

Original Article

Male reproductive evidence for the importance of gastropods as a sentinel species from Libong Island, Thailand

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Abstract: A unique marine productive ecosystem of the seagrass beds on Thailand's Libong has been identified, which may raise environmental concerns. However, limited data on the health status of marine animals, especially gastropods, as a good sentinel species, has been found. Therefore, the present study provides a detailed observation of the male reproductive health and spermatogenesis of three gastropod species, including *Polinices mammilla*, *Cerithidea cingulata*, and *Nerita balteata* as sentinel species, which are investigated using morphology and histological methods. All samples were collected randomly from seagrass areas with healthy and unhealthy conditions in April 2021. The samples' male gonads were then investigated using histological methods. The results indicate that the species shared testicular structure and spermatogenesis in different stages (spermatogonium to spermatozoa). Additionally, their spermatogenic stages were not significantly different in size and diameter. Consequently, the structure of brown cells was common and distributed among the spermatogenic stages. Brown cells have been reported in animals that are under stress or unhealthy, such as in seagrass areas with unhealthy conditions. These results suggest that environmental and seagrass loss and the threatened seagrass habitats on Libong Island may impact aquatic animal health, necessitating long-term monitoring in further studies.

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Introduction

The male reproductive system of mollusks is typically composed of a testicular structure and a duct, like that found in vertebrates (Chen et al., 2015). Several investigations have shown that the spermatogenic stages are highly complex, with spermatogonia undergoing proliferation and differentiation (mitosis and meiosis), spermatocytes, spermatids, and finally producing mature spermatozoa (Franco et al., 2011; Shepardson et al., 2012; Chen et al., 2015). Lunetta and Damiani (2002) studied the spermatogenesis of *Dendropoma petraeum* in which five spermatogenic

stages (from spermatogonium to spermatozoa) were recorded in the follicle. There is a great number of studies on gastropod spermatogenesis (Shepardson et al., 2012; Chen et al., 2015), including *Trophon geversianus* (Giménez, 2013) and *Littorina saxatilis* (Demin et al., 2019). To develop reproductive knowledge, it is necessary to assess the reproductive strategies, sperm quality and quantity, and spawning events throughout the reproductive health status (Mylonas et al., 2010; Palma et al., 2019).

Aquatic animals can be used as sentinel marker species to monitor environmental health and change

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(Carew et al., 2013; El-Gammal et al., 2016). Because of their small size, persistence, and proclivity to bioaccumulate contaminants, aquatic invertebrates are widely accepted as an appropriate sentinel candidate species to monitor changes in ecological habitats (Lazorchak et al., 2003; Zorita et al., 2007; Chiarelli and Roccheri, 2014). In many earlier investigations of the environmental problems of coastal sites and estuaries, the gastropod has been proposed as a global regulatory framework for risk assessment (Sousa et al., 2018; Krupnova et al., 2018). Gastropod mollusks have been used to assess the effect of potentially toxic metals in all marine ecosystems (Zorita et al., 2007). Guidelines and recommended candidate species for use as biological markers or biomarkers have been reported (NRC, 1991). Several biomarkers are selected, with a focus on histopathological biomarkers. This biomarker is the most accurate, robust instrument for health and welfare monitoring of aquatic animals used in research (Ayas et al., 2007; Dietrich and Krieger, 2009; Senarat et al., 2015). Given their significance, the tissue alterations of mollusks are associated with the effects of numerous environmental stressors (Fernández San Juan et al., 2020).

One of the threats to Libong Island, Thailand, is the loss of seagrass in combination with the persistent properties of sediment microplastic pollution and marine debris (Pradit et al., 2020). The expected impact on the loss of marine invertebrate biodiversity in terms of their health and fitness loss has been proposed (Reynoldson and Metcalfe-Smith, 1992), however until present, the male reproductive health status of gastropods from the seagrass area of Libong Island has not been determined. Accordingly, the present study examines aspects of male reproductive health and a series of detailed spermatogenesis of gastropods, including *Polinices mammilla*, *Cerithidea cingulata*, and *Nerita balteata* as sentinel species, to compare the healthy and unhealthy conditions of Libong Island. These species are significant to the ecology of the Libong coast. Once baseline values are established, it is possible to provide adequate reproductive biology and project monitoring data to

manage the seagrass areas of Libong Island in support of fisheries productivity and food security.

