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Presence of amyloid protein in the yellow clam *Amarilladesma mactroides* (Reeve, 1854), an emerging species for aquaculture

Presencia de proteína amiloide en la almeja amarilla *Amarilladesma mactroides* (Reeve, 1854), una especie emergente para la acuicultura

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ABSTRACT | Amyloid proteins are proteic groups that comprise a superfamily of apolipoproteins, highly conserved in vertebrates, being involved in the modulation of numerous immune responses during infections, injuries or stress. In this study, the yellow clam *Amarilladesma mactroides* was used as an invertebrate model in the search for amyloid protein deposits in their tissues. Adult specimens of this bivalve were collected from the beach and transported to the Laboratory of Immunology and Pathology of Aquatic Organisms - LIPOA at the Federal University of Rio Grande – FURG. Their tissues were sectioned and stained either with Hematoxylin and Eosin, and examined using light microscopy, or with Congo Red and analyzed under polarized light microscopy. Congo Red stain revealed the presence of amyloid proteins in the foot and digestive glands of the yellow clam. The techniques used proved to be appropriate for this species and evidenced a complementary role of amyloid proteins in the immune response, indicating that they are useful as a marker for the innate immune system and health of invertebrates. The ability to assess the immunological condition can provide information related to the cultivation of emerging species for aquaculture.

Palabras clave
Rojo Congo
Proteína ameloide
Histologia
Invertebrados

RESUMEN | Las proteínas amiloides son grupos proteicos que comprenden una superfamilia de apolipoproteínas altamente conservadas en vertebrados, estando implicadas en la modulación de numerosas respuestas inmunes durante infecciones, lesiones o estrés. En este estudio, la almeja amarilla *Amarilladesma mactroides* se utilizó como modelo para invertebrados y demostrar depósitos de proteína amiloide en sus tejidos. Se recolectaron de la playa ejemplares adultos de este bivalvo y se transportaron al Laboratorio de Inmunología y Patología de Organismos Acuáticos (LIPOA) de la Universidad Federal de Rio Grande (FURG). Sus tejidos se procesaron para su estudio histológico y se tiñeron con hematoxilina y eosina, y Rojo Congo. Las secciones histológicas teñidas con hematoxilina y eosina se examinaron con microscopía óptica clásica. Además, las secciones fueron teñidas con Rojo Congo y se analizaron con microscopía de luz polarizada. La tinción con Rojo Congo reveló la presencia de proteínas amiloides en el pie muscular y en las glándulas digestivas de la almeja amarilla. Las técnicas utilizadas demostraron ser adecuadas para la determinación de proteína amiloide en esta especie y evidenciaron un papel complementario de las proteínas amiloides en la respuesta inmune, indicando que pueden servir como marcador del sistema inmunológico innato y la salud de los invertebrados. La capacidad de evaluar la condición inmunológica puede proporcionar información relacionada con el cultivo de especies emergentes para la acuicultura.

The yellow clam *Amarilladesma mactroides* (Reeve 1854), (syn. *Mesodesma mactroides*) is a native surf clam from southernmost Brazil, Uruguay and Argentina. This species has recently been the subject of studies (Santos *et al*. 2016; Carvalho *et al*. 2016; Santos *et al*. 2020a; Santos *et al*. 2020b) and stands out for its historical value as a significant fishing resource on the mentioned countries (Coscarón 1959).

Currently, the *A. mactroides* is endangered due to overexploitation with successive mass mortalities of unknown cause, which directly affect their stocks (Carvalho *et al*. 2013a; Carvalho *et al*. 2013b; Santos *et al*. 2016).

Massive mortality outbreaks can be related to variations of environmental parameters that affect the defence mechanisms of marine invertebrates, making them more susceptible to various diseases (Gagnaire *et al*. 2006; Carvalho *et al*. 2013a; Carvalho *et al*. 2013b). Although the interest and development of research for several species of bivalves has grown, information related to the immune system of these organisms is still limited (Bibby *et al*. 2008). Hence, complementary immune parameters have increasingly been used and explored as markers to improve its understanding.

The development of technologies to produce *A. mactroides* in the laboratory, has resulted in protocols for the broodstock conditioning, spawning, larviculture and settlement and growth until seed was ready to plant out in the sea (Santos *et al*. 2018). The culture systems proposed for the yellow clam provide different conditions from the place where these animals occur (Ayerbe *et al*. 2018; Silva 2019). The cultivation of *A. mactroides* has been carried out subtidally in bay regions (Silva 2019). Even showing promising results, the cultivation site differs greatly from the environment where they occur, intertidally in dissipative sand beach which could induce physiological imbalances and affecting the immune system response. In this sense, studies have focus on the examination of quick and unspecific mechanisms related to the innate immune system, which play an important role in the early defence of organisms against pathogens (Qu *et al*. 2014). One of these mechanisms entails the action of amyloid proteins.

