Population Dynamics and Behavioral Ecology of *Halimeda* slug *Elysia pusilla* (Bergh, 1872)

Apisara Nakpan

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology (International Program)

Prince of Songkla University

2022

Population Dynamics and Behavioral Ecology of *Halimeda* slug *Elysia pusilla* (Bergh, 1872)

Apisara Nakpan

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology (International Program)

Prince of Songkla University

2022

Thesis Title	Population dynamics and behavioral ecology of *Halimeda* slug *Elysia pusilla* (Bergh, 1872)
Author	Miss Apisara Nakpan
Major Program	Biology (International Program)

Major Advisor	**Examining Committee:**
....	....Chairperson
(Asst. Prof. Dr. Kringpaka Wangkulangkul)	(Asst. Prof. Dr. Phuripong Meksuwan)
Co-advisor	....Committee
	(Asst. Prof. Dr. Jaruwan Mayakun)
....	
(Asst. Prof. Dr. Jaruwan Mayakun)	....Committee
	(Asst. Prof. Dr. Kringpaka Wangkulangkul)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Master of Science Degree in Biology (International program)

....

(Prof. Dr. Damrongsak Faroongsarng)

Dean of Graduate School

This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

....Signature
(Asst. Prof. Dr. Kringpaka Wangkulangkul)
Major Advisor

....Signature
(Asst. Prof. Dr. Jaruwan Mayakun)
Co-advisor

....Signature
(Miss Apisara Nakpan)
Candidate

I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

....Signature

(Miss Apisara Nakpan)

Candidate

ชื่อวิทยานิพนธ์ พลวัตประชากร และนิเวศวิทยาพฤติกรรมของทากทะเลสาหร่ายใบมะกรูด *Elysia pusilla* (Bergh, 1872)

ผู้เขียน นางสาวอภิสรา นาคแป้น

สาขาวิชา ชีววิทยา

ปีการศึกษา 2564

บทคัดย่อ

Elysia pusilla (Bergh, 1872) เป็นทากทะเลในกลุ่ม Sacoglossa ซึ่งเป็นสัตว์กินพืช โดยกินเฉพาะสาหร่ายใบมะกรูด (*Halimeda* spp.) ทากชนิดนี้สามารถเก็บคลอโรพลาสต์จากสาหร่ายที่มันกินเข้าไปและนำมาใช้ประโยชน์ได้ คุณสมบัตินี้เรียกว่า Kleptoplasty การกระจายตัวของทากชนิดนี้จะอยู่ในบริเวณเขตร้อนของอินโด-แปซิฟิก มีการศึกษามากมายเกี่ยวกับวิวัฒนาการความสัมพันธ์แบบ symbiotic ของทากกับสาหร่ายที่เป็นแหล่งอาศัย และคุณสมบัติ Kleptoplasty แต่การศึกษาในด้านพลวัตรประชากรของทากในธรรมชาติในช่วงระยะเวลาหนึ่งรวมถึงความสัมพันธ์ระหว่างความชุกชุมของทากและสาหร่ายที่เป็นที่แหล่งอาศัยยังมีการศึกษาน้อย ดังนั้นในการศึกษาครั้งนี้จึงสนใจศึกษาพลวัตรประชากรของทาก *E. pusilla* ในธรรมชาติ ความสัมพันธ์ระหว่างความชุกชุมของทาก *E. pusilla* และสาหร่ายที่เป็นแหล่งอาศัย และนิเวศวิทยาพฤติกรรมของทาก *E. pusilla* กับสาหร่าย *Halimeda macroloba* โดยผลการศึกษาแสดงให้เห็นว่าความหนาแน่นของสาหร่าย *H. macroloba* และก้อนไข่ของ *E. pusilla* มีความแปรปรวนในช่วงระยะเวลาที่ทำการศึกษา แต่ความหนาแน่นของตัวทากกลับไม่มีความแปรปรวนในช่วงระยะเวลาดังกล่าวเนื่องจากจำนวนทากที่พบมีจำนวนน้อย ผลการวิเคราะห์คาดว่าความหนาแน่นและพื้นที่ผิวของ *H. macroloba* ที่ทากสามารถใช้ประโยชน์ได้ อาจจะเป็นตัวกำหนดการปรากฏของทาก *E. pusilla* และก้อนไข่ และจะมีโอกาสสูงมากที่จะพบทากในบริเวณที่มี หย่อมสาหร่ายที่หนาแน่นสูงเนื่องจากมีพื้นที่ใช้ประโยชน์ที่มากกว่า

ในการศึกษาครั้งนี้ อายุของสาหร่ายถูกจัดกลุ่มเป็น 4 ระยะ โดยระยะที่ 1 เป็นระยะที่สาหร่ายเพิ่งลงเกาะใหม่จนถึงระยะที่ 4 ซึ่งเป็นระยะที่สาหร่ายเจริญเติบโตเต็มที่ จำนวนสูงสุดของทาก ก้อนไข่ และต้นสาหร่ายที่ถูกกิน พบในสาหร่ายระยะที่ 4 ซึ่งเป็นระยะที่สาหร่ายเจริญเติบโต

เต็มที่ และมีพื้นที่ผิวที่กว้างที่สุดมากกว่าระยะอื่นๆ จำนวนสูงสุดของก้อนไข่ และแผ่นใบ (segment) ที่พบรอยกัดของทาก พบบนบริเวณปลายสุดของแผ่นใบของต้นสาหร่าย ซึ่งบริเวณนี้เป็นบริเวณที่มีความเข้มข้นของ secondary metabolites สูง สาร secondary metabolites เหล่านี้จะถูกทากนำไปใช้เพื่อปกป้องตัวเองจากผู้ล่า นอกจากนี้บริเวณปลายสุดของแผ่นใบเป็นบริเวณที่มีการสะสมหินปูนน้อยซึ่งจะช่วยให้ทากครูดสาหร่ายกินได้ง่ายขึ้น สัดส่วนของบริเวณด้านบนของแผ่นใบและบริเวณปลายสุดของแผ่นใบ มีพื้นที่กว้างกว่าบริเวณแผ่นใบส่วนฐาน ดังนั้นบริเวณด้านบนของแผ่นใบจะครอบครองพื้นที่มากที่สุดซึ่งแสดงถึงพื้นที่ผิวที่มากกว่า ซึ่งอาจจะเป็นอีกหนึ่งปัจจัยที่ส่งผลต่อการปรากฏของก้อนไข่และรอยกัดที่มากกว่า และพื้นที่ผิวใบของสาหร่ายอาจเป็นปัจจัยสำคัญในการเลือกที่อยู่อาศัยของทากชนิดนี้

คำสำคัญ : การพึ่งพาอาศัยกัน, *Elysia, Halimeda*

Thesis Title Population dynamics and behavioral ecology of *Halimeda* slug *Elysia pusilla* (Bergh, 1872).

Author Apisara Nakpan

Major Program Biology (International Program)

Academic year 2021

ABSTRACT

Elysia pusilla (Bergh, 1872) is a sacoglossan sea slug that feeds on calcified green algae, *Halimeda* spp. It can incorporate and maintain chloroplasts from its algal food in its digestive glands, exhibiting kleptoplasty. The slug distributes in tropical Indo-Pacific. Evolution, symbiotic relationship, and kleptoplastic ability of sea slug have been well documented. However, less is known about their temporal variation in natural populations including the relationship between abundance of slugs and its algal hosts. Therefore, in this study, population dynamics of *E. pusilla*, the relationship between abundance of *E. pusilla* and its algal hosts, *Halimeda macroloba*, and behavioral ecology of *E. pusilla* were investigated at Lidee Island, Satun province. The results show that there was temporal variation in density of *H. macroloba* and *E. pusilla* egg masses but there was no variation in density of *E. pusilla* individuals because the number of the slugs was low. The analysis suggested that density and total surface area of *H. macroloba* which is the habitat availability might determine the occurrence of the slugs and the egg masses. There was a higher occasion to find the slugs in dense patches of the algae in which it related to surface area of the algal host.

In this study, the age of algae was categorized into 4 stages, stage 1 is the new recruitment and stage 4 is mature plant. The highest number of slugs, egg masses and grazing marks were found on stage 4 which has the largest surface area comparing to the other stages. The highest number of egg masses and segments with grazing marks were found on the terminal segments. This part of the thalli has high concentration of secondary metabolites. These secondary metabolites reported to be utilized by slugs to deter their predators. In addition, terminal segments have thin calcification which helped the slugs graze easier. Moreover, the upper segments including terminal segments have larger surface area than the basal segments, reflecting the larger area of habitat use for the slugs. It might be another reason of a high occurrence of egg masses and grazing marks. Therefore, the amount of surface area which is related to the availability of habitat might be the important factor of habitat selection in *E. pusilla*.

Keywords : symbiosis, *Elysia*, *Halimeda*

ACKNOWLEDGEMENTS

First, I sincerely appreciate to Assistant Professor Dr. Kringpaka Wangkulangkul (my advisor) who inspire, guided and supported me throughout this thesis project and I am deeply grateful to Assistant Professor Dr. Jaruwan Mayakun (my co-advisor) who support and give the advice to throughout the work also.

I would like to appreciate to Professor Patrick J. Krug of Biology Science, California State University for guiding the work, sharing his experience and helping with slug identification.

I gratefully thank for members of the Coastal Ecology Research Lab, Prince of Songkla University, for their support, suggestions, encouragement and assistance with field work.

I also thank members of the Seaweed and Seagrass Research Unit, Prince of Songkla University, for their support, suggestions and assistance with field work.

I thank to my parents for the support

Finally, I thank to my friends for supporting other necessity

Apisara Nakpan

CONTENT

LIST OF TABLES

LIST OF FIGURES

Lists **Page**

LIST OF FIGURES (CONTINUOUS)

LIST OF FIGURES (CONTINUOUS)

Lists **Page**

LIST OF FIGURES (CONTINUOUS)

Lists **Page**

INTRODUCTION

Background and rationale

Elysia pusilla (Bergh, 1872) or *Halimeda* slug is a non-shelled sea slug in the order Sacoglossa (Subclass Heterobranchia) which is known as a marine suctorial herbivore (Jensen, 1997; Trowbridge & Todd, 2001; Baumgartner & Toth, 2014). This species was found throughout the tropical Indo-Pacific (Vendetti et al., 2012; Jensen, 2015; Krug et al., 2016). The radula of this slug group was specifically modified to feed on their algal host and *E. pusilla* only consumes the calcified green algae in the genus *Halimeda*. (Jensen, 1993; Händeler & Wägele, 2007; Ponder et al., 2020). They also use the algae as habitat and nursery ground where they lay eggs. *E. pusilla* has symbiotic relationship with its algal food called kleptoplasty similar to other species in this genus. *Elysia* can retain chloroplasts from algae and obtain sugars from photosynthesis when the food is lacking (Pierce et al., 1999; Vendetti et al., 2012; Middlebrooks et al., 2019; Ponder et al., 2020). Moreover, the slug's body color reflects the color of chloroplasts from algal food which help them camouflage (Brandley, 1984; Jensen 1997; Händeler et al., 2009; Krug et al., 2016; Ponder et al., 2020). Moreover, some non-shelled marine slug ingests the noxious chemicals for antipredator defense including *E. pusilla*. It ingests the diterpenoids from its algal host and the chemical is used to protect itself from the predators (Paul & Van Alstyne, 1988b; Ponder et al., 2020).

One of the largest *Halimeda* meadows in Thailand is on a mudflat located between Lidee Lek and Lidee Yai Island, Satun province which comprises only one species of *Halimeda*, *Halimeda macroloba* Decaisne, 1841 (Pongparadon, 2009; Mayakun & Pratep, 2019). In this algal species, amount of calcium carbonate accumulation and chemical composition vary with age (Paul & Van Alstyne, 1988a; Mayakun & Pratep, 2019). Chemical compositions (secondary metabolites) also vary between different layers of segment. Younger plants and new segments have more secondary metabolites than mature plants and older parts (Paul & Van Alstyne, 1988a). Calcium carbonate and secondary metabolites are used as feeding deterrents to herbivores (Paul & Van Alstyne, 1988a). As *Halimeda* is the only food source for *E. pusilla*, variation in characteristics of the algal host might affect habitat selection, feeding and spawning behavior of *E. pusilla*.

Because the slug in this group is cryptic, the biology of sacoglossans are poorly known (Jensen, 1997). There are studies on the relationship between other *Elysia* and algal hosts. *E*. cf. *furvacuada* and *E*. *papillosa* fed on different algal species in different seasons to maximize the benefit from photosynthesis by algal chloroplasts (Brandley, 1984; Gowacki, 2017; Middlebrooks et. al., 2019). *E*. *tricinuata* of different ages chose different species of *Codium* (Trowbridge et. al., 2008). Although *E. pusilla* distributes widely in the tropical Indo-Pacific region, population dynamics, habitat selection and feeding ecology of the species are not well understood including the data of the relationship between the slug and its algal host. Even though *E*. *pusilla* was extremely specific to *Halimeda*, the data of the relationship still lacking. Abundance of algal host might be a key determinant of the abundance of slugs. Thus, the objectives in this study are 1.) to investigate the population dynamic of *E*. *pusilla* 2.) to investigate the relationship between *H*. *macroloba* and *E*. *pusilla* in terms of habitat use 3.) to investigate whether the number of slugs, egg masses and grazing mark differ between different life-history stages of *H. macroloba* and 4.) to investigate whether different positions of *H. macroloba* segment influence number of *E. pusilla*'s egg masses and grazing marks found on the thalli.

Review of literature

1. Characteristics of sacoglossan

Order Sacoglossa are well-known marine herbivores (Trowbridge & Todd, 2001; Baumgartner & Toth, 2014). All of the slugs in this order are suctorial feeders (Jensen, 1997). The sacoglossan is separated into shelled and non-shelled groups. Both of them consume different genus of green algae (Class Ulvophyceae). Most sacoglossan are stenophagous, consuming one or two genera of green algae (Jensen, 1983, 1997; Trowbridge & Todd, 2001) and some are euryphagy, consuming more than two genera of green algae (Jensen, 1994; Curtis et al., 2006; Curtis et al., 2010). Most of non-shelled sacoglossa mainly consume siphonalean or septate green algae (Clark & Busacca, 1978; Clark et al., 1990; Clark, 1992; Jensen, 1993). The non-shelled sacoglossan genus *Elysia* can eat several species of algae. Some of them feed on seagrasses, diatoms and opisthobranch eggs (Jensen, 1997).

Life history of most sacoglossans was different between different climatic zones (temperate and tropical zones). Species in tropical zone such as *Elysia australis* spawn and recruit throughout the year while the reproduction of temperate species is seasonal. For example, the temperate species *E. furvacauda* and *E. papillosa* reproduce in spring and early summer (Clark & Defreese, 1987; Brandley, 1984).

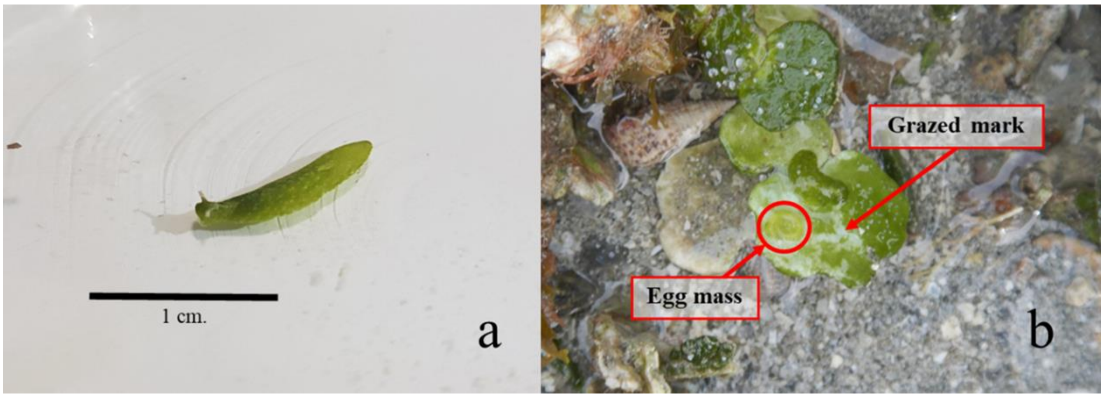

Fig. 1 a External morphology of *Elysia pusilla* **b** *Halimeda macroloba* was grazed by *E. pusilla*. An egg mass is also present.

2. General characteristics of *Elysia pusilla* (Bergh, 1872)

2.1 Classification

Kingdom	Animalia
Phylum	Mollusca
Class	Gastropoda
Subclass	Heterobranchia
Order	Sacoglossa
Family	Plakobranchidae
Genus	*Elysia* Risso, 1818
Species	*Elysia pusilla* (Bergh, 1872)

Elysia pusilla (Bergh, 1872) (*Halimeda* sea slug) was firstly described as *Elysiella pusilla* Bergh, 1872, and eventually relocated to genus *Elysia* as *Elysia pusilla* (Bergh, 1872) (Jensen, 1996; Bass & Karl, 2006; Händeler & Wägele, 2007). The synonym of this species is *Elysia halimedae* Macnae, 1954. *E. pusilla* is a species in the subclass Heterobranchia (=Opisthobranch) (Krug et al., 2016), order Sacoglossa (=Ascoglossan) and family Plakobranchidae which has approximately 350 species (MolluscaBase eds., 2021). In the case of *E. pusilla*, it only consumes *Halimeda* spp. (Jensen, 1993; Händeler & Wägele, 2007).