Materials and Methods

Gastropod samples and study areas: In April 2021, we selected categorizing sites from Libong Island, Thailand, namely unhealthy and healthy seagrass areas. Representative adult gastropods, including *P. mammilla*, *C. cingulata*, and *N. balteata* were randomly sampled by hand or visual observation. A total of 30 pooled samples from each site were obtained and then euthanized with a rapid cooling shock (Wilson et al., 2009). The Animal Care and Use Committee of the Faculty of Science and Fisheries Technology, Trang Campus, Rajamangala University of Technology Srivijaya (Protocol Review No. IAC 13-03-64) made strict recommendations regarding animal treatment.

Morphology and histological methods: All gastropod bodies were morphologically examined using a stereo microscope to observe the male reproductive localization. They were preserved for approximately 48 hours at ambient temperature with modified Davidson's fixative for histology and histopathological examination. Small pieces of fixed specimens were dissected and processed using standard histological procedures (Presnell and Schreiber, 1997; Suvarna et al., 2013). Paraffin blocks were sectioned at 4 μm using a standard rotary microtome (Leica, Germany) and routinely stained with Harris' hematoxylin and eosin (H&E and Periodic acid–Schiff (PAS)). A light microscope was used to examine the classification and histological structure of the testis, while their histopathology and photographs were taken using a Panoramic Digital Slide Scanner (3DHISTECH, Germany).

Data analysis and statistical analysis: It is important to note that the number of brown cells and size of spermatogenic stages (from the spermatogonia to spermatids) were identified within the seminiferous tubule at 40X magnification and oil immersion. Ten seminiferous tubules from each gastropod species were randomly chosen and presented as mean \pm SD. The nonparametric Kruskal-Wallis and Tukey–

