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Research article

Evidence of histopathological appearances in representative fishes and invertebrates from Libong Island, Thailand

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Abstract

The seagrass beds at Libong Island, Thailand, are a complex ecological habitat supporting many marine organisms. Unfortunately, the seagrass area is being lost, possibly exerting adverse impacts on aquatic life, but comprehensive aquatic monitoring and assessment efforts are still lacking. In this study, sentinel species were selected from two species groups commonly found in this area, pelagic species (*Ambassis nalua* and *A. vachelli*) and benthic species (*Amphibalanus amphitrite*, and *Alpheus* sp.). Specimens were collected from healthy and unhealthy seagrass areas around the island from April to June 2021. The health of the specimens was assessed using the histopathological approach together with the histological alteration index (HAI). Some histological alterations were identified that HAI values indicated were significantly more prevalent in the unhealthy seagrass areas ($P<0.05$). Among the invertebrates, *A. amphitrite* exhibited melanomacrophage centers while *Alpheus* sp. exhibited lamellar disorganization in gill and degeneration of hepatopancreatic cells. The two fish species exhibited vacuolar degeneration in the liver that was more pronounced in specimens from the unhealthy seagrass area. However, the HAI values calculated for all samples ranged from 0.1 to 1, indicating normal organs. These results suggest the emergence of environmental alteration in the threatened seagrass habitats at Libong Island, where there is a need to monitor impacts on flora and fauna health in further studies. It is also noted that fishes can be sensitive sentinel species of aquatic ecosystem health.

Keywords: Animal aquatic health, Histopathology, Seagrass area, Sentinel species, Thailand.

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INTRODUCTION

The use of aquatic animals as sentinel marker species to monitor the health of a population and environment has been well described (Viarengo 1993; Fowler et al., 2004; Hodkinson and Jackson 2005; Carew et al., 2013; El-Gammal et al., 2016). It is accepted that appropriate biological markers can be monitored using sentinel species to show evidence of environmental exposure. Invertebrates are suitable species because they are tiny, widespread, sessile, and have a great propensity to accumulate contaminants from their surroundings (Lazorchak et al., 2003; Chiarelli and Roccheri, 2014). Biomarkers of exposure are commonly utilized because the substances of interest can be quantified (National Research Council, 1991). Biomarkers of effects can also aid in estimating chemical exposure. Several studies have suggested that histopathological biomarkers are the most accurate, robust instruments for predicting fish health in the laboratory and field (Dietrich and Krieger, 2009) and can be used to diagnose and predict critical changes in tissue histology (Ayas et al., 2007; Dietrich and Krieger, 2009; Senarat et al., 2015).

Pathologists evaluate the effects of environmental stressors and contaminants by observing the degeneration and disruption of the more susceptible organs such as the brain, gill, gonad, kidney, and liver (Dietrich and Krieger, 2009; Organisation for Economic Co-operation and Development, 2009; Tillitt et al., 2010; Senarat et al., 2015). The information gained is used to address issues about animal health and environmental contamination (Blazer, 2002; OECD, 2009; Tillitt et al., 2010; Liebel et al., 2013). Field and laboratory researchers have raised health concerns for aquatic organisms by linking histopathological changes to chemical and heavy metal pollutants (Sarojini et al., 1993; Victor, 1993; Soegianto et al., 1999a; Soegianto et al., 1999b; Bhavan and Geraldine, 2000).

Libong Island is situated in the Andaman Sea off the southwestern coast of Trang Province in Thailand. The island features a rich ecosystem that includes seagrass beds covering about 27.2 km^2. Seagrass growth and expansion can be restricted by sediment load (Short and Wyllie-Echeverria, 1996) and massive silt loads can reduce the availability of light, killing seagrass and lowering its biomass (Short and Wyllie-Echeverria, 1996). Similar conditions exist around Libong Island together with microplastic pollution in the sediment and mudflat areas of the island (Pradit et al., 2020). These conditions can impact not just marine animals but also marine biodiversity. Benthic invertebrates and fish are especially susceptible to environmental changes (Reynoldson and Metcalfe-Smith, 1992) (Blazer, 2002; Liebel et al., 2013). However, for aquatic species inhabiting the seagrass area of Libong Island, no health status data exists.

Therefore, this study aimed to assess the structure and histopathological appearances of aquatic animals collected from healthy and unhealthy areas of the seagrass bed of Libong Island. Sentinel species were chosen from a diverse group of organisms that included pelagic species (*Ambassis nalua* and *A. vachelli*) and benthic species (*Amphibalanus amphitrite*, and *Alpheus* sp.). According to the NRC (1991), all selected species were abundant, easy to capture, and inhabited overlapping ranges. Morphological analyses and histological techniques were integrated. Baseline values from our results will provide data to help manage the seagrass areas of Libong Island that are under environmental stress.

