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Virginia Institute of Marine Science

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Shell disease in the gold lip pearl oyster, *Pinctada maxima* and the Eastern oyster, *Crassostrea virginica*

Frank O. Perkins

School of Marine Science, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062, U.S.A.

*Present address: Departments of Zoology and Microbiology, Pathology and Parasitology, North Carolina State University, Raleigh, NC 27695, U.S.A.*

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**Abstract**

A description is provided of the anomalous conchiolin deposits which are formed by *Pinctada maxima* and which are associated with unusual mortalities. Comparisons are made with brown ring disease found in *Ruditapes philippinarum* and juvenile *Crassostrea virginica*. In *P. maxima*, the deposits are not organized into a ring but rather are broad-based and result in retraction of the mantle with the deposits lying outside the edge of the mantle. *Vibrio* sp. have been implicated in causing the diseases of *P. maxima* and *R. philippinarum* whereas the etiological agent of the disease in *C. virginica* is unknown. It is suggested that the coccoïd bodies formed in the mantle of *C. virginica*, but not in affected *P. maxima* and *R. philippinarum*, are sequestered portions of cytoplasm formed as a result of autophagocytosis. Stimuli which could be responsible for inducing sequestration are discussed. The ultrastructure of the presumptive autophagosomes is described and compared to similar bodies found in *C. gigas* infected with a herpes-like virus.

**Keywords:** *Pinctada maxima, Crassostrea virginica*, shell disease, conchiolin, ultrastructure, light microscopy.

**Résumé**

Les anomalies de dépôt de conchioline secrétée par *Pinctada maxima*, associées avec des mortalités inhabituelles, sont décrites ici. Des comparaisons sont faites entre la maladie de l’anneau brun trouvé chez *Ruditapes philippinarum* et chez des jeunes *Crassostrea virginica*. Chez *P. maxima*, les dépôts ne sont pas organisés en anneau mais plutôt à partir d’une base assez large et résulte d’une rétraction du manteau avec des dépôts à l’extérieur du bord du manteau. Les *Vibrio* sp. ont été impliqués dans les maladies de *P. maxima* et *R. philippinarum* tandis que l’agent étiologique de la maladie est inconnu chez *C. virginica*. Les corps de forme cocoïde formés dans le manteau de *C. virginica*, mais n’affectant pas *P. maxima* ni *R. philippinarum* seraient des fractions de cytoplasme isolées, « sequestrées », résultant d’une autophagocytose. Les stimulus qui pourraient être responsables de ces fractionnements sont passés en revue. L’ultrastructure des autophagosomes présomus est décrite et comparée à des corps similaires trouvés chez *C. gigas* infecté par un virus de type herpès.

**Mots-clés :** *Pinctada maxima, Crassostrea virginica*, maladie de la coquille, conchioline, ultrastructure, microscopie optique.
Anomalous deposits of conchiolin on the nacre have been described in a diversity of species of marine bivalves, most notably in the result of irritation of the mantle by a variety of different microbes and metazoan parasites and commensals (literature reviewed by Paul et al., 1994). Particular species have been concerned with those cases where there are high mortalities of the bivalves involved. The most extensively studied is brown ring disease of the Indo-Pacific clam (Ruditapes philippinarum) and oysters (Crassostrea gigas) in waters of France and Spain. In that host the causative agent is a species of Vibri0, termed Vibri0 P1 or VPI which expresses itself between 21°C (Paull and Maes, 1990; Paul et al., 1994).

Another shell disease is associated with juvenile oyster mortalities during the summer months (22-25°C) and was first observed on Long Island Sound (Briceij et al., 1992). As in R. philippinarum, the conchiolin deposits are most pronounced in the form of a brown ring on the nacre, around the perimeter of the shell. Another characteristic in some individuals is a pronounced cupping and overgrowth of the left valve over the dorsal of the right valve.

In the search for the causative agent of juvenile oyster mortalities, a number of hypotheses have emerged; however, the agent has not been conclusively identified. At the same time, there have been numerous, around the possibility that a protist or bacterium initially causes retraction of the mantle, followed by abnormal conchiolin deposition. Further progression of the disease, resulting in death, involves bleeding from the mantle epithelium and detachment of the adductor muscle with deposits between the adductor and the shell. Secondary invasion of microorganisms into the lesions is believed to exacerbate the pathology. It has also been suggested that the disease is a biobubble caused by a toxic phytoplankton species or some chemical contaminant could be responsible for the pathological condition (Brieier et al., 1992). However, most of the early studies of juvenile oyster disease can be found in the present issue of this journal.