Amyloid proteins (e.g., serum amyloid protein A - SAA) are protein groups that include a superfamily of apolipoproteins highly conserved in vertebrates, being involved in the modulation of numerous immune responses during infections, injuries or stress (Jensen and Whitehead 1998). These proteins are found in mammals, birds and fish (Uhlar and Whitehead 1999), but rarely described in marine invertebrates such as: the echinoderm *Holothuria glaberrima* (Santiago-Cardona *et al*. 2003) and the species of bivalve molluscs *Crassostrea hongkongensis* (Qu *et al*. 2014) and *Meretrix meretrix* (Zou and Liu, 2015). Given these gaps of information and functionality, Rosani *et al*. (2016) searched for sequences of SAA types that could be present in the genomes of several invertebrates groups (including the bivalves *Mytilus galloprovincialis* and *Crassostrea gigas*), providing a phylogenetic panorama for these proteins.

During stress, such as an acute infection, SSA can be expressed up to 1000 times more in plasma levels, whereas in chronic stress, this protein can be deposited in large amounts (extracellular, in tissues or organs), generating a disease called amyloidosis (Woldemeskel 2012; Sipe *et al*. 2016). Thus, the activity of these proteins acts as a biomarker for monitoring the health status of animals, which can exhibit an acute or chronic immune response (Steel and Whitehead 1994; Jensen and Whitehead 1998; Uhlar and Whitehead 1999).

One technique to visualize deposits of amyloid proteins in tissues of organisms is using appropriate histopathological techniques. Therefore, the amyloid proteins can be demonstrated in sections stained with Hematoxylin and Eosin. The precision of this technique improved when the slides is submitted to chemical identification with Congo Red stain under polarized light (Puchtler *et al*. 1962; Woldemeskel 2012), according to the International Amyloidosis Society (Sipe *et al*. 2016).

Therefore, by using these techniques in tissues of the yellow clam *A. mactroides*, the present novel study aimed to enable the location of amyloid proteins deposits and the development of a potential marker for the health of *A. mactroides* as well as other invertebrates.

In January 2016, ten adult specimens of *A. mactroides* bivalves (with shell length ≥ 43 mm) were manually collected along the intertidal zone of Cassino Beach, in the state of Rio Grande do Sul (32°12'S 52°10'W). After collection, the shellfish were stored in thermal boxes (5L), with seawater under the same salinity (32) and room temperature (25° C) while they were transported to the Laboratory of Immunology and Pathology of Aquatic Organisms - LIPOA at the Federal University of Rio Grande - FURG in Rio Grande do Sul. In the lab, the bivalves were kept for a day in a water tank (20L) with constant aeration and seawater and temperature similar to that of the collection.

Subsequently, for histological procedures and analyses, ten bivalves were sacrificed, their tissues were

sectioned (gills, foot, digestive gland and gonads), fixed (20% formaldehyde), and processed in the Leica TP1020 automatic tissue processor with inclusion in Paraplast (Sigma).

After embedding, each tissue sample was sliced into 2 slides of 4µm by the microtome Leica RM2245. One slide was stained with Hematoxylin and Eosin (HE) and examined using light microscopy. For the second slide, the Congo Red technique was used and inspection for the presence of amyloid protein was performed by polarized light microscopy coupled with the biological microscope (ref. TA-0159), that was composed of two filters for polarization: F1 polarizing and F2 Analyzer (Nikon).

On tissues stained with Hematoxylin and Eosin, we observed eosinophilic deposits which were homogeneous referring to the foot muscles and digestive glands (Fig. 1). When using the Congo Red technique, these deposits were confirmed to be positive for the presence of amyloid proteins (Fig. 2) and the polarized light microscopy showed central and peripheral birefringence in the tissues, also allowing visualization of such deposits (Fig. 3). Patterns of these deposits were observed in both specific tissues (foot muscles and digestive glands) of all animals examined.

