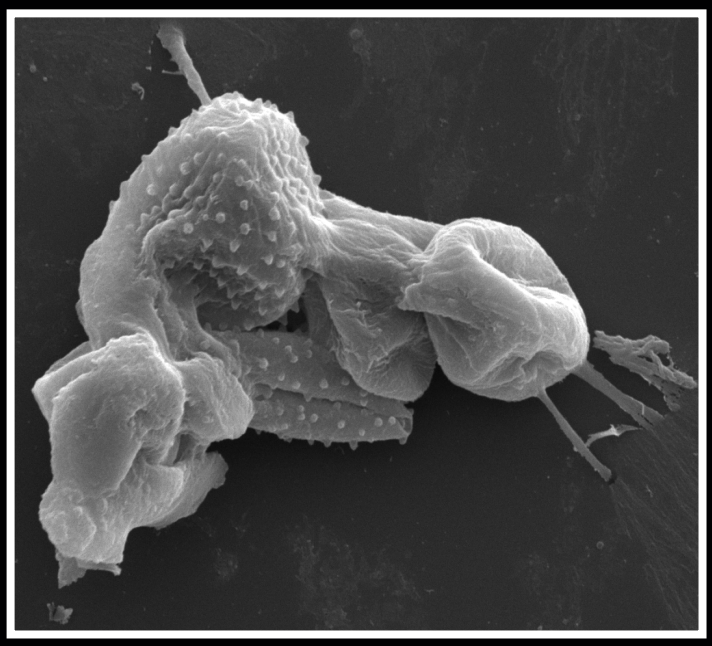
Universidade Vigo

DEPARTAMENTO DE ECOLOGÍA Y BIOLOGÍA ANIMAL

COCCIDIOSIS AND MOLECULAR BASIS OF THE IMMUNE RESPONSE OF COMMON OCTOPUS (*Octopus vulgaris* Cuvier, 1797)



TESIS DOCTORAL

SHEILA CASTELLANOS MARTÍNEZ

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DEPARTAMENTO DE ECOLOGÍAY BIOLOGÍA ANIMAL

COCCIDIOSIS AND MOLECULAR BASIS OF THE IMMUNE RESPONSE OF COMMON OCTOPUS (*Octopus vulgaris* Cuvier, 1797)

MEMORIA PRESENTADA POR

SHEILA CASTELLANOS MARTÍNEZ

PARA OPTAR AL GRADO DE DOCTOR POR LA UNIVERSIDAD DE VIGO

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Durante el desarrollo del presente trabajo Sheila Castellanos Martínez ha recibido una beca de estudios del Consejo Nacional de Ciencia y Tecnología (CONACyT) del gobierno de México.

La presente tesis doctoral ha sido desarrollada en el marco del proyecto "*Bases moleculares da resposta inmune do polbo comun, Octopus vulgaris*" financiado por la Xunta de Galicia (10PXIB402116P) y en las instalaciones del Instituto de Investigaciones Marinas del Consejo Superior de Investigaciones Científicas (IIM-CSIC).

Dña. **Ma. del Camino Gestal Mateo**, Doctora en Biología, científico titular del Consejo Superior de Investigaciones Científicas

INFORMA:

Que la memoria titulada "Coccidiosis and molecular basis of the immune response of common octopus (*Octopus vulgaris* Cuvier, 1797)" que presenta Dña. Sheila Castellanos Martínez para optar al grado de Doctor por la Universidad de Vigo, ha sido realizada bajo su dirección y considerándola concluida, autoriza su presentación a fin de que pueda ser juzgada por el tribunal correspondiente.

Y para que así conste, expiden y firman el presente informe en Vigo, a 25 de Abril de 2013

Fdo. Dra. Ma. Camino Gestal Mateo

D. Francisco Javier Rocha Valdés, Doctor en Biología e investigador del grupo de Zoología Marina del Departamento de Ecología y Biología Animal de la Universidad de Vigo

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SUMMARY

The common octopus, *Octopus vulgaris* Cuvier, 1797, is one of the most important species in worldwide fisheries and aquaculture. Galicia is the pioneer Autonomic Community in octopus culture, which is considered one of the most important alternative resources to diversify the aquaculture. One of the main constraints in this activity is the diseases caused by several pathogens. Therefore, in order to control and eradicate the main diseases, such as coccidiosis caused by *Aggregata octopiana,* it is highly important to develop studies focused on knowing the octopus immune response against pathogens. Those studies will allow us to establish the basis to develop strategies towards an appropriate sanitary practice in octopus aquaculture. Furthermore, supplementary studies of genes involved in immune response will contribute to establishing the molecular basis to identify and select octopuses resistant against the coccidia infection. Hence, the first study of the common octopus immune response and their interaction with the infection by the coccidia *A. octopiana* is herein presented.

The molecular characterization of *A. octopiana* from NE Atlantic (Ria of Vigo) using 18S rRNA gene has allowed the complementation and confirmation of the pre-existing morphological description. Likewise, the molecular characterization of *A. eberthi* that infects *Sepia officinalis* was also performed. The new sequences obtained were compared with the only sequences of *A. octopiana* and *A. eberthi* available in GenBak from the Adriatic Sea (Croatia). The low genetic divergence between *A. eberthi* species indicates that these coccidia infect two different populations of *S. officinalis.* In contrast, the high genetic divergence between *A. octopiana* from NE Atlantic and Adriatic Sea indicates that they correspond to different coccidia species. Therefore, according to previous morphological descriptions, host specificity and the molecular data herein obtained, *A. octopiana* from NE Atlantic (Ria of Vigo) is considered as the valid species.

The studies conducted through microscopy and flow cytometry allowed to characterize the hemocytes present in the octopus hemolymph. Two sub-populations or types of hemocytes were characterized, namely large granulocytes and small granulocytes. Using functional analysis it was demonstrated that both types of cells showed the ability to develop defensive activities in the organism. However, phagocytic ability and respiratory burst were higher in large granulocytes than in small ones. Nitric oxide (NO) production was measured in the total hemocytic population following challenge with zymosan, LPS and PMA in a time course. The highest NO production was reached after 3 h of incubation. There was confirmed that cellular immune defense is affected by the level of *A. octopiana* infection. The phagocytic activity increased according to the increase of the infection, mainly in autumn; whereas, respiratory burst (ROS) and NO decreased when the coccidia infection increase. The NO production decline was particularly notorious in low infected octopuses, but also in the heaviest individuals. In addition, a similar pattern in the cellular immune defense was observed in wild octopuses and in those reared in floating cages. In both cases, the phagocytic ability increase with the level of infection, but respiratory burst and NO decreased. Furthermore, NO production was significantly lower in wild octopuses than in those reared in floated cages, suggesting that the stressful culture conditions and

coccidia infection acts synergistically, and triggers a high cytotoxic response in those octopuses reared in floating cages.

The transcriptomic study of the hemocytes from *O. vulgaris* by construction of cDNA library using a high-throughput sequencing method, allowed for the identification of important immune pathways such as NFkB, complement, Toll-Like Receptors (TLR) and apoptosis. From the present study, most of the immune genes identified are reported for the first time in cephalopods. The transcriptome of hemocytes from octopuses harboring high and low infection by *A. octopiana* were compared. A total of 539 genes were found differentially expressed between both levels of infection. Q-PCR analysis of genes selected according to their importance in the host-pathogen interaction confirmed the previous expression pattern and corroborated the results obtained by the high-throughput sequencing. In the proteomic study of the octopus hemolymph, 42 significant spots were found in hemocytes from octopuses harboring high and low infection by *A. octopiana.* These spots were statistically analyzed by principal component analysis, from which 7 proteins are herein suggested as candidates of putative resistance biomarkers against the coccidia infection. Particularly, the proteins filamin, fascin and peroxiredoxin are highlighted because of their implication in the octopus immune defense. Considering the information obtained in this study, there is evidenced that coccidiosis by *A. octopiana* affects the proper functioning of the octopus cellular immune response. Phagocytosis is stimulated by the infection, however respiratory burst is suppressed. The molecular evidence agreed with functional assays. The respiratory burst reduction results in a down-regulation of antioxidant genes at both trancriptomic and proteomic level. Likewise, the increase in phagocytic ability of the hemocytes is consistent with the significant up-regulation of proteins like filamin and fascin (both related to phagocytosis) in highly infected octopuses. Therefore, the results exposed in the present work provide the first molecular insights into the molecular basis of host-pathogen relationship between *O. vulgaris* and *A*. *octopiana*.

RESUMEN

El pulpo común, *Octopus vulgaris* Cuvier, 1797, es una de las especies más importantes en las pesquerías mundiales y en acuicultura. Galicia es la Comunidad Autónoma pionera en cultivo de pulpo, el cual está considerado uno de los recursos alternativos más importantes para diversificar la acuicultura. Uno de los inconvenientes de la actividad acuícola son las enfermedades producidas por diversos patógenos. Por tanto, para controlar y erradicar las principales enfermedades, tal como la coccidiosis causada por *Aggregata octopiana*, es imprescindible desarrollar estudios que permitan conocer la respuesta inmune de este molusco ante patógenos. Dichos estudios permitirán establecer las bases para elaborar estrategias enfocadas a mantener una adecuada sanidad acuícola. Además, el estudio complementario de genes de respuesta inmune, contribuirá a establecer las bases moleculares para identificar y seleccionar individuos resistentes a la infección por el coccidio. En esta memoria se recoge el primer estudio sobre la respuesta inmune del pulpo común y su interacción con la infección por el coccidio *A. octopiana*.

La caracterización molecular de *A. octopiana* en el Atlántico NE (Ría de Vigo) utilizando el gen 18S ARNr, ha permitido complementar y confirmar la descripción morfológica ya existente. Asimismo, el estudio molecular se extendió al coccidio *Aggregata eberthi*, parásito del choco *Sepia officinalis*. Las nuevas secuencias generadas se compararon con las únicas secuencias de *A. octopiana* y *A. eberthi* disponibles en GenBank procedentes del Mar Adriático (Croacia). La baja divergencia genética observada entre especies de *A. eberthi* indica que este coccidio infecta a distintas poblaciones de la especie *S. officinalis*. En cambio, la alta divergencia genética observada entre *A. octopiana* del Atlántico NE y del Mar Adriático indica que corresponden a dos especies distintas de coccidios. Por tanto, con base en los estudios morfológicos previos, los datos específicos del hospedador y los datos moleculares ahora presentados *A. octopiana* del Atlántico NE (Ría de Vigo) es considerada la especie válida.

Los estudios mediante microscopía y citometría de flujo permitieron la caracterización de los hemocitos presentes en la hemolinfa del pulpo. Se caracterizaron dos subpoblaciones o tipos de hemocitos denominados granulocitos grandes y granulocitos pequeños. Mediante análisis funcionales se demostró que ambos tipos celulares presentan capacidad para desarrollar actividades involucradas en la defensa celular del organismo. Sin embargo, la actividad fagocítica y el estallido respiratorio fueron mayores en granulocitos grandes que en granulocitos pequeños. La producción de óxido nítrico (NO) se midió en la población total de hemocitos ante distintos estímulos como zimosán, LPS y PMA durante un transcurso de tiempo, alcanzando mayor producción de NO a las 3h de incubación. Se confirmó que la defensa inmune celular es afectada por el grado de infección por *A. octopiana*. La actividad fagocítica de los hemocitos se incrementó conforme aumentó la infección, principalmente en otoño; mientras que, el estallido respiratorio (ROS) y NO disminuyeron con el aumento de la infección. La disminución en la producción de NO fue particularmente notable en pulpos poco infectados y en los individuos de mayor peso. Se observó un patrón similar en la defensa inmune celular tanto de pulpos salvajes como de pulpos engordados en batea. En ambos casos, la capacidad fagocítica se incrementó con el grado de infección, pero el

estallido respiratorio y el NO disminuyeron. Asimismo, la producción de NO fue significativamente menor en pulpos salvajes que en pulpos de batea, lo que sugiere que las condiciones estresantes de cultivo y la infección por el coccidio actúan sinérgicamente, y desencadenan una alta respuesta citotóxica en los pulpos cultivados en batea.

El estudio transcriptómico de los hemocitos de *O. vulgaris*, mediante la generación de una librería de cDNA utilizando técnicas de secuenciación masiva permitió la identificación de importantes cascadas de señalización implicadas en la respuesta inmune como NFkB, complemento, receptores tipo Toll (TLR) y apoptosis. Mediante este estudio, muchos de los genes registrados involucrados en estas cascadas se han identificado por primera vez en cefalópodos. A partir de la comparación del transcriptoma de los hemocitos de *O. vulgaris* con alta y baja infección por *A. octopiana*, se identificaron 539 genes diferencialmente expresados entre ambos grados de infección. El análisis mediante q-PCR de genes seleccionados por su importancia en la interacción hospedador-patógeno confirma el patrón de expresión y corrobora los resultados obtenidos mediante la secuenciación masiva. En el estudio proteómico de la hemolinfa de pulpos, 42 spots resultaron significativos en muestras de hemocitos de individuos con alto y bajo grado de infección por *A. octopiana.* Estos spots se analizaron mediante un análisis de componentes principales a partir cual, se proponen 7 proteínas como candidatas a posibles biomarcadores de resistencia a la infección por el coccidio. Particularmente, las proteínas filamina, fascina y peroxiredoxina se proponen como las más relevantes debido a su implicación en la defensa inmune del pulpo.

Tomando en cuenta la información generada en este estudio, se evidencia que la coccidiosis ocasionada por *A. octopiana* afecta al funcionamiento adecuado de la respuesta inmune celular del pulpo. La fagocitosis se ve estimulada por la infección, sin embargo, el estallido respiratorio es suprimido. La evidencia molecular concuerda con los análisis funcionales. La reducción del estallido respiratorio se traduce en menor expresión de genes antioxidantes tanto en el transcriptoma como en el proteoma. Asimismo, el incremento de la actividad fagocítica es acorde con la expresión significativa de las proteínas filamina y fascina (ambas implicadas en fagocitosis) en pulpos con alta infección. Por tanto, los resultados expuestos en el presente trabajo aportan las primeras bases moleculares de la relación hospedador-parásito entre *O. vulgaris* y *A. octopiana*.

I. General introduction

Octopus vulgaris reared in floating cages in the Ria of Vigo (Galicia, Spain) (From: Rubén Chamorro).

I.1 Overview of the Octopus vulgaris fishery

Cephalopods are molluscs with their primary skeletal features, a cranium, and in most cases, a mantle/fin support (cuttlebone or gladius). Their central nervous system is highly developed, especially the well-organized eyes. Likewise, the circulatory system is restricted to vessels and arteries. Due to the well developed nervous and closed circulatory system, cephalopods are considered as the most specialized class of molluscs. There are four groups of cephalopods: squids, cuttlefishes, octopuses and nautiluses. Almost all of them are fast swimmers, living pelagically or in the bottom. They all are active carnivore's predators upon shrimps, crabs, fishes, bivalves and even other cephalopods (Roper et al., 1984).

The interest in cephalopods has increased considerably in the last 40 years, mainly because of their introduction into the world market as a major fishery resource. Cephalopods are a valued seafood for human consumption because of their high protein content, and fatty acids DHA (docosahexaenoic acid) and EPA (eicosapentaenoic acid). DHA and EPA are highly important to humans for the prevention of coronary heart disease. They are both found in higher content in cephalopods than in fish species. Moreover, there is a high content of both fatty acids even if cephalopods under culture conditions are feed using inadequate diets (Zlatanos et al., 2006; Ozogul et al., 2008; García-Garrido et al., 2010).

Currently, cephalopod (squids, octopus, and cuttlefishes) fisheries in European waters are of substantial importance. Total annual cephalopod landings in the ICES area reached 27,620 tons in 2011. From these, the landing proportion by groups was 15,440 tons of cuttlefish (*Sepia officinalis*); 7,859 tons of long-finned squid (*Loligo forbesi, L. vulgaris, Alloteuthis subulata* and *A. media*); 1,580 tons of short-finned squids (*Illex coindetii* and *Todaropsis eblanae*) and 2, 741 ton of octopods (*Octopus vulgaris* and *Eledone spp.*) (Fig. 1). Regarding to octopus landings, Spain and Portugal were the most important countries with more than 95% of octopus landing shared by two nations.

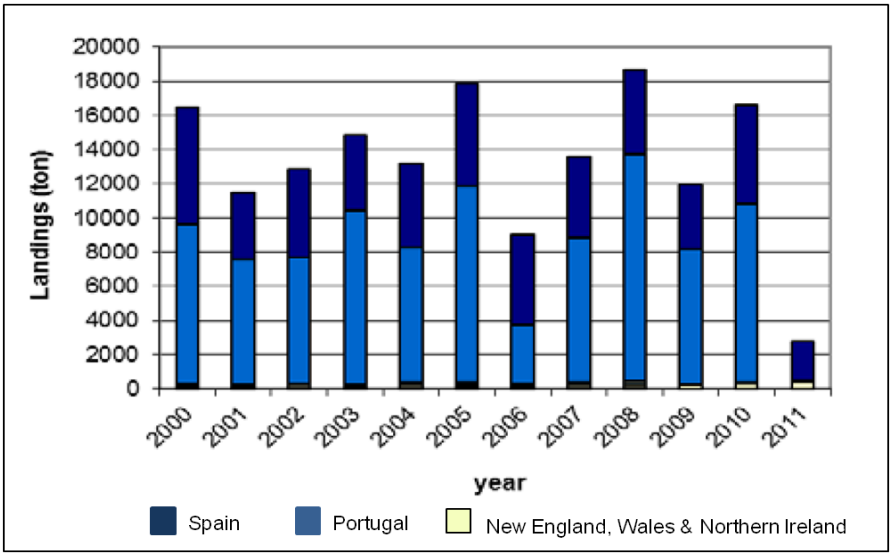


Fig. 1. Total annual octopus landings (in tones) in whole ICES area showing the most important countries (From: ICES, 2012).

Galicia is the main fishing region in Spain and one of the largest in the European Union. The artisanal fishery is the most numerous within the inshore fishery and is multi-specific and multi-gear, exploiting a diverse array of invertebrates. From an economic point of view, the most important are crustaceans (spider crab, prawns, goose barnacle), bivalve molluscs (clams, razor clams, scallops) and cephalopods (octopus, squid, cuttlefish) (Freire and García-Allut, 2000). The cephalopods depicts a highly valuable resource which profits reached 26.96 million € in 2008. The cephalopod target species are the common octopus, *Octopus vulgaris*; common cuttlefish, *Sepia officinalis;* and European squid, *Loligo vulgaris* with major fishery volume coming from the *Rias Baixas* (Fig. 2) (García-Tasende et al., 2009). In 2011 the total cephalopod species fished in Galicia reached 11.66 tons, with profits of 39 million €. From all these species, 3.40 tons were *O. vulgaris* with a reported value of 20.43million € (Anuario de Pesca, 2011). Due to the proved value of cephalopod fishery, in Galicia, a minimum legal capture size of 1000 g in weight for *O. vulgaris*; 8 cm mantle length (ML) for *S. officinalis* and 10 cm ML for *L. vulgaris* were established in order to protect marine resources and to develop a controlled exploitation of stocks (Anuario de Pesca, 2011).

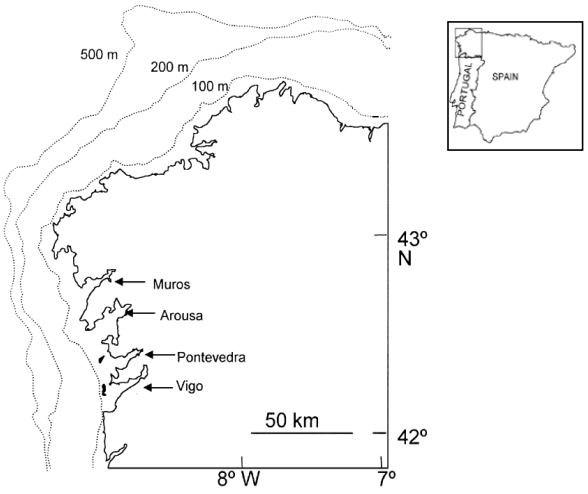


Fig. 2. The Iberian Peninsule and Galicia showing the location of the *Rias Baixas* (Modified from Freire and García-Allut, 2000).

I.2 The Octopus vulgaris aquaculture

Cephalopods are important model organisms for neuroscience, physiology and ethology research. For that reason, the initial trials of cephalopod's maintenance were mainly to provide live specimens for research or aquariums (Boletzky and Hanlon, 1983). However, the importance of cephalopods for human consumption was rising. Consequently, their commercial importance has also risen substantially in recent decades. It is obvious to expect the increasing cephalopod harvest in order to satisfy the growing demand. Nonetheless, we must not forget that cephalopods are also valuable as forage for commercial fishes and therefore, a trade-off between the commercial and ecological value must be encouraged (Hunsicker et al., 2010). In this context, aquaculture offers a reduction of fishing pressure on wild cephalopod stocks and a constant supply of the product to the market. This is particularly important in countries like Spain, Italy and Japan, which are the largest consumers and importers of cephalopods (FAO, 2012). Particularly in Spain, the fishing sector demanded the diversification of the marine farming industry, based on mussels (*Mytilus galloprovincialis*), turbot (*Scophthalmus maximus*), sea bass (*Dicentrarchus labrax*) and sea

bream (*Sparus aurata*) (Chapela et al., 2006; Vaz-Pirez et al., 2004). Thus, farming trials were developed using *O. vulgaris* as a new species target due to their high value in the market, short life cycle (12-18 months), high fecundity (100, 000- 500,000 eggs per female), high food conversion rates (assimilating 40-60% of ingested food), its rapid and easy adaptation to captivity conditions and acceptance of frozen food (Iglesias et al., 2000; Vaz-Pirez et al., 2004).

To date, the octopus aquaculture is established as a successful activity that in 2011 produced 2,755 kg equivalent to 19,330€ (Anuario de Acuicultura, 2011). However, the first trial to evaluate the viability of this culture in laboratory conditions started during 1995-1999 (Iglesias et al., 2000). The results demonstrated the feasibility of fattening octopuses mainly with crustaceans (80% of the diet). Individuals weighing 300 g achieved 2,200 g in weight in 4 months, whereas octopuses weighing 1,300 g reached 12,300 g after 10 months of fattening (Iglesias et al., 2000). Parallel fattening research was conducted in floating cages in the Ria of Muros (Galicia). After feeding octopuses with fishes of low economic value, similar results in octopus growth rates (0.3-0.8 kg/month) were obtained (Rama-Villar et al., 1997). The potential of *O. vulgaris* culture was thus evident and promoted the establishment of five companies for intensive on-growing. Octopuses of 750 g (minimum legal weight in 1995) were reared in cylindrical or square shaped cages (Fig. 3), providing individual dens with a total capacity of 150 octopuses. The fattening program lasted 4 months and three fattening cycles were initially conducted during the year (Iglesias et al., 2000). However, wide variation in weight and profitability were obtained. The economic analysis of this activity revealed that supply of juveniles produced in laboratory and availability of artificial diets (for paralarvae and sub-adults) are needed to reduce costs and make it a profitability activity (García-García et al., 2004). Nevertheless, to complete the octopus life cycle in captivity is still a challenge.

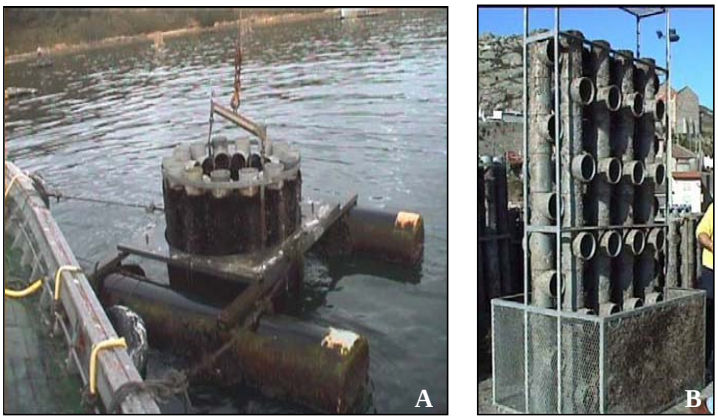


Fig. 3. Culture system (A) cylindrical and (B) square shaped cages used for rearing *O. vulgaris* in Galicia (From: Iglesias et al., 2000).

The main constraint to complete commercial rearing of the common octopus is the high paralarvae mortality during the first weeks of life (Iglesias et al., 1997). The hatchling cephalopods are planktonic and carnivorous, and require live prey of suitable size with high protein content and swimming behavior (Villanueva, 1994). Successful rearing experiments of the common octopus from paralarvae to juveniles have been developed using zoeae of crustaceans like *Liocarcinus depurator* and *Pagurus prideaux,* reaching a survival rate of 34.6% as individuals reached 30 days old (Villanueva, 1994; 1995). An *Artemia* based diet complemented with zoeae of *Maja squinado* has also been used, but only 0.2% of juveniles (individuals of 52 days) reached survival (Moxica et al., 2002). Iglesias et al. (2004) used the same mixed diet *Artemia–M. squinado* and reared paralarvae of up to 8 months old. However, from an economic point of view, the use of zoeae is not suitable for large-scale cultivation. Hence, additional prey to be reared commercially or artificial diets are required (Fuentes et al., 2011). To complete such goal, the paralarval feeding needs should be elucidate (Iglesias et al., 2006). To date, molecular detection of prey in wild paralarvae's stomach has contributed to reveal the range of prey that paralarvae typically consume in the sea (Roura et al., 2010). Meanwhile, biochemical studies have pointed out that a deficiency of polyunsaturated fatty acids in prey like *Artemia,* also poor in protein content, is the cause of low growth and low survival in captivity (Navarro and Villanueva, 2000; Iglesias et al., 2007). Co-feeding techniques tried in paralarvae included a combination of life prey *Artemia* and microcapsules

with 84-91% moisture. However, low or no growth in paralarvae was obtained when compared to the single *Artemia* diet (Villanueva et al., 2002). Fuentes et al. (2011) demonstrated that *Artemia* enriched with the microalgae *Nannochloropsis* sp. produces a higher paralarvae growth, in average, 1.61 mg and 46% of survival at day 30, than feeding individuals with *Artemia* enriched with sand eel (*Hyperoplus lanceolatus*) or crushed wild zooplankton. Nonetheless, results reported to date do not surpass the mean weigh value of 17.4 mg reported on paralarvae at 60 days (benthic phase) by Villanueva (1995). Presumably, the minimum nutritional requirements are covered in the planktonic phase but have not yet been established for the planktonic settlement phase. However, additional nutritional factors implicated in growth and survival are needed and therefore, further research is yet to be done (Fuentes et al., 2011).

The rearing of octopus in suspended cages in the sea depends on the supply of sub-adults captured by fishermen, but also on the availability of formulated diets that will support the commercial production of the species and will make the octopus culture a profitably activity (Lee, 1994; García-García et al., 2004; García-García and Cerezo-Valverde, 2006; Cerezo-Valverde et al., 2008). The octopus diet in the wild is mostly composed by crustaceans, but also by fishes and molluscs (Guerra et al., 1978). Crabs have provided better results than fish or molluscs in octopus growth (Cagnetta and Sublimi 2000). However, because crab supply could be expensive, discarded or low market value fish, such as *Boops boops, Sardina pilchardus, Sardinella aurita* or *Trachurus mediterraneus),*are usually used to feed octopus (Socorro et al., 2005; García-García and Aguado-Giménez, 2002; Rodríguez et al., 2006). To date, the economic viability of the octopus culture is still in progress and will be certainly achieved once the full biological cycle can be reproduced under controlled conditions, and the formulated diets and the necessary technology for a rearing system have been developed (García-García et al., 2004).

I.3. Pathogens affecting wild and cultured Octopus vulgaris

Cephalopods are secondary, third or paratenic hosts for trematodes digenea, cestodes and nematodes (Hochberg, 1990). Those parasites are transmitted to the definitive host: fishes, marine mammals or birds (Clarke, 1996; Gonzalez et al., 2003). Nevertheless, the effects of parasites on growth, reproduction and survival of the cephalopod hosts are still poorly studied (Abollo et al., 2001; Pascual et al., 1996; 2007).

Despite the benefits of aquaculture, one of the disadvantages is the increase in the incidence of pathologies produced by pathogens. Parasites have a negative effect on specimens in high population densities such as under culture conditions. Culture is associated with stress, which favours the development of infectious diseases leading to severe economical losses (Berthe, 2005). Hence, the knowledge and management of diseases affecting species under culture is a priority for the aquaculture. Moreover, in the European Union, comercialization of fishing products from the wild or aquaculture facilities must be free of parasites before making them available to the consumers. To avoid the transmission of parasites like *Anisakis* spp., the fishing products must be frozen, at least 24 h, previous to commercialization. Additionally, in order to ensure this statement, all the fishing products are visually examined. When macroparasites are found, the fishing products are rejected for human consumption (CE No. 853/2004). Therefore, because *O. vulgaris* is one of the most promising products, one of the main goals of the marine aquaculture program in Spain is the study of pathologies suffered by *O. vulgaris*. The specific objectives are i) the development of diagnostic tools for pathogens, ii) the development of methods for preventing infections under culture conditions and iii) to establish the basis to identify resistant octopuses that would allow the development of breeding programs with resistant specimens.

To date, the study of octopus parasites and pathologies has received less than 54% of research effort (Pascual and Guerra, 2001). Consequently, since the contributions by Hanlon and Forsythe (1990a,b) and Hochberg (1990) scarce data has been added. Several pathogens including virus, bacteria and protozoa parasites have been identified in *O. vulgaris* from both, wild and reared in on-growing cages (Pascual et al., 1996; 2007; 2010; Castellanos-Martínez and Gestal, 2013). Among the pathogens recently identified, two of them are highlighted, the Gram negative bacteria *Vibrio lentus* (Farto et al., 2003); and the gastrointestinal coccidia *Aggregata octopiana* (Gestal et al., 2002a,b; Gestal et al., 2007). Hence, both pathogens will be discussed herein.

I.3.1. Pathogens of bacterial etiology

All species of cephalopods are susceptible to bacterial infections on the skin, derived from secondary infections by opportunistic pathogens. The cephalopod's skin has a thin epidermis and a thicker dermis that covers the muscle layers beneath. The epidermis is micro-villous and it is composed by a monolayer of simple cuboidal cells interspersed with mucous-secreting cells that collaborate to retain and remove potential pathogens from the skin (Fig. 4). Nonetheless, the cephalopod skin is frail and susceptible to infections (Hanlon et al., 1984; Forsythe et al., 1987).

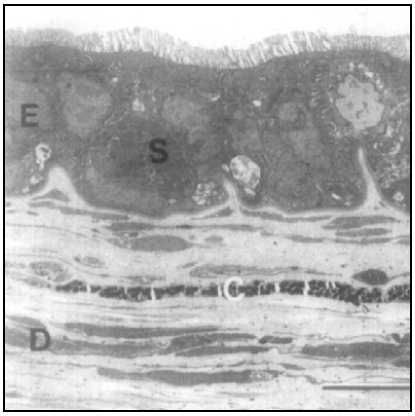


Fig. 4. Transmission electron microscopy section of skin from a young *Octopus joubini*. Epidermis (E) showing the thick layer and interspersed secretory cells (S). Dermis (D) including a portion of an expanded chromatophore (C). Barr=10 μm. (From Hanlon et al., 1984).

One of the most recent records of bacterial infections in wild cephalopods was reported in *O. vulgaris* kept in floating cages at the Ria of Vigo (Farto et al., 2003). The specimens showed lesions in the mantle and some of them died once in the laboratory. Lesions on the mantle have been attributed to the bacteria *Cytophaga*-like and *Pseudomonas*, which were isolated from the damaged tissue. However, the Gram negative bacteria *Vibrio lentus*, originally isolated from reared Mediterranean oysters (Macían et al., 2001), was isolated for the first time from the branchial heart of octopuses. Experimental infections performed by a challenge bath of *V. lentus* (72h, 2×10^8 cfu/ml) induced mortality in 50% of octopuses after the first six hours. The lesion showed a typical round pattern in the arms or

the head. No variations in mortality rate were recorded after nine hours post-infection, which is assumed to be a result of inter-individual differences in the immune system (Farto et al., 2003). Low salinity (29%) reported in the area was suggested to be a stressor that may impair the immune response of animals against the opportunistic pathogen *V. lentus* (Farto et al., 2003; Ford et al., 1986).

I.3.2. Pathogens of protozoan etiology: the eimeriorin coccidia Aggregata spp.

Coccidians of the genus *Aggregata* spp. (Apicomplexa: Eimeriorina) are a cause of severe disease in cephalopods. The protozoan infects the digestive tract of the host (Hochberg, 1990), mainly the caecum, thus impairing the absorption of nutrients (Boucher-Rodoni et al., 1987).

The genus *Aggregata* is distributed all around the world. A total of 10 species have been described to date (Gestal et al., 2010) infecting cuttlefishes, squids and octopuses, even those inhabiting deep-sea hydrotermal vents (Gestal et al., 2010).

Traditional identification and characterization of *Aggregata* species has relied primarily on differences in morphological features such as size and shape of sporogonial stages and host specificity. Nowadays, molecular techniques provide useful methods for taxonomic studies, and are important tools in solving problems of species delimitation. So far, molecular characterization of *Aggregata octopiana* and *Aggregata eberthi* has been carried out by sequencing the 18S rRNA gene (Kopečná et al., 2006; Gestal, *pers. comm*.). New molecular data from *Aggregata* spp. from the Ria of Vigo, their phylogenetic affinity and validation of morphologic characters for coccidia identification is presented in Chapter 1.

The intracellular protozoan *Aggregata* spp. has a heteroxenous life cycle (Fig. 5) which requires a crustacean intermediate host to develop its merogonic stage, while cephalopods are the definitive hosts in which the parasite develops its gamogony and sporogony stages (Hochberg, 1990). In Spain the infection by *Aggregata* spp. has been recorded in *O. vulgaris* and *Sepia officinalis* (Pascual et al., 1996), reaching a high prevalence and infection intensity. In addition, a new species was described in the ommastrephid squid *Todarodes sagittatus*, but showing a less prevalence of infection. Taking in consideration the elevated infection

prevalence and intensity, and the important pathological effects, parasites of the genus *Aggregata* are known to be the main epizootiological agents in wild and cultured octopus stocks (Gestal et al., 2007).

Infections by *Aggregata* initiate in the mucosal folds where the tissue ruptures at the basal membrane and a detachment of the epithelial cells is produced. As a consequence, the mucosal folds of the intestine and caecum suffer atrophy; at the intracellular level, displacement of the nucleus host cell to one side is visible (Gestal et al., 2002a; Poynton et al., 1992). All of the infected tissues show hemocytic infiltration and a pericyst reaction in both (gamogony and sporogony) infective stages (Gestal et al., 2002a; Licciardo et al., 2005; Mladineo and Jozić, 2005). The capsule formed is originally composed of flattened hemocytes and then connective tissue elements appear (Tripp, 1974). In senescent octopuses, the infection is predominantly by sporogonial (few merogonial) stages that extend widely in the tissue, showing scarce hemocyte infiltration or fibrotic reactions which are signs of a weak immune system (Pascual et al., 2010). During severe infective episodes the pathology is even extended to the mantle and gill's connective and epithelial tissues with similar signs of damage (Mladineo and Bočina, 2007).

The injury caused by the protozoan also has an effect at a biochemical level. The infection produces a decrease of the pH in the infected digestive tissue and, as a consequence, an inaccurate functioning of the digestive enzymes, such as maltase and leucin-aminopeptidase occurs, thus producing a malabsorption syndrome (Gestal et al., 2002b). In addition, heavily infected specimens show poor conditions reflected in Fulton's condition index, low DNA/RNA ratio, RNA/protein conversion and even a decrease of the number of circulating hemocytes (Gestal et al., 2007).

Although pathologies induced by this protozoan are not fatal, they severely weaken the cephalopod host making it more vulnerable to other biotic and abiotic stressors (Pascual et al., 2007).

A chronic infection by the coccidia *Aggregata* spp. in the cephalopod host offers the opportunity to study the immune response mechanisms in cephalopods at different developmental stages of the host life cycle, and different intensities of infection. Then, this should be established as a study model of the host-parasite relationship.

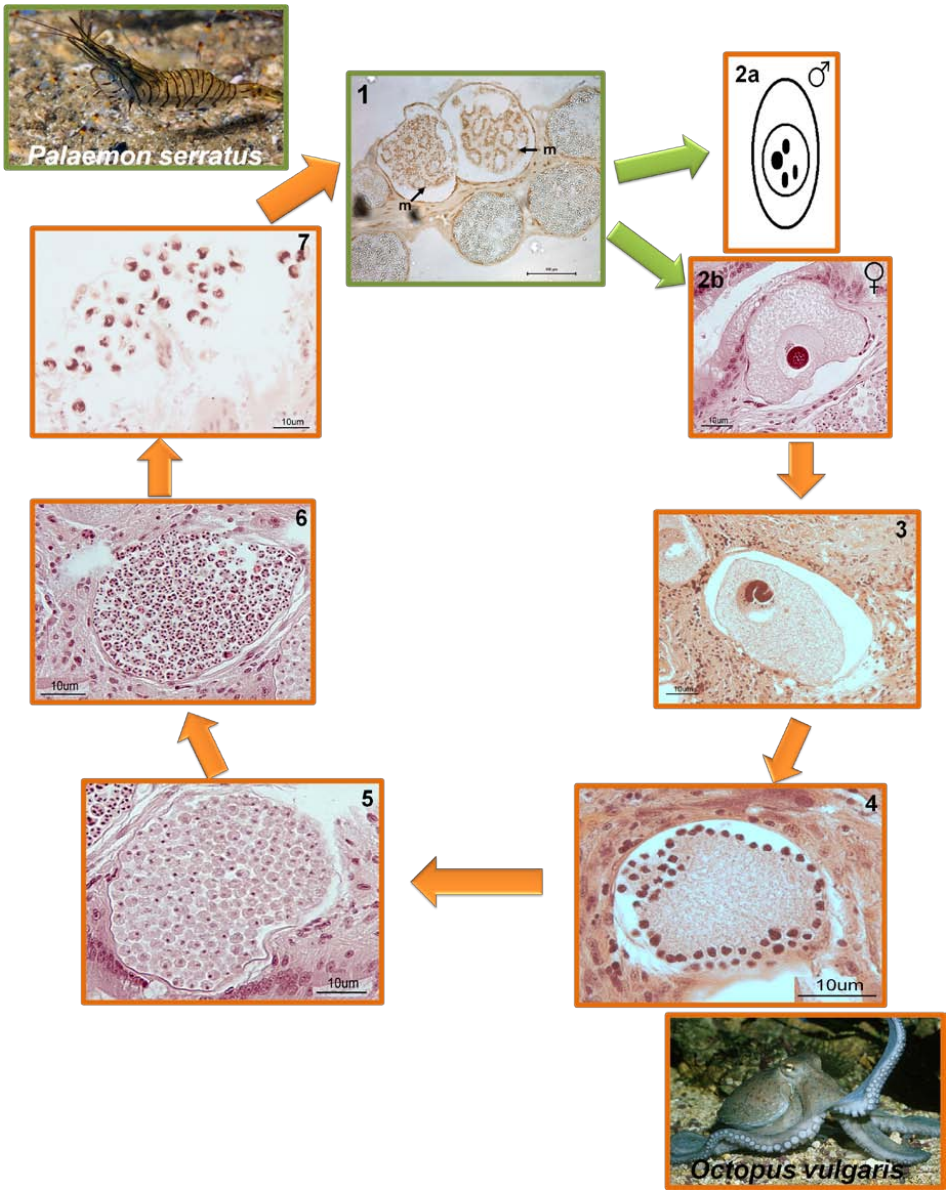


Fig. 5. Diagram of the life cycle of *Aggregata octopiana* (According to: Hochberg, 1990). **Merogony**: 1, asexual development of the parasite inside the intermediate crustacean host, giving rise to merozoites (m). **Gamogony**: after ingestion by the crustacean host and once in the cephalopod digestive tract, merozoites divide into (2a) male (microgametes) and (2b) female (macrogametes) gametes. 3, fertilization of gametes gives rise to a zygote. **Sporogony**:

4, zygote suffers multiple fission, thus starting the sporogony stage. 5, subsequent development, originates uninucleate sporoblasts. 6, several sporocysts develop inside oocysts. 7, once the sporocysts are completely mature, they are freed from the infected tissue and released to the sea with the octopus feces.

I.4. Overview of the cephalopod immune system

Cephalopods are molluscs with a well developed circulatory system; a systemic and two accessory hearts (branchial hearts) distribute the hemolymph through arteries and capillaries to the whole body (Schipp, 1987; Wells and Smith, 1987). Branchial hearts contribute to the production of hemocyanin, and the elimination of particles (Beuerlein et al., 1998; Beuerlein et al., 2002). Similarly to other molluscs, cephalopods have a non adaptive (or innate) immune system, which is the most rapidly acting. The general strategy of innate immune detection includes numerous receptors dedicated to recognize microbial and parasite molecules that are conserved across broad taxa. The receptors must detect pathogen molecules to avoid pathogen proliferation, dissemination and finally overwhelming the host. The term Pattern Recognition Receptors (PRR) has been applied to denote host molecules that recognize microbial infection; whereas, the term Pathogen-Associated Microbial Patterns (PAMPs) is used to denote the structural features of microbes that are recognized (Beutler, 2004). A far as is known, cephalopods do not have immunoglobulins and therefore they do not have extended protection against pathogens for future infections. Thus, the cephalopod immune system works on the basis of 'cellular factors'. The hemocytes respond by phagocytosis, encapsulation, infiltration or cytotoxic activities to infections and they destroy or isolate pathogens. In addition, molecules dissolved in the serum (opsonins, agglutinins, lysozyme) (Fig. 6) also contribute to the immune response (Ford, 1992).

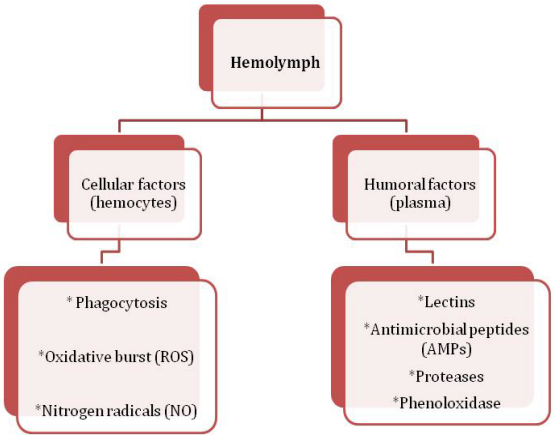


Fig. 6. Diagram of cephalopod innate immune defences against pathogens.

I.4.1. Cellular factors

Hemocytes play a major role in the internal defense by recognizing and eliminating foreign materials. They are also involved in shell and wound repair (Cheng, 1975). In cephalopods the hemocytes (also named leukocytes) are produced in the white bodies, in the orbital pits of the cranial cartilages, located behind the eyes (Cowden, 1972). The white body is constituted by two primary lobes, of unequal size, organized into several secondary lobes and a large number of small lobules providing a glandular appearance to the organ (Claes, 1996). Its embryonic development has not been studied yet (Cowden and Curtis, 1973). Inside the organ, strings of leukopoietic cells are found at different developmental stages (Cowden, 1972). Their leukopoietic function is deduced from the ultra-structural similarities between the putative final stage of their cells and the circulating blood cells, which correspond to a single cell line; hence, only one type of hemocytes is found in the peripheral hemolymph (Claes, 1996; Cowden and Curtis, 1973; Cowden and Curtis, 1981).

At least two kinds of hemocytes have been identified in bivalves and gastropods (hyalinocytes and granulocytes) according to the presence or absence of granules, which are characterized by staining affinity (Chu, 2000; López et al., 1997a; Salimi et al., 2009). Furthermore, there is an agreement about the role of hemocytes to repair damaged tissue, to

transport nutrients, in digestion, and in the internal defense against non-self material (Cheng, 1975; Chu, 2000). However, in the case of cephalopods, those 'tasks' are performed by the only type of cell present in the circulating hemolymph (Claes, 1996; Cowden and Curtis, 1981). The number and type of hemocytes in the common octopus is the subject of Chapter 2.

I.4.2. Phagocytosis of hemocytes

As in their molluscan relatives (López et al., 1997a) the cellular defense by cephalopod hemocytes involves phagocytosis (Ford, 1992; Malham et al., 1997; Malham and Runham, 1998; Rodríguez-Domínguez et al., 2006). Phagocytosis of microbial agents or any other non-self material is an important defense reaction (Cheng, 1975; Chu, 2000) that involves recognition, binding and internalization of pathogens (Fig. 7). Following non-self recognition and adhesion to the hemocyte membrane promoted by physical forces and ligand receptor interactions, hemocyte membrane invagination at the site of foreign particle adhesion results in the phagocytosis of the pathogen in a vesicle called the primary phagosome. Subsequently, the lysosomal granules move toward the primary phagosome and fuse with the membrane to form a secondary phagosome or phagolysosome. Finally, they discharge their enzymes such as phosphatases, hydrolases, esterases and amidases into the vacuole, starting the intracellular killing and digestion of the pathogen (Carballal et al., 1997, López et al., 1997b; Canesi et al., 2002; Donaghy et al., 2009b).

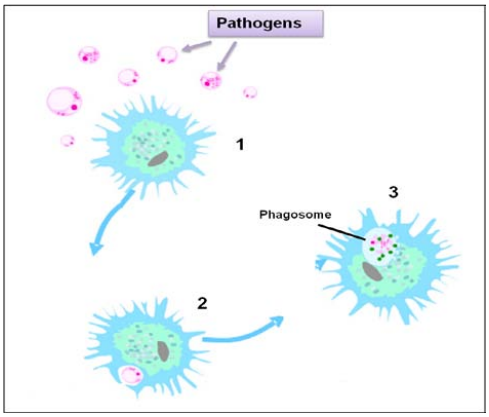


Fig. 7. Phagocytosis stages performed by hemocytes to engulf pathogens. 1: Chemotaxis, attraction and migration towards pathogens; 2: Recognition and attachment to pathogen, 3: Internalization of pathogen and subsequent destruction into phagosome through enzymatic and cytotoxic activity (Modified from Donaghy et al., 2009b).

Phagocytosis capability has been demonstrated in *Eledone cirrhosa* (Malham et al. 1997; 2002) and *O. vulgaris* (Novoa et al., 2002; Rodríguez-Domínguez et al., 2006). The hemocytes of both octopus species are able to internalize bacteria and yeast cells, respectively. However, different and not conclusive results have been recorded. Malham et al. (1997) recorded 80% of phagocytic hemocytes in *E. cirrhosa* when challenged with non opsonized *Vibrio anguillarum*. The longest exposure to the bacteria led to a higher percentage of phagocytosis; mainly if the bacteria were pre-incubated in cephalopod hemolymph free of cells at low temperature. This suggests that phagocytosis is assisted by opsonizing elements. In contrast, variable results have been recorded in *O. vulgaris*. Novoa et al. (2002) found 19% of phagocytosis in hemocytes challenged with zymosan at 18°C, whereas Rodríguez-Domínguez et al. (2006) recorded 50% of phagocytosis in hemocytes of *O. vulgaris* treated with anticoagulant buffer and challenged with zymosan. The results demonstrated that cells without the presence of an opsonic factor from hemolymph had a higher phagocytic ability; however the incubation time had no effect (Rodríguez-Domínguez et al., 2006).

When an invading organism is larger than single hemocytes to be phagocytosed, then encapsulation occurs. Under such circumstances hemocytes surround the pathogen forming various layers of cells, isolating it and limiting the potential damage (Chu, 2000), but even this defensive response is not capable of eliminating the intruder (Tripp, 1963). Encapsulation is

performed by cephalopods infected by helminthes and nematodes, due to their large size (even when in larval forms), but it is also usually observed in octopuses, where hemocytes encapsulate *Aggregata* spp. at gamogonic stages (Fig. 8) (Sardella et al., 2000; Gestal et al., 2002a).

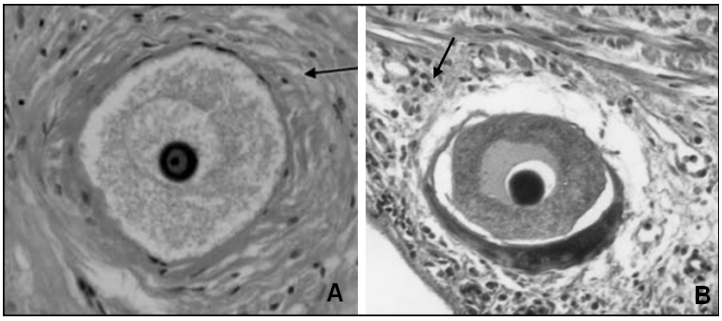


Fig. 8. Encapsulation observed in the octopus digestive tissue infected by *Aggregata* spp. (A) Encapsulation of *Aggregata octopiana* gamogonic stage in the *O. vulgaris* intestine (From: Gestal et al., 2002a). (B) Gamogonic stage of *Aggregata patagonica* infecting the intestine of *Enteroctopus megalocyathus* (From: Sardella et al., 2000). The arrow indicates infiltration of hemocytes surrounding gamogonic stage.

I.4.3. Production of Reactive oxygen species (ROS)

Destruction of pathogens through phagocytosis or under hemocyte stimulation is complemented with the production of oxidative chemicals. Most frequently, these are represented by reactive oxygen species (ROS), collectively known as respiratory burst. In these conditions, an increased uptake and consumption of oxygen and stimulation of NAPDPH oxidase occurs (Chu, 2000). The initial metabolite is superoxide anion (O_2^-), which is dismutated to hydrogen peroxide (H_2O_2), and converted to other toxic ROS such as hydroxyl radical (OH^-) and single oxygen $^1O_2$ (Fig. 9) (Buggé et al., 2007). ROS are usually measured in the cellular fraction; this allows the investigation of any change in hemocyte functionality mediated by bactericidal or pathogen activity (Ellis et al., 2011). The most common assays utilized to measure ROIs are based on nitroblue tetrazolium (NBT) reduction and luminol-dependent chemiluminiscence (LDCL). In the first case, oxygen radicals (O_2^-) can reduce yellow, water soluble, NBT to an insoluble dark blue formazan visible under the microscope or spectrophotometer after extracting formazan from the cells (Anderson, 1994; Pipe, 1992).

