

Population Ecology and Habitat use of the Sea Slug *Elysia pusilla* (Bergh, 1872) (Sacoglossa) in A Tropical *Halimeda macroloba* Decaisne Meadow

Apisara Nakpan¹, Jaruwat Mayakun² and Kringpaka Wangkulangkul^{1,*}

¹Division of Biological Science, Faculty of Science, Prince of Songkla University, Songkhla 90110, Thailand

²Molecular Evolution and Computational Biology Research Unit, Division of Biological Science, Faculty of Science, Prince of Songkla University, Songkhla 90110, Thailand

(*Corresponding author's e-mail: kringpaka.w@psu.ac.th)

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Abstract

Relationship between abundance of specialist marine herbivores and their food sources is poorly known because these herbivores are relatively rare in marine systems. The relationship between the cryptic sea slug *Elysia pusilla* (Bergh, 1872) and its host alga, *Halimeda macroloba* Decaisne, was evaluated in terms of spatial association and habitat utilization in a tropical algal meadow in southern Thailand that exclusively comprised of *H. macroloba*. The density of *H. macroloba* and of *E. pusilla* egg masses varied temporally throughout the sampling period, but temporal variation was not detected in the density of *E. pusilla* individuals, which was generally low with a maximum of 4 individuals per 400 cm². Analysis suggests that the occurrence of the slug and its egg masses might be determined by the availability of the algal host. The slug was more likely to be observed in dense patches of algae that which offered a large total algal surface area. Occurrence of the slug was also higher when the algae were abundant. The numbers of slugs, egg masses and grazing marks were higher on mature thalli, which have larger surface areas than younger thalli. Egg masses and grazing marks were observed more often on segments at terminal positions on thalli. According to previous works, these segments contain low levels of accumulated calcium carbonate and high levels of secondary metabolites, which are sequestered by the slug and used to deter predators. The findings provide an insight into the life history of *E. pusilla* and variations in a natural population which that were previously little known.

Keywords: Plant-animal interactions, Sacoglossa, Algal host, Symbiosis, Elysia, Halimeda

Introduction

Generally, the abundance of specialist herbivores is positively related to the abundance of their food sources [1]. However, this relationship is understudied in marine systems because specialist marine herbivores are relatively rare compared to their terrestrial counterparts. One group of almost exclusively herbivorous sea slugs comprises the organisms known as sacoglossans (Subclass Heterobranchia: Superorder Sacoglossa). Sacoglossans consume algae with the aid of highly modified radulae [2] and find refuge from predators and physical disturbance among the algal meadows in which they graze [3]. Moreover, many species of sacoglossans exhibit a kleptoplastic relationship with their algal host in which the slug eats the alga, retains functional chloroplasts undigested and then utilizes the photosynthetic products of the sequestered chloroplasts [3-7].

The sea slug *Elysia pusilla* (Bergh, 1872) is a sacoglossan that feeds exclusively on a few species of the calcified green alga genus *Halimeda* J.V. Lamouroux, 1812 [8-10]. The slug probably spends its whole benthic life stage on *Halimeda* thalli. It utilizes the thalli as a feeding ground and a nursery where jelly-like egg masses are laid. When the slug feeds on the algae, it strips off cell walls with its triangular radular teeth and sucks out the cytoplasm [8]. Even though *E. pusilla* is widely distributed throughout the tropical Indo-Pacific [10], little is known about the life history and populations of the species in its natural habitats or the relationship between the slug and host species.

Along the Andaman coast of Thailand, extensive meadows of *Halimeda macroloba* Decaisne can be found at several locations, including the Lidee Islands, Libong Island, Similan Island, and Tang Khen Bay [11]. At the Lidee Islands, an algal meadow, comprised solely of *H. macroloba*, occupies around 500 m²

of an intertidal area of sandy mud flat. *E. pusilla* has occasionally been observed on *H. macroloba* thalli at this location. Given that there are known spatial and temporal variations in the abundance of *H. macroloba* at this location [12], the abundance of *E. pusilla* might vary in correlation to its algal host abundance.