2.2 Biology

Elysia can sequester chloroplasts from algae and utilize sugars from photosynthesis (Pierce et al., 1999; Vendetti et al., 2012; Middlebrooks et al., 2019; Ponder et al., 2020). Thus, it could live without food for a long time (Middlebrooks et al., 2019). This phenomenon was described for the first time by Clark et.al. (1990) and is called kleptoplasty (Jensen, 1997). *E. pusilla* can retain plastids for a few weeks (Vendetti et al., 2012). It was reported that *E. chlorotica* can live normally without food for 9 months (Middlebrooks et al., 2019). *Elysia pusilla* has been found throughout the tropical Indo-Pacific such as Guam USA, Queensland Australia, Okinawa Japan and north of Singapore (Vendetti et al., 2012; Jensen, 2015; Krug et al., 2016).

Most heterobranchs are hermaphrodite (Schmitt et al., 2007; Ponder et al., 2020) including *Elysia* (Jensen, 1992; Schmitt et al., 2007). Reproduction of *Elysia pusilla* is unique because it can produce two distinct larval morphs, both planktotrophic and lecithotrophic larva, which is uncommon in invertebrates. The slug is the only example from tropical Indo-Pacific which was found by Vendetti et al. (2012) that has larval dimorphism. The character of sacoglossa slug's egg mass is a coiled cord (LaForge, 2006). One side of the egg mass was attached to the substrate. Its eggs were in membranous capsules enveloped by gelatinous matrix. Its extra-capsular yolk (ECY) is yellow-orange or orange ribbon inside transparent jelly matrix (Vendetti et al., 2012).

3. Characteristics of *Halimeda* J.V.Lamouroux, 1812

Classification

Kingdom	Plantae
Division	Chlorophyta
Class	Ulvophyceae
Order	Bryopsidales
Family	Halimedaceae
Genus	*Halimeda* J.V.Lamouroux, 1812

Halimeda J.V. Lamouroux, 1812 is a green calcified alga distributed in warm-temperate and tropical zone (Paul & Van Alstyne, 1988a; Sinutok, 2008; Pongparadon, 2009; Mayakun et al., 2012). Its thallus consists of several segments containing a multinucleate tubular cell with deposited calcium carbonate (Pongparadon, 2009). The algae developed the new segment at the terminal segment. Calcification starts after 12 to 36 hours (Paul & Van Alstyne, 1988a; Sinutok, 2008; Pongparadon, 2009). Calcium carbonate inside *Halimeda* thallus was deposited in form of aragonite crystal in the interfilament space (Borowitzka & Larkum, 1976).

Morphological characteristics, shape of thallus and segment, and type of holdfast are used for identification in the field (Pongparadon, 2009). *Halimeda* is dioecious having male and female gamete on different thalli (Pongparadon, 2009).

There are two types of reproduction in this genus, sexual and asexual reproduction. Asexual reproduction happens when a new thallus grows from fragmented thallus or segments or a new thallus is formed at the end of uncorticated siphons growing out either from segments or from filaments of the holdfast. Life span of *Halimeda* is different between species. There are 8 species of *Halimeda* reported in Thailand by Pongparadon (2009) which are *Halimeda discoidea*, *H. opuntia*, *H. heteromorpha*, *H. tuna*, *H. borneensis*, *H. gigas*, *H. macroloba* and one unknown species. In Thailand, *H. macroloba* is the most dominant and only species which was found in both Andaman Sea and Gulf of Thailand (Pongparadon, 2009; Mayakun & Pratep, 2019).

4. General characteristics of *Halimeda macroloba* Decaisne 1841

Halimeda macroloba Decaisne 1841 processes erect thallus with a bulbous holdfast. Most of them have fan-shape. The shape of segments is cuneate, rounded or ovate. Moreover, its irregular circle surface view of utricle is the differential diagnostic character of this species (Pongparadon, 2009). Its life span was around 8 to 12 months (Sinutok, 2008; Mayakun & Pratep, 2019). One to two segments were growing every day but this rate was varied and influenced by environmental factors (Sinutok, 2008; Mayakun et al., 2012). This species was found in Africa, Islands in Indian Ocean, Middle East South-east Asia, Asia, Australia, New Zealand and Islands in Pacific (Guiry & Guiry, 2021). The extensive meadows in Thailand located in Lidee Island, Satun; Libong Island, Trang; Similan Island, Phang Nga; Tang Khen Bay, Phuket; Pun Wa Promontory, Phuket in Andaman Sea (Pongparadon, 2009) and Ko Rab, Suratthani in Gulf of Thailand (Mayakun, Unpublished). At Lidee Island, Satun province, only one species *H. macroloba* Decaisne was reported. The highest abundance of the green alga in Thailand has been reported in intertidal zone at the Lidee Island (Mayakun & Pratep, 2019).

Life-history stage of *H. macroloba* was categorized into 6 stages by Sinutok (2008). New recruitment was defined as first stage. The second stage was young and non-calcified plant. The stage that accumulation of calcium carbonate is found in some segments is called 3rd stage. The 4th stage is mature plants which accumulation of calcium carbonate is found in every segment. A fertile plant is defined as 5th stage. In this stage, the plant produces gametangia clusters which are small green dots at the tips on upper segment. Dead plant was without chloroplast is called 6th stage.

Different amounts of calcium carbonate accumulation are found among different life-history stages. This accumulation of calcium carbonate is well-known as a morphological defense against herbivores (Paul & Van Alstyne, 1988a). There is more calcium carbonate accumulated in older stages (4-6) than young stages (1-3) (Mayakun & Prathep, 2019). In addition, the older alga has a longer and roundish needle than a younger alga (Mayakun & Prathep, 2019). The other variation of this species is a chemical composition. There are two major metabolites: halimedatrial and halimedatetraacetate. The character of these metabolites is feeding deterrents to herbivores (Paul & Van Alstyne, 1988a). The difference of chemical compositions occurs at the different ages of algae and layers of segment. The highest layer of segment is terminal segments and the youngest segments as well. There are more secondary metabolites in younger plant and newly segment. The effect of higher concentration from the youngest segments were influenced by lower accumulation of calcium carbonate (Paul & Van Alstyne, 1988a).

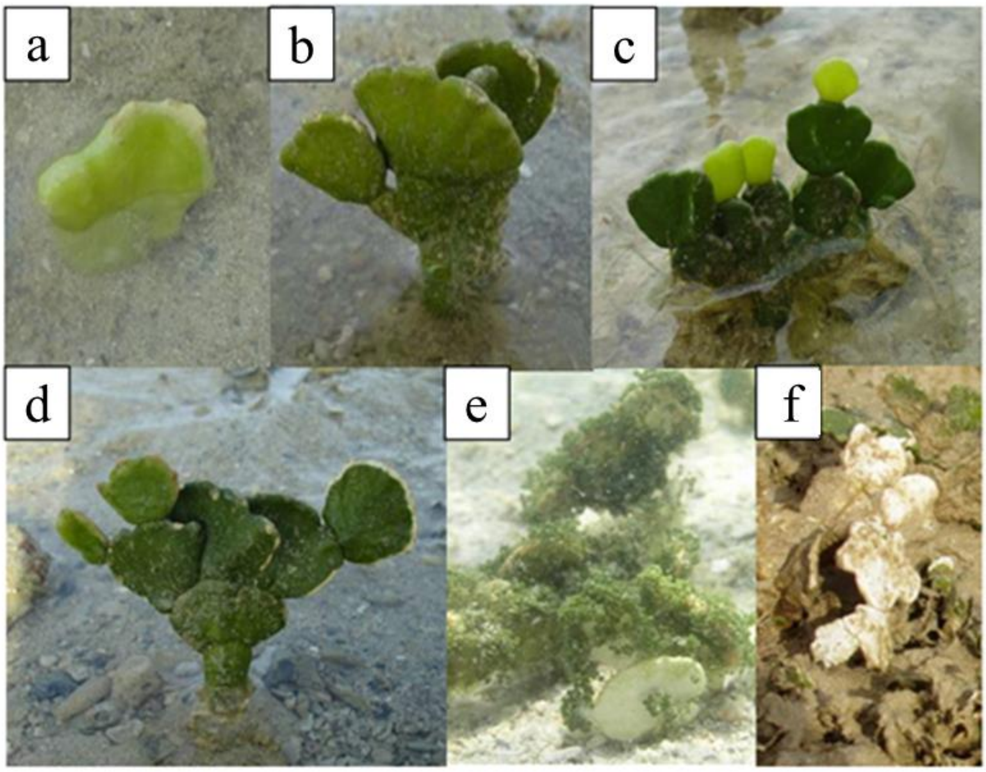

Fig. 2 Life-history stage of *Halimeda macroloba* (Sinutok, 2008; Mayakun & Prathep, 2019)

a stage 1: newly recruited plant

b stage 2: young plant

c stage 3: partially-calcified plant

d stage 4: mature plant

e stage 5: fertile plant

f stage 6: dead plant

5. Relationship between Sacoglossa and macroalgae

Relationship between sacoglossan and their algal host was recognized as the predator and prey interaction (Jensen, 1997). Radula teeth of sacoglossans were designed for different types of algae (Jensen, 1993). So, there are a number of research focused on the specificity of algal group and the slugs (Jensen, 1993, 1997; Krug et al., 2016). Even though the habitat and food of the slugs were known, it was hard to see the slug in the field because the color of them is similar to their host. Some sacoglossa species has low density. Moreover, most of this sacoglossa group has a small size

(Jensen, 1997). *Elysia* uses the algal host as a habitat and food. The clumps of the algal host are used as the shelter (Jensen, 1993; Händeler & Wägele, 2007; Krug et al., 2016). Normally, the body of this slug is flat and it well griped the flattened surface of algal host (Krug et al., 2016). Moreover, the co-adaptation of slug color and the host might help them to disguise themselves from predators (Händeler et al., 2009; Krug et al., 2016). Because of these reasons, the sacoglossans are difficult to study in terms of ecology (Jensen, 1997). There are reports about the relationship between the slugs and algal host on many scales: part of algal preference for different parts of algae (Paul & Van Alstyne, 1988b; Trowbridge, 1993), different species of the algae in the same genus that the slugs prefer (Trowbridge et. al., 2008), preference for different algal genera (Trowbridge et. al., 2008) and the fluctuation in population of slug and its host (Brandley, 1984; Gowacki, 2017; Middlebrooks et. al., 2019).

Objectives

1. To investigate population dynamics of *Elysia pusilla* in a *Halimeda macroloba* meadow at Lidee Island, Satun province by examining temporal variation in abundance of slugs with different age classes. The presence of egg masses was monitored.
2. To investigate whether abundance of the slug *E. pusilla* is a host-density-dependent. The abundance of the slug might be regulated by density of *H. macroloba*.
3. To test whether abundance of the slug *E. pusilla* varies according to the life-history stages of *H. macroloba*. In this study, the different ages of algae are divided into number of life-history stages. The abundance of the slugs might be regulated by the life-history stage of *H. macroloba*.
4. To test whether life-history stage of *H. macroloba* and position of segments influence number of the egg masses. The abundance of egg masses might be regulated by life-history stage and position of segments of algae.
5. To investigate whether the grazing marks of the slug were influenced by different positions of segments of *H. macroloba* and life-history stage of the algae. The different positions of segment and each life-history stage might influence number of grazing marks.

Research goal

The goal of this study is to investigate population dynamics of *Elysia pusilla* in a *Halimeda macroloba* meadow at Lidee Island, Satun province. Relationship between *E. pusilla* and *H. macroloba* in terms of habitat use was studied. In addition, there are variations in characteristics of *H. macroloba* thalli between life-history stages and characteristics of segments at different positions along thalli. These variations may affect the slug's feeding behavior and selection of place to lay egg masses. Therefore, the algal life-history stage influencing the presence of slugs, their egg masses and grazing marks were investigated. Position of egg masses and grazing marks on thalli of *H. macroloba* were also examined.

MATERIALS AND METHODS

1. Study site and field collection

The study site was located in *Halimeda macroloba* meadow between Lidee Lek and Lidee Yai Island (6° 46’58” N, 99° 46’10” E), Satun province, southern Thailand (Fig. 3a). The meadow was an intertidal sandy-mud flat where *H. macroloba* was the dominant species (Fig. 3b-e). Moreover, five species of seagrasses were observed including *Halophila ovalis* (R. Brown) J. D. Hooker, *Thalassia hemprichii* (Ehrenberg) Ascherson, *Cymodocea rotundata* Ascherson & Schweinfurth, *Syringodium isoetifolium* (Ascherson) Dandy, and *Enhalus acoroides* (L. f.) Royle (Mayakun & Prathep, 2019). *Elysia pusilla* was the only sacoglossa slug that was found on this *H. macroloba* meadow (personal observations). The *E. pusilla* and *H. macroloba* were collected monthly by random sampling from September 2019 to October 2020 except in February and September 2020 when field work was canceled because of rough sea, and from April to July 2020 because logistic difficulties during the COVID-19 pandemic in Thailand.

Eighteen of 0.04 m^2 (20 × 20 cm) quadrats were randomly placed in the *H. macroloba* meadow. The size and number of quadrats were determined as the sampling was practical in a limited time period of low tide. All *H. macroloba* thalli inside the quadrats were carefully collected. The holdfasts of each thallus were cut off to reduce the interference in searching for the slugs due to sediment and debris. Each thallus of the algae inside the quadrat was kept in a plastic bag and brought back to laboratory for counting the number of slugs. The samples were brought back because the slug was very small (1-19 mm) and the color of slugs was similar to their algal host. Accordingly, the slug cannot easily be seen in the field. The plastic bags with samples were placed in the undisturbed area for 1-2 hours to let the slug crawl out so the slug can be observed easier. After the number of the slugs was counted, the algae in the plastic bags were used to calculate the algal surface area.

The grazing mark on segment of *H. macroloba* was observed in the field before collection of the algal thalli. A grazing mark was bleached, white continuous area on *H. macroloba* thallus where *E. pusilla* was seen nearby. Several photos of segments/thalli which were grazed were taken immediately (Fig. 5a) because the marks faded away rapidly (personal observation). In the field, the boundary of grazing marks was not usually clear, so the number of grazing marks sometimes could not be counted. The number of segments of different positions along a thallus and the number of thalli of different life-history stages with grazed marks were counted instead.

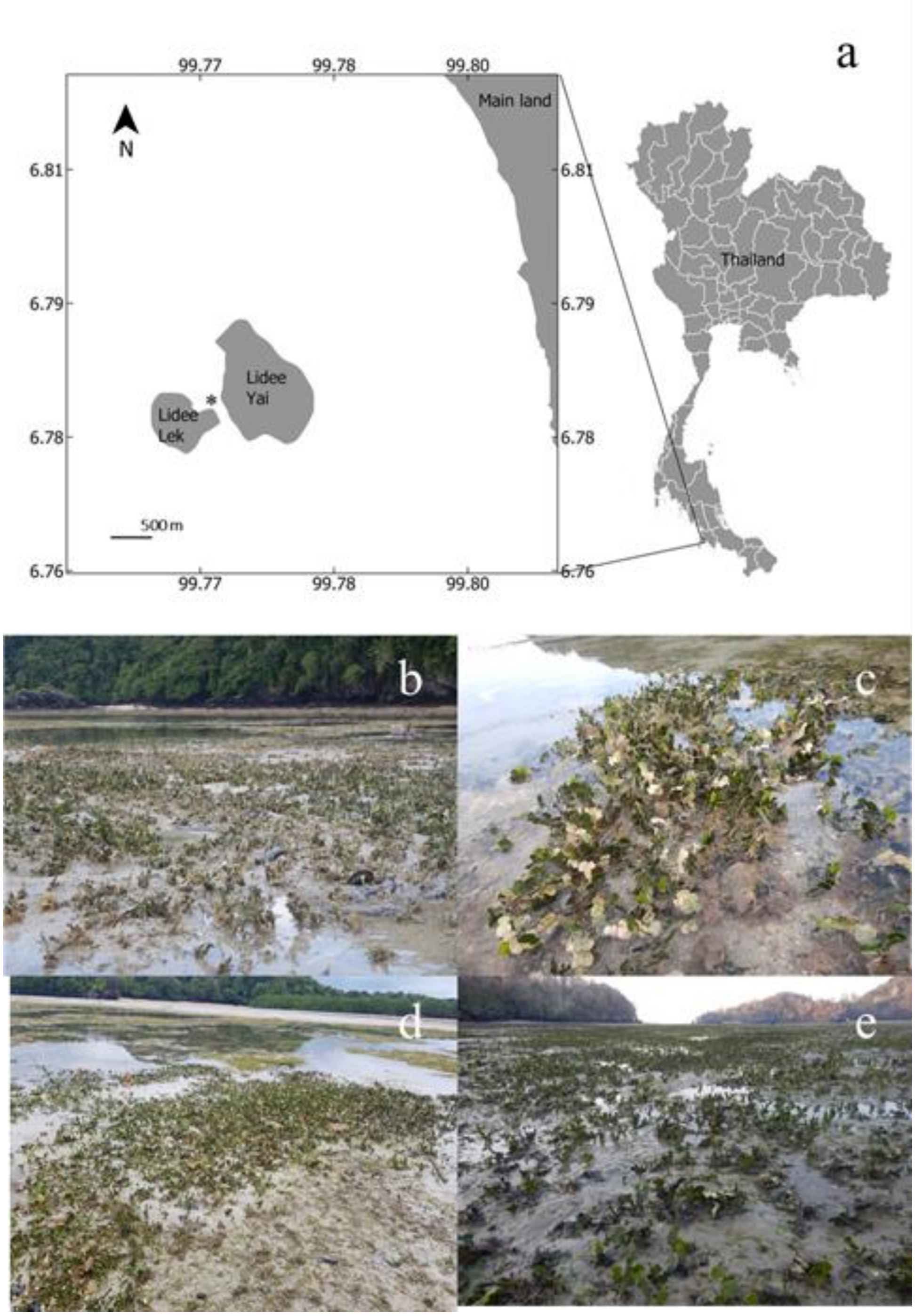

Fig. 3 **a** Location of sampling area (*) which is occupied by *Halimeda macroloba* meadow between Lidee Lek and Lidee Yai, Satun province in Andaman Sea, Thailand **b-e** *H. macroloba* meadow at low tide.

