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Health evaluation of the sexually mature *Crassostrea belcheri* (G.B. Sowerby II, 1871) in an enclosed rearing condition

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ABSTRACT: Studies on the health status of a variety of oyster species in aquaculture have been widely reported, except that of *Crassostrea belcheri* despite its commercial significance. The quality of the oyster depends greatly only on the physiological conditions of the broodstock. The present study aimed to evaluate ultrastructures, presence of apoptotic cells, and other histopathology of sexually mature *C. belcheri* to provide basic information of this commercial oyster species. The oyster specimens of each sex were randomly sampled ($n = 5$) and cultivated under the aquaculture laboratory and the water quality within acceptable limits for broodstock oyster culture. The specimens were processed using the histology, ultrastructure, and terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay. We demonstrated that the selected organs including gill, mantle, and gonads showed no abnormal histopathological findings, as commonly found in the oyster histology. No apoptosis was observed in general. The ultrastructure of gametes appeared normally chartered. However, with high magnifications, brown cells were observed in the lamina propria of the stomach, indicating that the sampled oysters were under certain physiological stress. Our findings and reports on the ultrastructure and histopathology of *C. belcheri* could provide baseline information on health conditions for aquaculture design and maintenance to pursue higher reproductive success of the oyster in aquaculture settings.

KEYWORDS: white-scar oyster, histopathology, oyster health, Thailand, TUNEL assay

INTRODUCTION

Knowledge of histopathological studies has been mainly acquired from the cellular and tissue alteration, which is a critical tool to evaluate the health status and physiological conditions of animals. It was then reported that an examination of the internal organs could reveal abnormalities such as shrinkage and necrosis of tissues when the aquatic animals were exposed to hazardous chemicals or other environmental stress [1, 2]. Demonstrating pathological signs including hypertrophy and hyperplasia of digestive diverticula, presence of granulocytomas and granulation tissue of a cultured oyster species, *Crassostrea iredalei*, was reported in relation to their increased mass mortalities [3]. Information of histopathological study in oysters was often used to better understand the effects of potentially unstable environments under laboratory conditions and those of pollutants in the environment [4–9]. In addition, *Crassostrea virginica* from Apalachicola Bay, Florida was studied and atrophy of its digestive gland after being exposed to low salinity was reported [4]. The increased digestive tubule atrophy of *Crassostrea gigas* was identified with the potential functions including the poor nutritional condition and spawning stress [10]. Furthermore, the research of *Saccostrea cucullata* from Libong Island, Thailand reported that temperature and available nutrients are strongly connected to morphometric features [11]. This evidence suggested that these symptoms could be used as indicators of the environmental health conditions as well as evaluation of overall health status of the oysters [8, 9].

The white-scar oyster, *C. belcheri*, is widely found in the marine-estuarine water and considered an economically important species in a global and local distribution that is in relatively high demand, especially as a luxury seafood menu. In recent years, the increasing demand for oysters has been satisfied by the expansion of aquaculture and managed fishery. Nevertheless, there has been a decline, due to overfishing, environmental problems, and unstable of natural broodstock along the coasts of Thailand [12]. In Thailand, most oyster farms have been set up with the artificial seed production of this oyster at the Marine Shellfish Breeding Research Unit, Faculty of Science and Fisheries Technology, Rajamangala University of Technology Srivijaya, Trang Campus, to ensure the sustainability of its aquaculture [13–15]. Many researchers have

worked on artificial reproduction of the oyster larvae in hatcheries of *C. belcheri* [16], gamete stripping [15, 17], and the formulation of mixed single-celled algae for diets [18]. However, general knowledge of the health status of *C. belcheri* in the captivity was missing.

In this work, we assessed the overall health status of the sexually mature *C. belcheri* broodstock in a rearing condition in aquaculture using integrated methods including the histopathology, ultrastructure, and TUNEL assay. This can provide a tool to implement a broodstock selection and other information to determine the reproductive success under the hatchery production and conservation efforts for sustainable oyster production.