MATERIALS AND METHODS

Aquatic animal collection and study areas

The two study sites chosen at Libong Island (Figures 1a-1c) were not adjacent. The health assessment of the seagrass beds was based on seagrass density and coverage (Wirachwong and Sudtongkung, 2020). Both areas were populated by *Halophila ovalis*. The healthy seagrass area (Figure 1d) had a density of 786–4,250 samples/m^2 and a seagrass coverage of 50–100%. The unhealthy seagrass area (Figure 1e) had a density of 18–500 samples/m^2 and a seagrass coverage of 5–25%. From April to June 2021, *Ambassis nalua* and *A. vachelli* from the pelagic group and *Amphibalanus amphitrite* and *Alpheus* sp. from the benthic group, were sampled by hand or visual observation. We collected 30 individuals of each representative species from each study site. Water physicochemical parameters including water temperature, salinity, pH and dissolved oxygen (DO) were measured at each site with a multiparameter water quality meter (U-50 – Horiba, HORIBA Advanced Techno Co., Ltd., Japan). Ethical approval for the work was provided by the Animal Care and Use Committee of Rajamangala University of Technology Srivijaya, Thailand (ID#IAC 13-03-64).

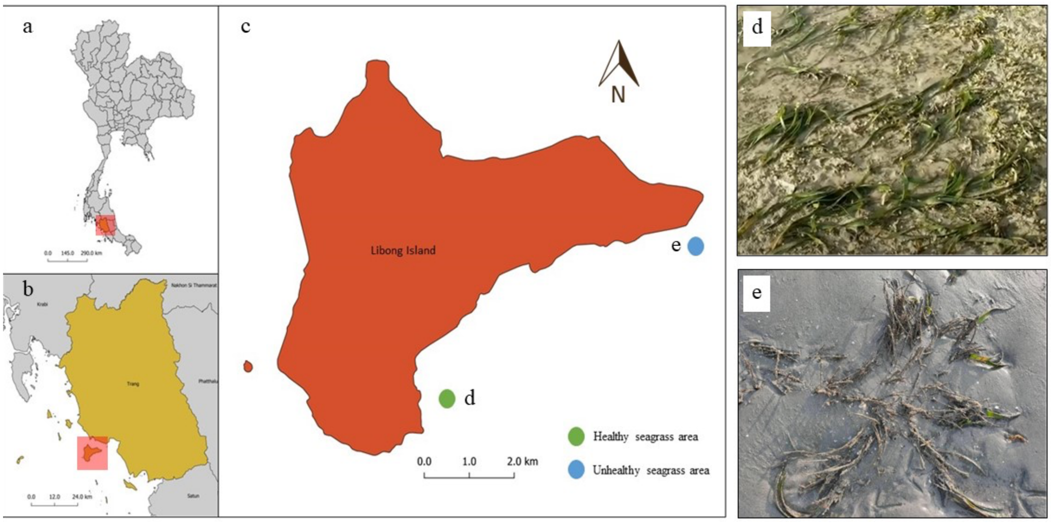

Figure 1 Locations in Thailand (a) of Trang Province (b) and the study area of Libong Island (c) including the study sites of healthy (d) and unhealthy (e) seagrass areas.

Morphology and histological observations

Specimens were euthanized with a rapid cooling shock (Wilson et al., 2009). They were then assessed under a stereo microscope for abnormal morphological features and the presence of external parasites. Specimens were preserved in Davidson's fixative for approximately 48 h at room temperature. Fixed specimens were subjected to standard histological procedures (Presnell and Schreibman, 1997; Suvarna et al., 2013). The paraffin blocks were sectioned at a thickness of 4 µm, and sections were stained with Harris' hematoxylin and eosin (H&E). Stained slides were examined under a light microscope to observe structural and histological alterations. The slides were scanned and photographed, and images were viewed using the 3DHISTECH Pannoramic Viewer (3DHISTECH, Hungary).

Semi-quantitative analysis

All histopathological alterations were semi-quantitatively classified with a histological alteration index (HAI) (Poleksic and Mitrovic-Tutundzic, 1994). The HAI was evaluated according to the degree of alteration and damage to each tissue (Table 1) (adapted from Poleksic and Mitrovic-Tutundzic, 1994; Paulo et al., 2012; Dos Santos et al., 2018; Barbierl et al., 2019). The HAI was calculated using the equation $HAI = 1 \times \sum I + 10 \times \sum II + 100 \times \sum III$, where I, II, and III correspond to stages of histopathological alteration (Table 1), respectively. The average HAI is divided into five categories (I: 0 to 10 (normal organ functioning), II: 11 to 20 (slight alteration in the organ), III: 21 to 50 (moderate alteration in the organ), IV: 51 to 100 (severe alteration in the organ) and V: values above 100 (irreparable alteration in the organ) (Poleksic and Mitrovic-Tutundzic, 1994).

