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

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Abstract

Health of wild and reared *Octopus vulgaris* is threatened by the most dangerous parasite for the octopus, the protozoan coccidia *Aggregata octopiana*. This host-parasite relationship was studied to analyze the effect of *A. octopiana* on the cellular immune parameters of *O. vulgaris*. *A. octopiana* sporocysts infecting the digestive tract of octopuses were counted in order to confirm two octopus groups of infection: 1) healthy (low parasite load, without histological caecum damage) and 2) sick (high parasite load and strong histological caecum damage). Cellular defense parameters (phagocytosis, respiratory burst (ROS) and nitric oxide (NO) production) were measured in the octopus hemolymph. In addition, i) infection degree (total parasitic infection or distributed by groups of infection (sick/healthy)), ii) octopus origin (wild or reared in floating cages), biometric data (sex, length, weight, maturity) and iii) season of collection were tested to know their contribution to the octopus cellular response. Results showed that season of collection and total parasitic infection were the most important factors affecting the phagocytic ability of hemocytes. Phagocytosis increased accordingly to the infection intensity and was particularly higher in Autumn ($P<0.01$) relative to Winter and Spring. ROS ($P<0.01$) and NO ($P<0.1$) production decreased with the *A. octopiana* infection increase. Related to biometric data, a markedly decrease in NO was observed in heaviest octopuses ($P<0.01$). 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.

Statement of relevance

To date, the octopus rearing is still dependent of wild specimens for fattening. Wild octopuses harbor the coccidia *Aggregata octopiana*, which is proved to affect the octopus wellbeing at tissue and molecular level. The present study provides functional data that complement previous studies and show that coccidiosis modify the octopus cellular immune defense.

Keywords: *Octopus vulgaris*, hemocytes, *Aggregata octopiana*, innate immune response, wild and reared octopuses.

1. Introduction

Octopus vulgaris is a world's economically important species, subject of active fisheries (García et al., 2014). The stocks present wide annual fluctuations due to their own biological characteristics like non-overlapping generations 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 easy acclimatization to captivity conditions and their rapid growth (Vaz-Pires et al., 2004).

The internal defense of cephalopods relies on their innate immune system composed by humoral (diluted molecules in plasma) and cellular (carried out by hemocytes) factors that impede pathogens grow (Castillo et al., 2015; Sykes and Gestal, 2014; Gestal and Castellanos-Martínez, 2015). Cellular mechanisms comprise encapsulation, phagocytosis and production of free toxic radicals such as reactive oxygen and nitrogen species (ROS and NOS, respectively). Cytotoxic defensive mechanisms are known in bivalves (Comesaña et al., 2012; Ray et al., 2013) and gastropods (Hahn et al., 2001; Zelck et al., 2005), but also in the cephalopods *Eledone cirrhosa* (Malham and Runham, 1998), *Euprymna scolopes* (Davidson et al., 2004; Nyholm et al., 2009) and *Octopus vulgaris* (Novoa et al., 2002; Rodríguez-Domínguez et al., 2006; Castellanos-Martínez et al., 2014b).

Studies conducted mainly on bivalves, showed that molluscan cellular immunity is sensitive to environmental factors (Chu, 2000; Soudant et al., 2004; Flye-Sainte-Marie et al., 2009; Dang et al., 2012a), reproduction (Duchemin et al., 2007; Dang et al., 2012b), pollutants (Hannam et al., 2010; Luna-Acosta et al., 2010), parasites and all

of these factors are capable to induce changes in total and differential hemocyte counts (Da-Silva et al., 2008; Comesaña et al., 2012). Parasites, in particular, are capable to mediate its own entry to hemocytes through phagocytosis (Da-Silva et al., 2008; Morga et al., 2009). In addition, parasites can modulate the apoptosis (Hughes et al., 2010) and cytotoxic defense of hemocytes in favor to the parasite survival (Anderson et al., 1995; Volety and Chu, 1995; Goedken et al., 2005), causing severe threats in aquaculture (Paperna, 1991; Alvarez-Pellitero et al., 2009; Perrigault and Allam, 2012).

