteristics of *Megasyllis*, namely, eyes, prostomium, palps and tentacles. **(B)** Posterior compound chaetae show distinctly subdistal serration on shafts. **(C)** Body segments are tri-annulated.

Table 1. Periods required for the postembryonic development in *Megasyllis nipponica*. EG: egg, MT: metatrochophore, JV: juvenile, BS: black-spotted individual; see text for details.

	days (mean ± s.e.)	n
EG ~ MT	2.67 ± 1.25	87
MT ~ JV	2.69 ± 2.07	71
JV ~ BS	23.26 ± 11.36	38
EG ~ BS	26.49 ± 15.02	45

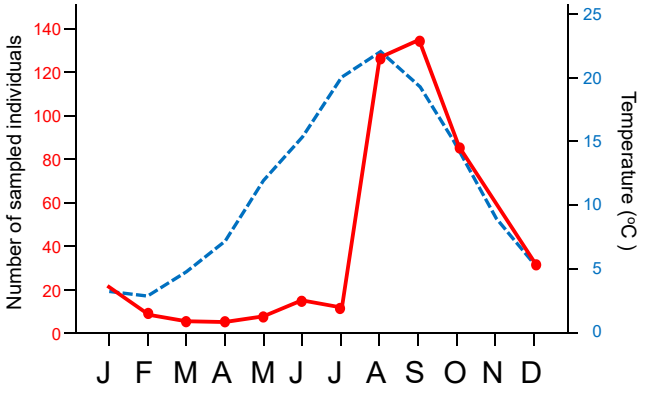

Fig. 4. Seasonal transitions of the number of *M. nipponica* individuals (red line) that were collected at Oshoro Bay. The number of sampled individuals was normalized by by the number of collectors who participated in each sampling (See Materials and Methods for details). The average temperature transition of seawater during three years of collection at Oshoro Bay (from 2016 to 2018) is also shown (dotted blue line). The sampling results suggest that the seasonal dynamics of the syllid population largely depends on water temperature.

cies at the sampling site. They dwell among kelp roots or Rhodophyta algae, by coiling their bodies onto them. As the distribution of the focal species is known to range from Hokkaido to Shikoku Island (Imajima, 1996), the authors sampled the same species also from Muroran, Tateyama, Misaki, and Manazuru (data not shown).

The number of collected individuals clearly showed a seasonal trend, although the survey was not precisely quantitative. Namely, many individuals were found from August to October, while few individuals were collected from December to July. Therefore, the best sampling season for *M. nipponica* at Oshoro Bay was from August to October (Fig. 4). A previous survey on the annual transition of water temperature at the area reveals that the temperature range from August to October is approximately 16–22°C (Nakata et al., 2001). The largest number of sampled individuals was in September, probably as a response to the temperature increase in August.

Feeding habits

Although very little is known about syllid feeding habits (Jumars et al., 2014), some species in Autolytinae and Syllinae have been considered carnivorous, while certain species in Exogoninae and Eusyllinae are supposed to be herbivorous or detritivorous (Malaquin, 1893; Allen, 1921; Okada, 1928; Korringa, 1951; Hammond, 1969; Hughes, 1975; Fauchald and Jumars, 1979). A recent extensive study on fecal pellet contents of syllid species revealed that many of the syllid species were omnivorous or detritivorous (Giangrande et al., 2000). As most of Syllinae species, our observations suggest that *M. nipponica* is omnivorous in nature, since they fed not only on plant tissues like spinach powder or *Nannochroropsis*, but also on dead bodies of other individuals and fish food.

Stolonization process

The stolonization process in laboratory conditions of *M. nipponica* was recorded (Fig. 5A–F). The presence of a stolon at the time when the specimens were collected seemed to depend on the seasons, being more abundant during warmer months. However, when reared in laboratory conditions, most individuals developed stolons and released them regularly.

Almost all individuals fed well on spinach powder and fish diet. Their digestive tracts full of food materials were clearly visible through their transparent body walls. The posterior part of the digestive tract was constricted and kinked in some adult individuals that have not developed gonads and stolons yet (Fig. 5A). This constriction was seen in the posterior part of the body, suggesting that this is a previous stage to stolonization. However, the precise causal relationship with stolonization is unraveled.