The thallus of *H. macroloba* forms fronds of flat segments that consist of multinucleate coenocytic filaments that retain deposits of calcium carbonate crystals [11-14]. As well as accumulating calcium carbonate, *Halimeda* produces secondary metabolites, 2 of which, halimediatrial and halimedatetracetate, are recognized as deterrents to herbivores. Since calcium carbonate accumulation and secondary metabolite content vary between life history stages and the position of segments along the thallus [12,15], the occurrence of *E. pusilla* and utilization of the host by the slug might vary between the life history stages of *H. macroloba* and the location of segments on the thallus.

This study focuses on 4 main investigations. Firstly, we investigate temporal variation in the population abundance of *E. pusilla* and its algal host, *H. macroloba*, at Lidee Islands. Secondly, we establish whether the distribution of *E. pusilla* is determined by the available habitat provided by *H. macroloba*, using the density and total surface area of *H. macroloba* as indicators of habitat availability in a given area. Thirdly, we determine the influence of algal life history stages on the abundance of slugs, their egg masses and grazing frequency. Lastly, we determine the influence of the position of thallus segments on the occurrence of egg masses and grazing marks. Information obtained from this work helped us gain more insight into the population dynamics of this cryptic sea slug and its life history, especially with regard to its relationships with the host alga.

Materials and methods

Field collection

Elysia pusilla and *Halimeda macroloba* were collected at an *H. macroloba* meadow located on an intertidal mudflat between Lidee Lek and Lidee Yai Islands (6°47'01.3"N 99°45'59.5"E) in Satun Province, southern Thailand (**Figure 1**). Two dominant seasons, a rainy season dominated by the southwest monsoon (May to October) and a dry season dominated by the northeast monsoon (November to April), influence the climate of this study site. The maximum tidal range is approximately 2.5 m. This algal meadow is composed only of *H. macroloba*; there are no other dominant macroalgal species in the area. Five species of seagrass were also present at this site: *Halophila ovalis* (R. Brown) J. D. Hooker, *Thalassia hemprichii* (Ehrenberg) Ascherson, *Cymodocea rotundata* Ascherson & Schweinfurth, *Syringodium isoetifolium* (Ascherson) Dandy, and *Enhalusa coroides* (L. f.) Royle [12], but mixed patches of *H. macroloba* and seagrasses were rare as they usually occupy different areas of the mudflat. Sampling was carried out monthly from September 2019 - January 2020, then in March, August and October 2020. Although monthly sampling was planned for the entire year, field work sometimes had to be cancelled due to rough sea and logistical difficulties encountered during a local COVID-19 pandemic lockdown.

The fieldwork proved problematic in other ways. Because samples could only be collected when the tide was low and we needed to remove all thalli from every quadrat in the few hours available, the size of quadrats and number of samples collected were largely determined by practical considerations. On 1 occasion, we were able to collect samples from 18 quadrats of 0.04 m² (20×20 cm) that we randomly set out in the *H. macroloba* meadow. As many as 75 thalli were found in 1 quadrat. In addition, grazing marks had to be examined in the field because the marks faded away rapidly (personal observation). All the *H. macroloba* thalli in a quadrat were carefully pulled out of the substrate and each thallus was placed in a plastic bag. Holdfasts were cut off because sediment and debris from this part of the thallus could interfere with the search for slugs. The recorded observations of each collected thallus included the life history stage of the plant and the position on the thallus of segments where grazing marks were present. Photographs of grazing marks were taken in the field. A grazing mark was defined as a bleached, white continuous area on the thallus. Since boundaries between grazing marks were not usually clear, we did not count the number of grazing marks. Instead, we counted the numbers of thalli and segments that presented grazing marks. To count slugs, collected thalli were brought back to the laboratory and left undisturbed for 1 - 2 h to let the slugs emerge.