The gastrointestinal protozoan *Aggregata octopiana* is one of the most dangerous pathogen affecting wild and cultured octopuses (Pascual et al., 1996; Gestal et al., 2007). Because the coccidiosis effect is not completely known on the host, the parasite might alter the results of experiments related to the digestive system therefore, the coccidian *A. octopiana* is considered a confounding factor (Sykes et al., 2017). This parasite is causative of digestive tissue rupture and atrophy of the intestinal mucosa, reducing the nutrient absorption area (Gestal et al., 2002a). However, harboring coccidia is not always causative of coccidiosis. In livestock, diagnose coccidiosis is often based on oocyst counts and observation of conspicuous clinical signs such as intestinal hemorrhage, diarrhea and malabsorption (Yun et al., 2000). In wild marine animals, diagnose coccidiosis is limited to the oocyst count and assessment of histopathological alterations of digestive tract (Gestal et al., 2002a, b). This way, it has been demonstrated that *O. vulgaris* severely infected by *A. octopiana* (up to 3×10^6 sporocyst/g infected tissue) shows a decline in the number of circulating hemocytes and suffer malfunction of absorption enzymes, which is reflected in a poor octopus weight gain (Gestal et al., 2002b; Gestal et al., 2007). In addition, proteins associated to the octopus cellular immune defense are weakly expressed in those specimens suffering severe coccidiosis (Castellanos-Martínez et al., 2014a).

To date, the host-parasite relationship established between *O. vulgaris* and *A. octopiana* is starting to be understood. However, fundamental aspects about the host-parasite interaction are highly required to face infections in the octopus aquaculture, which still depends on wild specimens (Iglesias et al., 2000). Most of these specimens are naturally infected by this coccidian parasite exhibiting a high prevalence (Pascual et al., 1996). In addition, infected octopuses are commonly subject of experimentation ignoring whether the coccidiosis have some effect on the results (Sykes et al., 2017). Therefore, the aim of the present study is to understand the effect of *A. octopiana* infection on the *O. vulgaris* cellular defense capabilities. Thus, phagocytosis, respiratory burst and nitric oxide radical's production were measured in octopuses harboring low and high degrees of coccidian infection. The octopus cellular defensive activities were also compared between wild and reared octopuses in floating cages in order to determinate the effect of culture conditions on the octopus immune defense performance.

2. Material and methods

2.1 Biological material and hemolymph collection

In all, 110 live *O. vulgaris* (65 males and 45 females) with an average eviscerated body weight of 935 g and mantle length of 80–220 mm (Table 1) were randomly collected from 2009–2011 by traditional traps, an artisanal gear used by local fishermen, from the Ria of Vigo, Spain (24 ° 14.09' N, 8 ° 47.18' W). From these, 40 live octopuses (25 males and 15 females) ranging 780–1,915 g wet body weight and 110–200 mm mantle length (Appendix A) were collected at random off an on-growing 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 m³ each one

and 250 PVC dens harboring 200 animals per cage. Specimens were daily fed *at libitum* with mussels (*Mytilus galloprovincialis*) and discard of fish (*Trachurus trachurus*, *Sardina pilchardus*, *Micromesistius poutassou*, *Scomber scombrus*, *Boop boops*). In the laboratory, octopuses were kept in culture tanks of open seawater system at 15 °C for 24 h to acclimate before experimentation.

Taking into account that the European Animal Directive was available for cephalopods at 2009, the experiments were firstly performed in accordance with recommendations to minimize suffering following Moltchanivskyj et al. (2007). Further experiments were carried out in strict accordance with the principles published in the European Animal directive (2010/63/EU) for the protection of experimental animals, including cephalopods, and approved by the Consejo Superior de Investigaciones Cientificas (CSIC) ethics committee (Project number 10PXIB402116PR). Before hemolymph extraction, each octopus was anaesthetized by immersion in a solution of 7.5% magnesium chloride (MgCl₂) according to 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. The sacrifice was performed by overdose of anesthetic.

2.2 Maturity index

Octopus sex was confirmed by visualization of gonad after dissection. Maturity stage was assigned according to the Hayashi Maturity Index (MI) following Guerra (1975). Thus, the following data were recorded: ovary and testis weight, oviducal and

Needham's complex weight. The sex and maturity from wild and reared octopuses were pooled together for statistical analysis.

2.3. Cell counting and hemocyte viability

Cell counting was carried out immediately after bleeding 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.

2.4 Counting of *Aggregata octopiana* sporocysts

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 $1000 \times g$, 4 °C, 5 min in a centrifuge Beckman GS-15R. Total infection (number of sporocyst infecting the digestive tract) was assessed individually by counting the number of sporocysts in Neubauer chamber and standardized as the number of parasites infecting a unit gram of each octopus digestive tract (spor/g). The mean intensity of infection was calculated following Bush et al. (1997). The intensity of infection was confirmed through observation of caecum sections. For histological observation, a sample of the caecum tissue was fixed in 10% buffered formalin, embedded in paraffin wax, sectioned at 5 µm using a Microm HM-340 E microtome (Microm, Walldorf, Germany) and stained with hematoxylin-eosin for microscopic analysis (Humason, 1972). Taking into account the number of sporocyst per gram of digestive tract tissue and the histopathology observed, octopuses were classified into two groups: healthy ($0-5 \times 10^5$ spor/g), without histological caecum damage; and sick ($5 \times 10^5-2,08 \times 10^7$ spor/g), showing a strong caecum damage.