Stolonization process started with the development of gonads. In adult specimens, around 100 segments, a pair of gonads can be seen in each of the last ~30 segments (Fig. 5B). The size of gonads was smaller at the beginning, but became larger, almost filling the segment (Fig. 5C). Simultaneously with the gonad development, newly-formed eyes were observed at the most anterior segment of the gonad-developing segments (Fig. 5D). During stolonization, the

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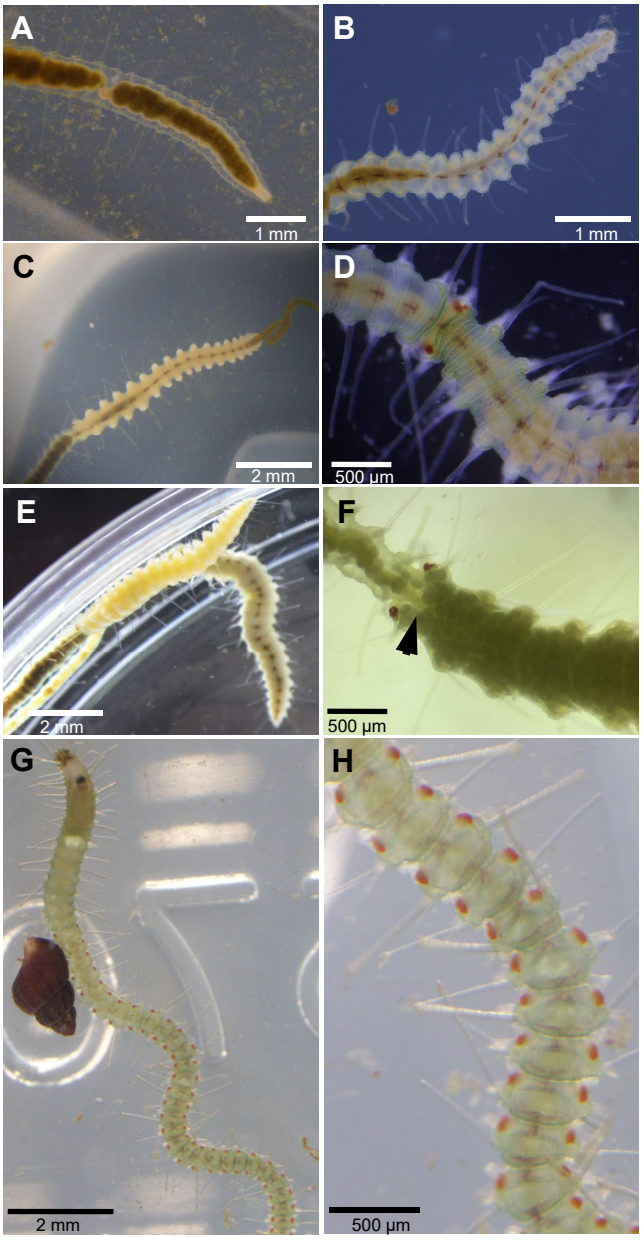

Fig. 5. The transformation of posterior segments during stolonization. **(A)** Before the beginning of stolonization, a kinked structure is seen in the digestive tract, relatively at the posterior region. **(B)** A pair of gonad primordia is seen in each of the posterior segments. **(C)** Even when gonads develop, the digestive tract inside the stolon still works, so that fecal pellets were expelled from the posterior part of the stolon. **(D)** Eyes are then formed, and the border between the stock individual and the stolon becomes apparent. **(E)** Sexes of fully developed stolons can easily be distinguished by their body color: female stolons are greenish and males are yellowish. **(F)** Prior to the detachment of the stolon, the new stock tail has already been formed under the stolon head. **(G, H)** Abnormal individuals sometimes appear. The most frequent type is the individuals with numerous eyes in all the posterior segments.

stolon head and gonads develop as well as the modification of swimming chaetae (Franke, 1999). Our observations indicated that the gonad development occurs before the formation of eyes (Fig. 5B–D), as has been observed in other species (Aguado et al., 2015b). The position where the gonad development started, i.e., both sides of each segment, would indicate the location of gonad primordia at both sides of each segment (Fig. 5B). In annelids, the proliferation of peritoneum cells is suggested to lead the formation of gametogonia (primary gametocytes), which are released in the coelom (e.g., Anderson and Huebner, 1968; Eckelbarger, 1979). These primary gametocytes are suggested to form the gonad primordia on both sides of future stolon segments.

