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Recommended Citation
https://doi.org/10.1080/07924259.2017.1287781

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Obligate planktotrophy in the Göttes larva of *Stylochus ellipticus* (Platyhelminthes)

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**ABSTRACT**

Polyclad flatworms are a diverse and emerging model system for developmental biologists, yet development remains poorly understood for many species. One limitation of polyclads as a model system has been the lack of reliable methods for culturing planktotrophic polyclad larvae to metamorphosis. There are conflicting statements in the literature about which types of polyclad larvae require food to complete development. We developed simple methods for rearing planktotrophic flatworms to metamorphosis and tested the effects of food type and concentration on development. The flatworm *Stylochus ellipticus* develops from small (~65 µm) eggs into an obligately planktotrophic Götte's larva. In this species, development to metamorphosis requires high concentrations
(50,000 cells/ml) of the unicellular alga *Rhodomonas lens* as a food source. High concentrations of two other algal species (*Dunaliella tertiolecta* and * Isochrysis galbana*) were successfully ingested by larvae, but failed to result in development to metamorphosis. We provide estimates of larval development times and initial descriptions of late larval and early juvenile forms in *S. ellipticus*. These data provide an important contrast between larval development in obligate planktotrophs with non-feeding species. Other indirect developing flatworm species may also be reared using these methods, allowing broader examination of polyclad developmental patterns than previously possible.

**KEYWORDS**
Müller's larva, Götte's larva, planktotrophy, polyclad, flatworm

**Introduction**
Polyclad flatworms have recently been proposed as model systems for examining the evolution of development in lophotrochozoans because of the diversity of developmental modes and larval types that they exhibit (Lapraz et al. 2013; Rawlinson 2014). However, the development of most species of polyclad flatworms is poorly understood, and recent reviews highlight the need for improved methods for studying larval development and metamorphosis of indirect-developing larvae in particular (Martin-Duran & Egger 2012; Rawlinson 2014). Indirect-developing polyclad flatworms progress through development as one of two heavily ciliated swimming larval forms: Müller's larvae and Götte's larvae.
The relationship of Müller's and Götte's larvae to one another and to the feeding requirements (if any) for completing larval development is unclear.

Götte's larva, a 4-lobed free-swimming larval form, was originally described as a precursor to the 8-lobed Müller's larval form, although that hypothesis is no longer accepted (Lang 1884 as reported in Ruppert 1978), and the two forms are thought to represent distinct developmental trajectories (Gammoudi et al. 2012a; Rawlinson 2014). The larval nutritional mode of Götte's larvae is especially cryptic in the literature, having been alternately described as non-feeding (Kato 1940; Anderson 1977), feeding (Ballarin & Galleni 1987; Murina et al. 1995) and facultatively feeding (Rawlinson 2014). Given the diversity of larval nutritional modes found among marine invertebrates (Allen & Pernet 2007), and even among close relatives (e.g. Allen & Podolsky 2007), it is possible that Götte's larvae vary in their larval nutritional requirements and that all three feeding types correctly describe some subset of cases.

To clarify the nutritional requirements of Götte's larvae, we studied the larval development of the polyclad flatworm *Stylochus ellipticus* (Girard, 1850). This species is abundant in Chesapeake Bay, where as an adult it is an important predator of oysters and barnacles (Newell et al. 2000; Campbell et al. 2011). *Stylochus ellipticus* has previously been described as developing via Götte's larvae (Girard 1854; Rawlinson 2014) but no information exists on its nutritional requirements. Here we test the larval nutritional requirements of *S. ellipticus* and show that larval development in this species is obligately planktotrophic. We further argue that our data, combined with the small egg size of other species known to produce Götte's larvae, suggest that obligate planktotrophy may be typical for this larval form. The relatively simple methods described here for culturing the
larvae of *S. ellipticus* may be extended to other species of polyclad flatworms to facilitate the study of the evolution of development in the indirect-developing species in this important group of spiralians.