MATERIALS AND METHODS

Preparing and cultured condition of the *C. belcheri* broodstock

The sexually mature *C. belcheri* broodstock (with average shell length: 8.5 ± 0.34 cm, $n = 5$ for each sex) were used in the experiment. The study was carried out at the Marine Shellfish Breeding Research Unit, Faculty of Science and Fisheries Technology, Rajamangala University of Technology Srivijaya, Trang Campus, Thailand. The collected oysters were cultivated in a re-circulation system in 0.096 m^3 fiberglass tanks with sand-filtered seawater (30 psu) and through a UV filter. All samples were fed with an algal mixture containing 6% *Chaetoceros calcitrans* and *Tetraselmis suecica* per mg oyster (dry algal weight/dry meat weight). The environmental factors including salinity, pH, and temperature (°C) were measured twice a day with Salino meter (Atago MASTER-URC/NM Clinical Refractometer, LEGA, Japan) and multiparameter probe (LAQUA 200 Series Handheld Water Quality Meters, HORIBA Instruments, Singapore). All procedures were performed under the Rajamangala University of Technology Srivijaya Animal Care and Use Committee under process number IAC 13-02-2023.

Histological analysis of cell death (apoptosis) with terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay

The bodies of the sampled *C. belcheri* were dissected and fixed in Davidson's fixative for 24 h at room temperature. They were then preserved in a 70% alcohol solution until further processing. The samples were morphologically observed under a Canon EOS R50 Mirrorless Camera (Canon, Japan). The samples were cut in approximately 5 mm longitudinal section from the dorsal to ventral views. All sections were processed based on the standard guidelines of histological method [19, 20]. They were subsequently dehydrated in sequential alcohol concentrations, cleared in xylene, and embedded in paraffin. The trimmed paraffin blocks were cut to 3-µm thickness with a microtome (Leica®, Germany) and subsequently stained with Harris' hematoxylin and eosin (H&E) and Grocott's methenamine silver stain (GMS) for further analysis.

The unstained slides from the histological method were prepared to investigate the presence of apoptotic cells with TUNEL Assay Kit – HRP-DAB (ab206386, Abcam, USA). The samples were dehydrated with ethanol and washed with 1xTris buffered saline pH 7.6 (1xTBS). After that, approximately 100 µl of Proteinase K solution was added per sample. In this process, the activity of endogenous peroxidases was inhibited by adding 3% hydrogen peroxide (H_2O_2). The tissue slides were incubated with terminal deoxynucleotidyl transferase (rTdT) at 37 °C for 90 min, and then the labeling reaction was terminated by adding 100 µl of the Stop Buffer. Then, the slides were washed with 1xTBS before adding 100 µl of the Blocking Buffer and incubated for 10 min, followed by adding horseradish peroxidase (HRP) to the slide and incubated for 30 min. After that, the slides were stained with diamino benzidine system (DAB) and followed by Methyl Green Counterstain before washing with ethanol and xylene and covering with a thin glass cover slip. Then, cells were observed for apoptosis under a light microscope.

To validate antibody specificity, we used a hepatocellular carcinoma (HCC) section as the positive control, which is donated from the Buddhachinaraj Phitsanulok hospital, Thailand. All histological sections and the TUNEL-positive cells from the stained sections were analyzed using a light microscope and scanned with a PANORAMIC Digital Slide Scanner (3DHISTECH, Hungary).

Ultrastructure observations

Fragmented cubic samples (1×1 mm) of *C. belcheri* were collected, dissected out, and intermediately fixed in 2.5% glutaraldehyde in phosphate buffer with a pH of 7.4 as the pre-fixative period for 24 h. They were then post-fixed in 1% osmium tetroxide for 1 h. The osmium-fixed tissues were dehydrated and embedded in Epon resin (812 epoxy resin). Afterward, the thin sections with a thickness of 90 nm were cut and examined under a transmission electron microscope (Philips/FEI Tecnai G2 F20, FEI Co., Eindhoven, Netherland).