When eyes started to develop (Fig. 5C), the digestive tract inside the developing stolon is still connected with that of the stock individual, since fecal pellets came out from the tail of the developing stolon. The digestive tube in the stolon gradually became smaller, as the coelum became occupied by gonads. At the end of gonad development, the sex of stolons could easily be distinguished by the color of testis and ovary tissues (Fig. 5E): male stolons were yellowish white while females were more greenish with visible eggs (Figs. 1B, 5E). Interestingly, before the stolon detachment, a new tail (pygidium and posterior segments) of the stock already started to be formed under the head of the stolon, i.e., on the ventral side of the stolon head (Fig. 5F). The onset process of posterior regeneration is different from the normal regeneration process which is initiated by amputation of body parts (Bely and Nyberg, 2010; Brockes and Kumar, 2008; Ribeiro et al., 2018). This situation has been previously reported in *Megasyllis* and *Alcyonosyllis* species (Aguado and San Martín, 2009; Aguado et al., 2012).

Among cultured individuals, abnormal individuals occasionally appeared. One of the most frequent morphotype showed numerous orange-reddish lateral spots in the posterior segments, which resembled eyes (Fig. 5G, H). The possible development of lateral eyes suggests that every posterior segment possesses the potential to become a stolon head. These individuals appeared when environmental conditions were poor, suggesting that some physiological regulations are involved in the stolonization process; however, careful and extensive investigations would be required to prove this.

The number of male and female stolons produced after sampling (28 October 2016) were counted (Supplementary Figure S1). Although we could not distinguish the first, second, or third production of stolons from each individual, the results suggest that a maximum of three rounds of stolonization per stock individual occurred. Male stolons were produced at 77.64 ± 21.35 days (n = 126) after sampling, while female stolons were produced at 73.03 ± 22.48 days (n = 123). There was no significant difference between the sexes (*t*-test; $P = 0.098$).

Spawning and fertilization

When the opposite sex stolons encountered, they began to actively vibrate to spawn eggs and sperms (Fig. 6A). Eggs were spawned as a cluster or spread on the bottom of the container (Fig. 6A, B). Female stolons often held the eggs under the laboratory condition, and also male stolons occasionally did so. The diameter of an egg was about 100 µm, and the color was whitish (Fig. 6B, C). The sperm head was oval-shaped and the sperm was strait shaped and ca. 40–50 µm in length (Fig. 6D). Parthenogenesis is not likely, since unfertilized eggs did not undergo embryogenesis (data not

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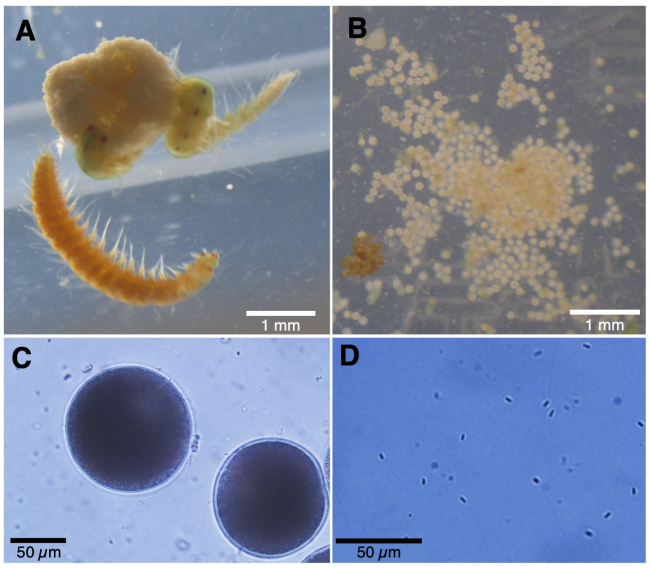

Fig. 6. Spawned eggs and sperms. **(A)** Stolons were located alongside the spawned eggs. Both male and female stolons sometimes held the egg mass. **(B)** Eggs were often laid on the bottom of the plastic case. **(C, D)** By DIC microscopic observations, eggs and sperms were recognized.

shown). Furthermore, artificial fertilization was attempted by dissecting mature detached stolons, but none of the eggs hatched thereafter. Some activation processes appear to be required at spawning.