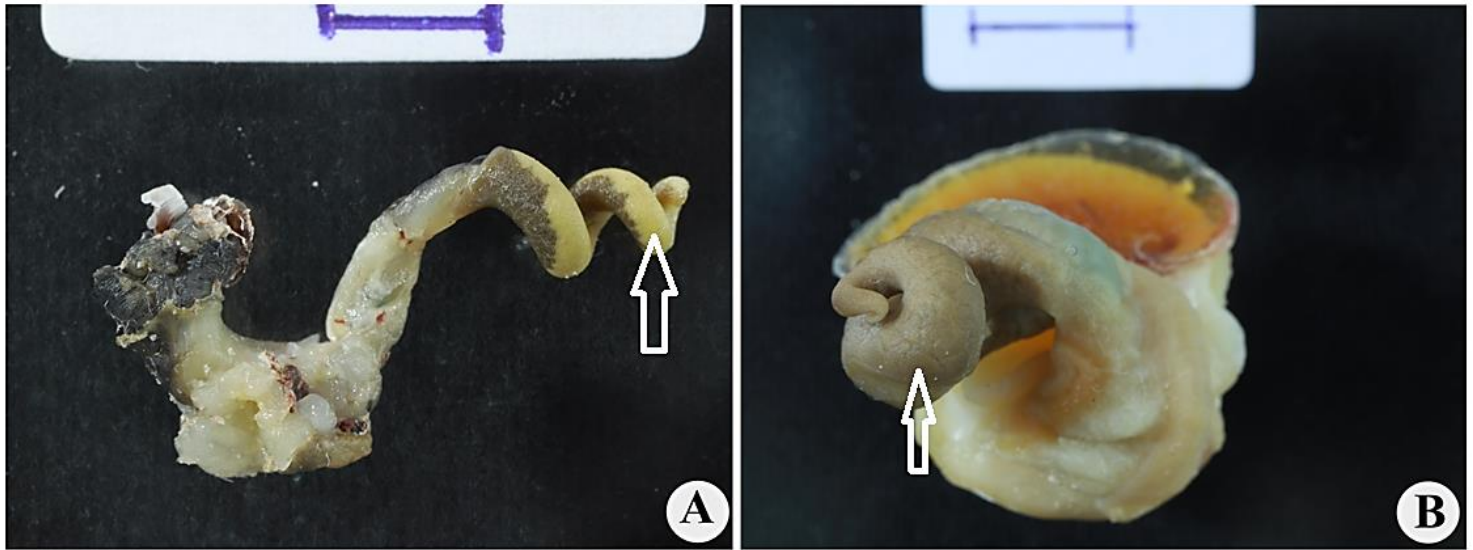


Figure 1. The internal anatomy of the gonad location (arrows) between (a) *Cerithidea cingulata* and (b) *Polinices mammilla* (Scale bar = 1 cm).

Kramer HSD tests were used to compare gastropod species. The level of significance was set at $P \leq 0.05$.

Results and Discussions

Testicular structure and cell morphometry: The testicular structure of mollusca has been reported to follow a linear development pattern similar to that of mammals. It is generally classified into testicular acinus and spermatogenesis, which has five stages: spermatogonium, primary spermatocyte, secondary spermatocyte, spermatid, and spermatozoon (Healy, 1991; Franco et al., 2011; Shepardson et al., 2012; Chen et al., 2015).

The results showed that the testicular morphology of the sampled gastropods was similar to the previous description (Figs. 1A-B). It was possible to clearly distinguish the testicular morphology as a white color from the digestive gland (termed the hepatopancreas) in the gastropod coiling due to the color difference, which is referred to as “the gonad-digestive-gland complex” (Figs. 2A-B). This feature is similarly found in *Tritia mutabilis* (Mallet et al., 2021), *Spurwinkia salsa* (Hershler and Ponder, 1998), and *Pomacea canaliculata* (Wu et al., 2011).

Histologically, the testis is surrounded by the testicular capsule, which consists of numerous seminiferous tubules or acini (High magnification, hermaphroditic features (Figs. 2A, C). Clusters of

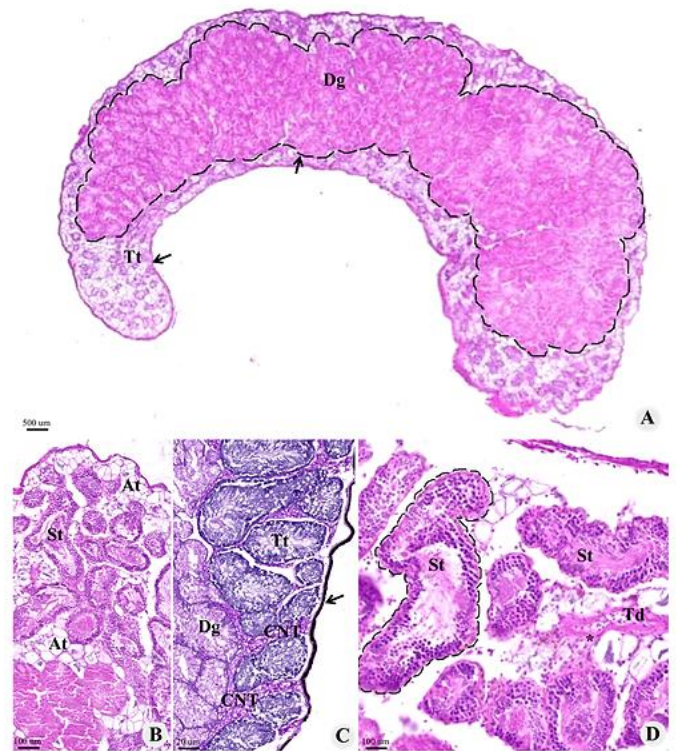


Figure 2. Light microscopic level showing the male reproductive system of the representative gastropod *Nerita balteata*. (A) The overall histology of the testis (Tt) was located close to the digestive gland (Dg) with its area (inner arrow). This organ was surrounded by the testicular capsule (outer arrow). (B-C) The seminiferous tubules (St) in the testicular tissue (Tt) were among the adipose tissue (At). Between the seminiferous tubules was separated with the connective tissue (CNT). (D) The testicular duct was found with the seminiferous tubules (St). Abbreviation: Dg = digestive gland, Td = testicular duct [Stanning method: A-B, D = Harris' hematoxylin and eosin (H&E); C = Periodic acid-Schiff (PAS)].