RESULTS AND DISCUSSION

Environmental parameters

These results reported the physical parameters of the *C. belcheri* broodstock culture. Water salinity in hatchery ranged from 25 to 32 ppt (mean = 28.18 ± 2.34 ppt), pH ranged from 7.20–7.50 (mean = 7.35 ± 0.10), and water temperature ranged from 26.50 to 31.25 °C (mean = 28.76 ± 1.68 °C). However, water quality factors such as lower temperature, higher salinity, and pH were correlated to oyster health due to the fact that it is stressful and may enhance dis-

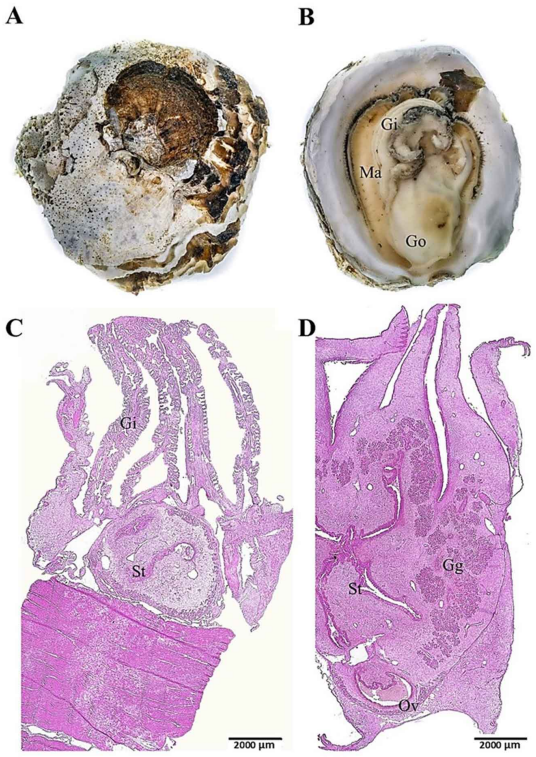

Fig. 1 The external shell (A), gross visceral morphology (B), and histology (C–D) of *C. belcheri* in longitudinal section. Several organs including the gills (Gi), mantle (Ma), gonad (Go), stomach (St), digestive gland (Dg), and ovary (Ov) were clearly identified. Staining method: C–D = H&E staining method

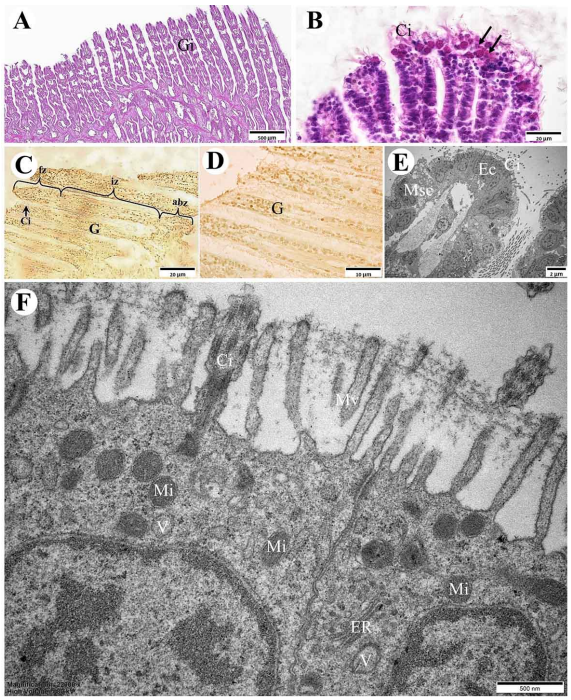

Fig. 2 Light microscopic level (A–D) and Transmission electron micrograph (TEM) (E–F) showing the gill composition of *C. belcheri*. (A–B) Structure of gill (Gi) lined with the cilia epithelium (Ci) and mucous secreting cell (arrows). Along the gill structure, it was composed of 3 parts: frontal zone (fz), intermediate zone (iz), and abfrontal zones (abz). (E–F) TEM study revealing that the microvilli (Mv) in epithelial cell (EC) close to the mucous secreting cell (Msc) was found. Several mitochondria (Mi), endoplasmic reticulum (ER), and vacuole (V) were also described. Staining method: A–B = H&E staining method and C–D = TUNEL assay

eases [21]. Moreover, oysters show a high performance under salinity of 15–25 ppt. [22].