2. Data collection

2.1 Population dynamics and abundance of egg masses

Mean density of *Halimeda macroloba*, *Elysia pusilla* and egg masses of slug were examined monthly as described in (1.). Moreover, all slug specimens were measured for their body length (mm) in the laboratory to examine temporal variation in abundance of slugs with different age classes. The length was defined as the longest length of the body when the slug was crawling.

2.2 Relationship between abundance of slugs, egg masses and their habitat

Relationship between abundance of *E. pusilla*, its egg masses and their algal host, *H. macroloba*, was investigated. Density and surface area of algae were used as proxies of availability of habitat. Density of slugs, density of egg masses and density of algae (number of individuals per 400 cm^2) were assessed in each quadrat and the surface area of each thallus in each quadrat was combined to obtain a total surface area of algae per 400 cm^2.

To estimate the surface area of each algal thallus, the thallus was flattened on a tray (Fig. 4) and the photos of each thallus were taken. The photos were used to calculate and analyze the surface area using ImageJ© program.

2.3 Occurrence of slugs, egg masses and grazing marks on *Halimeda macroloba* of different life-history stages

In this study, life-history of *Halimeda macroloba* was categorized into 6 stages which were referred from Sinutok (2008) and Mayakun and Prathep (2019). Newly recruit plant with only one segment was classified into stage 1 (Fig. 4a). Stage 2 was young plant that has one or two layers of segment with three or four segments (Fig. 4b). Partially calcified plant which has three or four layers of segment was grouped into stage 3 (Fig.4c) and stage 4 was mature calcified plant (Fig. 4d). Fertile plant which produced gametophores along the outer rim of segments was stage 5 and dead plant which have all segments bleached and brittle was stage 6. In this work, data from the thalli of stage 5 and stage 6 were not collected. Thalli of stage 5 were barely found on the date of collection and the slug has never been observed on this stage. Stage 6 was a dead plant and there was no slug was found (personal observation). The thallus where slugs, egg masses and grazing marks were found was categorized into the above mentioned stages and counted for numbers.

There was a report about *E. pusilla* laid jelly-like egg masses on *Halimeda* (Vendetti et al., 2012). In this study, the egg mass of *E*. *pusilla* on the segments of *H*. *macroloba* was found. There are three types of ECY (extra-capsular yolk) which were found in this research (Fig. 5b,c,d,e,f). There is a variation of egg mass found in one species. The 1^{st} type is a thin light green ribbons ECY between some capsules (Fig. 5d). The 2^{nd} type is a spiral orange ribbon of ECY in the jelly matrix (Fig. 5e) which was the same type as Vendetti et al. (2012) reported and these two types of egg mass were found in the container which has only *E*. *pusilla* inside (personal observation). The 3^{rd} in this study is a transparent jelly matrix with white blobs of ECY on the upper face of the egg mass (Fig. 5f). The last type of egg mass might possibly be *E*. *pusilla* egg mass. The personal observation found that its fertilized ova were deposited within a string of membranous capsules containing albumen (Vendetti et al., 2012). Although egg masses appeared to have various morphs, they were not categorized in the present study.

In addition, the surface areas of *H. macroloba* thallus at different life-history stages were compared to evaluate potential differences in the availability of the space that can be used by *E. pusilla*.

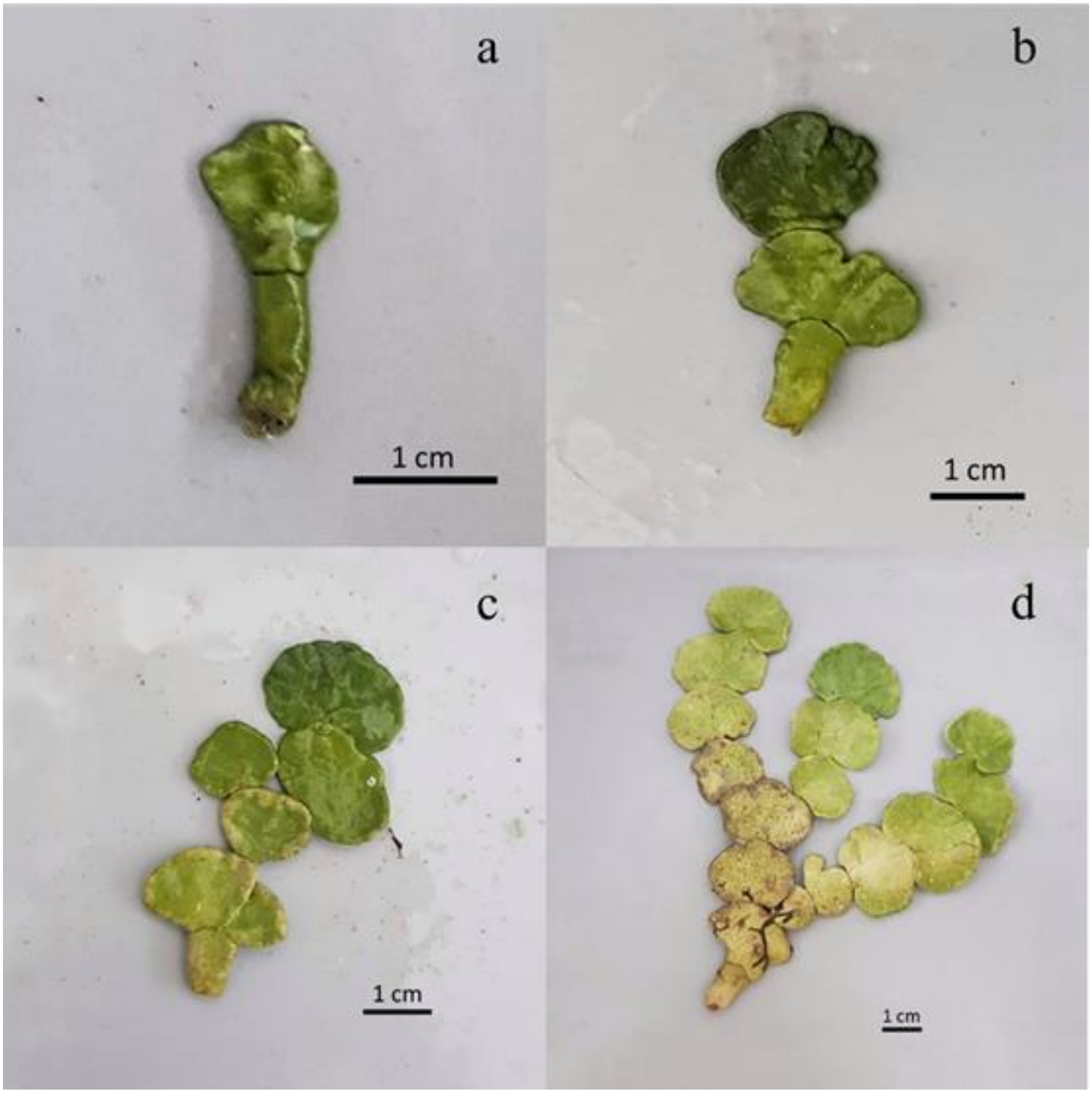

Fig. 4 *Halimeda macroloba* thalli with different life-history stages; **a**-**d** = stage 1-4, respectively.

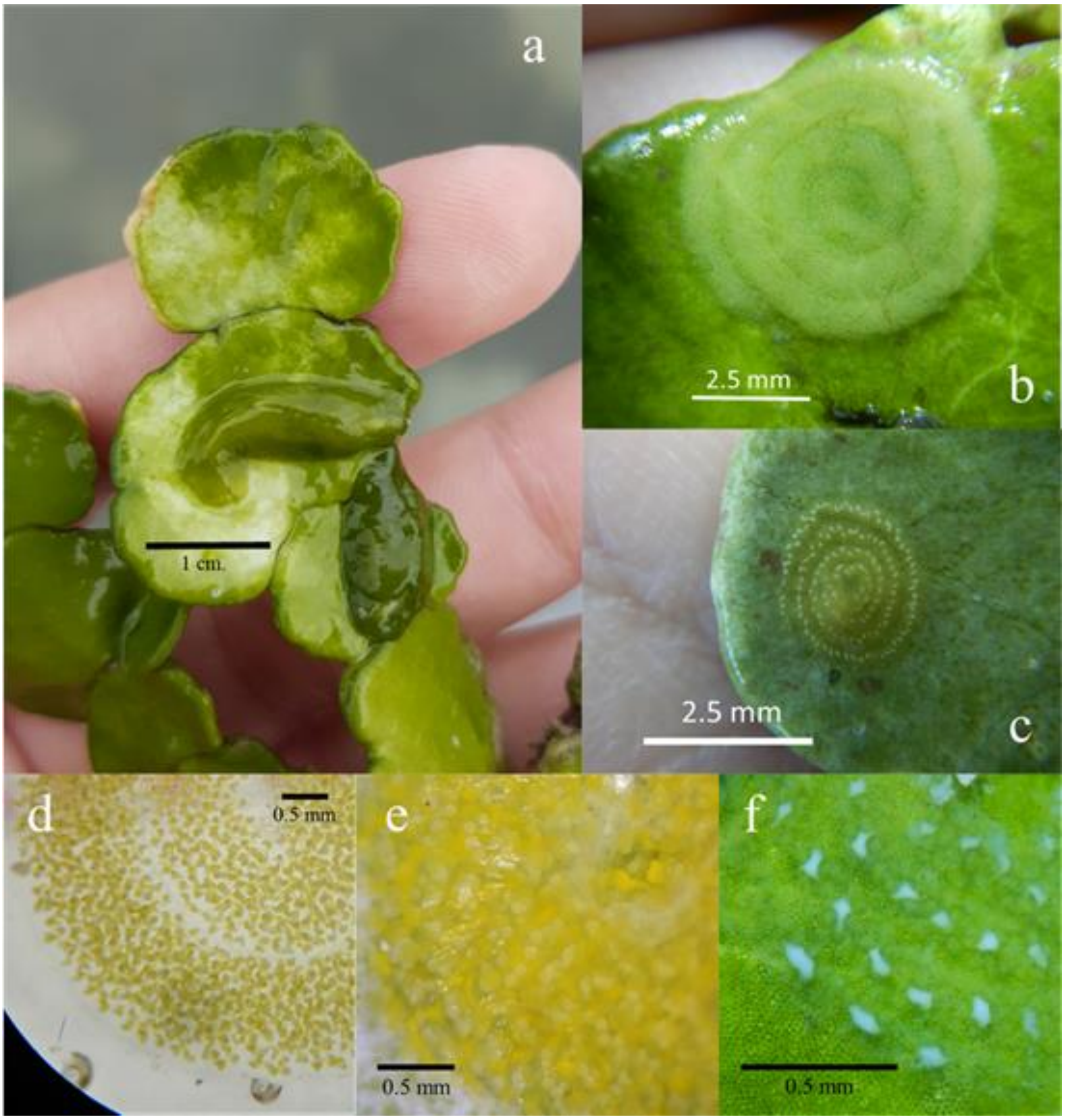

Fig. 5 a Two *Elysia pusilla* can be seen on a mature *Halimeda macroloba* (stage 4) thallus. Grazing marks are visible as white areas on the terminal and second segments. Photograph **b-f** The egg masses with **-d** thin light green ribbons ECY (extra-capsular yolk) between some capsules **-e** spiral orange ribbon of ECY in the jelly matrix **-f** transparent jelly matrix with white blobs of ECY on the upper face of the egg mass.

2.4 Occurrence of egg masses and grazing marks on different positions along *Halimeda macroloba* thalli

Position of *Halimeda macroloba* segments in each thallus was determined by referring from the longest branch of algae. Terminal segment of the longest branch was sorted as 1^{st} segment. The lower segments in the same branch were respectively categorized as 2^{nd}, 3^{rd}, 4^{th} segments. The position of segments on other branches was referred to the longest branch which is used as the reference (Fig. 6). The number of egg masses occurrence and the number of segments with grazing marks on the different positions of segment were recorded.

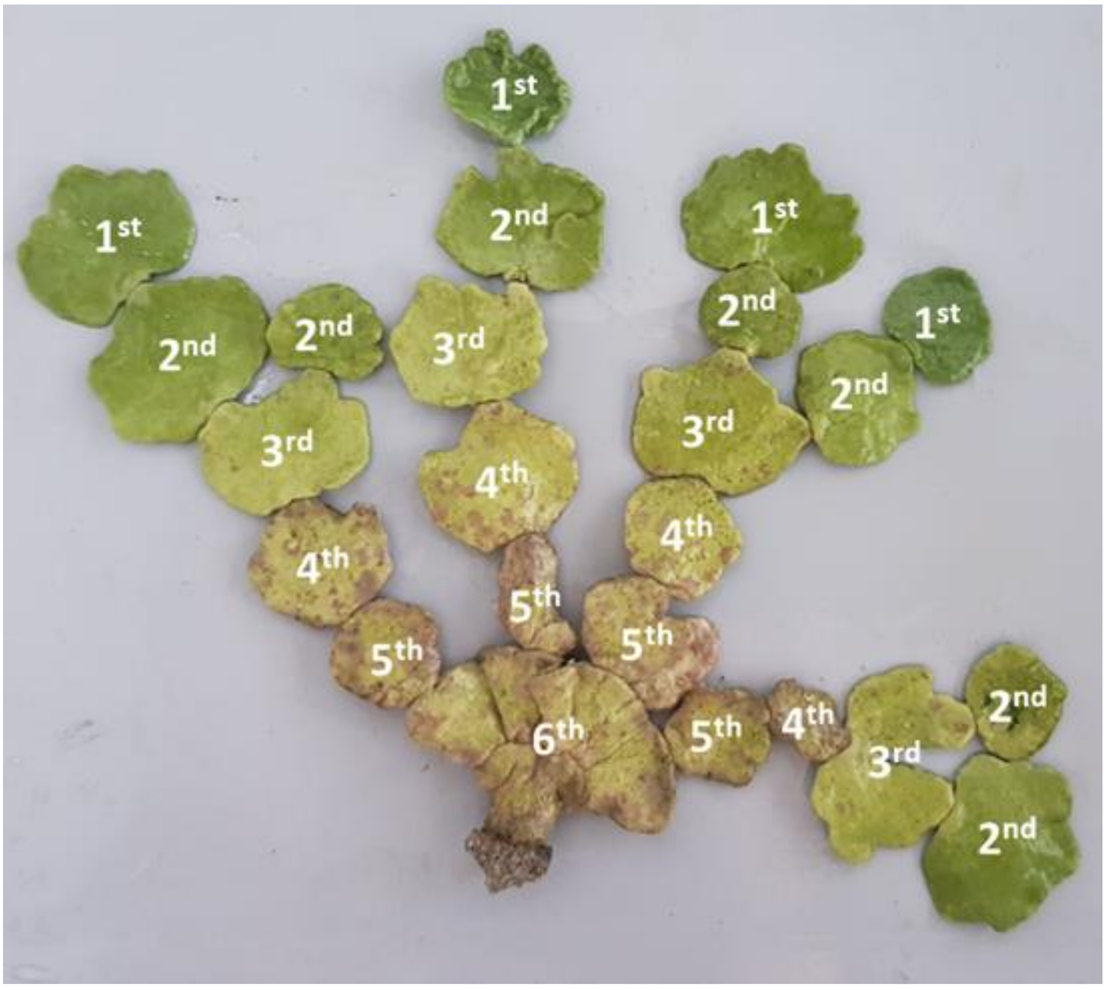

Fig. 6 Schematic diagram of *Halimeda macroloba* segments defined in this study.