Table 1 Classification of the severity of histopathological alterations in the representative fish and invertebrates from Libong Island, Thailand

Representative aquatic animals	Histopathological alterations	Stage
Amphibalanus amphitrite	Melanomacrophage centers (MMCs)	I
Alpheus sp.	Lamellar disorganization Degeneration of hepatopancreatic cells	I I
Ambassis nalua and *A. vachelli*	Vacuolar degeneration of hepatocyte (or hepatocellular lipidosis) in liver Melanomacrophage centers (MMCs) Lamellar aneurysm in gill Renal degeneration in kidney	I I II II

Statistical analysis

Water physicochemical parameters were expressed as means ± SD, whereas the HAIs between areas were compared by an independent Student's t-test ($p < 0.05$) (GraphPad Prism for Windows version).

RESULTS

Environmental factors

Water temperature, pH, salinity, and DO were monitored at both areas (Table 2). All water physicochemical parameters, except pH, were significantly different between areas ($P<0.05$).

Table 2 Observation of environmental factors from Libong Island, Thailand

Water physicochemical parameters (n = 3 per area)	Healthy seagrass condition	Unhealthy seagrass condition	Reference (Lohaluksanadech et al., 2008)
Temperature (° C)	31.69 ± 0.13*	30.98 ± 0.08*	29.25-30.61
pH	7.67 ± 0.02	7.62 ± 0.05	7.74-8.18
Salinity (ppt)	29.97 ± 0.23*	31.10 ± 0.17*	19.67-30.46
DO (mg/L)	8.01 ± 0.10*	5.94 ± 0.13*	5.63-6.98

Note: The asterisk indicates a significant difference ($P < 0.05$) when the data are compared with other groups

Morpho-histology and histopathology

No abnormal morphological features were observed, and no parasites were found in any of the sampled species. However, some structural and histopathological alterations were found and are described below (Figures 2–4).

Amphibalanus amphitrite

The main body, female reproductive tissue (Wang et al., 2018), and the cement glands (Lin et al., 2021) of this acorn barnacle have been observed and the important histology reported. Our whole-body investigation of *A. amphitrite* revealed that the main body had cirri, muscle, testis, and a digestive tract comprising a U-shaped stomach and intestine (Figures 2a–2b).

The testicular follicle in the sub-mantle cavity was normally surrounded by connective tissue (Figure 2c). Sperm at different stages of development could be observed within the follicles (Figure 2d), including spermatocytes, spermatids, and spermatozoa (Figures 2d–2e). Melanomacrophage centers (MMCs), as highly pigmented phagocytes (Figure 2e), were found close to testicular follicles only of specimens from the unhealthy seagrass area. Large ganglia contained a normal prominent neuronal cell (Figure 2f). At high magnification, the simple columnar epithelium of the stomach mucosa and intestinal mucosa could be observed (Figures 2g–2h).

***Alpheus* sp.**

Histological examination of a longitudinal section revealed the male reproductive organ, muscle, gill, and ganglion (Figure 3a). The hepatopancreas was a yellowish-brown organ located in the cephalothorax (Figure 3a), formed by numerous oval or circular acini (tubules) (Figure 3b). The cross-section also revealed the star-shaped lumen of an acinus (Figure 3b). Based on the detailed attributes reported in other crustaceans (Ceccaldi, 1989; Maharajan et al., 2015), the four major cell types observed in our study of the acinus were identified as E- (embryonic), R- (reserve), B- (secretory), and F- (fibrillar) cells (Figures 3b–g). At the center of cytoplasm, E-cells with round to oval nuclei were found near the basement membrane (Figure 3b). R-cells had a tall columnar structure featuring an apical border of microvilli and a basal nucleus (Figure 3b). Many tiny lipid vacuoles could be observed in R-cells (Figure 3b). B-cells were large, containing a large and single secretory granule (Figure 3b), whereas the F cells were long without vacuoles in the cytoplasm (Figure 3b). Some samples from the unhealthy seagrass area showed some degeneration of hepatopancreatic cells (Figure 3c). Gill lamellae were covered with a thin cuticle layer (Figure 3d). Spaces were observed within pillar cells parallel to the surface of lamellae (Figure 3d), while interlamellar and haemocoelic spaces were uniform and normal, containing hemocytes (Figure 3d). Additionally, the seminiferous tubule in the testis contained sperm at different stages of development, including spermatocytes (Figure 3e) and spermatozoa (Figure 3f).