Data collection

In this study, the life history stages of *H. macroloba* were categorized into 6 stages, following Sinutok [13], Mayakun and Prathep [12]. Stage 1 consisted of newly recruited plants with only 1 segment (**Figure 2(a)**); stage 2 of young plants with 3 or 4 segments and 1 or 2 layers of segments (**Figure 2(b)**); stage 3 of partially calcified plants with 3 or 4 layers of segments (**Figure 2(c)**); stage 4 of mature calcified plants (**Figure 2(d)**); and stage 6 of dead plants with bleached segments only. Thalli of plants at stages 5 and 6

were not included in this study. Thalli of plants at stages 5 and 6 were not included in this study. Only one thallus of stage 5 was found in our samples and there was no slug, no egg mass and no grazing mark on this thallus. No dead plants were observed among our samples.

The slugs, egg masses and *H. macroloba* thalli collected from each quadrat were counted in the laboratory and the densities of slugs, egg masses and *H. macroloba* were calculated. Slug specimens were identified according to Jensen [16]. To evaluate the surface area of a thallus, photographs were taken of the thallus flattened on a tray and the area was calculated from the image, using the ImageJ © program. *E. pusilla* lays jelly-like egg masses on the segments of *H. macroloba*. Although larval dimorphism has been reported in this species [11] and egg masses have various morphs (**Figures 3(b) - 3(c)**; unpublished data), egg masses were not categorized in the present study.

The surface areas of *H. macroloba* individuals at different life history stages were compared to evaluate potential differences in the habitat space available to *E. pusilla*. To determine the position of segments along the *H. macroloba* thalli, the longest branch of a thallus was used as the reference. The terminal segment on this branch was classified as the first segment and the preceding segments were classified as 2nd, 3rd, 4th and so on. The positions of segments on other branches were determined outward from the basal segment of the reference thallus (**Figure 3(d)**). When counting egg masses, the segment number was noted where each egg mass was found. The numbers of segments that showed grazing marks and the positions of these segments on the thallus were recorded. Sampling was conducted in accordance with a permit issued by the Institutional Animal Care and Use Committee, Prince of Songkla University (MHESI 68014/361).

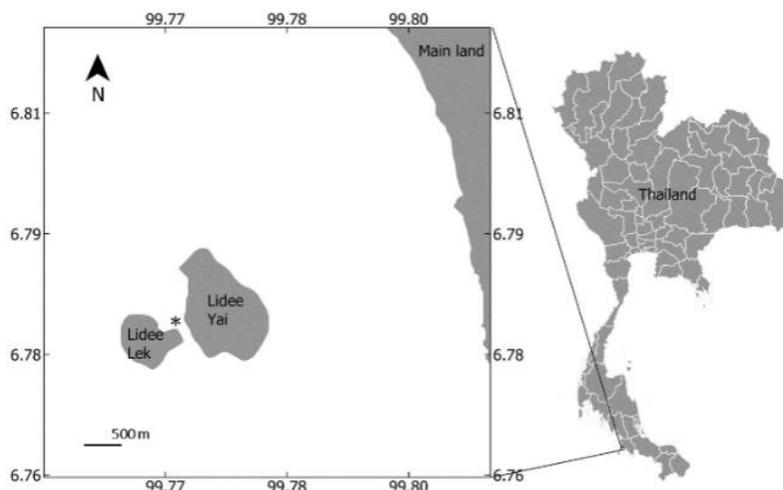


Figure 1 The sampling area (*) was located between Lidee Lek and Lidee Yai Islands in the Andaman Sea, Satun Province, Thailand.

