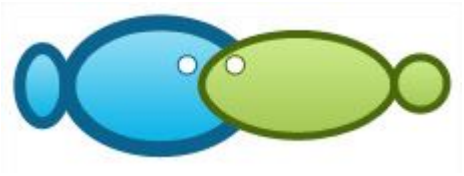

Histopathology conditions of cultured oyster, *Crassostrea iredalei* from southern and east Malaysia

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Abstract. This is a histopathologic survey of cultured oysters, *Crassostrea iredalei* in Southern Malaysian state and East Malaysia, where incidents of mass mortalities of oysters occurred in 2012. A total of 48 samples of C. *iredalei* were sampled from both locations. The samples were examined for macroscopic symptoms and were processed for histological study. The collected oysters had similar average shell length between Johor (8.73±0.46 cm) and Sabah (8.75±0.66 cm), respectively. The histopathology examination revealed hypertrophy, hyperplasia, necrosis, thinning of structure such as digestive diverticula, presence of granulocytomas and granulation tissue. "Lesion", "Accumulation of brown cells" and "Hemocyte accumulation" were more severe in Sabah samples compared to Johor samples, except for "Oocysts of *Nematopsis* sp.". The only pathogen found in this study was *Nematopsis* sp. and higher prevalence of *Nematopsis* sp. was found in Johor samples. This study for the first time, documented the histopathology of the sub-population of cultured oysters from two main culture areas in Malaysia, despite the low number of samples.

Key Words: histopathology, *Crassostrea iredalei*, *Nematopsis* sp., Johor, Marudu Bay, Sabah.

Introduction. As the human population grows, exceeding seven billions nowadays, the supply for protein especially aquatic food supplies such as fish, mollusks, crustaceans and others in the wild are being overexploited (FAO Fisheries and Aquaculture Department 2016). The overexploitation is due to the advancement of facilities such as vessels with high output with minimum effort and other new technologies of fish farming over the last five decades. In 2014, Asia produced about 21.8 million tonnes in the marine and coastal aquaculture section and the production of mollusks is comprised at 66.5% (FAO Fisheries and Aquaculture Department 2016). The main oyster species reported in the FAO database include *Crassostrea madrasensis* (Preston, 1916), *Crassostrea gigas* (Thunberg, 1793), *Crassostrea iredalei* (Faustino, 1932) and others (FAO Fisheries and Aquaculture Information and Statistics Service 2012). In Malaysia, the states that are involved in the production of oysters include Kedah, Johor, Penang, Terengganu, Kelantan and Sabah. Sabah is the highest contributor of oysters in Malaysia producing about 668.65 tonnes out of the total of 697.93 tonnes in 2013 (Department of Fisheries Malaysia 2013); lower compared to the 2010 production of 713.53 tonnes.

Oysters are filter feeder organisms and they are known to filter the food particles from surrounding environment including plankton, virus, bacteria and others (FAO Fisheries and Aquaculture Department 2005; Wang et al 2008; Elston et al 2008; Ueki et al 2010). Hence, oysters can be easily infested with pathogens as a host or a carrier. In Malaysia, insufficient research has been done and published and hence the knowledge on oyster's health conditions is scarce (Tan et al 2014). Therefore, it was vital to conduct

disease related studies in Malaysian oyster industry. Occurrence of low yield of production was observed due to mass mortalities of oysters (*C. iredalei*) and mussels (*Perna viridis*) in the affected Sabah farms in 2012 (Tan et al 2014). A preliminary study has been conducted by researchers from Universiti Malaysia Terengganu (UMT), Universiti Malaysia Sabah (UMS) and Department of Fisheries Sabah (DOF Sabah) on the mussels and the presence of iridoviruses, bacteria and parasites was found (Julian Ransangan, unpublished data). Mass mortality of oysters and mussels in Sabah was mentioned but no further study has been done to investigate the incidents hereafter (Tan et al 2014). Thus, this study was carried out using histological method to survey the health status of oyster reared at Marudu Bay, Sabah and Johor, southern state, another intensive oyster culture area in Malaysia.