Structure, fine structure, and histopathology

The survival rate and mortality of *C. belcheri* have been well-reported [13–15]; however, information on its structure and histopathology is still lacking [23, 24], when compared to other commercial oyster species [25–27]. This study, therefore, aimed to provide information on structure and determine histopathology in selected organs of *C. belcheri* using several biomarkers from organ to organelle levels (Figs. 1–5).

Gills

Histological characteristics of the gills in *C. belcheri* normally showed V-shaped demi-branch structure. Each was separated from the central axis with gill filament extending from the central gill axis, which was lined with ciliated epithelium and mucus-producing cells (Fig. 2A,B). Each 2 rows of gill lamina were divided into 3 locations: frontal, intermediate, and abfrontal zones (Fig. 2C). At the apical tip of the frontal zone, short cilia were found, which consisted of frontal cells. The nucleus was oval-shaped (Fig. 2D). The middle area, the intermediate zone, was covered with ciliated epithelium. The nucleus was flattened. The base of the abfrontal zone consisted of ciliated cells, and hemolymph blood vessels in the interlamellar space (Fig. 2E). This can be clearly confirmed at the detailed structure level that no apoptosis was observed in the gills. In addition, it was found that there was a clear arrangement of epithelial cells with cilia and microvilli. Within the cytoplasm, there was a large distribution of mitochondria and a great extension of endoplasmic reticulum (Fig. 2F).

Stomach

The stomach of *C. belcheri* consisted of 3 parts: the gastric shield, the sorting, and the sac regions. Histological study showed that the tall gastric shield region was distinguished by cilia on top and supported by lamina propria consisting of epithelium and lamina propria

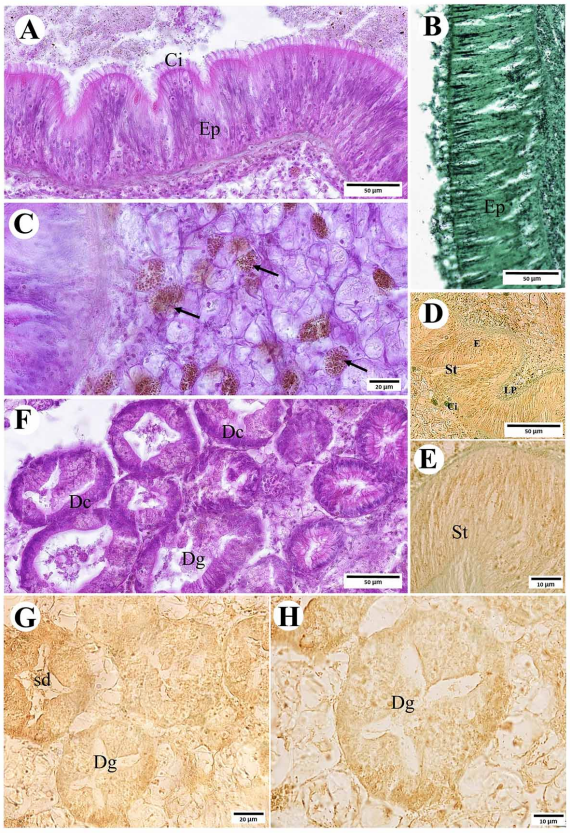

Fig. 3 Light microscopic level of the stomach of *C. belcheri*. (A–B) The epithelial stomach (Ep) with cilia (Ci). (C) The prominent brown cells (arrows) in lamina propria identified. The absence of apoptotic cells found in both the stomach (D–E) and the digestive gland (G–H). (F) Several digestive glands (Dg) having the digestive cell (Dc) clearly observed. Staining method: A, C, and, F = H&E staining method; B = GMS; and D–E and G–H = TUNEL assay.