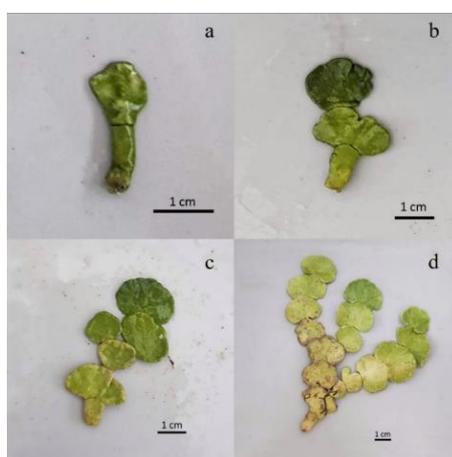


Figure 2 (a) - (d) Life history stages 1 - 4, respectively, of *Halimeda macroloba*.

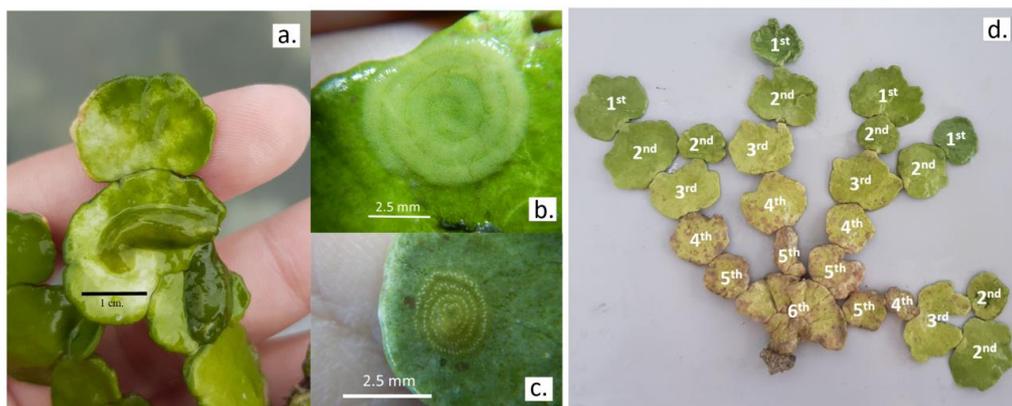


Figure 3 (a) Two *E. pusilla* individuals can be seen on a mature *H. macroloba* (stage 4) thallus. The 2 white areas visible on the terminal and second segments are grazing marks. (b) - (c) Egg masses of *E. pusilla*. (d) The photograph of a complete *H. macroloba* thallus shows how the positions of thallus segments were determined in this study.

Statistical analysis

Temporal variations in the abundance of *H. macroloba* over 8 months (fixed factor, 8 levels) were identified by ANOVA and the results were analyzed by the Student Newman Keuls (SNK) test for *post hoc* pair wise comparisons. The homogeneity of variance was tested by the Cochran's C test. Due to high variances among months, temporal variations in abundance of *E. pusilla* and its egg masses were analyzed by the Kruskal-Wallis test. Pearson correlation coefficients were calculated to evaluate relationships between the population density and total surface area of *H. macroloba* and the population density of *E. pusilla* and between the population density and total surface area of *H. macroloba* and the density of egg masses. Data from all quadrates was pooled across months for each variable in this analysis. After that, values of these 4 variables both for the slug and the algae from each month when sampling occurred were averaged to obtain monthly averages. The correlation coefficients were then calculated from these data. Differences in the surface area of *H. macroloba* between the 4 life-history stages taken into account were analyzed by one-way PERMANOVA based on Euclidean similarity. A chi-squared test was used in order to determine whether the numbers of *E. pusilla*, egg masses and grazing marks displayed any association with a particular life history stage of *H. macroloba*.

Results and discussion

Symbiotic relationships between sacoglossans and their macroalgal hosts have been studied mostly from the perspective of plant-animal co-evolution at the physiological level [2,7-9,17]. This study sought to quantify whether the abundance of an algal host *H. macroloba* is a determinant of the abundance of the sacoglossan sea slug, *E. pusilla*. How the slug utilizes the biogenic structure of *H. macroloba* thalli as feeding and nursery grounds was also examined.