Material and Method

Sampling. The oysters (*C. iredalei*) were collected from Marudu Bay, Sabah, east Malaysia (6°45'00"N, 116°55'00"E) on 2nd January 2014 and 8th May 2014 (total number of samples = 15), during dry and wet seasons. Whereas, the Johor samples from southern Malaysia (1°29'02.3"N, 103°49'02.4"E) were collected on 25th November 2013 and 8th July 2014 (total number of samples = 33). Low number of samples were collected due to the previous mass mortality incident at the local farms. The collected oysters from the sampling sites were cleaned first to remove debris and dirt on the oyster surface. Macroscopic examination was recorded and then the oysters were processed for histological study.

Histology study. The histological methods were conducted according to modified methods of Histological Techniques for Marine Bivalve Mollusks by Howard (1983). A transverse section cut (5 mm) was cut between the palps and gills containing intestine, connective tissue, stomach, digestive diverticula, gonad, kidney, gills and mantle. The dissected sections were fixed in Davidson's fixative for 24 hours, subsequently dehydrated and embedded in molten paraffin wax. The prepared paraffin blocks were sectioned at 5 µm and stained with haematoxylin and eosin. The slides were examined for histopathological conditions and the presence of pathogens were examined under Leica DM LB2 light microscope equipped with Leica DFC 450C camera. The conditions and the intensity of the histopathology were classified and recorded according to the scale suggested by Lassudrie et al (2014). The histopathological conditions were categorized in this study as: "Lesion"; "Accumulation of brown cells"; "Hemocyte accumulation" and "Oocysts of *Nematopsis* sp.". The relationship between each histopathological condition were analyzed statistically using Statistical Package for the Social Sciences (SPSS) software version 22 (IBM) between each histopathological conditions intensity among samples in respective to sampling location. The length, width and number of the oocysts of *Nematopsis* sp. (Apicomplexa) observed were recorded for descriptive statistics analysis from each sampling location.

Results

Macroscopic examination. The average length of the Johor and Sabah samples was 8.73±0.46 cm and 8.75±0.66 cm, respectively. The shell of the oyster was occupied with barnacles-like organisms (Figure 1).

Figure 1. External and internal morphology of C. *iredalei*, with presence of barnacles-like organisms on the oyster shell.

Histological study. The histological signs observed in samples from both locations include hypertrophy, hyperplasia, necrosis, thinning of structure such as digestive diverticula, presence of granulocytomas and granulation tissue and others (Figure 2). The histopathological conditions observed were grouped into "Lesion", "Accumulation of brown cells", "Hemocyte accumulation" and "Oocysts of *Nematopsis* sp." (Table 1).

Table 1

The definitions of histopatological conditions categorized in this study

Histopathological conditions	*Definitions*
Lesion	Any damages and abnormality of the structures of the organs, which includes hypertrophy, hyperplasia, necrosis, thinning of structure such as digestive diverticula, presence of granulocytomas and granulation tissue organs (Kim et al 2006; Farlex Partner Medical Dictionary 2012)
Accumulation of brown cells	Accumulation of brown cells, which are phagocytes containing brown pigments in a large surface area (Humphrey & Norton 2005)
Hemocyte accumulation	Accumulation of hemocytes, which is the immune-competent cells that play a role in immune defense system of oyster, over large surface area of connective tissue of oyster (Labreuche et al 2006)
Oocysts of *Nematopsis* sp.	The presence of the oocysts of protozoan parasite gregarine *Nematopsis* sp. in the sample by having one or few oocysts observed within phagocyte, where the sporozoite was enveloped in the parasitophorous vacuole (Clopton 2002)