**Material and methods**

Adult *Stylochus ellipticus* were collected from Chesapeake Bay in Virginia, east of the entrance of Indian Field Creek into the York River (37°16'05"N, 76°33'11"W). At low tide, barnacle and oyster shells were collected from riprap along the banks of the river and transported back to our laboratory in Williamsburg, VA where they were kept unaerated in buckets. Over the course of 24 h, adult *S. ellipticus* emerged from the collected shell material and were transferred to glass bowls containing artificial seawater (ASW). ASW was generated from a commercially available mixture of salts (Instant Ocean, Spectrum Brands, Blacksburg, VA) and dissolved in deionized water to achieve a salinity of 26 ppt for both adult and larval rearing. Flatworms were identified as *S. ellipticus* and distinguished from another common oyster predator in Chesapeake Bay, *Euplana gracilis* (Girard 1853), on the basis of three morphological criteria: *S. ellipticus* are 1) larger in size (> 10 mm) 2) possess anterior marginal eyes on the anterior of the body and 3) possess middorsal tentacles on the anterior portion of the body as described in Pollock (1998). All experiments were conducted at room temperature (~22°C).

Adult worms were maintained in glass bowls filled with 1 l of ASW and monitored daily for signs of egg laying. In general, newly collected *S. ellipticus* began laying eggs in masses on the sides of the glass bowls within 2-3 days following collection. When sufficient material for experiments was deposited (generally 2-3 masses from 2-4
adult worms) adults were removed, the ASW in the glass bowls was replaced and developing embryos were held until hatching, approximately 5 d after egg deposition.

Three independent larval feeding trials were conducted in the springs of 2014, 2015, and 2016. For the first feeding experiment, larvae were separated into four groups, each consisting of three replicate beakers, and given one of four unique feeding regimes: unfed, fed *Dunaliella tertiolecta* (Butcher 1959), fed *Isochrysis galbana* (Parke 1949) or fed *Rhodomonas lens* (Pascher & Ruttner 1913). Algal cultures of *D. tertiolecta* were obtained from UTEX Alga Supply (Austin, TX) and cultures of *I. galbana* and *R. lens* were obtained from the National Center for Marine Algae and Microbiota (East Boothbay, ME). Algae were cultured in sterilized ASW at room temperature under constant artificial light conditions using modified Guillard's f/2 medium (Florida Aqua Farms, Inc.). Algae were separated from culture medium by centrifugation prior to resuspension in larval cultures. For each of the feeding treatments, algal cells were provided at a concentration of 50,000 cells ml\(^{-1}\). Multiple pilot studies (results not shown) suggested that lower algal densities of 7,500 cells ml\(^{-1}\) were insufficient for rearing larvae to metamorphosis. For each feeding treatment, there were three replicates consisting of a 250 ml glass beaker filled with 200 ml of ASW seeded with larvae at a density of approximately 1 larva ml\(^{-1}\). Every other day 50% of the ASW was replaced by reverse filtration and fresh algal food was provided to maintain food concentrations. Larval cultures were kept stirred at a rate of 10 rpm using standard methods (Strathmann 1987).

The second feeding experiment was identical to the first with three exceptions: 1) it included two additional feeding treatments of 30,000 cells *R. lens* and 10,000 cells *R. lens*, 2) initial larval densities were 5-6 ml\(^{-1}\) and 3) each treatment was replicated five
times. The third feeding experiment was identical to the first except samples of ~20 larvae were fixed (see below for fixation protocol) from each replicate beaker every week to assess larval growth over time. Fixed larvae were mounted on slides with a cover slip supported by clay feet to prevent compression and then photographed at a magnification of 200X using an Olympus BX51 light microscope mounted with a DSLR digital camera to capture images. All images were subsequently measured for length, width and plan area using ImageJ software (Schneider et al. 2012). Differences in length, width and plan area were assessed using a three-way ANOVA with food treatment and age modeled as fixed factors and beaker modeled as a random factor. Following analysis, the residuals of the model were tested for normality using the Shapiro-Wilks test to confirm the assumption of a normal distribution. Statistical analysis was completed using IBM SPSS version 23.

For SEM imaging, larvae and juveniles were cultured as above and fixed at multiple stages of development. Samples were fixed overnight at 4°C in 2.5% glutaraldehyde in 80% ASW, then postfixed with 1% osmium tetroxide in ASW. After being dehydrated in a standard ethanol series, larvae and juveniles were critical point dried in a Tousimis Samdri-PVT-3B critical point drier, then mounted and sputter coated with gold palladium in a Hummer 6.2 sputter coater. Samples were imaged in an Amray 1810 with Orion digital grabbing system.

