A new pathology in the red abalone *Haliotis rufescens* (Mollusca: Gastropoda) cultured in Baja California, Mexico, associated with macro-crystal inclusions in gonadal tissue

Una nueva patología en el abulón rojo *Haliotis rufescens* (Mollusca: Gastropoda) cultivado en Baja California, México, asociada con inclusiones de macro-cristales en tejido gonadal

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ABSTRACT

A new severe pathological alteration in the gonadic tissue of red abalone was observed, which compromises reproduction of affected organisms. The alteration is related to the presence of numerous extracellular macro-crystal inclusions (Mci) reaching up to 300 µm in size. These Mci may be surrounded by hemocytes, cellular debris, and deposition of fibroblasts. Moreover, hypertrophy of the nuclei of cells in the top germinal epithelium and presence of brown cells in trabeculae were observed. The normal architecture of the tissue looks entirely altered and destroyed in some cases. No gonadal development was observed. The chemical nature of the Mci is unknown, but it is possibly related to inorganic toxic compounds. No previous records of similar alterations in abalone are known to exist. This record could help determine if this pathology has been observed in other abalone culture areas in the world, its possible origin, and if control is necessary.

Key words: Extracellular crystal inclusions, *Haliotis rufescens*, pathology, red abalone.

RESUMEN

Se observó una nueva alteración patológica severa en el tejido gonadal de abulón rojo que compromete la reproducción de los organismos afectados. Esta alteración está asociada con la presencia de numerosas inclusiones extracelulares formadas por macro cristales (Mci) de hasta 300 µm de longitud. Estas Mci pueden estar rodeadas por hemocitos, restos celulares y acumulación de fibroblastos. Además, se observó hipertrofia del núcleo de las células del extremo externo del epitelio germinal y presencia de células cafés en las trabéculas. La arquitectura normal del tejido se vió completamente alterada y en algunos casos destruida. No se observó desarrollo gonadal. La naturaleza química de las Mci no se conoce, pero posiblemente está relacionada con compuestos tóxicos inorgánicos. No se conocen registros previos de alteraciones similares en abulón. Este registro podría ayudar a determinar si esta patología se ha observado en otras áreas de cultivo a el abulón en el mundo, así como su posible origen y si es necesario su control.

Palabras clave: Abulón rojo, *Haliotis rufescens*, inclusiones extracelulares de cristales, patología.

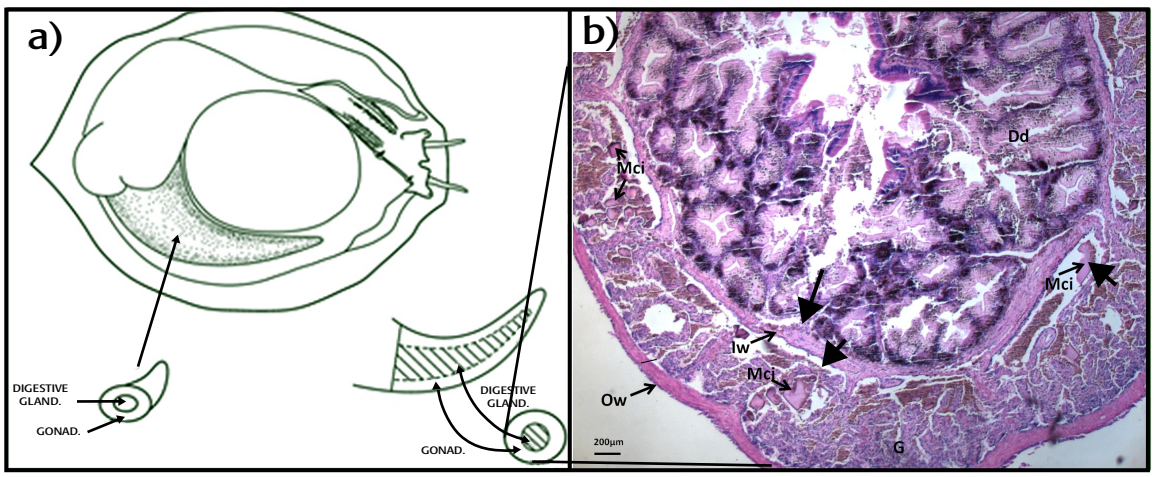
Interest in the culture of red abalone *Haliotis rufescens* (Swainson, 1822) has increased in Baja California, Mexico, in part due to the decrease in the commercial fishery of blue abalone *Haliotis fulgens* (Philippi, 1845) and yellow abalone *Haliotis corrugate* (Wood, 1828), available culture technology, and market demands (Ramade-Villanueva *et al*., 1998; Searcy-Bernal *et al*., 2010). This species is also cultivated in California, USA (McBride, 1998) and has become a fast-growing industry in Chile where red abalone was introduced in 1977 (Flores-Aguilar, 2007). One of the main challenges for successful production is maintaining a healthy culture population. In this sense, sanitary surveillance must be permanent. Red abalone *H. rufescens* can be affected by different symbionts and parasites, such as ciliates in the esophageal pouch, ciliates in branchial filaments, the renal coccidian *Margolisiella haliotis* (Desser & Bower, 1997) that causes abalone coccidiosis, and the intracellular prokaryote *Candidatus* Xenohaliotis californiensis (Friedman, Andree, Robbins, Shields, Moore, Beauchamp, & Hedrick, 2000), a causal agent of withering syndrome (WS) in abalone that is flagged by the World Organisation for Animal Health (OIE). Descriptions of histopathological