Ambassis nalua* and *A. vachelli

The two species shared histological structures and *A. nalua* was chosen to represent both species (Figure 4a). When viewed in the longitudinal section, gill, heart, liver, kidney, stomach and intestine could be observed (Figure 4a). The gill was made up of a comb-like structure. The gill filament was composed of primary and secondary lamellae having clusters of erythrocytes (Figure 4b). Although numerous histological changes were observed, lamellar disorganization was the most frequent among specimens from both sampling areas (Figure 4b). The liver tissue comprised many hepatocytes and hepatic sinusoids (Figure 4d). Foamy features, or empty vacuoles, associated with hepatocellular lipidosis were observed in specimens from the healthy and unhealthy seagrass areas. Close to the hematopoietic tissue, signs of renal tubule degeneration were identified (Figure 4f).

Semi-quantitative analysis of histopathological alterations

The HAI value of *A. amphitrite* was significantly different between two areas ($P<0.05$), showing 0.1 (n = 30) of healthy seagrass area and 0.6 (n = 30) of unhealthy seagrass area (Figure 5b); however, the observed HAI value of gill (0.1, n = 30) in *Alpheus* sp. was only found in the unhealthy seagrass area (Figure 5b). The HAI values of the hepatopancreas in *Alpheus* sp. were also similarly observed between areas (0.1, n = 30 per area, Figure 5c). *Ambassis nalua* and *A. vachelli* exhibited tissue alterations in various organs, including liver (Figure 5d), gill (Figure 5e) and kidney (Figure 5f), but the HAI values were not significantly different (Figures 5d-5f).