Results
In all three feeding trials, the Götte's larvae of *S. ellipticus* were observed to ingest particulate algal food of all three species that were offered (Figure 1). However,
development proceeded to metamorphosis only in beakers that were provided with *R. lens* as the algal food source. Larvae fed *R. lens* developed a pinkish-reddish hue throughout their body (Figure 2A) and grew considerably in size as larval development proceeded. Late-stage larvae were not only much larger in size but also possessed large numbers of presumptive lipid droplets throughout the larval body. No metamorphic cue was provided, but individual larvae began to spontaneously metamorphose 18 days post-hatching. Metamorphosing individuals were characterized by a reduction in the size and number of lobes associated with larval swimming and feeding, and began to elongate into an ovoid shape as metamorphosis proceeded (Figure 2B). In general, metamorphosing individuals completed their transition to the juvenile form (Figure 2C,D) within 24 hours after larval lobes visibly began to reduce in size. SEM images of larvae revealed uniform and heavy ciliation across the entire body, with the exception of an apical and posterior tuft of elongated cilia that were approximately twice the length of all other cilia (Figure 3A,B). SEM images of juvenile flatworms also revealed a uniform covering of cilia across the entire body surface (Figure 4A). In one juvenile, the pharynx was everted, possibly as an artifact of preservation, and presumptive lipid droplets were visible emerging from the everted structure (Figure 4B, compare with lipid droplets visible in Figure 2D).

In the first feeding trial, time to metamorphosis ranged from 18 to 38 days, with an average time to metamorphosis of 27 days (Figure 5). In the second feeding trial, time to metamorphosis ranged from 22 to 48 days with a mean time of 30 days to metamorphosis (Figure 6). Flatworms again reached metamorphosis only in beakers provided with *R. lens*, and all but one juvenile was found in the highest food treatment
(the lone exception being found in a low food treatment of *R. lens*). These data strongly suggest that development in *S. ellipticus* is best characterized as obligately planktotrophic. A mixed algal diet was not assessed but, given the ability of *S. ellipticus* larvae to ingest all three algal species offered, might be recommended for future culturing efforts as mixed diets have been found to improve culturing success in other marine invertebrate groups (e.g. Pechenik 1987; Schiopu & George 2004). Similarly, we did not assess the biochemical make-up of the algal species tested here, but prior studies have and found that *R. lens* has the highest protein content (Seixas et al 2009) and concentrations of stearidonic and eicosapenanoic acids (Schiopu et al 2006). There was no evidence that juvenile flatworms ingested any of the unicellular algae following metamorphosis. No attempt was made to provide an alternate food source for juveniles following metamorphosis and they eventually starved to death following our experiments.

In the third feeding trial, larvae were successfully cultured for the first three weeks of development. In the fourth week of larval culture the beakers fed high levels of *R. lens* became infested with a high density of unidentified ciliates. These ciliates were likely introduced to all of the cultures from remains of the egg mass when larvae were initially added. Based on our direct observations of their consumption of algae, these ciliates were excellent competitors for the algal food that we provided to the flatworms. By the fourth week, the ciliates were at high enough densities that they appeared to be negatively affecting growth of the flatworms, despite our attempts to remove them (Figure 7). Nevertheless, through the first three weeks of development, it was clear that larvae feeding on *R. lens* were longer, wider and had a greater area than those fed other algal types or than those that were unfed (Figure 7). In our statistical analysis of these
three variables, we found that food treatment consistently had the strongest effect on all size measures and was always a significant effect (Table 1). The main effect of age significantly affected only larval width, while the interaction between age and food significantly affected both larval width and larval area (Table 1). Posthoc comparisons of food treatments were conducted, using a Bonferroni adjustment for multiple comparisons, and showed that larvae fed *R. lens* were significantly larger in all size metrics than any other food treatment (*p* < 0.010). No other food levels were significantly different from one another (*p* > 0.500).

Immediately preceding, during, and following metamorphosis we also made observations of the number of eyespots in larval and juvenile *S. ellipticus*; eyespot numbers in larval and juvenile polyclads are sometimes useful taxonomic characters (e.g. Gammoudi et al. 2012b). We recorded the number of eyespots for 10 individuals at a variety of stages. Late stage larvae that had not yet begun to reabsorb their larval lobes consistently had a single pair of eyespots. Larvae that had begun to absorb their larval lobes (e.g. Figure 2B), but had not yet completed metamorphosis, also consistently had two eyespots. Early juveniles, within 1 to 3 d post-metamorphosis consistently had four eyespots present, whereas later juveniles 4 to 5 d post-metamorphosis were more variable in the number of eyespots with 4 to 8 eyespots present (e.g. Figure 2C). These observations suggest that during metamorphosis larval lobes are lost first, followed by multiplication of the eyespots during metamorphosis and further addition of paired eyespots in the days following metamorphosis.