2.5 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) and Castellanos-Martínez et al. (2014b). Cell viability was determined by Trypan blue exclusion test (Weeks-Perkins et al., 1995) in crude hemolymph and treated with Squid Ringer Solution (SRS) and Filtered Sea Water (FSW). From 110 specimens, the 78% (n=86) were used to perform more than a single functional assay whereas, in 22% octopuses (n=24) the hemolymph withdrawn was not enough to perform more than one assay.

2.5.1 Phagocytic capability of hemocytes

The hemolymph withdrawn from each octopus was centrifuged $300 \times g$, 4°C , 5 min. Plasma was discarded and replaced with the same volume of 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 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 fluorescein-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.

2.5.2 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 H₂O₂ 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.

2.6 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 microliter 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 assay was performed on 89 octopuses.

2.7 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. Data of phagocytic capability of hemocytes were normalized by square-root arcsine transformation previous to statistics. The Kolmogorov–Smirnov (K–S) was used to test normality of variables. The Levene test was used to check homogeneity of variances before statistical analysis. Results are expressed as the mean $\pm$ standard error of the mean (SEM). Differences were considered significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.1$.

In order to know the relationship between the standardized octopus cellular responses (defined as response: phagocytosis, ROS or NO/hemocytes per ml), the octopus biometric factors (weight, length, sex and maturity), season of collection, total infection, infection group (sick/healthy) and octopus origin (wild or reared), a multiple linear regression analysis was performed. 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. The Student *t*-test of significance is shown for each variable in the obtained

models. All the statistical analyses were performed using the R software (R Development Core Team, 2006).

3. Results

3.1 General pattern of the coccidia infection

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

3.2 Cellular immune parameters

3.2.1. Total hemocyte count

Total circulating hemocyte counts ranged from $2 \times 10^5-3 \times 10^7$ cells/ml (Table 1). Hemocyte viability achieved through the Trypan blue exclusion test was $\geq 95\%$ in crude hemolymph and $\geq 90\%$ in samples treated with SRS and FSW.

3.2.2. Phagocytic capability of hemocytes

Phagocytosis showed wide variations ($1\text{--}56\% \pm 1.1$) being higher in sick than in healthy octopuses ($P=0.00294$, $P<0.05$) (Fig. 3A). The multiple regression analysis performed showed that an increase of total infection leads to a significant increase of phagocytosis ($P=0.0454$, $P<0.05$) (Table 2). The interaction between total infection and group of infection (sick/healthy) was not significant. Regarding the remaining variables, only season of collection and octopus sex stayed in the final model, although the variable sex was not significant. Phagocytosis in Autumn was significantly higher than Winter ($P=0.00155$, $P<0.05$) and Spring ($P=0.00251$, $P<0.05$ not shown). The model provided through the automatic backwards stepwise AIC criterion retained the variables total infection, sex and season. These variables explained the 27.65% of the variance observed in the phagocytic ability of the hemocytes.

3.2.3 Respiratory burst

Respiratory burst measured in octopuses showed a mean of $12 \text{ A.U.} \pm 0.94$. When comparing groups of infection, ROS production was slightly higher in sick than in healthy octopuses ($P=0.3343$, $P>0.05$) (Fig. 3B). Multivariate regression analysis showed a significant ($P<0.01$) and negative relationship between the total coccidian infection and ROS production (Table 2), while the interaction between total infection and group of infection (sick/healthy) was not significant. In addition to infection, the variable origin (reared/wild octopuses) was the only retained by the model ($P=2.86e^{-5}$, $P<0.001$) and showed that wild octopuses produced less ROS than that reared in floating cages. The model obtained explained 24.35% of the variance observed in ROS production with the variables total infection and octopus origin (reared/wild) as the most important.

3.2.4 Nitric oxide (NO)

There were no differences in the mean production of NO between hemocytes of sick and healthy octopuses ($P=0.4378$, $P>0.05$) (data not shown). The multivariate regression analysis reveals a significant interaction (at 10% of significance level) between intensity of total infection and group of infection ($P=0.0617$, $P<0.1$). According to this, a significant ($P<0.1$) and negative relationship between NO production and infection is only noticeable in healthy (lowly infected) octopuses (Table 2). 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 (Table 2). Regarding octopus weight, the NO production was significantly ($P<0.1$) reduced in heaviest octopuses. When comparing wild and reared specimens, a significantly ($P<0.01$) lower NO production was yielded by wild than octopuses reared in floating cages (Table 2). The variables retained by the model were infection, octopus weight and origin, which explained 17.25% of variation in NO production.