In this study, variations in *H. macroloba* abundance within and between seasons were evident. The density of *H. macroloba* was found to be highest in December 2019, August, and October 2020, and lowest in September to November 2019 and March 2020 ($F = 6.52$, $p < 0.001$; SNK; **Figure 4(a)**). In contrast to our findings, Mayakun and Prathep [12] reported the highest density of *H. macroloba* at this site in March and the lowest in September. It seemed that the monsoon seasons did not have an effect on the population of *H. macroloba* and that some other unknown factors may have driven the fluctuations in algal density. *E. pusilla* was found on thalli collected on every sampling occasion except March 2020. The population density of the slug was not different between months ($H = 10.18$, $p = 0.17$; **Figure 4(b)**). However, it is worth noting that the variation between months was high. The highest density was 4 slugs per quadrate and in numerous quadrates no slugs were observed. Most sea slug species have been reported to be rare [18]; therefore, the pattern of variation observed in our samples can be seen as a typical pattern of sea slug occurrence. The density of egg masses varied significantly between months ($H = 33.25$, $p = 0.001$; **Figure 4(b)**). Egg masses were found in November 2019, and in March, August and October 2020 (**Figure 4(b)**). In March 2020 egg masses were found, but no slugs were observed.

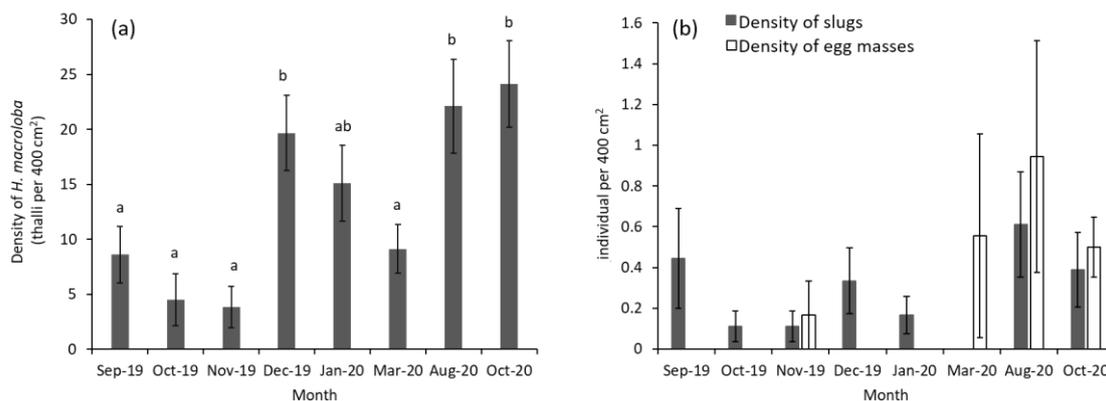


Figure 4 (a) Monthly density (Mean \pm SE) of *H. macroloba* and (b) *E. pusilla* and its egg masses from September 2019 to October 2020. Field work was cancelled due to rough seas and pandemic lockdown in some months (Data was not shown in the graphs). Different letters above bars = significant differences between months.

When data from different months were combined, the population density of *E. pusilla* and its egg masses showed small to moderate positive correlations with the population density and total surface areas of *H. macroloba* ($r = 0.12 - 0.36$; **Figure 5(a)**). Analysis of the pooled data suggested that the slugs inhabit areas of dense algal growth with high total surface areas of *H. macroloba*, but it is worth noting that the correlation suggested by the coefficient values was not strong. Middlebrooks *et al.* [19] found that spatial associations were not significant between the sea slug *Elysia clarki* and its food source in mixed patches of macroalgae. A number of *E. clarki* were found on other algal species that were not in its diet. For *E. pusilla* at this locality, though, *H. macroloba* is the only macroalgae substrate that the slug can utilize. They have also never been observed on seagrass previously.