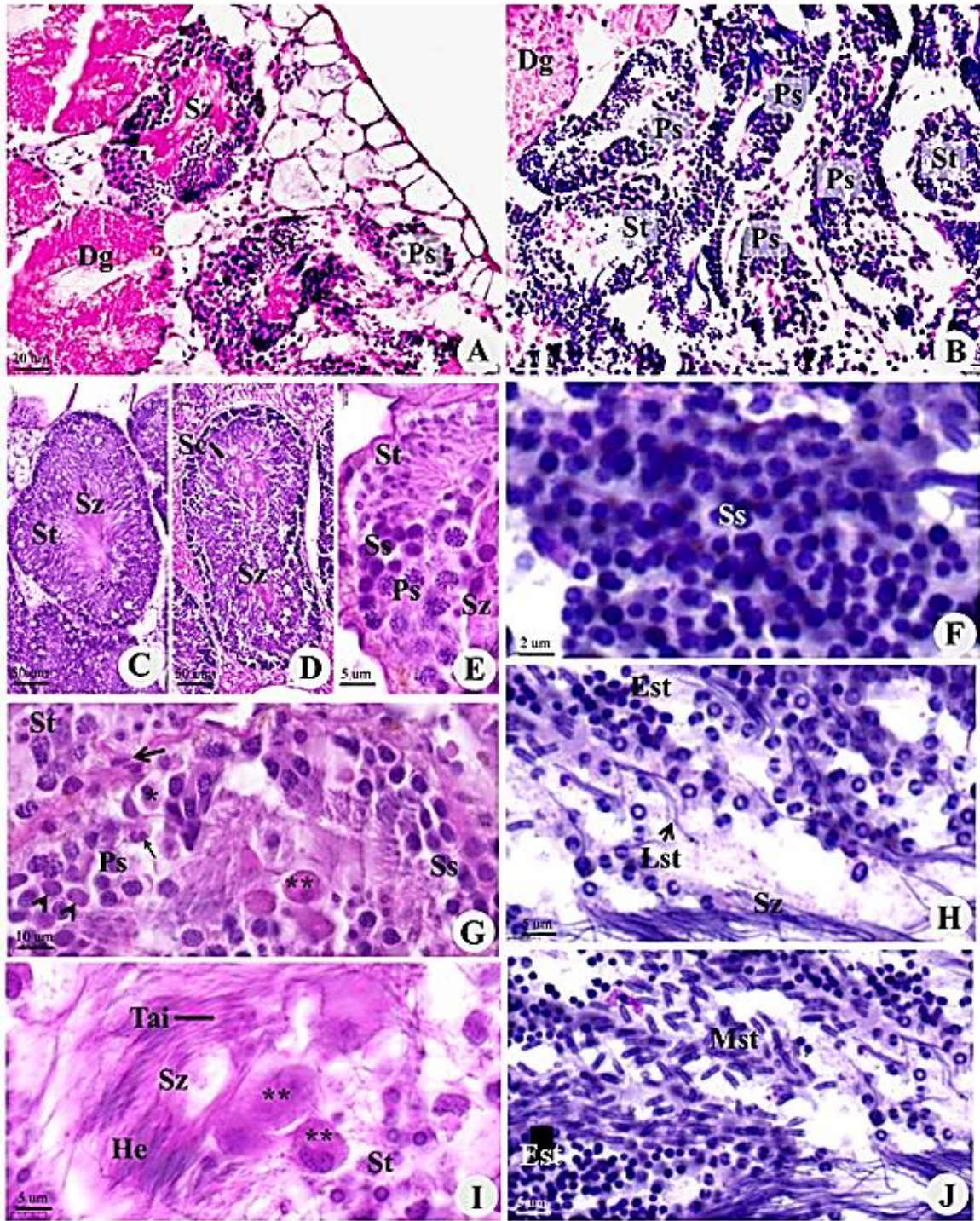


Figure 3. Light microscopic level showing the comparative testicular tissue including *Polinices mammilla* (A), *Cerithidea cingulata* (B) and *Nerita balteata* (C-J). (A-D) The prominent of spermatozoa (Sz) in the seminiferous tubules was visible. (E-J) The spermatogenesis was considered to be a germinal epithelium. High magnification showed that it is classified into the spermatogonium (Head arrows), primary spermatocyte (Ps), secondary spermatocyte (Ss), spermatids (St) having three sub-stages including the early spermatid (Est), middle spermatid (Mst) and late spermatid (Lst) and spermatozoa (Sz). Sperm morulae (double asterisk) were identified. Abbreviation: Dg = digestive gland, He = head, Sc = spermatogenic cell, Tai = tail, large arrow = Sertoli cell, asterisk = cytoplasm, small arrows = cell division [Staining method: A-J = Harris' hematoxylin and eosin (H&E)].

spermatogenic cells in the seminiferous tubules (Fig. Figs. 3B-D). The studied gastropods lacked 2A, C)

were separated by the adipose and connective tissues (Fig. 2C). A comparable testicular structure was