3. Data analysis

One-way Analyses of variance (ANOVA) and Student Newman Keuls (SNK) test were used to examine temporal variation in abundance of *Halimeda macroloba* between eight months (fixed factor, 8 levels). Homogeneity of variance was tested by Cochran's C test. These analyses were done using the GMAV5 program. Temporal variation in abundance *Elysia pusilla* and its egg mass were analyzed by Kruskal-Wallis test due to high variance among months. Kruskal-Wallis test was done using RStudio program. Size frequency data of the slug in each month were presented as histograms.

Pearson correlation coefficient was used to test relationship between animals, egg masses and the algae. The relationship investigated was 1) density of *E. pusilla* and density of *H. macroloba*; 2) density of *E. pusilla* and total surface area of *H. macroloba* in a quadrat; 3) density of egg masses and density of *H. macroloba* and; 4) density of egg masses and total surface area of *H. macroloba* in a quadrat. Moreover, Analysis for relationships was divided into two parts; 1) all the data were pooled across months and; 2) the data were separated in each month and averaged to analyze for monthly relationship studying. Pearson correlation coefficient was performed using Analysis ToolPax in Excel, Microsoft office 365. Cohen's (1988) conventions were used to categorize the effect size. A correlation coefficient of 0.1,0.3 and 0.5 was classified as a small, moderate and strong correlation respectively.

One-way PERMANOVA which based on Euclidean similarity was used for the difference in surface area of *H. macroloba* between life-history stages. Stage of algal thalli was a fixed factor. One-way PERMANOVA was analyzed by Past4 (Hammer, Harper, & Ryan, 2001).

RESULTS

Population dynamics and abundance of egg masses

In this study, there was a temporal variation in abundance of *Halimeda macroloba*. The lowest density of *H. macroloba* was found from September to November 2019 and in March 2020 while the highest density of algae was found in December 2019, August and October 2020 ($F = 6.52$, $p < 0.001$; SNK; Fig. 7a). The density of *Elysia pusilla* was not different between months ($H = 10.18$, $p = 0.17$; Fig. 7b). There was a difference between density of egg masses in each month ($H = 33.25$, $p < 0.001$; Fig. 7b). The highest density of the slugs and egg masses was in August 2020. There was no slug in March 2020 and no egg mass in September, October 2019 and January 2020.

Mean length of the slug was 8.51±0.81 mm (n=39). The smallest was 1 mm which was found in September 2019 (Fig. 8a) and the largest was 19 mm recorded in December 2019 (Fig. 8d).

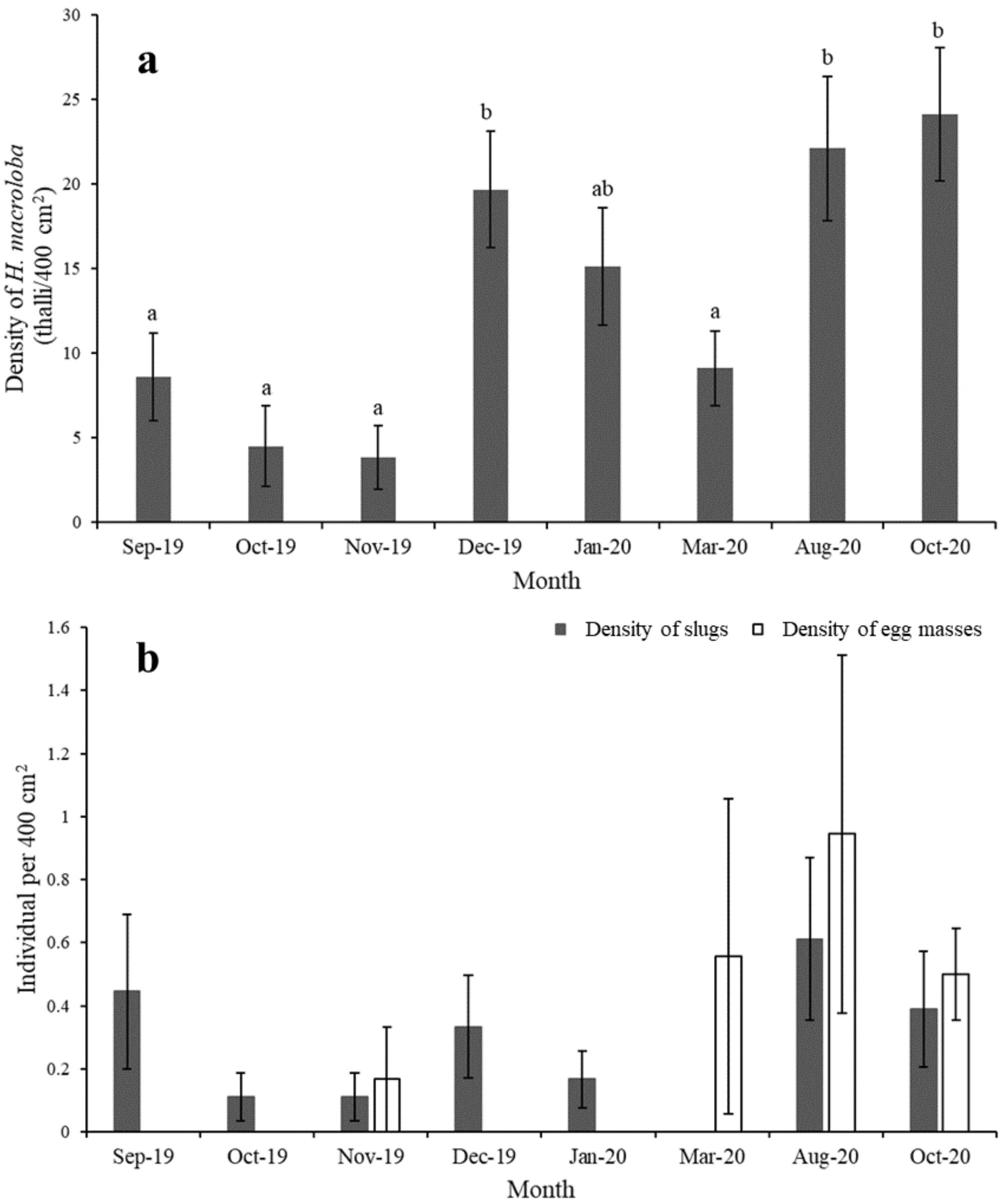

Fig. 7 a Density (Mean ± SE) of *Halimeda macroloba* and **b** *Elysia pusilla* and its egg masses in September 2019 to October 2020. In February and September 2020 field work was cancelled due to rough sea and in April to July 2020 because prevention of COVID-19 pandemic. Letters above bars: different letters = significant difference between months.

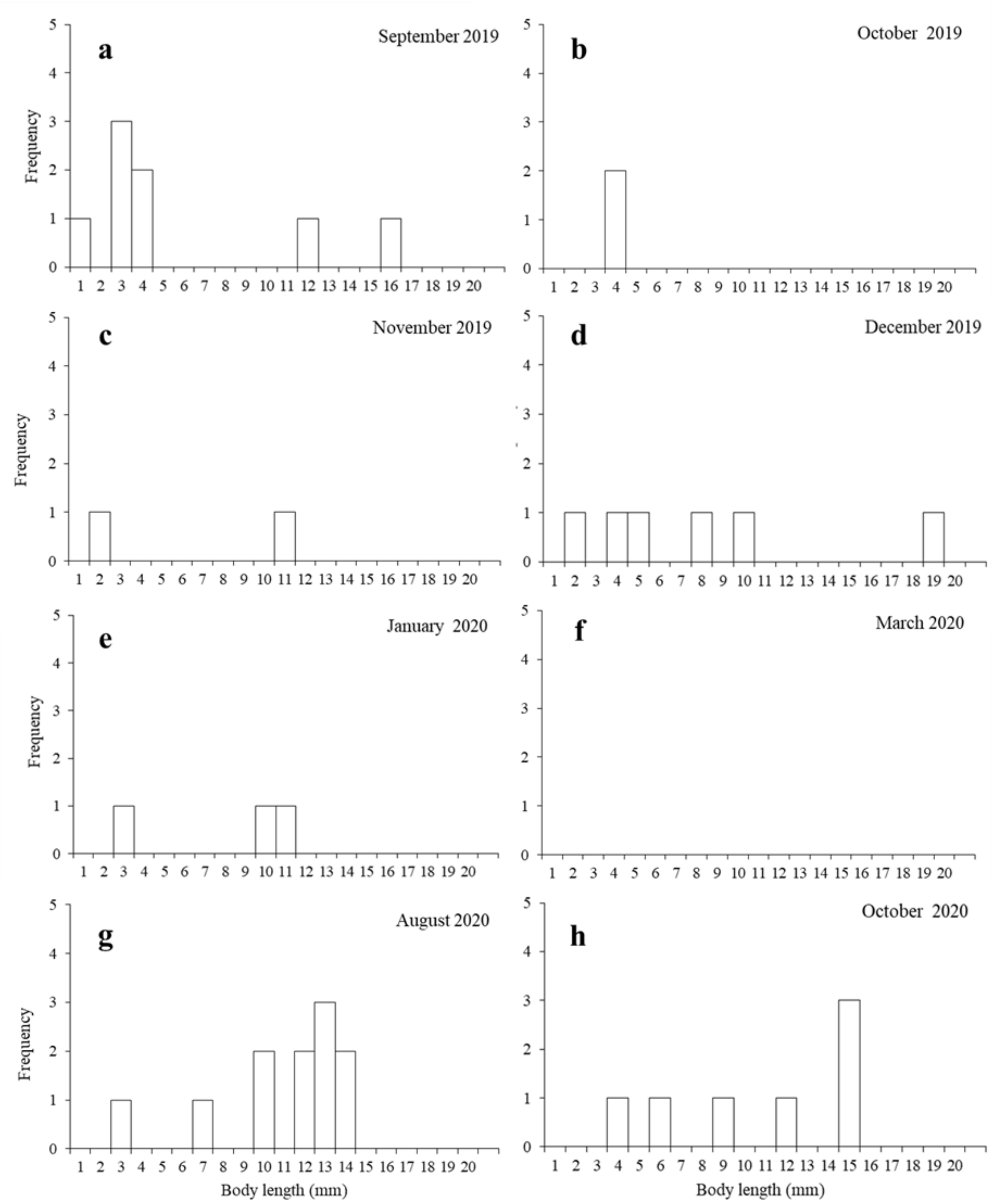

Fig. 8 Size frequency distribution of *Elysia pusilla*. Thirty nine *E. pusilla* specimens were found. Animals were collected in **a** September 2019 (n=8) **b** October 2019 (n=2) **c** November 2019 (n=2) **d** December 2019 (n=6) **e** January 2020 (n=3) **f** March 2020 (n=0) **g** August 2020 (n=11) and **h** October 2020 (n=7) In February and September 2020, the field work was cancelled due to rough sea and in April to July 2020 field work was cancelled because of COVID-19 pandemic.

Relationship between abundance of slugs, egg masses and their habitat

When data were pooled across months, there were a small to medium positive correlations between density of *E*. *pusilla*, its egg masses and density, total surface area of *H*. *macroloba* (r = 0.12 to 0.36; Fig. 9a-d). When monthly averaged data were analyzed, the results show that there was a medium positive correlation between mean density of the slugs, egg masses and mean density of the algae (r = 0.43,0.32; Fig 9e,g) as the relationship between mean density of the egg masses and mean total surface area of algae (r = 0.12; Fig. 9f). There was a strong positive correlation between mean density of the slugs and mean total surface area of the algae (r = 0.56; Fig. 9f).

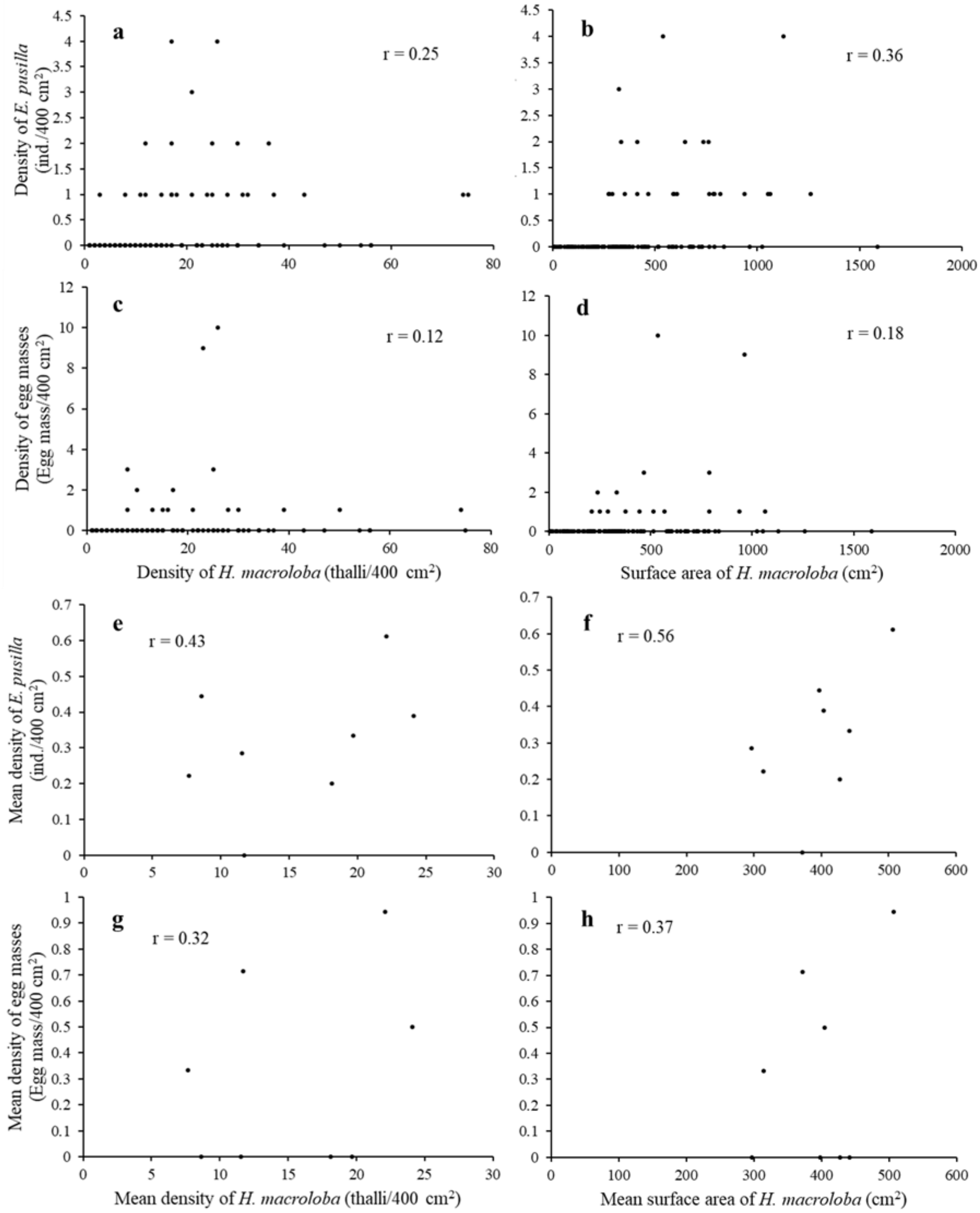

Fig. 9 Relationships between *Elysia pusilla*, its egg masses and *Halimeda macroloba*; **a**-**d** relationships between slug, its egg mass and algal host were analyzed from data pooled across months (September 2019 to January 2020, March 2020, August 2020 and October 2020); **e**-**h** relationships were analyzed from monthly averages of all variables.

Occurrence of slugs, egg masses and grazing marks on *Halimeda macroloba* of different life-history stages

A total of 39 *Elysia pusilla* specimens were found in this study. Analysis shows that the slug was significantly found on stage 4 *Halimeda macroloba* more than the other stages ($\chi2 = 12.35$; $p< 0.01$; Fig. 10a). The number of egg mass and the number of grazed *H. macroloba* were not influenced by the different life-history stages ($\chi2$ = 1.51, 2.56; p = 0.68, 0.46; Fig. 10b,c). However, the highest number of egg masses and grazing marks were observed on stage 4 thalli (Figure 10b,c). The average surface area of a thallus in each life-history stage was different (F = 143.69, p = 0.001). The thallus which has the largest surface area was stage 4 and the smallest surface area was stage 1 (Fig. 11).