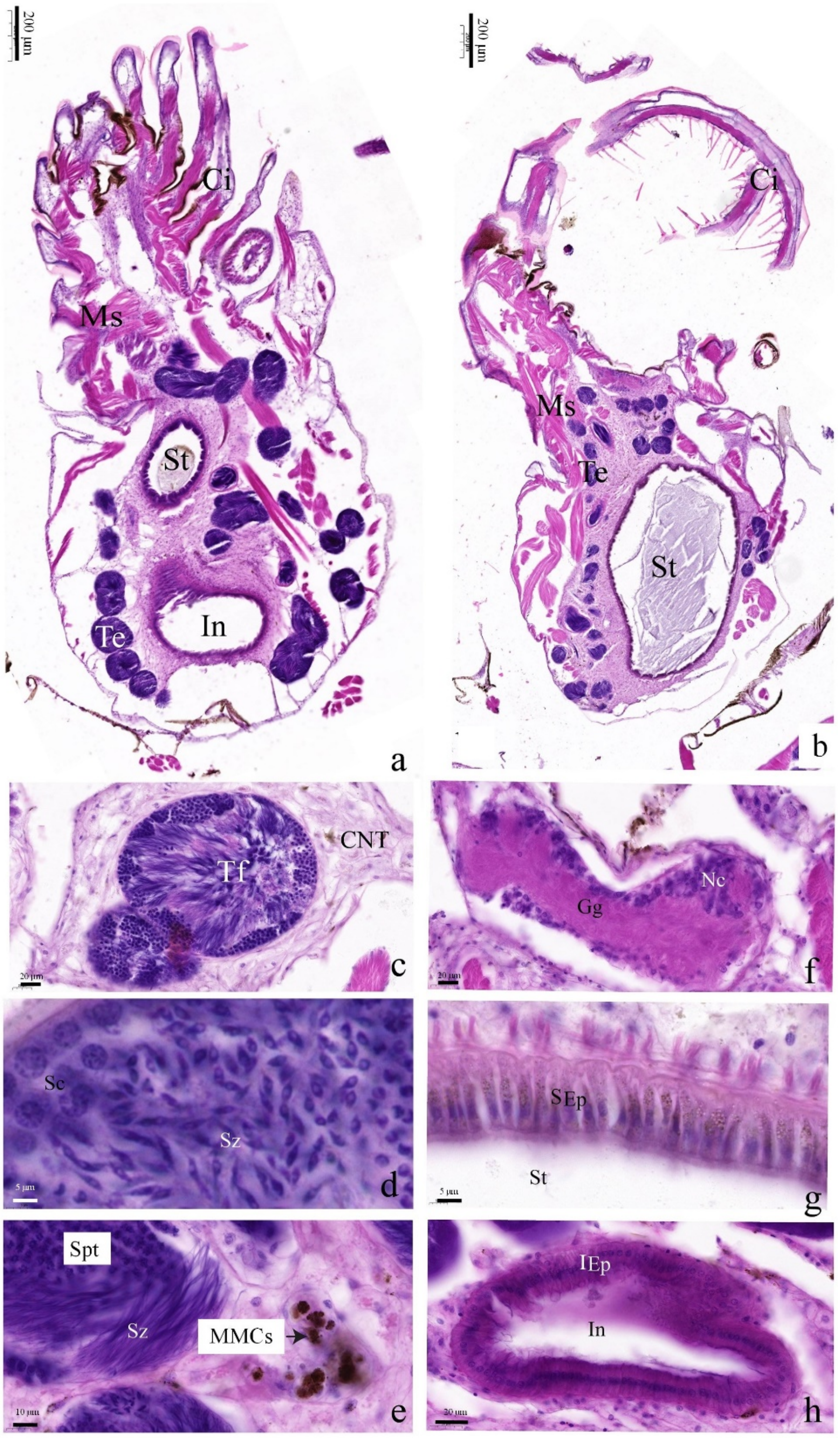

Figure 2 Stained slides under the light microscope produced the whole-body images of *Amphibalanus amphitrite* in longitudinal section (a-b). Prominent organs observed were cirri (Ci), muscle (Ms), stomach (St), intestine (In) and testis (Te). Images at higher magnification show the testicular follicle (Tf) containing spermatocytes (Sc), spermatids (Spt) and spermatozoa (Sz) (c-e). A cluster of melanomacrophage centers (MMCs) was identified (e). A ganglion (Gg) with neuronal cell (Nc) is shown (f), the stomach epithelium (SEp) was normal (g), with a structure similar to the intestinal epithelium (IEp) (h).