**Discussion**
The feeding abilities and requirements of polyclad flatworms are poorly understood (Rawlinson 2014), and simple techniques for rearing feeding polyclad larvae to metamorphosis are needed to facilitate careful comparative studies of the evolution of development in this important and developmentally diverse bilaterian group (Lapraz et al. 2013). Our results clearly show that the Götte's larvae of *S. ellipticus* both possess the ability to ingest algal food particles, and indeed require a high concentration of nutritionally appropriate algae (*R. lens*) to successfully complete development to metamorphosis. This is one of very few cases where the feeding larvae of polyclad flatworms of any type (Müller's or Götte's) have been reared to metamorphosis and where the type and level of algal diet have been manipulated to determine their effects on larval growth and development.

Prior descriptions of feeding by polyclad flatworms are scarce. Scarpa et al. (1996) provide one of the few direct descriptions of feeding in any polyclad larva, in the Müller's larva of *Maritigrella crozieri* (Hyman 1939). Similar to our results, Scarpa et al. (1996) found evidence for ingestion of particulate food at 50,000 cells ml\(^{-1}\), however no report of successful culture to metamorphosis is given. Within the genus *Stylochus*, there are two possible reports of planktotrophy in species that develop via Götte's larvae. First, Murina et al. (1995) describe methods for culturing *Stylochus tauricus* (Jacubowa 1909) that include a period of larval feeding. However, while food was provided to larvae during development, no mention is made of any direct observations of larval feeding or what happened if larvae were reared in the absence of food. Thus, while larvae that were fed completed development, we do not know whether algal food was a requirement for (or even contributed to) development to metamorphosis. Second, in another congener,
*Stylochus mediterraneus* (Galleni 1976), Ballarin and Galleni (1987) describe the ingestion of the unicellular green alga *Dunaliella* sp. In their report however, Ballarin and Galleni (1987) did not attempt to rear the larvae of *S. mediterraneus* to metamorphosis on this diet and so, again, it is unclear whether the Götte's larva of this species is obligately planktotrophic or merely possesses the ability to ingest particulate food.

One of the few prior descriptions of metamorphosis in polyclad flatworms with Götte's larva comes from *Notoplana australis* Shmarda 1859 (Anderson, 1977). Some of the details of metamorphosis appear similar to our own observations, notably that ciliated lobes are reduced prior to metamorphosis into a benthic juvenile. Unlike our own observations, however, Anderson's figures of late stage larvae suggest late stage larvae don't grow following hatching, but appear to shrink prior to settlement. There is no indication that these larvae are able to feed, although no mention is made of whether algal food was offered to these larvae and, if so, what types or levels of algae were provided. It is possible, therefore, that the larvae of *N. australis* may be facultative planktrotrophs, although food does not appear to be required for metamorphosis (Anderson, 1977). The large egg size of *N. australis* relative to other polyclads exhibiting Götte's larvae (Table 2) may explain the ability of this species to develop to metamorphosis in the absence of food while species developing from smaller eggs, like *S. ellipticus*, cannot.

The ability of *S. ellipticus* to ingest food particles, and the requirement of exogenous food for development has potentially significant implications for the ecology of this species. Our results suggest that larval development to metamorphosis only occurs at very high levels of algal feeding (50,000 cells ml\(^{-1}\)). In Chesapeake Bay, where we collected *S. ellipticus*, eutrophication has led to increasing phytoplankton abundance
since the 1950's (Harding & Perry 1997) with significant ecological consequences for many species that live in the bay (Kemp et al. 2005). One consequence of increasing phytoplankton densities for *S. ellipticus*, which appears to require high phytoplankton concentrations for effective lab culture, may be increasing recruitment of this species in Chesapeake Bay. Since *S. ellipticus* adults are known to be voracious predators of oysters in the bay (Newell et al. 2000), it is important to understand how changing phytoplankton concentrations in nature affect the survival and recruitment of *S. ellipticus* larvae. We are conducting experiments rearing *S. ellipticus* on natural algal diets at current and historical levels from Chesapeake Bay in an attempt to further elucidate the role that eutrophication may have on this important predator of an ecosystem engineer (Klompen & Allen, unpublished data).