Oocysts of Nematopsis sp. A total of 1010 oocysts of *Nematopsis* sp. were found in Johor sample at 78.8% (Figure 2J). The mean length of oocysts found was 12.451±0.088 µm and the mean width of oocyst was 7.599±0.070 µm. The intensity of oocysts in infected samples ranged from one to 30. Out of the sample examined, 22 samples showed the presence of phagocytes and area of encapsulation by hemocytes (Ph/AoE) along with the presence of oocysts of *Nematopsis* sp. A sum of 45 oocysts of *Nematopsis* sp. were found in Sabah samples with the prevalence of 13.3% (Figure 2I). The mean length of oocysts found was 13.087±0.230 µm and the mean width of oocyst was 7.318±0.188 µm. The intensity of oocysts in infected samples ranged from one to 7. Two samples showed the presence of phagocytes and area of encapsulation by hemocytes (Ph/AoE) along with the presence of oocysts of *Nematopsis* sp.

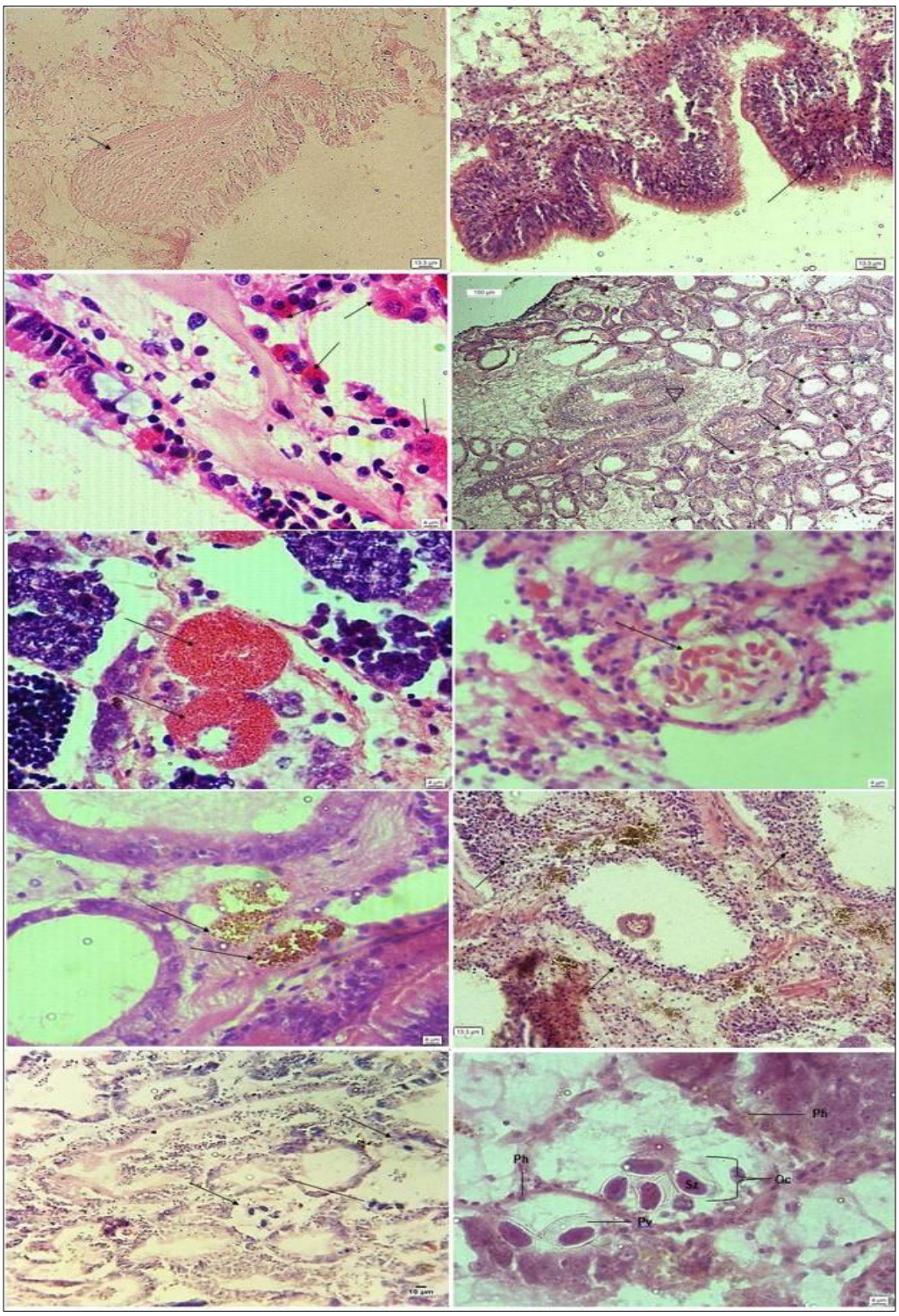

Figure 2. Histopathological conditions: (A) Hypertrophy of muscle fibre of mantle (arrow) of C. *iredalei* from Johor (hematoxylin and eosin, 200X total magnification); (B) Hyperplasia of mantle epithelium (arrow) of oyster from Sabah (H & E, 200X total magnification); (C) Gills necrosis (arrow) of oyster collected from Sabah (H & E, 400X total magnification); (D) Thinning (arrow), hemocyte accumulation and vacuolation (triangle) of the digestive diverticula of oyster from Sabah (H & E, 100X total magnification); (E) Presence of granulocytomas (arrow) at the connective tissue of oyster from Sabah (H & E, 400X total magnification); (F) Presence of granulation tissue (arrow) at gills of oyster from Sabah (H & E, 400X total magnification); (G) Accumulation of brown cells (arrow) at the connective tissue of oyster from Sabah (H & E, 400X total magnification); (H) Hemocyte accumulation (arrow) at the connective tissue of oyster from Sabah (H & E, 200X total magnification); (I) Presence of oocysts of *Nematopsis* sp. in phagocyte (arrow) and being freely (line) at the connective tissue of oyster from Sabah (H & E, 200X total magnification); (J) Presence of two phagocytes (Ph) with four and two oocysts (Oc) respectively, each one in a parasitophorous vacuole (Pv) with a sporozoite (Sz) at the connective tissue of oyster from Johor (H & E, 400X total magnification).