For *E. pusilla*, there are several advantages to living in dense patches of *H. macroloba* thalli. Water velocity is generally reduced by algal patches [20], which reduce the dislodgement of slugs, and a high density of thalli provides a greater food supply. We observed that *E. pusilla* in captivity moves between thalli from time to time and this behavior might be triggered by the depletion of a food source. Moreover, inhabiting a dense algal patch may increase reproductive success. There was no evidence of territoriality in this slug species, but from observation of captive *E. pusilla*, we noted that many mating occasions happened just after slugs were placed together in confined spaces.

When data were averaged and mean values were analyzed, the density of *E. pusilla* was found to be strongly correlated with the total surface area of *H. macroloba* ($r = 0.56$). Degrees of correlation were moderate for relationships between the density of *E. pusilla* and the density of *H. macroloba* ($r = 0.43$), as well as the density of egg masses and the density ($r = 0.32$) and total surface area of *H. macroloba* ($r = 0.37$). Results suggest that when the abundance and total surface area of the algae in a given area were high, there was a higher chance to finding *E. pusilla* in the samples. The availability of the habitat provided by *H. macroloba* might be a limiting factor for populations of the slug at this temporal scale. Middlebrooks *et al.* [12] also found that the numbers of *Elysia papillosa* were affected by the biomass of its main food source, *Penicillus capitatus*.