alterations associated with these symbionts and parasites are relatively well described (Friedman *et al.*, 1995; Gardner *et al.*, 1995; Moore *et al.*, 2000; Cáceres-Martínez & Tinoco-Orta, 2001; Alvarez-Tinajero *et al.*, 2002; Cáceres-Martínez *et al.*, 2011; OIE, 2014). However, the emergence of new infectious and non-infectious pathologies must be permanently recorded due to their potential negative impact in production. To date, reports on non-infectious diseases or descriptions of tissue alterations not associated to biologic agents in abalone are practically unknown. This note describes a new pathological condition associated with the presence of macro-crystal inclusions (Mci) of unknown composition located in the tissue between the inner and exterior walls of the gonad of cultured red abalone.

In August 2004, six red abalone of a mean size of 72.5 mm in shell length, reared in an aquaculture facility located in Eréndira, Baja California, Mexico, were sent to the biology and pathology laboratory of aquatic organisms at the Centro de Investigación Científica y de Educación Superior de Ensenada, B.C. Mexico (CICESE) for health analysis. The abalone arrived live packed in ice. Two abalones had an externally healthy appearance; the others showed weakness, lethargy, mantle retraction, inability to adhere tightly to the substrate, and their foot muscle appeared shrunken. After an external analysis, the shell was detached from the soft body and tissues were individually fixed in Davidson´s fixative for 24 h. Four transverse sections containing portions of the digestive tract, gonad, kidney, muscle, and gills were processed for conventional histology. Deparaffinized 5 µm sections were stained with hematoxylin and eosin (Howard *et al.*, 2004). Slides were reviewed under the microscope at magnifications of 100X to 630X looking for parasites and tissue alterations. Prevalence of parasites was estimated as the number of infected animals in the sample expressed in percentage (Olivas-Valdez & Cáceres-Martínez, 2002). The intensity of the infection was determined using specific scales previously established by Friedman *et al.* (1997) and Cáceres-Martínez and Tinoco-Orta (2001) for *Candidatus X.* californiensis and Friedman *et al.* (1995) for *M. haliotis*. A photographic record documented results. In February 2014 a sample of 30 fixed red abalone tissues was obtained from animals of a mean size of 80 mm in shell length, from a different aquaculture facility located in Bahía Falsa, Baja California, located approximately 100 km south of Eréndira, B.C. These mollusks were analyzed for health status using the conventional histological procedure described above; in this case, the record from the aquaculture facility indicated that all the abalone had a healthy external appearance before fixation. Similarly, slides were reviewed and documented photographically.

All abalone from the first sample were infected by *Candidatus* X. californiensis. Cell and tissue alterations agree with the characteristics of WS at a histological level that went from host cell hypertrophy in light infections, to metaplasia of digestive diverticula, and detachment of some colonies to the lumen of the post-esophagus in severe ones. Three abalones were females, two were males, and one was undefined; this latter abalone had a light infection caused by *Candidatus* X. californiensis. This abalone also had abundant extracellular macro crystal inclusions (Mci) among the tissue placed between the inner and outer wall of the gonad, where the germinal epithelium growths and gametogenesis take place (Fig. 1). The shape of Mci varied from irregular to polygonal and their size, measuring along the larger axis, reached 300 µm (Figs. 2-3). The gonadal tissue was entirely affected by the Mci; the connective tissue and fibers lost their normal architecture (Fig. 2a). In some cases, the Mci were surrounded by hemocytes, cellular debris and deposition of fibroblasts (Fig. 2b). In others, the Mci seemed to be in degradation among hemocytes and cellular debris with brown granules (Fig. 2c).