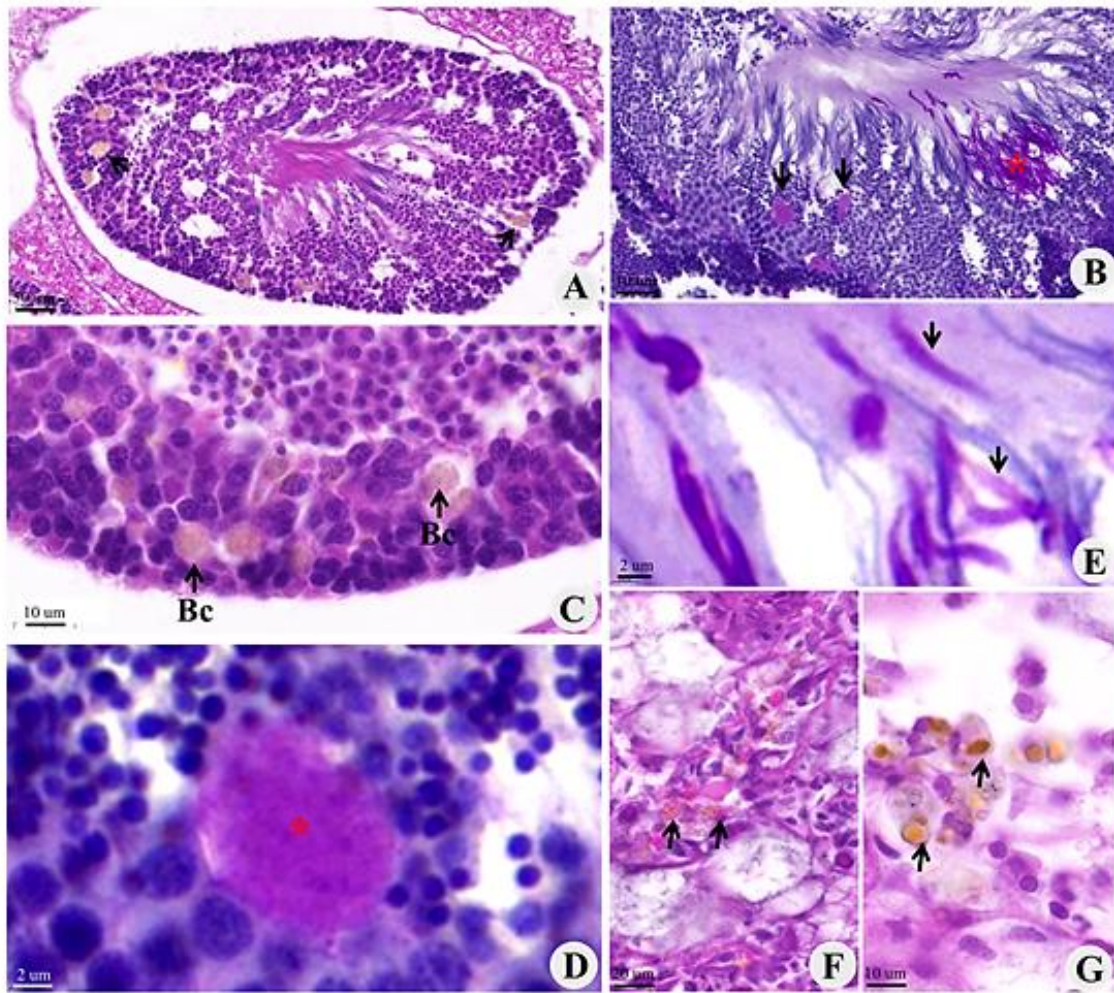


Figure 4. Light microscopic level showing the feature of the brown cell in the representative gastropod *Nerita balteata* (A) The distribution of the oval shape of the brown cell (Bc) among the spermatogenic stages. (B) The PAS reaction method was shown in the brown cell (arrows) and some spermatozoa (red asterisk). (C-D) High magnification revealed that the brown cell (Bc) had brown pigments, which also reacted to the PAS reaction. E: PAS reaction with the head spermatozoa (arrows). F-G: The Melanomacrophage centers (arrows) with highly pigmented color were aggregated. [Staining method: A, C, and E-F = Harris' hematoxylin and eosin (H&E); B, D, and E = Periodic acid–Schiff (PAS)].

described for *Trophon geversianus* (Giménez, 2013). Moreover, the testicular duct and its prominent smooth muscle were observed (Fig. 2D).

Figure 2D clearly shows the spermatogenic differentiation in the clusters of varying tubules between the sampled gastropods. Different spermatogenetic stages were identified, reflecting male reproductive maturation (Figs. 3A-E). The spermatogenic epithelium was arranged disorderly along the periphery of the tubule (Fig. 3E), as was also observed in *Radix balthica* and *Lymnaea stagnalis* (Tair-Abbaci et al., 2017).

Spermatogonia were formed and lined with the basement membrane near the Sertoli cells (Fig. 3G).