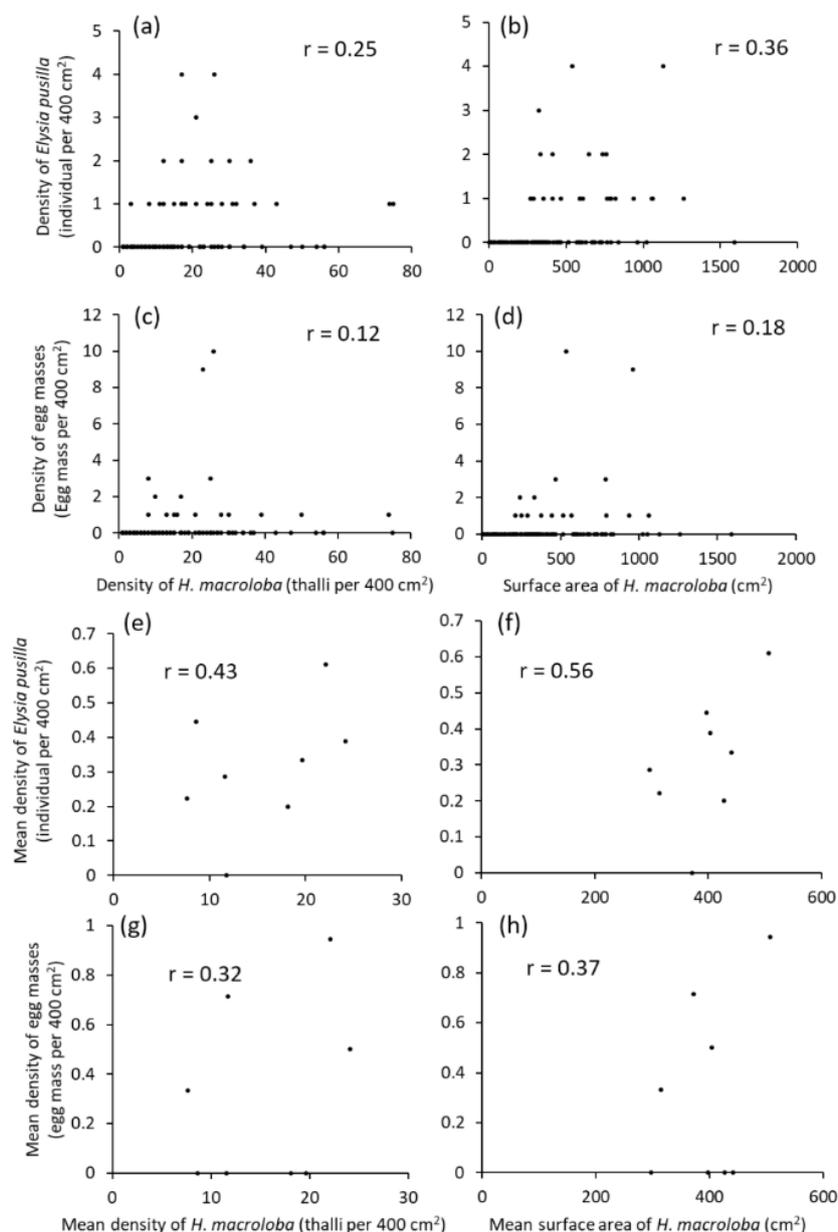


Figure 5 Relationships between the density of *Elysia pusilla*, its egg masses and *Halimeda macroloba* density and surface area (a) - (d) and between mean densities of *E. pusilla*, its egg masses and *H. macroloba* mean density and surface area (e) - (h). Analyzed data were pooled across months. For (e) - (h), relationships were analyzed from monthly averages of all variables.

A total of 39 slugs were found in this study (**Figure 6**). Analysis suggested that slugs were significantly more likely to be found on stage 4 of *H. macroloba* thalli than on other stages ($\chi^2 = 12.35$; $p < 0.01$; **Figure 6(a)**). The influence of algal life history stage on the occurrence of egg masses ($\chi^2 = 1.51$; $p = 0.68$) and grazing marks ($\chi^2 = 2.56$; $p = 0.46$) was not significant. However, the highest numbers of egg masses and grazing marks were observed on stage 4 thalli (**Figures 6(b) - 6(c)**). The surface area of *H. macroloba* individuals at different life history stages varied ($F = 143.69$; $p = 0.001$; **Figure 6(d)**). Individual thalli of stage 4 showed the largest average surface area and individual thalli of stage 1 showed the smallest average surface area. This finding suggests that larger, mature thalli were preferred as shelter, nursery, and feeding grounds. The amount of space (surface area of *H. macroloba*) might be important in determining habitat use by the slug.

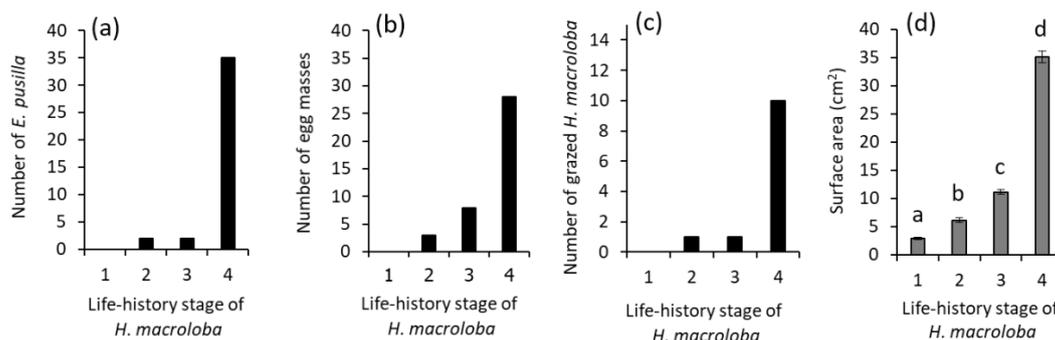


Figure 6 (a) Numbers of *E. pusilla*, (b) and numbers of egg masses found on thalli of *H. macroloba* at each different life-history stage. (c) Numbers of *H. macroloba* thalli at each stage that presented grazing marks and (d) surface area (mean \pm SE) of *H. macroloba* thalli at each life-history stage (stage 1, n = 85; stage 2, n = 172; stage 3, n = 500; stage 4, n = 1169).