(Fig. 3A). No fungi were observed (GMS, Fig. 3B). Brown cells with round or oval shape were found scattered throughout the connective tissue. Cytoplasm of the brown cells contained small brown granules (Fig. 3C). However, the absence of apoptotic cells was observed (Fig. 3D,E).

Digestive glands

The digestive glands of *C. belcheri* were clearly visible organs located near the digestive tract. It had the appearance of a sac with blind-ending tubules. Histological characteristics of the digestive cells in the tropical oyster consisted of cells with a spherical shape. It also consisted of tall digestive cells. The nucleus was found at the base of the cell, whereas the cytoplasm was heavily stained with hematoxylin (H&E, Fig. 3F), but it was without the apoptotic cells (Fig. 3G,H).

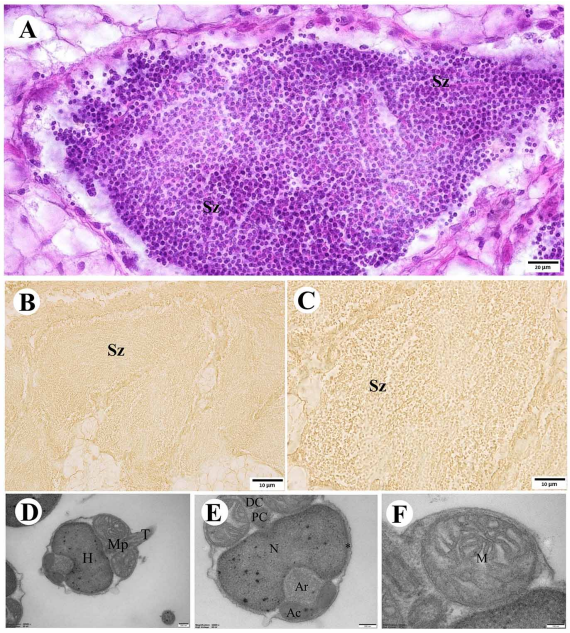

Fig. 4 Light microscopic level of testes and sperm ultrastructure of *C. belcheri*. (A) The testis with the prominent spermatozoa (Sz) found. (B–C) The absence of apoptotic cells found in the testes. (D–F) High magnification showing the sperm ultrastructure composed of head (H), mid-piece (Mp), and tail (T). Abbreviations: Ar = axial rod, Ac = acrosome, DC = distal centriole, M = mitochondria, N = nucleus, PC = proximal centriole, and asterisk = nucleus membrane. Staining methods: A = H&E staining method.

Testis

Testicular follicles of *C. belcheri* were widely distributed and immersed in connective tissues. The process of sperm production occurred within the follicle where sperm cells prominently developed (Fig. 4A). High magnification showed that the small nucleus was stained dark purple of hematoxylin, but it was difficult to distinguish the structure of the sperm tail (Fig. 4A). However, the absence of apoptotic cells was observed (Fig. 4B,C). When compared to sperm ultrastructure, it was easy to identify with 3 parts: head, middle (or mid-piece), and tail (Fig. 4D). With high magnification, the fine structure of the head was composed of an acrosome, an axial rod, and a nucleus. It is also described that the arrangement of the proximal centriole and distal centriole was surrounded by mitochondria (Fig. 4E,F).

Ovary

The ovarian structure consisted of a large number of follicle sacs, and the inside contained the development of oocytes (Fig. 5A), which can be divided into 2 stages (immature and mature oocytes). The immature oocyte had a round shape with a round or oval shaped nucleus