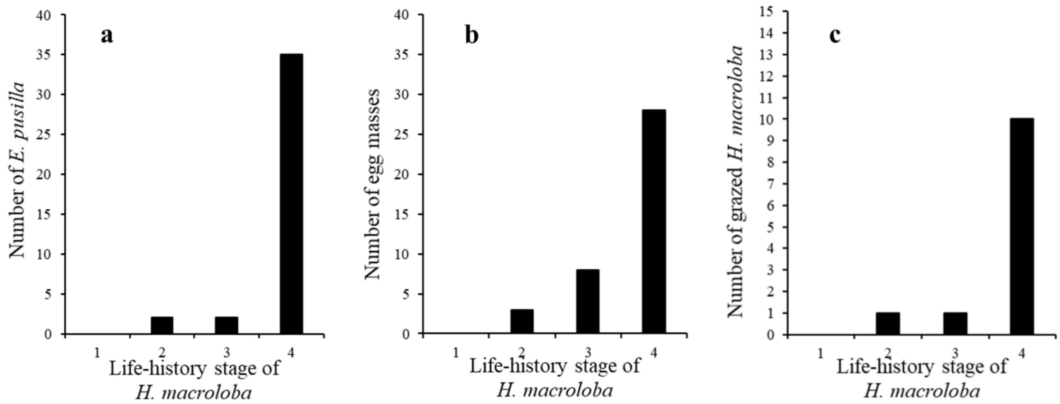

Fig. 10 a Numbers of *Elysia pusilla* (n=39), **b** numbers of egg masses (n=39) found on thalli of *Halimeda macroloba* at each different life-history stage; and **c** numbers of *H. macroloba* thalli at each stage that grazing marks were present (n=13).

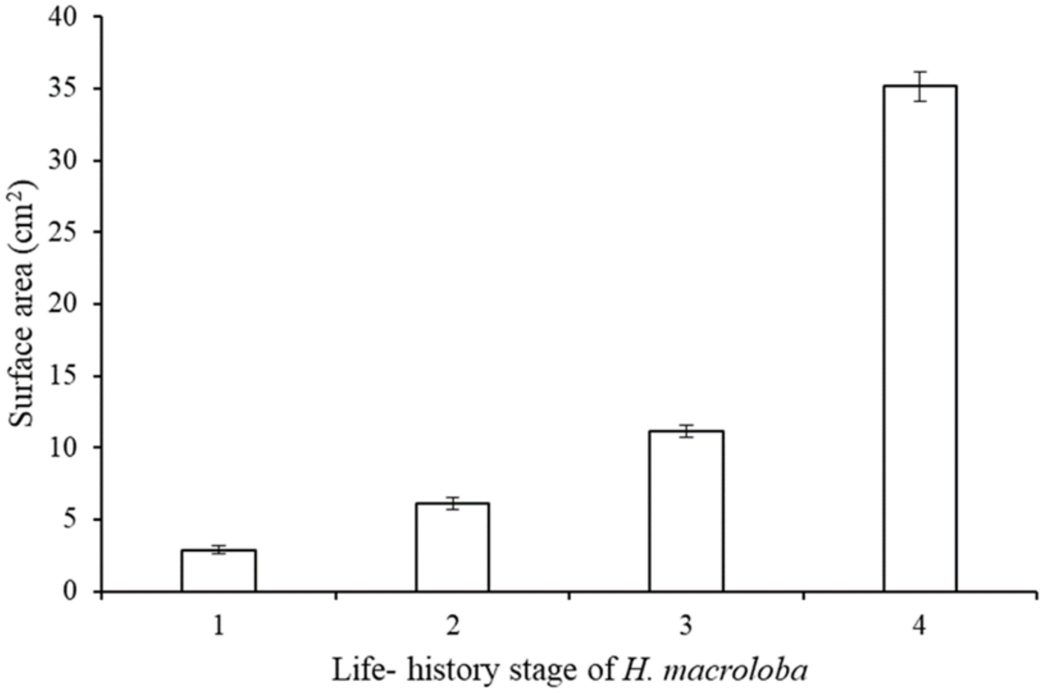

Fig. 11 Surface area (mean ± SE) of *Halimeda macroloba* thalli at each stage (stage 1, n=85; stage 2, n=172; stage 3, n=501; stage 4, n=1,168).

Occurrence of egg masses and grazing marks on different positions along *Halimeda macroloba* thalli

Numbers of egg masses and the segments with grazing marks were found only on the 1st to 3rd segments. The number of egg mass and the segments with grazing marks respectively decreased from the 1st to 3rd segments. (Fig. 16a, b).

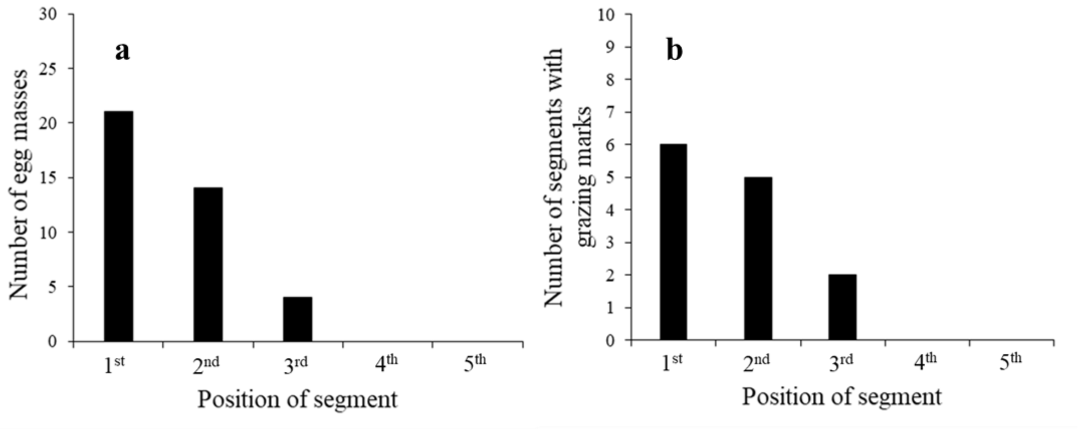

Fig. 12 a Numbers of egg masses on segments at different positions along *Halimeda macroloba* thalli (n=39); **b** Numbers of segments with grazing marks at different positions (n=12).

DISCUSSION

Population dynamics and abundance of egg masses

In this study, there was a variation in abundance of *Halimeda macroloba* between months. In September to November 2019 and March 2020, density of the algae was low. The high density of the algae was found in December 2019, August and October 2020. This result was different from Mayakun and Prathep (2019) reported that the lowest density of *H. macroloba* in the same site was found in September and the highest was found in March and the peak density of *H. macroloba* occurred in the dry season from February to April. The monsoon seasons appeared to have no effect on the population of *H. macroloba* in this study, and the fluctuation of algal density might be driven by other factors such as occasional high sedimentation or sudden increase in number of seagrasses (Sinutok, 2008).

The variation in abundance of *Elysia pusilla* between months was not found. There was no slug found in March 2020. The highest number of slugs was only four individuals per quadrat. There was a variation in abundance of slug's egg masses. The egg masses were found in November 2019, March, August and October 2020. Unfortunately, there was a logistic difficulty during the COVID-19 pandemic, thus the samples data were not collected continuously. In March 2020, there was a high density of the egg masses, but the slug was not found. The slugs might die after egg laying because of high energy expenditure during reproduction, However, the hypothesis was not investigated. The highest abundance of *E. pusilla* and its egg mass was found in August 2020 and October 2020. Moreover, the density of *H. macroloba* was also highest in August and October 2020. The higher abundance of algal hosts provides more habitat availability and more food sources. It might affect the number of *E. pusilla* in this population because egg production of the female in hermaphrodite species was limited by resources (Schmitt et al., 2007). Moreover, the higher density of algae can decrease water velocity within the patch more than the lower density (Panyawai et al., 2019). The decreased water velocity might reduce the risk of detachment of the slugs from their habitat due to wave action.

There were a few works that reported the size of slug in this species. In Japan, the range of slug length was 2-8 mm (Vendetti et al., 2012). In Singapore, the range size of the living samples was 5-7 mm, and the preserved animal size was 3 mm. (Jensen, 2009; Jensen, 2015). In this study, the biggest slug was 19 mm ever the biggest size which was recorded. The different sizes of animals in the same species might be from geographic variation. In the investigation of population dynamics which was examined by temporal variation in abundance of slugs with different age classes, the small size of *E. pusilla* was found every month except March 2020. This result suggested that there are recruitments throughout the year.

Relationship between abundance of slugs, egg masses and their habitat

The results show that there were small to moderate correlations between habitat availability (density and surface area of the algae) and abundance of slugs and their egg masses when data of the population were pooled across the months. The coefficient values were not strong, and it was similar to a work by Middlebrook et al. (2014). They show that spatial associations were not significant between *Elysia clarki* and its algal diets in macroalgal mixed patches because there was occurrence of *E. clarki* on other algae which were not its diets. In this study area, there was the only algal host, *H. macroloba*, with five species of seagrasses. The slugs might appear on the seagrasses which were not the slug's food. However, there was no report that this slug was found on the seagrasses. On the other hand, there were medium to strong correlations when data analyzed were the monthly averages. These results demonstrate that there was a higher chance to find *E. pusilla* and its egg mass when there were higher density and higher total surface area of *H. macroloba*. At this temporal scale, the density and total surface area of *H. macroloba* which represented the habitat availability might be the factor that limits the number of slugs in the populations. This suggestion was similar to the result from Middlebrook et al. (2019) who reported that the main host, *Penicillus capitatus*, affected the numbers of *E. papillosa* on the date of data collection.

Occurrence of slugs, egg masses and grazing marks on *Halimeda macroloba* of different life-history stages and different positions along thalli

The highest number of slugs, number of egg masses and number of grazed *H. macroloba* were found on the mature plant, stage 4, which represented the largest surface area of thallus. This indicated that the mature algae may be used as shelter nursery and feeding ground. It also implied that the amount of surface area of *H*. *macroloba* might determine habitat use of slug.

The different characteristics between younger plants and mature plants were not only by means of surface area of thallus but also by the accumulation of calcium carbonate (Mayakun & Prathep, 2019) and chemical composition (Paul & Van Alstyne, 1988a). The younger plants contained less calcium carbonate accumulation than the older plants (Mayakun & Prathep, 2019). Calcium carbonate inside the plant was a physical defense that can deter the herbivore (Paul & Van Alstyne, 1988a). The softer thalli may be easier to graze. The slug rasped the surface of the plant by its specific radula which consisted of triangular cusps and lateral denticles (Jensen, 1993). The surface of plant is exposed then the sap was sucked. Moreover, the younger plant which had lightly calcified contained a higher concentration of diterpenoids, halimedatetraactate and halimidatrial, which was an algal toxin (Paul & Van Alstyne, 1988a). The higher amount of toxin might be modified more converted toxin of slug and slug's egg mass because the mucus of *E*. *pusilla* and its egg mass was modified from one of major secondary metabolites of *H*. *macroloba* which was halimedatetraactate (Paul & Van Alstyne, 1988b). The chemical compound of the mucus can deter the slug and its egg mass from the predator like other heterobranch (Roesener & Scheuer, 1986; Paul & Van Alstyne, 1988b) as the algal toxin deterred itself from herbivores (Paul & Van Alstyne, 1988a).

Even though there were advantages from younger plants, the slug chose the mature plant. Therefore, the amount of toxins or soft segments might not be important as availability of habitat. There was no report noted about the different quality of food or net energy between young and old plants which might be habitat selection factors.

The position of segments was one of the determinants which influenced the variation of some characters within a thallus such as chemical composition and accumulation of calcium carbonate (Paul & Van Alstyne, 1988a). The terminal segments contained more diterpenoids and more halimidatrial which was one of major metabolites than the lower segments (Paul & Van Alstyne, 1988a,b). Paul & Van Alstyne (1988a) reported about the efficacy of defended compound between two major secondary metabolites, halimedatrial and halimedatetraacetate, that halimedatrial can deter the herbivore feeding better. The higher toxin and more effective toxin from algal host may influence the selection of egg mass laying. Most egg masses that were laid on the terminal segments in this study. The egg mass may be protected by the higher amount of toxin from algae and more halimedatrial which was more effective toxin from the predator (Paul & Van Alstyne, 1988a,b). Furthermore, the terminal segments which were new segments were lightly calcified. So, the terminal segments were softer and easier to graze than the lower segments. Combination with higher quantity of toxins, the slug can sequester the terminal segments easier and can utilize the defensive compounds more than the lower segments. These reasons might influence feeding behavior. Therefore, the grazing marks appeared on terminal segments in this study. Avoidance of sediment disturbance at lower segments might be the one reason that affected the egg-laying behavior on terminal segments also. Because there was more chance that the egg mass was covered up by sediment at lower part of thalli. Another reason was the greater proportion area of terminal segments which means the area of terminal segments had a greater total surface area. The larger availability habitat might get more occurrence of egg masses. According to the results from the relationship between algal and slug, a higher number of egg masses may be found on larger surface areas of algal also.

CONCLUSION

In this study, there was a variation in abundance of *Halimeda macroloba* between months. The density of *H. macroloba* was variable. The monsoon seasons appeared to have had no effect on the population of *H. macroloba* and the fluctuation of algal density might drive by the other unknown factors. The variation in abundance of *Elysia pusilla* between months was not found but the variation between months was high. There is no slug found in March 2020. The highest number of slugs was only four individuals per quadrat same as the most sea slug species which are rare. There is a variation in abundance of slug's egg masses. The highest abundance of *H. macroloba*, *E. pusilla* and egg masses was found in August and October 2020. More habitat availability and more food sources might influence the production of eggs which affected reproduction success. Moreover, the higher density of *H. macroloba* can decrease the water velocity within the patch which might reduce the expulsion of slugs from its habitat. The range of slug's size in this study is 1 to 19 mm. The small size of slugs was found every month except March 2020. Thus, the suggestion was *E. pusilla* in this area might recruit thought out the year

The results of the relationship between abundance of slugs, egg masses and their habitat show that the coefficient values were not strong when the data of population were pooled across the month. The analysis was similar to Middlebrook et al. (2014). The report demonstrates that spatial associations were not significant between slug and its algal diets. Conversely, there were medium to strong correlations when data were averaged which means that there was a higher chance to find *E. pusilla* and its egg mass in the area which has a higher density and higher total surface area of *H. macroloba*. Moreover, habitat availability might be the factor that limits the number of slugs in the populations at this temporal scale.

There were different surface areas in different ages of alga. In this study, the age of alga was separated into 4 stages. Stage 1 to stage 4 determined the age of alga respectively, stage 1 was newly recruited plant and stage 4 was mature plant which was the oldest plant in this study. The largest area was found on stage 4 which was mature plant, and the smallest area was investigated on stage 1 which was the youngest plant. Moreover, different stages of alga have different calcium carbonate accumulation and chemical composition. The higher age of alga accumulated more calcium carbonate which was harder to consume. In addition, the higher stage of alga had fewer secondary metabolites which reduced the deterrent feeding from predators. These factors demonstrated the inappropriate habitat availability of slugs. On the other hand, the results show that there was more opportunity to find the slug on stage 4 which displayed the largest surface area. Therefore, the larger area was an important factor that determined the number of slugs.

The different positions of segments of *H. macroloba* thalli had different calcium carbonate accumulation and chemical composition also. The slug chose the terminal segments which accumulated less calcium carbonate and had higher concentrations of secondary metabolites. These factors were appropriate for feeding and egg-laying. Moreover, the egg mass on terminal segments had a higher chance to avoid the sediment than the basal segments. Furthermore, the proportion area of upper segments was larger than the lower segments. Thus, there was a larger availability habitat for the slug.

These discussions can be concluded that the availability of habitat might determine the number of slugs and their egg masses. There was a higher chance to find the slug and its egg mass on the upper position of segments in the higher density of mature *H. macroloba* which shows the behavior of habitat selection in *E. pusilla*. Hence, the population density of *H. macroloba* might regulate the population density of *E. pusilla*. In this study, there were unknown factors that influenced the fluctuation of *H. macroloba*. These factors might affect the density of slugs indirectly.

REFERENCES

Bass, A. L., & Karl, S. A. (2006). Molecular phylogenetic analysis of genera in the family Plakobranchidae (Mollusca: Opisthobranchia: Sacoglossa). *Records of the Western Australian Museum Supplement*, *69*, 61-68.

Baumgartner, F. A., & Toth, G. B. (2014). Abundance and size distribution of the sacoglossan *Elysia viridis* on co-occurring algal hosts on the Swedish west coast. *PloS one*, *9*(3), e92472.

Borowitzka, M. A., & Larkum, A. W. (1976). Calcification in the green alga *Halimeda*: III. The sources of inorganic carbon for photosynthesis and calcification and a model of the mechanism of calcification. *Journal of Experimental Botany*, *27*(5), 879-893.

Brandley, B. K. (1984). Aspects of the ecology and physiology of *Elysia* cf. *furvacauda* (Mollusca: Sacoglossa). *Bulletin of Marine Science*, *34*(2), 207-219.

Clark, K. B., & Busacca, M. (1978). Feeding specificity and chloroplast retention in four tropical Ascoglossa, with a discussion of the extent of chloroplast symbiosis and the evolution of the order. *Journal of Molluscan Studies*, *44*(3), 272-282.

