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Life history traits and ecological conditions influencing the symbiotic relationship between the flatworm *Stylochoplana pusilla* and host snail *Monodonta labio*

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Clarification of the life-history traits of a symbiont and its host is necessary to evaluate the relationship between them. Stylochoplana pusilla*, a polyclad, inhabits the mantle cavity of the eulittoral snail* Monodonta labio*. This study elucidated the life-history traits of* S. pusilla *relative to those of* M. labio *in a tide pool, which retained seawater, and a boulder shore, which was dry at low tide, in Mutsu Bay, northern Japan, from 2010 to 2012. In these habitats,* S. pusilla *was semelparous with a 1-year life cycle; it reproduced in midsummer and inhabited the shell of* M. labio *except for the early juvenile stages, which were planktonic. More juvenile* S. pusilla *newly settled on hosts in the tide pool than on the boulder shore, however, their survival rate was higher on the boulder shore compared with the tide pool. The growth rate of juvenile* S. pusilla *was higher in the tide pool than on the boulder shore. The higher growth rate of* S. pusilla *at the tide pool suggested that they were more actively feeding. Stable isotope analysis showed that* S. pusilla *acquired food resources outside the host snail both in the tide pool and on the boulder shore. Thus, it was likely that* S. pusilla *engaged in feeding outside the host snails but resulted in lower survival rates at the tide pool. These results suggest that* S. pusilla *is semi-free living and uses* M. labio *as a refuge in the eulittoral zone for protection against desiccation.*

Keywords: eulittoral, flatworm, life history, *Monodonta labio*, Polyclad, *Stylochoplana pusilla*, symbiosis

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INTRODUCTION

Symbiosis is a frequently observed phenomenon. In this relationship, the host provides the habitat or food source, and occasionally a reproduction site, for the symbionts (Castro, 1991; Combes, 1991; Crompton, 1991). Symbionts have altered their life-history traits to increase their fitness within the constraints imposed by the host species and the symbionts themselves (Trouve et al., 1998). Therefore, to evaluate the strength of the relationship between a host and symbiont, it is essential to examine their life histories, which are generally investigated by analysis of the population dynamics of them.

Stylochoplana pusilla Bock, 1924, a polyclad commonly found from southern Hokkaido to Kyushu, Japan, is a species showing symbiotic associations with several other organisms (Kato, 1965). Indeed, this polyclad has been found in the mantle cavity of various snails such as *Monodonta labio* (Linnaeus, 1758), *Chlorostoma xanthostigma* Adams, 1853, *Chlorostoma turbinatum* Adams, 1853, *Omphalius rusticus* (Gmelin, 1791), and *Thais clavigera* (Kuster, 1860) (Kato, 1933; Fujiwara *et al.*, 2014). Of these snails, *S. pusilla* shows a high preference for *M. labio* as the host species (Fujiwara *et al.*, 2014). Unlike the other snails that normally inhabit the sublittoral zone, *M. labio* inhabits the eulittoral zone (Takada, 1995, 1996). Therefore, it is likely that the host preference of *S. pusilla* is related to the habitat differences of the host snails (Fujiwara *et al.*, 2014). However, it is not clear why *S. pusilla* dwells in snails living in the eulittoral zone.

Kato (1933) hypothesized that *S. pusilla* fed on the excrement of *M. labio* within the shell. If this is the case, the relationship between *S. pusilla* and *M. labio* should properly be regarded as commensalism because *S. pusilla* would be harmless to the host snail (Kato, 1933). Alternatively, Fujiwara *et al.* (2014) hypothesized that, because polyclads do not have any external means of protecting their bodies from drying, *S. pusilla* developed the habit of dwelling within the mantle cavities of eulittoral snails to avoid desiccation at low tide. If this is the case, the abundance and population dynamics of *S. pusilla* would differ between solely aquatic habitats such as tide pools and well-drained habitats such as boulder shores because the diel migration of patterns of *M. labio* differ between these locations; *M. labio* were observed in exposed areas of boulder shores, and also submerged in seawater in tide pools (Fujiwara *et al.*, 2014).