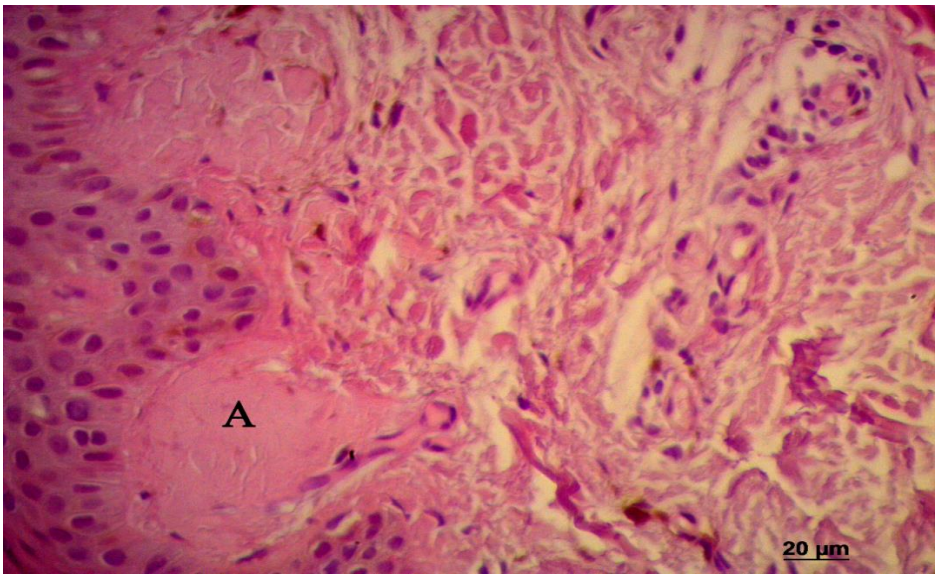

Figure 1 Deposits of eosinophilic material, homogeneous (A) on the digestive glands of yellow clam Amarilladesma mactroides stained with Hematoxylin and Eosin (HE). 20 µm scale.

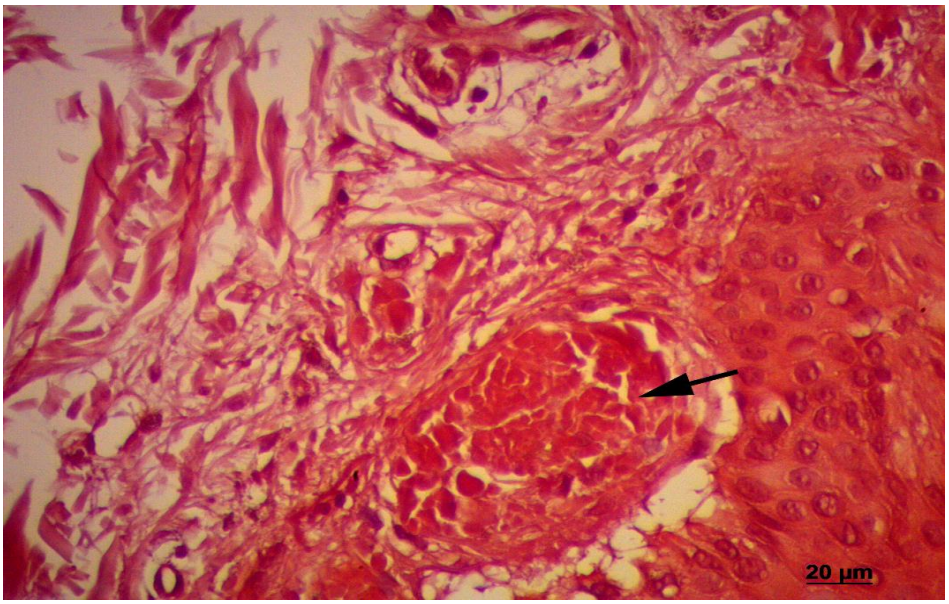

Figure 2 Homogeneous deposits (arrow), on the digestive glands of yellow clam Amarilladesma mactroides, positive for the Congo Red, without polarized light. 20 µm scale.