Clark, K., Jensen, K., & Stirts, H. (1990). Survey for functional kleptoplasty (chloroplast symbiosis) among West Atlantic Ascoglossa (Sacoglossa). *Veliger*, *33*, 339-345.

Clark, K. B. (1992). Plant-like animals and animal-like plants: symbiotic coevolution of ascoglossan (= sacoglossan) molluscs, their algal prey, and algal plastids. *Algae and symbioses: plants, animals, fungi, viruses, interactions explored. Bristol: Biopress*, 515-530.

Clark, K. B., & DeFreese, D. (1987). Population ecology of Caribbean ascoglossa (Mollusca, Opisthobranchia) a study of specialized algal herbivores. *American Malacological Bulletin*, *5*(2), 259-280.

Cohen, J. (1988). *Statistical power analysis for the behavioral sciences* (2nd ed.). Hillsdale, NJ: Lawrence Earlbaum Associates.

Curtis, N. E., Massey, S. E., & Pierce, S. K. (2006). The symbiotic chloroplasts in the sacoglossan *Elysia clarki* are from several algal species. *Invertebrate Biology*, *125*(4), 336-345.

Curtis, N. E., Schwartz, J. A., & Pierce, S. K. (2010). Ultrastructure of sequestered chloroplasts in sacoglossan gastropods with differing abilities for plastid uptake and maintenance. *Invertebrate Biology*, *129*(4), 297-308.

Drew, E. A., & Abel, K. M. (1988). Studies on *Halimeda*. II. Reproduction, particularly the seasonality of gametangia formation, in a number of species from the Great Barrier Reef Province. *Coral reefs (Print)*, *6*(3-4), 207-218.

Gowacki, W. A. (2017). Taxonomy, Ecology, and Behavior of the Kleptoplastic Sea Slug *Elysia papillosa* (Master's thesis). University of South Florida, Florida, United States.

Guiry, M.D. & Guiry, G.M. (2021). *Halimeda macroloba Decaisne 1841: AlgaeBase*. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org/search/species/detail/?species_id=3807

Händeler, K., & Wägele, H. (2007). Preliminary study on molecular phylogeny of Sacoglossa and a compilation of their food organisms. *Bonner Zoologische Beiträge*, *55*(3-4), 231-254.

Händeler, K., Grzymbowski, Y. P., Krug, P. J., & Wägele, H. (2009). Functional chloroplasts in metazoan cells-a unique evolutionary strategy in animal life. *Frontiers in Zoology*, *6*(1), 1-18.

Jensen, K. R. (1983). Factors affecting feeding selectivity in herbivorous Ascoglossa (Mollusca: Opisthobranchia). *Journal of Experimental Marine Biology and Ecology*, *66*(2), 135- 148.

Jensen, K. R. (1992). Anatomy of some Indo-Pacific Elysiidae (Opisthobranchia: Sacoglossa (= Ascoglossa)), with a discussion of the generic division and phylogeny. *Journal of Molluscan Studies*, *58*(3), 257-296.

Jensen, K. R. (1993). Morphological adaptations and plasticity of radular teeth of the Sacoglossa (= Ascoglossa) (Mollusca: Opisthobranchia) in relation to their food plants. *Biological Journal of the Linnean Society*, *48*(2), 135-155.

Jensen, K. R. (1994). Behavioural adaptations and diet specificity of sacoglossan opisthobranchs. *Ethology ecology & evolution*, *6*(1), 87-101.

Jensen, K. R. (1996). Phylogenetic systematics and classification of the Sacoglossa (Mollusca, Gastropoda, Opisthobranchia). *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, 351*(1335), 91-122.

Jensen, K. R. (1997). Evolution of the Sacoglossa (Mollusca, Opisthobranchia) and the ecological associations with their food plants. Evolutionary Ecology, 11(3), 301-335.

Jensen, K. (2009). Sacoglossa (Mollusca: Gastropoda: Opisthobranchia) from Singapore. *Raffles Bulletin of Zoology*, 207-223.

Jensen, K. R. (2015). Sacoglossa (Mollusca: Gastropoda: Heterobranchia) from northern coasts of Singapore. *Raffles Bulletin of Zoology*.

Krug, P. J., Vendetti, J. E., & Valdes, A. (2016). Molecular and morphological systematics of *Elysia Risso*, 1818 (Heterobranchia: Sacoglossa) from the Caribbean region. *Zootaxa*, *4148*(1), 1-137.

LaForge, N. L. (2006). Larval development and metamorphosis of *Berthella californica* (Gastropoda, Heterobranchia, Pleurobranchoidea) including phylogenetic implications (Master's thesis). University of Victoria, British Columbia, Canada.

Mayakun, J., Kim, J. H., Lapointe, B. E., & Prathep, A. (2012). Gametangial characteristics in the sexual reproduction of *Halimeda macroloba Decaisne* (Chlorophyta: Halimedaceae). *Songklanakarin Journal of Science & Technology*, *34*(2).

Mayakun, J., & Prathep, A. (2019). Calcium carbonate productivity by *Halimeda macroloba* in the tropical intertidal ecosystem: The significant contributor to global carbonate budgets. *Phycological Research*, *67*(2), 94-101.

Middlebrooks, M. L., Gowacki, W. A., Bell, S. S., & Pierce, S. K. (2019). Annual occurrence and algal preferences of the kleptoplastic sea slug, *Elysia papillosa*. *Symbiosis*, 1-8.

MolluscaBase eds. (2021). *Sacoglossa*. MolluscaBase. http://www.molluscabase.org/aphia.php?p=taxdetails&id=167

Panyawai, J., Tuntiprapas, P., & Prathep, A. (2019). High macrophyte canopy complexity enhances sediment retention and carbon storage in coastal vegetative meadows at Tangkhen Bay, Phuket, Southern Thailand. *Ecological research*, *34*(1), 201-212

Paul, V. J., & Van Alstyne, K. L. (1988a). Chemical defense and chemical variation in some tropical Pacific species of *Halimeda* (Halimedaceae; Chlorophyta). *Coral Reefs*, *6*(3-4), 263-269.

Paul, V. J., & Van Alstyne, K. L. (1988b). Use of ingested algal diterpenoids by *Elysia halimedae* Macnae (Opisthobranchia: Ascoglossa) as antipredator defenses. *Journal of Experimental Marine Biology and Ecology*, *119*(1), 15-29.

Pierce, S. K., Maugel, T. K., Rumpho, M. E., Hanten, J. J., & Mondy, W. L. (1999). Annual viral expression in a sea slug population: life cycle control and symbiotic chloroplast maintenance. *The Biological Bulletin*, *197*(1), 1-6.

Pongparadon, S. (2009). Diversity, distribution and variations within species of genus *Halimeda* J.V. Lamour (Chlorophyta) in Peninsular Thailand (Doctoral dissertation) Prince of Songkla University, Songkla, Thailand

Ponder, W.F., Lindberg, D.R., & Ponder, J.M. (2020). Biology and Evolution of the Mollusca (1st ed.). CRC Press. https://doi.org/10.1201/9781351115667

Roesener, J. A., & Scheuer, P. J. (1986). Ulapualide A and B, extraordinary antitumor macrolides from nudibranch eggmasses. *Journal of the American Chemical Society*, *108*(4), 846-847.

Schmitt, V., Anthes, N., & Michiels, N. K. (2007). Mating behaviour in the sea slug *Elysia timida* (Opisthobranchia, Sacoglossa): hypodermic injection, sperm transfer and balanced reciprocity. *Frontiers in Zoology*, *4*(1), 1-9.

Sinutok, S. (2008). Seasonal variation in distribution, density, and life stage of *Halimeda macroloba* Decaisne at Tankhen Bay, Phuket Province, Thailand (Master's thesis). Prince of Songkla University, Songkhla, Thailand.

Trowbridge, C. D. (1993). Interactions between an ascoglossan sea slug and its green algal host: branch loss and role of epiphytes. *Marine Ecology-Progress Series*, *101*, 263-263.

Trowbridge, C. D., & Todd, C. D. (2001). Host-plant change in marine specialist herbivores: ascoglossan sea slugs on introduced macroalgae. *Ecological Monographs*, *71*(2), 219-243.

Trowbridge, C. D., Hirano, Y. J., & Hirano, Y. M. (2008). Sacoglossan opisthobranchs associated with the green macroalgae *Codium* spp. on Pacific rocky shores of Japan. *Venus (Journal of the Malacological Society of Japan)*, *66*(3-4), 175-190.

Vendetti, J. E., Trowbridge, C. D., & Krug, P. J. (2012). Poecilogony and population genetic structure in *Elysia pusilla* (Heterobranchia: Sacoglossa), and reproductive data for five sacoglossans that express dimorphisms in larval development. *Integrative and Comparative Biology*, *52*(1), 138–150

APPENDIX

Manuscript (Accepted)

WALAILAK JOURNAL
http://wjst.wu.ac.th https://doi.org

Population ecology and habitat use of the sea slug *Elysia pusilla* (Bergh, 1872) (Sacoglossa) in a tropical *Halimeda macroloba* Decaisne meadow.

Apisara NAKPAN[1], Jaruwan MAYAKUN[2] and Kringpaka WANGKULANGKUL[1]*

[1]*Division of Biological Science, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand 90110*

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

Received: xxx, Revised: xxx, Accepted: xxx

Running title

Relationship between sea slug and macroalga

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 cm^2. 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 Heterobrachia: 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 m^2 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 [11,13,14]. As well as accumulating calcium carbonate, *Halimeda* produces secondary metabolites, two 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 four 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 to 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 sand 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 quadrate in the few hours available, the size of quadrates and number of samples collected were largely determined by practical considerations. On one occasion, we were able to collect samples from 18 quadrats of 0.04 m^2 (20 × 20 cm) that we randomly set out in the *H. macroloba* meadow. As many as 75 thalli were found in one quadrate. 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 quadrate 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 hours to let the slugs emerge.

Data collection

In this study, the life history stages of *H. macroloba* were categorized in to six stages, following Sinutok [13] and Mayakun and Prathep [12]. Stage 1 consisted of newly recruited plants with only one segment (Figure 2a); stage 2 of young plants with three or four segments and one or two layers of segments (Figure 2b); stage 3 of partially calcified plants with three or four layers of segments (Figure 2c); stage 4 of mature calcified plants (Figure 2d); 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 (Figure 3b-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 3d). 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).

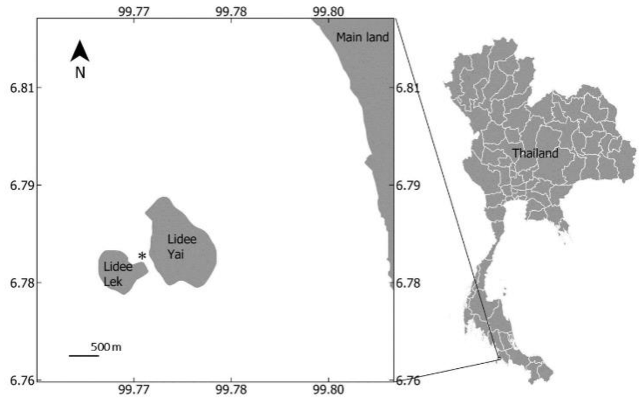

Figure 1. The sampling area (*) was located between Lidee Lek and Lidee Yai Islands in the Andaman Sea, Satun Province, Thailand

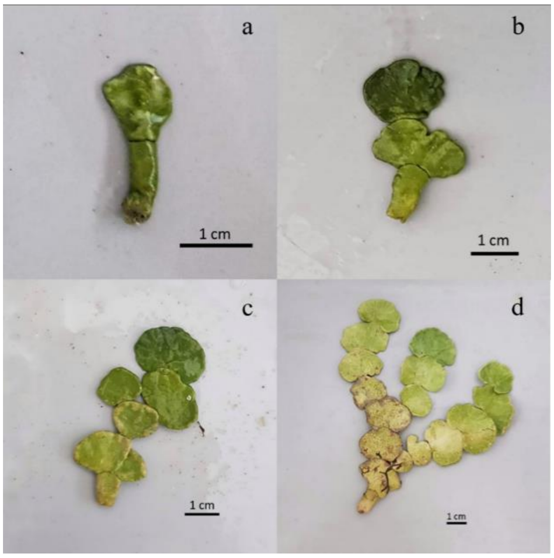

Figure 2. (a)-(d) Life history stages 1-4, respectively, of *Halimeda macroloba*.

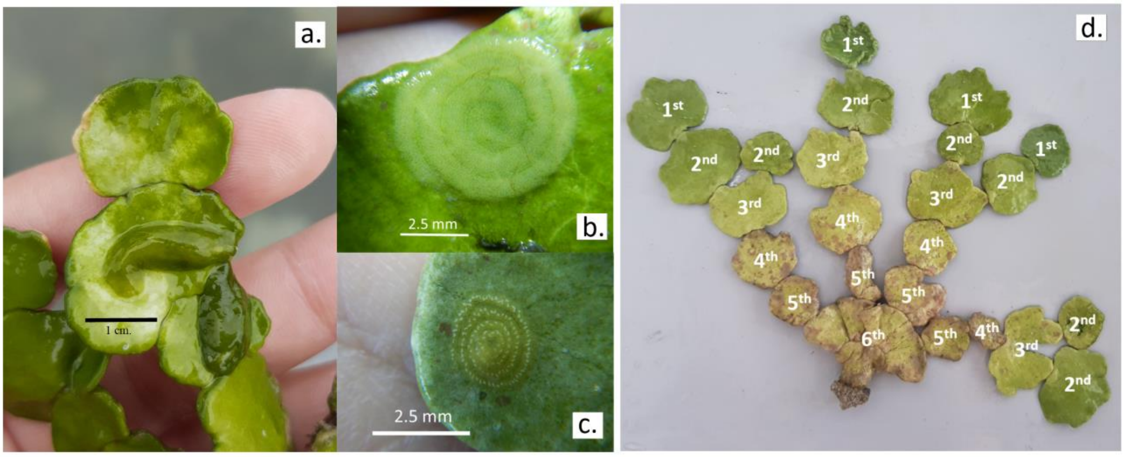

Figure 3. (a) Two *E. pusilla* individuals can be seen on a mature *H. macroloba* (stage 4) thallus. The two white areas visible on the terminal and second segments are grazing marks. (b)-(c) Egg masses of *E. pusilla*. (d) The photograph of a complete *H. macroloba* thallus shows how the positions of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

Statistical Analysis

Temporal variations in the abundance of *H. macroloba* over eight 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 four 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 four 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 [2,7,8,9,17]. 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 4a). 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 4b). However, it is worth noting that the variation between months was high. The highest density was four 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 4b). Egg masses were found in November 2019, and in March, August and October 2020 (Figure 4b). In March 2020 egg masses were found, but no slugs were observed.

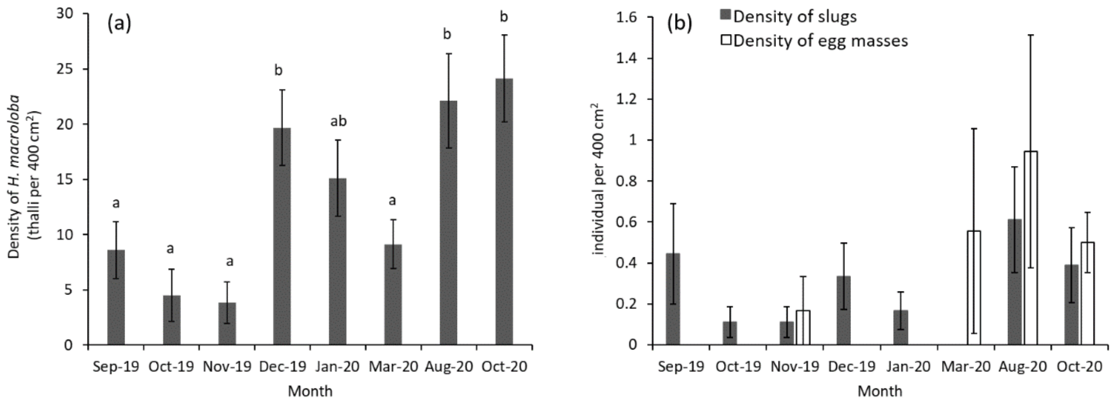

Figure 4. (a) Monthly density (Mean ± SE) of *H. macroloba* and (b) *E. pusilla* and its egg masses from September 2019 to October 2020. Field work was cancelled due to rough seas and pandemic lockdown in some months (Data was not shown in the graphs). Different letters above bars = significant differences 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 to 0.36; Figure 5a). 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*.