Intensity level of histopathological conditions. Most of the histopathological conditions observed in Johor samples based on the intensity level ranged from absence (0) to low (1.0). Whereas, "Accumulation of brown cells" and "Oocysts of *Nematopsis* sp." showed moderate (1.5) level of intensity in the collected samples (Table 2). Based on the correlation of presence of histopathological conditions in each sample, a total of 54.55% of the samples showed the presence of "Accumulation of brown cell" and "Oocysts of *Nematopsis* sp." in the same sample (Table 3). However, only 24.24% of the samples showed the presence of all the conditions in the same sample.

Table 2

The intensity level of each histopathological conditions of Johor samples (n = 33)

Intensity level	*Lesion*	*Accumulation of brown cell*	*Hemocyte accumulation*	*Oocysts of Nematopsis sp.*
0 (absence)	16	11	18	7
0.5 (very low)	15	11	11	5
1.0 (low)	2	10	4	17
1.5 (moderate)	0	1	0	4

Table 3

The correlation of presence of histopathological conditions in samples from Johor

Histopathological conditions	*Percentage (%)*
Lesion + accumulation of brown cells	48.48
Lesion + hemocyte accumulation	30.30
Lesion + oocysts of *Nematopsis* sp.	42.42
Accumulation of brown cells + hemocyte accumulation	39.39
Accumulation of brown cells + oocysts of *Nematopsis* sp.	54.55
Hemocyte accumulation + oocysts of *Nematopsis* sp.	36.36
Lesion + accumulation of brown cells + hemocyte accumulation	30.30
Lesion + hemocyte accumulation + oocysts of *Nematopsis* sp.	39.39
Accumulation of brown cells + hemocyte accumulation + oocysts of *Nematopsis* sp.	30.30
Lesion + Accumulation of brown cells + hemocyte accumulation + oocysts of *Nematopsis* sp.	24.24