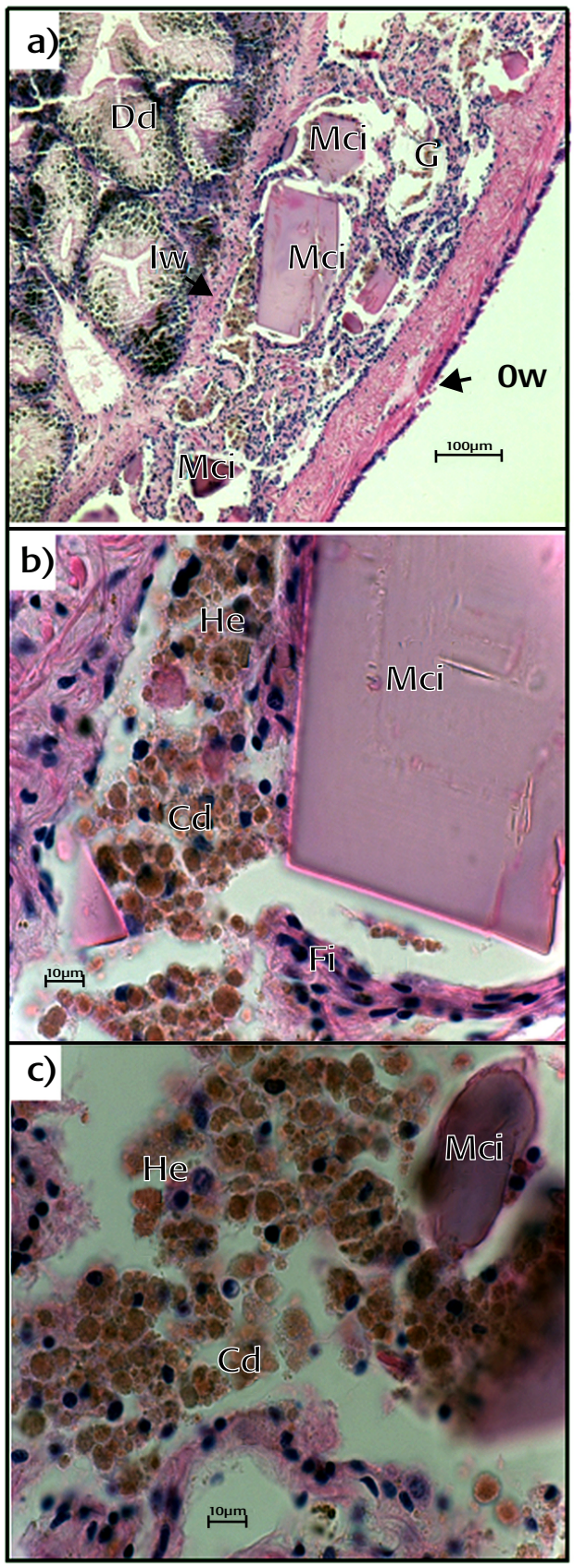
The prevalence of *Candidatus* X. californiensis in abalone from the second sample (30 animals) was 60%, the tissue damages were similar to those described above. In this case, the presence of *M. haliotis* was recorded at 30%; this parasite invades the right and left kidney occupying some areas of the tissue, but no haemocytic reaction was observed. In this sample 63% of abalone were females, 10% males and 27% were undifferentiated. In one of the undifferentiated abalones, a light infection caused by *M. haliotis* was observed, but it was not infected by *Candidatus X.* Californiensis this animal showed a pathology associated with Mci similar to those found in the individual from the sample taken in 2004. The shape and sizes of the Mci were in the range previously determined; however, in this case, there was a major development of