Beyond the implications of our work for *S. ellipticus* and its congeners, it is generally unclear how broadly distributed planktotrophy is among polyclads. A recent review (Rawlinson 2014) suggests that the more complex form of Müller's larvae, as reflected in the number of larval lobes (8 to 10), suggests they are more likely to be planktotrophic than the relatively simpler shaped Götte's larvae that possess only four lobes. However, our own review of the literature suggests that Götte's larvae generally develop from smaller eggs (61–125 µm) than do species developing via free-swimming Müller's larvae (90–330 µm; Table 2). While these ranges overlap, the mean egg size of Götte's larvae (97.0 ± 7.3 µm) is significantly smaller than the mean egg size of Müller's larvae (153.3 ± 24.9 µm; Mann-Whitney U test, U = 56.5, p = 0.046) although this analysis would clearly benefit from greater taxonomic sampling. If one assumes, however that mean egg sizes truly are smaller in Götte's larvae, then planktotrophy may also be
more likely in Götte's than in Müller's larval forms, as small egg size is typically associated with obligate planktotrophy in marine invertebrates (Wray 1995; Allen & Pernet 2007). In the absence of evidence to the contrary, our work, combined with other recent studies, suggests that planktotrophy is likely widespread in both Götte's and Müller's larval forms (Scarpa et al. 1996; Younossi-Hartenstein & Hartenstein 2000; Rawlinson 2008; Lapraz et al. 2013). The simple methods for larval culture presented here should allow widespread testing of the feeding mechanisms, needs and abilities of polyclad flatworms, and raise the potential for detailed comparative studies of the evolution of development.

**Acknowledgments**

We thank K. Pickering and S. Ziegler for their contributions to pilot studies of larval flatworm rearing. We also thank B. Brown and B. Pernet for general encouragement and help discovering the flatworm literature.

**Funding**

This work was supported by the National Science Foundation under Grant No. 1257039, awarded to JDA; and the Department of Biology at the College of William and Mary through a Mary E. Ferguson Award to AMK.

**Disclosure statement**

The authors declare that they have no competing interests.
References


FIGURE CAPTIONS:
Figure 1: Early larval stages of *Stylochus ellipticus* showing particulate algal food in the guts (arrowheads) for each of three species: A) *Isochrysis galbana* B) *Dunaliella tertiolecta* C) *Rhodomonas lens*. Scale bar = 100 µm.

Figure 2: Developmental stages of *Stylochus ellipticus* fed *R. lens*. A) Late stage larvae showing apical extension of cilia. B) Larva mid-metamorphosis showing resorption of larval lobes (arrowheads. C) Newly metamorphosed juvenile. D) Close-up of juvenile pharynx with presumptive lipid droplets visible. Scale bars = 100 µm A-C. Scale bar = 10 µm D.

Figure 3: SEM images of larva with apical (A) and posterior (B) tufts of elongated cilia visible, as marked by arrows. The matted cilia appearing on the posterior of the larval lobe reflect a preparation artifact. Scale bar = 10 µm.

Figure 4: SEM images of juvenile exhibiting uniform ciliation (A) and close-up of a juvenile with the pharynx everted (B). The spherical structures visible in the pharynx in B are thought to be lipid droplets being expelled from the pharynx and their expulsion is likely an artifact of the fixation process. Scale bar = 50 µm in A and 10 µm in B.

Figure 5: Cumulative settlement of juvenile *S. ellipticus* over time in feeding trial 1. Data represent the means ± SE of three replicates, each fed 50,000 cells ml⁻¹ of *Rhodomonas lens*.

Figure 6: Larval densities and cumulative juvenile settlement over time in feeding trial 2. Data represent the means ± SE of five replicates for each feeding treatment: unfed, DH = *Dunaliella tertiolecta* @ 50,000 cells ml⁻¹, IH = *Isochrysis galbana* @ 50,000 cells ml⁻¹, RH = *Rhodomonas lens* @ 50,000 cells ml⁻¹, RM = *R. lens* @ 30,000 cells ml⁻¹, RL = *R. lens* @ 10,000 cells ml⁻¹. RHJ = Cumulative number of juveniles from RH feeding treatment.

Figure 7: Larval growth over time in feeding trial 3. A) Larval length over time. B) Larval width over time. C) Larval area over time. Data represent the means ± SE of three replicates for each feeding treatment. Unf = unfed, Dun = *Dunaliella tertiolecta* @ 50,000 cells ml⁻¹, Iso = *Isochrysis galbana* @ 50,000 cells ml⁻¹, Rho = *Rhodomonas lens* @ 50,000 cells ml⁻¹.
Supplemental Figure 1: Unfed larva of *S. ellipticus* at 25 days post-hatching. Scale bar = 10 μm. Compare with larva fed high levels of *R. lens* in figure 2A.
Mean Cumulative Number of Juveniles vs. Days Post Hatching