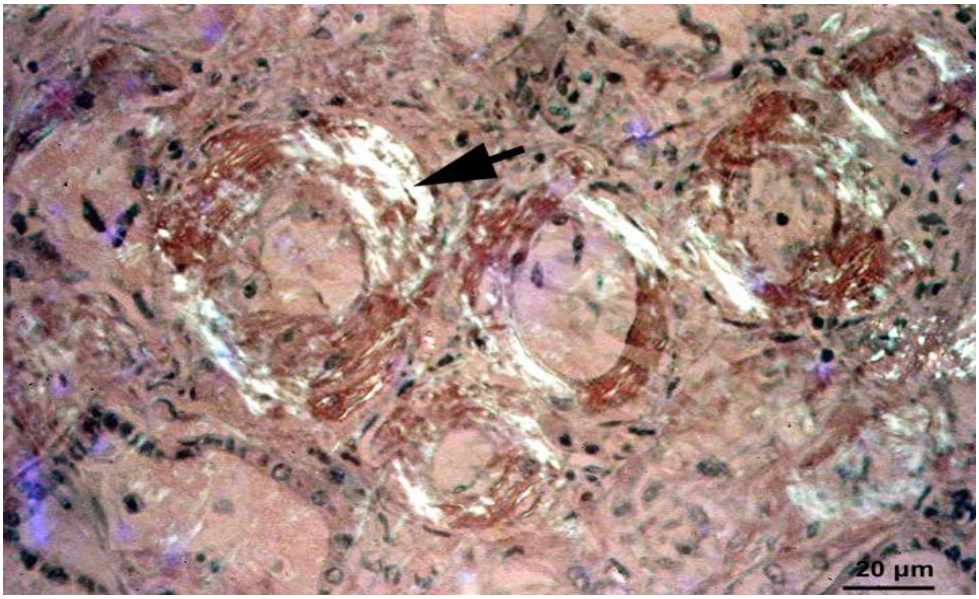

Figure 3. Areas of homogeneous deposits, within the digestive glands of the yellow clam *Amarilladesma mactroides*. Presence of amyloid protein on slides stained with Congo Red, under polarized light microscopy showing central and peripheral birefringence in the tissues (arrow). 20 µm scale.

Depending on the bivalve mollusc species, amyloid protein can be detected in different tissues (Rosani *et al*. 2016). In the current study, this protein was observed by using the technique of chemical identification of the Congo Red, in the foot and digestive glands tissues of the yellow clam *A. mactroides*.

According to Steel and Whitehead (1994), the systemic immune response is activated by a series of factors, such as infections, trauma or tissue injuries. It is notable that the *A. mactroides* is susceptible to continuous stresses, arising from environmental changes and human actions such as human trampling, vehicle traffic and pollutants. Therefore, amyloid protein accumulations in the tissues of *A. mactroides* may be related to the area in which the animals were collected and their specific stressors (Carvalho *et al*. 2013a; Carvalho *et al*. 2013b; Santos *et al*. 2016).

Furthermore, the incidence of this protein in the foot tissues of the yellow clam can be explained by its sand-burrowing behavior. Being biologically conditioned to bury itself to more than 15 cm in depth (Coscarón 1959) and using its muscular foot to perform this mechanism can make this section tissues particularly susceptible to chronic injuries and traumas, thus generating amyloid accumulation (Woldemeskel 2012).

In the vertebrates, the immune response can be performed by plasma specific groups of proteins, predominantly, for these produced by hepatocytes (Bayne and Gerwick 2001). The SSA is one of these highly conserved proteins in vertebrates and it is involved in the modulation of several immune responses during infections, injuries or stress (Jensen and Whitehead 1998). It may justify the pattern of amyloid protein presence, that was scored positive staining and found to be homogeneously central and peripheral in the tissues of digestive glands of the species *A. mactroides*. A similar arrangement was found for the hard clam *M. meretrix* (bacteria challenge), showing high expression of SSA in hepatopancreas and foot tissues (Zou and Liu 2015). However, the *M. galloprovincialis* revealed greater expression in the mantle, which was limited to the digestive gland and null in the foot (bacteria challenge) (Rosani *et al*. 2016). Moreover, the oysters *C. hongkongensis* (fungi and bacteria challenges) (Qu *et al*. 2014) and *C. gigas* (bacteria challenge) (Rosani *et al*. 2016) showed greater expression in the gonads and mantle.

Another factor to be considered is that the Cassino beach has salinity fluctuations (Odebrecht *et al*. 2010; Santos *et al*. 2016) and such fluctuations could trigger histological traumas and changes in the digestive gland of the yellow clam, as shown by Carvalho *et al*. (2015).

The evidence and associations above need further clarifications, nevertheless, this work was primarily conceived of as an attempt to develop an immunological monitoring tool adding information to other studies that have already been reported. For instance, previous molecular studies have identified and characterized the increased expression of genes encoding SAA in various tissues exposed to pathogens as fungi and bacteria. Besides improving the understanding of the evolution of this protein and how it can act in the resistance of organisms against pathogens during their growth, more importantly, such findings contributed to increase the knowledge about the innate immune response to aquaculture of these invertebrates (Qu *et al*. 2014; Zou and Liu 2015; Rosani *et al*. 2016). On the other side, the present study brings the application of histopathological techniques with the *A. mactroides* as a supportive approach to explore the complex innate immune system of invertebrates. The organisms are constantly challenged by biotic and abiotic environmental stressors, therefore they require a highly coordinated systemic response to cope with these stressors (Qu *et al*. 2014) and the immunological tool presented here is a potential health marker and an instrument to enhance this effort.

This study evidenced the presence of amyloid protein in the tissues of the yellow clam *A. mactroides* and showed evidence that it can have an important complementary role for the immune response in face of their different physiological situations and even culture systems. However, more detailed quantitative and qualitative studies still required to elucidate these mechanisms.

Conflict of interest

Authors declare no conflict of interest

Declaration of best practices in the use of animals

According to Brazilian law, authorization for the use of invertebrates, including clams, is not required in the conduct of scientific experiments.

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