To clarify the validities of these hypotheses, the feeding habits and population dynamics of *S. pusilla* were examined in this study. To elucidate their food resources, stable

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carbon and nitrogen isotope analyses of *S. pusilla* were performed with its potential food sources. In addition, the size and number of eggs, and seasonal changes in the body size of *S. pusilla*, were determined over a 3-year period in two habitats with different water conditions, i.e. a tidally drained boulder shore and constantly immersed tide pools. The proportions of *M. labio* having *S. pusilla* in their shells (i.e. prevalence) and the number of individual *S. pusilla* per host (i.e. abundance) in the two habitats were also investigated. From these data, we estimated the growth rate and survival rate of this flatworm species *in situ* and discussed why *S. pusilla* used *M. labio* as a host.

MATERIALS AND METHODS

Sampling sites

Sampling was carried out at the tide pool (40°56′41.4″N 140°51′31.1″E) and boulder sites (40°56′44.6″N 140°51′36.0″E) in Mutsu Bay, northern Japan. The boulder site was composed of one to three layers of boulders with a maximum diameter of ~30 cm on coarse sand. It completely drained at low tide but *M. labio* remained on the surface of the boulders even though uncovered by seawater. At the tide pool site, seawater remained in small shallow depressions (~1 m × 5 m × 10 cm deep) in the bedrock at low tide. Snails were usually in seawater at the tide pool site, unlike those at the boulder site. The two sites were about 200 m apart. Further information on the sampling sites and benthic fauna are detailed elsewhere (Fujiwara *et al*., 2014).

Samplings, counts and body size measurements

To clarify how the life history traits of *S. pusilla* were related with those of *M. labio*, sampling was carried out monthly from October 2010 to November 2012 at the lowest spring tide during daytime hours. During the period, the abundance of *M. labio*, its shell diameter, and the prevalence and number of *S. pusilla* per host snail were recorded in both the tide pool and boulder sites. At each study site, three quadrats of 50 × 50 cm^2 were placed randomly and all individuals of *M. labio* in each quadrat were collected. Shell diameter (major axis) of these snails was measured by calipers and recorded immediately after sampling. In one of the three quadrats, *M. labio* were collected, and each individual was separately stored in a plastic bag and transported to the laboratory. Shells of these individuals were then broken open carefully so as not to damage the soft body and the number of *S. pusilla* counted. All *S. pusilla* found were anaesthetized in 0.35 M MgCl_2 seawater (Deguchi *et al*., 2009) and measured for length (minimum distance between the anterior and posterior edges) to the nearest 0.1 mm under a binocular microscope.

Because *M. labio* with a shell diameter <12 mm rarely had *S. pusilla* within their mantle cavities, the prevalence and number of *S. pusilla* per *M. labio* with a shell diameter >12 mm alone were analysed (Fujiwara *et al*., 2014). The separation of cohorts for sampled *S. pusilla* was carried out by a normal quantile–quantile plot and, within each cohort, the variation in body size with growth was examined.

Apart from these samplings, *M. labio* were collected every week from August to September 2012 at the boulder and tide pool sites to determine changes in the number and size of juvenile flatworms just after settlement. *Stylochoplana pusilla* were removed from *M. labio* as described above, and body size measured.

Food source of *S. pusilla*

To examine the food resource of *S. pusilla*, stable carbon and nitrogen isotope analyses of *S. pusilla* and potential food sources were performed. The whole body of *S. pusilla*, the muscle tissue and excrement of *M. labio*, and the periphyton, which was trimmed with a brush on boulder surface, were collected in July 2013 both at the boulder and tide pool sites. The periphyton collected were those on the surface of boulders and rocks where *M. labio* adhered. Five separate samples were used for analysis. Each sample was treated with 1 N hydrochloric acid five times (a few seconds at each time) to remove carbonate, dried at 60°C for 24 h after washing with distilled water, wrapped in tin foil, and preserved in desiccators. For analysis, the ^{13}C and ^{15}N content of the samples was measured with a continuous-flow stable isotope ratio mass spectrometry (CF-IRMS; ANCA-GSL, Sercon). Stable isotope ratios are expressed in δ notation as the difference in parts per thousand from the standard:

$$\mathrm{d}X = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000, \qquad (1)$$

where X is ^{13}C or ^{15}N, R is $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$, and appropriate standards were Pee Dee Belmnite and atmospheric nitrogen for carbon and nitrogen, respectively.