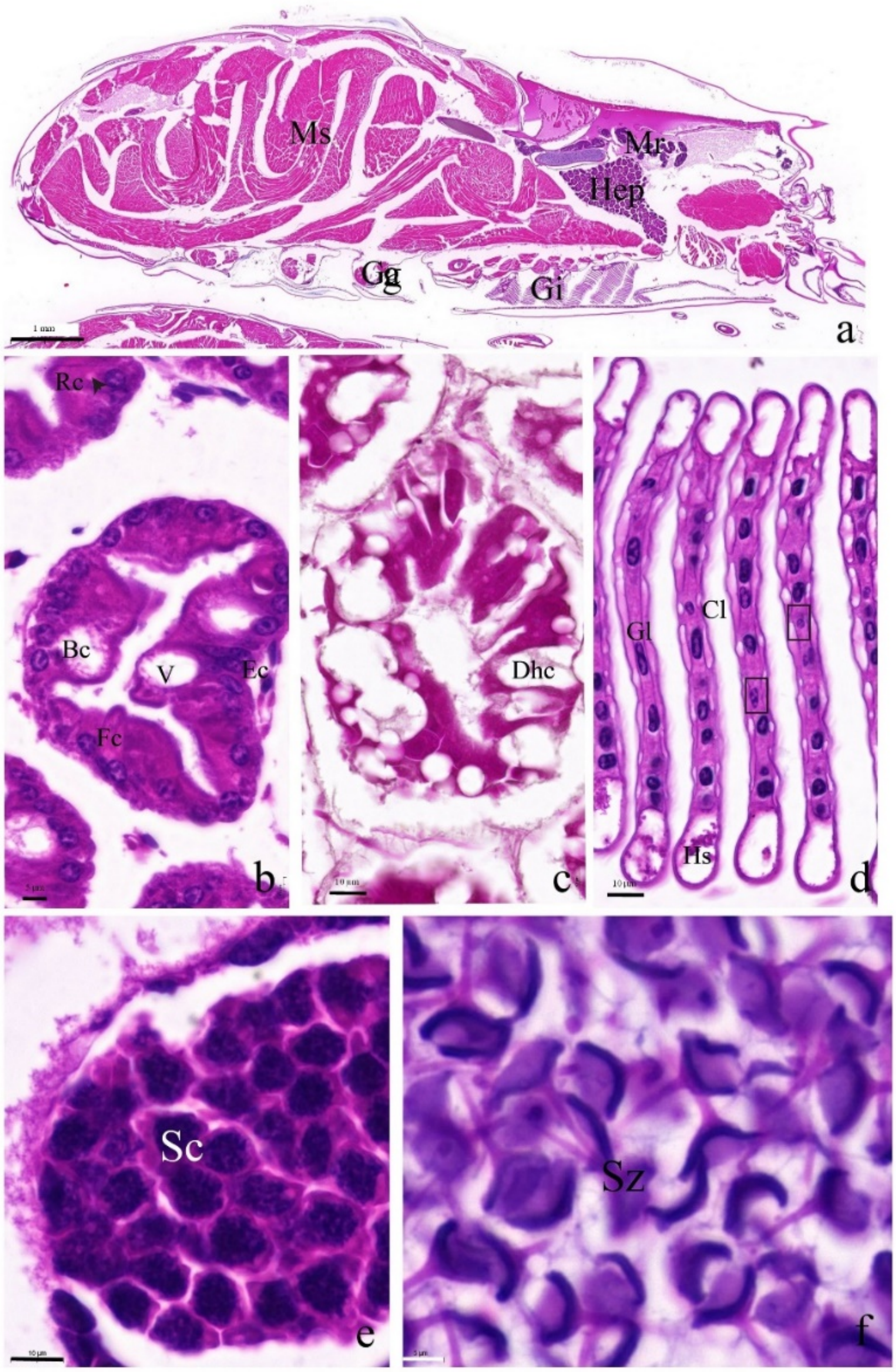

Figure 3 The whole body of *Alpheus* sp. is shown in the longitudinal section. The observed organs include muscle (Ms), ganglion (Gg), gill lamella (Gi), male reproductive organ (Mr) and hepatopancreas (Hep) (a). High magnification revealed E (Ec), R (Rc), B (Bc), and F (Fc) hepatopancreatic cells around a vacuole (V) (b). In some specimens, the degeneration of hepatopancreatic cells (Dhc) was observed (c). The cuticle layer (Cl) covering lamellar disorganization had occurred. Haemocoelic spaces (Hs) were normal, while pillar cells (indicated by squares) were not (d). Spermatocytes (Sc) were oval and contained a basophilic nucleus in parallel to the presence of spermatozoa (Sz) (f).