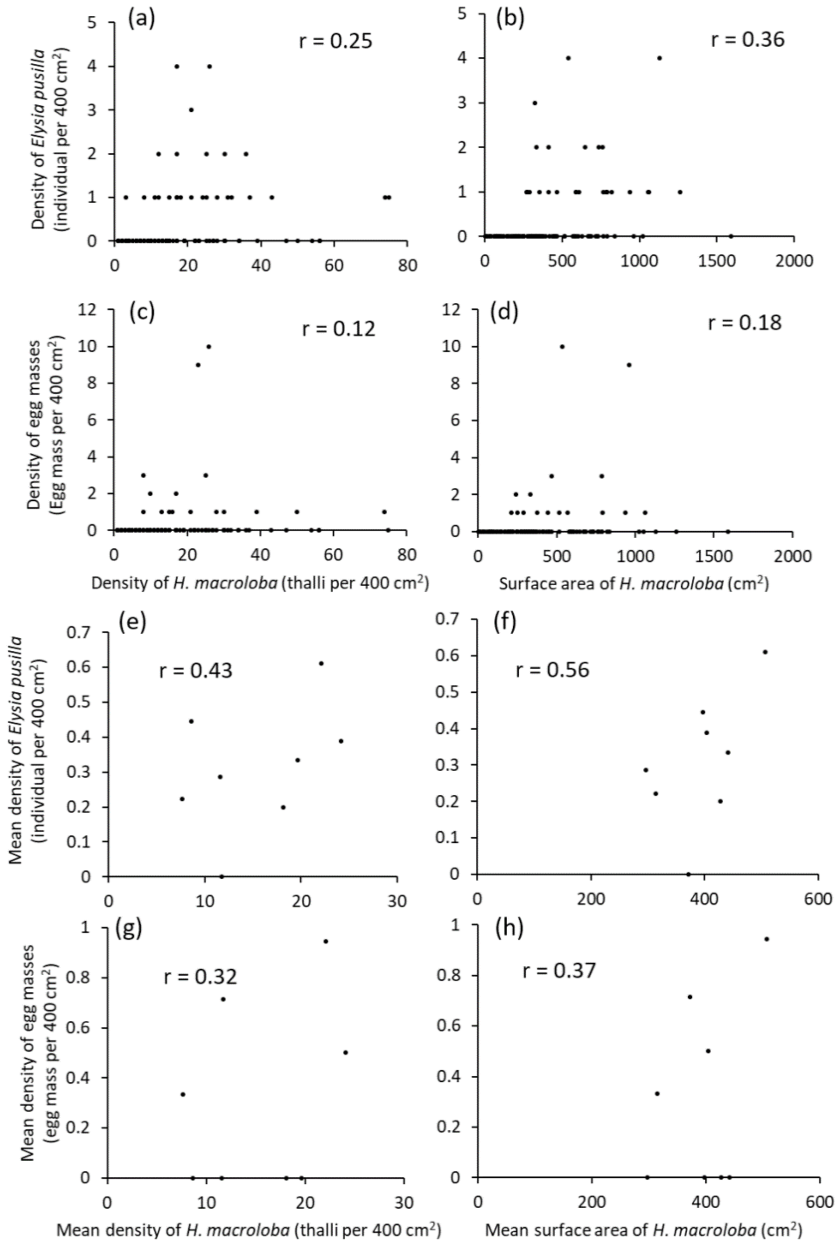

Figure 5. Relationships between the density of *Elysia pusilla*, its egg masses and *Halimeda macroloba* density and surface area (a)-(d) and between mean densities of *E. pusilla*, its egg masses and *H. macroloba* mean density and surface area (e)-(h). Analyzed data were pooled across months. For (e)-(h), 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 6a). 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 (Figure 6b,c). The surface area of *H. macroloba* individuals at different life history stages varied ($F = 143.69$; $p = 0.001$; Figure 6d). 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.

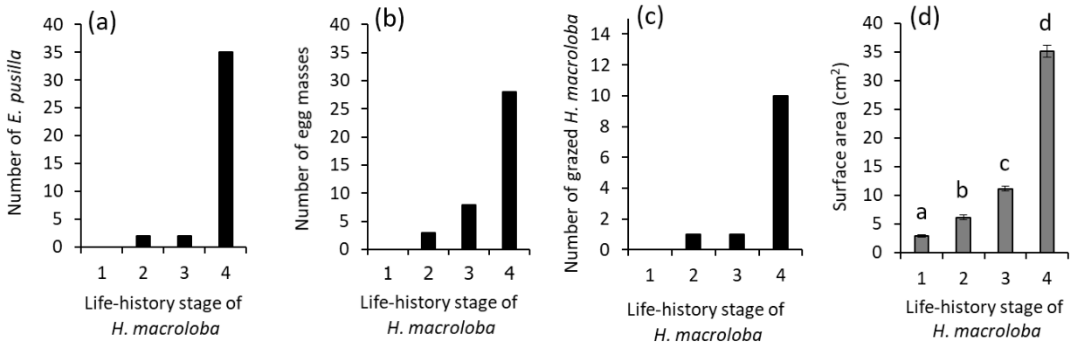

Figure 6. (a) Numbers of *E. pusilla*, (b) and numbers of egg masses found on thalli of *H. macroloba* at each different life-history stage. (c) Numbers of *H. macroloba* thalli at each stage that presented grazing marks and (d) surface area (mean ± SE) of *H. macroloba* thalli at each life-history stage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

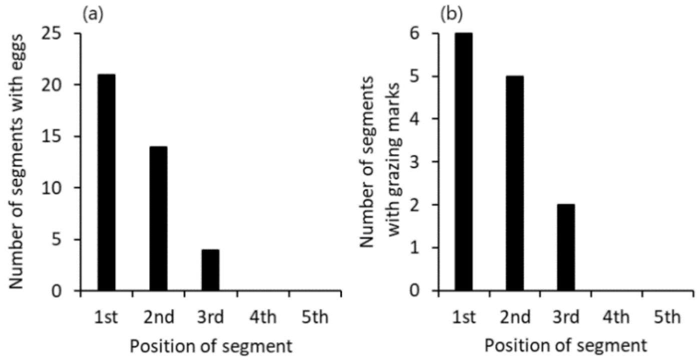

Figure 7. (a) Numbers of thallus segments at different positions along *H. macroloba* thalli that present egg masses: (b) Numbers of segments at different positions that presented grazing marks.

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 (Figure 7a,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. halimedae*) 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 one 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.

Acknowledgements

This study was partially supported by grants from the Graduate School and Faculty of Science, Prince of Songkla University. We would like to thank Professor Patrick J. Krug of Biology Science, California State University for helping with slug identification and sharing his experience. We gratefully thank members of the Coastal Ecology Research Lab, Prince of Songkla University, for their support and suggestions. We also thank members of the Seaweed and Seagrass Research Unit, Prince of Songkla University, for assistance with field work.

References

[1] JE Duffy and ME Hay. Herbivore resistance to seaweed chemical defense: the roles of mobility and predation risk. *Ecology*. 1994, DOI: 10.2307/1937456

[2] KR Jensen. Evolution of the Sacoglossa (Mollusca, Opisthobranchia) and the ecological associations with their food plants. *Evol. Ecol*. 1997; 11, 301-335.

[3] ML Middlebrooks, WA Gowacki, SS Bell and SK Pierce. Annual occurrence and algal preferences of the kleptoplastic sea slug, *Elysia papillosa*. *Symbiosis*. 2019; 77, 217-224.

[4] S Weaver and KB Clark. Light intensity and color preferences of five ascoglossan (= sacoglossan) molluscs (Gastropoda: Opisthobranchia): a comparison of chloroplast-symbiotic and aposymbiotic species. *Mar. Freshw. Behav. Phy.* 1981, DOI: 10.1080/10236248109386991

[5] BK Brandley. Aspects of the ecology and physiology of *Elysia* cf. *furvacauda* (Mollusca: Sacoglossa). *B. Mar. Sci*. 1984; 34, 207-219.

[6] NE Curtis, SE Massey and SK Pierce. The symbiotic chloroplasts in the sacoglossan*Elysiaclarki* are from several algal species. *Invertebr. Biol*. 2006, DOI: 10.1111/j.1744-7410.2006.00065.x

[7] MA Phuong. 2010, Solar powered slugs: biology of sacoglossans on Mo'orea, French Polynesia. University of California Berkeley, California, United States.

[8] KR Jensen. Morphological adaptations and plasticity of radular teeth of the Sacoglossa (= Ascoglossa) (Mollusca: Opisthobranchia) in relation to their food plants. *Biol. J. Linn. Soc*. 1993, DOI: 10.1111/j.1095-8312.1993.tb00883.x

[9] K Händeler and H Wägele. Preliminary study on molecular phylogeny of Sacoglossa and a compilation of their food organisms. *Bonn. zool. Beitr*. 2007; 55, 231-254.

[10] JE Vendetti, CD Trowbridge and PJ Krug. Poecilogony and population genetic structure in *Elysiapusilla* (Heterobranchia: Sacoglossa), and reproductive data for five sacoglossans that express dimorphisms in larval development. *Integr. Comp. Biol*. 2012, DOI: 10.1093/icb/ics077

[11] S Pongparadon. 2009, Diversity, distribution and variations within species of genus *Halimeda* JV Lamour. (Chlorophyta) in Peninsular Thailand, Master's thesis. Prince of Songkla University, Songkhla, Thailand.

[12] J Mayakun and A Prathep. Calcium carbonate productivity by *Halimeda macroloba* in the tropical intertidal ecosystem: the significant contributor to global carbonate budgets. *Phycol. Res*. 2019, DOI: 10.1111/pre.12361

[13] S Sinutok. 2008, Seasonal variation in distribution, density, and life stage of *Halimedamacroloba*Decaisne at Tangkhen Bay, Phuket Province, Thailand, Master's thesis. Prince of Songkla University, Songkhla, Thailand.

[14] AW Larkum, A Salih and M Kühl. Rapid mass movement of chloroplasts during segment formation of the calcifying siphonalean green alga, *Halimeda macroloba*. *PLoS One*. 2011, DOI: 10.1371/journal.pone.0020841

[15] VJ Paul and KL Van Alstyne. Chemical defense and chemical variation in some tropical Pacific species of *Halimeda* (Halimedaceae; Chlorophyta). *Coral Reefs*. 1988; 6, 263-269.

[16] KR Jensen. Anatomy of some Indo-Pacific Elysiidae (Opisthobranchia: Sacoglossa (= Ascoglossa)), with a discussion of the generic division and phylogeny. *J. Mollus. Stud*. 1992, DOI: 10.1093/mollus/58.3.257

[17] VJ Paul and KL Van Alstyne. Use of ingested algal diterpenoids by *Elysiahalimedae*Macnae (Opisthobranchia: Ascoglossa) as antipredator defenses. *J. Exp. Mar. Biol. Ecol*. 1988, DOI: 10.1016/0022-0981(88)90149-9

[18] J Schubert and SDA Smith. Sea slugs—"rare in space and time"—but not always. *Diversity*. 2020, DOI: 10.3390/d12110423

[19] ML Middlebrooks, SS Bell, NE Curtis and SK Pierce. Atypical plant–herbivore association of algal food and a kleptoplastic sea slug (*Elysiaclarki*) revealed by DNA barcoding and field surveys. *Mar. Biol*. 2014; 161, 1429-1440.

[20] J Panyawai, P Tuntiprapas and A Prathep. High macrophyte canopy complexity enhances sediment retention and carbon storage in coastal vegetative meadows at Tangkhen Bay, Phuket, Southern Thailand. *Ecol. Res*. 2019, DOI: 10.1111/1440-1703.1066

The study of embryonic development of *Elysia pusilla*

In preparation for next manuscript

The study of embryonic development of *Elysia pusilla*

Reproduction and embryonic development method

In this study, twelve egg masses were observed (Fig. 13a). There are two egg masses from the temporary aquarium in laboratory (Fig. 13a,b) and nine egg masses from Lidee Island (Fig. c-m). The temporary aquarium which was filled with seawater was used for resting the samples before released to nature. The air pump was used in the temporary aquarium for keeping the concentration of oxygen. The egg masses which were laid in the temporary aquarium were observed for larval development. After mating (Fig. 14) and oviposition of *Elysia pusilla* in temporary aquarium, two egg masses were moved to another aerated plastic container which was filled with seawater from Lidee Island as the aquarium. The reproductive characteristics of these egg masses; diameter of egg mass (mm), number of eggs in clutch, larval shell length (µm), time hatching (day) and pattern and color of extra-capsular yolk (ECY) deposition were observed and noted. Number of eggs was evaluated the number by counting the egg from some part of egg mass. In each egg mass, the shell length of ten larvae were measured by ocular micrometer. Shell length was the length of shell across the aperture after the hatching. Pattern and color ECY, Number of egg mass in each clutch and diameter of egg mass were observed and measured under the stereomicroscope.

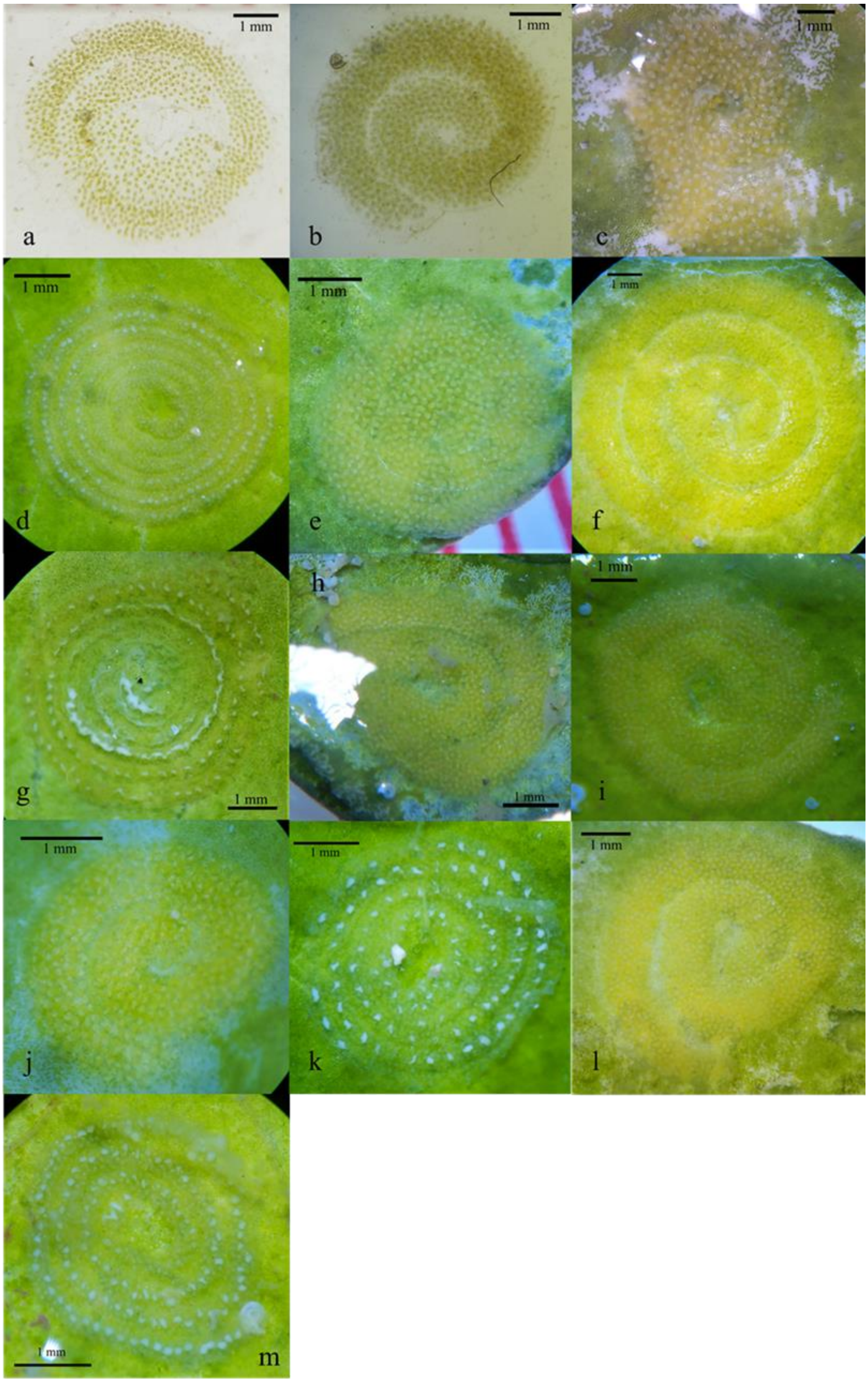

Fig. 13 Twelve egg masses were used for observation.

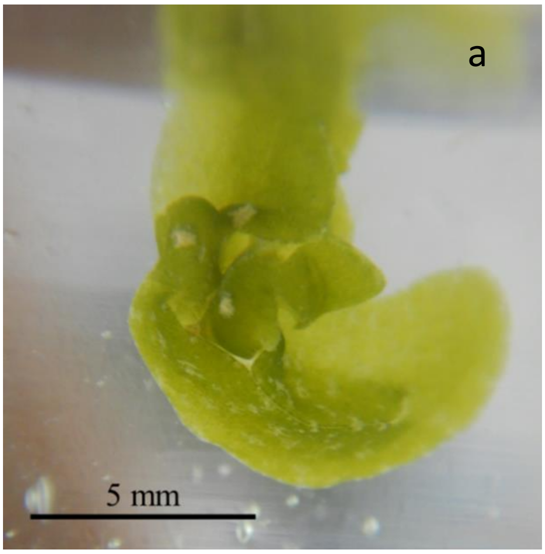

Fig. 14 Mating of *Elysia pusilla*.