LDCL is used to measure the activity of myeloperoxidase/hydrogen peroxide (MPO/H_2O_2) system; Luminol generates excited aminophthalate anions that relax to the ground state with the production of light (Anderson, 1994).

Radicals are released into the extracellular medium to kill pathogenic agents. ROS production is a common defense mechanism noticed in bivalves as *Mytilus edulis* (Pipe, 1992), *Crassosstrea virginica* (Anderson, 1994), *M. galloprovincialis* (Arumugam et al., 2000) and *Mercenaria mercenaria* (Buggé et al., 2007). However, few records are available from cephalopods. Malham et al. (2002) showed that hemocytes of *E. cirrhosa* produce intracellular superoxide in response to stress. The superoxide production increases after octopus exposure to the air for five minutes, indicating that this radical is also produced by the animal in response to this kind of stress (Malham et al., 2002). By applying the reduction of ferricytochrome C, Novoa et al. (2002) measured the production of superoxide after stimulation of the circulating hemocytes and white body cells with *Escherichia coli* lipopolysacharide (LPS), zymosan and PMA. The response was obtained in the white bodies and in the circulating hemocytes using PMA and LPS, but the highest reaction was recorded when stimulated with zymosan (Novoa et al., 2002).

Currently, flow cytometry is a widely used tool to measure molluscan hemocytes immune response through the detection of fluorescence produced by each cell (Buggé et al., 2007). Flow cytometry is advantageous since it allows almost real-time measurement of the response and also to analyse the response of each cell within a big sample (Davey, 2002).

I.4.4. Production of Nitric oxide (NO)

Nitric oxide (NO) is considered part of the innate immune response and is synthesized after parasite infection (Rivero, 2006). NO results from the oxidation of L-arginine to citrulline by the enzyme nitric oxide synthase (NOS) (Fig. 9), which is present in mammals as neuronal, inducible and endothelial isoforms. NO is a signaling molecule with a physiological function in vasodilatation, secretor control, intestinal relaxation, macrophage cytotoxicity, regulation of developmental processes, neurotransmission and neuro-modulation (Jacklet, 1997). Furthermore, NO has been detected in the central nervous system of polyplacophora, gastropods and cephalopods (Palumbo, 2005). In cephalopods, nitric oxide synthase has been suggested to play a role in tactile learning (Robertson et al., 1994); the presence of NOS in the

brain of *S. officinalis* let to hypothesize the role of NO as a messenger molecule (Di Cosmo et al., 2000; Di Cristo et al., 2007). NO is a highly-reactive free radical gas that is not stored and it readily diffuses through membranes (Jacklet, 1997), therefore it is an effective agent against pathogens.

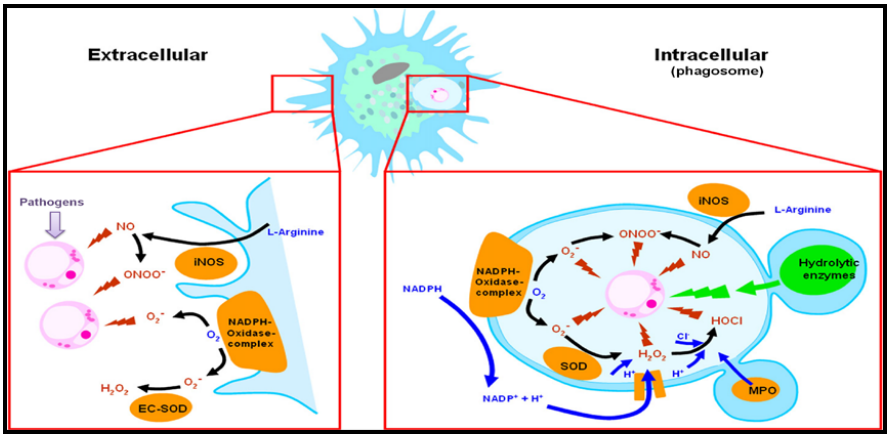


Fig. 9. Pathways involved in the production of reactive oxygen species (ROS) and nitrogen species (NO) outside the cell membrane (extracellular) and inside phagosome (intracellular) in molluscs' hemocytes. (From: Donaghy et al., 2009b).

I.4.5. Cellular factors as tools for assessing octopus health

Changes in the number, morphology, viability and functional defense mechanisms of hemocytes can be used as an indicator of the organism's health (Ellis et al., 2011) since variations have been found in relation to parasitic infections (Beckmann et al., 1992; Ford et al., 1993; Cochenec-Laureau et al., 2003; Allam et al., 2006; da Silva et al., 2008; Comesaña et al., 2012), seasonal variations (Villalba et al., 2004; Duchemin et al., 2007; Flye-Sainte-Marie et al., 2009) or contamination by toxic chemicals (Galloway and Depledge, 2001; Mayrand et al., 2005; Latire et al., 2012).

The immune parameters are widely used to assess whether the immune system is capable of performing the immune functions efficiently against parasitic infections (Chu, 2000). While parameters such as total and differential hemocyte count, phagocytic ability of

hemocytes and ROS production have been the subject of a considerable number of investigations in bivalves (Allam et al., 2006; da Silva et al., 2008; Cochenec-Laureau et al., 2003; Comesaña et al., 2012), similar studies in cephalopods have received little attention. At present, the only study considering immune parameters corresponds to Malham et al. (2002), who studied the immune changes in *E. cirrhosa* after the stress produced by exposing the octopuses to the air during 5 min. A significant decrease of the total circulating hemocyte count was observed. The hypothesis is that the reduction in the hemocyte's number is due to a migration of cells to tissues that are prone to injury or infection. With regard to functional immune assays: the phagocytic ability and intracellular superoxide production by hemocytes showed an increase during the 5 min of stress. As the octopus immune response was different from that of bivalves and gastropods, it was suggested that cephalopod immune functions may perform differently from such molluscs and could even be more complex than expected (Malham et al., 1998).

In this respect, the effect of the parasite *A. octopiana* on the octopus cellular response was studied in Chapter 3. Hence, phagocytic ability of hemocytes, ROS and NO production were measured to try to answer a question: how does the intensity of infection by the coccidia impact on the octopus defensive activities?

I.4.6. Humoral factors

Research published so far has shown that marine molluscs lack an specific immune response and immunoglobulins. Instead, they have factors with agglutinating, opsonic, lytic, antimicrobial and protease-inhibition activities present in the serum. Those factors are part of the mollusc humoral defense (Chu, 2000). Humoral factors complement the cellular activity. After the internalization of a particle or pathogen, it is enclosed in a vacuole (phagosome) where killing and destruction takes place by toxic radicals (oxygen or nitrogen) or enzymes like acid phosphatase, peroxidase, β-glucuronidase, NADH oxidase and lysozyme (Cheng, 1975; Chu, 2000).

The cephalopod cell-free hemolymph is the carrier of oxygen, which is delivered to the whole organism (Wells and Smith, 1987), but it is also the carrier of humoral components like lectins. Lectins are proteins or glycoproteins that bind specifically to carbohydrates. They cause agglutination of particles or serve as opsonins creating bridges between intruders and

immune cells (Horák and van der Knaap, 1997). A 260-kDa lectin was described in *O. vulgaris* by Rögener et al. (1985). Recently, Alpuche et al. (2010) described a new lectin of 66 kDa (OmA) found in *Octopus maya*. It was found to be a homologue to the type A hemocyanin from *Octopus dofleini*. Due to the specificity of the lectin to galactosamine, mannose and fucose, it was suggested that it could play a role in the immune response by recognizing and agglutinating oligosaccharides from cells and perhaps also from pathogens (Alpuche et al., 2010). In the presence of rat erythrocytes the hemagglutinating activity of the new OmA lectin from *O. maya* was elevated (Alpuche et al., 2010). Antibactericidal activity of sera from *O. maya* was confirmed using beef erythrocytes, but sera from other cephalopods such as the cuttlefish *S. officinalis* and the squid *Sepioteuthis lessoniana* resulted to have higher agglutinating success over a wide range of bacteria (Fisher and Dinuzzo, 1991).

Lectins are intimately related to the complement system, a potent humoral factor composed of about 30 distinct plasma soluble proteins and cell surface receptors. The complement is activated by three different pathways: classic (activated by antibody release after a humoral response), lectin (activated after the recognition and binding of pathogen associated molecular patterns [PAMPs] by lectins) and alternative (which binds to a wide range of suitable acceptor sites and spontaneously activates C3). All of these pathways converge in the central component C3 that can be cleaved into fragments that may interact with different receptors residing on different cells. A product of C3 activation is the C3b, which opsonizes the pathogen's surface, leading either to the formation of the membrane attack complex or to phagocytosis by cells (Vasta et al., 1999; Carrol et al., 2004). To date, the factor C3 has been identified and characterized in cephalopods but only in tissues from the squid *Euprymna scolopes* (Castillo et al., 2009).

Enzymes like lysozyme are also part of the mollusc defense mechanism. Its effectiveness against a broad variety of bacteria is due to the catalyzed hydrolysis of *N*-acetylmuramic acid (1-4) *N*-acetylglucosamine links of the polymeric chains in the bacterial cell wall. Lysozyme, arylsulphatase and β-glucuronidase are closely involved in digestive and defensive processes, taking an active role in the destruction of microorganisms. This group of enzymes is highly concentrated in leukocytes, neutrophilic granulocytes and macrophages (Grossowicz et al., 1979), but some of them like lysozymes have also been found in the serum of *C. virginica* (McDade and Tripp, 1967) and in hemocytes and tissue from the octopus *E. cirrhosa* (Malham et al., 1998). The lysozyme activity was higher in hemocytes of octopuses

infected by *V. anguillarum* when measured immediately after injection; activity that was reduced after 4 h and 24 h (Malham et al., 1998).

The antimicrobial peptides (AMPs) also comprise one of the main humoral components of the innate immune system. Presumably, AMPs are produced and stored in granular hemocytes. They are released into the serum after bacterial stimulation in order to destroy the pathogen (Mitta et al., 2000). AMPs have little or no functional specificity and possess a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, yeast and in some instances viruses and protozoa (Ellis et al., 2011). Although AMPs have already been studied in hemocytes and in the serum of bivalves (Mitta et al., 2000), cephalopods, among the marine invertebrates, are particularly attractive to seek potential antimicrobial drugs. Thus, the antimicrobial activity from the body tissue of cephalopods such as *Sepioteuthis lessoniana, Octopus aegina, Sepia kobiensis* (Ramasamy et al., 2011) or the cuttlebone of *Sepia aculeate, Sepia brevimana* (Shanmugam et al., 2008) or *Sepiella inermis* (Vairamani et al., 2012) have been successfully tested against important human bacteria like *Vibrio chlolerae, Salmonella sp., Escherichia coli* or *Klebsiella pneumoniae*.

Enzymes like proteases are present in the serum as well. Proteases have multiple actions, regulate the fate, localization and activity of many proteins, modulate protein-protein interactions, take part in cell proliferation and differentiation, angiogensis, neurogenesis, inflammation, immunity, necrosis and apoptosis. Thus, alterations in proteolytic systems underlie multiple pathological conditions (López-Otín and Bond, 2008). Parasites possess their own proteases, and are considered virulence factors because they may contribute to the production of disease in the host by acting as toxins or agents that over-stimulate the host immune defenses. They are used by the pathogens to combat the host defense mechanisms allowing the pathogen invasion into the host (La Peyre and Faisal, 1995, Brown and Reece, 2003). In consequence, the host produces inhibitors that are capable of inactivating the proteases involved in parasitic invasion (Armstrong, 2006). Apicomplexa parasites induce intra-membrane proteolysis via proteases to infect the cells, but also to egress from parasitic vacuole (Gorman, 2001; Sibley, 2011). The protease and inhibitory protease activity has been showed in protozoans and farmed bivalve molluscs, respectively (Faisal et al., 1998; 1999; Ordás et al., 2001; Romestand et al., 2002). In cephalopods, protease activity has been demonstrated in the hemolymph of the octopus *O. vulgaris* (Thøgersen et al., 1992) and in *E. cirrhosa* hemocytes and tissues after *V. anguillarum* challenge (Malham et al., 1998).

Phenoloxidase (PO) is an important enzyme for pigmentation and sclerotization of many tissues, but it also has an active role in innate immunity. After mechanical injuries or the presence of microorganisms and parasites, the melanin deposition around the damaged tissue or intruding pathogen takes place. Thus, the melanin will physically prevent or retard the intruder's growth. The activation of this enzyme is brought about by the proPO activating system that consists of proteins capable of binding to lipopolysaccharides. The bacteria triggers proPO activation through induction of proteolysis by native serine proteases (Cerenius and Söderhäll, 2004). The presence of proPO has been reported in the blood of a range of invertebrates (Smith and Söderhäl, 1991; Asokan et al., 1997). Thus, PO has been characterized in the ink sac of *Illex argentinus* (Naraoka et al., 2003) and *Octopus ocellatus* (Fan et al., 2009), as well as in the hemocyanin of *O. vulgaris* (Salvato et al., 1998) and *S. officinalis* (Siddiqui et al., 2006). In addition, PO activity was detected in *S. officinalis* embryo at the end of organogenesis, suggesting that PO could work in the innate defense system (Lacoue-Labarthe et al., 2009). Nonetheless, the study of the PO activity in the innate immunity of *O. vulgaris* as well as other cephalopod species is still deficient.

I.5. Study of the cephalopod host/pathogens interaction in the 'omics' era

For many pathogens of cephalopod molluscs, current diagnostic techniques are rather limited, and screening has been restricted to histological and ultrastructural examination. Protozoans and anisakids are the most studied cephalopod pathogens. Most recently, molecular techniques for detecting and identifying pathogens in cephalopods have been developed as valid and suitable tools (Mattiucci and Nasceti, 2008). They are expected to be increasingly used in pathogen monitoring programs. In addition, molecular techniques could be useful to determine whether different strains of a pathogen could demonstrate genetic and/or virulence variations (Gestal et al., 2008). However, the routine use of DNA-based diagnostic tools is hampered by a number of major concerns. Not all regions of the pathogen DNA are equally useful as targets for molecular detection, and therefore, it is necessary to identify regions of the genome that may prove useful for species differentiation.

Expressed sequence tags (ESTs) have been sequenced from non-redundant, normalized cDNA libraries and are currently used as a valuable molecular tool. They have

been successfully applied to find genes which are involved in different physiological processes (e.g. respiratory chain, cell communication, cell defense). ESTs have also been successful to determinate genes differentially expressed, mainly in bivalves of aquaculture interest naturally infected by parasites such as the clam *Ruditapes decussatus* infected by *Perkinsus olseni* (Prado-Alvarez et al., 2009a) or in those experimentally challenged with bacteria such as *R. decussatus* (Gestal et al., 2007a) or the mussel *M. galloprovincialis* (Li et al., 2010; Pallavicini et al., 2008) in order to identify potential genetic biomarkers of resistant individuals against pathogens and thus, improve the aquaculture production.

ESTs have been successfully applied to study the symbiotic relationship between the cephalopod squid *E. scolopes* and the bacteria *Vibrio fischery*. In recent years 11 cDNA libraries have been generated from light organs of a pool of juvenile sepiolids *E. scolopes* with and without the colonizing bacteria *V. fischeri*. A total of 13,962 non-redundant ESTs were characterized. 6,061 correspond to annotated ESTs; 2,793 to hypothetical ones and 874 to unknown proteins (Chun et al., 2006). The data available from the cDNA library has lead to identify genes related to the immune system such as the complement factor C3 in tissues (light organ without core, central core, mantle, arm muscle, gills and white body) of juvenile and adult squids. The lowest level of C3 transcript was detected in arm tissue and in hemocytes even though those cells are the primary site of its synthesis (Castillo et al., 2009). Transcripts encoding proteins in the Toll/NF-B pathway have also been identified. The analysis showed only one Toll-Like Receptor (TLR) that probably works as a global microbe receptor. This result suggests a similar Toll/NF-kB pathway to those present in other molluscs, but further investigation is required (Goodson et al., 2005).

Despite the highly evolved plan of cephalopods, their importance in world fisheries and neurobiology model research, cephalopod genomics is poorly understood. The knowledge of full sequence from cephalopod proteins, genes and their regulation would contribute to understand biological processes involved in the development of disease, to track population migration or to assess how climate change affects cephalopods at molecular level. Therefore, *Sepia officinalis, Loligo pealei* and *Euprymna scolopes* are the first species chosen for genome sequencing. Then, it is expected that novelty genes are discovered in the few next years providing valuable genomic information for applying in medicine, ecology or aquaculture (Albertin et al., 2012).

Nowadays, innate immunity is recognized as a complex network of interconnected pathways with activities dependent on many factors, including the pathogen virulence (Gardy et al., 2009). To study such complex network, current advances in technologies provide a catalog of high-throughput methodologies (also called Next Generation Sequencing technologies, NGS) that lead to the generation of a mass of informative data, even from non-model organisms and at low cost (Gayral et al., 2011). Hence, in the "omics" era, the fields of genomics to study DNA variations, transcriptomics for genome-wide characterization of gene expression by measuring mRNAs, proteomics to assess the cell and tissue-wide expression of proteins, and metabolomics for global assessment of metabolite concentration, have been developed (Ju et al., 2010; Prieto-Alamo et al., 2012).

Databases of "omics" comprise an essential toolbox that provides detailed molecular information and is important for integrative biology (Prieto-Alamo et al., 2012). The huge amount of data files generated ranges from gigabytes to terabytes in size. Consequently, huge amounts of nucleic acid sequences have flooded public databases. Such information must be processed by sophisticated computational methods and powerful computers (Cantacessi et al., 2012). Therefore, bioinformatics is becoming more critical about the integration of data from the systems analyzed. Hence, in the future, bioinformatics will allow the transformation of descriptive biology to a predictive science based on "omics" databases (Shinozaki and Sakakibara, 2009).

I.5.1 Transcriptomics

The transcriptome is the total RNAs produced in one cell or in a population of cells, which includes various protein-coding and non-coding RNAs. Thus, the transcriptome represents a small percentage of the genetic code that is transcribed into RNA molecules (Adams, 2008; Geng et al., 2011).

In general terms, there are two strategies for reconstructing the transcriptome. The first one is the "genome guided" approach that first maps all the transcriptome sequencing reads to the reference genome and assembles the reads into transcripts or fragments according to the read mapping information. However, this strategy needs a relatively complete and high-quality reference genome from the organism of interest, which is not available for most organisms. The second strategy is the "genome-independent" approach that

does not need a reference genome. It directly assembles reads into transcripts and therefore, is the most viable strategy for *de novo* assembling transcriptomic projects when no reference genome is available (Marguera and Bahler, 2010; Geng et al., 2011).

The commonest platforms used are 454 Life Sciences (Roche), Illumina/ (sequencing by synthesis, Solexa), AB SOLiD system (sequencing by ligation, Life Technologies), and Pacific BioSciences (RS). In the four platforms, cDNA fragments are sequenced in parallel and produce several short sequences of "reads". The read lengths range from 30-100 bp with Illumina and SOLiD, to 200-500 bp when sequenced with 454 (Marguerat and Bahler, 2010; Wheat, 2010; Cantacessi et al., 2012). Longer reads (like those obtained with 454) are preferred to reduce the complexity of the assembly. However, when short reads are used, the problem of length can be alleviated by using a paired-end protocol, in which 75-150 bp are sequenced from both ends of short cDNA fragments (100-250 bp). Then, the overlapping reads are computationally joined together to form a longer read (Martin and Wang, 2011), as it occurs with Illumina. In fact, this strategy using the paired-end protocol, is the most recommended for "*de novo*" sequencing projects. Regarding the cost and length of reads from each platform, a full 454 run produces around one million reads whereas, Illumina and SOLiD platforms produce close to 20 million reads per lane. Nonetheless, Illumina sequencing approach is considerably cheaper than 454. Thus, a trade-off between length and cost of the transcriptomic project must be evaluated (Feldmeyer et al., 2011).

Unlike the genome, the transcriptome dynamically changes in response to the environment or to intrinsic programmes. Therefore, it is a useful approach that enables the discovery of processes and pathways. Because the expression level of transcripts is related to the number of reads mapped on them, transcriptomic (also called RNA-seq method) allows the study of differentially expressed genes for several cell types or physiological conditions (Adams, 2008; Gardy et al., 2009).

To date, most *de novo* transcriptomes have been generated using 454 (Roche) technology (Vera et al., 2008; Wang et al., 2010; Zhuang et al., 2012; Hoffman et al., 2013). However, Illumina technology is becoming popular and proved to be reliable even for non-model organisms which also lack of a genome sequencing database (Crawford et al., 2010; Reid et al., 2011; Riesgo et al., 2012; Feldmeyer et al., 2011). In molluscs, *de novo* assembled transcriptomes have been employed to characterize the whole transcriptome (Hou et al., 2011), to analise gene expression (McGinty et al., 2012), for evolutionary studies (Pante et al.,

2012), to study the central nervous system (Sadamoto et al., 2012), to study the mollusc response against stress (Zhao et al., 2012), and also to investigate the immune response against pathogens (Deleury et al., 2012; Moreira et al., 2012). In contrast, despite the economic and ecologic importance of cephalopods, transcriptomic studies using cephalopods are scarce (Albertin et al., 2012). To date, transcriptomic studies have been applied to study the *O. vulgaris* central nervous system (Zhang et al., 2012) and for comparative biology and evolutionary studies of other invertebrates (Riesgo et al., 2012). Regarding the role of cephalopod hemocytes, the only available transcriptomic study has focused on the role of the sepiolid *E. scolopes* hemocytes in recognizing the symbiont bacteria *V. fischery* (Collins et al., 2012).

I.5.2 Proteomics

The full protein expressed by the genome of one organism, tissue or cell at a specific time is defined as proteome. Proteomics is the field developed to study the proteome through protein quantification, protein-protein interaction, protein function and posttranslational modifications (PTMs) (Diz et al., 2012).

The proteomes of cells are extremely complex, consisting of several thousand proteins. Two-dimensional poly-acrylamide gel electrophoresis (2-DE) has been used as the standard protein separation method. Proteins are separated according to their isoelectric point (p*I*) in the first dimension and molecular mass (M_r) in the second dimension by coupling isoelectric focusing (IEF) and sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE). Separated proteins are commonly visualized by staining with Coomassie blue dye and silver stain. Theoretically, 2-DE is capable of resolving up to 10,000 proteins simultaneously and provides information about their M_r and p*I*. Additionally, the spots of interest are excised from 2-DE gel in order to identify the protein by mass spectrometry (MS) (López, 2007). One of the major advantages of 2-DE gel is its robustness, which has been tested in inter-laboratory comparisons as well as the influence of various parameters in the intra-laboratory reproducibility. Improvements have also been made in the image production through the new fluorescent stains, colloidal Coomassie blue and modern silver staining that are also mass-spectrometry compatible. In fact, the most critical variable nowadays is the sample preparation. The IEF is very sensitive to many interfering compounds already present in

biological samples. Thus, sample preparation must be adapted to each sample type (Rabilloud et al., 2010).

In proteomics, two general strategies can be followed: 1) the bottom-up that analyses the peptides and therefore proteins are usually digested with trypsin enzyme; and 2) the top-down which analyses the proteins without any modification. Additionally, when quantitative information is needed, two main approaches can be followed: 1) relative quantification, which is usually used in 2-DE analysis. When using this approach, the protein spots are visualized through different stains facilitating their matching and quantification with the aid of specific software. Then, the intensity of normalized protein spots from different gels is used to apply statistics, and candidate protein spots (up or down regulated) are determined. On the other hand, 2) absolute quantification of proteins is usually used in Liquid Chromatography coupled with Mass Spectrometry (LC-MS). In this case, synthetic peptides of known concentration are added to the samples of interest for further quantification of the proteins (Diz et al., 2012).

Linking the information available from transcriptomics to protein expression would lead to an improved understanding of cellular processes. However, proteomic and transcriptomic data have a low convergence as some proteins detected do not show their corresponding transcript (Diz et al., 2012). The information of transcripts provides a static overview of the ways in which a cell might use its proteins, whereas the life of the cell is a dynamic process (Diz et al., 2012; Vogel and Marcotte, 2012). Hence, it is not possible to predict the number of proteins, abundance or function based on RNA transcripts (Diz et al., 2012). Some reasons for the absence of correlation have been attributed to i) the regulatory mechanisms during the gene expression like post-transcriptional mechanisms involved in turning mRNA into protein, ii) the nature of proteins may differ significantly *in vivo*, iii) technical problems measuring transcript and proteins (Greenbaum et al., 2003).

Proteomics is a powerful tool applied to study host parasitic manipulation (Biron et al., 2005a,b; Nelson et al., 2008; Lutz et al., 2011), host resistance/susceptibility against parasites (Vergote et al., 2005; Bouchut et al., 2006), tumor biomarkers (Álvarez-Chaver et al., 2011) and biomarkers of environmental pollutants (Riva et al., 2011), speciation (Martínez-Fernández et al., 2008), and differential expression of proteins against biotic or abiotic stress (Dheilly et al., 2011; Tomanek, 2011). In addition, important contributions have been made by proteomics to aquaculture, helping the industry to reach its main goal: high productivity of a better quality product. Farmed seafood organisms are susceptible to a wide range of factors

that can threaten the aquaculture industry with considerable economical repercussions. Hence, considerable efforts have been made to study the innate immune response in some aquaculture animals using comparative proteomics (Zhou et al., 2012). Thus, the changes in protein expression patterns between healthy and infected oysters *Crassostrea gigas* and three different stocks of *Ostrea edulis* were studied to find the bases of tolerance/resistance to *Bonamia ostreae* (Cao et al., 2009). To understand the immune response of crustacean hemocytes to bacteria, a proteomics approach was used to investigate the differential expression of proteins in the hemocytes of the prawn *Penaeus monodon* after *Vibrio harveyi* infection. The results showed proteins differentially expressed involved in the host defense like prophenoloxidase, serine proteinase-like or heat shock protein 90 (Somboonwiwat et al., 2010). The differential expression of proteins applied in the squid *E. scolopes* demonstrated that *V. fischeri* induced changes in the host light organ proteome after colonizing the light organ (Doino and Mc Fall-Ngai, 2000). Recently, important proteins involved in the immune defense of this squid were identified in their light organ (Schleicher and Nyholm, 2011; Collins et al., 2012). Most of these proteins have been previously identified in gastropods, bivalves and even in sea urchins, suggesting that cephalopods also possess highly conserved proteins in a wide range of marine animals. Therefore, a similar immune defense pattern could be hypothesized.

I.6 Justification and objectives

Although cephalopods are important molluscs for ecology, fisheries and aquaculture, there is still a lack of knowledge about the functional and molecular basis that regulates the cephalopod immune defense. In light of the evident importance of *O. vulgaris* for aquaculture, the present work studies the basic immune defense activities carried out by the octopus. The hemocytes are the main effectors of defensive activities therefore this work is principally focused on the cellular immune defense: phagocytosis ability, respiratory burst and nitric oxide production. In addition, this study intends to establish the first molecular bases of the octopus immune response. New technological advances were used to study the transcriptome and proteome of octopuses with low and high infection by *A. octopiana* in order to find genes and proteins that could be used as biomarkers of resistance to the coccidia infection. Hence, the aim of the present study is to contribute to the establishment of the basis for the identification of host resistant/susceptibility to the *A. octopiana* infection that in turn, will provide valuable information for achieving a successful octopus aquaculture.

The present dissertation has the following objectives:

1. The molecular characterization of the coccidia *Aggregata octopiana* infecting the common octopus in NE Atlantic Ocean.

2. To characterize at morpho-functional level the *Octopus vulgaris* hemocytes.

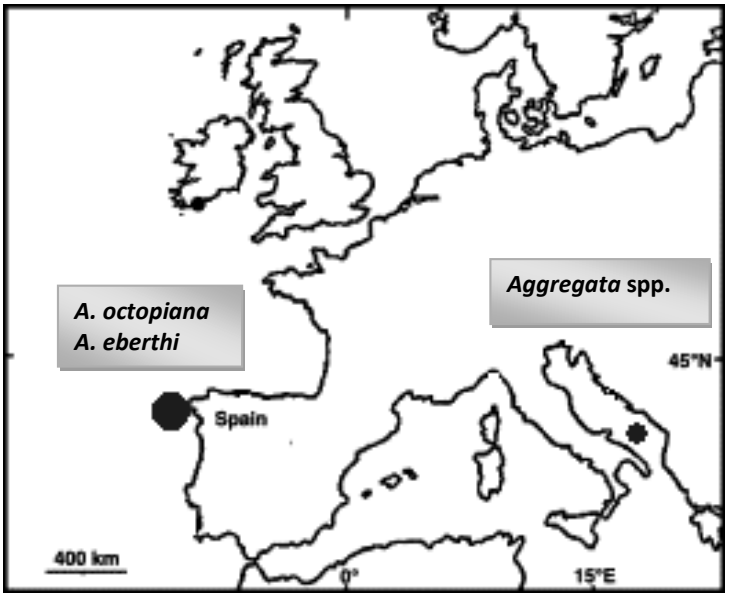
3. To study the effect of the parasite *Aggregata octopiana* on the cellular response of the common octopus, *Octopus vulgaris*.

4. To perform a transcriptome analysis of the *Octopus vulgaris* hemocytes using high-throughput sequencing technology. The identification of genes differentially expressed in octopuses infected by *Aggregata octopiana.*

5. To characterize the protein profile of the hemolymph of *Octopus vulgaris*. The analysis of proteins differentially expressed in octopuses infected by *Aggregata octopiana*.

Chapter 1

Molecular phylogenetic analysis of the coccidian cephalopod parasites *Aggregata octopiana* and *Aggregata eberthi* (Apicomplexa: Aggregatidae) from the NE Atlantic coast using 18S rRNA sequences



Abstract

The coccidia genus *Aggregata* is responsible for intestinal coccidiosis in wild and cultivated cephalopods. Two coccidia species, *Aggregata octopiana*, (infecting the common octopus *Octopus vulgaris*), and *A. eberthi*, (infecting the cuttlefish Sepia officinalis), are identified in European waters. Extensive investigation of their morphology resulted in a redescription of *A. octopiana* in octopuses from the NE Atlantic Coast (NW Spain) thus clarifying confusing descriptions recorded in the past. The present study sequenced the 18S rRNA gene in *A. octopiana* and *A. eberthi* from the NE Atlantic coast in order to assess their taxonomic and phylogenetic status. Phylogenetic analyses revealed conspecific genetic differences (2.5%) in 18S rRNA sequences between *A. eberthi* from the Ria of Vigo (NW Spain) and the Adriatic Sea. Larger congeneric differences (15.9%) were observed between *A. octopiana* samples from the same two areas, which suggest the existence of two species. Based on previous morphological evidence, host specificity data, and new molecular phylogenetic analyses, we suggest that *A. octopiana* from the Ria of Vigo is the valid type species.

1. Introduction

Coccidians are obligate intracellular parasites that cause severe injuriesmainly in poultry and livestock (Levine 1985), but are also able to infect marine fishes and molluscs causing a detrimental effect on their physiological condition (Kent and Hedrick 1985; Lom and Dyková 1992). Cephalopods are specifically infected by coccidians of the genus Aggregata (Hochberg, 1990), which are heteroxenous parasites transmitted through the food web. Sexual stages (gamogony and sporogony) occur inside the digestive tract of the definitive cephalopod host, whereas asexual stages (merogony) can be found inside the digestive tract of the intermediate crustacean host (Hochberg 1990).

The genus *Aggregata* has a complex taxonomic history. It was first described by Lieberkuhn (1854) as a gregarine infecting *Sepia officinalis*. Schneider (1875) described a similar parasite infecting *Octopus vulgaris*, whereas the later genus was correctly classified as a coccidium (Schneider, 1883). Then, the genus *Aggregata* was assigned by Frenzel (1885), who described merogonic stages of the parasite in *Portunus arcuatus.* Finally, the cephalopod coccidia were classified into the family Aggregatidae by Labbé (1899). The taxonomy of the *Aggregata* species has been controversial (Hochberg, 1990), and confusing descriptions have been recorded in the past. The species *Aggregata octopiana* was first described by Schneider (1875) in *O. vulgaris* from the English Channel and Western Mediterranean Sea (Banyuls sur-Mer, France), and redescribed in samples from the NE Atlantic Ocean (Gestal et al., 1999b). Comparative ultrastructural studies revealed that the taxon described by others as *Aggregata spinosa* in the same host and locations using light microscopy (Moroff, 1908), was synonymous to *A. octopiana* (Gestal et al., 1999b). Consequently, 10 *Aggregata* species have been described to date (see Table 1), and three of them are found in European waters: (1) *A. eberthi*, which is the representative type-species of the genus *Aggregata* and infects the cuttlefish *S. officinalis* from the Mediterranean Sea, English Channel and NE Atlantic Ocean (Dobell, 1925); (2) *A octopiana*, which infects the common octopus *O. vulgaris* and has been re-described in hosts from the NE Atlantic Ocean (Gestal et al., 1999b); and (3) *A. sagittata*, which infects the flying squid *Todarodes sagittatus* (Gestal et al., 2000).

Understanding cephalopod pathogens is particularly relevant to the worldwide aquaculture of octopus species, which has to satisfy the global demand of cephalopods for human consumption (Iglesias et al., 2004; Domingues et al. 2007; Solorzano et al., 2009). The coccidian *A. octopiana* is known to cause heavy infections in the digestive tract of *O. vulgaris* (Pascual et al., 1996). Gamogonic and sporogonic stages cause the host's digestive

tissue to rupture (Gestal et al., 2002a).Malabsorption syndrome is a secondary effect of high infection rates, reducing the growth and condition of infected octopuses (Gestal et al., 2002b) and negatively impacting octopus culture (Gestal et al., 2007b). Moreover, food sanitary regulations forbid commercialization of parasitized fishery and aquaculture products. Hence, despite *Aggregata* spp. not being zoonotic parasites, the octopus will be withdrawn from human consumption circulation as soon as oocysts are detected in muscle (Peñalver et al., 2008).

Due to the increasing importance of coccidian diseases, particularly those caused by *Aggregata* species, the use of highly sensitive molecular methods for parasite diagnosis becomes crucial. Furthermore, molecular approaches are also useful to characterize parasites, complementing morphological descriptions, and phylogenetic classification (Jirků et al., 2009; Rueckert et al., 2011). The species *A. octopiana* and *A. eberthi* have been identified and characterized in the NE Atlantic coast according tomorphological characters and host specificity (Gestal et al., 1999b, 2002c; Gestal and Pascual, 2002). In contrast, very little is known about their molecular classification and phylogenetic position, which could confirm their taxonomic affiliation within the genus and validate conservative and robust phenotypic characters used for species diagnosis Kopečná et al. (2006) generated the first 18S rRNA sequences for *A. octopiana* and *A. eberthi* from Croatia (Adriatic Sea); however, the phylogenetic position of both coccidians remained unresolved.

In this study, we generated new 18S rRNA nucleotide sequences for *A. octopiana* and *A. eberthi* from the NE Atlantic coast (Galicia, NW Spain) to assess their phylogenetic position, complement existing morphologic descriptions and validate their phenotypic characters.

Table 1. *Aggregata* species recorded from cephalopod hosts. Length and width measurements are given as ranges in µm (- denotes no data available).

***Aggregata* species**	**Host**	**Locality (Ocean/Sea)**	**Sporocysts**			**Sporozoites**		**References**
			length	**width**	**cyst wall**	**nº**	**length**	
octopiana	*Octopus vulgaris*	NE Atlantic, W Mediterranean	11-15	11-15	spiny	8	16-24	Gestal et al. (1999b) Schneider (1875)
Aggregata sp.	*O. vulgaris*	E Mediterranean (Adriatic Sea)	-	-	-	4-5 8	-	Mladineo and Jozić (2005) Mladineo and Bočina (2007)
dobelli	*Enteroctopus dofleini*	NE Pacific	18-31	15-27	smooth	9-22	18-23	Poynton et al. (1992)
millerorum	*O. bimaculoides*	NE Pacific	12-20	11-17	smooth	8-10	18-31	Poynton et al. (1992)
patagonica	*E. megalocyatus*	SW Atlantic	13	12	smooth	8	18	Sardella et al. (2000)
valdesensis	*O. tehuelchus*	SW Atlantic	10	10	-	4-8	17	Sardella et al. (2000)
bathytherma	*Vulcanoctopus hydrothermalis*	NE Pacific	27-32	24-32	smooth; thick	14-17	49	Gestal et al. (2010)
sagittata	*Todarodes sagittatus*	NE Atlantic	17	15	smooth; thick	4-8	12	Gestal et al. (2000)
andresi	*Martialia hyadesi*	SW Atlantic	9.7	8.2	smooth; thick	3	16-20	Gestal et al. (2005)
eberthi	*Sepia officinalis*	NE Atlantic, W Mediterranean	8-9	-	smooth	3	15-17	Labbé (1895)
kudoi	*S. elliptica*	NW Indian	9-14	-	smooth	6-12	16-18	Narasimhamurti (1979)

2. Material and Methods

2.1 Sampling and microscopic identification

A. octopiana was isolated from a pool of 10 infected octopuses of the species *O. vulgaris*, while *Aggregata eberthi* was isolated from a pool of 10 infected cuttlefishes of the species *S. officinalis*. Both cephalopod species were collected by traps, an artisanal gear used by local fishermen from the Ria of Vigo, Spain (24°14.09″N, 8°47.18″ W). The oocysts are easily observed as white spots on the digestive tract. Thus in the laboratory, the presence of *Aggregata* was assessed macroscopically in each of the cephalopod hosts, white oocysts were extracted from fresh caecum and intestine. Coccidians were identified using light microscopy and scanning electronmicroscopy (SEM) to analyzemorphology and dimensions of the fresh sporocysts and by histological analysis of the caecum, which is the target organ of the infection. The infected tissue was fixed in Davidson, embedded in paraffinwax and sectioned using aMicromHM-340 Emicrotome. Sections at 4 μm were stained with H–E according to standard procedures (Humason, 1979). For scanning electron microscopy (SEM), purified oocyst suspensionwas fixed for 4 h in 2.5% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4) at 4° C and washed for 30min in the same buffer. After dehydration in ethanol series, samples were critical point-dried in CO_2 using a Polaron E3000 and sputter-coated in a Polaron SC500 using 60% gold–palladium. Analysis was performed with a Philips XC30 SEM operated at 10–20 kV.

2.2 Isolation and purification of the parasite

The infected digestive tract of cephalopods was dissected and homogenised in 10ml of filtered sea water (FSW) 1% Tween 80 using an electric tissue grinder (IKA-Ultra Turrax T-25). Tissue homogenates were filtered twice with nylon meshes of 100 and 41 μm, respectively, to remove tissue fragments. The filtrate was then centrifuged at 1000×*g* for 5min in a centrifuge Beckman GS-15R. The sporocysts were purified by means of 142 the density gradient centrifugation method according to Gestal et al. (1999a), and counted in a Neubauer chamber to standardise the sample at 2×10^6 sporocysts/ml. Finally, sporocysts were preserved in 70% ethanol.

2.3 DNA extraction

Genomic DNA was extracted from *A. octopiana* and *A. eberthi* sporocysts. Sporocysts were resuspended in 500 µl of extraction buffer (NaCl 100 mM, EDTA 25 mM pH 8, SDS 0.5%) and opened by sonication on ice (5 cycles, 40W, 50 s) to release sporozoites. After Proteinase K (Sigma) digestion (1 mg ml−1) at 37 °C overnight, the DNA was purified following the phenol:chloroform:isoamil alcohol extraction method, as described by Sambrook et al. (1989). DNA was precipitated with ethanol and sodium acetate overnight at −20 °C. The precipitated pellet was resuspended in 50 µl of Tris–EDTA (TE) buffer.

2.4 DNA amplification, cloning and sequencing

The small subunit 18S rRNA gene of both coccidia species was amplified by PCR using conserved primers designed for *Aggregata* spp. (Kopečná et al., 2006) and derived from GenBank sequences: (Aggregata 1-F: 5'-ATGATGAAACTGCGAAGAGC-3'; Aggregata 2-R: 5'-CGACGGTATCTGATCGTCTT-3'; Aggregata 3-F: 5'-GGGGGTATTTGTATTTAACAAGCA-3'; Aggregata 4-R: 5'- CCTACGGAAACCTTGTTACGA-3'). Aggregata primers 1–2 (positions 76–1008) amplify the initial 970 bp of the 18S rRNA gene, whereas Aggregata primers 3–4 (positions 871–1781) amplify the next 915 bp. PCR reactions were performed in a total volume of 25µl containing 1µl 10 mM dNTP mix, 0.25 µl *Taq* DNA polymerase (Roche), 2.5µl *Taq* 10× buffer, 1µl 2.5 mM MgCl2, 1µl of each primer (10 µM) and 1µl of template DNA at 100 ng μl^{-1}. The temperature profile for primers 1–2 included an initial denaturation at 94 °C for 10 min; 35 cycles of 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min, and a final extension at 72 ◦C for 10 min. For primers 3–4, we used an annealing temperature of 55 °C. PCR products were separated on 1% agarose in TAE 1× buffer gels (w/v), stained with ethidium bromide including a 100-bp ladder size standard (Invitrogen) and visualised using ultraviolet (UV) light. Fresh PCR products were cloned using a TOPO TA Cloning Kit (invitrogen) according to the protocol supplied by the manufacturers and transformed in TOP 10 F_competent bacteria *Escherichia coli* (invitrogen). Screening of clones carrying 18S rRNA-coding region fragments was performed by PCR adding the positive colony directly to the PCR mixture reaction using the corresponding Aggregata primers. Positive clones were purified by digestion with the enzymes exonuclease I and shrimp phosphatase (SAP) (Amersham Pharmacia Biothech) for 1 h at 37 °C. The enzymes were then denatured for 15 min at 80 °C. The purified PCR products were bi-directionally sequenced using the proper *Aggregata* pair of primers and

using ABI 3130 Genetic Analyzer according to the manufacturer's directions (Applied Biosystems, Carlsbad, CA, USA).

Sequenced fragments from multiple clones belonging to each of the two *Aggregrata* species were then assembled together into two consensus sequences (see below). Based on the obtained cloned sequences, the specific primers RV-F: 5'-GCTTATTAAATCAGTTATAGTT-3'204; RV-R: 5'-ATATTTACACACATTCTAATTC-3' (positions 20–1619) were designed and used to amplify almost complete 18S rRNA sequences for each species (annealing temperature of 54 °C). Primers Aggregata 5-F:

5'-AAGCTCGTAGTTGCAGTTTTGA-3'; Aggregata 6-R: 5'-AACTAAGAACGGCCATGCAC-3' (positions 544–1178) equivalent to 662 bp were designed to amplify the internal sequence at an annealing temperature of 54 °C. All sites available in these new two sequences were also present in the two consensus sequences assembled from multiple Aggregata clones.

2.5 Phylogenetic analysis

In addition to the new 18S rRNA sequences generated in this study for *A. octopiana* and *A. eberthi* from Ria of Vigo, sequences of 33 Apicomplexa taxa available at GenBank were used in the phylogenetic reconstruction. The GenBank accession numbers of the18S rRNA gene sequences used are as follows: *Theileria buffeli* (AF236097), *Theileria* sp. (U97055), *Babesia* sp. (AY048113), *Babesia conradae* (AF158702), *Eimeria alabamensis* (AF291427), *Eimeria bovis* (U77084), *Eimeria falciformis* (AF080614), *Eimeria arnyi* (AY613853), *Cyclospora cayetanensis* (AF111183), *Cyclospora papionis* (AF111187), *Cyclospora colobi* (AF111186), *Isospora belli* (U94787), *Isospora felis* (L76471), *Goussia janae* (AY043206), *Goussia carpelli* (GU479640), *Goussia metchnikovi* (FJ009244), *Sarcocystis gracilis* (FJ196261), *Sarcocystis neurona* (U07812), *Toxoplasma gondii* (L37415), *Neospora caninum* (GQ899206), *Neospora* sp.(BPA1 U17345), *Hepatozoon canis* (EF622096), *Hepatozoon catesbianae* (AF130361), *Calyptospora spinosa* (FJ904637), *Calyptospora funduli* (FJ904645), *Adelina grylli* (DQ096836), *Adelina bambarooniae* (AF494059), *Adelina dimidiata* (DQ096835), *Tridacna* hemolymph apicomplexan (AB000912), *Klossia helicina* (HQ224955) clon 43, *K. helicina* (HQ224956) clone 26, *A. octopiana* from the Adriatic Sea (DQ096837), and *A. eberthi* from the Adriatic Sea (DQ096838). Representative species of *Babesia* and *Theileria* were used as outgroups.

All sequences were aligned in MAFFT v6 (Katoh et al. 2005; Katoh 2008) under the Q-INS-i algorithm, which takes into account RNA secondary structure. Ambiguous regions in the resulting alignment were identified and removed using GBlocks 0.91b (Castresana 2000). *Aggregata* phylogenetic relationships were inferred using maximum likelihood (ML) and Bayesian inference coupled with Markov chain Monte Carlo (BMCMC). ML trees were built in RAxML v7.2.0 (Stamatakis et al., 2008) using 1,000 searches and 10 runs. JModelTest v1.0.1 (Posada, 2009) was used to select the appropriate model of evolution under the Akaike Information Criterion (Posada and Buckley, 2004). The general time reversible (GTR) model (Tavaré, 1986), with invariable sites (I = 0.13) and gamma distribution (G= 0.63) to account for the among site rate heterogeneity was chosen. Clade support was assessed using the non-parametric bootstrap procedure (Felsenstein, 1985) with 5,000 bootstrap replicates run in the portal CIPRES Science Gateway portal (Miller et al., 2010). BMCMC trees were built in MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). Three independent BMCMC analyses were run in CIPRES with each consisting of four chains. Each Markov chain was started from a random tree and run for 5×106 cycles, sampling every 1000th generation. Model parameters were unlinked and treated as unknown variables with uniform default priors. They were estimated as part of the analysis. Convergence and mixing were monitored using Tracer v1.5 (Rambaut and Drummond, 2009). All sample points prior to reaching stationary were discarded as burn-in. The posterior probabilities for individual clade obtained from separate analyses were compared for congruence and then combined and summarized on a 50% majority-rule consensus tree.

3. Results

Phenotypic identification of both *A. octopiana* and *A. eberthi* was performed by light microscopy, histology and SEM (Fig. 1). The morphed as part of the analysed oocysts, sporocysts and sporozoites was consistent with that previously described as the type species from the NE Atlantic (Dobell, 1925; Gestal et al., 1999b) (see Table 1). A total of 13 and nine 18S rRNA partial sequence clones of *A. octopiana* and *A. eberthi*, respectively, were sequenced and assembled to obtain two overlapping 50% majority-rule consensus partial 18S DNA sequences of 1624 bp for *A. octopiana* and 1686 bp for *A. eberthi*. Variation among *A. octopiana* clones was <0.55%, whereas variation among *A. eberthi* clones was <0.25%. In addition, single sequences of similar length taken in one single PCR amplification were taken for each species in order to confirm the assembled fragments.

We used the consensus sequences in all phylogenetic analyses to take into account intra-species genetic variation. The consensus sequences of *A. octopiana* and *A.eberthi* were deposited with GenBank under the accession numbers KC188342 and KC188343, respectively.

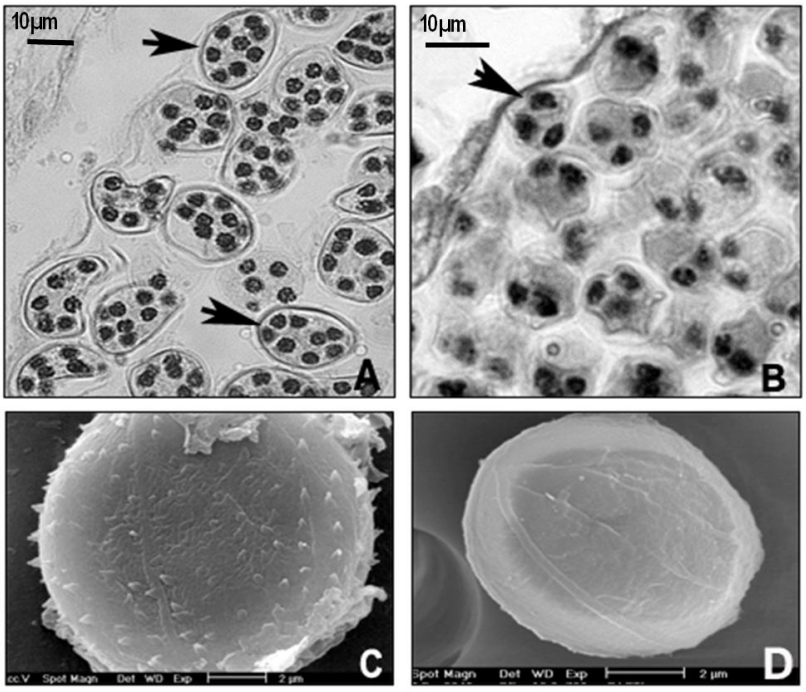


Fig. 1. Morphology of *A. octopiana* and *A. eberthi*. (A) Histological section of the digestive tract of *O. vulgaris* showing sporocyst of *A. octopiana* containing 8 sporozoites. (B) Histological section of the digestive tract of *S. officinalis* showing sporocyst of *A. eberthi* containing 3 sporozoites. (C) SEM photograph of *A. octopiana* sporocyst showing the spiny wall. (D) SEM photograph of *A. eberthi* showing the smooth sporocyst wall.