Most of the histopathological conditions observed in Sabah samples based on the intensity level (Table 4) showed low (1.0) level of intensity while the presence of "Oocysts of *Nematopsis* sp." were absent (0) in most of the samples. The presence of "Accumulation of brown cells" showed moderate (1.5) level of intensity (Table 4). About 80% of the samples showed the presence of "Accumulation of brown cells" and "Hemocyte accumulation" in the same sample (Table 5). However, only 6.67% of samples showed all the conditions in the same sample.

Table 4

The intensity level of each histopathological conditions of Sabah samples (n = 15)

Intensity level	*Lesion*	*Accumulation of brown cell*	*Hemocyte accumulation*	*Oocysts of Nematopsis sp.*
0 (absence)	4	3	2	13
0.5 (very low)	3	1	6	1
1.0 (low)	8	9	7	1
1.5 (moderate)	0	2	0	0

Table 5

The correlation of presence of histopathological conditions in samples from Sabah

Histopathological conditions	*Percentage (%)*
Lesion + accumulation of brown cells	66.67
Lesion + hemocyte accumulation	73.33
Lesion + oocysts of *Nematopsis* sp.	6.67
Accumulation of brown cells + hemocyte accumulation	80.00
Accumulation of brown cells + oocysts of *Nematopsis* sp.	13.33
Hemocyte accumulation + oocysts of *Nematopsis* sp.	13.33
Lesion + accumulation of brown cells + hemocyte accumulation	66.67
Lesion + hemocyte accumulation + oocysts of *Nematopsis* sp.	6.67
Accumulation of brown cells + hemocyte accumulation + oocysts of *Nematopsis* sp.	13.33
Lesion + accumulation of brown cells + hemocyte accumulation + oocysts of *Nematopsis* sp.	6.67

Discussion. This is the first health survey conducted on oyster species, C. *iredalei* cultured at Johor and Sabah through histological study, despite the low number of samples obtained throughout the study. Overall, the oyster samples collected from Sabah displayed severe histopathological conditions compared to Johor samples. Symptoms such as hypertrophy, hyperplasia, necrosis of gills cells, granulation tissue, granulocytomas, and dilation of digestive diverticula followed by flattening and thinning of epithelium were observed in the sample in low occurrence and hence, they were grouped together as “Lesion”. Hypertrophy and hyperplasia were defined as the increase in cell size and cell number respectively; a response to either physiological or pathological stimuli (Wheater et al 1985). These two signs were observed at the epithelium of mantle and digestive organs of samples from Sabah while only mantle muscle fibre hypertrophy was observed in a few samples from Johor. Similar symptoms were reported in other bivalve species where hypertrophy, hyperplasia and vacuolization of cell and epithelia of gills and mantle of zebra mussels, *Dreissena polymorpha w*ere observed due to high infestation of ciliate species, *Sphenophrya dreissenae* (Laruelle et al 1999). Hypertrophy of epithelial cells of digestive gland in mangrove mussels, *Mytella guyanensis* has been reported with the presence of rickettsia-like organisms (Boehs et al 2010). Apart from that, hypertrophy was observed in gill tissue of razor clam, *Ensis arcuatus* due to presence of unknown copepod (Darriba et al 2010). These evidences suggested that these symptoms occurred along with the presence of foreign bodies or pathogens; the only pathogen observed through histological study was the presence of oocysts of *Nematopsis* sp. at the connective tissue of gills and digestive diverticula.