Reproduction of *S. pusilla*

To examine if the reproductive traits of *S. pusilla* differed between the two sites with different water conditions, the relationship between maternal body size and number of eggs per individual was examined using individuals collected between July and September 2012 at the boulder (12 individuals) and tide pool sites (20 individuals). At each sampling date, mature *S. pusilla* were initially selected, which were easily judged from the presence of stored oocytes and white seminal receptacles in the body. These individuals were then carefully and individually transferred to plastic containers (7.5 × 9 × 4.5 cm^3 deep) filled with seawater. The containers were placed in an incubator at a constant temperature of 23°C under dark conditions. *Stylochoplana pusilla* were observed every day to confirm if they spawned an egg plate on the wall of the container. When spawning was noticed, the egg plate was removed to a Petri dish and photographed under a binocular microscope to estimate the number of eggs. The body size of maternal individuals was also measured under the binocular microscope.

Mature *S. pusilla* were also taken from *M. labio* collected randomly in the boulder and tide pool sites during July–September 2012 to examine the relationship between maternal body size and egg size. In the laboratory, body size was measured and then the eggs were picked out from the ventral side of mature *S. pusilla* using a needle to make sure that the eggs were not yet developed. Since the diameters of eggs changed according to the developmental stage, we used undeveloped eggs for the measurements. In each site, at least 10 eggs from each of 15 maternal individuals were photographed and the diameters were measured by Image J.

Statistical analyses

Repeated-measures analysis of variance (ANOVA) was used to examine the significant differences in the abundance of *M. labio* with a shell diameter >12 mm, the length and abundance of *S. pusilla*, and the body size of juvenile *S. pusilla* after settlement between the boulder and tide pool sites. When a significant difference was observed, a Tukey HSD test was used to detect significant difference between the two sites at each sampling date. Significant differences in the prevalence of *S. pusilla* between the boulder and tide pool sites were examined using a Chi-squared test, and between the two sites at each sampling date using the Fisher's exact probability test with a Bonferroni adjustment of the significance level. Significant differences in egg size between the boulder and tide pool sites were examined by a *t*-test. The relationship between egg number and maternal body size of *S. pusilla* was examined by simple linear regression analysis. Finally, significant differences in the regression lines between the boulder and tide pool sites were tested by analysis of covariance (ANCOVA). All statistical analyses were performed using R ver. 2.15.1 (R Development Core Team, 2005).

RESULTS

Food resources

According to stable isotope analysis, δ^{15}N values were similar between *S. pusilla* and *M. labio* regardless of site, but δ^{13}C values differed greatly between these species, especially at the tide pool site (Figure 1). In addition, δ^{13}C values of *S. pusilla* was ~7‰ lower than *M. labio* excrement collected at the tide pool site, and was ~4‰ higher at the boulder site. In *S. pusilla*, δ^{13}C values were ~2‰ higher and δ^{15}N values ~5‰ higher than the periphytic material on the surface of boulders in the tide pool site. On the other hand, δ^{15}N values of the periphytic material of the boulder site was not detected due to the small content of N in the samples.

Host abundance and prevalence of host snails

The abundance of *M. labio* with a shell diameter >12 mm varied from 5 to 15 individuals per quadrat in both sites, except for several dates in 2011 at both sites (Figure 2). However, there was no seasonal variation in their abundance, and no significant difference was detected between the boulder and tide pool sites (repeated-measures ANOVA: $F_{1,\ 176} = 0.107$, $P = 0.744$).

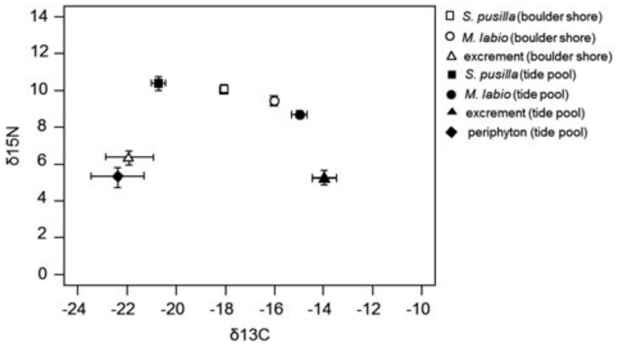

Fig. 1. Stable isotope analysis (±SD) of *Stylochoplana pusilla* body tissue, *Monodonta labio* body tissue, excrement of *M. labio*, and periphytic material collected from surfaces at the boulder or tide pool sites.

At the boulder site, the prevalence of *S. pusilla* within *M. labio* with a shell diameter >12 mm varied seasonally, between 40 and 90% with a mean of 74.7%. At the tide pool site, prevalence was generally higher than 80% with a mean of 91.2%, which was significantly higher than that at the boulder site (Chi-squared test: $P < 0.001$) (Figure 3).