ML and BMCMC phylogenetic searches generated identical topologies, hence only the ML tree with corrected branch lengths is presented (Fig. 2). In our analysis, two main coccidian clades were recognised, and one of them, the adeleorinids clade, included *A. octopiana* and *A. eberthi* (Fig. 2). The aggregatids from the Ria of Vigo and the Adriatic Sea formed a highly supported monophyletic group [(bootstrap proportion (bp) = 100%, posterior probability (pP) = 1.0)].

In our 18SrRNAMLtree, the minimum genetic divergence (corrected branch lengths) observed between different recognized coccidian species pairs ranged from 0.1%

to 15.1%, with most cases above 3%. A genetic divergence of 15.9% was observed between *A. octopiana* from the Ria of Vigo and *A. octopiana* from the Adriatic Sea, whereas a genetic divergence of only 2.4% was found between *A. eberthi* from the Ria of Vigo and *A. eberthi* from the Adriatic Sea (Fig. 2).

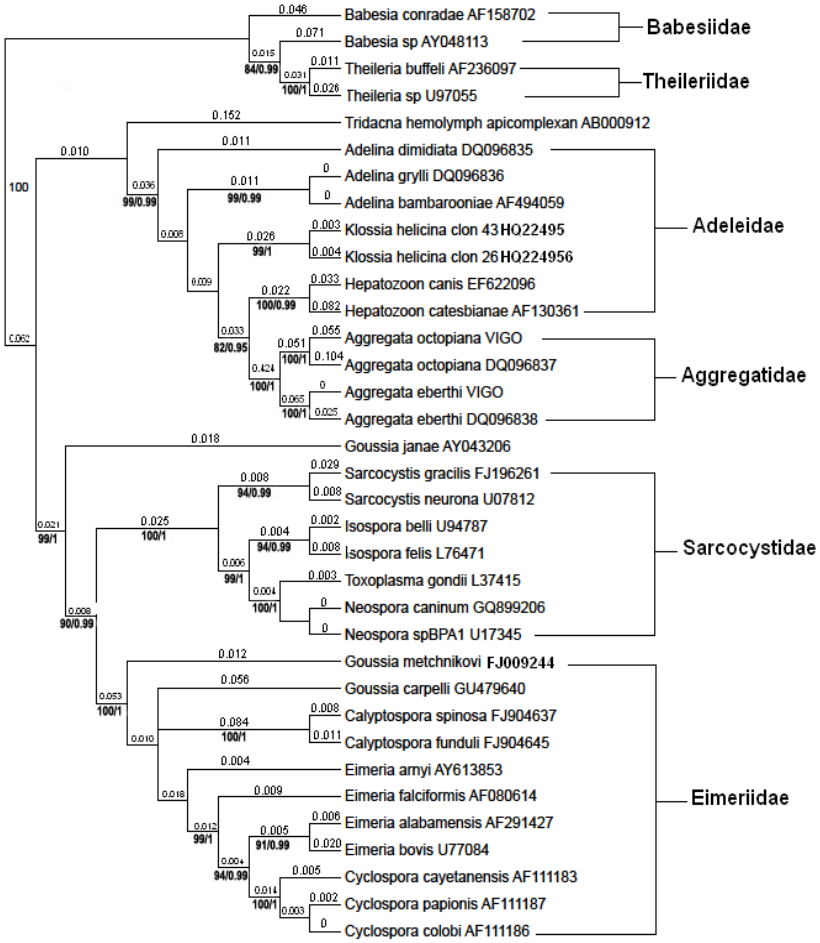


Fig. 2. Maximum likelihood cladogram of Apicomplexa evolutionary relationship. Corrected branch lengths estimated under the GTR+G+I evolutionary model are shown above branches and bootstrap proportions (if ≥70%)/posterior probability (if ≥0.95) are shown in bold below branches.

4. Discussion

According to their histological and ultrastructural features, *A. octopiana* and *A. eberthi*, have been successfully characterized in samples from the NE Atlantic Ocean by Gestal et al. (1999b, 2002c) and Gestal and Pascual (2002). Now, the molecular characterization carried out in this work showed a broad similarity to the two *Aggregata* sequences available at GenBank. Our phylogenetic analyses were consistent with what is known from previous coccidian studies, where Eimeriidae and Sarcocystidae families formed well supportedmonophyletic groups (bp = 100%; pP = 1.00). Both ML and Bayesian phylogenies strongly support (bp = 99%, pP = 0.99) a clade formed by Aggregatidae and Adeleidae species. Within this clade, *Aggregata* species are evolutionarily close to the adeleorinid *Hepatozoon*, *Klossia* and *Adelina*, the last being the most basal group, as also suggested by the ML tree in Kopečná et al. (2006). This makes adeleorinids the most primitive group of the Eucoccidiorida, as stated by Levine (1985), sharing with aggregatids the formation of the sporocyst and the excystation through a longitudinal suture (Gestal et al., 1999b; Kopečná et al., 2006). As in Kopečná et al. (2006), however, our 18S rRNA tree cannot accurately discriminate the basal relationships and position of the genus *Aggregata*. As previously suggested for other Apicomplexa (Barta et al., 2012), additional taxa and new genetic markers will be required to resolve the relationships among these parasites.

Our tree shows that *A. octopiana* and *A. eberthi* from the Ria of Vigo cluster with their respective counterparts *A. octopiana* and *A. eberthi* from the Adriatic Sea (Fig. 2); however, the high genetic divergence (15.9%) observed between the two *A. octopiana* samples suggests that they represent different species (congeneric divergence). On the contrary, the genetic divergence estimated between *A. eberthi* samples (2.5%) falls within the range observed among populations from the same species (conspecific divergence).

Coupled with molecular data, phenotypic characters are also required to classify coccidians (Tenter et al., 2002). Among them, one of the most conspicuous characters is the number of sporozoites per sporocyst (Lom and Dyková, 1992). From the Adriatic Sea, scarce and confusing records about the sporozoite number of coccidians infecting cephalopods exist. Mladineo and Jozić (2005), for example, reported *O. vulgaris* infected by coccidian of the genus *Aggregata* with four to five sporozoites. The Adriatic coccidia fit with the usual size range of A. octopiana from the NE Atlantic (Gestal et al., 1999b). There are differences, however, with regard to the number of sporozoites (eight sporozoites per sporocyst for *A. octopiana*), and the spiny sporocyst wall, which are the most noticeable

specific features. Based on the number of sporozoites, the *Aggregata* sp. from Adriatic Sea resembles *A. sagittata*, which infects only the squid Todarodes sagitattus (Gestal et al., 2000), or *A. valdesensis*, which infects *O. tehuelchus* in SW Atlantic (Sardella et al., 2000). Interestingly, a second record by Mladineo and Bočina (2007) also mentions coccidia with eight sporozoites infecting the Adriatic *O. vulgaris*, which suggests the presence of *A. octopiana*. Thus, following morphologic characters, these records suggest two different *Aggregata* species infecting *O. vulgaris* in the Adriatic Sea. In addition, the absence of consistent and reliable morphological information about the coccidia sequenced by Kopečná et al. (2006) makes it difficult to identify the Adriatic *Aggregata* sp. correctly. Therefore, detailed morphological characterisation and accurate identification of the *Aggregata* species occurring in the Adriatic Sea are needed.

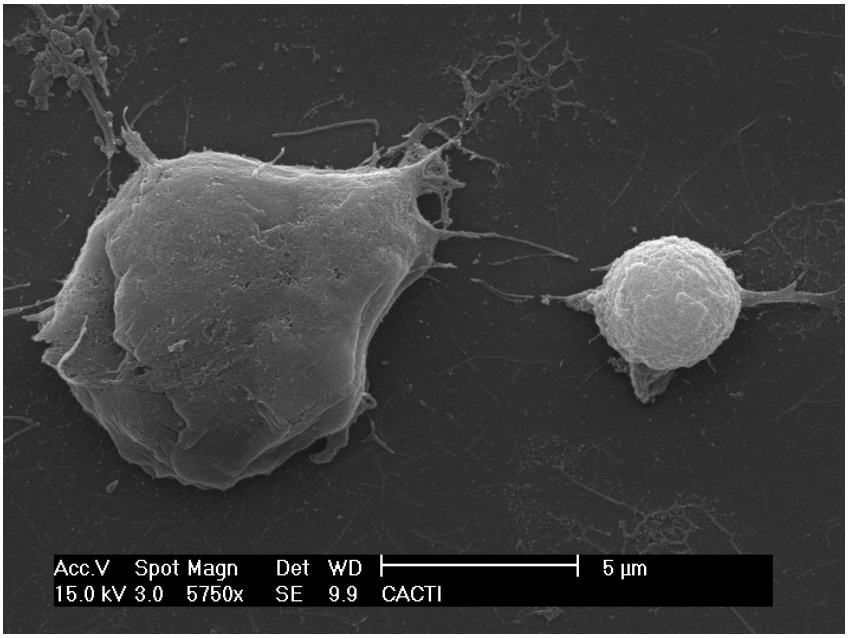
O. vulgaris has a worldwide distribution including the Southern Indian Ocean (Roper et al. 1984; Guerra et al. 2010). This octopod is now considered to form different populations with differences in reproductive structures and parasite specificity (Mangold 1998; Guerra pers. comm.). Because coccidia are host-specific parasites, the distinct number of sporozoites in coccidia recorded from the Adriatic Sea (Mladineo and Jozić, 2005; Mladineo and Bočina, 2007) suggests the possibility of different octopus populations harboring different *Aggregata* parasites.

Therefore, based on previous morphological evidence (Gestal et al., 1999b; Gestal and Pascual, 2002; Gestal et al., 2002c), host-specificity data and the new molecular phylogenetic analyses presented herein, we conclude that the *Aggregata* species parasitising the common octopus *O. vulgaris* from the Ria of Vigo (NW Spain, NE Atlantic) is *A. octopiana*, the valid type species. We also confirm the identification of *A. eberthi* infecting the cuttlefish *S. officinalis* from the same locality, validating the known phenotypic characters as useful diagnostic tools.

Further effort is needed to sample cephalopod hosts harboring *Aggregata* species at different geographic locations in the NE Atlantic and worldwide. Moreover, new genetic markers need to be combined with the 18S rRNA gene to improve phylogenetic analysis and complement the morphological taxonomy and classification of this poorly understood coccidian group.

Chapter 2

Morphologic, cytometric and functional characterization of the common octopus (*Octopus vulgaris*) hemocytes



Two different morphotypes of hemocytes observed in the *O. vulgaris* hemolymph.

Abstract

Mollusc hemocytes are responsible for several immunological functions including killing and eradication of microorganism. Therefore, characterization of the circulating hemocytes and their immune activities are required to assess the octopus health. In the present work, the hemocytes of *Octopus vulgaris* were morphologically and functionally characterized. Light microscopy, electron microscopy (TEM and SEM) and flow cytometry analyses revealed the existence of two hemocyte populations. Large granulocytes showed U-shaped nucleus, numerous granules in the cytoplasm and measured a mean of 11.6 μm ± 1.2 in diameter. Stained hemocytes showed basophilic granular inclusions and some vacuoles. Small granulocytes showed a mean of 8.12 μm ± 0.74 in diameter, a round nucleus occupying almost the entire cell and few or not granules in the cytoplasm. Large granulocyte presented different types of granules by TEM. In addition, histochemical stains showed glycogen deposits and lysosomic activity. Phagocytosis and the associated respiratory burst were measured by flow cytometry. Large granulocytes are the principal cells that develop both phagocytosis and ROS. Octopus hemocytes showed high variable ability to engulf latex beads, rising up to 56% after 2 h of incubation. Zymosan induced effectively higher ROS production than controls. Nitric oxide (NO) was measured in hemocytes against different stimuli during a time curse. Zymosan induced the highest NO production, following by PMA and LPS. The maximum response was recorded at 3 h of incubation. This study is the first tread towards understanding the *O. vulgaris* immune system by applying new tools to provide a most comprehensive morpho-functional study of their hemocytes.

1. Introduction

Cephalopods are molluscs with a close circulatory system, where the hemolymph is restricted to blood vessels and capillaries (Cowden and Curtis, 1981). Molluscs hemocytes, the circulating cells of the hemolymph, are involved in several functions such as wound repair, nutrient digestion, transport and excretion. In addition, they play an important role in the internal defense (Cheng, 1975; Chu 2000). Although studies focused on bivalve and gastropod mollusc's diseases and defense mechanisms have been performed, little attention has been placed on the defense mechanism of cephalopods.

Cephalopods are the subject of important fisheries and biomedical studies, serving as host for parasites like *Anisakis* sp. that can infect humans (Hochberg, 1990). In addition they have a great potential as aquaculture species (Iglesias et al., 2007). However, the characterization and functionality of these mollusc hemocytes has not been fully studied (Ford, 1992). The general agreement differentiates two types of cells in bivalves and gastropods, granulocytes and hyalinocytes, according to the presence of cytoplasmic inclusions (Cheng, 1975; Donaghy et al., 2010). In contrast, available studies performed by classical microscopic techniques recognize a single cell type in cephalopods. These cells are round shaped, with several cytoplasmic inclusions and U-shaped nucleus resembling mammalian monocytes (Cowden and Curtis, 1981; Malham and Runham, 1998). The leukopoietic organ of cephalopods is certainly known as the white body, located behind the eyes in the orbital pits of the cranial cartilages (Cowden, 1972). Studies performed by light and electron microscopy on *Octopus vulgaris* (Cowden, 1972), *Octopus briareus* (Cowden and Curtis, 1973) and *Sepia officinalis* (Bolognari, 1949; Claes, 1996) described the morphology of precursor stages of cells developed in the white body before maturation and release to the circulating hemolymph.

The non-adaptive immune system of invertebrates is composed by cellular and humoral components, which in a combined action control the infection by pathogens. Among these mechanism, phagocytosis, encapsulation, and production of toxic radicals of oxygen and nitrogen are developed to combat pathogens (Ellis et al., 2011).

Up to date, few studies have been conducted to identify the hemocyte function in cephalopods (Ford, 1992). Cephalopods' hemocytes phagocytosis of the bacteria *Vibrio anguillarum* and zimosan A was assayed using traditional light microscopy techniques in *Eledone cirrhosa* and *Octopus vulgaris*, respectively (Malham et al., 1997; Novoa et al., 2002; Rodríguez-Domínguez et al., 2006). Linked to phagocytosis is the release of oxidative chemicals like reactive oxygen species (ROS), named respiratory burst; and

reactive nitrogen species (RNS) like nitric oxide (NO), both acting as killing agents (Tiscar and Mosca, 2004).

Conventional methods applied to bivalves for measuring ROS, as reduction of nitroblue tetrazolium, that measures intracellular radicals (Anderson et al., 1994) or cytrochrome-C to measure extracellular oxygen radicals (Wootton et al., 2003) have been also used in cephalopods. Intracellular ROS was measured in *E. cirrhosa* hemocytes after exposure the octopuses to the air for 5 minutes (Malham et al., 2002). In *O. vulgaris*, extracellular ROS was observed in hemocytes following stimulation with zymosan. The same stimuli was effective to induce significant NO production, whereas *Escherichia coli* lypopolysacharide (LPS) induced a weak reaction (Novoa et al., 2002).

Differences in methods used for hemocytes classification or measurement techniques to study the molluscan immune response could rise to disparities in the results. Therefore, automated methods that can remove that bias are valuable for further research in the field (Ashton-Alcox and Ford, 1998). Flow cytometry is used to compliment time consuming techniques based on physical separation of cells. This methodology provides information about different characteristics including their size and granularity, very valuable parameters to characterize cell populations and has been widely used to characterize bivalve and gastropod hemocyte populations (Ashton-Alcox and Ford, 1998; Allam et al., 2002). In addition, flow cytometry have been used to measure cellular defense parameters as phagocytosis, respiratory burst and production of nitric oxide simultaneously, in *Mytilus galloprovincialis* (García-García et al., 2008), *Haliotis tuberculata* (Travers et al., 2008), *Crassostrea ariakiensis* (Donaghy et al., 2009a), *Haliotis discus discus, Turbo cornutus* (Donaghy et al., 2010) and *Ruditapes decussatus* (Prado-Alvarez et al., 2012).

O. vulgaris is an important mollusc for commercial fisheries worldwide and aquaculture. Therefore, understanding the immune system of this cephalopod is required to assess the effect of environmental factors and natural pathogens, which will help in the future to prevent economical looses in aquaculture. The purpose of the present study is the morphological and functional characterization of the circulating hemocytes of *O. vulgaris* by light and electron microscopy complimented for first time with flow cytometry.

2. Material and methods

2.1 Biological material and hemolymph collection

O. vulgaris from 65-220 mm mantle length were collected by traps, an artisanal gear used by local fishermen, from the Ria of Vigo, Spain (24° 14.09'N, 8° 47.18'W) and transported to the laboratory. Octopuses were placed in culture tanks of open seawater system at 15 °C for 24 h to acclimate before experimentation.

Following ethical procedures (Directive 2010/63/EU) octopuses were anesthetized using 7.5% magnesium chloride ($MgCl_2$) according to Messenger et al. (1985). Hemolymph was withdrawn from the cephalic aorta using a disposable syringe (1 ml) containing different solutions depending on the procedure. Hemolymph from each octopus was used immediately or transferred into a vial and kept on ice until use.

2.2. Morphological characterization of O. vulgaris hemocytes

2.2.1 Light microscopy: fresh and fixed hemocytes

2.2.1.1 Cells in suspension

Prefilled-syringes with 0.22 µm filtered seawater (FSW), 4% paraformaldehyde solution (in 0.1 M phosphate-buffered saline (PBS), pH 7.4) or 2.5% glutaraldehyde (in 0.2 M sodium cacodylate buffer pH 7.4) were used to dilute (1:1) the hemolymph. A subsample of crude hemolymph (without dilution and without anti-aggregating solution) was centrifuged for 5 min at 300 × *g* and 4 °C). The hemocytes were then re-suspended in Squid Ringer Solution (SRS: 530 mM NaCl, 10 mM KCl, 25 mM $MgCl_2$, 10 mM Ca Cl_2 and 10 mM HEPES buffer, pH 7.5). Preparations of 100 µl of SRS re-suspended hemocytes and diluted hemolymph samples were immediately observed at light microscopy DM2500 (Leica) equipped with Nomarsky contrast to enhance the contrast of fresh non-stained hemocytes. Hemocyte diameters were measured (at least 200 cells) using Leica Application Suite software v4.

2.2.1.2 Hemolymph cell monolayer

Hemolymph cells monolayer were prepared by cell adhesion and cyto-centrifugation. For both cases crude hemolymph (without anti-aggregating solution), hemolymph diluted 1:1 in FSW and cells re-suspended in SRS were used. To perform spontaneous cell adhesion 100 µl of each hemocyte solution was settling onto a glass slide and allowed to adhere for 20 min in a moist chamber at 15 °C. Cyto-centrifugation was carried out with 100 µl of hemocyte solution at 200 × *g* for 5 min in a Cytospin 4 cytocentrifuge (Thermo Scientific). Hemolymph cell monolayers were fixed in 100% ethanol for 1 min and stained for 1 min with each of the two solutions included in the rapid Hemacolor® kit (Merck). Glass slides were gently washed in distilled water and mounted in DPX resin (BDH, Chemicals). Hemocyte diameters were measured as previously mentioned.

2.2.2. Electron microscopy analysis

Fresh hemolymph (100 µl) was fixed for 4 h in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4) at 4 °C and washed for 30 min in the same buffer for scanning electron microscopy (SEM) study. Samples were then dehydrated in an ethanol series, critical point dried in CO_2 using a Polaron E3000 and sputter-coated in a Polaron SC 500 using 60% gold-palladium. Hemocytes were examined with a Philips XL 30 scanning electron microscope operated at 5 kV.

Transmission electron microscopy (TEM) was applied to circulating hemocytes and white body tissue. Small portions of white body and fresh hemolymph were fixed in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer pH 7.4. After 4 h at 4 °C, hemocytes and tissue were washed for 12 h at 4 °C in 0.2 M sodium cacodylate buffer pH 7.4 and, post-fixed in buffered 2% OsO4 for 3 h at the same temperature. Hemocytes were pelleted and pre-embedded in 4% agar at 40 °C. Small agar pieces containing fixed hemocytes and white body samples were dehydrated in a graded ethanol series and embedded in Epon resin. Semi-thin sections obtained by diamond knife were stained with methylene blue. Ultrathin sections were double stained with uranyl acetate and lead citrate, and visualized using a JEOL 100CXII TEM operated at 60 kV.

2.2.3. Cytochemical characterization of the white body and circulating hemocytes

2.2.3.1. Periodic Acid Schiff (PAS) in circulating hemocytes

Periodic acid Shiff (PAS) technique was applied for detection of polysaccharide in semi-thin sections obtained from Epon resin embedded pieces (see above). Oxidation of sections was carried out with 1% periodic acid for 10 min. Tissue sections were washed with water, stained with Schiff reagent during 20 min and a final washed was done. Periodic acid was avoided for controls (Lobo-da-Cunha et al., 2010).

2. 2.3.2. Arylsulphatase

Samples were obtained as previously mentioned for TEM assays (see above). For lysosomal enzymes detection, circulating hemocytes and white body tissue were fixed for 1 h at 4 ˚C in 2.5% glutaraldehyde (diluted in 0.4 M cacodylate buffer pH 7.4). Samples were washed in acetate buffer 0.2 M pH 5.0 for 30 min, incubated during 60 min at 35 ˚C in medium containing $BaCl_2$, p-nitrocatechol sulphate in acetate buffer 0.1 M pH 5.0. For controls, p-nitrocatechol sulphate was omitted. Post-fixation was done during 2 h at room temperature in 1% OsO_4 plus 1% potassium ferrocianure in cacodylate buffer (Hopsu-Havu et al., 1967).

For all the electron microscopic samples, the semi-thin sections obtained were stained with metylene blue and azur II.

2.2.4. Flow cytometry (FCM) analysis

Hemocyte population was determined in fresh crude or FSW diluted hemolymph using a FACScalibur flow cytometer (Becton Dickinson; Franklin Lakes, NJ) equipped with cell-sorting and cell-concentrator modules. Hemocyte population was discriminated in density plots according to the relative flow-cytometric morphological parameters: side scatter (SSC) that measure internal complexity, and forward scatter (FSC) that measure cell size, using a logarithmic and lineal scale, respectively. Fixed hemolymph in 4% paraformaldehyde solution (in 0.1 M PBS, pH 7.4) was used to isolate the population of interest by cell sorting. Cells were centrifuged 300 × *g*, 4 °C, 10 min and placed under glass coverslips for optical identification using a microscope Leica DM 2500.

2.3. Functional characterization of O. vulgaris hemocytes

2.3.1. Cell counting and hemocyte viability

Immediately after bleeding, cell counting was carried out using a Neubauer chamber. Cell viability was determined by Trypan blue exclusion test (Weeks-Perkins et al., 1995) in fresh and SRS re-suspended hemocytes. Cell mortality was assessed by flow cytometry labeling cells with 5 µl of 7-amino actinomycin D (7-AAD, BD Biosciences). After 15 min of incubation at 15 °C in the dark, the fluorescence was detected in the FL-3 channel of the flow cytometer using Cell Quest software (BD Biosciences).

2.3.2. Phagocytosis assay

Flow cytometry phagocytosis protocol was adapted from García-García et al. (2008) and assayed on 92 individuals. From each octopus, 1 ml hemolymph was extracted and centrifuged 300 × *g*, 4 °C, 5 min. Supernatant was discarded and replaced with the same volume of SRS anti-aggregating solution to avoid clotting. The hemocyte solution was maintained on ice for 10 min, while cell counting was performed. Hemocyte samples were centrifuged again as described above, re-suspended in FSW and 100 µl were dispensed in triplicates into 96-wells plate. After 30 min for cell adhesion at 15 °C in the dark, 100 µl of flourescein-labelled 1.2 µm latex beads (Molecular Probes, Invitrogen) were added at a ratio of 1:10 (hemocyte:beads). Control hemocytes were exposed to FSW. After 2 h incubation at 15 °C in the dark, excess of beads was removed by gently washing twice with 100 µl PBS. Attached cells were collected in 200 µl PBS supplemented with 20 µl of 0.8% trypan blue (in PBS) to quench external fluorescence. A total of 50,000 events were measured through the FL-1 channel. Results were expressed as the percentage of cells with at least one internalized bead. In addition, to confirm the phagocytic ability of cells, 100 µl of crude and washed (SRS) hemolymph were used to prepare hemolymph cell monolayers by cell-adhesion in presence of 100 µl of zymosan A (1 mg/ml) following the protocol yet described. After 30 min of incubation at 15 °C, excess of particles was eliminated by gently washing twice with PBS. Cell monolayers were fixed in 100% ethanol and stained using Hemacolor® kit (Merck).

2.3.3. Respiratory burst (ROS production)

Production of oxygen radicals was measured by flow cytometry using the CM- 2',7'-dichlorofluorescein diacetate probe (DCFH-DA, Molecular Probes). DCFH-DA diffuses into the cells where the intracellular esterases cleave to DCFH, which is oxidized by reactive oxygen species (ROS) to the highly fluorescent DCF. Subsequent oxidation yields fluorescence proportional to intracellular ROS (Hégaret et al., 2003). One hundred microlitres of FSW diluted hemolymph (1:1) were placed into 96-wells plate. Cells were incubated 30 min at 15 °C in dark for cell adhesion. The supernatant was removed and 100 µl of FSW containing 5 µM CM-DCFDA and 0.4% DMSO final concentration were added per well. Cells were incubated 10 min on ice in the dark. The supernatant was removed and hemocytes were washed twice with 100 µl FSW before being stimulated adding 100 µl zymosan (1 mg/ml). Superoxide dismutase (SOD, 15 µl at 300 U/ml, Sigma) or of N^G-methyl-L-arginine acetate salt (NMMA, 10 µl at 1mg/ml, Sigma) inhibitors were added to determine whether H_2O_2 and NO, respectively, contributes to the oxidation of DCF-DA. Controls were exposed to the same volume of FSW. After 60 min of incubation at 15 °C in the dark, ROS were measured by flow cytometry in cells re-suspended in PBS. A total of 50,000 events were measured and data were collected as mean fluorescence of the sample. The oxidative activity is expressed as mean fluorescence in arbitrary units (A.U.). The assay was performed in the hemolymph of 57 octopuses.

2.3.4. Nitric oxide production (NO)

The NO production was assayed in 89 octopus hemolymph by the Griess reaction (Green et al., 1982) that quantifies nitrites (NO_2^-), a stable product that result from the degradation of NO. Briefly, 100 µl of the withdrawn crude hemolymph were exposed to 100 µl zymosan (1 mg/ml final concentration), while 100 µl of FSW was added to controls. After 2 h of incubation at 15 °C, 50 µl of supernatants were carefully removed from each well and placed in new ones. Afterwards, 100 µl of 1% sulfanilamide (Sigma) in 2.5% phosphoric acid were added to each well followed by 100 µl of 0.1% N-naphthyl-ethylenediamine (Sigma) in 2.5% phosphoric acid. After 5 min of incubation at room temperature, the optical density at 540 nm was measured (Multiscan Spectrophotometer, Labsystems). An additional assay was performed in order to test different stimuli: zymosan (2 mg/ml), phorbol myristate acetate (PMA) and *Escherichia coli* lipopolysaccharide (LPS, Sigma) at 2 µg/ml final concentration, respectively, at different incubation times (30 min, 1, 3, 6, 24 and 48 h). A total of three pools of 4 octopuses each

were assayed. The molar concentration of nitrite in all samples was determined from standard curves generated using known concentrations of sodium nitrite.

2.4. Statistical analyses

One-way analysis of variance (ANOVA) and Tukey test, when needed, were carried out to compare phagocytic ability, respiratory burst and nitric oxide production. The phagocytic percentage data were transformed to arc sine of the square root before ANOVA. The mean fluorescence intensity value of oxidative activity and nitrite concentration of samples were logarithmic (LN) transformed before ANOVA. The Kolmogorov-Smirnov (K-S) was used to test normality of variables whereas Levene test was used to check homogeneity of variances before statistical analysis. NO measured in pool samples were compared using a Student's *t* test. Results were expressed as the mean ±E.S. and differences were considered significant at $p<0.05$. All the analyses were performed with the software Statistica v6.0.

3. Results

3.1 Morphological and cytometric characterization of hemocytes

3.1.1 Light microscopy: fresh and fixed hemocytes

Live cells and cytological analyses of fixed hemocytes revealed a predominant type of circulating hemocytes in the *O. vulgaris* hemolymph, measuring a mean of 10.57 µm ± 0.41 in diameter (10-12.57) (Table 1). Examined by light microscopy, fresh and fixed hemocytes were rounded in form, with visible numerous cytoplasmic inclusions and refringent vacuoles (Fig. 1A,B). In addition, a second type rounded or ovoid hemocytes with fewer or even without granules in cytoplasm and nucleus occupying almost the entire cell were also observed (Fig. 1C). The mean diameter of these cells was 9.27 µm ± 0.68, but showed a wide range of variation (5.55-9.98) (Table 1). Fresh cells, FSW-diluted or cyto-centrifuged hemocytes extend thick pseudopodia; while hemocytes re-suspended in SRS sent out many thin pseudopodia. Hemocytes in SRS showed pseudopodia with time delay, whereas fresh hemocytes in crude hemolymph rapidly sent out pseudopodia, showing a quickly cell to cell association and forming dense cellular clots as soon as 20 min of

observation. Hemocytes in FSW spread and attach to the slide forming cellular clots around 30 min of observation (Fig. 1D).

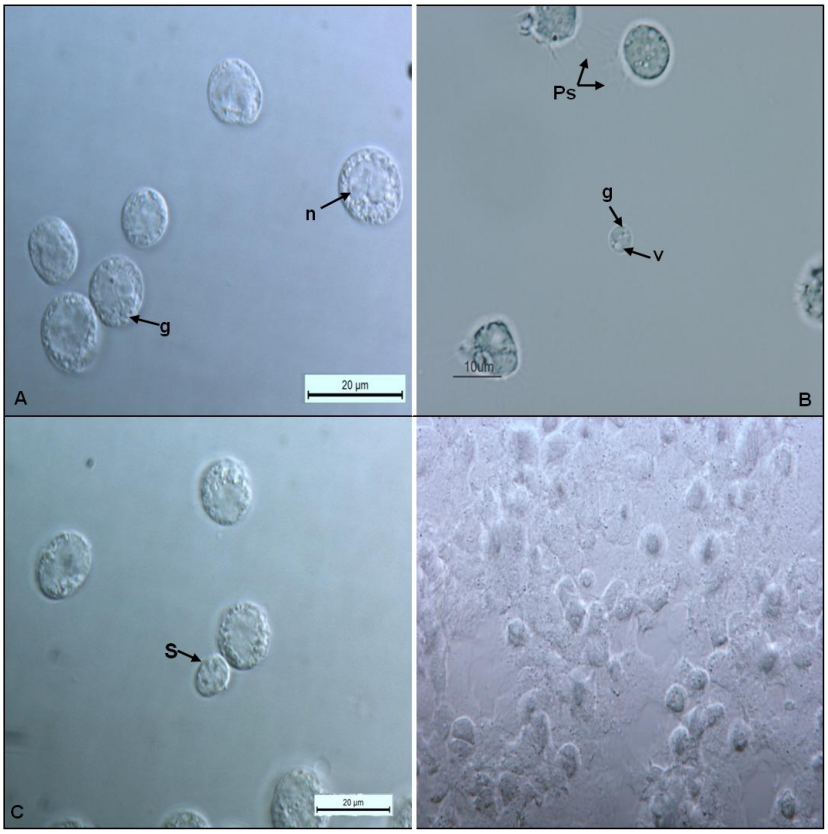


Fig. 1. Live hemocyte on fresh hemolymph of *O. vulgaris*. (A) Hemocytes showing (n) U-shaped nuclei and granules (g) in the cytoplasm. (B) Hemocyte showing granules, refringent vacuole (v) and thin pseudopodia (Ps) formation. (C) Small hemocyte in hemolymph (g). (D) Hemocytes in FSW forming cellular clots (20x).

After staining with Hemacolor® most of the cells were round to ovoid in shape and measuring a mean of 12.5 µm ±1.10 in diameter (10.23-14.97) (Table 1). The U-shaped nucleus was eccentric in the abundant cytoplasm. The nuclei/cytoplasm ratio had a mean of 0.72 ± 0.08 (0.59-0.87). All hemocytes presented visible basophilic granules in cytoplasm (Fig. 2A). Additionally, a second type of rounded or ovoid hemocytes was observed. They measured a mean diameter of 9.12 µm ± 0.71 with wide variations ranging 7.98 – 9.9 µm (Table 1). The nucleus was large round to ovoid with few or thin cytoplasm and scarce granules or totally absent (Fig. 2B, C). The nuclei/cytoplasm ratio had a mean

of 0.75± 0.06 (0.65–0.84). Stained cells demonstrate the aggregating ability and formation of filaments constituted by cell to cell association.

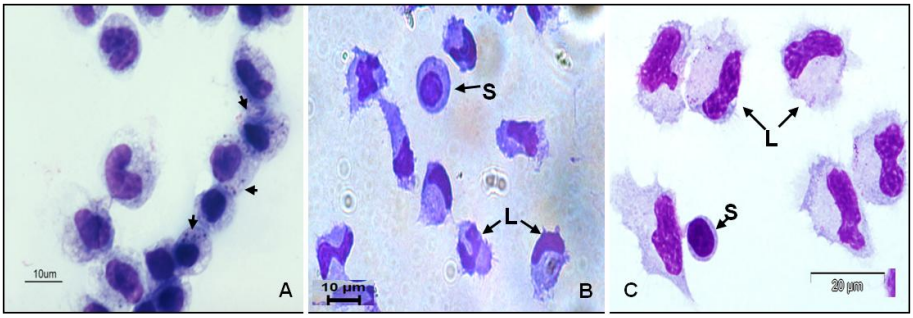


Fig. 2. Hemolymph cell monolayers stained with Hemacolor®. (A) Large cells showing U-shaped nuclei and basophilic granules (arrows). (B) Two hemocyte types could be distinguished: large (L) and small (S) granulocytes. (C) Detail of Large and small granulocytes.

3.1.2 Electron microscopy

Most of the hemocytes observed by SEM were large (11 µm) and round, with shallow surface indentations and many thin pseudopodia. These cells showed a high ability to extend completely and attach to the surface (Fig. 3A). Small hemocytes (3.7-8 µm) with irregular surface, shallow indentations or protrusion and few thin pseudopodia were also distinguished (Fig. 3B). Thus, two types of cells with differences in surface and pseudopodia were found (Fig. 3C).

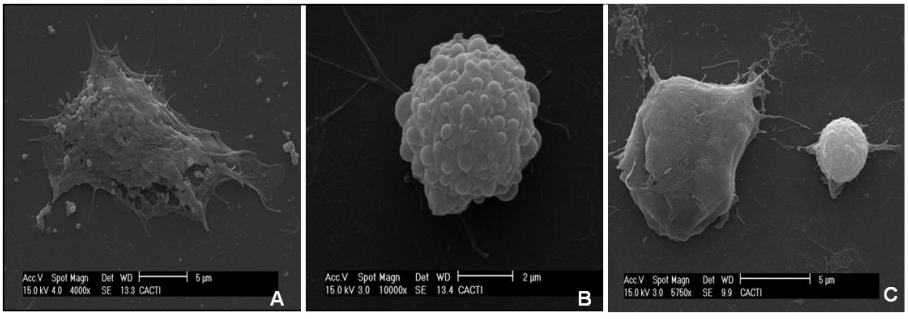


Fig. 3. Scanning electron transmission of *O. vulgaris* hemocytes. (A) Large extended hemocytes of irregular surface and numerous pseudopodia. (B) Small hemocytes with shallow indentations and thin pseudopodia. (C) Comparative of large and small hemocytes showing differences in surface and pseudopodia.

One morphotype of cell was found by TEM on circulating hemocytes. The cells were round in shape, with a plasmatic membrane that displays a smooth undulating surface and short pseudopodia. Chromatin is accumulated in electron-dense clumps in the periphery of nucleus (Fig. 4A). Cells exhibit endoplasmic reticulum around the nucleus. Numerous inclusions corresponding to granules of different sizes were observed in the cytoplasm (Fig. 4B). Inclusions were mostly spherical. Some of them displayed irregular outlines and occasionally small rod-like inclusions. Small and large inclusions showed a configuration of different electron-density core and medium electron-density, corresponding to different types of granules (Fig. 4C,D). Some of the dense inclusions could putatively contain glycoproteins.

The white body tissue analyzed by TEM showed cells at two different developmental stages. One of the cell stages fit to secondary leucoblast, an intermediate stage of development between primary leucoblast and mature hemocytes. The cell surface seemed to be covered by a thin layer of electron-dense material. Chromatin was condensed through the entire nucleus with some small clumps. The cytoplasm presented very few medium electron-dense and electron-dense inclusions (Fig. 4E). The second developmental stage could correspond to a transitional cell previous to hemocyte maturity, showing a nucleus most compact and less condensed chromatin. The nucleus shape became irregular and a noticeable increase of electron-dense and medium lectron-density granules was distinguished in the cytoplasm (Fig. 4F).

3.1.3 Cytochemistry characterization of the white body and circulating hemocytes

The PAS technique performed in semithin sections showed a positive reaction in hemocytes' granules of different size. The reaction was observed as an intense magenta stain in a faint magenta cytoplasmic background while other cells were weakly stained (Fig. 5A). The PAS positive reaction observed demonstrates the presence of polysaccharides in circulating hemocytes.

A positive reaction was found for arylsulphatase in ultrathin sections of circulating hemocytes observed by TEM, confirming that the specifically filled electron-dense bodies observed corresponded to lysosomes (Fig. 5B). In controls, all structures were free of barium deposits (data not shown). Cells in the white body showed also a positive reaction, confirming the presence of lysosomes. However it was not observed in all cells, which could be attained to differences in the developmental stages present inside the white body.

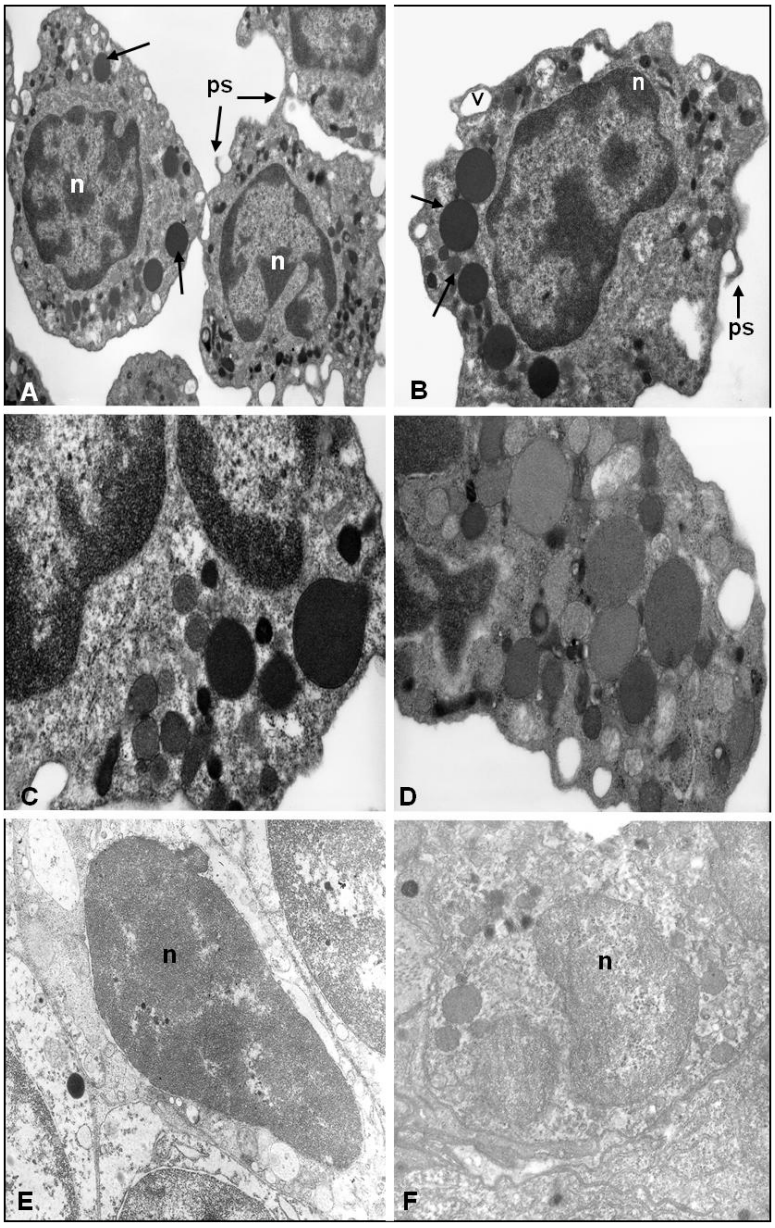


Fig. 4. TEM micrographs of *O. vulgaris* hemocytes and white body cells. (A-B) Circulating hemocytes in the *O. vulgaris* hemolymph. U-shaped nucleus, dense and medium-dense cytoplasmic inclusions (arrows), vacuoles (v) and pseudopods (ps) are observed (A: 5,300x; B: 8,000x). (C-D) Detail of granules in the cytoplasm of circulating hemocytes (10,000x). (E-F) Cells developed inside the *O. vulgaris* white body: (E) secondary leucoblast showing a large nucleus (n), chromatin condensed (5,300x). (F) Transitional cell, previous to become a mature circulating hemocyte, the nuclei is starting to acquire their typical U-shape and few granules are observed in cytoplasm (5,300x).

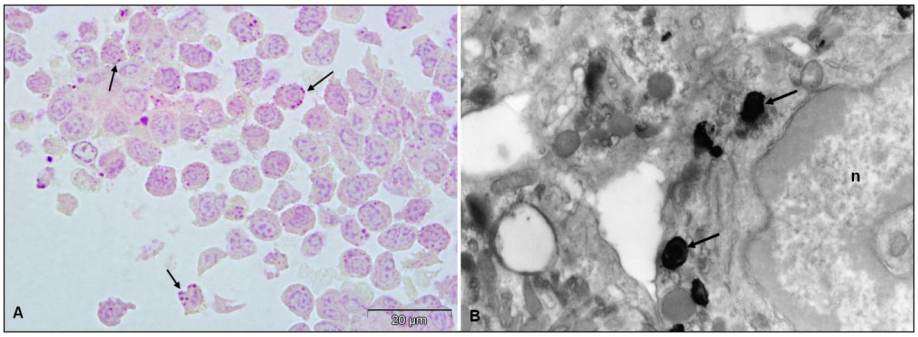


Fig. 5. Cytochemistry of *O. vulgaris* hemocytes. (A) Periodic acid Schiff (PAS). Light micrograph of circulating hemocytes showing stained glycogen deposits (arrows) in the cytoplasm. (B) TEM micrograph showing positive reaction of arylsulphatase (5,300X). Lysosomes are visible in the cytoplasm of circulating hemocytes (arrows).

3.1.4 Flow cytometry

According to the two-parameter plots, size (FSC) and granularity (SSC), two populations were recognized in octopus hemolymph by flow cytometry and were designated as R1 and R2. Hemocytes of the R1 fraction with high FSC and SSC are large sized cells with high granularity, representing the 82% (ES ± 2.47) of the hemocytic population and were designated large granulocytes. Hemocytes of the R2 fraction with low FSC and low SSC value were small to medium sized cells with fewer granules, constituted the 18% (ES ± 2.13) of the hemocytic population and were designated as small granulocytes (Fig.6A). Fixed hemocytes (in 4% paraformadehyde) were sorted and immediately observed by optical microscopy. Isolated cells from R1 population were large, with a mean diameter of 11.6 µm ± 1.2 (9.32–15.56), round or ovoid in shape, visible U-shaped nuclei and numerous granules in cytoplasm (Fig. 6B, C) (Table 1). Hemocytes isolated from R2 population were small to medium sized, with a mean diameter of 8.12 µm ± 0.74 and wide variations (6.69–9.99 µm) in size (Table 1). They were round in shape, the round nucleus occupies the cell almost entirely, and showed a thin cytoplasmic layer and few or absent granules in cytoplasm (Fig. 6D-E).

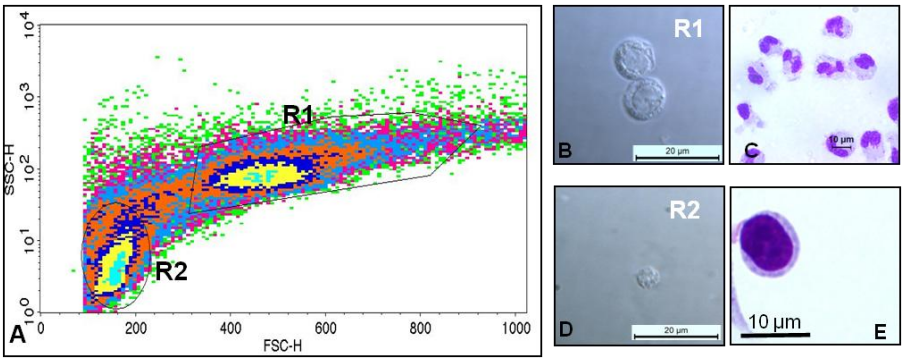


Fig. 6. Flow cytometric determination of the hemocyte populations of *O. vulgaris*. (A) Size (FSC) and internal complexity (SSC) density plot showing two hemocyte populations. Phase contrast micrographs and stained hemocytes isolated by cell sorting from R1 (B-C) and small hemocytes from R2 (D-E).

Table 1. Microscopic characterization of the hemocyte populations of the *O. vulgaris* hemolymph (at least 200 cells were measured)(mean ± DS).

	Hemocytes in suspension	**Stained hemocytes**	**Hemocytes isolated by sortin**
Large granulocyte	10.57 µm ± 0.41 (10-12.57)	12.5 µm ±1.10 (10.23-14.97)	11.6 µm ± 1.2 (9.32–15.56)
Small granulocyte	9.27 µm ± 0.68 (5.55-9.98)	9.12 µm ± 0.71 (7.98 – 9.9)	8.12 µm ± 0.74 (6.69 – 9.99)

3.2 *Functional characterization of the O. vulgaris hemocytes*

3.2.1. *Cell counting and hemocyte viability*

The mean number of total circulating hemocytes counted in individual octopus was 10.3×10^6 (SE ± 8.1×10^5) varying from 4.9×10^5 to 32×10^6 (SE ± 8.1×10^5). The hemocyte mortality assessed by flow cytometry reached the 7% of the total cells. In order to check the effect of the anti-aggregant solutions used in the experiments, the hemocyte mortality was analyzed. Thus, the hemocyte viability achieved through the Trypan blue exclusion test was ≥95% in crude hemolymph and close to 90% in samples treated with SRS and FSW (Fig.7).

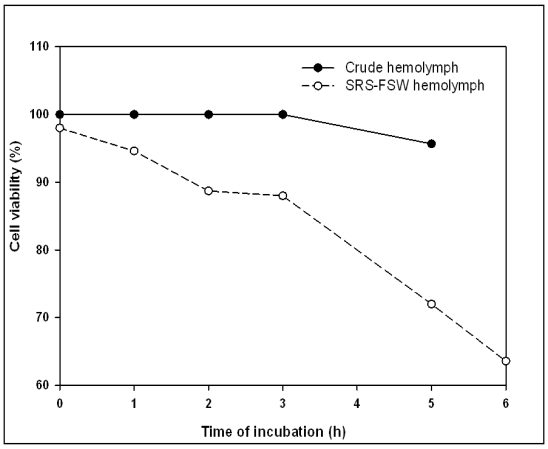


Fig.7. Comparative hemocyte viability in (A) crude hemolymph and (B) treated with anti-aggregating solution (SRS and FSW) in a time course.

3.2.2. Phagocytic ability

Light microscopy observations showed that hemocytes of *O. vulgaris* were able to engulf zymosan A and fluorescent latex beads (Fig. 8A, B). By flow cytometry, the phagocytosis of latex beads was detected in the hemocytes as an increase of the fluorescent level registered in the FL1-H channel (Fig. 8C). The two cell populations showed different ability to phagocyte fluorescent latex beads. The mean percentage of phagocytosis was 13% reaching up to 56% in R1. The mean percentage of phagocytosis in R2 was 3% reaching up to 9% after incubation of 120 min. Phagocytic activity recorded from both cell populations showed a wide variability among individuals.

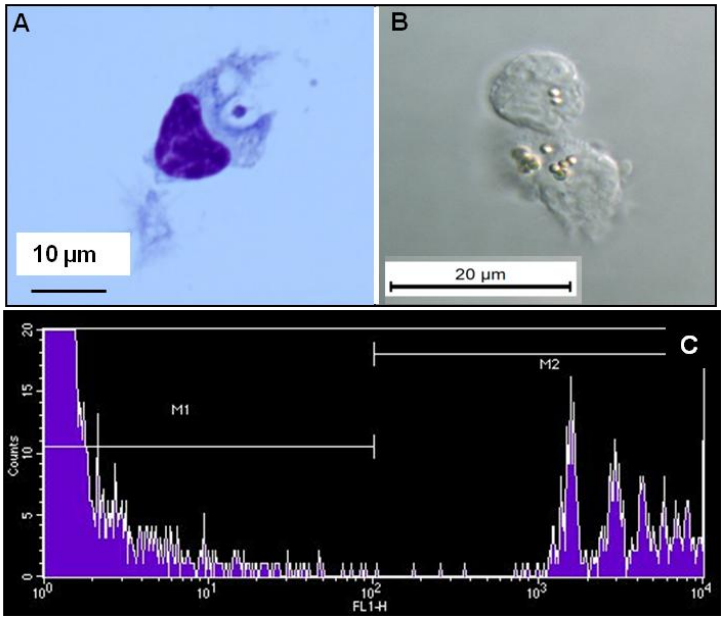


Fig. 8. Phagocytic ability of hemocytes. Light micrographs of hemocytes phagocyting zymosan (A) and fluorospheres (B). (C) Phagocytic activity recorded by flow cytometry. The histogram of fluorescence in FL1-H channel represents the phagocytic activity recorded in the *O. vulgaris* hemocytes: M1, hemocytes that not engulfed fluorospheres; M2, hemocytes that engulfed one or more fluorospheres.