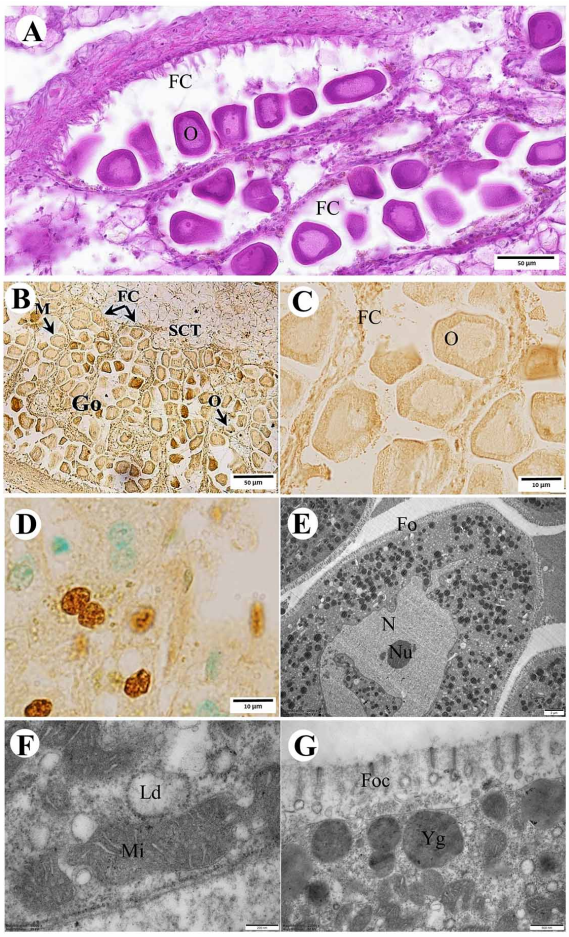

Fig. 5 Light microscopic level of ovarian structure (A–D) and oocyte ultrastructure (E–G) of *C. belcheri*. (A) Several stages of oocyte (O) developed in the follicle (FC). (B–C) Clear observation of the absence of apoptotic cell in the gonad-ovary (Go) found. (D) The presence of apoptotic cells as a positive control. Ultrastructural evidence was shown in the oocyte that contained in the nucleolus (Nu), nucleus (N), and follicular oocyte (Fo). (E–G) High magnification showing several components including yolk (Yg), lipid droplet (Ld), mitochondria (Mi), microvilli (Mv) in the ooplasm, and follicle cell (Foc). Abbreviations: Go = gonadal ovary and SCT = storage connective tissue. Staining method: A = H&E staining method and B–D = TUNEL assay.

and surrounded by dark purple cytoplasm. The mature oocytes also became larger. The large nucleus of the mature oocytes had a polyhedral shape. The absence of apoptotic cells in the ovary was found (Fig. 5B,C). For the positive controls, TUNEL immunoreactivity was observed in liver cancer sections (HCC) (Fig. 5D). Ultrastructure showed that the mature oocytes contained one nucleolus and a large amount of yolk and lipid droplet (Fig. 5E,F). A clear distribution of mitochondria was clearly identified (Fig. 5F). A single layer of follicular cells close to the yolk formation was described (Fig. 5G).

Our study provided basic information on the arrangement of tissues and composition of various organs including gills, stomach, digestive glands, and reproductive organs of *C. belcheri*. In general, our findings were similar to those reported in general bivalves [25–30]. However, we found a structure containing a mass of brown cells with the preeminent brown granule (H&E staining method). This cell type has been reported in other bivalves such as *Mercenaria mercenaria* [31] and *Mytilus galloprovincialis* [32].

Brown cells have been used as a biological indicator of environmental problems in bivalves [33]. The presence of brown granules in the brown cell contained fine lysosome as a component that seemed responsible for the elimination of toxins and foreign substances [34, 35]. It could be related to the exposure to environmental pollutants [31, 36, 37]. The number of brown cells increased when parasites were found in the bivalves [33]. So, the brown cells may be associated with defense mechanisms of the oyster as in other bivalves [29, 32, 38–40]. This may be related to environmental factors in the hatchery for the reasons discussed above. Overall, we believed that the health of the *C. belcheri* oyster was still in good condition. Regular and proper monitoring of the environmental parameters was required to maintain the quality of the tropical oyster production to be within the standard criteria.

CONCLUSION

Histopathological information has been used to determine the health status of the *C. belcheri* oyster. As for the biomarker evaluations, although signs of histopathology of the sampled oysters were found, we suggested that the sampled *C. belcheri* were still considered a healthy condition. This information should provide baseline data necessary for the further design of aquaculture and could help increase the reproductive success under a permanent monitoring program of *C. belcheri*.

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