Reproduction and embryonic development results

In this study, diameter of *Elysia pusilla*'s egg mass was 5.12 ± 0.41 mm. Mean number of eggs in clutch was 1369.92 ± 214.85. In each egg mass, mean larval shell length was 155.218 ± 3.25 µm, 151.202 ± 4.35 µm and 146 ± 3.46 µm, respectively (Table 2). Egg masses number 1 and 2 which were used for larval development studying developed in 8 days before hatch (Table 2). There were three types of ECY which are thin light green ribbon between capsules, orange ribbon between capsules and white blobs in the upper face of the egg mass (Table 2; Fig. 15a-c). The egg masses with light green ribbon hatched in 8 days (Table 2). There is no reduction of light green ECY while the embryos grow (Fig. 16a-b). But after hatching, some larva that stuck in the jelly matrix fed light green ECY granules (Fig. 16c). Whereas the orange ribbon disappeared while the developing embryos (Fig. 17a,b).

Table 1 Reproductive characters of *Elysia pusilla* egg masses.

Number of egg mass	diameter of egg mass (mm)	Number of eggs in clutch	Larval shell length (µm) (n=10)	time hatching (day)	ECY
1	7	934	155.218 ± 3.25	8	light green ribbon
2	6	953	151.202 ± 4.35	8	light green ribbon
3	6	520	146 ± 3.46	-	orange ribbon
4	4.5	3,250	-	-	white blobs
5	4	1,350	-	-	orange ribbon
6	8	2,500	-	-	orange ribbon
7	5	815	-	-	white blobs
8	6	1,850	-	-	orange ribbon
9	5	1,430	-	-	orange ribbon
10	3	728	-	-	orange ribbon
11	4	844	-	-	white blobs
12	5	1,500	-	-	orange ribbon
13	3	1,135	-	-	white blobs

The two egg masses used for larval development observation have light green ECY. They are coil and attached to the substrate along one side.

The observation of egg masses was started on day 3 to day 8. On the 3rd day, in each capsule contained one green embryo. Most of embryos rotated. The embryos were observed by stereomicroscope and there are no cilia. By day 4, embryos were observed by microscope. Thin cilia appeared and moved. Larval shell (protoconch) became visible (Fig. 18a). During 5th day the perceptible cilia on velum beat in metachronal rhythm. Internal organs could distinguish. Larval shell clearly appeared. The intestine was visible. Operculum and statocyst were visible on day 6 (Fig. 18b). On the 8th day, the veliger hatched (Fig. 18c) from the outer terminal of the egg mass coil to the inner of the egg mass cord.

Another egg mass contained orange ECY. Laying day of the egg mass was not noted but there is the observation since the day which was found until the hatching day. The larva hatched in 4 days after the egg mass was found and the ECY of this egg mass decreased every day.

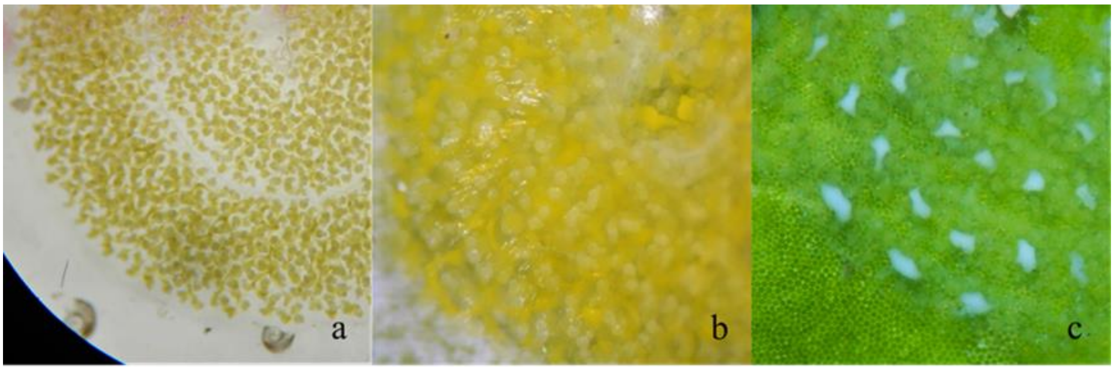

Fig. 15 Variation of *Elysia pusilla* egg mass in this study **a** the thin light green ribbon ECY between capsules **b** the orange ribbon ECY between capsules and **c** the white blobs ECY on the upper face of the egg mass.

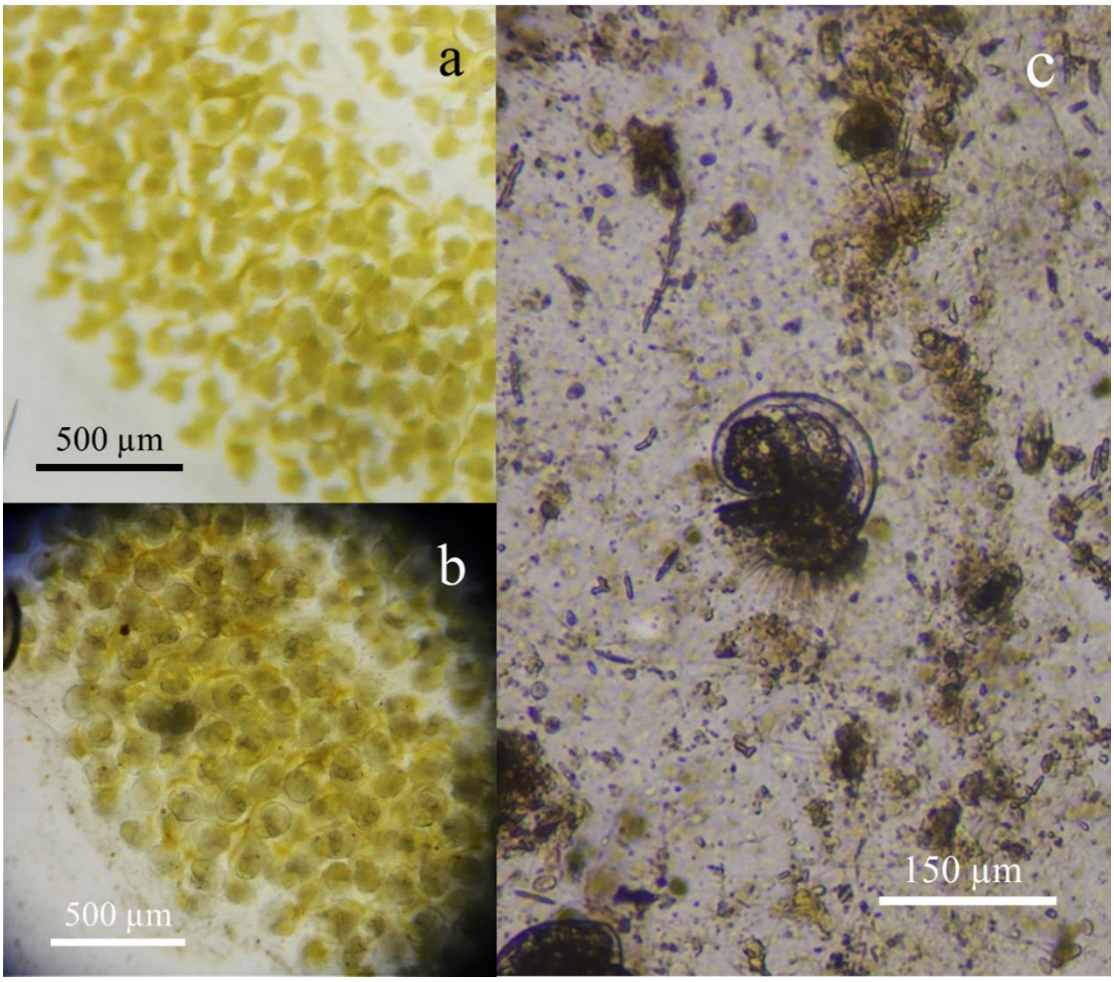

Fig. 16 Quantity of light green ECY in 4 days **a** ECY on 22 August 2020 **b** ECY on 25 August 2020 **c** larva which stuck in jelly matrix fed light green ECY granules.

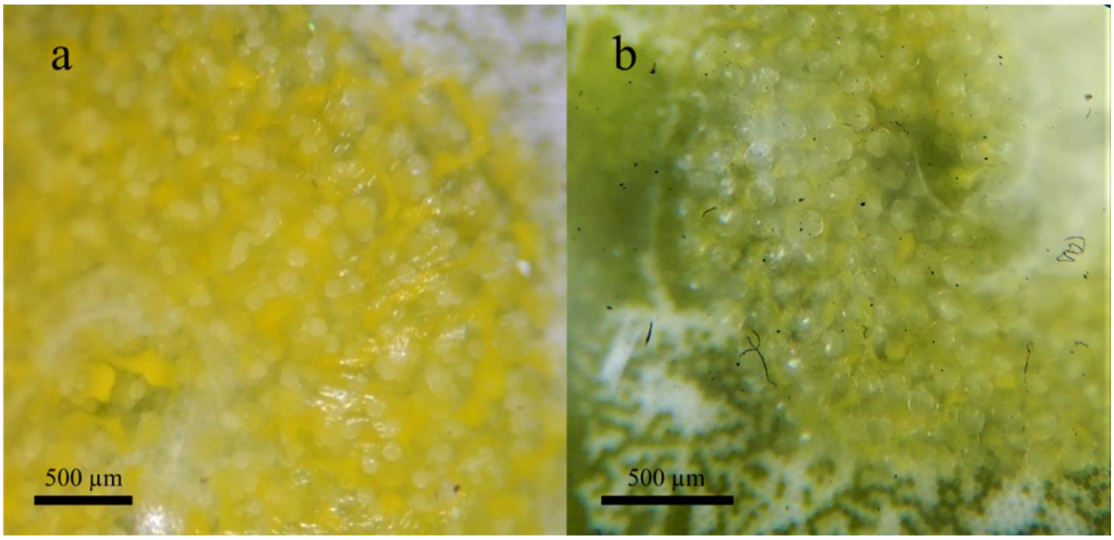

Fig. 17 Reduction of orange ribbon ECY in 4 days **a** ECY on 28 August 2020 **b** ECY on 31 August 2020.

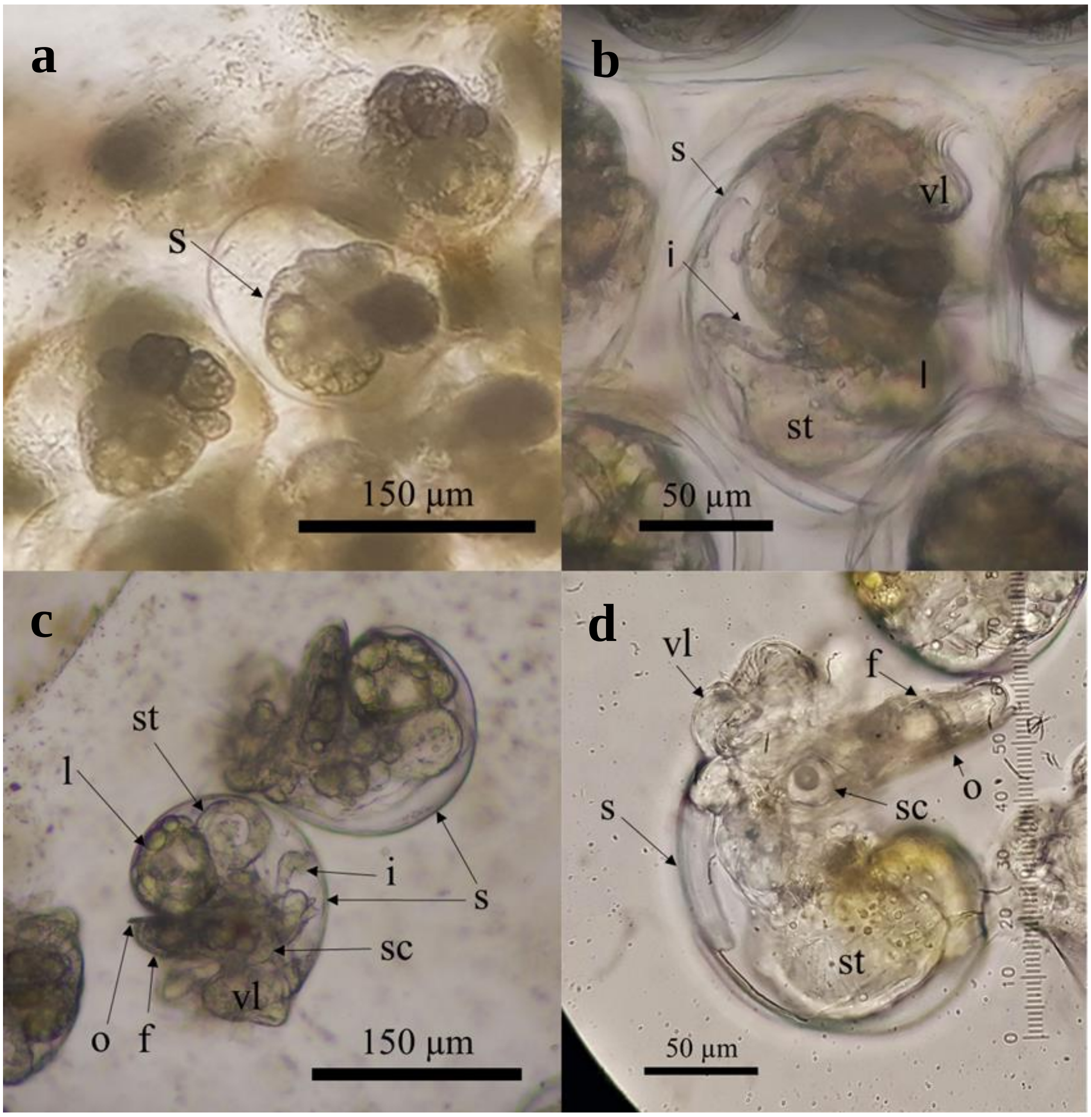

Fig. 18 Observable characters and internal organs of embryos of *Elysia pusilla* **a** embryos on day 4 after oviposition showing moving cilia and larval shell (s) (protoconch) **b** embryos on day 6 after oviposition showing perceptible cilia on velum lopes (vl), intestine (i), stomach (st) and liver (l) **c** newly hatch showing foot (f), operculum (o) and statocyst (sc) **d** larvae after hatching 2 days.

Discussion on reproduction and embryonic development

All the egg masses of *Elysia pusilla*, which were observed for larval development, had light green ECY. The larvae in the observed egg masses were assumed as planktotrophic larva type because the size of the larval shell length are 155.218 ± 3.25 µm, 151.202 ± 4.35 µm and 146 ± 3.46 µm which were similar to the larval shell size of planktotrophic larva from Vendetti et al. (2012) report. There was no report noted about hatching time of planktotrophic larva, but there is a report mentioned to hatching time of lecithotrophic larva that they developed in 15-16 days before hatched (Vendetti et al., 2012). In this study, the larvae which were assumed as planktotrophic larva type hatched in 8 days. There was a paper from Vendetti et al. (2012) that noted about *E. pusilla* ECY that there were two characters which were a winding ribbon of orange ECY and a yellow-orange ribbon ECY. In this work, there were three types of ECY, thin light green ribbon between capsules, orange ribbon between capsules and embed white blobs on the upper inside face of egg masses found in this area. Similar to the other slugs in this genus, there is a variation of ECY in the same species in the same place such as *E. mercusi* from Jamaica and *E. conigera* from Florida (Krug et al., 2016).

In this study, three egg masses were followed up for larval development study. The egg mass with orange ECY was not observed since the first day that it was laid. Within three days, ECY inside the egg mass was vanished, similar to the lecithotrophic larva from Queensland, Australia (Vendetti et al., 2012). Vendetti et al. (2012) suggested that the larvae absorbed the orange ECY because the ECY was gradually disappeared, and its digestive gland turned orange. Therefore, this egg mass was suggested as lecithotrophic larva. Two egg masses which contained light green ECY were observed. ECY was not disappeared until the larva hatched. After hatching, the light green ECY was beaten to be light green granules by the larva. Moreover, the hatched larva which stuck inside the jelly matrix was found that they fed the light green granules. Thus, the larvae from egg mass number 1 and 2 were suggested that they may be planktotrophic larva. The range of egg mass diameter in this work was 3-8 mm.

VITAE

Name Miss Apisara Nakpan

Student ID 6210220063

Educational Attainment

Degree	**Name of Institution**	**Year of Graduation**
Bachelor of Science (Biology)	Prince of Songkla University	2018