The examinations showed that the Sertoli cells had a triangular nucleus (Fig. 3G). As previously indicated, Sertoli cells are essential to the nutrition and production of steroid hormones from spermatogenic cells (de Jong-brink et al., 1977). The spermatogonia presents an oval nucleus shape with slight chromatin and is surrounded by a slightly acidophilic cytoplasm (Fig. 3G). In the next stage of spermatogenesis, the primary spermatocyte is processed under the meiotic stage. This is characterized by cells with increasing nucleus size, chromatin compaction, and a small cluster of chromatins (Fig. 3E). The transformation process of secondary spermatocytes was found (Figs. 3E-F). An increased condensation of chromatin can be

Table 1. The morphometric features of the spermatogenic stages of gastropods including *Polinices mammilla*, *Cerithidea cingulata*, and *Nerita balteata* from unhealthy and healthy seagrass sites.

Gastropods	Spermatogenesis stage (Mean ± SE)			
	Spermatogonia	Primary spermatocytes	Secondary spermatocytes	Spermatids
<i>Polinices mammilla</i>	4.71±0.07	3.84±0.03	2.77±0.04	1.69±0.03
<i>Nerita balteata</i>	4.76±0.07	4.14±0.07	2.60±0.05	1.25±0.03
<i>Cerithidea cingulata</i>	4.28±0.09	4.30±0.07	3.05±0.06	1.28±0.04

observed in this stage. However, its size was moderately reduced (Fig. 3F). Some cells were separated during the mitotic division close to the end of the process (Fig. 3G).