Postembryonic development: Metatrochophore

The process of postembryonic development was observed in detail, and the period required for each developmental stage was also recorded (Table 1). After spawning, embryogenesis immediately started. Female stolons sometimes spawned eggs even without male stolons, although those eggs did not develop. It took 2.67 ± 1.25 days (n = 87) after fertilization until hatching. After hatching, metatrochophore larvae crawled on the bottom (Fig. 7A, B). Although the detailed process of embryogenesis was not observed in this study, the larvae hatched from eggs were not planktonic ones, but rather benthic metatrochophores, exhibiting direct development, as previously described in other species, such as *Typosyllis prolifera* (Franke, 1980). Only a few syllid species, such as *Typosyllis pulchra* (Heacox, 1980), have been reported to have swimming trochophore larvae. In *M. nipponica*, the developmental phase of trochophore seems to take place inside the extraembryonic membrane, and the hatched metatrochophores immediately crawl by ventral ciliation.

Postembryonic development: Juvenile

The metatrochophore period (MT) until the appearance of a complete digestive tract (including the proventricle) was 2.69 ± 2.07 days (n = 71). After the appearance of the digestive tract, these individuals fed on spinach powder and *Nannochloropsis*, which were visible inside the digestive tract (Fig. 7C, D). These individuals were termed "juveniles (JV)," as they did not possess some of the adult characters, such as the black spot. Later, the body size and the number

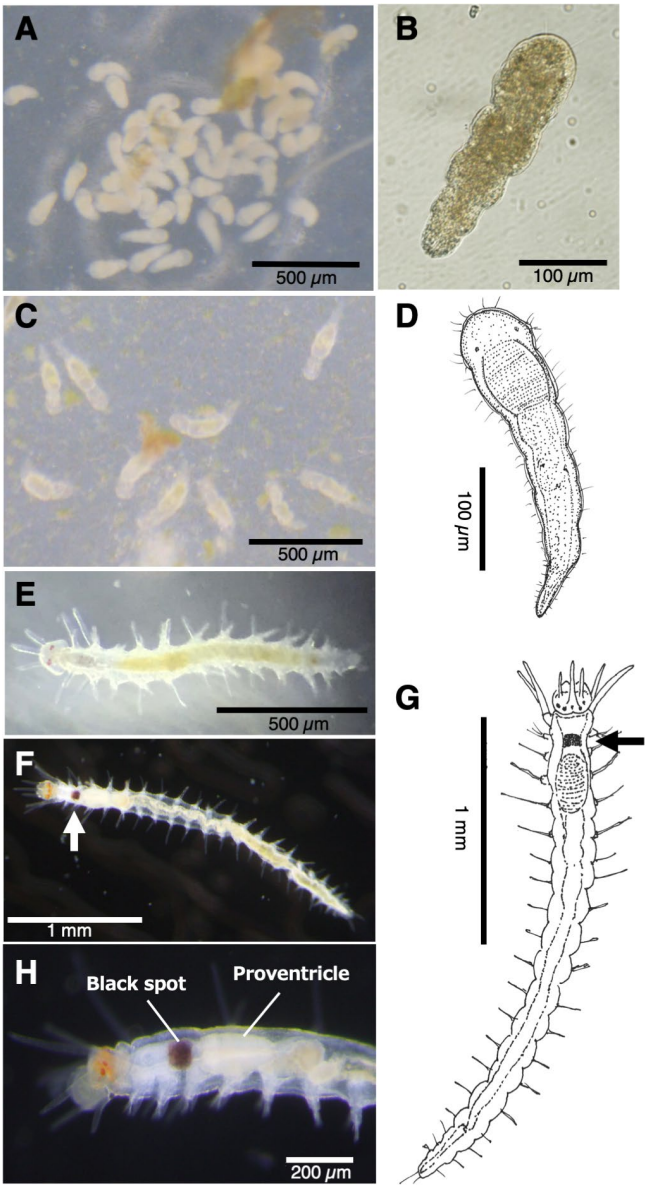

Fig. 7. Postembryonic stages. **(A, B)** Metatrochophore larvae. **(C, D)** Juveniles with apparent digestive tracts. **(E)** Large juveniles in which a black spot is about to appear. **(F, G)** Black-spotted individual which possesses adult-like characters. Arrows indicate the black spot. **(H)** Lateral view of the head of a black-spotted individual. The black spot is actually a band located at the posterior end of the pharynx.

Table 2. Periods from appearance of black-spot to stolon detachment.

	days (mean ± s.e.)	n
Male stolonization	34.87 ± 15.03	15
Female stolonization	36.27 ± 13.05	15
	(t-test: P = 0.394)	

of segments increased (Fig. 7E). The JV stage with a clearly detectable digestive tract before the appearance of a black spot was relatively long (23.26 ± 11.36 days: n = 38).