3.2.3. Respiratory burst (ROS production)

ROS production was measured using the oxidation of nonfluorescent DFCH to the highly fluorescent DCF (Fig 9A). Significant differences (p<0.05) were found between the mean fluorescence value of non-stimulated (controls) hemocytes compared to stimulated samples. After 60 min, oxidative activity in stimulated hemocytes of R1 increased to 12 A.U. (3-31 A.U. ± 0.94). Whereas, the oxidative activity in hemocytes from R2 increased up to, in average, 5 A.U. (2-17 A.U. ± 0. 32). Hemocytes pre-incubated with SOD before stimulation with zymosan showed a slight inhibition of the respiratory burst (p>0.05); while pre-incubation with the nitric oxide synthase inhibitor NMMA did not suppress respiratory burst (p<0.05) in *O. vulgaris* hemocytes (Fig. 9B).

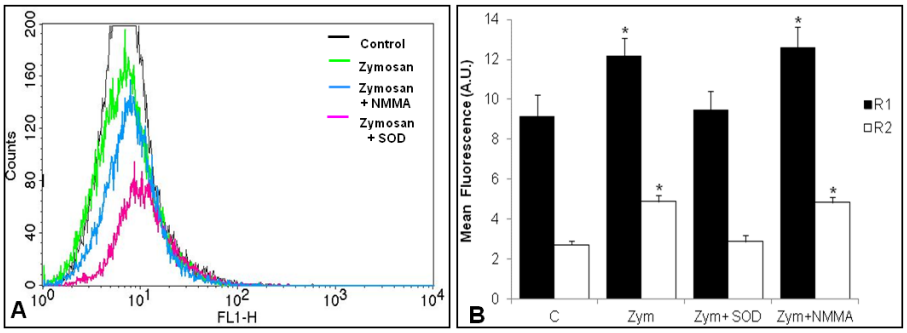


Fig. 9. Respiratory burst measured in *O. vulgaris* hemocytes. (A) Representative oxidative activity in the main hemocyte population (R1). (B) Respiratory burst measured in the hemocytes populations following stimulation with zymosan and treated with oxygen and nitrogen radicals inhibitors SOD and NMMA (p<0.05) (mean ± SE). (*) Differences are considered significant at p<0.05.

3.2.4. Nitric oxide (NO) production

The concentration of nitrite (NO_2^-) produced by zymosan A- stimulated hemocytes was significantly (p<0.05) higher compared to the basal level detected in control hemocytes. The mean nitrite concentration obtained in unstimulated hemocytes was 16 µM (SE ± 2), whereas stimulated hemocytes produced 31 µM (SE ± 3) nitrite (p<0.05) (Fig. 10A).

The differential ability of *O. vulgaris* hemocytes to release NO during a course of time showed that challenge of hemocytes with zymosan resulted in the highest NO production (33 µM SE ± 4) (p<0.05), followed by PMA and LPS. The maximum value of nitrite recorded using PMA as stimulus was 31 µM (SE ± 3) after 3 h of incubation (p>0.05). LPS reached 21 µM (SE ± 6) after 6 h of incubation (p>0.05) (Fig. 10B).

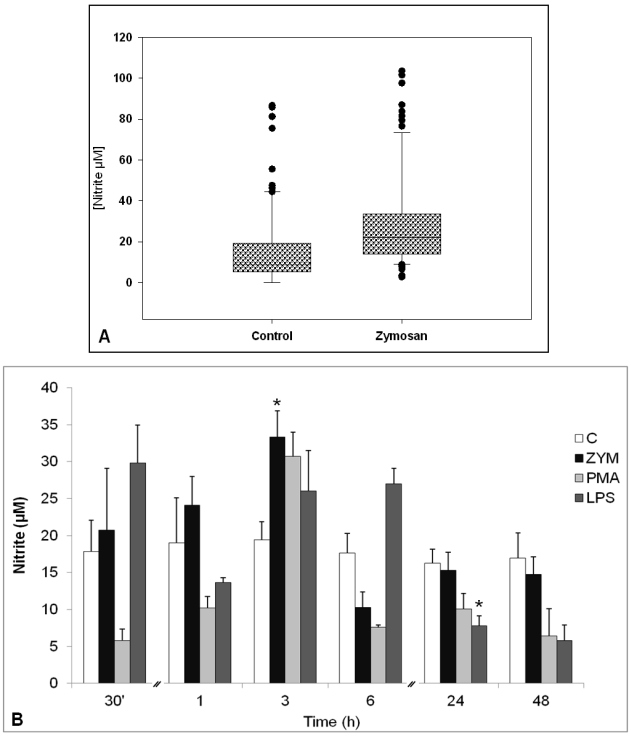


Fig. 10. Nitric oxide production in *O. vulgaris* hemocytes. (A) NO measured in hemocytes incubated with zymosan during 2h, 15 °C (p<0.05). Time course evolution of NO production using different stimuli (mean ± SE). (*) Differences are considered significant at p<0.05.

4. Discussion

In molluscs, characterization of the hemocyte populations is useful to evaluate their capacity to respond against pathogens and environmental stress (Hégaret et al., 2003). Hence, the present work is the first attempt to examine the populations and immune related activities of the circulating hemocytes of *O. vulgaris* combining light and electron microscopy with flow cytometry analysis. The advantage of flow cytometry to analyze a large number of cells in short time and gating cellular subpopulations became this method a more precise tool to study cell populations (Ashton-Alcox and Ford, 1998). Consequently, recent studies have been used to classify molluscan hemocytes using flow cytometry (Donaghy et al., 2009a,b; 2010; Prado-Alvarez et al., 2012; Ray et al., 2013).

Previous studies carried out on light and transmission electron microscopy in *Octopus briareus* (Cowden and Curtis, 1973) and the sepiolid *Euprymna scolopes* (Nyholm and Mc Fall-Ngai, 1998; Koropatnick et al., 2007) indicates the only occurrence of one type of granular cells measuring, in average, 10 μm in the circulating hemolymph. Based on light and electron microscopy, and flow cytometry analysis, our results indicate that two morphological hemocyte types occur in the *O. vulgaris* hemolymph. Flow cytometry analysis allowed identifying accurately two different cellular populations that were confirmed by sorting, after cytocentrifugation and light microscopy observation. Thus, a new classification scheme can be established referring them as large granulocytes, with a mean diameter of 11.6 μm, U-shaped nucleus, abundant cytoplasm and high granularity; and small granulocytes, with a mean of 8.12 μm in diameter, nucleus occupying almost the entire cell and lower granularity. Stained cell monolayers revealed that most hemocytes possess basophilic granules as in bivalves (Chu, 2000), and previously described in the single type of hemocytes recognized in *O. vulgaris*, *O. minor* and *O. ocellatus* (Kondo et al., 2003). However, our results demonstrated the presence of a second type of small hemocytes with very few or without granules, easily identified by flow cytometry due to their low complexity and corroborated by stained techniques of light microscopy.

Hemocytic populations and activities in common octopus could vary with inter and intra individual factors as well as the methodology used for study. A similar pattern has been stated in other molluscs (Ashton-Alcox and Ford, 1998). Stained large granulocytes showed a slight increase in cell diameter related to the size observed by other techniques, similar to those observed in the hemocytes of *Ruditapes decussatus* (López et al., 1997a). The small granulocytes (R2) showed a wide range of cells with high variability in diameter and occasionally including hemocytes smaller than 5 μm. These smallest cells were not

easily recovered from sorting neither observed by TEM, suggesting that they could be easily lost during microscopic methods (cytospin, TEM procedure).

Malham et al. (1998) suggest that new maturing hemocytes could be released from the white body to the hemolymph to compensate the blood loss. Then, a sequence of cell maturity similar to that suggested for *Crassosstrea rizophorae* (Rebelo et al., 2013) could be present in the octopus hemolymph. We hypothesize that small granulocytes (R2) could be starting maturing cells with a thin cytoplasm and absent granules. As the hemocytes mature, cells might enlarge developing more granules in the cytoplasm, and increasing the cell complexity. However, this hypothesis must be confirmed in the future.

The ultrastructural study of *O. vulgaris* hemocytes agree with Cowden and Curtis (1981) description, since two types of granules were found in the hemocytes. The assays herein performed demonstrated that lysosomes are present in the *O. vulgaris* white body cells and circulating hemocytes. The scarce observation of lysosomes in white body cells agreed with the low (9%) phagocytic activity reported by Novoa et al. (2002). Circulating hemocytes also showed lysosomes which reveal their phagocytic ability as occurs in bivalve hemocytes (Cheng, 1975). Glycogen granules were also observed in octopus hemocytes, with some cells weakly or not stained. The variation in glycogen deposits in the hemocytes of the intertidal *Littorina littorea* were attained to the need of energy store for using during anoxia (Gorbushin and Iakovleva, 2007). Similarly, Travers et al. (2008) explained the large glycogen deposits observed in *Haliotis tuberculata* hemocytes as a common strategy in intertidal molluscs. Glycogen was reported in *O. vulgaris*, but not in *O. briareus* hemocytes. However, despite octopuses are able to displace in the intertidal zone, such glycogen variations might be attained as a response to seasonal or environmental variation (Cowden and Curtis, 1981).

Total hemocyte count was highly variable in *O. vulgaris*, similar to those previously recorded by Rodríguez-Domínguez et al. (2006). Whether individual variability in octopus hemocytes is common or due to (biotic or abiotic) stressors remains to be elucidated. The percentage of viable cells in crude hemolymph was slightly higher than in anti-agregating solution. However, our results showed that crude hemolymph is disadvantageous for functional essays because the quickly formation of cellular clots. Clots formation neutralizes invaders (Cowden and Curtis, 1973) and prevents bleeding (Féral, 1988), which hampers the measurement of functional responses in cephalopod's hemocytes.

Considering different hemocyte subpopulations imply putative different functionality. Therefore, the defensive activities like phagocytosis and production of

cytotoxic factors like respiratory burst and nitric oxide of each subpopulation is required to evaluate the health of individuals (da Silva et al., 2008; Comesaña et al., 2012). Phagocytosis is a highly important mechanism implied in the invertebrate internal defense. Therefore, the proportion of hemocytes that is phagocytically active in the hemolymph provides relevant information for assessing the health of the organisms (Ellis et al., 2011). Functional studies performed on bivalve hemocytes showed that mainly, but not only granulocytes have a high ability to phagocytose latex beads or zymosan (García-García et al., 2008; Travers et al., 2008; Donaghy et al., 2009a,b; 2010). Until now, the phagocytic ability of *O. vulgaris* hemocytes has been measured by conventional methods of microscopic assessment (Novoa et al., 2002; Rodríguez-Domínguez et al., 2006). Our results verified the ability of *O. vulgaris* hemocytes to phagocytose zymosan and demonstrate also by flow cytometry that octopus hemocytes are capable to ingest fluorescent latex beads. Large granulocytes were the most active cells showing a higher phagocytic capability than small granulocytes, which showed a positive but very low phagocytosis. A high variability in the phagocytic ability of the hemocytes was observed, that could be derived from natural fluctuations in the octopus hemocytes. Nonetheless, the results obtained are similar to that reported for other molluscs, such as *Haliotis discus discus* (Donaghy et al., 2010) *Haliotis tuberculata* (Travers et al., 2008) and *Ruditapes decussatus* (Prado-Alvarez et al., 2012) in both, phagocytic percentage and hemocyte capability.

Reactive oxygen species ($^1O_2$, H_2O_2, HO), derived from the oxidative metabolic event named respiratory burst, are employed for antioxidant defenses to combat pathogenic infections (Chu, 2000; Lesser, 2006). The generation of oxygen radicals has been investigated in bivalves by different methodologies such as luminol or lucigening chemiluminescence (Volety and Chu, 1995; Bramble and Anderson, 1999); citrochromo-C reduction (Wootton et al., 2003); and reduction of nitroblue tetrazolium (Anderson et al., 1992; Pipe, 1992). The extracellular ROS production was measured in populations of *O. vulgaris* hemocytes in response to LPS and zymosan using ferricytochrome C (Novoa et al., 2002). The same procedure was applied to *E. cirrhosa* hemocytes in response to phorbol myristate acetate (PMA), bacteria and LPS, but no ROS production were detected (Malham and Runham, 1998). In contrast, intracellular ROS were accurately measured by reduction of NBT in response to air exposure stress in the same species (Malham et al., 2002). Flow cytometry has been demonstrated to be very sensitive in the detection of respiratory burst activity compared with the classic luminol-dependent chemiluminiscence or NTB reduction (Goedken and De Guise, 2004). This methodology allowed us to demonstrate that *O. vulgaris* hemocytes were capable to produce ROS, measured through DCF

fluorescence. The fluorescence yielded by large granulocytes was higher than small granulocytes. This is consistent with a higher rate of phagocytosis in large granulocytes compared to the small ones. Thus, as has been described in other molluscs, large granulocytes could be the most effective cells to engulf pathogens (Hégaret et al., 2003; Goedken and De Guise, 2004). Respiratory burst was inhibited by SOD, indicating that the probe is oxidized by H_2O_2. The NMMA inhibitor of NOS did not induced a decrease in ROS production, which lead us to conclude that oxidation of DCFH-DA was derived from ROS (Possel et al., 1997; Buggé et al., 2007; Lesser, 2006).

Nitric oxide is involved in immune defense inactivating pathogens after diffusion through cell membranes (Rivero, 2006). *O. vulgaris* hemocytes showed a high ability to release NO in response to zymosan after 2 h of incubation, obtaining higher values than those observed in other molluscs (Tafalla et al., 2002; 2003) and thus, suggesting that octopus could have a strong response against potential pathogens. Our results showed that zymosan induced the highest NO production followed by PMA and LPS. Zymosan has been successfully used to stimulate NO production in *O. vulgaris* and other molluscs (Novoa et al., 2002; Tafalla et al., 2003; García-García et al., 2008). PMA is capable to cross the cell membrane for inducing NO production. In octopus hemocytes, the highest values reached after 3 h of incubation suggest a slow, but strong reaction. A previous study reported that LPS induced a weak stimulation in *O. vulgaris* hemocytes (Novoa et al., 2002). However, the present results showed that LPS also induced a strong reaction after 30 min and up to 6 h of incubation. After that time, NO dropped to low values similar to those recorded in the white body cells at 24 h of incubation (Novoa et al., 2002). To our knowledge, this is the first study reporting several stimuli and incubation times to induce NO production in *O. vulgaris* hemocytes. Further studies are needed to reveal whether pathways activated by zymosan, PMA and LPS are similar to those reported in *Lymnaea stagnalis* (Wright et al., 2006) or *Mytilus galloprovincialis* (García-García et al., 2008). Hence, having a framework to reveal the pathways activated by parasites.

In conclusion, in the present study the morphological and functional characterization of the hemocytes of *O. vulgaris* was performed for the first time combining microscopic and flow cytometry methodologies. By cytometric analysis two subpopulations were characterized in the hemolymph of *O. vulgaris*: large granulocytes and small granulocytes. Microscopic studies confirmed that both types of cells differed in cell size and complexity attained to the quantity of granules present in the cytoplasm. Particularly, small granulocytes showed a wide range in diameter. However, they differ markedly in granularity from large granulocytes, which are the major cells in the octopus

hemolymph. Large granulocytes possess numerous lysosomes making them the main effectors of phagocytosis and ROS production. In contrast, small granulocytes have a limited phagocytic ability, and consequently, a limited respiratory burst. The present results establish the basis for developing studies focus on the involvement of *O. vulgaris* hemocytes in the ability of controlling pathogen infections as well as the influence of environmental factors in the octopus cellular response. Further investigations using fluorescent antibodies would improve the hemocyte classification and would be valuable to understand the specific immunological functions of each hemocyte subpopulation and to distinct if additional hemocyte differences exist.

Chapter 3

Immune parameters in the common octopus (*Octopus vulgaris* Cuvier, 1797) naturally infected by the gastrointestinal protozoan *Aggregata octopiana*

O. vulgaris reared in floating cages at Ria of Vigo, Spain (Original: Rubén Chamorro)

Abstract

Octopus vulgaris is an important cephalopod species in world fisheries and in recent years it has been gained importance as an emergent aquaculture species. The protozoan *Aggregata octopiana* has been recorded as one of the most dangerous parasite that threats the health of octopuses in both wild and reared conditions. The *A. octopiana* infection intensity was measured in the digestive tract of octopuses and two groups of infection, one showing a low parasite load, corresponding to healthy individuals; and a second one, showing a high parasite load, corresponding to sick individuals were performed. Cellular defense parameters (phagocytosis, respiratory burst (ROS) and nitric oxide (NO) production) were measured in the octopus hemolymph. In addition, its relationship to i) the infection degree (measured as total amount of infection or distributed by groups of infection (sick/healthy) and ii) to the octopus origin (wild or reared in floating cages) were measured. Moreover, octopus biometric data (sex, length, weight, gonadic development) and season of collection were also tested to know their contribution on the octopus cellular response. Results indicated that season of collection and total parasitic infection were the most important factors affecting the phagocytic ability of hemocytes. The infection intensity was significantly and positively associated with the increase in phagocytosis, which was little higher in autumn relative to winter and spring. Total infection had a negative effect on cytotoxic reaction of hemocytes. ROS and NO production decreased with the *A. octopiana* infection increase. Particularly, a markedly decrease in NO was observed in heaviest octopuses. Moreover, comparing wild and reared octopuses, the cytotoxic activity notably decreased in the former group. The present results evidenced for the first time that the intensity of infection by *A. octopiana* severely weaken the octopus cellular immune response. Additionally, here is showed that the negative coccidia effect seems to be maximized in octopuses reared in floating cages.

1. Introduction

The common octopus (*Octopus vulgaris*) is one of the world's economically important species, subject of active fisheries and highly appreciated as food. The stocks present wide annual fluctuations due to their own biological characteristics like non-overlapping generations and thus, lack of buffering of the population; but also, due to the influence of environmental conditions like temperature that impact on spawning and recruitment success (Pierce et al., 2008). Hence, the economic importance of *O. vulgaris* and the need to diversify marine aquaculture products have encouraged the research of cephalopod culture taking advantage of its rapid and easy acclimatization to captivity conditions, their rapid growth, and their high protein content and high food conversion rate (Vaz-Pires et al., 2004). However, the stress developed in farmed facilities promotes parasitic outbreaks (Berthe, 2005). The gastrointestinal protozoan *Aggregata octopiana* is one of the most dangerous pathogen affecting wild and cultured octopuses (Pascual et al., 1996; Gestal et al., 2007b). This intracellular coccidian produces a strong hemocytic infiltration, followed by fibrosis, necrosis and rupture of the basal membrane inducing atrophy of the intestinal mucosa (Gestal et al., 2002a). The acidification lumen of infected tissues originated by the coccidia causes the malfunction of absorption enzymes (Gestal et al., 2002b). Furthermore, octopuses highly infected (up to 3×10^6 sporocyst/g infected tissue) show a decline in the number of circulating hemocytes, plasmatic protein and the octopus condition, which is reflected in the reduction of the octopus weight (Gestal et al., 2007b).

In cephalopods, as other mollusk, the internal defense against pathogens relies on their innate immune system, composed by humoral (diluted molecules in plasma) and cellular (developed by hemocytes) factors that impede the pathogen grow (Ford, 1992). Cellular defense is carried out by the hemocytes (Malham and Runham, 1998). Defensive role of hemocytes includes mechanisms like encapsulation, mainly for large particles; nacrezation, phagocytosis or production of free toxic radicals. Phagocytosis is the main mechanism to remove pathogens, whereas the generation of toxic radicals like reactive oxygen and nitrogen species (ROS and NO, respectively) are used to destroy the engulfed pathogens (Cheng, 2000).

The formation of oxygen radicals is called respiratory burst. The source of these oxidants is the superoxide anion (O_2-) that undergoes enzymatic dismutation to produce hydrogen peroxide (H_2O_2), giving rise to other highly toxic radicals (Cheng, 2000). The nitric oxide (NO) is synthesized by the enzyme nitric oxide synthase through the oxidation

of L-arginine to citrulline. In vertebrates and invertebrates, NO participate in physiological processes as neurotransmitter and cardiovascular system (Palumbo, 2005), but also it's a free radical produced by hemocytes as microbicidal and antiparasitic molecule. NO react with superoxide anion and produces peroxinitrite ($ONOO^-$), which is highly toxic for cells (Ottaviani et al., 1993; Rivero, 2006).

The cellular defense mechanisms are crucial to eradicate pathogens through phagocytosis and its further destruction by ROS and NO. This defensive mechanism has been detected in bivalves like *Crassostea gigas* (Goedken et al., 2005) or *Crassosstrea virginica* infected by *Perkinsus marinus* (Volety and Chu, 1995; Anderson et al., 1995, 1999). The enzymes involved in defense function of *Mytilus galloprovincialis* have been studied by Carballal et al. (1997), whereas the phagocytic ability of mussels infected by *Perkinsus atlanticus* have been studied by (Ordás et al., 1999). NO production by *M. galloprovincialis* hemocytes was also demonstrated (Tafalla et al., 2002) as well as in *Ruditapes decussatus* (Tafalla et al., 2003) and the gastropod *Biomphalaria glabrata* (Hahn et al., 2001). In addition, cellular defense mechanism have been demonstrated through *in vitro* experiments in the cephalopod *Eledone cirrhosa* (Malham and Runham, 1998; Malham et al., 2002), *Euprymna scolopes* (Davidson et al., 2004; Nyholm et al., 2009) and *Octopus vulgaris* (Novoa et al., 2002; Rodríguez-Domínguez et al., 2006).

In the present work, we have studied the cellular response of *O. vulgaris* naturally infected by *A. octopiana*. The aim of the study is to understand the effect of the infection on the octopus cellular defense capability. Thus, phagocytosis, respiratory burst and nitric oxide radical's production were measured in octopuses harboring low and high degrees of coccidian infection. In order to determinate whether culture conditions have an additional effect on the octopus immune defense, the cellular defensive activities were also assessed between wild and reared octopuses in floating cages.

2. Material and methods

2.1 Biological material and hemolymph collection

In all, 110 live *O. vulgaris* (55 males and 45 females) with an average body weight (BW) of 935 g and mantle length of 80-220 mm (Table 1) were randomly collected by traditional traps, an artisanal gear used by local fishermen, from the Ria of Vigo, Spain (24 º 14.09´ N, 8 ° 47.18´ W). From these, 40 live octopus (15 males and 25 females) ranging 780-1915 g BW and 110-167 mm ML (Table 2) were collected at random off an ongrowing floating cage system in the Ria de Aldan, Galicia, Spain (NE Atlantic: 42° 15'N 8°48'W). The floating cage system is formed by 12 cylindrical cages of 10 m^3 each one and 250 PVC dens harboring 200 animals per cage. In the laboratory, octopuses were kept in culture tanks of open seawater system at 15 °C for 24 h to acclimate before experimentation.

According to ethical procedures for experimentation with cephalopods (Moltschaniwskyj et al., 2007), the animals were anesthetized whit 7.5% magnesium chloride ($MgCl_2$) (Messenger et al., 1985). A dorsal incision was made through the mantle muscle and hemolymph was withdrawn from the cephalic aorta. For each individual, a disposable syringe (1 ml) containing different solutions depending on each procedure (see below) was used. Hemolymph from each octopus was used immediately or transferred into a vial and kept on ice until use. Octopuses were dissected; sex and weight of reproductive organs were recorded to determine the gonadic stage according to Hayashi Index following Guerra (1975).

2.2. *Cell counting and hemocyte viability*

Crude hemolymph was withdrawn through the cephalic aorta using a disposable syringe (1ml). Immediately after bleeding, cell counting was carried out using a Neubauer chamber. Viability of octopus hemocytes was determined by Trypan blue exclusion test (Weeks-Perkins et al., 1995) in samples of fresh hemolymph at 15 °C.

2.3 Counting of coccidia Aggregata octopiana

The digestive tract of each octopus was dissected, weighted and homogenized in 10 ml of filter sea water (FSW) 1% Tween80 using an electric tissue grinder (IKA-Ultra Turrax T-25). Homogenates were filtered twice using a nylon mesh of 100 µm and 41 µm,

respectively to remove tissue. The filtrate was then centrifuged 1000 × *g*, 4 °C, 5 min in a centrifuge Beckman GS-15R. The number of sporocyst was counted in Neubauer chamber and standardized as number of parasites infecting a unit gram of each octopus digestive tract (spor/g). The intensity of infection was confirmed through observation of caecum sections processed by standard histological methods (Humason., 1979). According to the level of infection determined by the number of sporocyst per gram of digestive tract tissue, and the analysis of the histopathology observed in caecum sections, octopuses were classified into two groups: healthy (0 to 5×10^5 spor/g), without histological caecum damage; and sick (5×10^5 to $2{,}08 \times 10^7$ spor/g), showing a strong caecum damage.

Table 1. Biometric data recorded from total *Octopus vulgaris* specimens used for measuring cellular immune parameters (- denotes no data). Data are expressed as mean (minimum–maximum value recorded).

Season	BW [a] (g)	DML[b] (mm)	spor/g[c]	hemocytes/ml[d]	Sex[e]		Gonadic stage[f]			
					F	M	I	D	M	Ps
Winter	694 (175-118)	118 (80-160)	2.17×10^{6} (0.0×10^{0}–2.08×10^{7})	8.13×106 (2.00×10^{5}–2.30×10^{7})	19	25	11	6	26	1
Spring	1087 (168-775)	130 (85-220)	7.94×10^{5} (1.04×10^{3}–3.06×10^{6})	1.47×10^{7} (2.50×10^{6}–3.20×10^{7})	9	16	7	–	18	–
Summer	850 (332-915)	133 (90-200)	1.32×10^{6} (5.20×10^{5}–3.68×10^{6})	1.00×10^{7} (1.16×10^{6}–2.82×10^{7})	7	6	2	–	10	1
Autumn	1220 (536-824)	141 (110-190)	4.61×10^{6} (3.41×10^{5}–1.54×10^{7})	8.21×10^{6} (1.76×10^{6}–2.71×10^{7})	10	18	7	2	17	2

[a] Total body weight
[b] DML: Dorsal mantle length
[c] Spor/g: *A. octopiana* sporocyst per gram of octopus digestive tissue counted by Neubauer chamber.
[d] Hemocytes/ml: hemocytes counted by Neubauer chamber.
[e] Number of specimens classified per sex F: female, M: males.
[f] Number of individuals per gonadic stage according to Hayashi index following Guerra (1975). Stages of gonadic development: (I) immature, (D) developing, (M) mature, (Ps) post-spawning.

Table 2. Biometric data recorded from wild *Octopus vulgaris* and reared in floating cages (- denotes no data). Data are expressed as mean (minimum–maximum value recorded).

Origin	BW(g) [a]	DML[b] (mm)	spor/g[c]	hemocytes/ml[d]	Sex[e]		Gonadic stage[f]			
					F	M	I	D	M	Ps
Floating cages	1221 (780-1915)	138 (110-200)	4.36×10^6 (2.55×10^4–2.08×10^7)	7.21×10^6 (2.00×10^5–2.82×10^7)	30	40	7	3	29	2
Wild	772 (168-4775)	123 (80-220)	1.24×10^6 (0.0×10^0 –3.86×10^6)	1.14×10^7 (1.86×10^6–3.20×10^7)	15	25	20	5	43	2

[a] Total body weight
[b] DML: Dorsal mantle length
[c] Spor/g: *A. octopiana* sporocyst per gram of octopus digestive tissue counted by Neubauer chamber.
[d] Hemocytes/ml: hemocytes counted by Neubauer chamber.
[e] Number of specimens classified per sex F: female, M: males.
[f] Number of individuals per gonadic stage according to Hayashi index following Guerra (1975). Stages of gonadic development: (I) immature, (D) developing, (M) mature, (Ps) post-spawning.

2.4 Flow cytometry (FCM) assays of the immune-related activities of hemocytes

Flow cytometry protocols of phagocytosis and detection of respiratory burst were adapted from García-García et al. (2008). Cell viability was determined by Trypan blue exclusion test (Weeks-Perkins et al., 1995) in crude hemolymph and treated with SRS and FSW. The three functional essays were performed on each octopus when possible.

2.4.1 Phagocytic capability of hemocytes

The hemolymph withdrawn from each octopus was centrifuged 300 × *g*, 4 °C, 5 min. Plasma was discarded and replaced with the same volume of Squid Ringer Solution (SRS-25mM $MgCl_2$, 10mM $CaCl_2$, 10mM KCl, 530mM NaCl and 10mM HEPES buffer, pH 7.5) to avoid aggregation. Antiaggregant solution SRS was then discarded by centrifugation as mentioned above and re-suspended in filtered sea water (FSW). From this hemocyte solution, 100 µl were dispensed in triplicates into 96-wells plate. After 30 min of cell adhesion at 15 °C in the dark, 100 µl of flourescein-labelled 1.2 µm latex beads (Molecular Probes, Invitrogen) were added at a ratio of 1:10 (hemocyte:beads). Control hemocytes were exposed to FSW. After 2 h incubation at 15 °C in the dark, excess of beads was removed by gently washing twice with 100 µl PBS and attached cells were collected in 200 µl PBS supplemented with 20 µl of 0.8% trypan blue (in PBS) to quench external fluorescence. A total of 50,000 events were measured through the FL-1 channel. Results were expressed as the percentage of cells with at least one internalized bead. The experiment was performed in the hemolymph of 89 octopuses.

2.4.2 Respiratory burst assay

Production of oxygen radicals was measured by flow cytometry using the CM- 2',7'-dichlorofluorescein diacetate probe (DCFH-DA, Molecular Probes). DCFH-DA diffuses into the cells where the intracellular esterases cleave to DCFH, which is oxidized by reactive oxygen species (ROS) to the highly fluorescent DCF. Subsequent oxidation yields fluorescence proportional to intracellular ROS (Hégaret et al., 2003). One hundred microlitres of FSW diluted hemolymph (1:1) were placed into 96-wells plate. Cells were incubated 30 min at 15 °C in dark for cell adhesion. The supernatant was removed and 100 µl of FSW containing 5 µM CM-DCFDA and 0.4% DMSO final concentration were added per well. Cells were incubated 10 min on ice in the dark. The supernatant was removed and

hemocytes were washed twice with 100 µl FSW before being stimulated adding 100 µl zymosan (1 mg/ml). Fifteen microliters of SOD (300 U/ml) or 10 µl of N^G-methyl-L-arginine acetate salt (NMMA, Sigma M7033) inhibitors were added to determine whether H_2O_2 and NO, respectively, contributes to the oxidation of DCF-DA. Controls were exposed to the same volume of FSW. After 60 min of incubation at 15 °C in the dark, ROS were measured by flow cytometry after cells re-suspended in PBS. A total of 50,000 events were measured and data were collected as mean fluorescence of the sample. The oxidative activity is expressed as mean fluorescence in arbitrary units (A.U.). The assay was performed in the hemolymph of 71 octopuses.

2.5 Nitric oxide production (NO)

Production of nitric oxide was measured through the quantification of nitrites by the Griess reaction (Green et al., 1982). One hundred of hemolymph was placed into 96-well plates per triplicate. For each sample, hemocytes were stimulated with 100 µl of zymosan (1 mg/ml final concentration) and 100 µl of FSW were added to controls. Cells were incubated for 2 h at 15 °C in the dark. Then, 50 µl of each sample were placed in individual wells. One hundred of 1% sulfanilamide (Sigma) in 2.5% phosphoric acid, followed by 0.1% N-naphthyl-ethylenediamine (Sigma) in 2.5% phosphoric acid was added to each well. The optical density at 540 nm was measured after 5 min of incubation at room temperature using a Multiscan spectrophotometer (Labsystems). The molar concentration of nitrite in the sample was determined from standard curves generated using known concentrations of sodium nitrite. The essay is based on 89 octopuses.

2.6 Statistical analysis

The mean data of immune parameters (phagocytosis, ROS, NO) were compared between groups of healthy and sick octopuses using a Student's *t*–test. Results are expressed as the mean ± standard error of the mean (ESM). Differences were considered significant at $p \leq 0.05$.

The octopus cellular responses (phagocytosis, ROS and NO production) were standardized for each individual as follows: cellular response +/ number of circulating hemocytes per ml. In order to determinate the effect of *A. octopiana* infection on the standardized immune parameters, a multiple linear regression analysis was performed

including total amount of infection and groups of infection (healthy/sick) as independent variables. Possible interactions between amount and group of infection were included. In addition, octopus biometric factors (sex, weight, length and gonadic stage), season of collection and origin (wild or reared in floating cages) were also included in the analysis to study their relationship with the octopus immune parameters. A stepwise (backward) procedure based on the Akaike's information criterion (AIC) was used to select the final model; that is at each step, the variable leading to the minimum AIC was identified and removed from the model. All the statistical analyses were performed using the R software (R Development Core Team, 2006).

3. Results

3.1 General pattern of prevalence and intensity of infection

A total of 99% (109/110) of octopuses analyzed were found infected by the protozoan *A. octopiana*. The intensity of infection ranged from 0 to 2×10^7 spor/g. Gamogony and sporogony life stages were observed in sick octopuses. Both stages were occasionally surrounded by pericyst reactions of connective tissue, and strong hemocytic infiltration and big tissue distension was observed in the caecum and intestine, causing rupture of the basal membrane (Fig.1A). In cases of heavy infection host cells undergo necrosis. Most of the infected tissue is replaced by parasites, which leads to a loss of intestinal epithelium and destruction of the tissue organ architecture (Fig. 1B). In contrast, no significant inflammation neither pericyst reaction was found in caecum tissue of healthy octopuses (Fig. 1C, D).

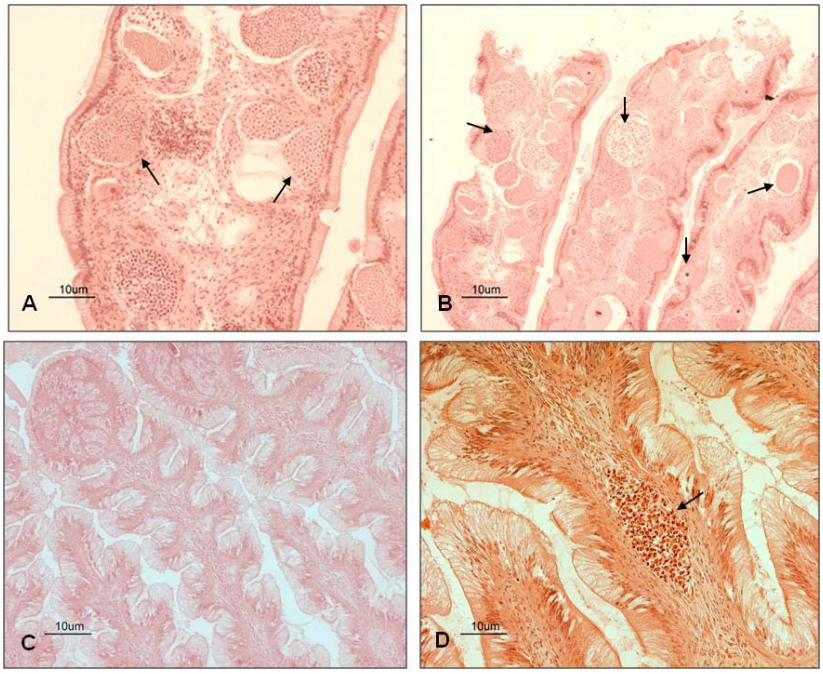


Fig. 1. Histological sections of *O. vulgaris* digestive tract. (A) Infected tissue showed distention. (B) Rupture of tissue organ architecture showing sporogonial stages. (C) Digestive tissue from lowly infected octopus. (D) Single oocyst infecting digestive tissue of lowly infected octopus.

3.2 *Cellular immune parameters*

3.2.1. Hemocyte density

Total circulating hemocyte counts ranged between 2×10^5 and 3×10^7 cells/ml. Hemocyte viability achieved through the Trypan blue exclusion test was ≥95% in crude hemolymph and ≥90% in samples treated with SRS and FSW.

3.2.2. Phagocytic capability of hemocytes

Phagocytosis measured from octopus hemocytes showed wide variations (1-56%, SEM ± 1.1). When comparing groups of infection, the percentage of phagocytic hemocytes was higher in sick than healthy octopuses (Fig. 2A). The multiple regression analysis performed showed that an increase in the intensity of total amount of infection leads to a

significant increase of phagocytosis (Table 3). The interaction between infection amount and group of infection (sick/healthy) was not significant. Regarding the remaining variables, season of collection and octopus sex also stayed in the final model, although the influence of sex was not significant; phagocytosis in autumn was significantly higher than in winter and spring (p=0.00155). Thus, the group of variables total infection, sex and season included in the model through the automatic backwards stepwise AIC criterion explained the 27.65% of the variance observed in the phagocytic ability of the hemocytes.

3.2.3 Respiratory burst

Respiratory burst measured in total octopuses showed a mean of 12 A.U. (ESM± 0.94). When comparing groups of infection, ROS production was slightly higher in sick than in healthy octopuses (Fig. 2B). An increase in the total coccidian infection leads to a significant decrease of ROS production (Table 3), while the interaction between infection amount (total infection) and group of infection (sick/healthy) was not significant. In addition to infection, the variable origin was the only retained by the model (p=2.86e^{-5}), showing that wild octopuses produced less ROS than those reared in floated cages. The group of variables total infection and origin (reared/wild octopuses) retained by the model explained the 24.35% of the variance observed in ROS production.

3.2.4 Assay of nitric oxide (NO)

The NO produced by octopus hemocytes was similar between sick and healthy individuals (Fig. 2C). The regression analysis performed showed that there is a significant interaction (at a 10% of significance level) between infection amount and group of infection (p=0.0617), indicating that the increase of infection causes a decrease in NO production only for lowly infected octopuses (Table 3). Concerning additional variables, octopus weight and origin were included by the model, being both of them significant at $p\leq$ 0.1 and $p\leq$ 0.01, respectively. The NO production decreases in heaviest octopuses. When comparing wild and reared specimens, a lower NO production is yielded by wild octopuses than reared in floating cages (Table 3). Thus, according to the variables retained by the model, infection, octopus weight and origin explained the 17.25% of variation in NO production.

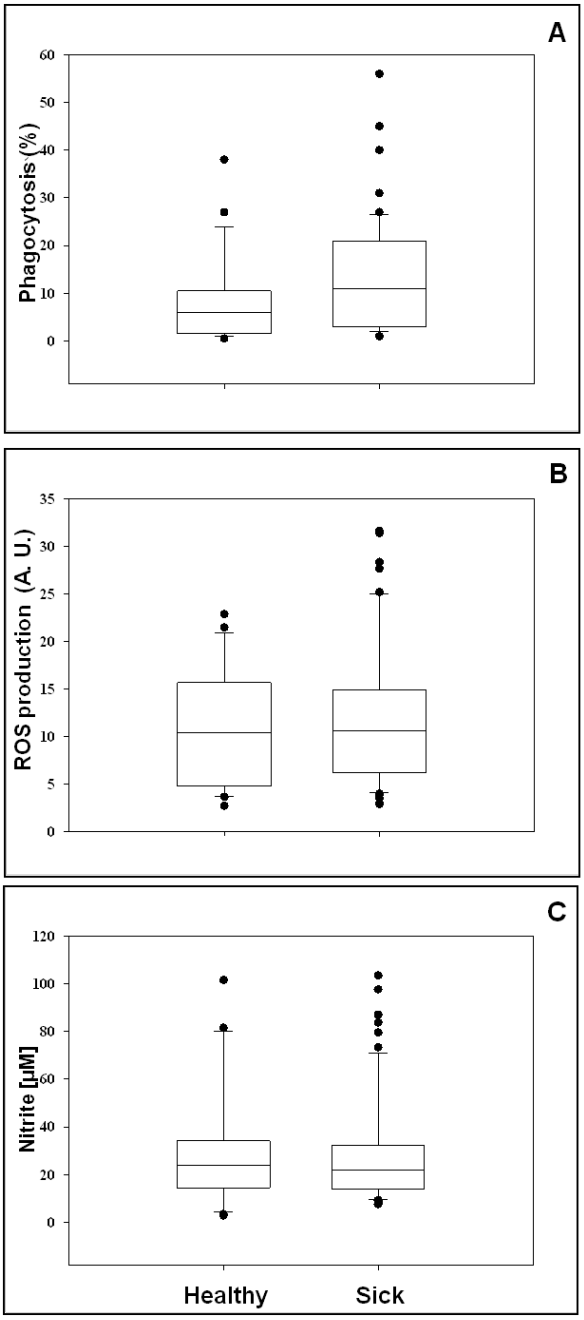


Fig.2. Cellular defensive activities measured in healthy (low infection) and sick (high infection) *O. vulgaris* hemocytes. (A) Phagocytic percentage recorded in hemocytes. (B). Respiratory burst, and (C) Nitric oxide (NO) produced by hemocytes from both octopus infection group.

Table 3. Relationship between octopus cellular immune response, total infection (recorded from whole specimens), infection by group and gonadic development.

		Estimate	Std. Error	*t*-value	P (>|*t*|)
Phagocytosis	Intercept	$3.446e^{-7}$	$4.925e^{-7}$	0.700	0.4860
	Total infection	$1.496e^{-13}$	$7.366e^{-14}$	2.031	0.0454 *
	Autumn	$2.080e^{-6}$	$6.353e^{-7}$	3.274	0.00155 **
	Spring	$-3.237e^{-7}$	$6.529e^{-7}$	-0.496	0.62135
	Summer	$1.049e^{-6}$	$8.526e^{-7}$	1.230	0.22204
	Sex (Male)	$8.010e^{-7}$	$4.933e^{-7}$	1.624	0.1082
ROS	Intercept	$7.198e^{-6}$	$1.145e^{-6}$	6.285	$2.68e^{-8}$ ***
	Total infection	$-5.746e^{-13}$	$2.110e^{-13}$	-2.724	0.0082 **
	Wild	$-4.928e^{-6}$	$1.098e^{-6}$	-4.487	$2.86e^{-5}$ ***
NO	Intercept	$2.004e^{-5}$	$5.379e^{-6}$	3.726	0.00035 ***
	Total infection	$-6.450e^{-13}$	$5.804e^{-13}$	-1.111	0.2697
	Healthy (Low infection)	$1.932e^{-5}$	$6.576e^{-6}$	2.938	0.0042 **
	Weight (g)	$-5.518e^{-9}$	$3.118e^{-9}$	-1.770	0.0805 ·
	Wild	$-1.409e^{-5}$	$4.473e^{-6}$	-3.150	0.00228 **
	Interaction (total infection: low infection)	$-4.959e^{-11}$	$2.618e^{-11}$	-1.895	0.06170·

**P<*0.05

***P<0.01*

****P<0.001*

·P<0.1

4. Discussion

The coccidian *A. octopiana* is a common parasite recorded in *O. vulgaris* at high prevalence and variable intensity of infection (Pascual et al., 1996). This coccidia causes severe digestive tissue rupture, strong hemocytic infiltration and affects the correct nutrient absorption (Gestal et al., 2002a,b). The infection is as much a cause of malnutrition as a consequence, and therefore, it could be difficult to ascribe the cause and effect once a severe infection is established. Nonetheless, the infection plays a major role in presentation of malnutrition (Hughes and Kelly, 2006) that affects the cell-mediated immune response (Fekete and Kellems, 2007). Because the recognized negative impact of coccidiosis in the octopus nutrition, the present study assesses the effect of the coccidian *A. octopiana* on the cellular immune defense of *Octopus vulgaris*.

Quantification of the strength of associations is valuable to understand the relative importance that different factors and forces have on pathologies. In the current work biometric variables (sex, weight, length, gonadic stage), season of collection, infection and origin (wild/reared in floating cages) were tested to known their relationship with the octopus cellular immune response.

Season of collection, particularly autumn, was the most important variable associated to phagocytic ability of hemocytes and related to *A. octopiana* pathology. According to Gestal (2000) the highest intensity of coccidia infection in the Ria of Vigo are recorded from autumn to spring, when the putative *A. octopiana* intermediate host *Palaemon serratus* is highly available. According to the data, here is verified that the highest intensity of coccidian infection is recorded in autumn, but season itself is important for phagocytic ability even when comparing specimens with the same amount of infection. Seasonality determinates the intensity of infection in the octopus and here is showed that phagocytosis is significantly stimulated by the infection of coccidia, mainly in autumn. Phagocytosis is an effective mechanism developed by hemocytes against pathogen invaders (Cheng, 2000) and thus this mechanism could acts against coccidia infection. According to the obtained results, an increase in the intensity of infection induces an increase in the phagocytic activity of hemocytes. In *O. vulgaris*, coccidiosis results in a self-limiting disease. Therefore, the gradual increase of infection by *A. octopiana* does not disrupt the phagocytic ability of hemocytes, except in severe outbreaks (infection of 2×10^7 spor/g) when phagocytosis was almost inhibited. At this level of infection malabsorption syndrome causes a gradual onset of host weakness, being

favorable for concurrent parasitic and bacterial secondary infections (Gestal et al., 2002b, 2007).

Internalization and following destruction of pathogens are accomplished by a cytotoxic response through the production of oxygen and nitrogen radicals (Cheng, 2000). The respiratory burst assay measures the reactive oxygen species (ROS) elicited by hemocytes for pathogen destruction, through fluorescence proportional to radicals produced by cells (Hégaret et al., 2003). Thereby, our results showed that hemocytes from sick octopuses were not able to yield a high respiratory burst. Hence, it is suggested that *A. octopiana* infection restricts the capability of ROS production, which is a mechanism of resistance used by parasites and bacteria to prevent the oxidative burst associated with phagocytosis (Canesi et al., 2002). Whilst ROS is efficiently produced in healthy octopuses, a decline started in octopuses infected by 4×10^6 spor/g with subsequent decrease according to the infection degree increase. The present result is consistent with Volety and Chu (1995) that recorded not significant reduction in respiratory burst, measured by chemiluminiscence, in *Crassosstrea virginica* hemocytes exposed to 3×10^6 live *P. marinus* cells. However, respiratory burst was suppressed in oyster hemocytes exposed to 7.5×10^6 – 5×10^7 live *P. marinus* cells (Volety and Chu, 1995; Anderson et al., 1999). Probably, a similar suppression pattern is observed in *O. vulgaris* harboring 8×10^6 up to 1.2×10^7 *A. octopiana* spor/g, suggesting a similar strategy used by *A. octopiana* and *P. marinus* to infect the host cells without triggering the host' cell respiratory burst. Whether *A. octopiana* possess a mechanism to suppress ROS and how it acts on the octopus hemocytes, remains to be clarified. However, it could be achieved through the production of acid phosphatase or proteases like *P. marinus* (Volety and Chu, 1995; Garreis et al., 1996), *Pseudoperkinsus tapetis* (Ordás et al., 2000; 2001) and *Bonamia ostrea* (Morga et al., 2009).

Nitric oxide (NO) is a highly reactive and unstable free-radical gas used as antiparasitic by vertebrates and invertebrates because the strong reactivity of NO with oxygen and reactive oxygen species (Rivero, 2006). The capability for producing NO has been demonstrated in *O. vulgaris* hemocytes (Novoa et al., 2002) as in other molluscs like *Ruditapes decussatus* (Tafalla et al., 2003), *Lymnaea stagnalis* (Wright et al., 2006), *C. virginica* (Villamil et al., 2007) and *Ruditapes philippinarum* (Jeffroy and Paillar, 2011). However, no studies had been performed related to the association between the NO production and the level of cephalopod host infection by *A. octopiana*. Our results showed that healthy octopuses are capable to release NO. However, an increase in *A. octopiana* infection affects the NO production causing a decrease in cytotoxic activity. Differences in

NO production against parasitic infections reflect the role of this molecule in the host defensive response. For example, a study performed in *C. virginica* demonstrated that infection with *P. marinus* induces a rapid NO increase in oysters. Consequently, NO could be an important molecule for preventing proliferation of *P. marinus* in *C. virginica* (Villamil et al., 2007). Contrarily, any significant difference was found in NO produced by *Ostrea edulis* infected and non infected by *B. ostrea,* suggesting that this molecule is not an effective mechanism to eliminate the parasite (Comesaña et al., 2012). Taking in count differences in molluscan defensive response and that nitrite decrease according to *A. octopiana* infection increase, the obtained results showed that NO could be effective to avoid the proliferation of *A. octopiana*, but seems to be suppressed to favors its own survival. Moreover, decrease in NO is markedly in heaviest octopuses. A cumulative pattern of infection is commonly observed in filter feeder bivalves due to an increased filtration rate in biggest individuals (Villalba et al., 2005; Flye-Sainte-Marie et al., 2009). Similarly, the *A. octopiana* infection, which is transmitted through the food-web, have also a cumulative effect of the parasite during life span, leading to an increase of infection intensity with size and weight in octopuses caught in the Ria of Vigo (Gestal, 2000). Consequently, the current decrease of NO in heaviest individuals could be linked to the strong coccidia infection.