Interestingly, the acidophilic cells with their small nucleus were distributed as a cluster of about 3-4 cells, termed “multinucleated cells”, possibly the sperm morula (Figs. 3G-I). This work is the first report on gastropods, while most previous studies reported on bivalves (Heard, 1975; Chatchavalvanich et al., 2006; Shepardson et al., 2012). The occurrence of sperm morula may be due to several factors (Shepardson et al., 2012), such as a sign of microhermaphroditism (Grande et al., 2001) or as a potential way to supply nutrients (Coe, 1943). Shepardson et al. (2012) recommended that a multinucleated sperm morula is in an intrinsic process of an “atypical hermaphroditism”. In contrast, some freshwater mussels’ sperm morula production was related to stress due to ecophysiological pressures (Bauer, 1987; Ghiselin, 2006).

During spermatogenesis, steps of spermatids are represented in Figures 3H-J. The developmental stage of early spermatids is an oval shape with clearly condensed chromatin (Fig. 3H). It then transferred to the mid-spermatid. An elongation event of this stage began as the nucleus (Fig. 3J). Late spermatids have an extended head and a short tail (Fig. 3H). Conspicuous spermatozoa with a head and elongated tails were detected in the middle area of the tubule (Fig. 3I).

Gastropod species have a higher tendency for spermatogenesis, yet their sizes may be connected to the species, suggesting distinct structural and functional relationships (Roosen-Runge et al., 1977). In this study, we show the morphometric calculations

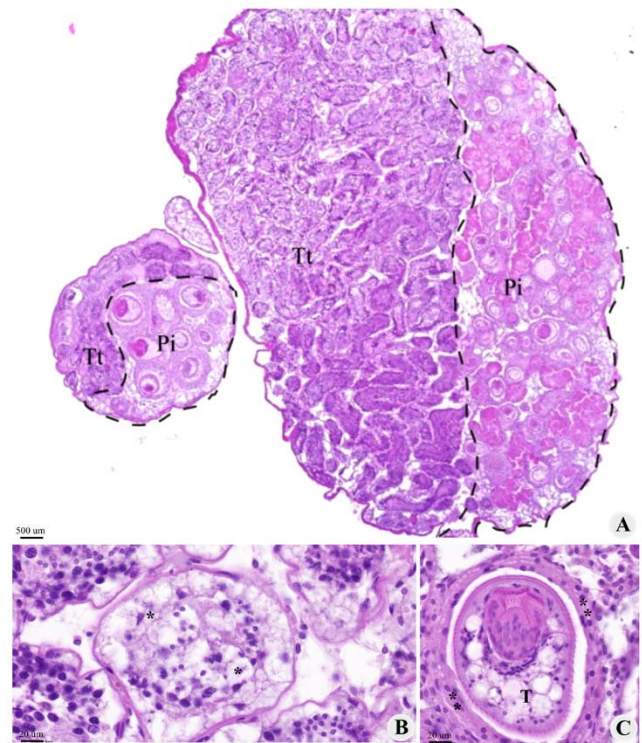


Figure 5. Light microscopic level showing the presence of parasites and histopathology of the representative gastropod *Nerita balteata* (A) Low magnification showed parasitic-populated infection (Pi). (B) The spermatogenic degeneration (asterisks) in the seminiferous tubule was observed. (C) The existence of the infected parasite, as possible to trematodes (T). This parasite was encapsulated by thin connective tissue (double asterisks) (Abbreviation: Tt = testicular testis, and dash line = The separated area between the parasitic infection (Pi); Staining method: A-C = Harris' hematoxylin and eosin (H&E)).

of spermatogenic stages (from spermatogonia to spermatids) in each sampled species, presented in Table 1, and the size of each dramatically decreased. There was no significant difference in spermatogenic stages among the studied gastropod species ($P < 0.5$, Table 1).