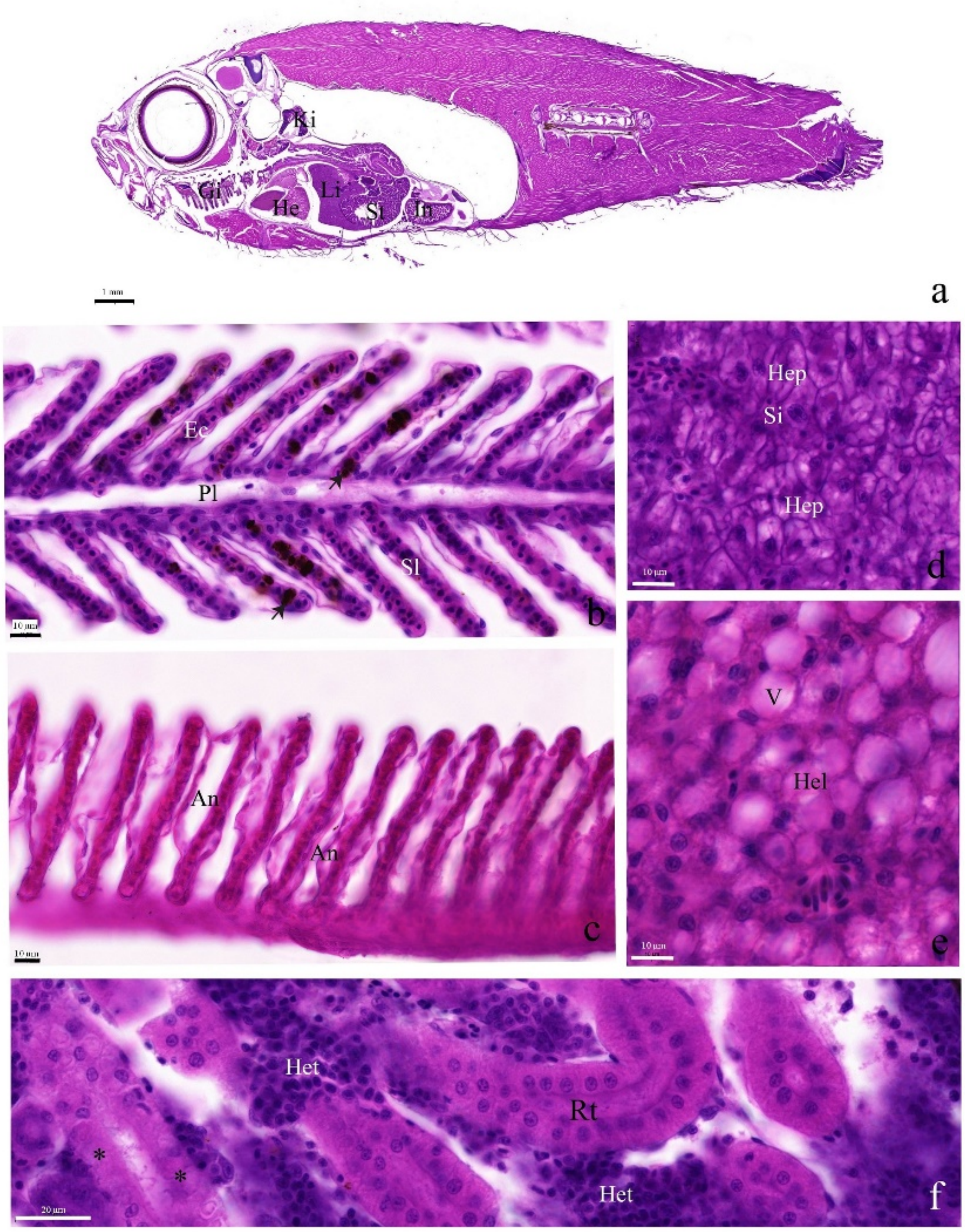

Figure 4 The stained slide (a) under the light microscope shows the whole body of *Ambassis nalua* in longitudinal section, representing both *A. nalua* and *A. vachelli*. The gill (Gi), heart (He), liver (Li), kidney (Ki), stomach (St) and intestine (In) were observed. The lamellar gill (b) comprised primary lamellae (Pl) and secondary lamellae (Sl). Several erythrocytes (Ec) were seen in the spaces of secondary lamellae, and melanomacrophage centers (MMCs, arrows) were also observed. Aneurysms (An) were identified (c). The liver structure generally contained hepatocytes (Hep) and hepatic sinusoids (Si) (d), whereas some livers showed a vacuolar degeneration of hepatocyte or a hepatocellular lipidosis (Hel), having prominent vacuoles (V) (e). Hematopoietic tissue (Het) and renal tubules (Rt) having the renal epithelium (*) were normal (f).

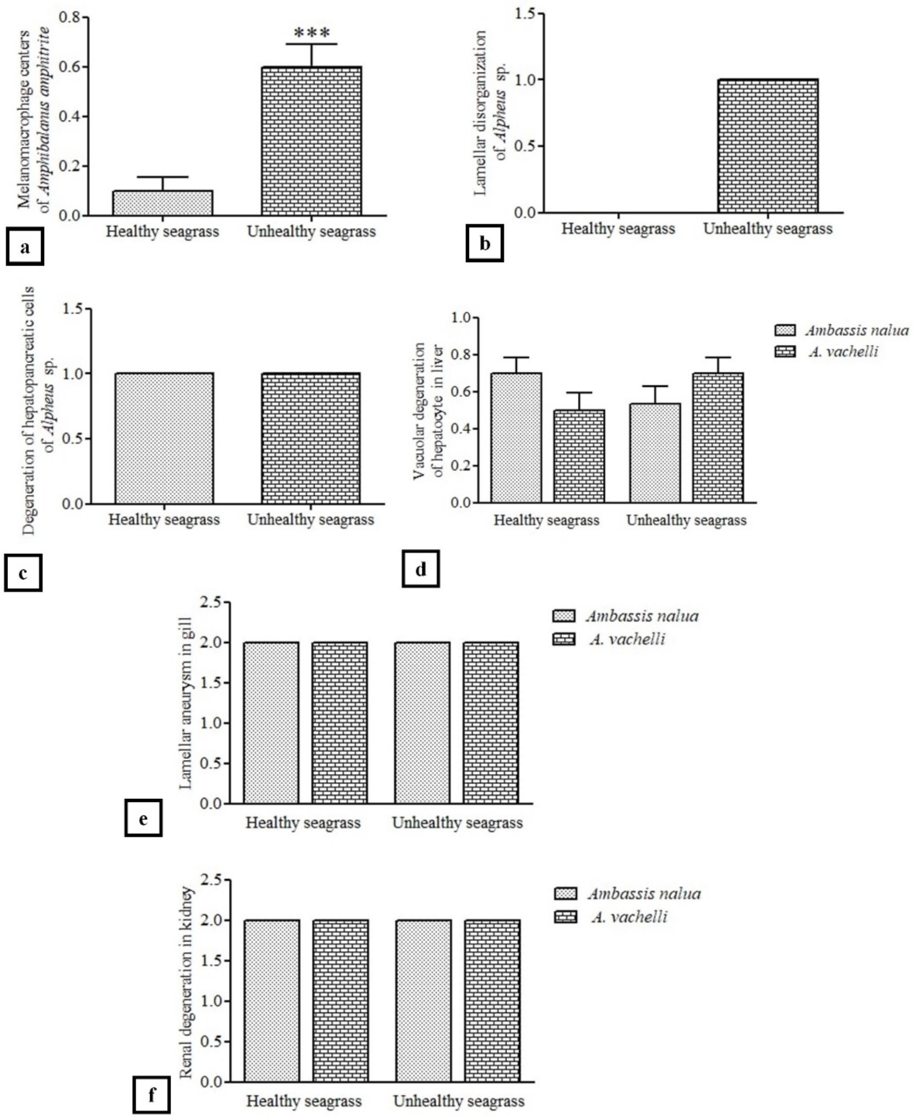

Figure 5 The charts (a-b) show means of the histological alteration indexes (HAI) of the invertebrates sampled at Libong Island, Thailand, including *Amphibalanus amphitrite*. For the representative fishes (a) and *Alpheus* sp. (b-c), the HAIs of various organs of *Ambassis nalua* and *A. vachelli* are compared, including the liver (d), gill (e) and kidney (f). The asterisk indicates a significant difference ($P < 0.05$) when the data are compared with other groups

DISCUSSION

The health of aquatic ecosystems can be monitored by observing the health status of aquatic animals. We used histopathology to observe biomarkers that indicated the health of marine aquatic animals from Libong Island, Thailand. The data acquired in this study showed that conditions at Libong Island were within the standard ranges for marine environmental resources according to Lohaluksanadech et al. (2008), and therefore it might be inferred that the representative samples were living in water of good quality.