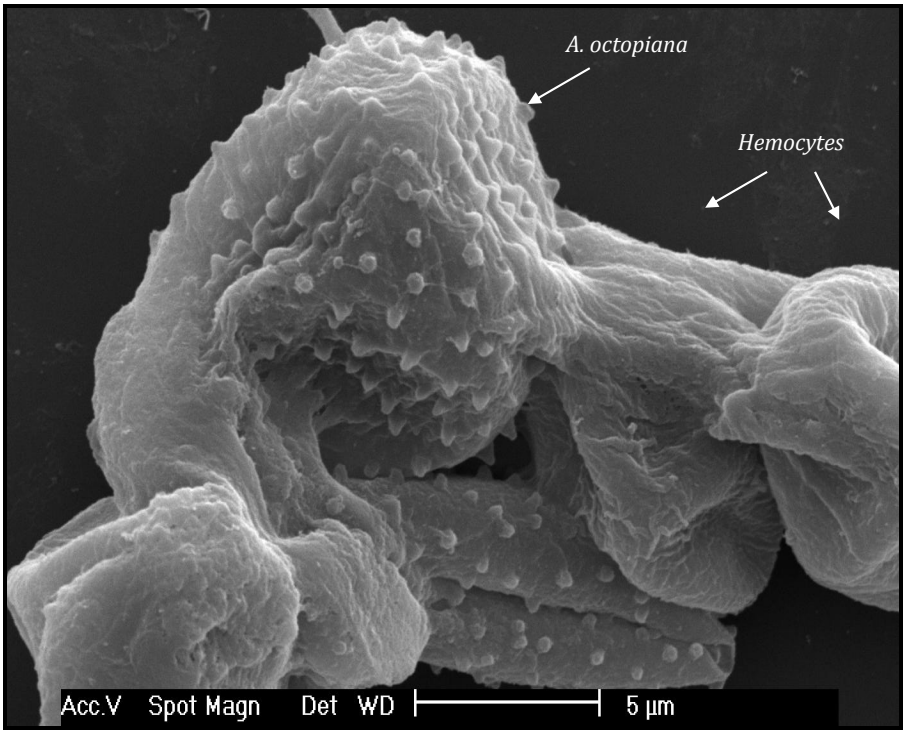
In addition to the infection effect, the cellular immune response between wild and reared octopuses in floating cages was studied. According to AIC, the condition of wild or reared in floating cages was only retained for models of cytotoxic cellular response. Wild octopuses exhibited lower ROS and NO production that those reared in floating cages. Thus, it implies that stressful conditions in floating cages make octopus trigger a highest ROS and NO production. Gestal et al. (2007b) recorded a decrease in plasmatic proteins and muscular protein concentration in octopuses reared in floating cages. The data herein provided complement those findings showing that stressful culture conditions could maximize the negative impact of coccidia infection on the octopus cellular immune response. Hence, these results also supports the fact that *A. octopiana* is a dangerous pathogen for octopus culture (Gestal et al., 2007b).

In conclusion, the present study investigated the effect of *A. octopiana* on the octopus cellular immune response. Here is evidenced that healthy octopuses (lowly infected) has higher phagocytic activity than sick (highly infected) ones, but respiratory burst and NO decreased when *A. octopiana* proliferation occurs. In addition, here is showed that a high cytotoxic response measured in octopuses reared in floating cages could be derived from the synergic effect of stress developed in culture conditions and

coccidia infection. The cellular immune parameters measured represented a suitable tool for assessing the immunological status of *O. vulgaris* naturally infected by *A. octopiana*. Additionally, the present study emphasizes the need for supplementary studies to complement the immunological assessment and to disclose the octopus genes responsible for coccidia resistance.

Chapter 4

Transcriptomic analysis of the *Octopus vulgaris* hemocytes: response to the *Aggregata octopiana* infection



Octopus vulgaris hemocytes attacking *A. octopiana* sporocysts (Original)

Abstract

Octopus vulgaris is an important organism for aquaculture; however, the octopus well-being is impaired by different pathogens, being one of the most important the gastrointestinal the coccidian parasite *Aggregata octopiana*. The coccidiosis induces malabsorption syndrome in octopus, which is reflected in economical losses. To date, the molecular basis of octopus resistance to pathogens in general, and coccidiosis in particular, are unknown. Therefore, the present study provides the first insights of the *O. vulgaris* resistant/susceptibility at the transcriptomic level using high-throughput Illumina sequencing technology. A comparative gene expression analysis of the hemocytes of octopuses showing high and low parasite infection degree by *A. octopiana,* corresponding to sick and healthy individuals, respectively, was performed. A total of 75,571,280 high quality reads were obtained for high infected octopus group, and 74,731,646 for low infected ones. The reads were assembled in 254, 506 contigs from which 48,225 contigs were successfully identified. The presence of putative immune-related genes involved in several immune pathways like NFkB, TLR signalling pathway, complement cascade and apoptosis were identified. In addition, a total of 539 genes were found differentially expressed between sick and healthy individuals. Of these, up and down regulation of different genes involved in immune recognition and host-pathogen interaction was confirmed by RT-qPCR in samples of high infected octopuses. New data herein provided establishes the molecular basis for searching biomarkers of octopus immune response and pathogen resistance that will contribute with valuable knowledge for improving the octopus aquaculture.

1. Introduction

Octopus vulgaris is the most important octopus species in worldwide fisheries (Boyle and Rodhouse, 2005; Globefish, 2007). It represents a major protein resource in most fish-eating countries, being of great commercial importance in Mediterranean, South American and Asian countries as well as Atlantic at NW Spain and Portugal (Otero *et al.*, 2005). The octopus culture on an industrial scale has gained increasing attention due to the declining of landings by fishery and the socio-economic relevance of this species (Iglesias et al., 2007). The octopus culture in tanks and in floating cages (Sendao, 1998; Chapela et al., 2006; Iglesias et al., 2007) have showed favorable results, however, mortality have also been recorded (García-García and Cerezo-Valverde, 2006; Prato et al., 2010; Estefanell et al., 2011), which has encouraged studies on the disease caused by different pathogens (Gestal et al., 2002b; 2007).

The gastrointestinal coccidian parasite *Aggregata octopiana* (Protozoa: Apicomplexa) has been noted as the most important epizootiological agent in wild and cultured octopus stocks from European waters (Pascual et al., 1996; Gestal, 2000). The infection by *A. octopiana* induces ulceration, partial destruction of the digestive tract and decrease or malfunction of absorption enzymes (Gestal et al., 2002a,b). Although enteritic coccidiosis is not a primary cause of death, it is likely that the malabsorption syndrome produced, may impair the octopus growth and health (Gestal et al., 2002b).

Mollusc hemolymph, specifically hemocytes, is the most important factor in different physiological functions, such as nutrition and detoxification. Moreover, mollusc hemocytes play also a major part in the cellular defence against pathogens (Cheng, 1975). Although molluscs lack a specific immune system, the innate response involving circulating hemocytes and molecular effectors seems to be an efficient defence method to respond to external aggressions by detecting the molecular signatures of infection. The role of the hemolymph and hemocytes in physiological functions and immune system of bivalve molluscs have been the objective of a large quantity of studies. However, few data have been published related to cephalopods until present. Thus, few studies are focussed on functional immune assays on the white octopus *Eledone cirrosa* (Malham et al., 1997; 1998), the Pacific sepiola *Euprymna scolopes* (Nyholm et al., 2009; Davison et al., 2004; Altura et al., 2011; Goodson et al., 2005; Koropatnick et al., 2007) and only a couple of works are found in *O. vulgaris* (Rodríguez-Domínguez et al., 2006; Novoa et al., 2002; Castellanos and Gestal, 2013 in press).

Cephalopods shares characters with lower invertebrates, but also shows advanced features such as no larval phase in ontogenesis, a vertebrate-like eye, a highly centralized nervous system and a close circulatory system, where the hemolymph is restricted to blood vessels and capillaries. Therefore, all these characters suggest that cephalopods are a special and high evolved branch of molluscs. In fact, cephalopods has served as models for neurobiological (Grant et al., 2006), learning (Robertson et al., 1994, 1996) and circulatory system studies (Wells and Smith, 1987). However, the development of molecular biology research in cephalopods and in *O. vulgaris* in particular has been scarce. To date, transcriptomic studies have been restricted to an ecological framework in the sepiolid *E. scolopes* through cDNA libraries (Chun et al., 2006) and EST collections (Goodson et al., 2005; Castillo et al., 2009). Related to the common octopus, the only genomic studies performed was a comparative analysis of gene expression carried out through an EST collection of the *O. vulgaris* ocular chamber (Ogura et al., 2004). Recently, Illumina Solexa sequencing technology was employed to characterize the transcriptome of the central nervous system of *O. vulgaris* (Zhang et al., 2012), and 454 pirosequencing have also been employed to understand the role of circulating hemocytes of *E. scolopes* (colonized by the symbiotic bacteria *Vibrio fischeri)* in the squid/*Vibrio* association (Collins et al., 2012).

Illumina short reads have been used to build transcriptomic datasets in non-model species (Feldmeyer et al., 2011; Riesgo et al., 2012). The combination of short inserts and longer reads increase the ability to fully characterize any genome or transcriptome. Thus, the assembly of short reads data into accurate, contiguous transcript sequences demonstrates that assembly of long, potentially full-length transcripts assemblies is indeed possible (Mizrachi et al., 2010). In addition, the relative low cost and good results obtained in *de novo* transcriptome sequencing from different organisms make the Illumina RNA-Seq technology (Paired-End, 100 bp reads) an useful tool for the study of the octopus immune response transcriptome.

In this study, we present the first hemocyte transcriptomic analysis of the cephalopod *O. vulgaris* by generation of a *de novo* sequencing an annotation of transcripts from the octopus hemocytes and thus, improving significantly the amount of mollusc data available to the specific community. In addition, the study here presented provides new information of the transcriptional expression pattern of the octopus hemocytes against the coccidiosis. Moreover, here we present new information for future research on the development of immune-related genetic markers, providing a useful tool for future

aquaculture applications on selective pathogen-resistant programs of this economically important species.

2. Materials and Methods

2.1. Animal sampling hemolymph extraction and counting of the coccidian A. octopiana

Specimens of *O. vulgaris* naturally infected by *A. octopiana* were collected by traps, an artisanal fishing gear used by local fishermen from the Ria of Vigo, Spain (24° 14.09'N, 8° 47.18'W). The octopuses were maintained in filtered sea water tanks at 15 °C during 24 h. Before hemolymph extraction, each octopus was anaesthetized using 7.5% magnesium chloride ($MgCl_2$) according to Messenger et al. (1985) and following ethical procedure (Moltschaniwskyj et al., 2007). A dorsal incision was made through the skin and mantle muscle behind the head to withdraw hemolymph with a disposable syringe (1 ml) directly inserted into the cephalic aorta. One milliliter of hemolymph of each octopus was centrifuged at 12000 × *g*, 4 °C for 5 min. The pellet of hemocytes was re-suspended in 1 ml of Trizol reagent (Invitrogen) and stored at -80 °C until proceed to analysis.

The digestive tract from each octopus was dissected and homogenized in 10 ml of filter sea water (FSW) 1% Tween80 using an electric tissue grinder (IKA-Ultra Turrax T-25). To remove tissue fragments, the homogenates were filtered twice with a nylon mesh of 100 µm and 41 µm, respectively. The filtrate was then centrifuged 1000 × *g*, 4 °C, 5 min in a centrifuge Beckman GS-15R. Finally, the number of sporocyst was counted in Neubauer chamber. The sporocyst number is referred as the number of parasites infecting a unit gram of octopus digestive tract (spor/g) in order to state the infection degree. The intensity of infection was confirmed through the observation of caecum sections processed by standard histological methods (Humason, 1979). Hence, taking into account the level of infection and the histopathological damage, two groups of infection were formed: the first one, with a high parasite load (6×10^6 to 2×10^7 spor/g; hereafter termed high infection group); and the second one, with a low parasite load (0 to 2×10^3 spor/g; hereafter termed the low infection group). Samples from the first group showed important caecum damage, and were considered as being from sick animals. The second group of samples with a light or no histological caecum damage was considered as being from healthy animals. Both groups of infection were confirmed using a Student's *t*-test analysis ($p<0.05$) performed in Statistica 6.0.

2.2. RNA isolation, paired-end mRNA Seq library preparation and sequencing

Total RNA from hemocytes of 5 highly and 5 lowly infected octopuses selected for each group was extracted according to the Invitrogen protocol. After RNA extraction, samples were treated with Turbo DNase free (Ambion) to eliminate DNA. The RNA samples were purified using RNeasy Mini Kit (Quiagen), quantified using a NanoDrop ND1000 spectrophotometer and the RNA quality was assessed in a Nano and Pico Chips Bioanalyzer (Agilent). A total of 1.5 μg of RNA from each of the 5 animals per group was pooled to construct the mRNA libraries according to Illumina standard protocol. Thus, two mRNA libraries (one from the pool of octopuses with high infection, and one from the pool of low infection) were performed in a Genome Analyzer (GAII) at the Functional Genomics Service (Progenika Biopharma Company, Vizcaya, Spain). Briefly, mRNA was purified using oligo (dT) probes and then fragmented into small pieces using divalent cations under elevated temperature. The cleaved RNA fragments were used for first strand cDNA synthesis using random primers. This was followed by second strand cDNA synthesis using DNA polymerase I and RNaseH. The products were purified and enriched for attachment to the Illumina flow cell. The two hemocyte libraries were generated using the Paired-end Cluster Generation kit v4. The libraries were validated by processing an Agilent DNA 1000 chip on a 2100 Bioanalyzer (Agilent) and quantified by qPCR using complementary primers of the library adapters with the KAPA SyBR FAST Universal qPCR kit (KAPA Biosystems). The cDNA libraries were sequenced on the Illumina sequencing platform (GA III) equipped with a paired-end module performing 105 cycles per read on two flow cell lanes.

2.3. De novo Transcriptome generation: transcript assembly

Prior to assembly we applied filters to remove low quality reads and bases, by using Condetri (Smeds and Künstner, 2011). Base trimming was done from the 3´end of each read to remove bases with a quality less than Q20 up to a minimum length of 80 bases. Reads not reaching the 80 nucleotide length were removed from further analysis. ConDeTri allows filtering in a paired manner. The filtered Illumina paired-end and remaining orphan reads for both sequenced samples were used together for assembly. First, an initial assembly was performed using Trinity (Grabherr et al., 2011). The Trinity assembly was then used as a long sequence to help re-assembly with VELVET (Zerbino and Birney, 2008). Finally, Oases is used to produce transcript clusters called contig (Schulz et al., 2012). The use of these two pieces of software allows us to test a wide range of K-mer lengths (25 for Trinity and 31, 35, 39, 43 for Velvet) and algorithms for assembly,

and to obtain a consensus transcriptome that may cover the hemocyte transcriptome spectrum. Sequence assembly was performed as shown in Fig. 1.

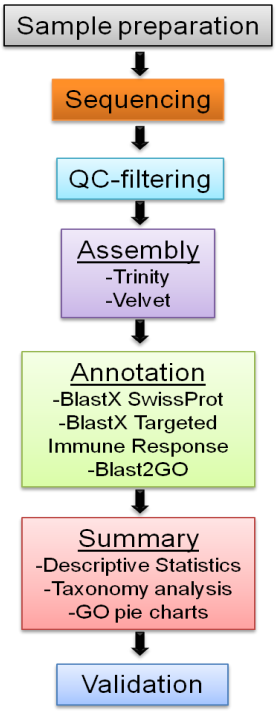


Fig. 1. Sequence of tasks and data processing for obtaining the cDNA library of the *O. vulgaris* hemocytes.

2.4. Assembly validation and Functional annotation

To assess the coverage of the assembly, homology search of the assembled transcriptome was performed against the Swissprot using BLASTx with an E-value threshold of $1e^{-3}$. BLASTx results were passed through a custom Perl script that merged the assembly Fasta sequence and summarized information to produce a table. Functional annotation was performed using Blast2GO v2.5.0 (Conesa et al., 2005; Conesa and Götz 2008; Götz et al., 2008) using the default annotation parameters (Blast e-value threshold of $1e^{-3}$, Gene Ontology annotation threshold of 55). The Gene Ontology (GO) terms associations for "Biological process", "Molecular function" and "Cellular component" were performed using BLASTx algorithm against the Uniprot database.

2.5. Comparative analysis

The library of the *O. vulgaris* hemocytes here generated was compared with sequences of the cephalopods *Euprymna scolopes* (35,420 ESTs) and *O. vulgaris* (31,929 ESTs)*;* and the bivalves *Mytilus galloprovincialis*, (19,617 ESTs), *Crassostrea gigas* (206,388 ESTs) and *Ruditappes philippinarum* (23,649 ESTs) deposited in NCBI public database. BLASTn algorithm was carried out for testing the sequence similarity with a threshold e-value less than $1e^{-5}$. The sequences were compared with the longest contig from each of the transcripts identified in *O. vulgaris hemocytes*.

2.6 Identification of immune-related genes

To identify the putative genes involved in the immune response, the sequences obtained in this study were screened using the GO terms at level 2 assigned to each sequence after annotation and confirmation of its relationship with the immune response. They were also revised based on an immune system process and response to stimulus keyword list elaborated in our lab. BLASTx was used to identify the putative immune related transcripts looking for these specific keywords in the hit descriptions of proteins of the NCBI database which had been demonstrated to have involved in immune response. The immune-related genes identified from our high-throughput sequencing results were grouped in 4 different pathway related to complement, Toll-like receptor, NF-κB and apoptosis.

2.7. Transcripts differentially expressed against the infection

Differential expression of transcripts between conditions (sick and healthy animals) was evaluated with TopHat (Trapnell et al., 2009) and Cufflinks (Trapnell et al., 2010) using the generated assembly as reference for mapping of reads for each condition and determining the relative transcript abundance by measuring FPKM (expected fragments per kilobase of transcript per million fragments). All *p*-values were adjusted with a false-discovery rate (FDR) correction for multiple testing by the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). The transcripts were considered significant at $p<0.05$.

2.8. Expression analysis of selected genes by quantitative real time PCR (RT-qPCR)

The differential expression of eight genes selected from the transcriptomic library and related to the innate immune response were analysed by RT-qPCR in three different tissues. Total RNA was extracted from hemocytes, caecum and gills of 5 individual octopus

from each infection group (showing high and low infection by *A. octopiana*) using TRIZOL reagent (Invitrogen) and following the manufacture's instruction. The RNA concentration was quantified using a NanoDrop ND2000 spectrophotometer (Thermo Scientific). First strand cDNA was synthesized using Maxima First Strand cDNA Synthesis Kit for RT-PCR (Thermo Scientific) using 1 μg of total RNA, treated with DNAse (QUIAGEN) to remove remaining genomic DNA. For each of the selected genes, forward and reverse primers were designed using primer 3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) (Table 1). PCR efficacy (*E*) was calculated for each primer pair by determining the slopes of standard curves according to Pfaffl (2001). The β-actin gene was determined as the best reference gene (HKG) through NormFinder (Andersen et al., 2004), geNorm (Vandesompele et al., 2002) and Bestkeeeper (Pfaffl et al., 2004) algorithms. RT-qPCR reactions were performed in triplicate and in a total volume of 25 μl using a 7500 FAST Thermocycler (Applied Biosystems) sequence detector in 96-microwell plates. Each well contained 1 μl of cDNA (dilution 1/10), 12.5 μl of SYBR green PCR master mix (Thermo Scientific) and 0.5 μl of each diluted primer (10μM). The standard cycling conditions were two steps method: 95 °C for 10 min; 40 cycles of 95 °C 15 s, and 60 °C for 1 min. The expression of selected genes was normalized using β-actin gene and analyzed following the Pfaffl method (2001). Results were expressed as the mean ± standard deviation. Fold units were calculated dividing the normalized expression values of tissues samples in sick individuals by the normalized expression values of healthy ones. Data were analyzed using a Student's *t*-test and differences were considered statistically significant at $p<0.05$.

Table 1. Primer sequences used in RT-qPCR.

Primer name	Primer sequence 5´– 3´	Amplicon bp
TLR F	TATGGGTACCTGCAGATGGT	137
TLR R	TGAAAGCTGCTCATGTGAAA	
Caspase F	AAGGTTCGTGGTTTATGCAA	115
Caspase R	TGGAATTCTAAAGAGGCAACA	
Galectin-F	TCCCTCTCCATCTCAATCCAA	100
Galectin-R	ACAGGCAATGGATGTCGTTCT	
C1q-F	ACCAAGGTGGCACTGAGA	130
C1q-R	TCGCCCTCATGGAGAGT	
PGRP-F	GAGCTGCTCCACAACTGC	119
PGRP-R	CGACACCATTTCCACCA	
Serpin-F	TGACAAATGCTGAGAAGACAAGAAT	111
Serpin-R	GAACCGATTGAGGTGTCAAACTT	
LITAF-F	CGGCCCAGAACCAAAAGAA	100
LITAF-R	TCCAGAGACCAGCCATGTTAAA	
PRDX-F	CCAGTGCCAGTCTCTTTGAACA	100
PRDX-R	AGTGCACCTGGTACACCAAAAA	

3. Results and Discussion

The general goal of this study was to generate a representative set of genes expressed in hemocytes, the cells responsible for cellular defence of the common octopus. The information herein obtained would be useful for further analyses of comparative immunology and cephalopod's immune response against pathogens.

3.1. Illumina sequencing and reads assembly

To obtain the *de novo* transcriptome of the circulating hemocytes from adult octopuses, and to analyze the octopus gene expression profile against the infection by the parasite *A. octopiana*, the paired-end Illumina sequencing platform was used. A total of 150,302,926 raw reads with an average length of 105 bp were produced (Table 2). The

Q20 percentage (sequences of high quality indicator) was of 97.6% (75,571,280 reads) for the pool of high infected individuals, and 97% (74,731,646 reads) for the pool of low infected individuals. After filtering to remove low quality reads, a total of 127,019,711 (84.5%) clean reads were obtained from both pools of samples. Reads from both levels of infection were assembled together sequentially with Trinity (Grabherr et al., 2011) and VELVET (Zerbino and Birney, 2008). In this manner, the transcriptome reflects specific genes from each infection group plus additional genes putatively common to both groups of infection. Further alignment of sequences belonging to each infection group against the entire transcriptome generated allowed us detecting the gene expression from each case. Trough assembly, 254, 506 contigs were generated with a mean length of 669 bp and a maximum of 19,120 bp (Table 2). Hence, the theoretical transcriptome length for *O. vulgaris* was 170.24 Mb. The distribution of contig length and the number of contigs by cluster are shown in Figure 2.

Table 2. Summary statistics of the transcriptome sequencing and assembly for *O. vulgaris* hemocytes library.

Sequences before Filtering	
Number of reads	15,030,2926
Total Megabases	15781,8
Sequences after Filtering	
Number of reads	127019711
Total Megabases	13180,8
Assembly Statistics	
Number of reads assembled	42826899
Number of contigs	254506
Total consensus Megabases	170,24
Average contig length	669
N50 contig length	1632
Range contig length	100-19120
Number of contigs >500bp	87408
Number of clusters	228314
Number of clusters with 1 contig	214607
Number of clusters with >1 contig	13707
Percentage of contigs annotated by SwissProt	18,9%
Percentage of contigs functionally annotated	13,7%

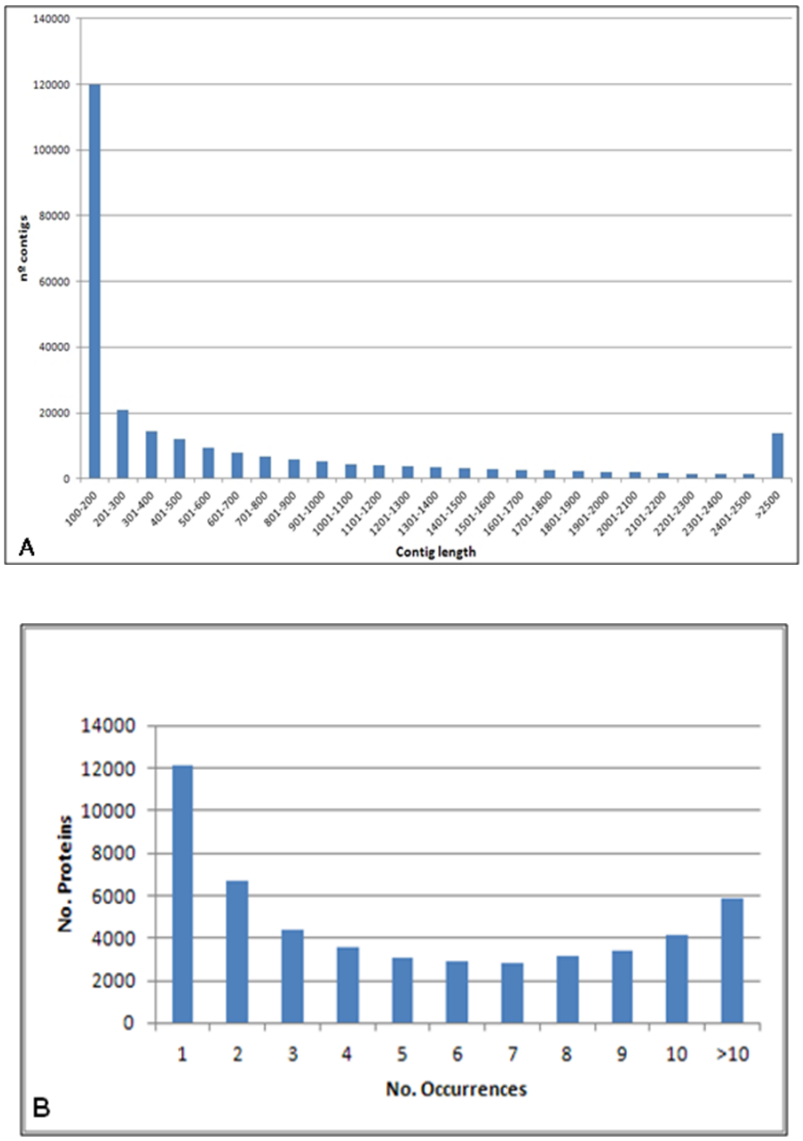


Fig. 2. (A) Length distribution of contigs obtained from *O. vulgaris* hemocytes transcriptome library. (B). Distribution of occurrences (frequency) of protein identified in NCBI.

3.2. BLASTx search in SwissProt database

Contig gene annotation was performed through BLASTx search against the SwissProt database using a cut-off E-value of 10^{-3}. Using this approach, a total of 48,225 (18.95%) contigs presented a significant BlastX hit (E-value<$1e^{-3}$). In contrast, 81.05% of assembled sequences did not matched to know proteins probably because the lack of molecular data of cephalopod species. Therefore, a high number of potentially novel genes are herein presented. Figure 3 indicates that sequences with highest number of matches were *Homo sapiens* with 1,073,995 occurrences, whereas the sea urchin *Strongylocentrotus purpuratus* (with 2,088 at position 35) was the single marine invertebrate homologue to common octopus sequences. These results clearly indicate the limited representation of cephalopods in public databases. In fact, for *O. vulgaris* only 32, 279 records in nucleotide sequence databases; 35 in ESTs, 251 in proteins and 13 in gene databases are deposited in GenBank. The majority of the sequences belong to taxonomic and central nervous system studies. Consequently, the present results highlight the need of contributions to increase the number of annotated sequences of cephalopods in public databases, which will help to discover new genes that allow understanding their entire biology, and such is the case in this work, genes related to the octopus innate defence that would be valuable for future applications in aquaculture.

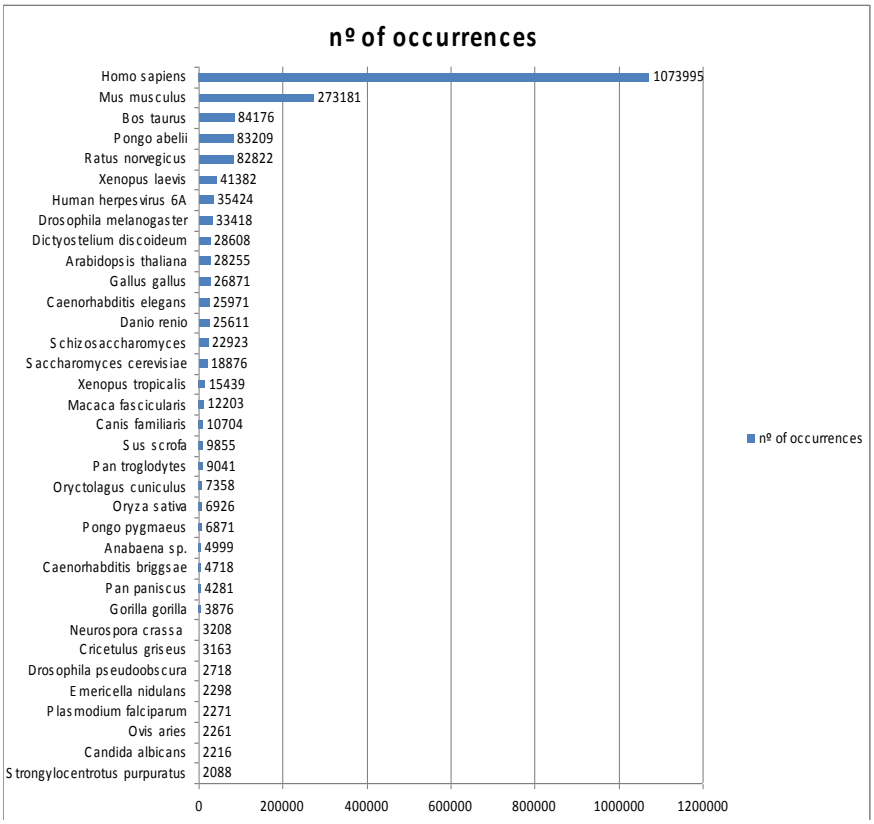


Fig. 3. Top 35 hit sequences matching *O. vulgaris* assembled sequences.

3.3. Functional annotation based on GO

Gene Ontology (GO) assignments were carried out at level 2 to classify the functions of the proteins differentially expressed between levels of infection and blasting the UniProt database. Based on sequences homology, three main categories were identified: cellular component, molecular function and biological process. Relative to cellular components (Fig. 4A), the highest percentage of GO corresponded to cell and organelle proteins, with 38% and 32% respectively. Within the molecular function classification (Fig. 4B), binding and catalytic activity were the most represented groups, with 57% and 29% respectively. Related to the biological process (Fig. 4C) cellular (17%) and metabolic process (15%) were the highest represented groups; in addition, biological (12%) and response to stimulus (8%) showed also a high percentage.

3.4 Comparative analysis

Comparison between the transcriptome of the *O. vulgaris* hemocytes and sequences available in NCBI for *E. scolopes* showed a 0.85% (301 hits) shared by both cephalopod species. In the comparison with *O. vulgaris* 20% (6402 hits) of sequences were coincident, whereas 0.06% was shared with *C. gigas* (135 hits), 0.40% with *M. galloprovincialis* (79 hits) and 3.80% with *R. philippinarum* (900 hits).

According to the present results, a low percentage of transcripts are shared among these molluscan species. This result stated again that a high number of potentially novel genes are herein presented. However, it should be noted that mollusc nucleotide and EST sequences available in the public databases and used for the comparison analysis are obtained from different tissues, and few are from hemocytes. This is the case of *E. scolopes*, where most of the information relative to this squid belongs to the light organ (Goodson et al., 2005; Chun et al., 2006; Castillo et al., 2009). Consequently, a low percentage of sequences are shared between our *O. vulgaris* database and *E. scolopes*. As it was expected, the major percentage was coincident when comparing *O. vulgaris* sequences. However, the comparison also includes sequences available from varied tissues as arms (Riesgo et al., 2012) or the central nervous system (Zhang et al., 2012), but no specifically from hemocytes. Regarding bivalves, the highest percentage was obtained after comparing *O. vulgaris* and *R. philipparum*. Considering that up-dated molecular data is available for bivalve's hemocytes (Zhang et al., 2009; Moreira et al., 2012;Philipp et al., 2012), the present results suggest that a set of transcripts is highly conserved between the octopus and the manila clam. Nevertheless, additional molecular data is needed to perform appropriate comparisons among mollusc species and specific tissues as hemocytes.

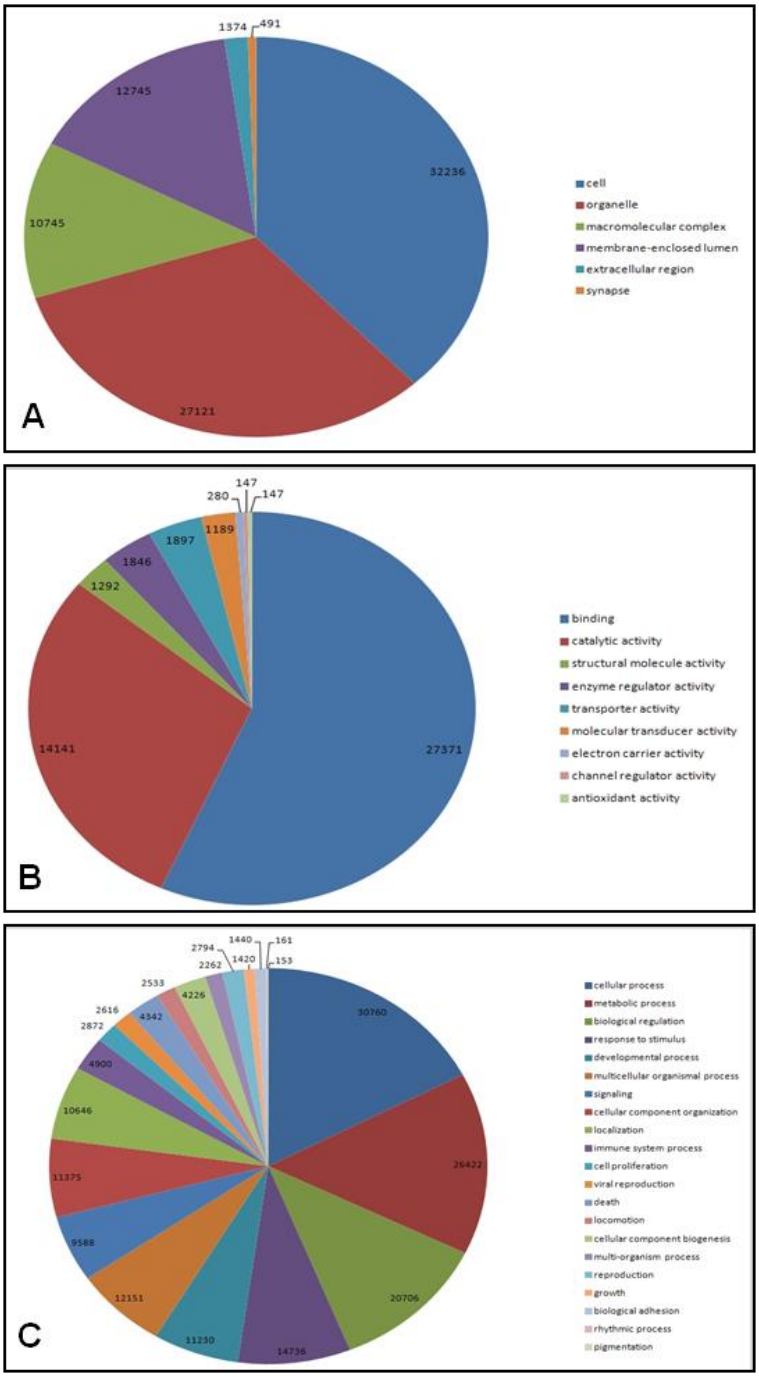


Fig. 4.Distribution of second level GO annotation in three categories: (A) cellular component, (B) molecular function and (C) biological process.

3.5. Immune transcriptome analysis

Hemocytes are the key effectors of cellular defence activities against invader agents. When challenged by pathogens, the octopus raises a strong and effective innate immune response (Malham and Runham, 1998) and therefore, immune genes are of particular interest to understand i) how the host-cell biological processes are altered by pathogens, and specifically by the *A. octopiana* infection; in consequence, ii) how the host faces the infection. The *O. vulgaris* immune system was stimulated by the natural infection of the coccidia *A. octopiana*. Differences in intensity of parasitism allowed to identify different gene expression in response to coccidiosis. A selection of GO immune-related terms allowed us to identify more than 3% of the predicted proteins with a possible immune function. This relative low annotation is probably due to the lack of molecular data of cephalopods in public databases and the high potential novel genes. Among the different transcripts identified, a significant number of putative immune-related genes involved in several immune pathways like NFκB, TLR signalling pathway, complement cascade and apoptosis were recognized (Fig. 5, 6, 7), suggesting that similar ancient mechanisms are shared with other molluscs.

3.6. Complement pathway and related proteins

The complement system is a complex pathway comprised by more than 30 plasma and membrane-associated proteins that interact from direct cell lysis to the enhancement of cellular responses. Consequently, it is a vital component of innate immunity. Complement is activated by three different pathways: classical, lectin and alternative. All three share in common the component C3 as the central molecule where known activation pathways converge (Carroll, 2004; Dunkelberger and Song, 2010). Homologs to complement C3 have been identified in diverse marine organisms like the horseshoe crab *Carcinoscorpius rotundicauda* (Zhu et al., 2005), the sea urchin *Strongylocentrotus purpuratus* (Al-Sharif et al., 1998), the carpet-shell clam *Ruditapes decussatus* (Prado-Álvarez et al., 2009) and the sea cucumber *Apostichopus japonicus* (Zhou et al., 2011). To date, the complement component C3 known in cephalopods was identified and characterized in the sepiolid *E. scolopes* (Castillo et al., 2009; Schleicher and Nyholm, 2011; Collins et al., 2012). Additionally, Collins et al. (2012) identified components of the complement cascade like CR1, CR2, C4b or C1qbinding protein in the proteome of *E. scolopes* hemocytes.

The *O. vulgaris* database herein presented contains putative homolog molecules of the complement signaling pathway (Fig. 5) such as C3, C3R, C5R, C1S, molecules MBL,

Ficolin and the C1q binding protein. C1q is a subcomponent of the complement C1 complex that plays a role in the classical pathway, but also in the recognition of microbial surfaces (Wang et al., 2013).

α_2-Macroglobulin (3 transcripts) is present in our *O. vulgaris* library. It is one of the most representatives of a group of plasma proteins that include complement components (Borth, 1992). This is a protein evolutionarily conserved in the innate immune system, operating as opsonin to bind and mark proteases for allowing subsequent recruitment of cells for endocytosis and intracellular proteolytic degradation of pathogens (Armstron, 2010). An α_2-macroglobulin protein about 180kDa capable to inhibit proteinases of different catalytic class was purified from the *O. vulgaris* plasma (Tørgersen et al., 1992), and represents the second most abundant protein in the hemolymph of *Sepia officinalis* (Vanhoorelbeke et al., 1993). Recently, an α_2-macroglobulin was identified in the proteome of *E. scolopes* hemocytes (Collins et al., 2012).

Fibronectin is a cell-adhesive protein that forms a complex with fibrin and mediated the adhesion of epithelial cells as a provisional matrix against skin wound healing in mammals. In invertebrates, fibronectin is involved in cell aggregation, migration and putatively correlates with invertebrate wound healing, and was isolated from the hemolymph of *Pinctada fucata* (Suzuki and Funakoshi, 1992). In cephalopods, the cell migration towards injured tissues is known (Féral, 1988). In fact, it has been suggested as a strategy to immobilize pathogens (Cowden and Curtis, 1981). Two fibronectin transcripts are here reported in the *O. vulgaris* library, providing molecular evidence of its presence in the octopus hemocytes. However, future studies of its molecular characterization would allow understanding how octopus hemocytes are capable to form clots quickly and thus to test whether cell aggregation is an effective defense activity. In addition, other putative immune molecules related to the complement and clothing pathway such as kalikrein (1 transcript) and Sushi, Von Willebrand factor (3 transcript) (VWF) has also been identified in *O. vulgaris* library. Similarly, the sushi domain has been identified in the proteome of *E. scolopes* hemocytes (Collins et al., 2012).

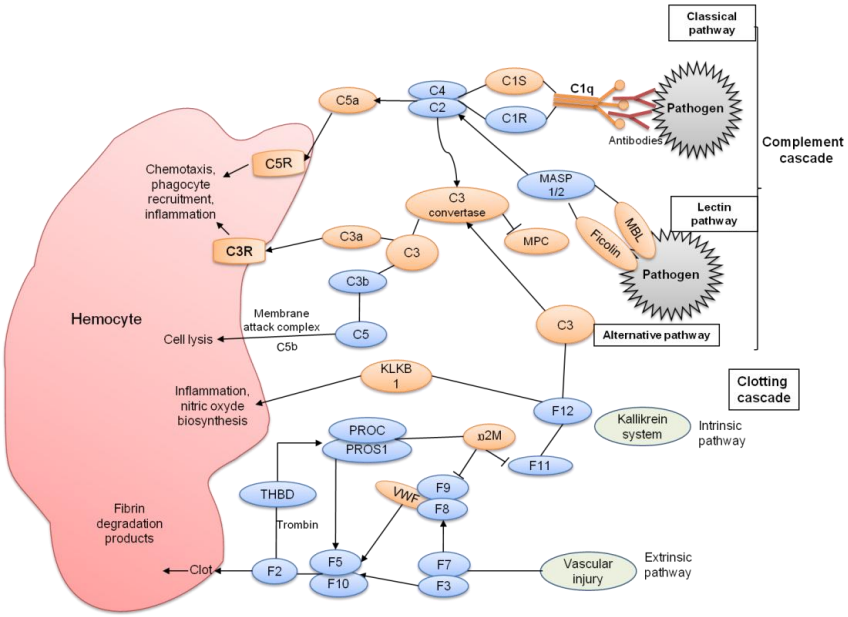


Fig. 5. Complement and clotting pathway. Brown figures indicate proteins identified in *O. vulgaris* library; and blue figures indicate the absent ones. C1q: complement C1q binding protein; C1R: C1r subcomponent; C1S: complement C1 subcomponent; C2: complement component 2; C4: complement component 4; C3: Complement component 3; C3a: anaphylatoxin subcomponent 3a; C3b: opsonin subcomponent 3b; C5: complement component; C3R: C3 receptor; C5R: C5 receptor; MBL: Manose-binding lectin. MASP1/2: mannan-binding lectin serine protease 1/2; F12: factor 12; F11: factor 11; α2M: alpha-macroglobulin; F2,3,5,7,8,9,10: coagulation factors 2,3,5,7,8,9,10; MPC: CD46, membrane cofactor protein VWF: von Willebrand factor; KLKB1: kallikrein B1; PROC: protein C; PROS1: protein S (alpha); THBD: trombomodulin.

3.6.1. Pattern recognition receptors (PRRs)

3.6.1.1 Lectins

Lectins are sugar-specific binding proteins with direct participation in innate immune functions as LPS-binding molecules, agglutination, recognition and phagocytosis through opsonisation and complement-activating factors. Therefore, lectins are valuable to recognize potential invaders and may be critical to the internal defence of marine molluscs (Vasta et al., 1999; Dodd and Drickamer, 2001). Few reports regarding the isolation and biochemical characterization of lectins and their role as non-self recognition molecules are available in cephalopods. The only ones identified and biochemically characterized are those from *O. vulgaris* (Rögener et al., 1985) and *Octopus maya* (Fisher and Dinuzzo, 1991; Alpuche et al., 2010). In the *O. vulgaris* library here reported, homolog sequences of mannose binding C-lectin (MBL) (2 transcripts), galectin (1 transcript) and a

different carbohydrate binding lectins (malectin) have been putatively found (1 transcript).

3.6.1.2 Peptidoglycan recognition proteins

Peptidoglycan recognition proteins (PGRPs) specifically recognize bacterial peptidoglycan from Gram-positive and Gram-negative bacteria. This protein is conserved from insects to mammals and has diverse functions in antimicrobial defence (Steiner, 2004; Royet et al., 2011). To date, four PGRP transcripts are known from cephalopods, all of them identified in a cDNA library from the sepiolid *E. scolopes*. According to the amino acid sequences it was predicted that EsPGRP1 have a cytosolic cellular localization, EsPGRP2 possess an N-terminal signal peptide that suggest EsPGRP2 is secreted into the extracellular environment. EsPGRP3 includes a glycosylphosphatidylinositol (GPI)-anchored protein that suggests EsPGRP3 is an extracellular extrinsic membrane protein, whereas EsPGRP4 putatively has two N-terminal transmembrane domains that suggest it is an integral membrane protein (Goodson et al., 2005). Recently, Collins et al. (2012) described a new PGRP (EsPGRP5) putatively capable of degrading bacterial peptidoglycan and its derivatives. The PGRPs are herein reported for the first time in the *O. vulgaris* library. The analysis led to the identification of three PGRPs in the circulating hemocytes of the octopus hemocytes. Further characterization of *O. vulgaris* PGRPs and studies to determinate their specific localization is required.

3.6.1.3. Toll-like receptors

Toll-like receptors are responsible for initiating inflammatory responses against invading pathogens in invertebrates and vertebrates. The Toll receptors provides the trans-membrane molecules linking the extracellular (where contact and recognition of pathogens occurs) and intracellular (where signalling cascades leading to cellular responses are initiated) compartments (Vasselon and Detmers, 2002; Engelmann et al., 2005). Some TLR are found in the cell membrane (TLR 1, 2, 4, 5, 6, 10); while others remain intracellularly due to they are anchored in the endosome (TLRs 3, 7, 8, 9). However, all TLRs contain (in the N-terminal end) a leucine-rich repeat (LRR) motif which mediates ligand binding. In addition, a highly conserved cytoplasmic domain termed Toll-IL-1R (TIR) is present and its function is as a binding site for downstream adaptor molecules (Ospelt and Gay, 2010). Toll-like receptors and some other genes involved in this pathway were described in the light organ of *E. scolopes*, where Toll-like receptor architecture is consistent with that known from mammals and insects (Goodson et al., 2005). The results obtained from our sequences showed transcripts encoding homologue

to the TLR-2, TLR-3 (1 transcript), TLR-4 and TLR-6 (1 transcript). In addition, most of the central proteins belonging to the TLR signalling pathway (the adaptor MyD88, IRAK, TRAF6 proteins) have been identified in this transcriptome analysis (Fig. 6). TLR-2 has the ability to recognize several ligands like peptigoglycan, bacterial lipopeptides, lipoteichoic acid and even protozoa like *Trypanosoma cruzi* (Campos et al., 2001; Beutler, 2004). Such versatility in binding several ligands has been explained by its ability to build heterodimers with other TLRs and to use co-receptors for the recognition of certain molecules (Ospelt and Gay, 2010). TLR-3 binds endogenous ligand from necrotic cells and double stranded RNA from virus, which in vertebrates induces the synthesis of type I interferons (IFN-α/β) with subsequent antiviral and immunostimulatory activities (Takeda and Akira, 2003). TLR-4 transduces the signals of lipopolysaccharide (LPS). Once the lipid is recognized, leads to the production of a wide range of immunostimulatory cytokines and chemokines mediated by mitogen-activated protein kinases (MAPKs) or NFκB pathways. In addition, several transcripts containing leucine rich repeat (LRR) domains and some immunoglobulin superfamily members containing also LRR have been identified in our library (48 transcripts in total). According to the different recognition roles of the TLRs found, the precise gene characterization and functional analysis of the identified TLRs in *O. vulgaris* have to be undertaken as the next step to understand their importance in the octopus immune system.

3.6.2 Cytokines

Cytokines are cell-signalling proteins that regulate inflammation and infection in the body (Miyajima et al., 1992). They can be released through complement receptor-mediated signaling or by pathogens through a wide array of pattern recognition receptors (PRR) (Lacy and Stow, 2011). Two transcripts of the putatively identified as IL-17 were found in our *O. vulgaris* library. IL-17 is involved in inflammatory process during infection and in the pathogenesis of chronic inflammation in autoimmune diseases. But also, is capable of activating NF-κB transcription factor in different cell types like macrophages or intestinal epithelial cells (Witowski et al., 2004; Roberts et al., 2008). In addition, the growth factors granulin (1 transcript), fibroblast growth factor 1 (FGF1) (1 transcript), fibroblast growth factor receptor 2 (FGRF2) (2 transcripts), transforming growth factor beta receptors (TGFβ) (2 transcripts), vascular endothelial growth factor (VEGF) (1 transcript), epidermal growth factor (EGF) (2 transcripts) and bone morphogenic protein (BMP) were found in the *O. vulgaris* transcriptome. To date, the molecular characterization of a single VEGF receptor ortholog have been performed in the bobtail squid *Idiosepius*

paradoxus. The homology with other VEGF receptors indicates that it could play an evolutionary conserved function in cardiac development (Yoshida et al., 2010).

3.6.3. NFκB pathway

The nuclear factor-κB (NF-κB) is rapidly activated by a wide diverse agents and cellular stress conditions including bacterial lipopolysaccharide (LPS), microbial and viral pathogens, cytokines and growth factors (Hatada et al., 2000). NF-κB pathway include molecule members as RELA (p65), NF-κB1, NF-κB2, c-REL and RELB that activates transcription from NF-κB binding sites in target genes (Li and Verma, 2002). Signal transduction is processed through the inhibitory proteins known as inhibitors of NF-κB (IκBs). There are several IκBs (IκBa, IκBb, IκBg, IκBε) with different affinities for individual NF-κB dimmers (Gilmore, 2006). Proinflammatory cytokines and pathogen associated molecular patterns (PAMPs) works through different receptors like the tumor necrosis factor (TNF) receptor (TNFR) and TLR–IL-1 receptor superfamilies. Those receptors activates the IκB kinase (IKK) complex consisting of catalytic kinase subunits (IKKα or IKK*β*) (Bonizzi and Karin, 2004). Following activation, NF-κB dimers are liberated from cytoplasmic complexes with ankyrin-repeats containing IκB. The released NF-κB translocate to the nucleus, bind DNA and activate transcription of genes encoding chemokines, cytokines that are important for innate immune response to invading microorganisms (Bonizzi and Karin, 2004). The NF-κB pathway seems an evolutionarily conserved innate immune pathway that is also present in mollusks. After bacterial challenge, a Rel protein of *C. gigas* was characterized in hemocytes, although it was also expressed in tissues like gills, heart or stomach (Montagnani et al., 2004). Likewise, the IκB gene was characterized in the pearl oyster *P. fucata*. In cephalopods, the NF-κB pathway was disclosed by the findings of molecules like IKKγ, TRAF6, IκB-like, IRAK4 and REL-like from juvenile *E. scolopes* light organs (Goodson et al., 2005). Now, transcripts found in this *O. vulgaris* library are coincident with such previous findings identified in *E. scolopes*, such as IRAK4, IκBα and TRAF6. Additionally, molecules like TRAF2, TRAF3, TRAF5, IKKα, IKKβ, RIP, TAK1, among others that had never been found in cephalopods, have been putatively identified in this work (Fig. 6).