The presence of brown cells and histopathology between sites: The brown cell distribution was clear in all sampled gastropods throughout the

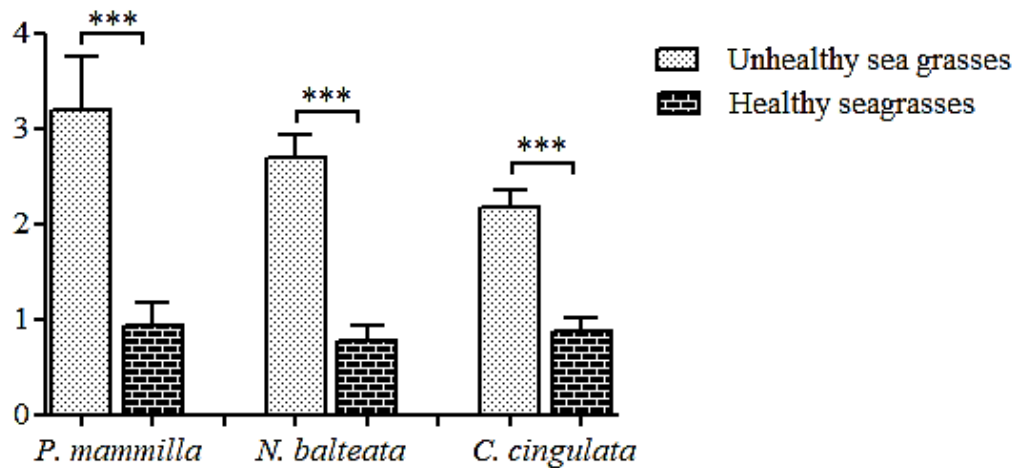


Figure 6. The number of brown cells of the gastropods, including *Polinices mammilla*, *Nerita balteata*, and *Cerithidea cingulata*, between the unhealthy and healthy seagrass. Values are represented as mean±SE (n = 30). Significant differences between 2 locations (***) $P < 0.05$). SE, standard error.

spermatogenic stage (Figs. 4A-C). High magnification showed that the round or oval brown cells had microscopic brown granules in their cytoplasm (Fig. 4C), which positively reacted with the PAS reaction (Fig. 4D).

Figures 4e and 5 compare the histopathological change (spermatogenic degeneration) and unidentified parasites (Figs. 5A-C) mentioned previously. The brown cells differed significantly ($P < 0.05$, Fig. 6). This cell is mostly used as a biological indicator of environmental problems in mollusks (Gosling, 2003). Brown granules in brown cells are well acknowledged as the primary proteolytic compartments for toxic elimination and foreign body removal (Gosling, 2003). This could be associated with exposure to environmental pollution (Zarogian et al., 1995, 2000; Zorita et al., 2006). The above-mentioned aspects may be related to environmental factors in the seagrass environment and the unhealthy seagrass site. Like many mollusca stocks in polluted areas (Zorita et al., 2006), we hypothesize that the seagrass environment could influence the health of the studied gastropods. Regular and proper monitoring of the environmental parameters on Libong Island is required.

According to both sample sites, some spermatozoa existed in Figures 4E, but the stage is still unclear. Melanomacrophage centers (MMCs) were also discovered as a distinctive grouping of pigment-

containing cells (Figs. 5F-G). One possibility is that the sampling gastropods would initially participate in both the innate and adaptive arms of the immune response (Roberts, 1975; Steinel and Bolnick, 2017), with each cell tasked with destroying, detoxifying, or recycling foreign components (Steinel and Bolnick, 2017). In this case, the stressful environment of seagrass areas on Libong Island may be due to pollution from fishery activities, as supported by the critical report (Pradit et al., 2020).

This study provides new information on the reproductive biology of gastropods dwelling on Libong Island. It revealed that the diameter of primary and secondary spermatocytes varied among the sampled gastropods, implying a reproductive function. Interestingly, prominent brown cells were found, especially at the unhealthy seagrass site. As prior observations have shown, this phenomenon is most likely linked to estuarine habitats' polluted and stressful conditions.

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