Body size and abundance of *S. pusilla*

In late August 2012, when numbers of *S. pusilla* per *M. labio* were high, their body sizes were small: 0.77 ± 0.19 and 1.04 ± 0.27 (SD) mm at the boulder and tide pool sites, respectively. Such small individuals of *S. pusilla* were also found in late August 2013 at both sites. Then, their body size increased gradually towards the following summer. With respect to size composition, *S. pusilla* appearing between October 2011 and August 2012 were considered to be the first cohort, while those appearing between August 2012 and August 2013 were considered the second cohort in both sites (Figure 4). From autumn to spring, the body sizes of *S. pusilla* collected in the tide pool site were larger than those in the boulder site (repeated-measures ANOVA: first cohort, $F_{1,\ 1440} = 83.040$, $P = 0.002$; second cohort, $F_{1,\ 1744} = 67.57$, $P = 0.009$). However, the mean length of *S. pusilla* in the boulder site reached the same size as those in the tide pool site in June and no significant difference was detected between the sites thereafter (see Figure 4).

Throughout the study period, the number of *S. pusilla* per *M. labio* with a shell diameter >12 mm varied from 0 to 90. The mean number of *S. pusilla* per *M. labio* was lowest in early to midsummer, with 1.00 ± 0.50 (SD) at the boulder site and 2.40 ± 0.55 at the tide pool site. However, it reached a maximum in late summer, with 8.20 ± 2.32 at the boulder site and 37.89 ± 24.18 at the tide pool site (Figure 4). Thereafter, the number of *S. pusilla* per *M. labio* decreased towards January. The number of *S. pusilla* per *M. labio* was greater at the tide pool than at the boulder site through the year (repeated-measures ANOVA: the first cohort, $F_{1,\ 259} = 30.86$, $P = 0.002$; the second cohort, $F_{1,\ 239} = 30.86$, $P = 0.013$).

Reproduction of *S. pusilla*

Mature *S. pusilla* laid one egg plate between August and September. All individuals examined had spawned within 10 days in the laboratory after collection in the field.

The number of eggs per plate increased proportionally with maternal body size (Figure 5). Both the slope (ANCOVA: $F_{1,\ 28} = 0.273$, $P = 0.605$) and elevation ($F_{1,\ 29} = 0.173$, $P = 0.679$) of the regression lines between the number of eggs and body size of maternal *S. pusilla* did not differ significantly between the boulder and tide pool sites. Accordingly, the regression line of the pooled data was expressed as number of eggs = 339.37 × body size − 818.55 ($N = 34$, $r^2 = 0.625$, $P = 0.003$; Figure 5). The diameter of eggs collected directly from mature individuals using a needle varied from 0.132 to 0.141 mm and did not significantly differ between the sites (t-test: $t = 0.018$, $P = 0.985$) regardless of body size of

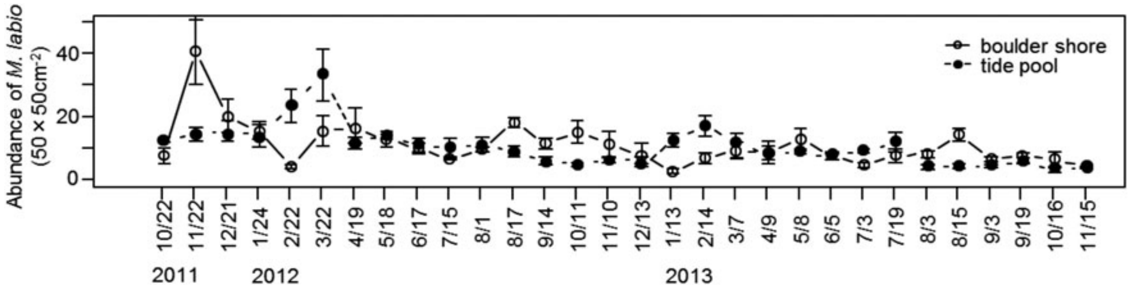

Fig. 2. Seasonal variations in the abundance of *Monodonta labio* with a shell diameter >12 mm.