4. Discussion

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

Phagocytosis is an effective mechanism carried out by hemocytes against pathogenic invaders (Cheng, 2000). In our data, the highest intensity of coccidian infection was recorded in Autumn as yet reported for the locality. It is presumably due to the major availability of the putative *A. octopiana* intermediate host, the prawn *Palaemon serratus* (Gestal, 2000). Thus, high coccidiosis (total infection) might trigger

the phagocytic ability of octopus hemocytes. However, season itself is important for phagocytosis even when comparing specimens with the same amount of infection. According to observations performed by the authors during the development of the present study, phagocytosis was almost inhibited in some octopuses severely infected (infection of $1.5\text{--}2 \times 10^7$ spor/g) (not shown). At this level of infection, malabsorption syndrome causes a gradual onset of host weakness (Gestal et al., 2002b) that might affect the cell mediated immune response (Fekete and Kellems, 2007). As a result, the octopus becomes favorable for concurrent parasitic and bacterial secondary infections (Gestal et al., 2002b, 2007; Andrews, 2013).

Internalization and following destruction of pathogens are accomplished by a cytotoxic response through the production of oxygen and nitrogen radicals (Cheng, 2000). Although no differences were found in ROS production between sick and healthy octopuses, our results showed that total infection (without groups) induces a decrease in ROS production. Thus, coccidiosis had a negative effect on the ability of octopus hemocytes for triggering this cytotoxic mechanism and that might be result of the octopus weakness (Gestal et al., 2002b) or perhaps, manipulated by the coccidia. Infecting cells without triggering or suppressing the host cytotoxic defense is common in parasites. For example, *Crassostrea virginica* hemocytes exposed to 3×10^6 live *Perkinsus marinus* are successfully infected without causing a significant reduction in respiratory burst. However, respiratory burst is suppressed in oyster hemocytes exposed to $7.5 \times 10^6 - 5 \times 10^7$ live *P. marinus* cells (Volety and Chu, 1995). From our observations, a weak ROS production was noticed in octopuses harboring 4×10^6 spor/g and agreed with a weak expression of the antioxidant protein peroxiredoxin, that protects host from oxidation caused by ROS, in sick octopuses (Castellanos-Martínez et al., 2014a). Additionally, octopuses harboring 8×10^6 to 1.2×10^7 *A. octopiana* spor/g

showed a similar suppression pattern than that mentioned in *C. virginica*. Thus, the present functional assays coupled to molecular evidence yet reported, suggest that *A. octopiana* probably use a similar strategy as *P. marinus* in order to infect the host hemocytes. However, the precise mechanism remains to be elucidated.

A similar suppressive pattern was also observed for nitric oxide (NO). This is an 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). In *C. virginica*, the infection by *P. marinus* induces a rapid NO increase, suggesting it as an important molecule for preventing proliferation of *P. marinus* in oysters (Villamil et al., 2007). NO production also increased in a dose dependent manner in *Ruditapes philippinarum* exposed to *Vibrio tapetis* (Jeffroy and Paillard, 2011). In contrast, *A. octopiana* caused a weak NO production in *O. vulgaris*, particularly in heaviest specimens. The largest specimens of *Octopus tehueltchus* showed a highest prevalence of infection by *Aggregata valdessensis* due to a cumulative pattern (Storero and Narvarte, 2013) such that observed in filter feeder bivalves (Villalba et al., 2005; Flye-Sainte-Marie et al., 2009). Consequently, it seems that *A. octopiana*, which is transmitted through the food-web, cause a cumulative effect that increase the intensity of infection and reduce NO production in large octopuses caught in the Ria of Vigo.

Concerning the cellular immune response between wild and reared octopuses, ROS and NO production were lower in wild than reared ones. Nevertheless, it seems to be derived from the season of collection since wild octopuses were mainly collected when coccidiosis is less intense in the Ria of Vigo (Gestal, 2000) and showed lower infection than reared ones. In contrast, because the own dynamic of the on-growing floating cage system, reared octopuses were mainly available from Autumn, when

coccidiosis is high in this locality (Gestal, 2000), which coincide with the highest intensity of infection recorded in this study.