Anomalous deposits of brown conchiolin have been reported on the inside surface of the shells of pearl oysters, Pinctada maxima, involved in high mortalities which occurred as part of culturred pearl operations on the western coast of Australia (Pass et al., 1987; Perkins, 1993). Pass et al. (1987) tentatively concluded that Vhri0 harveyi was responsible; at least in part, for the mortalities when the oysters were subjected to stressful conditions during handling and shipment.

With the recent increased interest in shell disease of bivalve molluscs as evidenced by the convening of the International Workshop on Shell Disease in Marine Invertebrates: Environment-Host-Pathogen Interactions in Broome, Western Australia, December 3-9, 1995, it appeared particularly appropriate to reexamine the morphology of shell disease in Pinctada maxima and juvenile oyster disease in hemispherical beaded cases of ACD and from without ACD. Lesions and from without ACD. Lesions and were not observed, and when observed (figs. 9 and 10) there was no correlation between their presence and the absence of ACD. Bacteria were not observed in the sections (all were hematoxylin and eosin). In an ultrastructural study Pass and Perkins (1988) found virus-like particles and intracellular inclusions in cell nuclei of the digestive gland. However, no correlation with ACD or mortalities was found.

The most closely associated with the ACD did not reveal any parasite which could be correlated with ACD. In addition, the organ samples examined after fluid thioglycollate medium treatment contained no Perkinsus sp. A description of the pathological conditions of ACD is provided (Perkins, 1993). Likewise mantle from samples from one oyster which had no anomalous conchiolin deposits were fixed for ultrastructural observations. The rest of the oysters' tissues were fixed in Davidson's fixative and dehydrated in methyl alcohol, the paraaffin-embedded sections were stained in hematoxylin and eosin or stained using the Feulgen technique to reveal DNA (Humason, 1962; light green used instead of fast green).

RESULTS

Pinctada maxima

The shells of P. maxima with no signs of anomalous conchiolin deposits (fig. 6) had nearly nacre on a periperal zone of brown conchiolin (fig. 1). The interface between the two is sharply defined and is distinct yellow-brown color. In the earliest or in mild cases of ACD, a wedge-shaped or a broad zone of thin deposits of conchiolin were found expanding from the paraaffin-embedded tissue toward the center of the shell with the broad base of the wedge on the pallial line (fig. 2). It appeared that if the pathological condition was not too severe then the lesions were not covered with a wrinkled layer of nacre (fig. 3). In the process of deposition the sharp interface between the nacre and peripheral conchiolin was blunted. In more severe cases of ACD, the conchiolin was formed in a broad zone and consisted of a thin compact-like layer (fig. 4). In the most severe cases of ACD, the nacre was blurred. In more severe cases of ACD, the nacre was blurred. In more severe cases of ACD, the nacre was blurred. In more severe cases of ACD, the nacre was blurred.

Spherical conchiolin deposits were not correlated with presence or absence of ACD. In the mantle samples examined, 37 were from oysters with ACD and 30 from oysters without ACD. Lesions and epithelial injuries were found in 68% of the former and 72% of the latter. Most oenhemcocytic cytolytic processes were not observed, and when observed (figs. 9, 10 and 11) there was no correlation between their presence and the absence of ACD. Bacteria were not observed in the sections (all were hematoxylin and eosin stained) but there were intracellular, spherical bodies observed, as
Figures 1 to 3.

- Pinctada maxima.

1. Apparently healthy oyster, examined a few days after harvesting from a wild population. The well-defined pallial line and interface between the nacre and conchiolin is visible (arrow). Visceral mass has been removed to expose most of nacre. Adductor muscle (A); pinnotherid crab (C); mantle edge (M); nacre overlying Polydora tunnel (N); Bar=20 mm. 2-3 Oysters harvested off Eighty-Mile Beach and held in Roebuck Bay for two months. 2 Early or mild case of anomalous conchiolin deposition (ACD) disorder in which wedge-shaped intrusions of ACD are found on the nacre (arrow). On one intrusion a calcareous deposit has been formed over the conchiolin (arrowhead), Bar=20 mm. 3 More advanced case of ACD in which mantle has retracted from the original pallial line (arrow) and in the process has deposited conchiolin (C) on the nacre. A layer of calcareous material has been formed over some of the ACD (Ca). Mantle edge (M); adductor muscle (A). Bar=10 mm.

2-3 Oysters harvested off Eighty-Mile Beach and held in Roebuck Bay for two months.

2. Early or mild case of anomalous conchiolin deposition (ACD) disorder in which wedge-shaped intrusions of ACD are found on the nacre (arrow). On one intrusion a calcareous deposit has been formed over the conchiolin (arrowhead), Bar=20 mm.

3. More advanced case of ACD in which mantle has retracted from the original pallial line (arrow) and in the process has deposited conchiolin (C) on the nacre. A layer of calcareous material has been formed over some of the ACD (Ca). Mantle edge (M); adductor muscle (A). Bar=10 mm.