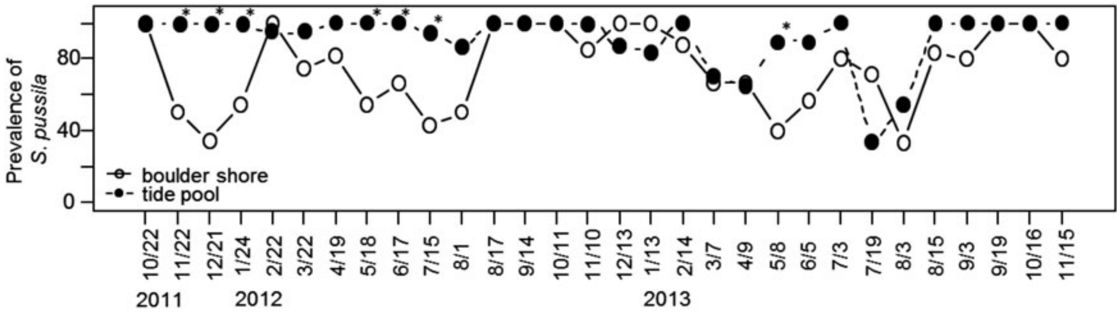

Fig. 3. Seasonal variations in the prevalence of *Stylochoplana pusilla* at each study site. Significant differences between the two sites at each sampling date via a Fisher's exact probability test ($P < 0.05$) are denoted by an asterisk.

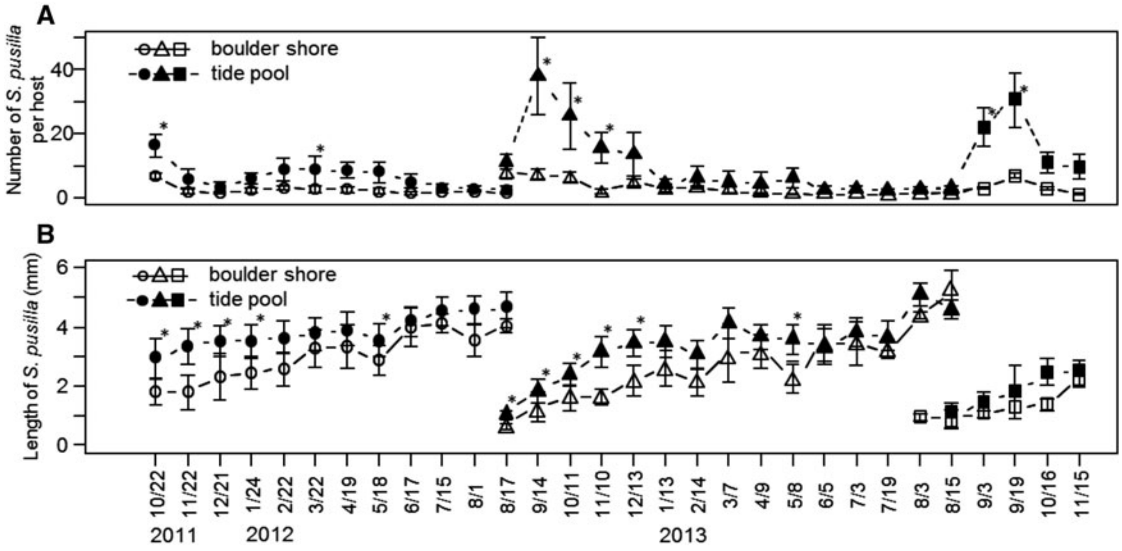

Fig. 4. Seasonal variations in the abundance (±SD) (A) and length (±SD) (B) of each *Stylochoplana pusilla* cohort at each study site (first cohort: circle symbol; second cohort: triangle symbol; third cohort: square symbol). Significant differences between the two sites at each sampling date by a Tukey test ($P < 0.05$) are denoted by an asterisk.

maternal individuals. However, as mentioned above, the number of eggs observed increased proportionally with maternal body size (Figure 5).

To examine the growth pattern of *S. pusilla* after settling into the shell, *M. labio* were collected weekly between August and September in 2012 at the boulder and tide pool sites. The body size of juvenile *S. pusilla* was significantly different between the boulder and tide pool sites (repeated-measures ANOVA: $F_{1,\ 420} = 10.290$, $P = 0.001$) during the study period. Body size of juvenile *S. pusilla*, presumed to have just settled in the shell, was 0.64 ± 0.04 (SD) mm ($N = 25$) at the boulder site, and 0.67 ± 0.05 mm ($N = 30$) at the tide pool site. Thus, there was no significant difference in body size between the sites just after settlement. However, 4 weeks after settlement, body size was 1.05 ± 0.90 mm at the boulder site and 2.04 ± 1.04 mm at the tide pool site (Figure 6), indicating a greater growth rate of juveniles at the tide pool than the boulder site.