Both Sabah and Johor samples demonstrated the presence of lesion, accumulation of hemocyte and brown cells. Both hemocyte and brown cells play a role in the defense system in bivalve species. Hemocyte functions as the primary cellular protection and actively involved in the processes of hemocytosis, phagocytosis and encapsulation (Gosling 2003; Coen & Bishop 2015). Brown cells are phagocytic cells enclosing brown pigment which are found usually at the interstitial tissue that acts on the degenerate tissue and cellular debris; where the intensity of accumulation of brown cell feasibly reveals prior tissue injury (Humphrey & Norton 2005). Severe hemocyte accumulation were observed in bivalve species under stress conditions (Sindermann 1984); high infestation of parasites (Spiers et al 2008) and in cases of inflammation (Kim et al 2006). Similarly, brown cells accumulation were observed under stress due to inflammation in bivalve species (Lassudrie et al 2015).

Nemaptopsis sp*.* was the only pathogen found in this study*. Nematopsis* sp. is a gregarine protozoa under Apicomplexa and can be identified by the presence of resistant oocyst containing sporozoites in mollusk host (Clopton 2002). In Malaysia, the oocysts of *Nematopsis* sp. were discovered in *Anadara granosa* from Straits of Malacca; *Perna viridis* and *C. iredalei* from Penang (Uddin et al 2012; Kua et al 2013). This is the first reported case of presence of oocysts of *Nematopsis* sp. in *C. iredalei* collected from Johor and

Sabah. The oocysts were found in the connective tissue of mantle, gills and digestive diverticula; these results were consistent with the previous discovery by other researchers (Uddin et al 2012; Sabry et al 2013).

The length and width of the oocysts found in the Sabah samples were 13.088±0.230 µm; 7.318±0.188 µm, and Johor samples were 12.451±0.088 µm; 7.600±0.070 µm, respectively. The length and width of oocysts from two different locations indicated possibility of similar species of *Nematopsis* based on the size. The size of the oocysts was found to be similar to *Nematopsis mytella* found in mangrove mussel in Brazil with an average length and width of 11.5 µm and 8.2 µm respectively (Azevedo & Matos 1999). The average length and width of *Nematopsis* sp. reported in *C. iredalei* collected from Penang were 15.04 µm and 12.22 µm respectively (Uddin et al 2012), which indicates that the average size of oocysts found in our study were relatively smaller.

Higher number of Johor samples showed the presence of oocysts of *Nematopsis* sp. The higher intensity in Johor samples was possibly due to high contaminant burden; limitation of water flow to the intertidal zone due to narrow and shallow geographic structure of Johore Strait with additional presence of Johore Causeway, thus led to the potential of heavy metal accumulation in the water (Yap et al 2010). Previously, occurrence of pollution of the intertidal sediment collected from Pantai Senibong, Johor (similar location from southern Malaysia) by copper (Cu) was found during an assessment study (Yap & Wong 2011). Water pollution has been reported in Masai River Johor due to industrial activities (Utusan Online 2016). Pollution and pathogens have direct influence on the aquatic mollusks diseases and studies done by National Oceanic and Atmospheric Administration (NOAA) showed significant relationship between intensity of *Nematopsis* sp. and contaminants: Polynuclear Aromatic Hydrocarbons (PAH) and mercury (Hg) (Kim et al 1998; Kim et al 2008; Morley 2010).

Conclusions. In conclusion, the oysters collected from Johor and Sabah were examined using macroscopic examination and histological study. The results showed that oysters sample in Sabah had severe intensity level of histopathological conditions including lesion, accumulation of brown cells and hemocyte accumulation compared to Johor samples. The only pathogen observed during histological study was the oocysts of gregarine parasites, *Nematopsis* sp. For future research, it is suggested that the complete life cycle of *Nematopsis* sp. and the influence towards the crustaceans hosts be investigated by study the crustaceans around the sampling site. Apart from that, the bacterial study on the microorganisms composition found in oysters can be further studied by identifying the strains through sequencing techniques. Besides that, application of Transmission Electron Microscope is suggested so that a better understanding on the histology of oysters can be achieved.

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