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Postembryonic development: Appearance of black spot

The end of the juvenile stage was defined as the stage at the appearance of a black spot, which is actually a black ring surrounding the pharynx (Fig. 7F–H). The black-spotted individuals (BS) possessed almost the same body structure as adult individuals. The total duration from spawning until the BS stage (EG-BS) was 26.49 ± 15.02 days (n = 45). After the appearance of the black spot, the individuals were maintained to measure the period until stolonization. The black spot was one of the key identifying traits of this species, but the adaptive significance of the pigmentation is totally unknown. When feeding, the black spot moves back and forth, showing that it surrounds the pharynx and moved by muscle contraction.

The period from the beginning of the BS stage until the first production of male stolons was 34.87 ± 15.03 days (n = 15), and until the first production of female stolons was 26.27 ± 13.05 days (n = 15) (Table 2). A significant difference between these two periods was not detected (*t*-test, $P = 0.394$).

Conclusion: *M. nipponica* as a study material

Based on the observation results, the life cycle of *M. niponica* is documented (Fig. 8). Under the rearing conditions (21°C, 16L:8D), postembryonic development up to the BS stage requires less than one month (~25 days). Then, another month is required until the detachment of a stolon (~35 days). In total, two months are suggested to be required for a generation, under favorable conditions. We have successfully cultured the focal strain in the laboratory up to four offspring generations (three full life cycles through four stolonizations; data not shown). The necessities for culturing this material species are food supply, maintenance of water quality, and collection of stolons for successful reproduction. Although they sometimes require careful handling, this species may be a suitable experimental animal that can be easily maintained in the laboratory. Since this animal possesses general biological features shared by Syllinae species, future developmental studies using *M. nipponica* will contribute to unraveling the shared mechanisms underlying the fascinating reproductive mode that has been acquired in this animal lineage.

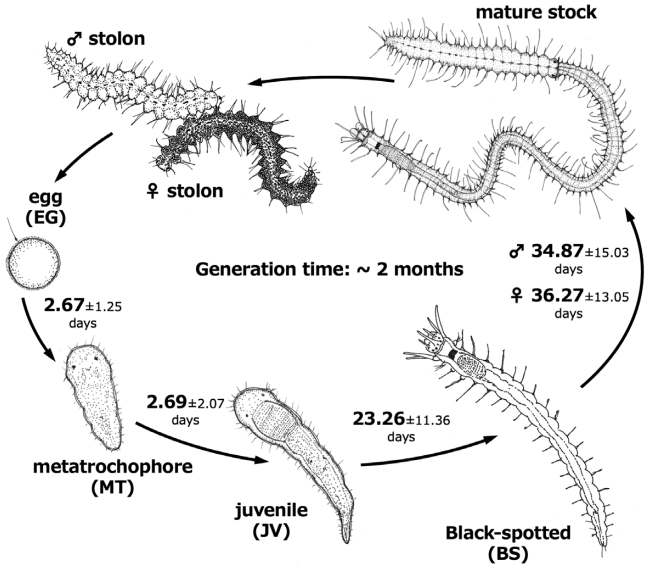

Fig. 8. Life cycle of *Megasyllis nipponica*. Number of days required for each postembryonic stage under the laboratory rearing conditions is indicated. The generation time for the focal species is suggested to be about two months.

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COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

TM conceived and designed the study. TM, KO, MN, SM, YH and SK performed field sampling. TM, KO, MN and SM performed rearing and observations. NJ and MTA identified the focal species. TM, KO, MN, NJ, SM, YH, SK and MTA wrote the paper.

SUPPLEMENTARY MATERIALS

Supplementary materials for this article are available online. (URL: https://bioone.org/journals/supplementalcontent/10.2108/zs190058/10.2108.zsj.36.372.s1.pdf)

Supplementary Figure S1. The number of stolons produced was counted after the collection date (27 October 2016). Stolons were continuously produced from the day 30 after the collection until at 128 days. Considering that the stolonization process requires about 30 days, a stock individual repeatedly produces stolons about three times during its life. No significant difference was detected between the number of male and female stolons ($P > 0.01$, *t*-test).

Supplementary Table S1. Number of sampled individuals of *M. nipponica* in each collection trial.

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