- Crassostrea virginica

In juvenile oyster disease (JOD) a well-defined brown ring of ACD was often deposited inside the shell margin on one or both valves (fig. 12) with a thin layer of ACD within the ring. In the absence of a ring, thin layers of ACD could be found in various places on the nacre of affected individuals. Since the morphological aspects of the condition have been described in some detail in Bricej et al. (1992), they will not be repeated herein, with the following exception. Due to the ultrastructural similarity of unusual intracellular bodies found in both juvenile Crassostrea gigas infected by a herpes-like virus (Renault et al., 1994a; 1994b) and juvenile C. virginica affected by JOD, it appeared appropriate to reexamine the ultrastructure of the bodies in C. virginica.

In epithelial and connective tissue cells of the mantle of C. virginica affected by JOD, unit membrane-bound, sequestered units of cytoplasm (SU) were observed. They were located in cytoplasmic vacuoles or cell-free in lesions (fig. 13). In the most complex form, they consisted of rough endoplasmic reticulum often in flexed parallel arrays, numerous mitochondria, lipid bodies, a finely granular and electron-dense body, myelin whorls, numerous small vesicles, a large electron-lucent region, and membrane-free ribosomes (figs. 14-17). Less complex forms lacked one or more of the characters listed. The SU were not
observed in the mantle of oysters lacking anomalous conchoidal deposits. In histological sections the SU appeared as spheroidal bodies of <1 to 6 μm diameter often with usually one, sometimes 2 or 3 internal basophilic, punctate regions of various sizes (fig. 15). Those punctate regions were also weakly Feulgen-positive indicating the presence of DNA. The smaller SU generally lacked visible basophilic inclusions. The electron-dense, granular regions, not delimited by unit membranes (figs. 16 and 17) probably correspond to the basophilic inclusions. There is no reason to suggest that the SU are protists since no nucleus was observed. It appeared that the oyster cells had subdivided into units containing many of the cytoplasmic organelles of the cell of origin. The mitochondria had mostly shell-like cristae like those of oysters (figs. 14 and 15) but a few had cristae which were more tubular. The rough endoplasmic reticulum was most often a dominant feature of the SU being found at the perimeter of the SU in an arc which extended around as much as 180° of the SU (figs. 14, 15 and 17). It was not unusual to find a high degree of organization in that the membranes were spaced equidistant and folded in stacks of sacculae with the ribosomes attached in rows on the outside of each sacculae. In some SU the rough E.R. was not as organized and could be found in various regions of the cytoplasm of the SU. Also in the ground cytoplasm of the SU were membrane-free ribosomes (fig. 17). In some SU there were numerous free vesicles and multivesicular bodies (figs. 15 and 16) scattered throughout the cytoplasm. In many SU the cytoplasm contained an electron-laden region centrally or eccentrically located and not membrane-bound (figs. 14 and 15). Lipoid bodies were often found in and around the perimeter of the lutein region (figs. 14 and 15).

Myelin whorls were found in a few of the SU, usually in the cortex (fig. 16). The whorls had the same structure one would expect to see in degenerating cells. A final structure observed were bodies in the ground cytoplasm which consisted of groups of electron-dense filaments in parallel arrays sometimes with two groups organized at right angles to each other (fig. 16). Unlike the SU observed by Renault et al. (1994; 1994) in C. gigas, herpes-like viruses were not found to be associated with the SU’s in juvenile C. virginica.

DISCUSSION

Differences in shell disease between that found in P. maxima and that found in R. philippinarum and C. virginica consists of the lack of a ring of anomalous conchoidal deposition (ACD) around the periphery of the inner surface of the shell (i.e., brown ring) in the former species and the presence of a ring of concholin in the other two species. A similarity involves higher than normal mortalities associated with the ACD. Mantle lesions were noted for affected C. virginica and R. philippinarum with unaffected individuals lacking such lesions (Bricelj et al., 1992; Paillard et al., 1994). However, in P. maxima there was not a clear difference in expression of lesions between unaffected and affected individuals. In fact, two of four freshly harvested and seemingly healthy pearl oysters had lesions in the mangle epithelium. This apparent lack of a distinction between affected and unaffected oysters may have been due to an inability to distinguish between artifacts or mechanical damage in handling the mantle samples and lesions associated with the ACD syndrome.