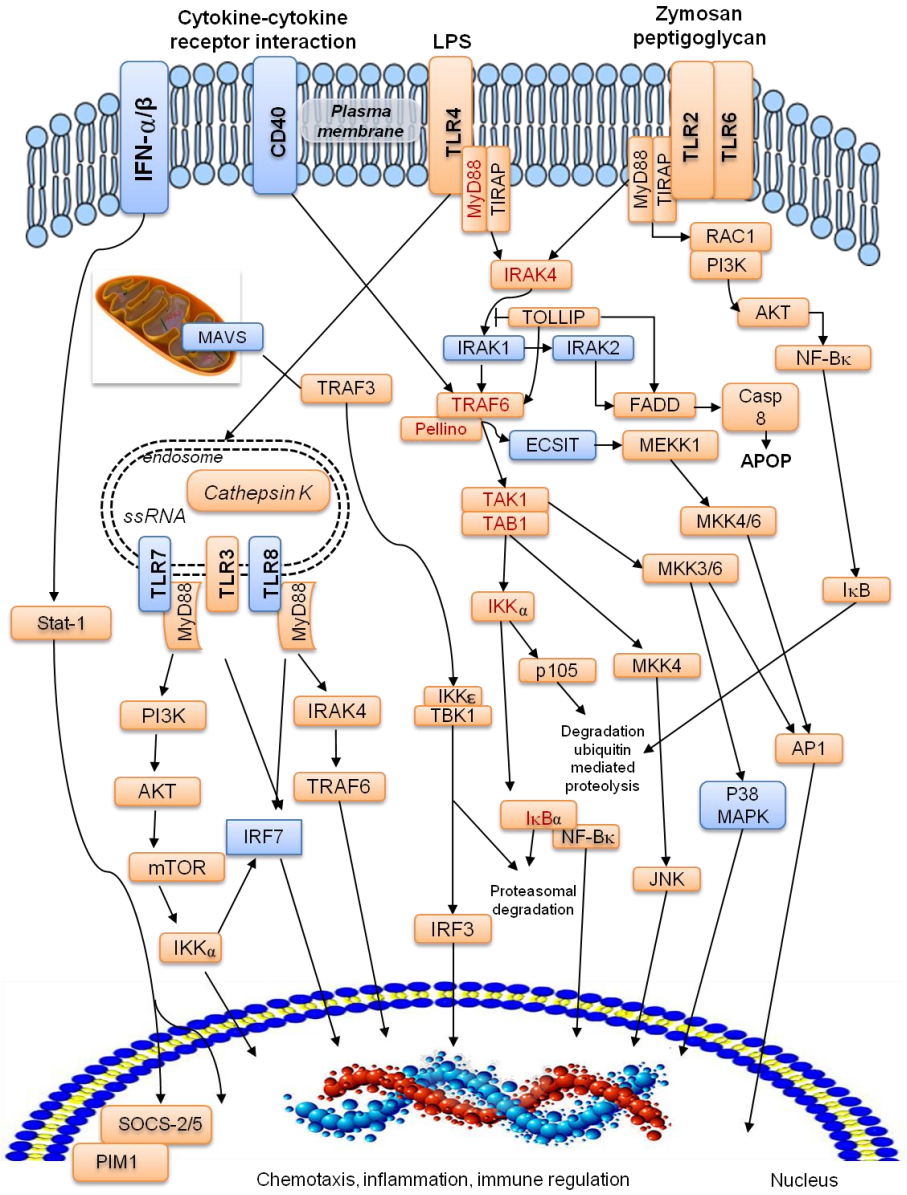


Fig. 6. TLR/NF-κB signaling pathway. Brown rectangles indicate proteins identified in the present *Octopus vulgaris* library and blue rectangles indicate the absent ones. Brown rectangles with red letters indicate proteins in the NF-κB pathway. AKT: RAC-alpha serine/threonine-protein kinase. API1: Transcription factor AP-1.Casp8: Caspase 8. FADD: FAS-associated via death domain. IκB: Inhibitor of NF-κB. IKKε: Inhibitor of nuclear factor kappa-B kinase subunit epsilon. IRAK4: Interleukin-1 receptor-associated kinase 4. IRF3: Interferon regulatory factor 3. IκBα: NF-kappa-B inhibitor alpha. JNK: c-Jun N-terminal kinase. MEKK1: Mitogen-activated protein kinase knase 1. MKK4/6: Mitogen-activated protein kinase kinase 4/6. MyD88: Myeloid differentiation primary response protein MyD88: Mtor: Mechanistic target of rapamycin. NF-Kb: Nuclear factor kappa-B. PI3K: Phosphatidylinositol 3 kinase. PIM1: Proto-onocogene serine/threonine-protein kinase pim-

1. p105: nuclear factor NF-kappa-B p105 subunit. RAC1: Ras related C3 botulinum toxin substrate. Stat-1: Signal transducer and activator of transcription 1. SOCS-2/5: Suppressor of cytokine signaling. TAB1: TAK1-binding protein1. TAK1: TGF-beta activated protein kinase kinase 1. TIRAP: Toll-interleukin 1 receptor domain-containing adaptor protein. TLR2: Toll-like receptor 2. TLR4: Toll like receptor 4. TOLLIP: Toll interacting protein (⊢direct inhibition). TRAF3: TNF receptor-associated factor 3. TRAF6: TNF receptor-associated factor 6. MAVS: Mitochondrial antiviral signaling protein that activates NF-kappa B and IRF 3. INFα/β: Interferon alpha/beta receptor. IRAK1-2: Interleukin receptor associated kinase 1, 2. IRF7: Interferon regulatory factor. P38MAPK: p38 mitogen-activated protein kinases. ECSIT: evolutionarily conserved signaling intermediate in Toll pathways.

3.6.4. Antimicrobial peptides (AMP)

Antimicrobial peptides are small molecular weight proteins with broad ability to kill or neutralize Gran-negative and Gram-positive bacteria, fungi, parasites or virus (Hancock and Scott, 2000). AMPs are capable to disrupt the pathogen membrane by pore formation or a detergent-like solubilization, leading to cell lysis (Izadpanah et al., 2005). Of these AMPs the bactericidal permeability-increasing protein (BPI) is produced by polymorphonuclear leukocytes, but also in epithelial cells. Mucosal epithelia that co-exist with microbes and microbial products express BPI and therefore, it probably contribute to the maintenance of immunologic homeostasis at mucosal surfaces (Canny and Levi, 2008). In contrast to common AMPs, BPI is notable for its high affinity to lipopolysaccharides (LPS) of gram-negative bacteria. Thus, the interaction with LPS triggers the BPI antimicrobial activity (Elsbach, 1998). At least three light-organ proteins in the BPI/LBP (lipopolysaccharide-binding protein) family were sequenced from *E. scolopes.* The role of these proteins in the context of the mutualism squid/*Vibrio* relationship is under study (Krasity et al., 2011). Now, a single transcript of BPI protein is provided from the *O. vulgaris* library. Additional studies are needed to understand the role of this protein in the octopus cellular defense.

3.6.5. Stress response genes

In addition to immune defense, the host system possesses mechanisms to reduce damaging effects of stress from diseases or environmental pressure (Kassahn et al., 2009). Pathogens induce stress to the organisms health, thus, reactive oxygen and nitrogen species are produced by host to control the infection. The use of the antioxidant system is part of the innate immune defense responsible to maintain those reactive species at low basal levels (Manduzio et al., 2004). Production of nitrogen species as nitric oxide is mediated by the enzyme nitric oxide synthase (NOS). One transcript of NOS and one transcript of the protein nitric oxide synthase trafficker (NOSTRIN) were putatively

identified in the *O. vulgaris* library. Superoxide dismutase (SOD), glutathione peroxidases (Gpxs), peroxiredoxins (Prxs) and catalases are involved in the antioxidant systems (Abele and Puntarulo, 2004; Lesser, 2006). SODs are metalloenzymes that removes O_2^- and avoids the subsequent dismutation of superoxide radical into hydrogen peroxide (Abele and Puntarulo, 2004), whereas Prxs are scavenger of hydrogen peroxide and alkyl hydroperoxides (Li et al., 2011). In this study, 3 transcripts homologous to SOD and 1 transcript of Prxs genes, respectively, were found. Other redox factors such as peroxisome (3 transcripts) were also observed. Peroxisomes cointain the enzyme catalase, which decomposes hydrogen peroxide by converting it to ware, or by using it to oxidize additional compounds (Cooper et al., 2000). Abundant transcripts of myeloperoxidase were found from a cDNA library of the *E. scolopes* symbiotic light organ (Tomarev et al., 1993). In addition, SOD, peroxirredoxins, peroxidases and glutathione peroxidase were also identified in the squid light organ proteome and transcriptome (Schleicher and Nyholm, 2011; Collins et al., 2012).

Heat-shock proteins (HSPs) serve as molecular chaperones that protect cells from the toxic effects of heat and modulate stress response (Lindquist and Craig, 1988; Tsan and Gao, 2009). In addition, their activity is close related to the immune innate response, involved in apoptosis, NF-κB regulation (Parcellier et al., 2003), antigen presentation, activation of lymphocytes and macrophages, and activation and maturation of dendritic cells (Tsan and Gao, 2009). From the *O. vulgaris* library HSP13, HSP27, HSP70, HSP71, HSP74, HSP76, HSP83, HSP85, HSP90 were identified.

3.6.6. Apoptosis

Apoptosis is a common physiological process for removal damaged or potential dangerous cells, but also a major defence mechanism against pathogens (Sahtout et al., 2001). Two pathways can be triggered: i) intrinsic (also named mitochondrial), initiated by internal cellular damage; and ii) extrinsic, initiated by environmental stimuli that lead to cell death. The central components of the apoptosis pathway are the proteases caspases. They are divided as initiator caspases (caspase 2, 8, 9, 10) that cleave and activate the effector caspases (3, 6, 7) (Sokolova, 2009). Apoptosis have been studied in marine invertebrate like the abalone *Haliotis diversicolor* (Huang et al., 2010), the mussel *M. galloprovincialis* (Romero et al., 2011) or the shrimp *Penaeus monodon* (Xian et al., 2013). However, apoptosis process has not been studied in cephalopods nor records exist for molecules of apoptosis pathway. Consequently, the first records of apoptotic components are herein provided. In *O. vulgaris* library the initiators: caspase 8 (3

transcripts) and 10 (1 transcript); and three effectors: caspases 3 (4 transcripts), 6 (1 transcript), 7 (4 transcript) were identified (Fig. 7).

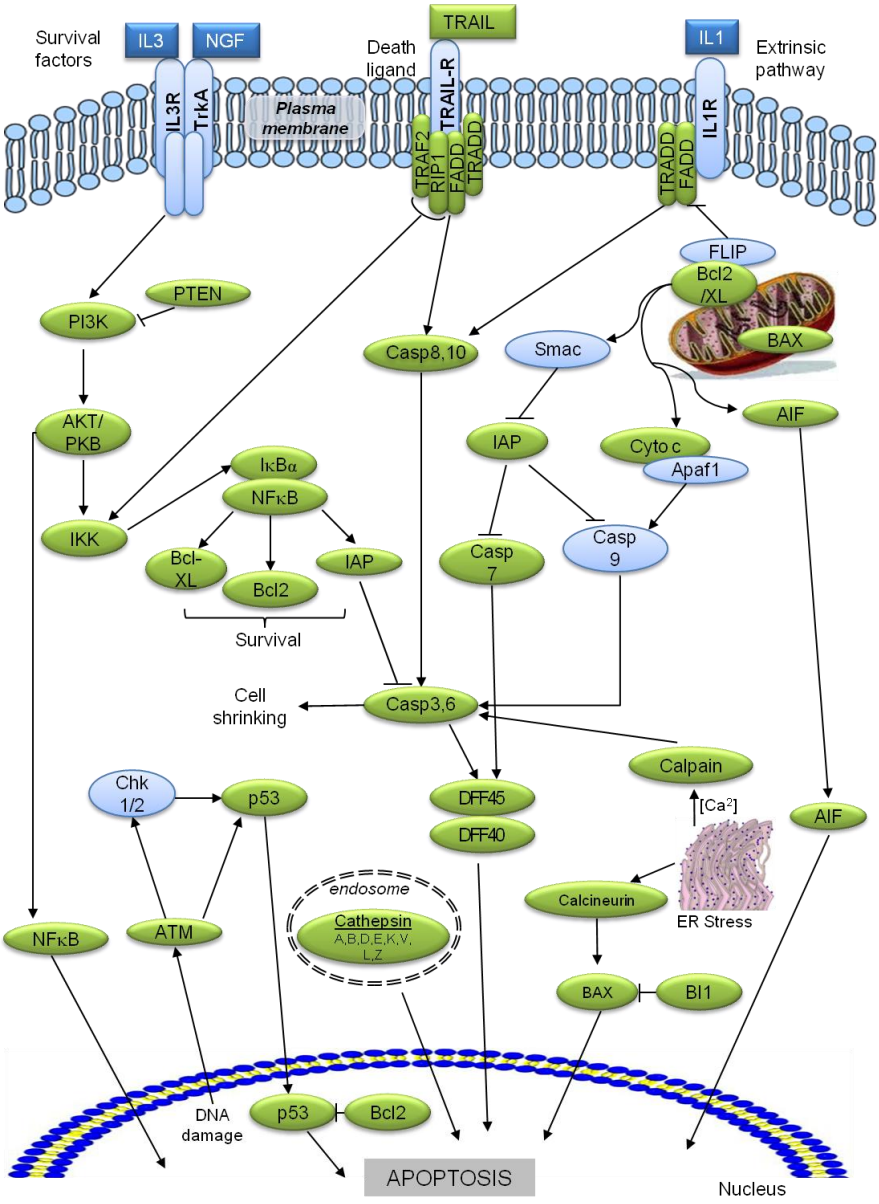


Fig. 7. Apoptosis pathway in *Octopus vulgaris.* Green ellipse indicates proteins identified in the present *O. vulgaris* library and blue ones indicate absence. (⊢ direct inhibition)AKT/PKB: RAC-alpha serine/thereonine-protein kinse/ Protein kinase B. AIF: Apoptosis-inducing factor 1 mitochondiral. ATM: Ataxia telangiectasia mutated protein. BAX: Apoptosis regulator BAX. Bcl2: Apoptosis regulator Bcl-2. Bcl-XL: Bcl-2 like protein 1. BI1: BAX inhibitor-1. Casp 3, 6, 7, 8, 10:

Caspase 3,6, 7, 8, 10. Cytc: Cytochrome c. DFF40, 45: DNA fragmentation factor of 40kD, 45kD. FADD: FAS-associated via death domain. IAP: Inhibitor of apoptosis. IKK: Inhibitor of nuclear factor kappa-Bkinase. IκBα: MyD88: Myeloid differentiation primary response protein MyD88. NF-kappa-B inhibitor alpha. IL3R: Interleukin 3 receptor. NFκB: Nuclear factor kappa-B. PI3K: Phosphatidylinositol 3-kinase. p53: Tumor suppressor p53. RIP1: Receptor interacting serine/threonine-protein kinase1. TRADD: TNF receptor superfamily 1 alpha-associated via death domain. TRAF2: TNF-receptor-associated factor 2. TRAIL: TNF-related apoptosis-inducing ligand. Apaf1: Apoptotic protease-activating factor. FLIP: FADD-like apoptosis regulator. PTEN: Phosphatidylinositol-3, 4, 5-trisphosphate 3 posphatase and dual specificity protein phosphatase PTEN. Smac: second mitochondria-derived activator of caspase. Chk1/2: checkpoint kinases 1, 2.

3.6.7. Other proteins

Serin protease inhibitor (SERPIN) proteins are important element of the host defense to inactivate proteases secreted by pathogens and to restrict their invasion (Armstron, 2006). Protease inhibitors have been found in *Crassostrea virginica* (Faisal et al., 1998) and *Chlamys farreri* (Wang et al., 2009), but it have not been described from cephalopods. A total of 6 transcripts corresponding to SERPIN were identified in the *O. vulgaris* library. Biochemical, functional and molecular characterization of SERPIN is needed to understand whether and how the octopus' hemocytes use this protein to counteract coccidiosis.

Angiopoietin is a protein that regulates angiogenesis, the process of formation of new blood vessel from other pre-existent ones (Muñoz-Chápuli, 2011). This protein is a Tie2 receptor agonist that is expressed in vertebrate's lung (Valenzuela et al., 1999). A protein putatively similar to angiopoietin-like 4 (2 transcripts) was identified in our *O. vulgaris* library, which is not surprising due to cephalopods possess the most complex circulatory system of all the invertebrates. Furthermore, the VEGF described in the squid *I. paradoxus* makes hypothesize that the processes of vascular growth in cephalopods could be comparable to vertebrates. The process of vascular growth involves endothelial cells in vertebrates. However, in *I. paradoxus* and in cephalopods, is unknown and therefore, additional evidence is required to clarify the process of vascular growth in these molluscs. Meanwhile, the vascular grow in invertebrates would not be comparable with the vertebrate angiogenesis (Muñoz-Chápuli, 2011).

Peroxisome proliferator-activated receptors (PPARS) termed α, β or γ belongs to the nuclear receptor superfamily involved in lipid metabolism and inflammation. They heterodimerize with retinoic X receptor for activation and alter the transcription of target genes after binding to response elements or peroxisome proliferator-response elements

(PPREs) (Schoonjans et al., 1996). PPARS are, in general, anti-inflammatory and can interact with transcription factors involved in inflammation such as NF-κB, activator protein-1 (AP-1) and STAT (Anthony et al., 2012). A total of 3 transcripts corresponding to PPARS were found in the *O. vulgaris* library.

Cluster of differentiation (CDs) are cell surface molecules expressed on various cell types in the immune system. They are markers that identify a particular lineage or differentiation stage, that has a defined structure and that is recognized by a group of monoclonal antibodies. Using this approach, certain CD markers have been revealed in small coelomocytes of the earthworm *Eisenia foetida* (Engelmann et al., 2002; 2011) and coelomocytes of the purple sea urchin *Arbacia punctulata* (Lin et al., 2001). In addition, CDs molecules similar to mammalian were found in the leech *Hirudo intestinalis* (Macagno et al., 2010), whereas CD63 molecule (3 transcripts) was found in the transcriptome of *E. scolopes* hemocytes (Collins et al., 2012). In the present *O. vulgaris* library, CD11b (1 transcript) CD13 (1 tanscript), CD26 (2 transcripts), CD36 (1 transcript), CD53 (4 transcripts), CD63 (1 transcript), CD81 (1 transcript), CD98 (2 transcript) were recorded. Finding these molecules suggest that some proteins marked by specific CDs and known to participate in vertebrate immune response could also be present in the common octopus. The commonest functions attributed to CD antigens are to promote cell-cell interactions and adhesion; and to transducer signals that lead to lymphocyte activation (Cooper, 2001). However, additional studies are needed to clarify whether similar functions are carried out in *O. vulgaris*. The useful of CDs herein found for identification and characterization of *O. vulgaris* hemocytes needs to be assessed.

LPS-induced TNF-α factor (LITAF) is a transcription factor that regulates inflammatory cytokines in response to LPS stimulation and thus, controls TNF-α expression. This gene have been identified in gastropods (Jiang and Wu, 2007; De Zoysa et al., 2009) and bivalves species (Zhu and Wu, 2012; Moreira et al., 2012; Philipp et al., 2012). In this study, we have found two transcripts similar to LITAF, which have not been reported from cephalopods before.

The Jaw1 (Lymphoid-restricted membrane protein, LRMP) is a protein localized in the cytoplasmic face of the endoplasmic reticulum (Behrens et al., 1996) with structural features of proteins that link the nuclear envelope to the cytoskeleton (Lindeman and Pelegri, 2012) and expressed in a developmentally regulated fashion in the B and T cell lineages (Behrens et al., 1994). A single transcript of Jaw1 protein was found in *O. vulgaris* hemocytes. However, B and T cell lineages have never been described in cephalopods.

Allograft inflammatory factor-1 (AIF-1) is a cytokine-responsive macrophage molecule, inducible by cytokines as IFN-g, IL1β or IL-18 (McDaniel et al., 2012). AIF-1 seems to develop a fundamental role in the processing of the inflammatory responses and is also associated to inflammatory diseases like bowel diseases (Morohashi et al., 2003; McDaniel et al., 2012). AIF-1 have been characterized in coelomocytes of Antarctic sea urchin *Sterechinus neumayeri* (Ovando et al., 2012) and the pearl oyster, *Pinctada martensii* (Li et al., 2013). In the present study, one transcript of AIF-1 is reported for first time in cephalopods.

Leukocyte receptor cluster (LRC) is located on human chromosome 19q13.4 and similar receptors are located on mouse chromosome 7. These genes, the killer immunoglobulin-like (KIR) receptors, expressed on natural killer cells (NK) and cytotoxic T cells; and the leukocyte Ig-like receptors (LILRs), expressed mainly on cells of the myeloid lineage constitute a subset of the Ig gene superfamily (IgSF) and some member mediate innate recognition (Barrow and Trowsdale, 2008). Knowledge of LRC is based on vertebrates (Yoder et al., 2001; Viertlboeck and Göbel, 2011), but nothing have been described before in cephalopods or any other invertebrate. In the present study, a total of 3 transcripts were found in the transcriptome of *O. vulgaris* hemocytes.

The results so far reported provide a general overview of the proteins encoded by the common octopus hemocytes. Until date, few transcriptome studies have been performed in cephalopod's hemocytes. Consequently, the present results proceed from a comparison with all the available sequences (belonging to different organisms) in public databases. Cellular components and proteins involved in metabolic processes were commonly found. However, here is also provided information relative to immune proteins expressed by the *O. vulgaris* hemocytes. Highly important receptors as Toll, lectins, cytokines; and proteins never recorded before in cephalopods as fibronectin, SERPIN or caspases suggest that the immune defense strategy in octopus is similar to other molluscs. However, proteins as leukocyte receptor cluster or allograft inflammatory factor-1, reported in vertebrates, were also found in the transcriptome of the octopus hemocytes. Further work must be carried out in order to characterize, at molecular and functional levels, such kind of proteins related to vertebrate immune defense and thus, confirm their identity. While doing this work, the present findings pave the way to reveal if cephalopods are markedly different from the rest of molluscs due to anatomical features, but also, due to differences in innate immunity among them.

3.7. Differentially expressed transcripts in response to coccidia infection

Cufflink program was used to analyze the reads of both infection condition and reports transcripts that are differentially expressed using a rigorous statistical analysis. From the two sample groups, the assembled contigs were transformed into RPKM (Reads Per Kilo bases per Million reads) to calculate abundance differences of each gene with further false discovery rate analysis. Thus, a set of 539 genes was differentially expressed ($p<0.05$) between sick and healthy octopuses. Of them, a total of 312 genes were successfully identified in public databases (a representative number of transcripts have been included in Table 3). The remaining 227 assembled sequences did not matched with known proteins probably due to the scarcity of the molecular representation of cephalopod species.

Table 3. Representative transcripts differentially expressed in sick *O. vulgaris* hemocytes.

Locus		E-value	Accession no.	Organism	Corrected[a] *p*-value	Fold change[b] (sick/healthy)
	Cytoskeleton					
Locus_7823_Transcript_2/3	Actin-1	0.0	P30162	*Onchocerca volvulus*	0.0438	7.40
Locus_1735_Transcrip40/41	Filamin	1E-47	Q14315	*Homo sapiens*	0.0201	0.11
Locus_2118_Transcript_15/13	Tubulin alpha-2	8E-92	P41383	*Patella vulgata*	0.0351	6.22
	Metabolism					
Locus27260_Transcript_13/15	NADH-ubiquinone oxidoreductase	2E-11	P48918	*Albinaria coerulea*	0.0048	4.15
Locus_44675_Transcript_1/1	ATP synthase	7E-20	P05496	*Homo sapiens*	0.0352	3.21
Locus_18667_Transcript_2/2	26S proteasome	4E-34	Q5RE15	*Pongo abelii*	0.0474	$1.79e^{308}$
Locus_2696_Transcript_38/45	Ubiquitin	0	Q2TL32	*Rattus norvegicus*	0.0396	4.50

[a]Value of significance after applying a false discovery rate multitest (Benjamini-Hochberg).

[b]Rate of change corresponding to sick octopuses respect to healthy ones. Values based on the abundance of transcripts in FPKM units. Those values higher than 1 are considered up-regulated. Those values lower than 1 are considered down-regulated.

Table 3. Representative transcripts differentially expressed in sick *O. vulgaris* hemocytes. (*Continue*)

Locus		E-value	Accession no.	Organism	Corrected p-value	Fold change (sick/healthy)
	Complement					
Locus_665_Transcript23/26	Sushi domain	1E-55	P0C6B8	*Rattus norvegicus*	0.0183	0.26
Locus_103759_Transcript1/1	Macrophage mannose receptor	8E-9	P22897	*Homo sapiens*	0.0380	0.21
Locus_4803_Transcript_3/5	Collectin-10	3E-13	Q9Y6Z7	*Homo sapiens*	0.0239	$1.79e^{308}$
	Toll-like receptor					
Locus_9596_Transcript_ 40/42	TLR2	2.0E-16	Q810C0	*Mus musculus*	0.0435	$1.79e^{308}$
	Apoptosis					
Locus_3296_Transcript_ 12/20	Caspase-3	5E-10	Q8MJC3	*Oryctolagus cuniculu*	0.0077	$1.79e^{308}$
Locus_2750_Transcript_ 1/1	Probable Bax inhibitor 1	8E-29	Q9IA79	*Paralichthys olivaceus*	0.0470	2.76
Locus_92598_Transcript_1/1	Calmodulin	1E-46	Q8STF0	*Strongylocentrotus intermedius*	0.0282	0.27
Locus_14244_Transcript 3/·	Calpain-5	2E-164	O08688	*Mus musculus*	0.0436	0.27

[a]Value of significance after applying a false discovery rate multitest (Benjamini-Hochberg).

[b]Rate of change corresponding to sick octopuses respect to healthy ones. Values based on the abundance of transcripts in FPKM units. Those values higher than 1 are considered up-regulated; while, those values lower than 1 are considered down-regulated.

Table 3. Representative transcripts differentially expressed in sick *O. vulgaris* hemocytes. (*Continue*)

Locus		No. transcripts	Accession no.	Organism	Corrected[a] p-value	Fold change[b] (sick/healthy)
	Stress					
Locus_1834_Transcript_ 9/10	HSP70	7E-63	P09446	*Caenorhabditis elegans*	0.0001	5.08
	Cytokine					
Locus_21297_Transcritp_ 2/2	Serine/threonine-protein kinase receptor R3	7E-128	Q61288	*Mus musculus*	0.0169	0.24

[a]Value of significance after applying a false discovery rate multitest (Benjamini-Hochberg).

[b]Rate of change corresponding to sick octopuses respect to healthy ones. Values based on the abundance of transcripts in FPKM units. Those values higher than 1 are considered up-regulated; while, those values lower than 1 are considered down-regulated.

3.8. Quantitative RT-qPCR of selected genes

To quantify differences in gene expression, RT-qPCR was performed for the selected genes as PRRs (galectin, PGRP, C1q, TLR), protease inhibitors (SERPIN), inflammatory response (LITAF), cell antioxidant system (PRDX-2), and apoptosis (Caspase-3). The gene selection was based, primarily, on their significant expression observed in the transcriptomic library; but also, additional genes were selected due to their implication in the host-immune response to pathogens despite they were not significant. Thus, for most of the genes tested, the mRNA expression showed the same trend of gene expression as in RNA-seq analysis, supporting thus the sequencing results and demonstrating the suitability of the method followed for the *de novo* sequencing of the *O. vulgaris* hemocytes.

In order to get a first insight on whether the tested genes reflect a mechanisms to combat the coccidia infection, RT-qPCR analysis of selected genes was performed in hemocytes, responsible for cellular defensive mechanisms (Chu, 2000); but also in caecum, which is the target organ of *A. octopiana* infection (Hochberg, 1990); and in gills, which are continually in contact to surrounding environment and potential pathogenic agents (Mladineo and Bočina, 2007).

In hemocytes, only galectin (1.02 fold increase) and TLR (0.73 fold increase) were up regulated in sick octopuses. In contrast, all the genes tested were up-regulated in caecum infected, but only some PRRs were up-regulated in gills (Fig. 8). Galectins play crucial roles in signaling and molecular recognition processes (Vasta et al., 1999). Kim et al. (2008) found higher expression of Manila clam galectin in tissues highly infected by *Perkinsus olseni*. The up-regulation of the galectin in hemocytes and caecum of sick octopus suggest that it could be acting as an opsonin. Consequently, could be involved in the recruitment of hemocyte to the caecum, where *A. octopiana* (gamogonic and sporogonic stages) occurs.

Toll-like receptor-2 (TLR-2) was up-regulated in all tissues tested, but mainly in the caecum from sick octopus group (Fig. 8). Up-regulation (0.73 fold increase) of TLR-2 in circulating hemocytes suggests that cells could detect parasite-derived ligands or endogenous molecules such as HSP and thus trigger an inflammatory response (Vabulas et al., 2001). The TLR-2 has a crucial role in tolerance against commensal flora to maintain gastrointestinal homeostasis, but is also important for recognition of pathogens (Ospel and Gay, 2010). Low levels of expression of TLR-2 are detected in digestive tissue of

healthy individuals, whereas a high expression has been related to chronic inflammatory diseases, such as inflammatory bowel disease (Candia et al., 2012). In octopus, TLR-2 was markedly up-regulated (2.58 fold increase) in caecum of sick octopuses compared to healthy ones, which is in agreement with the digestive tissue rupture and hemocytic infiltration described by Gestal et al. (2002a). The present finding indicates that coccidiosis induces the expression of TLR-2 in a disease specific manner as occurs in the inflammatory bowel disease.

Most of the genes tested were also involved in pathogen recognition, detoxification and apoptosis, and all of them were down-regulated in hemocytes (Fig. 8). Some reports found up-regulated C1q protein in hemocytes infected by *Perkisus marinus* (Prado-Álvarez et al., 2009b), whereas LITAF expression is up-regulated after bacterial challenge in *Haliotis discus discus* (De Zoysa et al., 2010). Bacterial challenge induces acute phase response and *P. marinus* give rise to a chronic infection similar to coccidiosis in octopus. C1q has been proposed involved in pathogen recognition, but there is not clear how chronic coccidiosis could interact with C1q, LITAF and additional genes down-regulated in the octopus circulating hemocytes. Contrarily, a markedly up-regulation of genes was found in the caecum from sick octopuses. An up-regulation was observed in C1q binding protein and PGRP showing 4.86 fold increase. C1q and TNF-α are known to be produced in response to infection as inducers of proinflammatory activators (Kishore et al., 2004). Thus, an assembly of functions of C1q and LITAF could be occurring in caecum of sick octopuses, activating both genes for inducing proinflammatory response (Kishore et al., 2004).

PGRP are effective PRR that recognize bacterial peptidoglycan (Steiner, 2004). This PRR was highly expressed in caecum. An up-regulation was also found in *E. scolopes* hemocytes (4.7 fold change) suggesting that PGRP are directly influenced to establish a symbiotic relationship with *V. fischery*. However, PGRP also regulate the microbiota inside the gut (Royet et al., 2001). Thus, an up-regulation (4.61 fold increase) in the octopus caecum suggests that microbiota is not longer controlled by PGRP, which could be attained to the severely damaged octopus digestive tissue and the impaired immune response. In addition, a slight but up-regulation of SERPIN (0.15 fold increase) was observed in the host caecum. This suggest that inhibitor of proteases are active. According to the gene expression results the high infection by *A. octopiana* also induces a strong ROS production. Consequently, up-regulation of antioxidant proteins like PRDX-2 is needed to regulate the levels of toxic radicals that can also damage the host tissue (Bandyopadhyay et al., 1999).

Thus, notably up-regulation of PRDX-2 was recorded in caecum of sick octopuses (Fig. 8). High coccidiosis provokes severe rupture of tissue (Gestal et al., 2002a). As a result, damaged cells must be eliminated to maintain homeostasis inside the organ. Therefore, Caspase-3 which is a type of effector caspase (Sokolova, 2009), was markedly up-regulated (1.02 fold increase) in caecum and can be attained to a task for eliminating tissue damaged. However, there is not clear how the Caspase-3 can regulate the progression of coccidiosis.

From gills, the highest up-regulated gene was C1q binding protein (2.93 fold increase), followed by PRDX-2 (1.32 fold increase) and SERPIN (1.09 fold increase) in sick octopuses. C1q could putatively work as an opsonising protein. Similar up-regulation was observed in protease inhibitors and PRDX-2, suggesting that the octopus cellular defence is acting against potential pathogens present in the sea water. The *A. octopiana* infection resides in the octopus digestive tract and gills are unusual sites infected by the coccidia (Pascual et al., 2006; Mladineo and Bočina, 2007). However, gills represent the main interface between aquatic organisms and surround environment. Therefore, in molluscs, gills are valuable not only for oxygenation; they are also an important defence against bacterial infection (Park et al., 2008) and thus, immune related genes can be found also expressed in octopus gills.

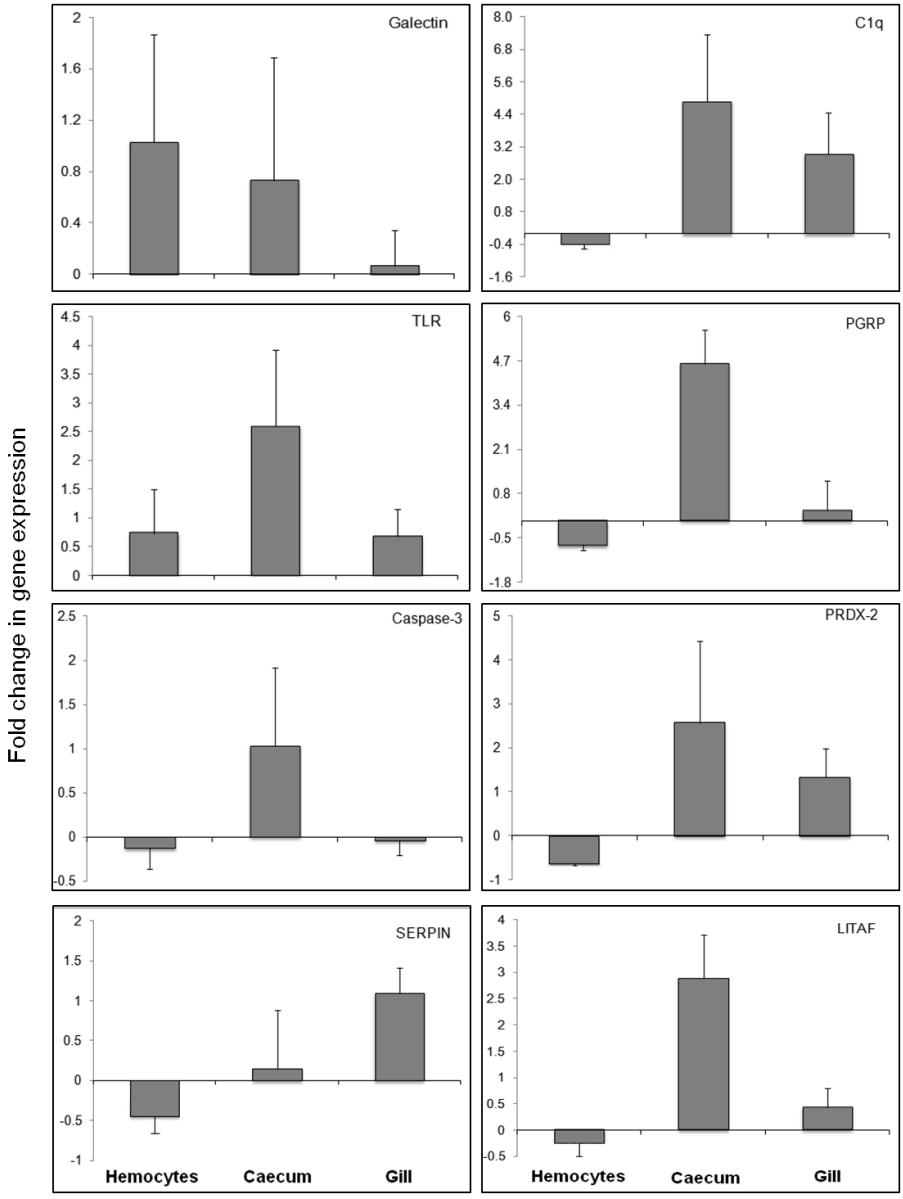


Fig. 8. Fold change in gene expression analysis by RT-qPCR. Tissue expression profiles of immune genes in *O. vulgaris*. β-acting gene was used as control transcript. Results are mean ± standard deviation.

4. Conclusion

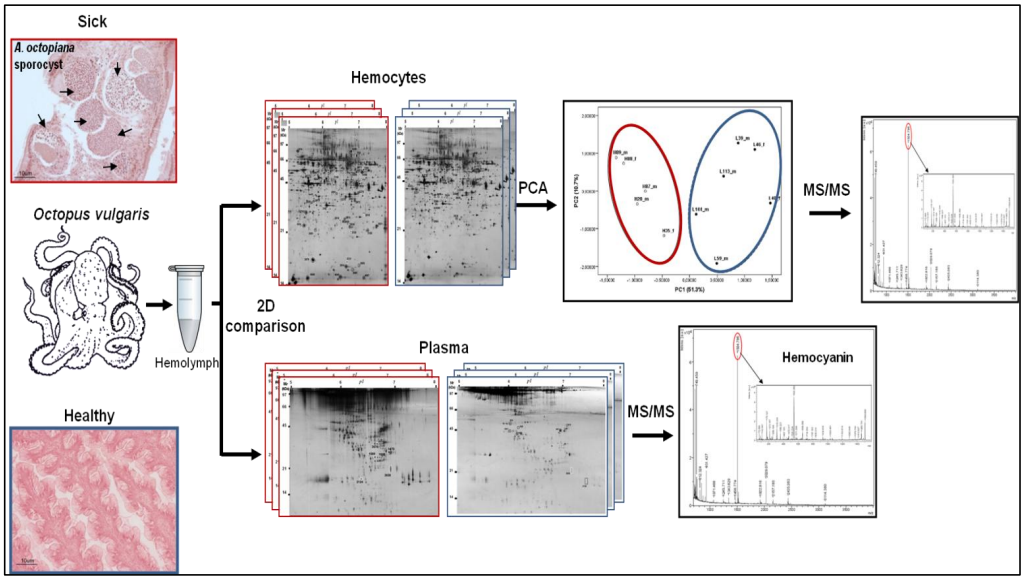
The present study applied Illumina technology to provide the first sequencing study of *Octopus vulgaris* transcriptome. The successful result allowed identifying several genes related to metabolic, functional and cellular components, but also transcripts of genes involved in the octopus immune response are herein provided for the first time. Highly important pathways for pathogen recognition and cellular homeostasis like complement, TLR and apoptosis were identified. Particularly, a high number of TLR pathway members were found, which indicates such pathway is also well-conserved in cephalopods like in other molluscs.

The inventory of *O. vulgaris* genes involved in the octopus immunity evidenced that coccidiosis by *A. octopiana* induces differential expression profiles. High intensity of infection induces over-expression on important PRRs like C-type lectins in hemocytes, but also apoptotic effectors molecules like caspase-3. Q-PCR assays complement and confirm the expression trend obtained from massive-sequence. Furthermore, here is demonstrated that all gene tested showed a noticeably over-expression in sick octopus caecum, which is the target organ of *A. octopiana*.

Altogether these results suggest a complex innate immune system in *O. vulgaris*. Thus, the first insights of the effect of *A. octopiana* infection at transcriptomic level, promoting the over-expression of some immune genes is herein provided. Moreover, the first molecular basis of the octopus resistance/tolerance to coccidiosis is herein established. The following efforts will be directed to characterize immune-relevant genes with a particular focus on those involved in octopus resistance, providing a better understanding of the octopus defense mechanisms.

Chapter 5

Proteomic characterization of the hemolymph of *Octopus vulgaris* infected by the protozoan parasite *Aggregata octopiana*



Abstract

One of the most important pathogens affecting *Octopus vulgaris* is the gastrointestinal parasite *Aggregata octopiana.* Despite its economic relevance and potential as an aquaculture species, the knowledge that exists about the defence mechanisms of this mollusc is limited. In this study, we used a proteomic approach to analyze the octopus immune response against the infection by the coccidia *A. octopiana*. A comparative proteomic analysis of the hemolymph of octopus with high and low parasite loads, corresponding to sick and healthy individuals, respectively, showed differences in relative abundance (p<0.05) in samples from hemocytes and plasma. As the octopus proteome is poorly represented in public databases, most of the proteins were identified from octopus' hemocytes RNA-seq database. The identities of 36 proteins from hemocytes and 5 proteins from plasma were determined by ESI-FTICR and MALDI-TOF-TOF. These proteins revealed changes in metabolism, cytoskeleton and antioxidant functions in hemocytes from high infected octopuses. Additionally, principal component analysis was used to select 7 proteins that were the major contributors to the overall difference between levels of infection and so could be considered as potential biomarkers. From these proteins, filamin, fascin and peroxiredoxin are highlighted because of their implication in octopus immune defense activity. From the octopus plasma proteome, the most abundant protein was identified as hemocyanin. The present results allow a description of the general protein profile for the common octopus hemolymph and suggest that infection by the parasite influences the activation of the *O. vulgaris* immune defense system. Moreover, the study supports the utility of proteomics in analyzing changes in the octopus proteome, and so contributes to understanding the basis of octopus tolerance-resistance to *A. octopiana*.

1. Introduction

The common octopus (*Octopus vulgaris* Cuvier, 1797) is the most significant cephalopod species in aquaculture worldwide. In particular, the autonomous community of Galicia in NW Spain has for long been the primary location for successfully rearing the common octopus in cages suspended in the sea until they reach the appropriate weight for selling (García-García et al., 2004). One of the main challenges of such aquaculture is to produce healthy animals with optimal growth rates. Nevertheless, the relative crowding of the animals in farmed environments also favors stress, which in turn promotes disease (Berthe, 2005).

One of the most important pathogens infecting cephalopod molluscs is the gastrointestinal parasite of the genus *Aggregata* spp. (Hochberg, 1990). This eimeriorin coccidian infects the digestive tract of cephalopods (Pascual et al., 1996). This coccidian is transmitted through the food-web and its merogonic (asexual) development takes place in crustaceans which are the intermediate host for a wide range of *Aggregata* species. The gamogonic and sporogonic stages (sexual stages) development of *Aggregata octopiana* occur in their final host, *O. vulgaris* (Hochberg, 1990). Because of this, development of gametes and oocysts of *A.octopiana* leads to hypertrophy and detachment of digestive epithelial cells in *O. vulgaris*. In heavily infected octopuses, this results in mucosal folds of caecum and ulceration of the gut (Gestal et al., 2002a). Moreover, chronic *A. octopiana* infection also causes malabsorption syndrome. The syndrome can be recognized by the malfunction of digestive enzymes, preventing correct nutrient absorption, which results in an abnormally low octopus weight (Gestal et al., 2002b). Furthermore, this chronic infection reduces the protein plasma and the DNA/RNA and RNA/ protein ratios in muscular tissue depending on the intensity of infection (Gestal et al., 2007b).

Disease outbreaks provoke serious reductions in production with potentially severe economic losses. For this reason, knowledge of the immune defense system of cultured species is required in order to develop disease prevention and eradication strategies (Roch, 1999). Currently, knowledge of immune mechanisms in invertebrates, and especially in cephalopods is limited. Despite they have an effective innate immune system, they lack an adaptive response. Thus, as with other molluscs, the internal defenses of cephalopods rely on their immune system composed of two factors: cellular (hemocytes) and humoral (Malham

and Runham, 1998). In the first case, phagocytosis results in reactive oxygen and nitrogen species production (ROS and RNS, respectively), which are the main defense mechanisms executed by hemocytes against pathogens (Cheng, 1975). In the second case, humoral components such as agglutinins, lysozymes, opsonins and lectins are diluted into the plasma. These humoral components are complementary to cellular defense and maintain the organism free of infections (Rögener et al., 1985; Ford, 1992; Alpuche et al., 2010).

Innate immunity is no longer seen as a series of simple signaling pathways activated by a pathogen binding to a receptor. Neither is the immune response considered only a function of the host as it is also regulated by the virulence of the pathogen (Gardy et al., 2009). Expressed sequence tags (ESTs) obtained by suppression subtractive hybridization (SSH) have been valuable in discovering host-defense genes and for screening differences in gene transcription against natural pathogens in different molluscs, including bivalves and gastropods (Gueguen et al., 2003; Perrigault et al., 2009; Prado-Álvarez et al., 2009a; Wang et al., 2009;Travers et al., 2010). Nowadays, the study of the host-parasite interaction is performed through specific fields of research that emerged with the arrival of high-throughput methodologies and it provides a wide range of information relative to genes (genomics), transcripts (transcriptomics), proteins (proteomics) and other molecules and their interactions (e.g., metabolomics and pharmacogenomics), even including non model species (Gardy et al., 2009).

The link between genes and their expression is the field covered by proteomics, which focuses on the proteins expressed by the genome of an organism, the proteome (López, 2007). Because the proteome is more dynamic than the genome, proteomics brings advantages such as analysis of the variation in gene expression, which allows visualizing protein expression under the influence of biological perturbations as potential parasites. Additionally proteomics enables quantitative and qualitative analysis of the protein pattern expressed by the host, tissue or cell and allows the study of protein interactions, their function and even post-translational modifications. Since several studies have reported a low correspondence (on average) between mRNA and protein levels due to post-transcriptional and post-translational modifications (PTM), it is not generally possible to predict the number of proteins, their abundance or function based on the DNA sequences and for this reason, proteomics provides valuable information complementary to genomics and transcriptomics (López, 2007; Diz et al., 2012).

In parasitology, the study of the proteome of host and parasites allows the identification of proteins that might be targets for new drugs (Biron et al., 2005b), the investigation of drug resistance dynamics (Barret et al., 2000), the description of manipulative mechanisms used by the parasite (Lefevre et al. 2007; Lutz et al., 2011), and the characterization of those proteins differentially expressed by the host against an infection (Biron et al., 2005a; Vergote et al., 2005). In cephalopods, proteins involved in the immune defense have been identified in the light organ exudate of the squid *Euprymna scolopes* containing the symbiotic bacteria *Vibrio ficheri* (Doino et al., 2000; Schleicher and Nyholm 2011). Recently, several proteins related to the host innate immunity were identified in the squid's circulating hemocytes (Collins et al., 2012). From these analyses, close coordination between host and symbiont systems for allowing the establishment of the *Euprymna /Vibrio* association was revealed.

In this particular study, a proteomic approach was used in the *O. vulgaris* hemolymph for the first time and it provides the identification of proteins expressed by octopus hemocytes using mass spectrometry analysis. In addition, the proteome of both the hemocytes and the plasma of *O. vulgaris* was analyzed in order to obtain the protein expression profile involved in octopus immune response to *A. octopiana* infection and so identifying possible candidate biomarkers. We compared the protein expression maps of octopuses with high and low parasitic load, corresponding to sick and healthy individuals, respectively, in order to explore the immune status of both conditions and to test whether significant changes attributed to the level of infection can be identified in the proteome profile. The present study represents the first step in understanding the immune defense mechanisms triggered by *O. vulgaris* against this protozoan parasite.

2. Materials and Methods

2.1 Specimens collection and hemolymph extraction

Specimens of *O. vulgaris* (n=11) were collected by traditional traps, artisanal fishing gear used by local fishermen from the Ria of Vigo (24˚ 14.09'N, 8˚ 47.18'W), Spain. The octopuses were maintained in filtered sea water tanks at 15 °C during 24 h. Before hemolymph extraction, each octopus was anaesthetized using 7.5% magnesium chloride ($MgCl_2$) according to (Messenger et al., 1985) and following ethical procedure

(Moltschaniwskyj et al., 2007). A dorsal incision was made through the skin and mantle muscle behind the head and, with a disposable syringe (1 ml) directly inserted into the cephalic aorta, hemolymph was withdrawn. One milliliter of hemolymph from each octopus was centrifuged at 12000 × *g*, 4 °C for 5 min. The plasma and hemocyte samples were stored separately at –80 °C until the analysis stage began.

2.2 Isolation and counting of the coccidian Aggregata octopiana

The digestive tract from each octopus was dissected and homogenized in 10 ml of filter sea water (FSW) 1% Tween80 using an electric tissue grinder (IKA-Ultra Turrax T-25). In order to remove tissue fragments, the homogenates were filtered twice with a nylon mesh of 100 and 41 μm respectively. The filtrate was then centrifuged 1000 × *g*, 4 °C, 5 min in a centrifuge Beckman GS-15R. Finally, the number of sporocyst was counted in a Neubauer chamber. The sporocyst number is measured as the number of parasites infecting a unit gram of octopus digestive tract (spor/g) in order to evaluate the parasite infection degree.