Although the HAI values obtained from all samples indicated normal organs and organ functioning, a few alterations were observed. The main histopathological appearances were more severe in specimens sampled from seagrass beds in unhealthy conditions. These appearances were probably related to the polluted and stressful conditions of the nearby estuary environment. Since the seagrass of Libong Island has decreased considerably in area, marine biodiversity, marine organisms, and some aquatic sentinel species may be affected.

A. amphitrite and fishes from the unhealthy seagrass bed exhibited clusters of yellowish-brown MMCs. Although numerous occurrences of MMCs have been recorded in fish species (Agius and Roberts, 2003; Dyková et al., 2022), there has been no previous report of the occurrence of MMCs in an invertebrate. Studies of the prevalence and intensity of MMCs in fish reveal indications of environmental stress, and evidence of contamination and pollution (Agius and Roberts, 2003; Steinel and Bolnick, 2017). MMCs have been proposed as an indicator of immune function in fish (Steinel and Bolnick, 2017) and of the impact of minute concentrations of pesticide contaminants (Manrique et al., 2014; Tjahjaningsih et al., 2017; Manrique et al., 2019). Unfortunately, there is little information on the environmental situation at Libong Island, where the critical reported issue is seagrass loss. Our observations suggest that the presence of MMCs in the affected species might be induced by environmental factors and stress. Fishelson (1996) showed that increased numbers of MMCs in fish are associated with environmental stress, rather than tissue catabolism. The present study provides more evidence that deteriorating seagrass beds present a stressful habitat for aquatic animals, which probably reduces the available habitat needed for aquatic animal health maintenance.

Micro-anatomically, *A. nalua* and *A. vachelli* showed evidence of vacuolar degeneration of hepatocytes, or hepatocellular lipidosis and cellular degeneration of hepatocytes. These pathologies were exhibited by specimens from seagrass beds in both healthy and unhealthy conditions. This type of cytoplasmic vacuolation in hepatocytes suggests a glycogen-type vacuolation (Wolf and Wheeler, 2018) and is categorized as liver damage (Greenfield et al., 2008), implying degeneration of the endoplasmic reticulum and Golgi apparatus (Braunbeck, 1998). Previous observations revealed that the occurrence of hepatocellular lipidosis could be induced by several factors, including chlorinated hydrocarbons and other contaminants that directly affect animals (Hinton et al., 1992; Robertson and Bradley, 1992; Schrank et al., 1997). Other causes of hepatocellular lipidosis are old age and poor nutrition (Hinton et al., 1992; Robertson and Bradley, 1992), as reported in a captive *Synanceja verrucosa* (Penrith et al., 1994). Ferguson (1989) experimentally found that diets high in polyunsaturated fats might be associated with lipid peroxidation. It is possible that the hepatic condition of the two fish species we collected from the unhealthy seagrass bed could be associated with environmental pollution, degraded food sources, and/or other factors mentioned above (Laurén and Wails, 1990). Fish are known to be susceptible to environmental change (Reynoldson and Metcalfe-Smith, 1992), and therefore this pathology may indicate a general deterioration in the health of fish at Libong Island.

CONCLUSIONS

Although we observed some histopathological appearances in some aquatic species, it is important to note that the presence of MMCs is an indicator of environmental stress as well as unhealthy seagrass conditions. The effects exerted by MMCs include alterations of the respiratory system. It is also recommended that fish be used to assess and monitor environmental problems. Further investigation of pollutants in the environment and aquatic animals should be performed in the field and laboratory with advanced techniques.

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AUTHOR CONTRIBUTIONS

Wikit Phinrub: Conception, data analysis and original writing. Nisreen Dahlan: Sampled collection, data analysis and manuscript editing. Sinlapachai Senarat: Conception, field study and manuscript editing. Atsuo Iida: Data analysis and manuscript editing. Natthawut Charoenphon: Data analysis and manuscript editing. Narit Thaochan: Data analysis and manuscript editing. Kitipong Angsujinda: Data analysis and manuscript editing. Supapong Imsonpang: Conception, data analysis, validation, and supervised data. All authors read and agreed on the final manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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