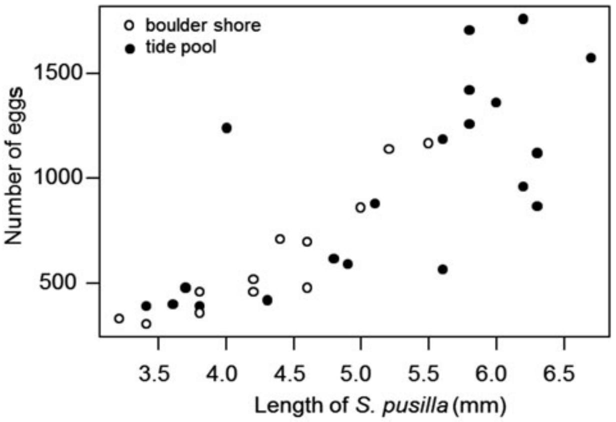

Fig. 5. Relationship between the number of eggs and body length of spawned *Stylochoplana pusilla.*

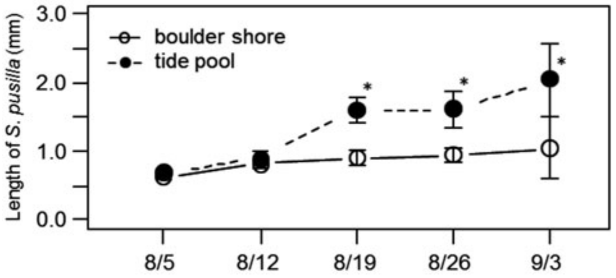

Fig. 6. Variations in the length of *Stylochoplana pusilla* (±SD) after settlement at each study site. Significant differences between the two sites at each sampling date by a Tukey's test ($P < 0.05$) are denoted by an asterisk.

DISCUSSION

Life history and feeding habits of *S. pusilla*

To the best of our knowledge, this is the first study on the life history of *S. pusilla*, a polyclad flatworm living in association with a snail species. In this species, individuals with a body size <1 mm were abundant in the mantle cavity of *M. labio* between August and September. These individuals grew to a size of 4–5 mm until July–August of the next year, and then disappeared. Together with the findings of Deguchi *et al.* (2009), who reported that almost all individuals collected in early summer had copulated and had preserved sperm in spermathecae, this study proves that *S. pusilla* is semelparous with a 1-year life cycle and reproduces in midsummer (mainly August).

During this study, eggs of *S. pusilla* were never found in the shell of *M. labio* and there was no evidence from laboratory observations that *S. pusilla* ate any eggs they produced (Fujiwara personal observation). Deguchi *et al.* (2009) found that, when reared at 23°C, the eggs of *S. pusilla* developed in a little over 2 weeks and hatched out as planktonic larvae. Individuals just after hatching were ~0.1 mm (Fujiwara personal observation) in body size and spent 1 week as plankters. After the pelagic stage, they began to crawl and settle, and to live in the host snail (Deguchi *et al.*, 2009). All the evidence implies that *S. pusilla* lays eggs outside the host. From our field studies, the period between the disappearance of mature individuals and appearance of numerous juveniles was a little over 1 month, suggesting that they reproduce over a similar time scale as that reported under laboratory conditions by Deguchi *et al.* (2009).

Our isotope analysis showed no major differences in $\delta^{15}N$ values between *S. pusilla* and *M. labio*, indicating that *S. pusilla* and *M. labio* have the same trophic level, and presumably assimilate primary producers. The result implies that this flatworm species neither feeds on the living body of *M. labio* nor material produced by *M. labio*, such as mucus secreted on the body surface, as food sources. In other words, the flatworm is not dependent on its host for or as a food resource. In the laboratory, *S. pusilla* can live by feeding on *Artemia* larvae as food (Deguchi *et al.*, 2009), suggesting that this flatworm may be omnivorous rather than an obligate herbivore. Although food resources were not determined in this study, there is no doubt that *S. pusilla* ingests food outside of the host snail. Thus, *S. pusilla* is considered to be semi-free living. This consideration conflicted with the hypothesis of Kato (1933) that the flatworm depended on the host snail for food resources.