A total of 11 octopuses were caught and the extracted plasma and hemocyte samples were divided into two groups: the first showing a high parasite load (4×10^6 to 2×10^7 spor/g; hereafter termed the high infection group) and the second having a low parasite load (0 to 5×10^4 spor/g; hereafter termed the low infection group) (Gestal et al., 2002b). The intensity of infection was confirmed through observation of caecum sections processed by standard histological methods (Humason, 1979). Samples from octopuses in the low infection group were considered as being from healthy animals and were grouped together. The other group with samples from octopuses with high infection and important caecum damage were considered as being from sick animals. The validity of both groups of infection were confirmed using a Student's *t*-test analysis ($p<0.05$) over the sporocyst number data, performed in Statistica 6.0 software.

2.3. Protein extraction

Protein from hemocytes and plasma were extracted in lysis buffer [7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 1% Bio-Lyte 5-8 ampholytes (Bio-Rad) for hemocytes and 2% Bio-Lyte 5-8 ampholytes (Bio-Rad) for plasma]. Samples were disrupted by sonication (Branson Digital Sonifier 250, Danbury, CT, USA) using 6 blasts of 10% amplitude, 5 s pulse on and 5 s pulse off keeping the samples on ice to avoid protein burning. Then, a centrifugation at 14000 rpm, 14 °C, 30 min was carried out. The supernatant was collected and protein concentration measured according to Bradford modified method (Ramagli et al., 1985).

Due to the well known problems of getting a well resolved protein spot pattern by 2-DE analysis when working with plasma (Pernemalm et al., 2009), we first tested different sample pretreatments before deciding the best way of proceeding with this type of sample. Trichloroacetic acid (TCA) and Amicon Ultra-4, 3K (Millipore) filters were used. However, the improvement in the resolution of the protein spot pattern came from using Nanosep 100K (PALL Life Sciences) devices. In this way, after plasma sonication, the protein concentration was standardized at 1.5 µg/ml in lysis buffer and filtered using Nanosep 100K (PALL Life Sciences) devices by centrifugation at 14,000 rpm, 15 °C, 10 min. The filtered fraction less than 100 kDa was recovered and the protein concentration was measured again as previously mentioned.

2.4 Two-dimensional electrophoresis (2-DE)

A total of 11 biological replicates from hemocytes and plasma, *i.e.* 5 with high infection (4×10^6 to 2×10^7 spor/g) and 6 with low (0 to 5×10^4 spor/g) infection individuals were analyzed by 2-DE in batches of six gels/samples per run according to a block design in order to control the described run-to-run variation (Diz et al., 2007). Four of these samples were technically replicated in order to assess the experimental noise in the samples. For analytical gels (hemocyte and plasma), 100 µg of total protein were used. Preparative gels (for protein identification by mass spectrometry) were performed using 300 µg of total protein (hemocytes and plasma).

The proteins were separated according to their isoelectric point through IEF, performed on immobilized pH gradient strips (pH 5–8/17cm, Bio-Rad Hercules, CA, USA) with

a horizontal electrophoresis apparatus Protean IEF System (BioRad) according to the manufacturer's instructions and including an active strip rehydration step (50V). After IEF, two steps of strip equilibration (2 × 15') were performed, one adding DTT and the other, IAA. Subsequently, the second dimension of protein separation was undertaken on 12.5% polyacrylamide gels (22 × 27 × 0.1cm^3) with an Ettan Dalt*six* electrophoresis system (GE Healthcare, Little Chalfont, UK) that allows loading a batch of six gels/samples per run. Electrophoresis was carried out at 20 °C, at 15W/gel ~ 6 h until the point at which the bromophenol blue front reached the bottom of the gel. Protein spots were visualized by the silver nitrate staining method compatible with mass spectrometry analysis (Shevchenko et al., 1996). Co-migrating broad-range standards (BioRad) were used in the second dimension to allow the estimation of molecular masses.

2.5 Image acquisition and analysis

Analytical gels were digitized with a calibrated densitometer (GS-800, BioRad) and the images were saved as TIFF files. The SameSpots v3.3 software (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK) was used to undertake semi-automatic alignment of gels, spot detection and volume measurements. All spots automatically detected by the software were exhaustively checked by manual verification of individual spots in order to discard those showing clear anomalies or artifacts such as specks as well as those saturated spots. Up to 70% of the initial spot detections were discarded for further analysis. The absolute spot volumes were normalized for each gel and transformed to logarithmic scale. Coefficients of variation (*CV*) and determination (R^2) were calculated using the whole protein spot dataset from technical replicates according to (Diz et al., 2011) for assessing the experimental reproducibility. One- way ANOVA was carried out using the normalized and logarithmic transformed volume of each protein spot (the dependent variable) to test for differences in the protein expression pattern previous verification of normality (Kolmogorov-Smirnov) and homocedasticity (Levene test). Principal Component Analysis (PCA) is a multivariate statistical method that allows the identification of the smallest group of principal components (PCs) capable of explaining the maximum variance from the original data. Therefore, significant spots from both tissue samples (hemocytes and plasma) were analyzed by PCA to assess whether or not these candidate spots might represent a confidence protein expression signature for discerning the level of infection (low *vs*. high) in this type of sample. In order to

reduce the number of false positive spots (Diz et al., 2011), a multitest correction SGoF+ (Carvajal-Rodríguez and de Uña-Álvarez, 2011) was applied to the *p-values* obtained from a priori tests (p<0.05). Calculations were carried out in SGoF+ v7.0 software (Carvajal-Rodríguez and Uña-Álvarez, 2011). SGoF+ is a new multiple test adjustment based on a sequential goodness of fit test (SGoF) (for more details consult Carvajal-Rodríguez and Uña-Álvarez, 2011; Carvajal-Rodríguez et al., 2009). Additionally, the fold change (FC) in the expression level between sample groups of both infection conditions was calculated for every protein spot of plasma and hemocytes. In order to test if an additional biological factor (sex) could have an effect on the expression levels observed in each protein spot (the dependent variable), a two-way ANOVA including infection and sex (both as independent variables) were performed. All the former statistical analyses were carried out in SPSS package (v14.0).

2.6 Mass spectrometry analysis for protein identification

The spots of interest were cut out from gels, destained using 15 mM potassium ferricyanide/50 mM sodium thiosulfate and washed sequentially with ammonium bicarbonate 25 mM and 50% ACN/ammonium bicarbonate 25 mM, in an ultrasonic bath. Then, proteins were reduced by treatment with 10 mM DTT for 1 h at 56 ºC and alkylated with 55 mM IAA for 30 min at room temperature. The trypsin digestion was accomplished with 15 µl (0.2 µg/µl) of trypsin (Promega) in 25 mM ammonium bicarbonate per spot at 37 ºC overnight. Tryptic peptides were extracted from the gel matrix in two steps with 0.1% (v/v) TFA and 100% (v/v) ACN, dried and re-dissolved in 2 µL TFA 0.5%/ACN 33% (v/v). The analysis of tryptic peptides was carried out by matrix-assisted laser desorption/ionization time of flight tandem mass spectrometry (MALDI-TOF/TOF MS) with an Autoflex III smartbeam (Bruker Daltonics). Typically, 1 µL were mixed with the same volume of CHCA matrix solution (3 mg/mL, Bruker) in acetone 50%/ethanol 50% (v/v) onto a disposable AnchorchipTM MTP-sized MALDI target. The data was acquired and analyzed using the flexControl v3.0 and flexAnalysis 3.0 software (Bruker Daltonics), respectively. The higher peaks obtained by MALDI-TOF were selected to be further characterized by TOF/TOF analyses. Data were generated in PKL file format, and were submitted for database searching by BioTools v3.2 software (Bruker Daltonics) through the version 2.3.0 of the MASCOT search engine (Matrix Sciences). Three searches were conducted; the first one against SwissProt 2012_05 (536029 sequences; 190235160 residues) and the second against NCBInr 20121216

(22069917 sequences; 7576678142 residues) databases. Search parameters were set as follows: taxonomy Metazoa; enzyme trypsin; allowance of one missed cleavage site; carbamidomethyl of cystein as fixed modification; oxidation of methionine as variable modification; monoisotopic mass values; 100 ppm of mass tolerance for precursor ions; 0.5 Da of mass tolerance for fragment ions and protein mass unrestricted. Protein hits were considered significant when scoring above the $p<0.05$ threshold. A third search was performed against an *O. vulgaris* protein sequence database generated from transcriptomic data (RNA-seq) of hemocytes from highly and lowly infected specimens and this aspect of the analysis will be the subject of a separate manuscript (in prep.). Search parameters were the same as mentioned, with taxonomy ignored.

Protein spots that showed significant differences ($p<0.05$) between infection groups were also analyzed by nano-electrospray ionization MS/MS (ESI-MS/MS) with a FTICR Apex-Qe (Bruker Daltonics). LC separations were performed using an Ultimate 3000 HPLC system (Dionex), operated at a flow rate of 250 nL/min onto a 75 mm x 15 cm, 3 micron particle size C18 reversed phase column (Acclaim PepMap 100 from Dionex). Peptides were eluted using a linear gradient starting at 98 % A (0.1 % formic acid in water) and ending at 50 % B (0.1 % formic acid in ACN) during 60 minutes. Mass measurements were taken from m/z 200 to 2000 and data-dependent MS/MS was performed on multiple charged precursors. A cell fill time of 0.5 s was used for MS measurements and 1 s for MS/MS. Data was acquired using the ApexControl v1.1 software (Bruker Daltonics). Data files were processed using the software Data Analysis v4.0 SP 2 (Bruker Daltonics) generating a Mascot generic file (mgf). The mgf files were submitted for database searching against the NCBInr 20121021 (21165401 sequences; 7253190834 residues) database using the MASCOT v2.3.0 search engine (Matrix science). All the parameters for database search were kept as described before, differing in 7 ppm of mass tolerance for precursor ions and 0.01 Da of mass tolerance for fragment ions.

3. Results

3.1 Two-Dimensional protein profile of O. vulgaris hemocytes

The protein spots from hemocytes were regularly distributed over the 2-DE gel map, and following a quick visual inspection, showed a similar pattern in samples from both groups of infection. More than 1000 spots per gel were observed but, after filtering, only 524 were retained for analysis. The one-way ANOVA over the normalized and logarithmic transformed spot volumes resulted in 42 significant (p<0.05) spots between high and low infection samples (Fig.1). After performing the SGoF+ correction a total of 20 spots remained significant which confirms these spots as candidate genes for further studies. Concerning the PCA carried out using the hemocytes dataset, the information from significant spots (p<0.05, before SGoF+ correction) was reduced to two components that described the 62% of variation observed in this proteomic data (Fig. 2). It can be observed that the information of this set of significant spots provides a proteomic signature capable of displaying samples in the PCA graph separately in two different groups according to their level of infection. From the two-way ANOVA, none of the spots was found significant (p<0.05) either for sex or for interaction between infection and sex. Thus, there is no evidence that the sex of octopuses has an effect over the proteome variation analyzed in this study and so discards any kind of bias in our result due to this factor. The coefficient of variation (*CV*) was computed for every spot between technical replicates and finally averaged for all spots and technical replicates. An average *CV* of 25% was obtained, which is lower than the 42% of biological variation found in our samples and the 30% quoted by (Molloy et al., 2003). The calculation of the coefficient of determination (R^2) is another good way to measure the technical error between replicates. It was calculated by comparing all normalized spot volumes in every pair of technical replicates analyzed. These values (R^2) ranged from 0.63 to 0.85, which is in agreement with those reported by (Diz and Skibinski, 2007).

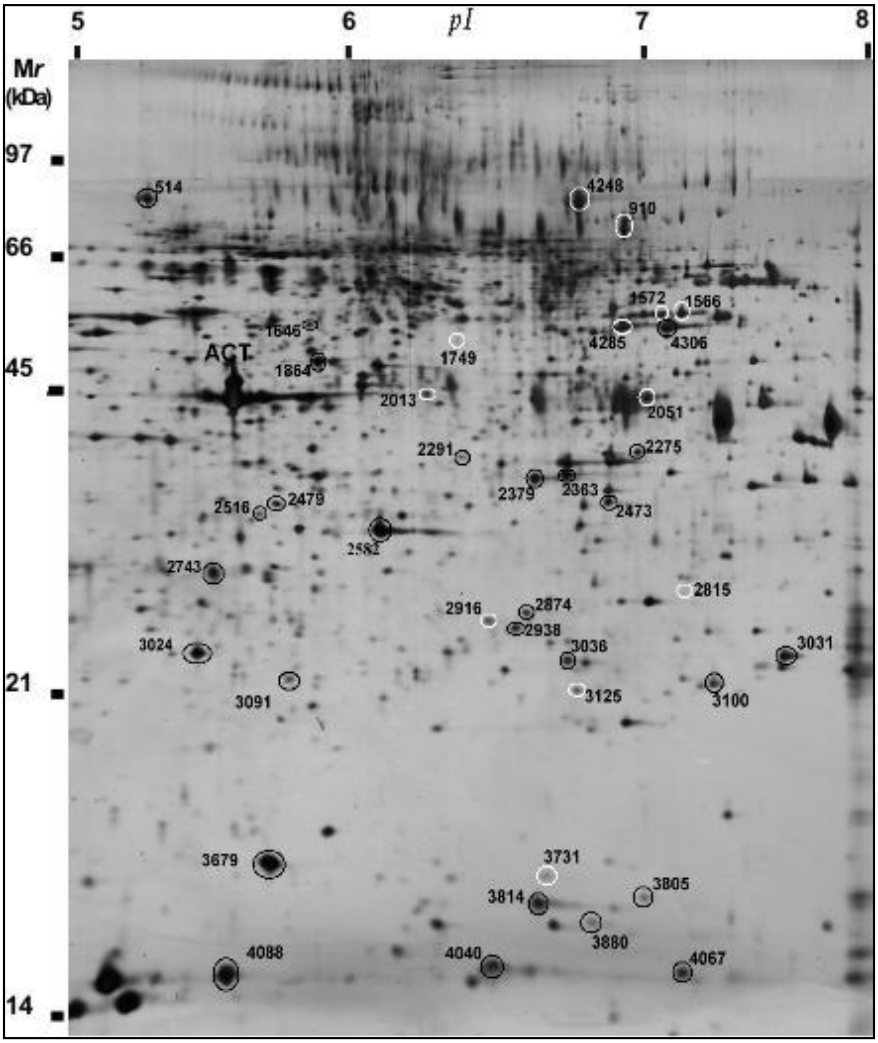


Fig. 1. Protein pattern spots of *Octopus vulgaris* hemocytes. Identified significant spots (before SGoF correction) are showed in white circle. Identified no significant spots are showed in black circle.

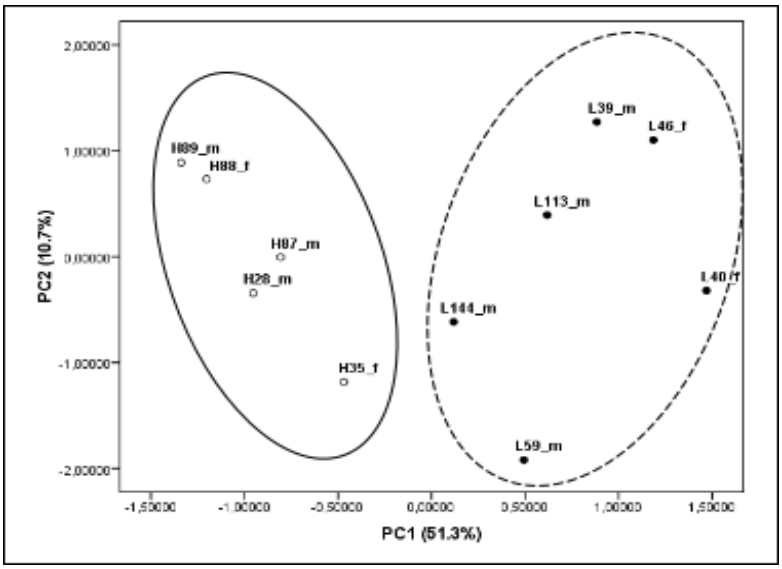


Fig. 2. Plot obtained after PCA of the 42 significant protein spots. Two groups corresponding to High (solid line) and Low (broken line) infection can be differentiated.

3.2 Two-Dimensional protein profile of O. vulgaris plasma

In line with hemocytes 2-DE proteome maps, the protein spot pattern observed was similar in plasma samples from both groups of infection. The 2-DE gel image analysis showed a total of 600 spots per gel. However, after manual verification, this number was reduced to 126 well-resolved spots (Fig. 3). One-way ANOVA analyses on these protein spots resulted in only 6 significant (p<0.05) spots between both groups of infection. After applying SGoF+ correction any spot remained significant. The average coefficient of variation (*CV*) across spots from technical replicates resulted in 47%. This high technical variation is due to the sample fractionation procedure, a necessary step in order to get an acceptable and well-resolved protein spot pattern after 2-DE. This high technical variation shows that despite the method used in the protein extraction (see m&m) to improve the resolution of the 2-DE protein map, compared to others methods, it performs quite poor enhancement in terms of reproducibility. This fact, makes difficult to detect any significant differences in protein expression between samples from both groups of infection through ANOVA analyses due to (on average) high noise-to-signal ratio. PCA analysis did not produce any meaningful result

(data not shown) unlike to hemocyte data. Therefore, quantitative results from plasma samples should be interpreted with great cautious.

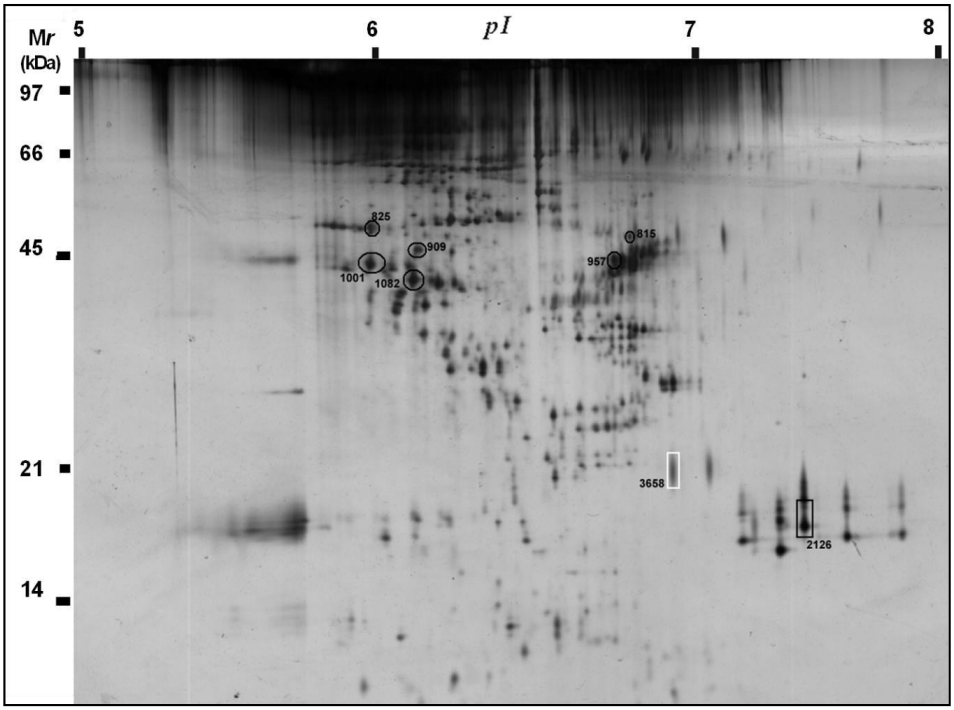


Fig. 3. Protein pattern of *Octopus vulgaris* plasma. Identified not significant spots are showed in black circle. Protein spots in rectangle were not identified but favorable spectra obtained. In white rectangle is showed a significant spot (before SGoF correction).

3.3. Identification of protein spots

Protein spots that showed significant differences (p<0.05) between infection groups, and few others representing the most intense spots, were subjected to in-gel trypsin digestion and mass spectrometry (MS) analysis for protein identification. Table 1 summarizes the data from 39 spots corresponding to 36 proteins from hemocytes. Only five spots (1749, 2013, 2815, 3125, and 4285) were successfully identified in public databases. In contrast, a total of 34 proteins were successfully obtained through the *O. vulgaris*' hemocytes transcriptome database (Table 1). Among the total spots identified, actin isoforms (spots 1749, 2013), peroxiredoxin (spot 3125), glutamate DH (spot 1572), fascin (spot 1566), isocitrate DH (spot

2051), filamin (spot 4248), Rho GDP (spot 3024) and peptidase c1-like protein (spot 2379) dissociation inhibitor were found. Most were significant protein spots and were found up-regulated in octopuses with high infection except actin, peroxiredoxin and 6-phosphogluconolacton (spot 2916), which were found down-regulated in octopuses highly infected by *A. octopiana* (Table 1).

In plasma samples, 23 spots were excised and 5 spots (825, 909, 1001, 1082, 947) were positively identified as the respiratory protein hemocyanin. Despite some other spots from hemocytes and plasma showed good spectra, they were not properly identified mainly due to the scarce representation of mollusc sequences in public databases and the absence of an assembled cephalopod genome.

Table 1- Protein spots identified by MS/MS in *Octopus vulgaris* hemocytes. Protein names according to SwissProt/NCBInr entries. SwissProt/NCBInr accession numbers correspond to protein identifications (--- indicates no identification in public databases or RNA-seq database)

Spot No.	**Fold change**[a]	***p-value***[b]	***p-value***[c] ***(SGoF+)***	**Protein name**	**Function/ Process**	**SwissProt/ NCBInr Accession number**	**Locus number *O.vulgaris* RNA-seq database**	**Overall process**
1749	0.61	0.022	0.048	Actin1B	ATP binding	ACTB_STRPU	1986_Transcript_ 1/10	Cytoskeleton
2013	0.59	0.018	0.014	Actin, adductor muscle	Cytoskeletal	ACT_BRUMA	1986_Transcript_ 1/10	Structural element
2815	1.5	0.025	0.192	Carbonic anhydrase 2	Catalyzes interconversion CO_2 and HCO^3	CAH2_BOVIN	---	Metabolism
3125	0.53	0.024	0.111	Peroxiredoxin	Cell redox homeostasis	TDX_ONCMY	28263_Transcript_ 4/5	Antioxidant
4285	1.2	0.031	0.531	ATP synthase subunit alpha, mitochondrial	Ion transport	ATPA_DROME	25711_Transcript_ 5/9	Metabolism
910	1.51	0.008	0.0009	Sodium channel protein type 4	Sodium ion trans-membrane transport	---	2455_Transcript_ 25/32	Ion transport

Table 1- Protein spots identified by MS/MS in *Octopus vulgaris* hemocytes. Protein names according to SwissProt/NCBInr entries. SwissProt/NCBInr accession numbers correspond to protein identifications (--- indicates no identification in public databases or RNA-seq database) (Continued)

Spot No.	**Fold change[a]**	***p-value[b]***	***p-value[c] (SGoF+)***	**Protein name**	**Function/ Process**	**NCBInr Accession code**	**Locus number *O. vulgaris* database**	**Overall process**
1566	1.70	0.044	0.889	Fascin	Actin organization	---	332_Transcript_ 9/13	Cytoskeleton
1572	1.45	0.020	0.023	Glutamate dehydrogenase	Cellular aminoacid	gi|458803	2222_Transcript_ 11/15	Metabolic process
2051	1.42	0.0003	0.0001	Isocitrate dehydrogenase [NADP], mitochondrial-like	Tricarboxylic acid cycle	---	1039_Transcript_ 17/25	Metabolism
2916	0.47	0.020	0.030	6-phosphogluconolacton	Hydrolase	---	10466_Transcript _2/2	Metabolism
3731	0.59	0.025	0.162	*	---	---	9716_Transcript_1 /9	---
4248	1.56	0.029	0.359	Filamin	Actin-binding protein	---	1735_Transcript_4 1/41	Cytoskeleton
4306	1.28	0.166	--	Enolase-2	Glycolysis	gi|6624237	8136_Transcript_1 /10	Metabolism

*Hypothetical protein (--- denotes no process identified)

Table 1- Protein spots identified by MS/MS in *Octopus vulgaris* hemocytes. Protein names according to SwissProt/NCBInr entries. SwissProt/NCBInr accession numbers correspond to protein identifications (--- indicates no identification in public databases or RNA-seq database) (Continued)

Spot No.	Fold change[a]	*p-value*[b]	*p-value*[c] *(SGoF+)*	Protein name	Function/ Process	NCBInr Accession code	Locus number *O.vulgaris* database	Overall process
4067	1.04	0.991	--	Ubiquitin conjugating enzyme	Catalyzes the covalent attachment of ubiquitin to other proteins	---	1689_Transcript_12/16	Ubl conjugation pathway
2938	0.81	0.439	--	Light organ C8 alpha proteasome subunit	Regulation of signaling events in light organ morphogenesis	gi|47607474	43364_Transcript_10/12	Ubiquitin-dependent protein catabolic process
2874	0.84	0.203	--	Proteasome subunit alpha type-4	Cleave peptide bonds	gi|53148461	---	Cell proliferation

Table 1- Protein spots identified by MS/MS in *Octopus vulgaris* hemocytes. Protein names according to SwissProt/NCBInr entries. SwissProt/NCBInr accession numbers correspond to protein identifications (--- indicates no identification in public databases or RNA-seq database) (Continued).

Spot No.	**Fold change[a]**	***p-value[b]***	***p-value[c] (SGoF+)***	**Protein name**	**Function/ Process**	**NCBInr Accession code**	**Locus number *O.vulgaris* database**	**Overall process**
2363	0.89	0.258	--	Mitochondrial mala dehydrogenase precursor	Tricarboxylic acid cycle	gi|30313535	28231_Transcript_ 13/13	Metabolism
2275	1.18	0.525	--	Proteasome 26S, non-ATPase, 7	Regulatory subunit of the 26S proteasome	gi|82524825	388_Transcript_2 /10	Proteasomal ubiquitin-dependent protein catabolic process
2473	0.38	0.206	--	26S proteasome, non-ATPase,14	Regulatory subunit of the 26S proteasome	gi|71895967	64443_Transcript _ 1/8	DNA damage response
1646	1.07	0.711	--	STE/STE20/PAKA protein kinase	Serine/threo nine proteina kinase	---	44_Transcript_ 12/14	Protein complex assembly
1864	0.95	0.730	--	Phosphoglycerate kinase	Glycolysis	---	13737_Transcript _ 3/10	Metabolism

Table 1- Protein spots identified by MS/MS in *Octopus vulgaris* hemocytes. Protein names according to SwissProt/NCBInr entries. SwissProt/NCBInr accession numbers correspond to protein identifications (--- indicates no identification in public databases or RNA-seq database) (Continued).

Spot No.	Fold change[a]	*p-value* [b]	*p-value*[c] *(SGoF+)*	Protein name	Function/ Process	NCBInr Accession code	Locus number *O.vulgaris* database	Overall process
2516	0.88	0.565	--	SNAP-type protein	Intracellular protein transport	---	35977_Transcript_ 13/13	Metabolism
2479	1.05	0.678	--	Proteasome subunit alpha type-1	Cleave peptides	---	30821_Transcript_ 3/11	Ubiquitin-dependent protein catabolic process
2582	0.91	0.363	--	Annexin	Calcium ion binding	---	103552_Transcript_ 1/4	Cell signalling
2743	0.93	0.619	--	UDP-N-acetylenolpyruvoylglucosamine reductase	Cell wall formation	---	1645_Transcript_ 10/12	Cell cycle
3024	1.17	0.384	--	Rho GDP dissociation inhibitor	Rho protein signal transduction	---	25157_Transcript_ 2/10	Actin filament organization
3091	0.91	0.338	--	Proteasome subunit beta type-2	Cleave peptides	---	35792_Transcript_ 1/1	Ubiquitin-dependent protein catabolic process

Table 1- Protein spots identified by MS/MS in *Octopus vulgaris* hemocytes. Protein names according to SwissProt/NCBInr entries. SwissProt/NCBInr accession numbers correspond to protein identifications (--- indicates no identification in public databases or RNA-seq database) (Continued).

Spot No.	Fold change[a]	*p-value*[b]	*p-value*[c] *(SGoF+)*	Protein name	Function/ Process	NCBInr Accession code	Locus number *O.vulgaris* database	Overall process
3679	1.15	0.255	--	Methylated-DNA-protein-cysteine methyltransferase	Cellular defense against the biological effects of O6-methylguanine in DNA	---	75617_Transcript_2/5	DNA damage, repair
4088	1.10	0.435	---	---	---	---	39173_Transipt_10/11	---
4040	1.37	0.225	--	Cytidine deaminase	Scavenge exogenous and endogenous cytidine	---	39567_Transcript_1/2	Cellular response
3814	1.21	0.122	--	GJ13471	Actin binding	---	9878_Transcript_1/10	Cytoskeleton
3880	0.62	0.283	--	Acriflavin efflux protein	Transporter activity	---	11013_Transcript_18/ 23	Cytoskeleton
3805	0.84	0.378	--	USP-like protein isoform 2	Response to stress	---	10424_Transcript_3/3	Stress response
3036	0.89	0.579	--	*	---	---	39204_Transcript_10	---

*Hypothetical protein (--- denotes no process identified)

Table 1- Protein spots identified by MS/MS in *Octopus vulgaris* hemocytes. Protein names according to SwissProt/NCBInr entries. SwissProt/NCBInr accession numbers correspond to protein identifications (--- indicates no identification in public databases or RNA-seq database) (Continued).

Spot No.	Fold change[a]	*p-value*[b]	*p-value*[c] *(SGoF+)*	Protein Name	Function/ Process	NCBInr Accession code	Locus number *O.vulgaris* database	Overall process
2291	0.80	0.379	--	Estrogen sulfotransferase	Catalyze the transfer of a sulfate group from a donor molecule	---	9965_Transcript_ 30/30	Metabolism
2379	0.76	0.339	--	Peptidase c1-like protein	Proteolysis	---	36179_Transcript_ 12/16	Immune response
3031	0.84	0.761	--	Glutathione s-transferase Mu	glutathione transferase activity	---	2672_Transcript_ 1/6	Detoxification of electrophilic compounds
3100	0.86	0.919	--	Glutathione s-transferase Zeta	glutathione transferase activity	---	40068_Transcript_ 11/11	Detoxification of electrophilic compounds
514	1.26	0.323	--	Glucose-regulated protein 94 (Grp94)/ Endoplasmin	Protein folding	---	27290_Transcript_ 10/10	Chaperon/ protein folding and response to stress

[a] Fold change for significant spots is given as the ratio of the average normalized spot volume low/high infected octopuses.
[b] *p-value* from 1-way ANOVA.
[c] *p-value* corrected for multiple hypothesis testing problem using SGoF+ method (data shown only for those spots with $p<0.05$).

4. Discussion

O. vulgaris is a valuable species for industrial aquaculture (Vaz-Pires et al., 2004). For many aquaculture species, diseases are the primary constraint. Octopuses like other molluscs present a powerful innate immune defence mechanism. However, they lack immune memory and it is therefore not possible to use vaccines for preventing diseases. Likewise drugs cannot be used, since octopuses are reared in suspended cages in the sea (García-García et al., 2004; Gestal et al., 2008). To date, functional and molecular mechanisms of cephalopod immune response are poorly studied. For this reason, better knowledge of octopus immune defense mechanisms will be valuable in managing diseases related to culture facilities and in identifying potential biomarkers of disease resistance that in turn will allow the selection of resistant traits for developing breeding programs.

Proteome changes have been found either in plasma or hemocytes of *Biomphalaria glabrata* (Bouchut et al., 2006), oyster *Ostrea edulis* (Cao et al., 2009), ascidian *Halacynthia roretzi* (Cha et al., 2011) and zebra mussel *Dreissena polymorpha* (Riva et al., 2011) after a period of exposure to pathogens or toxic chemicals. In cephalopods, a proteomic overview of the symbiotic relationship between the sepiolid *E. scolopes* and *V. fischeri* showed that bacteria induces differential expression of distinct protein spots once colonized the light organ (Doino and McFall-Ngai, 2000). Host and symbiont express proteins that allow only *V. fischery* colonizing the host. At the same time, the host expresses proteins to protect itself against cytotoxic damage triggered for avoiding the establishment of non-symbiotic bacteria (Schleicher and Nyholm, 2011;Collins et al., 2012).

In the present work, a proteomic approach was followed for the first time in the *O. vulgaris* hemolymph. In this way, the proteome expression signature of the octopus hemolymph (hemocytes and plasma) is presented. Additionally, the hemolymph protein pattern differentially expressed against the parasite *A. octopiana* is reported, providing evidence for the influence of the parasite over the octopus immune response. A comparative study was conducted in circulating hemocytes and plasma from octopuses with high and low parasite infection degree, corresponding to sick and healthy animals respectively, in order to detect changes in the protein expression and to identify those proteins potentially involved in the octopus immune defense.

The proteome of hemocytes and plasma presented in this study showed significant differences between groups of infection. All the octopuses analysed were sampled at the same locality, exposed to the same environmental conditions, showing similar length (DML: dorsal mantle length), weights and gonadic development (corresponding to

maturing or mature stages as reported by (Otero et al, 2007) to the same area). The only observed difference was the *A. octopiana* infection degree. Therefore, the changes observed in the expression of proteins between groups should be due to the effect of the parasite.

The PCA carried out using the normalized volume of 42 significant hemocyte spots gave rise to two main components that explained the 62% of variance. This result indicates that variances brought about the alteration of the significant spots volumes are attained to the *A. octopiana* infection. The separation of the groups by the PC1 was of 51%. Among the proteins identified with more contribution to PC1 were sodium channel, fascin, glutamate DH, 6-phosphogluconolacton, peroxiredoxin, filamin and ATP synthase. Therefore, such proteins are suitable to study as potential biomarkers involved in the octopus immune response against *A. octopiana.* Furthermore, significant differences (1-way ANOVA, $p<0.05$) in the protein expression pattern according to the low or high infection by *A. octopiana* reinforce the observed results. Our analyses rule out that these results are sex biased as no significant differences were found for this factor (2-way ANOVA, $p>0.05$).

Despite the fact that protein databases of cephalopods and specifically of *O. vulgaris* are scarce, metabolic (carbonic anhydrase), energetic (ATP synthase) and antioxidant (peroxiredoxin) proteins were successfully identified by homology-search to all sequence data available in public databases (Table 1). The identification of these proteins was confirmed by repeating the search against an unpublished protein database (RNA-seq database translation to 6-reading frames) of *O. vulgaris* hemocytes which is the subject of a separate manuscript (in prep.). Moreover, this latter approach allowed the additional identification of 34 protein spots, providing information related to proteins that regulate cell functions (protein kinase), cytoskeleton component (filamin) and proteins with a role in metabolic process like glycolysis (phosphoglycerate kinase) and tricarboxylic cycle (isocitrate dehydrogenase) (Table 1) not yet reported in octopus hemocytes. It was not possible to identify these proteins by the former approach which highlights the need to develop the cephalopod's genomic field in order to make an inventory of protein-coding and non coding genes available (Albertin et al, 2012). Finally, among the 39 hemocyte protein spots selected for identification by MS/MS, a total of 36 proteins were successfully identified. Of these, a total of four spots (1566, 1749, 2013, 4248) corresponded to actin isoforms and cytoskeletal proteins, two spots (2815 and 4285) were proteins related to cellular energy (ATP synthase and carbonic anhydrase),

while most of the spots were proteins involved in different metabolic process, and one of these (3125) corresponded to the antioxidant protein, peroxiredoxin (Table 1).

Actin is involved in cytoskeletal structure and is valuable for numerous cellular processes such as cell division, cell organization and motility, and because of this, it is commonly found in proteomic studies (Riva et al., 2011; Goodson and Hawse, 2002; Martínez-Fernández et al., 2008). Additional cytoskeletal proteins filamin (spot 4248) and fascin (spot 1566) were identified in *O. vulgaris* hemocytes. As a structural protein, filamin is related to cell functions as motility or maintenance of shape. The filamin gene was found over-expressed in *Ostrea edulis* hemocytes challenged with live *Bonamia ostrea*. After the hemocyte-parasite contact, the phagosome is formed resulting in an increase of cytoskeleton polymerization which internalizes and destroys the parasite (Morga et al., 2011). Filamin is recognized as a structural protein, though it can also play an important role as an interface in protein-protein interactions, usually interacting with membrane receptors for cell signaling molecules. This protein takes an active role in signaling pathways through the activation of NF-κB with a key function in regulating the immune response to infections (Vasselon and Detmers, 2002; Feng and Walsh, 2004). In relation to the cell structure, fascins represent a family of actin-bundling proteins responsible for membrane protrusions and formation of actin bundles present in filopodia. Because of this, fascins participate in extending membranes for cell motility and phagocytic defense (Yamashiro et al., 1998). Filamin and fascin were identified up-regulated in the proteome of highly infected octopuses, suggesting that they could be related to the host immune response and phagocytic process during the infection by *A. octopiana.* ATP synthase was also found in the proteome of the octopus hemocytes. ATP synthase is an integral membrane enzyme. It catalyzes ATP hydrolysis and supplies energy in order to maintain the electrochemical gradient, the regulation of cell volume, the synthesis of macromolecules, ion transport and ionic integrity, which also activates the immune defense carrying out defense actions as phagocytosis (Buttgereit et al., 2000; Huang et al., 2011; Coyne, 2011).

The antioxidant protein peroxiredoxin (spot 3125) was down-regulated (0.53 fold change) in octopuses highly infected by *A. octopiana.* Thus, a faint spot or completely absent was seen in this octopus group, while a strong spot was observed in octopuses with low infection. Similar to their mollusc relatives, octopuses depend on their innate immune defense to protect themselves against invaders (Ford, 1992). The hemocytes play a crucial role in the host's immune functions. They are involved in cytotoxic reactions when the organism is attacked by pathogens, which induce release of high levels of reactive oxygen

species (ROS) and consequently, oxidative stress (Chu, 2000). However, the host cells can also be damaged by ROS in membrane properties like ion transport, protein cross-linking, DNA strand scission, protein oxidation and lipid peroxidation (Bandyopadhyay et al., 1999). Therefore, an antioxidative system is needed to protect against oxidative stress. Such is the case of peroxiredoxins, antioxidant proteins present in prokaryotes and eukaryotes with a major common function of enzymatic degradation of hydrogen peroxide (Immenschuh and Baumgart-Vogt, 2005). In *Drosophila melanogaster,* detoxification by peroxiredoxin is the key to protecting the epithelia gut against the oxidative stress after bacterial infection (Ahn et al., 2012). Likewise, peroxiredoxin protein has been identified in *E. scolopes* light organ, where the symbiont (*V. fischeri*) must overcome an oxidative microenvironment to colonize the host (Schleicher and Nyholm, 2011), however, the precise role of peroxiredoxin in this symbiotic relationship has not yet been clarified.

The differences in peroxiredoxin expression level observed in our results suggest different ability of individuals to deal with ROS. The intense spot clearly observed in octopuses with low infection allude to their ability in attacking pathogens through cytotoxic response. In this case, consequences derived from oxidative stress can be handled by cells and this does not seem to occur in highly infected octopuses. According to functional assays, we have observed an inverse relationship among the octopus immune response and the infection intensity by *A. octopiana*. Nitric oxide (NO) and ROS showed a weak, but not significant decrease in highly (up to 3.6 x 10^6) infected octopuses (Castellanos-Martínez and Gestal, in press.). A noticeable but not significant decrease of ROS was also detected in *Crassostrea virginica* hemocytes exposed to 3.7 x10^6 *Perkinsus marinus* cells, unless the infection reached at least to 7.5 x 10^6 cells, in which case the ROS decreased significantly (Volety and Chu, 1995).

The inhibition or suppression of ROS is a mechanism commonly employed by parasites like *P. marinus* and *B. ostreae* to enter the host cells without triggering the respiratory burst (Morga et al., 2011; Volety and Chu, 1995). In case of *A. octopiana*, the parasite infects the octopus digestive tract which in turn, causes malfunction of digestive enzymes (Gestal et al., 2002b). However, a heavy *A. octopiana* infection also causes a decrease in the number of circulating hemocytes; this result indicates an effect of parasitosis on the octopus immune system (Gestal et al., 2007b). The absence of peroxiredoxin in circulating hemocytes from octopuses heavily infected could therefore be a suppressive mechanism of ROS. On the other hand, a second hypothesis may be stated: once *A. octopiana* is established in the caecum it starts a chronic infection. Typically, hemocytic infiltration and fibrotic reaction is observed in the tissue infected (Gestal et al.,

2002a). Hemocytes enclose parasites and become susceptible to nitrogen and oxygen radicals. After that, production of NO and ROS is continuously produced by infiltrated hemocytes to the point where oxidative stress is high enough to reach equilibrium despite not inducing irreversible cell damage (Novo and Parola, 2008). Because of this, peroxiredoxins may be almost inactive. Considering the expression differences in octopuses with low and high infection, gene encoding peroxiredoxin shows activation in the former group. In this case, the protein abundance and presumably differential transcription according to the infection remains to be elucidated.

Additional proteins that showed significant differences in expression between levels of infection were identified as glutamate DH, isocitrate DH, sodium channel and phosphogluconolactonase; the former three proteins were up-regulated in high infected octopuses, while phosphogluconolactonase was down-regulated in the same group, suggesting that cellular processes are differentially affected by the level of infection. Consequently, these proteins require further study in order to reveal their specific role during coccidiosis and to assess the validity of those proteins that, according to PCA, are potential biomarkers. Proteins such as enolase, ubiquitin and mitochondrial malate DH, highly conserved proteins with roles in metabolism and cellular functions, did not show significant differences in expression in high compared to low infected samples (Table 1).

Complementary to cellular factors, the octopus immune defense is carried out by molecules dissolved in the plasma, collectively named humoral factors (Malham and Runham, 1998). Some of these molecules are potentially useful biomarkers of infection, of disease resistance, or possible targets to combat parasites (Barret et al., 2000). Therefore, an attempt to search for differential expression of proteins present in the *O. vulgaris* humoral factors was undertaken. Nevertheless we encountered problems in obtaining a well-resolved proteome map by 2-DE. Plasma is a challenging biological material due to the high dynamic range of protein concentration, and is usually dominated by few highly abundant proteins that make good resolution difficult after protein separation by 2-DE. For this reason, a common approach is to reduce the high range of concentrations by fractionation, typically based on antibody affinity (Pernemalm et al., 2009) to eliminate those few highly abundant proteins. Nevertheless, no specific method has yet been developed for octopus plasma. Thus, pretreatment of samples using filters Amicon® Ultra-4, 3K (Millipore) and protein precipitation by TCA were assayed in the present study. However differences in the proteome obtained with both methods were observed, since a limited representation of proteins was obtained mainly using TCA. Therefore, pretreatment of octopus plasma was assessed using Nanosep devices 100K (PALL

Corporation). The major protein contained in the octopus plasma, hemocyanin (Rögener et al., 1985; Van-Holde and Miller, 1995) was partially retained, which help to observe those less abundant proteins in the 2-DE protein map. Certainly any golden standard method exists in plasma proteomics due to every method has its own limitations (Pernemalm et al., 2009), but in order to find those proteins poorly represented in the octopus proteome and putatively involved in the cephalopod immune response, a trade-off between improved sample quality and complete protein representation was carefully considered. Derived from the method followed to research differential expression of the *O. vulgaris* humoral factors, 23 spots including 6 significant (before multitest correction SGoF+) and others from the most intense spots were selected for identification. From these, only 5 spots were positively identified as hemocyanin, the main protein freely dissolved in the octopus hemolymph and responsible of oxygen transportation (Rögener et al., 1985; Van-Holde and Miller, 1995). Two additional spots (3658, 2126) with good spectra could not be identified because the lack of sequences in public databases. Further studies will be performed to clarify the identity of such unknown proteins and to determinate whether they are involved in the octopus humoral defense system.

5. Concluding remarks

The proteomic approach applied in this study revealed, for the first time, the protein composition of hemocytes and plasma of *O. vulgaris* and allowed the study of the relationship between *O. vulgaris* and its parasite *A. octopiana*. The proteome of the *O. vulgaris* hemocytes and plasma were successfully obtained from individuals with different levels of *A. octopiana* infection. Moreover, important proteins showed different levels of expression in response to the infection by *A. octopiana* allowing the discovery of a protein expression signature. A total of 36 proteins were identified from hemocytes, and seven of them are suggested as candidates for biomarkers by PCA. The proteins filamin, fascin and peroxiredoxin are highlighted because of their implication with defense activity. Despite the little available information concerning the role of hemocytes in the octopus immune response and its susceptibility or resistance to infection, the results stated here showed that octopus hemocytes are capable of fighting against pathogens activating several proteins. Moreover, the up- and down- regulated proteins identified in this study may establish the initial basis for the octopus susceptibility/resistance research in a natural host-parasite relationship, and are suitable for study as potential biomarkers for pathogen resistance. Future molecular characterization and functional studies should be aimed at determining the precise role of the proteins identified here in the coccidiosis and in the octopus immune defense.

II. RESUMEN

II.1. Introducción

Octopus vulgaris Cuvier, 1797 es una de las especies de cefalópodos más importantes tanto en pesquerías mundiales como en acuicultura. Galicia es la Comunidad Autónoma pionera en cultivo de pulpo el cual, es ya uno de los recursos alternativos más importantes para diversificar la acuicultura. Para obtener una buena producción acuícola también es importante mantener buenas prácticas sanitarias, lo que implica erradicar y prevenir enfermedades de los organismos en cultivo. Para ello, el primer paso es identificar los patógenos para poder combatirlos, y en segundo lugar es de gran importancia conocer cómo actúa la respuesta inmune del pulpo ante patógenos. De esta manera, se podrán elaborar estrategias enfocadas a mantener una adecuada sanidad acuícola, y se establecerán las bases moleculares para identificar y seleccionar pulpos resistentes a la infección. Uno de los patógenos mas importantes que afectan al pulpo *O. vulgaris* es el protozoo gastrotintestinal *Aggregata octopiana*. Por ello, en la presente tesis se abordó el estudio de la respuesta inmune celular del pulpo común frente a la infección por *A. octopiana,* analizando la interacción hospedador-patógeno tanto a nivel funcional, como transcriptómico y proteómico.

El presente resumen está enfocado en resaltar los resultados más relevantes de la tesis doctoral. Con el objetivo de facilitar la comprensión de los mismos, éstos serán descritos individualmente por capítulos.

II.2. Capítulo 1. Análisis filogenético molecular de los coccidios parásitos de cefalópodos *Aggregata octopiana* y *Aggregata eberthi* (Apicomplexa: Aggregatidae) del Atlántico NE usando secuencias del gen 18S rARN

En Europa hay tres especies reconocidas de coccidios: *Aggregata octopiana* es el coccidio que infecta a *Octopus vulgaris; Aggregata eberthi* es el coccidio que infecta a *Sepia officinalis* (Gestal et al., 1999); y *Aggregata sagittata*, que infecta a la pota *Todarodes sagittatus* (Gestal et al., 2000). Al igual que otros coccidios, las tres especies especies se identifican con base en caracteres morfológicos tales como diámetro del esporoquiste, estructura de la pared del mismo, el número de esporozoítos dentro de cada esporoquiste, así como la especie de cefalópodo que infectan, ya que los coccidios son altamente específicos de sus hospedadores definitivos (Hochberg, 1990; Gestal et al., 1999). El objetivo de este capítulo fue realizar la caracterización molecular de *A. octopiana* y *A. eberthi*, ya que ambos parásitos infectan a los cefalópodos con mayor importancia económica en la región. De esta manera, se complementa la descripción morfológica existente con información molecular del gen 18S rARN y a su vez, se confirma la afiliación taxonómica de ambos coccidios mediante un estudio filogenético.

Hasta ahora, las únicas secuencias disponibles en GenBank de ambos parásitos son las depositadas por Kopečná et al. (2006) usando el gen 18S rARN. Los parásitos fueron aislados del tracto digestivo de *O. vulgaris* y *S. officinalis* del Mar Adriático (Croacia) e identificados como *A. octopiana* y *A. eberthi*, respectivamente. Ambas secuencias se utilizaron como referencia para comparar con las especies de coccidios secuenciadas de la Ría de Vigo. Así, se determinó la afinidad filogenética de los coccidios de la Ría de Vigo y su similitud con las especies del Mar Adriático.

Los caracteres morfológicos observados mediante histología, microscopía óptica y microscopía electrónica de barrido (MEB) de *A. octopiana* y *A. eberthi* de la Ría de Vigo fueron consistentes con la información previamente descrita de ambas especies en el Atlántico NE (Dobell, 1925; Gestal et al., 1999b).

En el análisis filogenético, los coccidios de la Ría de Vigo formaron un grupo monofilético con los coccidios de Croacia, respaldado por un bootstrap del 100%. Los coccidios de la familia Aggregatidae formaron un clado con las especies del género *Klossia,*

Hepatozoon y *Adeleorina* sp. (bootstrap 100%). De éstas, los coccidios del género *Adelina* mostraron una posición basal, coincidiendo con los resultados obtenidos por Kopečná et al. (2006). De esta manera, los parásitos Adeleorinidos se ubican como los miembros más antiguos del grupo de los Eucoccidiorida, tal como lo establece Levine (1985), compartiendo con los aggregatidos algunas características como son la formación de esporoquistes y la presencia de una sutura longitudinal (Gestal et al., 1999b; Kopečná et al., 2006). Cabe mencionar que en el presente estudio, al igual que los resultados obtenidos por Kopečná et al. (2006), no fue posible discriminar con exactitud la posición y relación filogenética del género *Aggregata* debido a las escasas secuencias disponibles. Por tanto, se requiere aumentar el número de secuencias disponibles de diversos coccidios, así como encontrar nuevos marcadores genéticos que permitan resolver la relación filogenética entre estos parásitos (Barta et al., 2012).