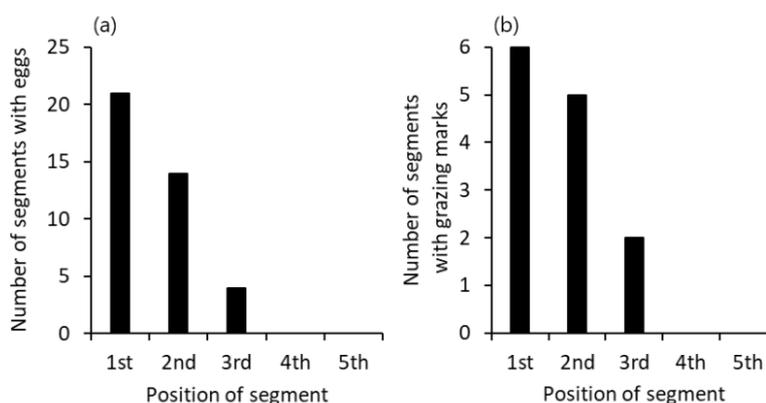


Figure 7 (a) Numbers of thallus segments at different positions along *H. macroloba* thalli that present egg masses: (b) Numbers of segments at different positions that presented grazing marks.

The chemical characteristics of *Halimeda* vary by age and by location on the thalli. These variations may also influence the behavior of slugs toward their hosts [15,17]. In our study, egg masses and grazing marks were present only on the 1st, 2nd, and 3rd segments of *H. macroloba*. The numbers of both variables decreased from the 1st segment to the 3rd (Figures 7(a) - 7(b)). The terminal segment of *H. macroloba* contains a higher concentration of secondary metabolites [15] that the slugs can utilize and has little accumulated calcium carbonate, which makes grazing easier [15]. Paul and Van Alstyne [17] also reported that *E. pusilla* (as *E. halimeda*) and its egg masses were found primarily on the terminal segments of *H. macroloba*. They suggested that the slug sequestered secondary metabolites from the algae and stored modified compounds for its own defense against predators. Not only the slug tissue but also the egg masses contained the compounds.

The prevalence of egg masses on terminal segments might result from selective behavior by the slugs. By depositing their eggs on terminal segments, they might reduce the disturbance of their eggs, as lower segments have a greater chance of being covered up by sediment. However, the higher occurrence of eggs on terminal segments might simply be due to the greater proportion of terminal segments, compared to other segments, in the total surface area of thalli. Moreover, it is important to note that the growth rate of *H. macroloba* is high, at 1 - 2 segments per day [12], and the position where an egg mass was found might not always indicate the position where it was laid. Egg masses on the 2nd and 3rd segments might have been laid on what were previously terminal segments.

This study increases our understanding of the relationship between the sacoglossan sea slug *E. pusilla* and its algal host, *H. macroloba*. It also reports the population dynamics of a slug species in a natural habitat. The descriptive ecological information and knowledge provided were gained from testing

hypotheses proposed for the habitat use of *E. pusilla*. The obtained descriptive ecological information concerning the life history of the slug and its relationship with the algal host can serve as a basis for future research on this species. Additional studies should be conducted to determine the effects of environmental factors on the population dynamics of the slug. Investigations of the life cycle and behavior of *E. pusilla* will also be required. Future research may also include more study sites where the examination of seasonal covariation between slug and algal abundance is more feasible.

Conclusions

Despite the wide distribution range of *E. pusilla* in the Indo-Pacific region, the study of this marine herbivore has been neglected and only a few published works have focused on the species. Here, with 1 exception in March 2020, *E. pusilla* was collected from the study site throughout the study period and temporal variation in its abundance was not detected. *E. pusilla* exhibits a habitat selection. The slug largely feeds and lays eggs on terminal segments of mature thalli of *H. macroloba*. An association between the abundance of *E. pusilla* and *H. macroloba* suggests that the population density of the slug is subject to change according to the availability of the algal host. Environmental stressors that influence the abundance of *H. macroloba* influence the abundance of the slug population indirectly.

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