Ecological traits and habitats of *S. pusilla*

Monodonta labio with a shell diameter >12 mm were abundant throughout the study, indicating that the number of hosts available to *S. pusilla* was stable throughout the year in both boulder and tide pool sites. However, the prevalence and number of *S. pusilla* in a host snail were greater at the tide pool site than at the boulder site, especially immediately after settlement of juveniles. These observations suggest that either the number of eggs produced per host snail, or the settlement rate of juveniles, was higher at the tide pool site. To examine these possibilities, the total number of eggs laid by *S. pusilla* dwelling in a host snail (birth rate) was calculated for each site using the linear regression of egg number to maternal body size, and the number of maternal individuals per host in early summer when most individuals were mature. The mean number of eggs was 2250 per host snail in 2011 and 2700 in 2012 at the tide pool site, and 900 per host snail in 2011 and 400 in 2012 at the boulder site. Then, the ratio of the maximum abundance of juveniles found in a host snail to the total egg number at each site was estimated as the settlement rate. The ratios were 0.0152 and 0.0303 at the tide pool site and 0.0071 and 0.0175 at the boulder site in 2011 and 2012, respectively. These estimates indicate that both birth rate and settlement rate from egg to juvenile were higher at the tide pool site than at the boulder site (Table 1).

However, during the period from winter to early summer, the numbers of *S. pusilla* per host snail were almost the same between the tide pool and boulder sites. This suggests that tide pools may not be an ideal habitat for *S. pusilla*. To examine this in more detail, the survival rate of *S. pusilla* from settlement to maturity (reached the following summer after initial settlement) was estimated by comparing the number of mature individuals in 2012 with that of newly settled individuals in host snails in 2011. The ratio was higher at the boulder site (0.1219) than at the tide pool site (0.0633) (Table 1). This result implies that the survival rate after settlement was higher at the boulder site than at the tide pool site. Thus, a tide pool is not necessarily an ideal habitat for young to adult individuals of *S. pusilla*, although it was a superior habitat for newly settled juveniles.

Why was the survival rate of *S. pusilla* lower in the tide pool site? The growth rate of the early juvenile stages suggests that food conditions were more favourable to *S. pusilla* at the tide pool site than at the boulder site. Probably, *S. pusilla* were

Table 1. Comparison of ecological traits and population parameters of *Stylochoplana pusilla* between the boulder and tide pool sites.

Population parameters	**2011**		**2012**	
	Boulder	**Tide pool**	**Boulder**	**Tide pool**
Mean length of mature *S. pusilla* (mm)	4.12	4.50	3.58	3.56
Mean number of mature *S. pusilla* per host	2.00	3.56	1.00	2.40
Estimated number of total eggs per host	1159.31	2522.67	396.39	1008.36
Mean number of newly settled juveniles per host	8.20	37.89	7.00	30.60
Settlement rate from egg to juveniles (%)[a]	0.71	1.52	1.75	3.03
Survival rate (2011–2012) (%)[b]			12.19	6.33

[a]Number of newly settled juvenile/total number of eggs.

[b]Number of mature *S. pusilla* in 2012/number of newly settled juveniles in 2011.

prevented from leaving the host snails during low tide on the boulder shores because of their susceptibility to desiccation (Sreeraj & Raghunathan, 2013). In the tide pools, however, seawater remained even at low tide and *M. labio*, the host snail, was always covered in water (Fujiwara *et al*., 2014). Under these environmental conditions, *S. pusilla* would have more opportunities to exit from the host shell and to spend time acquiring food outside the host shell. When *S. pusilla* were removed from the host snails and released into the tide pool, they were immediately preyed upon by gobies (Fujiwara personal observation). Apparently, increasing the time spent feeding outside the host shell increases the predation risk within the tide pool, which is similar to the high predation pressure of the sublittoral zone (Connell, 1972; Menge & Branch, 2001; Perez et al., 2009). Therefore, the survival rate of *S. pusilla* after the settlement would be lower in the tide pool site, although the growth rate was higher in the tide pool site.

To summarize, this study showed that differences in the ecological conditions of the host snails, and in the feeding conditions of the flatworms, affect the abundance of *S. pusilla* per host snail, through differences in the birth, growth, and survival rates of the flatworms. These results support the hypothesis by Fujiwara *et al.* (2014) that *S. pusilla* preferentially use the mantle cavity of *M. labio* as a refuge for protection from desiccation in the eulittoral zone, where predation pressure is lower. The relationship between *S. pusilla* and *M. labio* can be regarded as commensalism, although the former species does not depend on obtaining food resources from the latter.

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