Por otra parte, se observó una divergencia genética del 15.9% entre la especie *A. octopiana* de la Ría de Vigo y *A. octopiana* de Mar Adriático. Mientras que la divergencia genética fue de tan sólo 2.4% entre *A. eberthi* de la Ría de Vigo y *A. eberthi* del Mar Adriático. La divergencia genética entre especies de *A. eberthi* indica una divergencia conespecífica (diferencias entre poblaciones de la misma especie). En cambio, la divergencia genética entre especies de *A. octopiana* sugiere que éstas corresponden a especies distintas. Los escasos registros de coccidios que infectan a *O. vulgaris* en el Mar Adriático son confusos, por lo cual no permiten precisar la especie de la que se trata. Los registros disponibles del Mar Adriático indican que el diámetro de los esporoquistes de *A. octopiana* es similar al registrado para *A. octopiana* en el Atlántico NE. Sin embargo, las características más conspicuas de la especie como son la pared espinosa y 8 esporozoítos dentro de cada esporoquiste no coinciden. Una posible explicación es que el pulpo *O. vulgaris* en el Mar Adriático corresponda a una población distinta a la que habita en el Atlántico NE. De hecho, aún no está definido si *O. vulgaris* corresponde a una sola especie cosmopolita o es un "complejo de especies" formado por especies crípticas (Guerra et al., 2010). Por tanto, se considera que existen distintas poblaciones con diferencias en las estructuras reproductivas y parásitos específicos (Mangold, 1998; Guerra com. pers.). Debido a que los coccidios son altamente específicos de sus hospedadores definitivos, las diferencias que muestran los coccidios del Mar Adriático (Mladineo y Jozić, 2005; Mladineo y Bočina, 2007) sugieren que las distintas poblaciones de pulpo albergan distintas especies de parásitos del género *Aggregata*.

Por tanto, tomando en cuenta la evidencia morfológica previa (Gestal et al., 1999b; Gestal y Pascual, 2002; Gestal et al., 2002c), la especificidad hospedadora y la nueva evidencia molecular, se concluye que la especie *A. octopiana* que parasita a *O. vulgaris* en la Ría de Vigo (NW España, Atlántico NE) es la especie válida. Además se confirma que *A. eberthi* es la especie que infecta a *S. officinalis* en la misma localidad. Asimismo, los datos moleculares validan los caracteres morfológicos como herramientas útiles para identificar correctamente ambas especies de coccidios.

II.3. Capítulo 2. Caracterización morfológica, citométrica y funcional de los hemocitos del pulpo común (*Octopus vulgaris*)

Los hemocitos son células presentes en la hemolinfa circulante de los moluscos y están involucradas en funciones tales como reparación de tejido dañado, transporte de nutrientes, pero también tienen un papel importante en la defensa interna del organismo (Cheng, 1975; Chu, 2000). Hasta ahora, en cefalópodos, usando técnicas de microscopía óptica y electrónica, se reconoce sólo un tipo de hemocito de aspecto redondeado, con numerosos gránulos en el citoplasma y un núcleo en forma de U, que los asemeja a monocitos de mamíferos (Cowden y Curtis, 1981; Malham y Runham, 1998). El presente estudio es el primero en introducir la citometría de flujo para caracterizar los hemocitos del pulpo y medir la actividad fagocítica y producción de radicales de oxígeno en dichas células. Conjuntamente, se midió la producción de óxido nítrico en hemocitos ante distintos estímulos. El estudio se complementó con el análisis de los hemocitos mediante microscopía óptica, microscopía electrónica de barrido (MEB) y de transmisión (MET) combinadas con técnicas citoquímicas. La citometría de flujo separa las células en función del tamaño y la complejidad de las mismas, determinada por la cantidad de gránulos presentes en el citoplasma.

En el caso de la hemolinfa del pulpo, mediante la observación de células por microscopía óptica y electrónica (MEB y MET) se observaron dos tipos celulares. El primero de ellos es de tamaño grande (Tabla I), presenta el núcleo en forma de U, con numerosos gránulos en el citoplasma y se ha denominado granulocito grande. El segundo tipo celular fue denominado granulocito pequeño, presenta el núcleo redondeado u ovalado, poco citoplasma e incluso algunas células tienen una fina capa de citoplasma apenas visible, presentan pocos gránulos o bien pueden estar totalmente ausentes. Estas células son pequeñas aunque con una gran variabilidad de tamaños (Tabla 1). Una vez teñidas, se confirmaron las características

celulares antes mencionadas. Se distinguieron gránulos basófilos en el citoplasma de las células (Tabla 1). Ambos tipos celulares se observaron también mediante MEB. Los granulocitos grandes observados (11 µm) mostraron gran capacidad para adherirse a la superficie. Particularmente, los granulocitos grandes observados mediante MET presentaron numerosos gránulos electron-densos y gránulos electron-claros redondeados, y ocasionalmente se observaron algunos gránulos en forma de bastón. Entre los gránulos observados se distinguieron algunos depósitos de glicógeno y lysosomas. Por su parte, mediante MEB los granulocitos pequeños mostraron una superficie celular irregular y presentan pseudópodos mas finos que en el caso de los granulocitos grandes extendidos sobre la superficie. Sin embargo, no fue posible observar granulocitos pequeños mediante MET debido a que aparecen en menor proporción que los granulocitos grandes.

Tabla 1. Diámetro de al menos 200 hemocitos medidos en distintas muestras y técnicas (media ± DS).

Tipo de hemocito	Células en suspensión	Células teñidas	Células aisladas por Sortin
Granulocito Grande	10.57 µm ± 0.41 (10-12.57)	12.5 µm ±1.10 (10.23-14.97)	11.6 µm ± 1.2 (9.32–15.56)
Granulocito pequeño	9.27 µm ± 0.68 (5.55-9.98)	9.12 µm ± 0.71 (7.98 – 9.9)	8.12 µm ± 0.74 (6.69 – 9.99)

Mediante citometría de flujo se confirmó la presencia de dos poblaciones celulares con distintas características de tamaño y complejidad denominadas R1 y R2. De acuerdo con la citometría, la principal población de células (R1) conforma el 82% de la hemolinfa del pulpo, son células de tamaño grande y presentan numerosos gránulos, por lo cual tienen mayor complejidad. En cambio, las células de la segunda población (R2) conforman el 18% restante de la hemolinfa; son de menor tamaño que las células de R1 y presentan pocos gránulos. Por tanto tienen menor complejidad. Ambas poblaciones celulares se aislaron mediante sortin. De esta manera, se observó que el diámetro de los hemocitos de R1 (Tabla 1), el núcleo en forma de U, cantidad de citoplasma y granularidad es consistente con las características observadas en los granulocitos grandes. Por su parte, las células aisladas de R2 mostraron una amplia

variación de diámetros (Tabla 1), el núcleo es ovoide o circular y presentan pocos o ningún gránulo en el citoplasma. Por tanto, tales características los clasifican como granulocitos pequeños.

En cefalópodos, el órgano leucopoyetico se denomina cuerpo blanco y se localiza detrás de los ojos. Una vez que los hemocitos han completado su desarrollo son vertidos a la hemolinfa (Cowden, 1972). En el cuerpo blanco de *O. vulgaris* se observaron células en dos fases de desarrollo distintas. La primera de ellas concuerda con las características de un leucoblasto secundario. Éste se caracteriza por presentar cromatina condensada en todo el núcleo, pocas inclusiones citoplasmáticas y una delgada capa de material denso bordeando la célula. El segundo tipo celular corresponde a un hemocito en etapa intermedia entre leucoblasto primario y un hemocito maduro. La característica de esta fase de transición es un núcleo compacto, de forma irregular con poca cromatina condensada, y numerosas inclusiones electron-densas y electron-claras en el citoplasma.

Para determinar la capacidad fagocítica de los hemocitos, se verificó mediante microscopía óptica que los hemocitos de *O. vulgaris* podían fagocitar zimosán y fluoroesferas. La cuantificación de la capacidad fagocítica de los hemocitos se midió mediante citometría de flujo y utilizando únicamente fluoroesferas. Ésta se registró como un incremento en el nivel de fluorescencia en el canal FL1-H. En ambos tipos celulares (granulocitos grandes y granulocitos pequeños) se observaron amplias variaciones en el porcentaje de fagocitosis. No obstante, la fagocitosis media fue mayor en la región R1, correspondiente a los granulocitos grandes (13%), que alcanzaron hasta un 56% de fagocitosis. En cambio, el porcentaje medio de fagocitosis fue menor (3%) en la región R2, correspondiente a los granulocitos pequeños, que alcanzaron un máximo de 9% después de 2 h de incubación.

La producción de especies reactivas de oxígeno (ROS), también denominado estallido respiratorio, medida mediante la oxidación del compuesto DFCH al compuesto altamente fluorescente DCF, resultó significativamente (p<0.05) mayor en los las muestras estimuladas con zimosán que en los controles. Los granulocitos grandes mostraron, en promedio, mayor fluorescencia 12 A.U. que los granulocitos pequeños 5 A.U. En ambos casos, se observó que la producción de radicales de oxígeno era inhibida por el SOD, lo que indica que la sonda es oxidada por el radical peróxido de hidrógeno. Por el contrario, no ocurrió inhibición cuando se añadió el NMMA, lo que indica que los radicales de nitrógeno no son responsables de oxidar el compuesto DFCH (Possel et al., 1997; Buggé et al., 2007; Lesser, 2005).

De los resultados obtenidos, se comprobó que los hemocitos de *O. vulgaris* tienen la capacidad de fagocitar zimosán, tal como se ha registrado con anterioridad (Novoa al., 2002; Rodríguez-Domínguez et al., 2006). Sin embargo, se demostró mediante citometría de flujo que las células también tienen capacidad de fagocitar fluoroesferas. Ambos tipos celulares presentaron actividad, sin embargo, los granulocitos grandes mostraron mayor fagocitosis comparado con los granulocitos pequeños. En otros moluscos, los granulocitos suelen ser las células con mayor actividad fagocítica, aunque se ha demostrado que los hialinocitos también pueden fagocitar fluoroesferas o zimosan (García-García et al., 2008; Travers et al., 2008; Donaghy et al., 2009; 2010). El porcentaje de células fagocíticas registradas en este estudio en la hemolinfa de *O. vulgaris* mostró una alta variación que posiblemente sea debida a fluctuaciones naturales. Sin embargo, el porcentaje promedio es similar al registrado previamente en *O. vulgaris* utilizando zimosan (Novoa et al., 2002; Rodríguez-Domínguez et al., 2006) y a otros moluscos como *Haliotis discus discus* (Donaghy et al., 2010) *Haliotis tuberculata* (Travers et al., 2008) y *Ruditapes decussatus* (Prado-Alvarez et al., 2012). Puesto que los granulocitos grandes mostraron mayor fagocitosis, es consistente que éstos también produjeran un estallido respiratorio mayor. Por tanto, éstas células podrían tener mayor capacidad de fagocitar y eliminar patógenos (Hégaret et al., 2003; Goedken et al., 2004).

Por otra parte, la producción óxido nítrico (NO) resultó significativamente ($p<0.05$) más alta en hemocitos estimulados con zimosán que en los controles. Además, se comprobó que el PMA y LPS también estimulan la producción de este radical ($p>0.05$). El zimosán se ha utilizado exitosamente para estimular la producción de NO en otros moluscos (Tafalla et al., 2003; García-García et al., 2008) y también resulta efectivo ($p<0.05$) en *O. vulgaris* (Novoa et al., 2002), donde mantiene una producción elevada durante las primeras 3 h de incubación. No obstante, utilizando PMA también muestran mayor producción de NO a las 3 h, sugiriendo que la reacción es lenta, pero intensa. Un estudio previo sugiere que el LPS induce una débil producción de NO en hemocitos de *O. vulgaris* (Novoa et al., 2002). Sin embargo, los resultados obtenidos muestran que induce una reacción intensa después de 30 min y hasta 6 h de incubación. Estudios posteriores permitirán averiguar si las cascadas activadas por el zimosán, PMA y LPS son similares a las descritas para *Lymnaea stagnalis* (Wright et al., 2006) o *Mytilus galloprovincialis* (García-García et al., 2008).

En conclusión, los resultados obtenidos en el capítulo 2 de esta tesis doctoral demuestran por primera vez que la hemolinfa del pulpo común está constituida por dos tipos

de células: granulocitos grandes y granulocitos pequeños. Ambas células presentan capacidad de fagocitar fluoroesferas y producir radicales de oxígeno. Sin embargo, dichas actividades son mayores en los granulocitos grandes. Los datos aportados en el presente estudio establecen las bases para que en estudios posteriores i) se determine la capacidad de los hemocitos de *O. vulgaris* para luchar contra infecciones, y ii) cómo influyen los patógenos en la respuesta inmune celular. Además, se requieren estudios que empleen anticuerpos fluorescentes para mejorar la clasificación de los hemocitos y comprender las funciones inmunes particulares de cada tipo celular.

II.4. Capítulo 3. Parámetros inmunes en el pulpo común (*Octopus vulgaris* Cuvier, 1797) infectado naturalmente por el protozoo gastrointestinal *Aggregata octopiana*

En este capítulo se estudio el efecto de la infección de *A. octopiana* sobre la respuesta inmune celular de *O. vulgaris*. Para ello, se midió el porcentaje de fagocitosis, el estallido respiratorio y la producción de NO en los hemocitos de pulpos clasificados como: sanos, con poca o nula infección (0 a 5×10^5 esporoquistes/gramo de tejido digestivo [spor/g]) y sin daño histopatológico; y enfermos, con alta infección [5×10^5 a 2×10^7 spor/g] y daño histopatológico severo. También se calculó de índice de Hayashi, de acuerdo con Guerra (1975) para determinar la fase de madurez de los pulpos. La respuesta inmune celular se midió a través de la fagocitosis de los hemocitos y la producción de radicales de oxígeno y nitrógeno. A su vez, los resultados obtenidos se estandarizaron de la siguiente manera: valor de fagocitosis, ROS o NO / número de hemocitos circulantes (de cada individuo) por ml. Para determinar el efecto de la infección de *A. octopiana* sobre los parámetros inmunes estandarizados, se realizó un análisis de regresión lineal usando la infección total e infección por grupos (sano-enfermo) como variables independientes. Además, se incluyeron en el análisis factores biómetricos del pulpo (talla, peso, sexo y fase de madurez), estación del año y origen (salvaje o cultivado en batea). Se aplicó el Criterio de Akaike (AIC) para seleccionar el modelo final empleado así como las variables de mayor relevancia. Las variables con menor relevancia para explicar la variación en la fagocitosis y la producción de ROS y NO de los hemocitos presentaron menor AIC y por tanto, fueron eliminadas del modelo final. Asimismo, se aplicó un análisis de regresión múltiple para estudiar la relación entre la infección y la respuesta inmune celular.

La prevalencia de infección fue del 99%, mientras que la intensidad de infección varió entre 0 y 2 × 10^7 spor/g. La capacidad fagocítica de los hemocitos se vio estimulada por el incremento de la infección total. La estación del año y el sexo de los pulpos también resultaron ser variables importantes para explicar la variación en la capacidad fagocítica de los hemocitos y por tanto, se mantuvieron en el modelo final. Sin embargo, la variable sexo no resultó significativa (p=0.1082), mientras que la fagocitosis se incrementó significativamente en otoño (p=0.0015). Por lo tanto, el grupo de variables infección total, sexo y estación del año explicaron el 24.35% de la variación observada en la fagocitosis de los hemocitos. Al mismo tiempo, el aumento de la infección total provocó la disminución del estallido respiratorio (ROS) (p=0.0082) y la producción de NO (p=0.2697). En el caso del estallido respiratorio, además de la infección, la variable origen también fue incluida en el modelo. Se observó que la producción de ROS fue menor en los pulpos salvajes que en los de batea. Por tanto, el grupo de variables infección total y origen de los pulpos (salvajes) explicaron el 24.35% de la variación observada en el estallido respiratorio. Por su parte, la producción de NO disminuyó significativamente en pulpos sanos (p=0.0043) y en los de mayor peso corporal. Comparando entre individuos salvajes y de batea, la producción de NO resultó significativamente (p=0.00228) menor en los primeros. De esta manera, las variables: infección total, infección por grupo (sanos), peso de los pulpos y origen (salvaje) explicaron el 17.25% de la variación en la producción de NO.

Los resultados obtenidos muestran evidencia complementaria a los estudios previamente desarrollados por Gestal et al. (2002a,b). En estos estudios se evidencia que en los pulpos enfermos (con alta infección) el epitelio del tracto digestivo se encuentra severamente deteriorado por el crecimiento y liberación de los parásitos, lo cual causa la ruptura del tejido. Además, la acidificación del lumen digestivo impide la correcta absorción de nutrientes. Los hemocitos de cefalópodos son capaces de desplazarse para reparar el tejido dañado, o bien formar un "tapón" para evitar la pérdida de hemolinfa (Cowden and Curtis, 1981; Féral, 1988). A causa de ello, se observó una importante infiltración hemocitaria en el ciego de pulpos enfermos.

En relación a las actividades inmunes medidas, se observó que la capacidad fagocítica de los hemocitos se incrementó conforme lo hace la infección. Puesto que la coccidiosis es una infección auto-limitante, ésta no parece limitar la capacidad de fagocitosis de los hemocitos aún cuando la infección se incrementa. Otra variable con una importante influencia en la

fagocitosis de los hemocitos fue la estación del año, particularmente otoño. De acuerdo con Gestal (2000) la intensidad de infección por el coccidio en la Ría de Vigo ocurre de otoño a primavera, debido a la alta disponibilidad del hospedador intermediario de *A. octopiana* que es, presumiblemente, el camarón *Palaemon serratus*. Por su parte, la reducción del estallido respiratorio sugiere que el coccidio podría restringir la capacidad de los hemocitos para producir radicales de oxígeno. Puesto que la supresión del estallido respiratorio es una estrategia habitual en parásitos como *Perkinsus marinus* (Volety y Chu, 1995) es posible que el mismo patrón sea reflejado en la coccidiosis producida por *A. octopiana*. Por otro lado, la respuesta citotóxica de NO también disminuyó con el incremento de la infección. Asi, la producción de NO sólo resultó significativa en pulpos sanos, lo cual sugiere que éste grupo de individuos es capaz de producir NO adecuadamente, pero el incremento en la coccidiosis reduce paulatinamente su producción. Asimismo, la reducción en la producción de NO fue notable en los pulpos de mayor peso. Puesto que *A. octopiana* es transmitido a través del alimento ingerido, el presente resultado indica que los individuos de mayor peso (y mayor talla) presentan mayor acumulación de parásitos. En consecuencia, la disminución en la producción del radical NO podría estar asociada a una fuerte coccidiosis.

Finalmente, el análisis de la respuesta inmune celular entre individuos salvajes y pulpos engordados en batea, mostraron un patrón similar. En ambos casos, la capacidad fagocítica de los hemocitos se incrementó, mientras que el estallido respiratorio y la producción de NO disminuyó. Sin embargo, la respuesta citotóxica marca una diferencia entre los pulpos de ambas procedencias. En pulpos salvajes se observó una notable disminución en la producción de ROS con respecto a los pulpos de batea. Igualmente, la producción de NO fue significativamente baja en pulpos salvajes.

Por tanto, es claro que además del daño mecánico y en la absorción de nutrientes causado por la coccidiosis, la infección también altera el correcto desempeño de la respuesta inmune celular. Además, las diferencias observadas entre individuos salvajes y de batea, sugieren que el estrés generado por las condiciones de cultivo favorece el impacto negativo que *A. octopiana* causa a los pulpos engordados en batea.

II.5. Capítulo 4. Análisis transcriptómico de los hemocitos de *Octopus vulgaris* en respuesta a la infección por *Aggregata octopiana*

El estudio molecular de la respuesta inmune de organismos de interés en acuicultura permite identificar numerosos genes claves en distintos procesos biológicos. Establecer las bases moleculares de la respuesta inmune del pulpo implica un importante avance que permitirá conocer cómo éstos moluscos hacen frente a numerosos patógenos, lo cual redundará en útiles aplicaciones de los datos obtenidos para seleccionar individuos resistentes a infecciones; y por ende, en mayor producción acuícola de productos de calidad.

En el presente estudio se elaboró una librería de genes expresados en los hemocitos de *O. vulgaris* con diferentes grados de infección (alta y baja) por *A. octopiana*, usando la tecnología de secuenciación masiva de Illumina. A partir de la secuenciación y ensamblaje de los transcritos obtenidos de los hemocitos, se generaron 254,506 contigs de 669 bp en promedio. Así, el transcriptoma teórico calculado para *O. vulgaris* es de 170.24Mb. Utilizando como valor de límite de corte 10^{-3}, el 18.95% de los contigs presentaron homología con alguna de las secuencias depositadas en la base de datos pública BLASTx. En contraste, el 81.05% de las secuencias (contigs) no presentaron homología, posiblemente debido a la escasez de datos moleculares de cefalópodos disponibles en las bases de datos públicas como GenBank. Por lo tanto, esto sugiere que en la librería de *O. vulgaris* generada en este estudio existe una gran cantidad de genes identificados por primera vez en cefalópodos. De las proteínas identificadas, en la categoría de componentes celulares, el 38% y 32% se clasificaron como pertenecientes a las categorías "célula" y "orgánulo" respectivamente. Dentro del grupo función molecular, las proteínas relacionadas con adhesión y actividad catalítica conformaron el 57% y 29%, respectivamente. En la categoría de procesos biológicos, el 17% de las proteínas correspondió a procesos celulares, el 15% a procesos metabólicos, 12% a procesos biológicos y 8% a estímulos biológicos.

A partir de la comparación del transcriptoma de los hemocitos de *O. vulgaris* y las secuencias disponibles en NCBI se observó que éste comparte el 0.85% (301 secuencias) de similitud con las secuencias de *E. scolopes*. El transcriptoma generado en este estudio presentó un 20% (6402 secuencias) de coincidencia con las secuencias de *O. vulgaris* disponibles en NCBI; mientras que sólo se observó un 0.06% de secuencias compartidas con *C. gigas* (135), 0.40% con *M. galloprovincialis* (79) y 3.80% con *R. philippinarum* (900 secuencias). Si bien, los resultados muestran un bajo porcentaje de transcritos coincidentes

entre las especies de moluscos comparados, también sugieren que existen numerosos genes novedosos aportados por el presente estudio. Cabe resaltar que muchas de las secuencias disponibles en las bases de datos públicas han sido obtenidas de diferentes tejidos, pero pocas de ellas provienen de hemocitos. Tal es el caso de los cefalópodos. Las secuencias disponibles de *E. scolopes* provienen, en su mayoría, del órgano luminoso; mientras que en el caso de *O. vulgaris*, provienen fundamentalmente del músculo y del tejido nervioso. Por su parte, considerando la disponibilidad de secuencias correspondientes a hemocitos de bivalvos y el porcentaje de secuencias compartidas entre el transcriptoma de *O. vulgaris* (de este estudio) y *R. philippinarum*, sugiere que existen numerosos genes altamente conservados entre el pulpo y dicho bivalvo. Sin embargo, se requieren más estudios que permitan comparan apropiadamente entre especies de moluscos y entre tejidos, tal como los hemocitos.

Del total de proteínas identificadas, 3% de éstas presentaron homología con genes de posible función inmune. Este es un resultado bajo, sin embargo, refleja la carencia de datos moleculares concernientes a cefalópodos en las bases de datos públicas. Al mismo tiempo, indica que el presente estudio aporta una gran cantidad de genes novedosos aún no descritos en cefalópodos.

Entre los genes identificados y de implicación en la respuesta inmune del pulpo se encontraron proteínas relacionadas con importantes cascadas implicadas en inflamación (NFκB), reconocimiento de patógenos (cascada del complemento, Toll-Like Receptor (TLR)) e incluso apoptosis, lo cual indica que existen mecanismos de respuesta inmune similares entre cefalópodos y otros moluscos.

Además del análisis transcriptómico general, se realizó un análisis comparativo del transcriptoma de pulpos con alta y baja infección por *A. octopiana*, en el cual se encontraron 539 genes diferencialmente expresados entre ambos niveles de infección. De ellos, se identificaron un total de 312 genes relacionados con la estructura celular, implicados en procesos metabólicos así como en la respuesta inmune celular. Considerando tanto los genes expresados diferencialmente como aquellos obtenidos en el patrón transcriptómico general, se seleccionaron determinados genes en función de su expresión significativa en pulpos enfermos, pero en algún caso también en función a su implicación en la interacción hospedador-patógeno (es decir, independientemente de la significación obtenida en el análisis bioinformático). Por tanto, se seleccionaron genes relacionados con reconocimiento de patógenos (galectina, PGRP, C1q, TLR), inhibidores de proteasas (SERPIN), respuesta

inflamatoria (LITAF), antioxidantes (PRDX-2) y apoptosis (Caspase-3), para estudiar su expresión mediante q-PCR. El patrón de expresión observado en los genes con expresión significativa fue concordante con la tendencia de expresión que mostraron en el estudio de transcriptómica. Por lo tanto, confirman los resultados obtenidos en la secuenciación masiva y avalan su fiabilidad. Por otra parte, el análisis de estos genes mediante q-PCR aporta un panorama general acerca de la expresión de los mismos en los tejidos utilizados: hemocitos, por ser las células responsables de la defensa celular; ciego, por ser el órgano diana de la infección por *A. octopiana*; y branquia, las cuales están continuamente en contacto con el ambiente y por lo tanto, con potenciales patógenos.

De todos los genes estudiados, la galectina y TLR se observaron sobre-expresados en hemocitos de pulpos con alta infección. Puesto que ambas proteínas participan en el reconocimiento de una amplia variedad de patógenos, es posible que la expresión observada en estos genes indique un intenso estímulo de los hemocitos circulantes en actividades de defensa celular como fagocitosis. La mayor expresión de los genes estudiados se observó en el ciego. Al ser el órgano diana de la infección es evidente una alta expresión de genes relacionados con el reconocimiento de patógenos (TLR, PGRP, Galectina), inflamación (LITAF) e inhibidores de proteasas (SERPIN) en pulpos con alta infección. A raíz del incremento en el número de parásitos que infectan el ciego, las células epiteliales parecen expresar inhibidores de proteasas y de inflamación como estrategia para eliminar o aislar a los parásitos y así evitar que infecten más tejido. La respuesta inflamatoria en el órgano diana es evidente a nivel histopatológico y con los datos presentados en este trabajo, también es evidente a nivel molecular. Los mecanismos citotóxicos parecen asimismo ser desencadenados, y por tanto, se observa una alta expresión de la proteína antioxidante peroxiredoxina (PRDX-2) para combatir el exceso de radicales de oxigeno que pueden afectar a las células del hospedador. Del mismo modo, se observó una alta expresión de la caspasa 3, la cual es una caspasa de tipo efectora implicada en llevar a cabo la eliminación de células. En el caso del tejido del ciego, la alta expresión de la caspasa-3 sugiere que ésta podría actuar para eliminar el tejido dañado, aunque no es claro si tiene alguna implicación en eliminar células ya infectadas por el coccidio.

Por su parte, en la branquia, la expresión más alta observada fue de C1qbp, seguido por PRDX-2 y SERPIN. Puesto que estos genes tienen implicación en distintos procesos de la defensa inmune, sugieren que en pulpos altamente infectados por el coccidio, el tejido de la

branquia también podría ser más vulnerable a infecciones secundarias. Por tanto, el C1qbp podría indicar la activación del sistema del complemento debido a la presencia y reconocimiento de patógenos, la proteína antioxidante podría indicar un intenso estallido respiratorio mientras que, la proteína inhibidora de proteasas, podría actuar en contener infecciones adicionales a las que ya pudiera haber en la branquia.

En conclusión, el estudio de transcriptómica desarrollado en los hemocitos de *O. vulgaris* aporta la identificación de una gran cantidad de genes relacionados con distintos procesos biológicos, y que son descritos por primera vez en cefalópodos. Además, aporta evidencia de que la infección por *A. octopiana* induce la expresión diferencial de genes implicados en la respuesta inmune celular del pulpo. El papel específico de dichos genes ante la coccidiosis aún requiere estudios posteriores. Sin embargo, los resultados obtenidos establecen las primeras bases moleculares para estudiar la resistencia/tolerancia de *O. vulgaris* a la coccidiosis por *A. octopiana*.

II.6. Capítulo 5. Caracterización del proteoma de la hemolinfa de *Octopus vulgaris* infectado por el protozoo parásito *Aggregata octopiana*

La proteómica se enfoca en el estudio de todas las proteínas expresadas por el genoma de un organismo o tejido, es decir, el proteoma (López, 2007). Debido a que el proteoma es muy dinámico permite estudiar las variaciones en la expresión de genes bajo la influencia de perturbaciones biológicas como son los parásitos. Si bien la expresión de genes es posible estudiarla también a nivel transcriptómico, existe poca concordancia entre el número de transcritos de ARNm y las proteínas identificadas. Esto se debe a la presencia de modificaciones post-transcripcionales y post-traduccionales que impiden predecir el número de proteínas, su abundancia o función, sólo con base en secuencias de ADN. Por lo tanto, la proteómica aporta información valiosa acerca de los genes que están de hecho expresados. De ahí que sea una herramienta complementaria a la genómica y transcriptómica (Diz et al., 2012).

En este capítulo el objetivo fue describir el proteoma de los hemocitos y plasma que conforman la hemolinfa de *O. vulgaris* e identificar las proteínas mediante espectrometría de masas. Además, se comparó el proteoma de hemocitos y plasma obtenido de pulpos con alta y

baja infección por el coccidio *A. octopiana* con el fin de detectar cambios atribuidos a la infección y tratar de identificar potenciales biomarcadores de resistencia la infección.

Para establecer el nivel de infección en cada individuo se realizó el recuento en cámara de Neubauer de los esporoquistes. Además, se realizaron cortes histológicos del tejido infectado de los individuos para verificar que el daño histopatológico fuera acorde con el número de esporoquistes contados. Para evaluar el nivel de infección, el número de esporoquistes se ha referido al peso en gramos del tracto digestivo del pulpo (spor/g). Considerando tanto el daño histopatológico como el número de esporoquistes, los pulpos se dividieron en dos grupos. Aquellos individuos con alta infección (4×10^6 a 2×10^7 spor/g) y considerable daño en el tejido se denominaron "enfermos". En tanto que, los pulpos con baja o nula infección (0 a 5×10^4 spor/g) y leve daño al tejido digestivo se denominaron "sanos". De todos los pulpos se registraron los datos morfométricos (talla, peso) así como el sexo. Este último factor, se analizó mediante una ANOVA-2 vías para determinar su posible efecto sobre la expresión de las proteínas.

En el proteoma de los hemocitos se obtuvieron 524 spots totales. El volumen normalizado de cada spot se transformó logarítmicamente para realizar una ANOVA de una vía, en la cual 42 spots resultaron significativamente ($p<0.05$) diferentes entre individuos sanos y enfermos. Con el fin de reducir el número de falsos positivos, se aplicó el método de corrección multitest SGoF+. De acuerdo con el resultado, sólo 20 spot se confirmaron como genes candidatos para su estudio subsecuente y determinar si podrían ser válidos como biomarcadores. Además, se realizó un análisis de componentes principales (PCA) usando únicamente los datos de los spots significativos a partir del ANOVA. Se obtuvieron dos componentes que describen el 62% de la variación observada, lo cual indica que el conjunto de spots significativos presenta una firma proteica capaz de separarlos de acuerdo a su grupo de infección. También se estudió mediante una ANOVA de 2-vías si el sexo de los individuos pudiera tener alguna influencia sobre el proteoma en ambos grupos de infección. Sin embargo, el sexo no resultó una variable significativa ($p>0.05$), y tampoco mostró ninguna interacción con la infección. Para evaluar la eficacia del método se calculó el coeficiente de variación entre réplicas técnicas. Éste resultó ser de un 25%, manteniéndose por debajo del 42% de la variación biológica de las muestras, lo que significa que la variación observada en el proteoma de individuos sanos y enfermos es debido a las propias muestras y no a la técnica.

En el plasma se detectaron 126 spots bien definidos. Sólo 6 de éstos resultaron significativos (ANOVA $p<0.05$), sin embargo, al aplicar la corrección multitest SGoF+ ninguno de éstos resultó significativo ($p>0.05$). Se obtuvo un alto coeficiente de variación (47%). No obstante, dicho resultado se atribuye al uso de filtros empleados con el fin mejorar la resolución del mapa proteico. Si bien el uso de filtros supone una desventaja en términos de reproducibilidad, también es necesario para eliminar las proteínas más abundantes de la muestra (como es en este caso la hemocianina) que impiden la resolución de las proteínas minoritarias. Por su parte, el PCA no arrojó resultados significativos. Por todo lo expresado, los resultados de las muestras de plasma se deben interpretar con cautela.

Para realizar la identificación de los spots mediante espectrometría de masas se seleccionaron aquellos spots que mostraron diferencias significativas entre grupos de infección y algunos otros que se observaron más intensos. Así, de un total de 39 spots se lograron identificar 36 proteínas en muestras de hemocitos. Sin embargo, sólo 5 de éstos se pudieron identificar en bases de datos públicas. Por el contrario, los 34 spots restantes se identificaron en la base de datos del transcriptoma del pulpo generada en el capítulo 4, después de realizar la traducción de cada una de las secuencias obtenidas a los 6 posibles marcos de lectura que podrían codificar para proteínas. Algunas de las proteínas identificadas son actina, peroxiredoxina, glutamato DH, filamina, isocitrato DH entre otras. Entre el total de proteínas, se observó que las identificadas como: canal de sodio, fascina, glutamato DH, 6-fosfogluconolacton, filamina, peroxiredoxina y ATP sintasa presentaron mayor contribución a la componente 1 del PCA, distinguiendo ambos grupos de infección en un 51%. Por tanto, estas proteínas representan potenciales biomarcadores involucrados en la respuesta inmune del pulpo ante la infección por *A. octopiana*.

Las proteínas del citoesqueleto filamina y fascina se observaron sobre-expresadas en hemocitos de pulpos enfermos. Ambas proteínas son importantes para la motilidad y mantenimiento de la forma celular. Otros estudios muestran que el gen de la filamina también se observa sobre-expresado en hemocitos de *Ostrea edulis* infectados *in vitro* con células de *Bonamia ostrea* vivas. Debido a que los hemocitos necesitan formar el fagosoma para ingerir al parásito, se incrementa la polimerización del citoesqueleto, con lo cual, hay un incremento de filamina (Morga et al., 2009). Es posible que algo similar ocurra en los hemocitos de pulpos enfermos. Sin embargo, la filamina también podría interactuar con receptores de membrana y participar en la activación de cascadas como la de NF-κB con una función clave en la

regulación de la respuesta inmune a las infecciones (Vasselon y Detmers, 2002; Feng y Walsh, 2004). Por su parte, la proteína fascina participa en la formación de pseudópodos y por consiguiente, en la extensión de la membrana celular, ya sea para desplazamiento o para fagocitar partículas (Yamashiro et al., 19998). La sobre-expresión de fascina y filamina en hemocitos de pulpos enfermos sugiere que ambas proteínas podrían estar relacionadas con el proceso de fagocitosis durante la infección por *A. octopiana*. Otra de las proteínas identificadas con una importante función es la peroxiredoxina. Esta proteína se observó sub-expresada en pulpos enfermos. La peroxiredoxina es una proteína antioxidante. Cuando los hemocitos producen radicales de oxigeno para eliminar patógenos, los mismos radicales pueden ser nocivos también para el hospedador (Chu, 2000). Por tanto, la peroxiredoxina es necesaria para reducir los radicales de oxigeno a niveles basales y así evitar el daño generado por el estallido respiratorio (Immenschuh y Baumgart-Vogt, 2005). Ante la escasa expresión de peroxiredoxina en pulpos enfermos, es probable que el coccidio estuviera inhibiendo el estallido respiratorio en los hemocitos de estos pulpos. La inhibición de la respuesta citotóxica también se ha registrado en hemocitos de *Crassostrea virginica* expuestos a 3.7 $x10^6$ células de *Perkinsus marinus* (Volety y Chu, 1995). Sin embargo, esta hipótesis requiere ser contrastada.

Cabe mencionar que otras proteínas como glutamato DH, isocitrato DH, canal de sodio y fosfogluconolactonasa también resultaron interesantes. Las tres primeras proteínas se observaron sobre-expresadas en pulpos enfermos. En cambio, la fosfogluconolactonasa se observó sub-expresada en los mismos individuos. Sin embargo, las cuatro proteínas contribuyen significativamente con la componente 1 del PCA y por lo tanto son potenciales biomarcadores. Aunque no es claro el papel que desarrollan, la diferencia de expresión sugiere que hay procesos celulares afectados diferencialmente por el nivel de infección. Consecuentemente, se necesita realizar estudios posteriores para determinar el papel de estas proteínas durante la coccidiosis y evaluar su validez como potenciales biomarcadores.

En cuanto al plasma, se seleccionaron 23 spots para su identificación incluyendo los 6 spots significativos (ANOVA $p<0.05$) y otros que se observaron intensos y definidos. Sin embargo, sólo 5 spots se identificaron positivamente como hemocianina, la proteína más abundante en la hemolinfa del pulpo y encargada de transportar el oxígeno (Rögener et al., 1985; Van-Holde y Miller, 1995). Dos spots en particular mostraron buenos espectros, sin embargo, no fue posible identificarlos debido a la carencia de secuencias de cefalópodos las

bases de datos públicas. Debido a que la base de datos del transcriptoma de *O. vulgaris* (generado en el capítulo 4) se realizó a partir de hemocitos hay pocas proteínas plasmáticas identificadas. Por lo tanto, no fue posible identificarlas. Estudios posteriores enfocados en el proteoma del plasma del pulpo permitirán identificar ambos spots y determinar si pertenecen a proteínas implicadas en la respuesta inmune humoral.

En conclusión, el último capítulo de esta tesis doctoral describe por primera vez el proteoma de hemocitos y plasma de *O. vulgaris*. Además, se identificaron proteínas sub- o sobre-expresadas en pulpos enfermos, lo que sugiere que la infección por *A. octopiana* deja huella en la expresión proteica. Por lo tanto, aquí se establecen las bases moleculares para estudiar la susceptibilidad/resistencia del pulpo en una relación natural hospedador-parásito. Los estudios posteriores permitirán caracterizar las proteínas aquí identificadas y determinar su papel exacto en la defensa inmune del pulpo y su relación con la coccidiosis.

CONCLUSIONS

1. Based on previous morphological evidence, host-specificity data and the new molecular phylogenetic analyses presented in this work, it is concluded that the *Aggregata* species parasitizing *Octopus vulgaris* in the Ria of Vigo (NE Atlantic) is *Aggregata octopiana.* This coccidian species is considered the valid *A. octopiana* species.

   Con base en la evidencia morfológica previa, los datos de especificidad hospedadora y los análisis moleculares presentados en este trabajo, se concluye que la especie de *Aggregata* que parasita a *Octopus vulgaris* en la Ría de Vigo (NE Atlántico) es *Aggregata octopiana.* Esta especie de coccidio es considerada la especie *A. octopiana* válida.

2. The identification of *A. eberthi* infecting the cuttlefish *Sepia officinalis* in the Ria of Vigo (NE Atlantic) is here confirmed by molecular analysis. The phylogenetic analysis corroborates the classification of both species as belonging to the *Aggregata* genus. The phenotypic and genotypic characters are validated as useful diagnostic tools for both *Aggregata* species.

   Se confirma mediante *análisis m*olecular la identificación *de A. eberthi c*omo el parásito que infecta a *Sepia officinalis* en la Ría de Vigo (Atlántico NE). Se corrobora mediante análisis filogenéticos la clasificación de ambas especies dentro del género *Aggregata*, y se validan los caracteres fenotípicos y genotípicos descritos para ambas especies de *Aggregata* como herramientas de diagnóstico útiles.

3. Through the study of the *O. vulgaris* hemolymph using microscopic (light and electronic) and flow cytometry techniques, two subpopulation or types of hemocytes were identified: large granulocytes and small granulocytes. Large granulocytes constituted the 82% of octopus hemolymph cells. They have a mean diameter of 11.6 µm, U-shaped nucleus and numerous granules in the cytoplasm. Small granulocytes constituted the 18% of the hemolymph cells. They showed a mean diameter of 8.12 µm, round nucleus, and cytoplasm with few or totally absent granules.

   El estudio de la hemolinfa de *O. vulgaris* mediante microscopía (óptica y electrónica) y citometría de flujo permitió identificar dos subpoblaciones o tipos de hemocitos: granulocitos grandes y granulocitos pequeños. Los granulocitos grandes conforman el 82% de las células presentes en la hemolinfa del pulpo, el diámetro promedio es de 11,6 µm, presentan núcleo en forma de U y numerosos gránulos en el citoplasma. Los granulocitos pequeños constituyen el 18% de las células presentes en la hemolinfa del pulpo, el diámetro promedio es de 8,12 µm, el núcleo es redondeado, y presentan pocos gránulos o totalmente ausentes en el citoplasma.

4. Large and small granulocytes showed cellular immune response activity. Large granulocytes are the main effectors of phagocytosis and ROS production. In contrast, small granulocytes have a limited phagocytic ability, and consequently, a limited respiratory burst.

   Ambos tipos de hemocitos presentan actividad de respuesta inmune celular. Los granulocitos grandes son las células que presentan mayor actividad fagocítica y producción de ROS. En cambio, los granulocitos pequeños presentan limitada capacidad fagocítica y por lo tanto, limitada producción de ROS.

5. The *O. vulgaris* hemocytes are capable to yield nitric oxide (NO) following challenge with zymosan, LPS and PMA. These three stimuli induced the highest NO production at 3h of incubation.

   Los hemocitos de *O. vulgaris* son capaces de producir óxido nítrico (NO) al ser estimulados con zimosán, LPS y PMA. Los tres estímulos inducen la mayor producción de NO a las 3h de incubación.

6. The increase of the total amount of infection by *A. octopiana* influences significantly the increase in the phagocytic ability of hemocytes. According to Akaike information criterion (AIC), the season of collection (mainly autumn) and octopus sex, are also significant variables that contribute to explain the variation of the phagocytic ability of hemocytes. Thus, the group of variables total infection, octopus sex and season of collection explain the 27.65% of variation in the phagocytic ability of the octopus hemocytes. .

   El incremento en la infección total por *A. octopiana* influye significativamente en el incremento de la capacidad fagocítica de los hemocitos. De acuerdo con el Criterio de Akaike (AIC), la estación del año (principalmente otoño) y el sexo de los pulpos, también son variables significativas que contribuyen a explicar la variabilidad en la capacidad fagocítica de los hemocitos. Así, el conjunto de variables grado de infección, sexo y estación explican el 27.65% de la variabilidad en la capacidad de fagocitosis de los hemocitos del pulpo.

7. ROS production decrease when *A. octopiana* infection increases. Comparing between wild and reared octopuses, ROS production was significantly decreased in wild octopuses than those reared in floating cages. The stressful conditions in floating cages favors the negative impact that *A. octopiana* causes to reared octopuses. Then, reared octopuses trigger a higher ROS production than wild ones. According to AIC, the variables total infection and octopus origin (wild or reared in floating cages), are significant variables that contribute to explain the variation in the ROS production. Thus, both variables explain the 24.35% of variation in the citotoxic activity of hemocytes.

La producción de ROS disminuye cuando aumenta la infección por *A. octopiana.* Comparando entre pulpos salvajes y de batea, la producción de ROS es significativamente menor en pulpos salvajes. Por tanto, las condiciones estresantes de cultivo favorecen el impacto negativo que *A. octopiana* causa a los pulpos de batea y por ello producen mayor cantidad de ROS que los pulpos salvajes. En base al AIC, el grado de infección y origen de los pulpos (salvajes o de batea), son variables significativas que contribuyen a explicar la variabilidad en la producción de ROS. Por tanto, ambas variables explican el 24.35% de la variabilidad en la actividad citotóxica de los hemocitos.

8. The NO production decreases when the intensity of infection by *A. octopiana* increases. Decline in the cytotoxic response is notably significant for healthy octopuses and the heaviest individuals. Comparing wild and reared octopuses, NO production is significantly lower in wild specimens than in those reared in floating cages. According to AIC, the variables total infection, group of infection (healthy), octopus weight and origin (wild or reared in floating cages) are significant for explaining the variation in NO production. All these variables included in the final model explained the 17.25% of the variation in the NO produced by hemocytes.

   La producción de NO disminuye conforme se incrementa la intensidad de infección por *A. octopiana.* La disminución en la respuesta citotóxica es particularmente significativa en pulpos sanos y en los individuos de mayor peso corporal. Comparando entre pulpos salvajes y pulpos de batea, la producción de NO es significativamente menor en pulpos salvajes. De acuerdo con el resultado del AIC, el grado de infección, grupo de infección (sanos), peso de los pulpos y origen de los mismos (salvaje o de batea) son variables significativas para explicar la variabilidad en la producción de NO. Todas estas variables incluidas en el modelo final explican el 17.25% de la variación observada en la producción de NO.

9. The "*De novo*" transcriptome of the circulating hemocytes of *O. vulgaris*, performed by Illumina high-throughput paired-end sequencing technology, yielded 75,571,280 high quality reads for the pool of octopuses showing high infection by *A. octopiana* and 74,731,646 for the pool of octopuses showing low infection by *A. octopiana*. A total of 254, 506 contigs were assembled and 18% (48,225 contigs) were successfully identified. Most of the immune genes identified are reported for the first time in cephalopods. A significant number of putative immune-related genes involved in several immune pathways like NFkB, TLR signaling pathway, complement and apoptosis pathways were identified.

   El transcriptoma "*De novo*" de los hemocitos de *O. vulgaris* generado mediante la tecnología de secuenciación masiva "paired-end" de Illumina, permitió obtener 75.571.280 secuencias de alta calidad en pulpos con alta infección por *A. octopiana* y 74.731.646 secuencias en pulpos con baja o nula infección por *A. octopiana*. A partir del ensamblaje, se obtuvieron 254.506 contigs de los cuales, 18% se identificaron con éxito. La mayoría de las secuencias obtenidas en este trabajo corresponden a genes identificados por primera vez en cefalópodos. Entre éstos, se ha identificado un

importante número de genes relacionados con la respuesta inmune, e involucrados en distintas cascadas de señalización, como NFkB, TLR, complemento y apoptosis.

10. The comparative analysis of the transcriptome of healthy (low or absent *A. octopiana* infection) and sick (high *A. octopiana* infection) octopuses using bioinformatics tools allowed the identification of 539 genes differentially expressed in both conditions. The differential expression determined by RT-qPCR of a pool of selected genes involved in pathogen recognition (galectin, PGRP, C1qbp, TLR), protease inhibition (SERPIN), inflammatory response (LITAF), antioxidant system (PRDX-2), and apoptosis (Caspase-3) were consistent with the changing trends of gene expression in most cases, with the RNA-seq analysis, supporting the reliability of transcriptomic results. The expression level of the selected genes was also analyzed in the caecum and gills of octopuses showing high and low infection by *A. octopiana*.

    El análisis comparativo mediante herramientas bioinformáticas del transcriptoma de los hemocitos de pulpos sanos (poca o nula infección por *A. octopiana*) y enfermos (alta infección por *A. octopiana*), permitió la identificación de 539 genes expresados diferencialmente en ambas condiciones. La expresión diferencial determinada mediante RT-qPCR de un grupo de genes seleccionados por su implicación en reconocimiento del patógeno (galectina, PGRP, C1qbp y TLR), inhibidores de proteases (SERPIN), respuesta inflamatoria (LITAF), sistema antioxidante (PRDX-2) y apoptosis (Caspasa-3) fue consistente con la tendencia observada en el análisis de RNA-seq, lo cual confirma y avala la fiabilidad de los resultados obtenidos en el análisis transcriptómico de secuenciación masiva. La expresión de dichos genes fue también analizada en el ciego y en las branquias de pulpos con alta y baja infección por *A. octopiana*.

11. The proteomic study of the *O. vulgaris* hemocytes allowed the identification of 42 significant spots between sick and healthy octopuses. The subsequent principal component analysis shows 7 proteins as the major contributors to differences between both groups of infection and thus can be consider as candidates to potential resistance biomarkers against the coccidia infection. From these, fascin, filamin and peroxiredoxin are highlighted by their involvement in cellular response.

    El estudio proteómico de los hemocitos permitió la identificación de 42 spots significativos entre pulpos enfermos y sanos. El análisis de componentes principales posterior muestra que 7 de estas proteínas que contribuyen mayoritariamente a las diferencias entre grupos de infección y por tanto podrían ser consideradas como posibles biomarcadores de resistencia a la infección por el coccidio. De éstas se enfatizan fascina, filamina y peroxiredoxina debido a su implicación en la respuesta inmune celular.

12. The analysis of the transcriptome of the hemocytes of *O. vulgaris* allowed the successful identification of only 18% of the total sequences assembled in the RNA-seq library and only 36 of the proteins obtained from the octopus proteomic analysis.

From these, only 5 proteins were identified from public databases. The remaining 31 proteins were successfully identified in the transcriptome of the *O. vulgaris*' hemocytes presented in this study. Thus, the scarcity of the cephalopod molecular information in public databases is here evidenced and highlights the need to increase researches that allow to identify additional proteins; hence, contributing to understand several biological processes occurring in cephalopods.

El análisis del transcriptoma de los hemocitos de *O. vulgaris* permitió la anotación únicamente del 18% del total de las secuencias obtenidas en la librería de RNA-seq y solo 36 de las proteínas obtenidas en el análisis proteómico. De estas últimas, sólo 5 proteínas se identificaron en bases de datos públicas. Las 31 proteínas restantes se identificaron en la base de datos del transcriptoma de los hemocitos de *O. vulgaris* que se presenta en este estudio. De esta manera, se evidencian los escasos datos moleculares de cefalópodos disponibles actualmente en las bases de datos públicas, y al mismo tiempo se destaca la necesidad de incrementar la investigación que permita identificar nuevas proteínas y por tanto, contribuir a la comprensión de numerosos procesos biológicos que ocurren en cefalópodos.

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