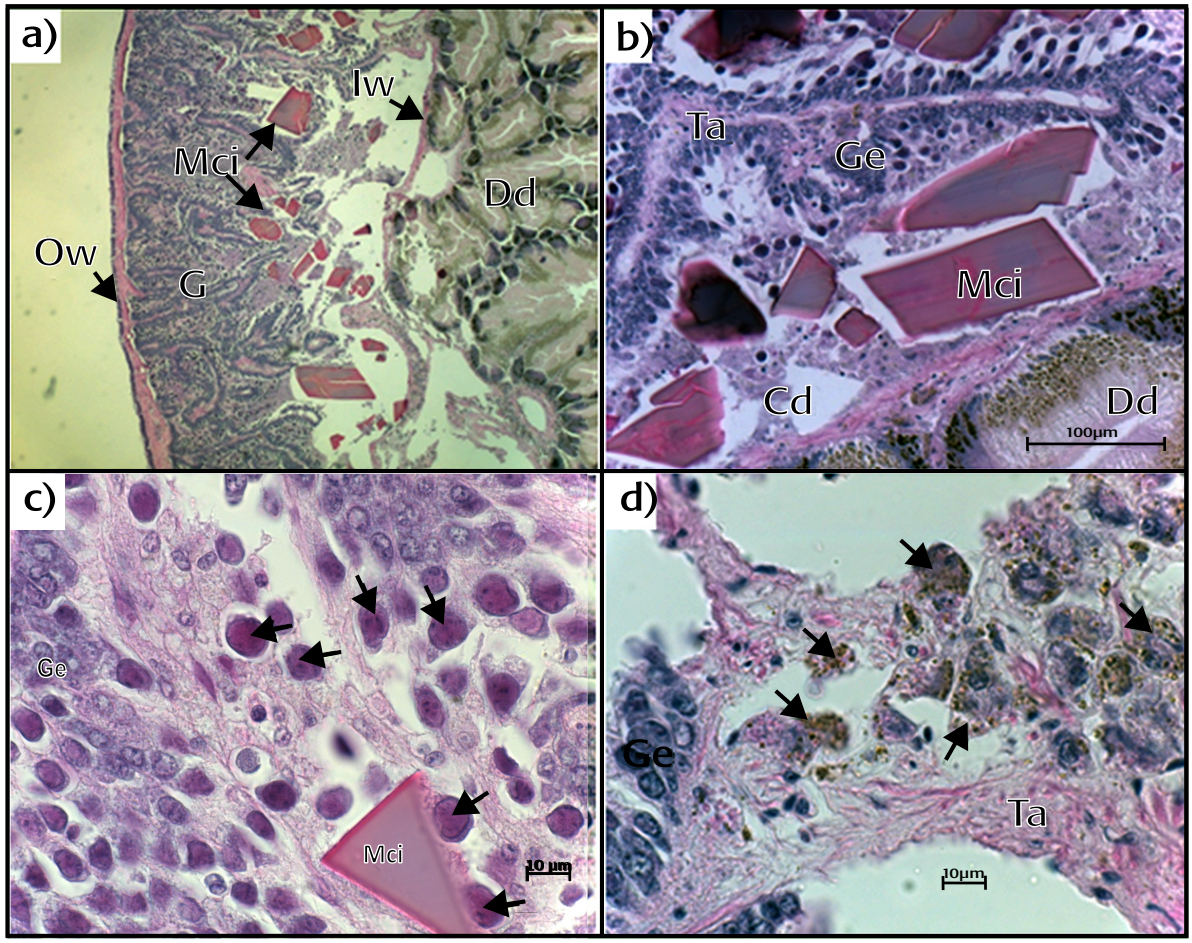
Figures 1a-b. a) Diagram showing the gonadal tissue around the digestive gland, where the macro-crystal inclusions (Mci) were observed (modified from Rogers-Bennett *et al.* 2004). b) Histological image of one transversal section of the tissue showing the gonad surrounding the digestive diverticula (Dd) where Mci are embedded, some of them are shown by arrows. Ow = outer wall of the gonad, Iw = inner wall of the gonad, G = gonad. Hematoxylin-eosin.

trabeculae accompanied by the loss of its normal architecture throughout the inner wall of the gonad (Fig. 3a). In the trabeculae, growth of germinal epithelium was detected, but it appeared deformed by the shape of the Mci (Fig. 3b). The nuclei of the top cells in the germinal epithelium were hypertrophied and strongly basophilic (Fig. 3c). As in the previous case, some brown cells were observed in the connective tissue of the trabeculae (Fig. 3d) and no development of gametes was observed.