Although bacteria were found in the affected mantle of P. maxima (Pass, et al., 1987) and in the mantle and associated ACD of C. virginica (Bricelj et al., 1992) and R. philippinarum (Paillard et al., 1994), the numbers seen in histological sections were comparatively small, there being no extensive invasion of the mantle except in moribund individuals. In sections of C. virginica-affected mantle less than 30% of the SU were found to have bacterial associated with the mantle. It appears that the bacterial lack of bacteria as seen in histological sections of tissues is a characteristic which is common to all three species of affected molluscs. As Paillard et al. (1994) have suggested for R. philippinarum, it may be that bacteria are generally not invasive and proliferate on the surface of the mantle epithelium, secreting toxic products which induce ACD. Formation of ACD represents a defense mechanism which serves to entrap bacteria between lamellae of the ACD and with the synthesis of melanin in the concholin some degree of bactericidal protection is provided (Paillard et al., 1994). However, only in R. philippinarum
Figure 14 to 16. - *Crassostrea virginica*. Sequestered unit in mantle epithelial cell cytoplasm containing parallel arrays of rough endoplasmic reticulum (E), mitochondria (arrow), lipid droplets (L), multivesicular body (arrow head) and an electron-lucent space (S) which is not membrane-bound. The units are membrane-bound and contained in a vacuole. Bar=0.1 µm. 16. - Sequestered unit containing an electron-dense, finely granular body (G) which is believed to be comparable to the basophilic, punctate bodies seen in some sequestered units in histological sections. Myelin whorl (W); mitochondrion (arrow); rough endoplasmic reticulum (E); multivesicular body (arrow head); body with parallel arrays of electron-dense rods (R). Bar=0.1 µm.

Bacteria, and more specifically *Vibrio* sp., may be involved in inducing ACD formation by *C. virginica*; however, efforts to find a bacterial causative agent have not been conclusive (Bricelj et al., 1992; see also other papers in this journal issue). The suggestion that the sequestered units (SU) with basophilic centers which are found in the mantle are prokaryotes is rejected since nuclei are not present (Bricelj et al., 1992). This situation is supported by ultrastructural studies which show that the SU is rounded with a membrane-bound electron-dense region with a few granularity (figs. 11). In both oysters the rough endoplasmic reticulum is often arranged in parallel arrays located around the perimeter of the body in which mitochondria are found as well as a finely granular electron-dense region with a rounded profile and without a delimiting membrane. Whether these similarities indicate that a virus is the causative agent of ACD in *C. virginica* was considered. However, no conclusive evidence of viruses being associated with the SU was found. It could be that the formation of SU can result from a diversity of stresses; however, the entities are unique enough to have not been reported in oysters prior to the papers by Bricelj et al. (1992) and Renault, Le Duff, Cochennec and Maffart (1994). Other than the present paper, they have not been reported since then.

Bricelj et al. (1992) suggested that the "bodies are secondary lysosomes resulting from an extreme example of autophagy" as described by Constantinides (1984). This suggestion may be valid since ultrastructurally similar bodies are found as a result of cellular sequestration (autophagocytosis) in a diversity of other species such as in rat pancreatic acinar cells (Swift and Hruban, 1964). The SU in *C. virginica* are ultrastructurally similar to those in the acinar cells in that parallel arrays of rough endoplasmic reticulum and mitochondria are present. The difference is that basophilic bodies have not been reported in the examples of autophagocytosis, found in the literature. This is not surprising since nuclear material is not sequestered during autophagocytosis in the cells previously described (Hirsimäki et al., 1983). It should be noted that not all of the bodies in *C. virginica* were found to have basophilic regions. Since they are Feulgen-positive, the basophilic regions in the oyster SU may be chromatin, derived from degraded oyster nuclei. A cytochemical test for acid phosphatase must be conducted to help substantiate whether the bodies are a result of autophagocytosis.

Constantinides (1984) stated that there are 5 situations in which "autophagocytosis with subsequent enzymatic lysis of the cell's own organelles is initiated". These situations include starvation, involution (degradation) of tissues, change of cell programming as during differentiation or dedifferentiation, sublethal injury of cells as in anoxia, and action of certain chemicals such as glucagon and phlorizin. Viruses were not cited as stimulatory factors for inducing sequestration.

In the absence of any ultrastructurally detectable agent of SU formation, it appears reasonable to consider the situations listed by Constantinides (1984). The action of a toxin or toxins warrants investigation. Such chemicals could be formed by microbes on the surface of the mantle epithelium or be present in the ambient water, either anthropogenically formed or formed by phytoplankton as suggested by Bricelj et al. (1992). Since the oysters used in this study were apparently not starved or exposed to anoxic conditions, those conditions are unlikely to have induced SU formation. Upon occasion when oysters were suffering high mortalities and were being held in high density in trays, lowered oxygen conditions probably prevailed for some of them (Bricelj et al., 1992); however, this was not the case for all oysters examined where SU were seen in histological sections. Obviously more work must be conducted to determine the causative agent or agents or juvenile oyster disease.

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