No record of extracellular Mci formation in the gonadic tissue of abalone species has been documented previously. However, a study exists regarding metal bioaccumulation as inorganic granules in the cytoplasm of cells from the digestive gland, gills and kidney of the blacklip abalone *Haliotis rubra* (Leach, 1814) (Hyne *et al*., 1992), and on the effect of iron on red abalone post larvae in a controlled-culturing system. Results indicate that *H. rufescens* post larvae accumulate iron granules in the stomach, digestive gland. and mantle, but not in the gills or in other tissues (Pino-Chandia *et al*., 2012). An observation of extracellular crystals formation of iron, chloride, and sulfur was recorded in the gonads of the sea-urchin *Paracentrotus lividus* (Lamarck, 1816), collected from a wastewater outfall near Marseilles, France. The gonads had a black and brown appearance (Delmas, 1990). Accumulation of iron crystal formation in animal tissues has been reported in dogs poisoned with ethylene glycol; calcium oxalate crystals in renal tubules and in the brain have been observed by histopathology and confirmed as ethylene glycol poison (García-Ortuño *et al*., 2006). In humans, hyperuricemia can be induced by a purine-rich diet producing uric-acid crystals in tissues (Chizyński & Rózycka, 2005). In accordance with the previous references, crystal formation in animal and/or in human tissues could be induced by pollution, poisoning, or by dietary composition. Those crystals have particular physical-chemical characteristics and a direct comparison among them is not possible. Mci in the studied abalone suggests the possible effect of pollution, poisoning, or nutritional alterations, but this must be determined by the physical-chemical characterization of the Mci. On the other hand, studies on the effect of contaminants at a histological level in mollusks are common in scientific literature; for example, Calabrese *et al*. (1984) studied the histopathological alterations of mussels exposed to silver and copper. Berthou & Balouët (1987) studied the presence of hydrocarbons and histopathological abnormalities in oysters after the *Amoco Cadiz* oil split. Lowe & Clarke (1989) studied the alterations in the digestive epithelial cells of the mussel *Mytilus edulis* (Linnaeus, 1798) following exposure to a mixture of hydrocarbons and copper. Gibbs and Bryan (2009) studied the reproductive failure in populations of the Dog-Whelk, *Nucella Lapillus* (Linnaeus, 1758), caused by imposex induced by tributyltin from antifouling paints. Jin Zhou *et al*. (2010) studied tributyltin (TBT) toxicity in abalone (*Haliotis diversicolor supertexta* Reeve, 1846) assessed by antioxidant enzyme activity, metabolic response, and histopathology. Kruatrachue *et al*. (2011) studied the snail *Poma-*



Figures 2a-c. a) General view of a transversal section of the gonad (G) and digestive diverticula (Dd) area from the first studied abalone (*Haliotis rufescens*), where several macro-crystal inclusions (Mci) are located between the outer (Ow) and inner (Iw) walls of the gonad, note the disruption of the trabeculae and connective tissue, with the absence of gametes. b) Close up of Mci surrounded by hemocytes (He), cellular debris (Cd) and fibroblast (Fi) note the brown granules. c) Close up of an apparent residual Mci surrounded by the He and abundant Cd. Hematoxylin-eosin.



Figures 3a-d. a) General view of a transversal section of the gonad (G) and digestive diverticula (Dd) area from the second studied abalone (*Haliotis rufescens*) where several macro-crystal inclusions (Mci) are located between the outer (Ow) and inner (Iw) walls of the gonad, note the presence of a large trabecula and its disarrangement through the Iw also showing the absence of gametes. b) Close up of a trabecula (Ta) with some growth of the germinal epithelium (Ge), which is in contact with Mci, note the cellular debris and the dark nuclei of the top cells in the Ge. c) Hypertrophied nuclei in the top cells (arrows in some of them) from the Ge around Mci. d) Connective tissue of trabecula with several brown cells shown by arrows. Hematoxylin-eosin.

cea canaliculata (Lamarck, 1828) which was exposed experimentally to contaminated sediments in Thailand. In any of these studies there is a mention of Mci formation that could be related to this study.

To determine the chemical nature of Mci, it is necessary to remove them from the tissue without modifying their structure and chemical composition so as to carry out X-ray and electron microscopy studies. Alternatives include the analysis for contaminants, poisons, or inorganic substances in gonadal tissues. The severe activation of the defense mechanisms of the host and damages in tissues suggests a high toxicity of Mci and an obvious impediment for reproduction of the individual.

In spite of this pathology, apparently of low prevalence or rare appearance, sampling is required to determine prevalence in aquaculture facilities, as are studies to establish potential negative effects in cultured red abalone populations from Baja California, Mexico. This pathology was found in an abalone infected by *Candidatus* X. californiensis, with the characteristic external signs of withering syndrome and in other abalone infected by *M. haliotis* with an external healthy appearance, showing an independent origin of this non-infectious pathology. These findings constitute the first record of a non-infectious pathology in the gonad of the cultured red abalone. Due to the lack of information on this alteration in abalone culture around the world, these results can be used to determine if this pathology could also be present in other abalone species and aquaculture facilities. Further research to determine its chemical composition and origin would help dimension the problem and would point to any appropriate sanitary measures needed to control this pathology.

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