In conclusion, the present study is an attempt to understand how the coccidiosis interacts with octopus cellular immune parameters and biological aspects of the host (sex, weight, maturity, wild or reared). The infection by *Aggregata* is highly prevalent and wide variations in the mean intensity of infection were recorded, leading to a wide variability in the cellular immune response of the host. However, here is evidenced that coccidiosis induces an increase in phagocytosis, but alters the ability of octopus hemocytes to yield respiratory burst and NO. This approach contributes to assess the immunological status of *O. vulgaris* naturally infected by *A. octopiana* and provides the bases to clarify the impact of the coccidian in experiments related to the diet and function of digestive system in reared cephalopods.

5. Conflicts of interest

The authors have no conflicts of interest to declare.

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8. Figure captions

Fig. 1. Seasonal variation of mean intensity of infection by *Aggregata octopiana* in *Octopus vulgaris* (n=110). The bars indicate standard error of the mean. Differences marked as (*) are significant ($P < 0.05$, Student's *t*-test).

Fig. 2. Histological sections of the *Octopus vulgaris* digestive tract. A-B: Sick octopuses (5×10^5 – $2,08 \times 10^7$ spor/g). (A) Rupture of tissue organ architecture by the infection of sporogonic (sp) and gamogonic (ga) stages. (B) Infected tissue showing distention (d), pericyst reaction (p) and strong hemocytic infiltration (h). C-D: Healthy octopuses (0 – 5×10^5 spor/g). (C) Digestive tissue from healthy octopus. (D) Single oocyst (Oc) infecting digestive tissue of healthy octopus.

Fig. 3. Functional immune assays performed in *Octopus vulgaris* hemocytes. (A) Mean percentage of phagocytic activity recorded in hemocytes from sick and healthy octopuses. (B) Respiratory burst measured in hemocytes from sick and healthy octopuses. Both assays are shown standardized as: cellular response/number of circulating hemocytes per ml. Differences marked as (*) are significant ($P < 0.05$, Student's *t*-test).

9. Tables legend

Table 1. Biometric data recorded from *Octopus vulgaris*, wild and reared in floating cages (- denotes no data). Data are expressed as mean (minimum–maximum value recorded).

Table 2. Models obtained (with the Student *t*-test of significance for each variable) according to the Akaike's Information Criterion (AIC) to determinate the relationship among the octopus cellular immune response with biometric, seasonal and origin of the hosts as variables.

Appendix A

Biometric data and *Aggregata octopiana* infection recorded from reared and wild *Octopus vulgaris* specimens used for measuring cellular immune parameters (– denotes no data).

Table 1. Biometric data recorded from *Octopus vulgaris*, wild and reared in floating cages (- denotes no data). Data are expressed as mean (minimum–maximum value recorded).

Origin	EBW(g)) ^a	DML _b (mm)	spor/g ^c	hemocytes/ml _d	Sex ^e		Female maturity stage (Hayashi index) ^f			
					F	M	I	D	M	P _s
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)	15	25	6	3	5	1
Wild	772 (168-4775*)	123 (80-220)	1.24×10^6 (0.0×10^0 – 3.86×10^6)	1.14×10^7 (1.68×10^6 – 3.20×10^7)	30	40	18	5	5	2

^a Total eviscerate 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 females per maturity stage according to Hayashi index following Guerra (1975). Maturity stages: (I) immature, (D) developing, (M) mature, (Ps) post-spawning.

*Single specimen with maximum EBW recorded.

Table 2. Models obtained (with the Student *t*-test of significance for each variable) according to the Akaike's Information Criterion (AIC) to determinate the relationship among the octopus cellular immune response with biometric, seasonal and origin of the hosts as variables.

		Estimate	Std. Error	<i>t</i> -value	P (> <i>t</i>)	Significance level
<i>Phagocytosis</i>	Intercept	3.446e ⁻⁷	4.925e ⁻⁷	0.700	0.4860	
	Total infection	1.496e ⁻¹³	7.366e ⁻¹⁴	2.031	0.0454	<i>P</i> <0.05
	Autumn	2.080e ⁻⁶	6.353e ⁻⁷	3.274	0.00155	<i>P</i> <0.01
	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	
<i>ROS</i>	Intercept	7.198e ⁻⁶	1.145e ⁻⁶	6.285	2.68e ⁻⁸	<i>P</i> <0.001
	Total infection	-5.746e ⁻¹³	2.110e ⁻¹³	-2.724	0.0082	<i>P</i> <0.01
	Wild	-4.928e ⁻⁶	1.098e ⁻⁶	-4.487	2.86e ⁻⁵	<i>P</i> <0.001
<i>NO</i>	Intercept	2.004e ⁻⁵	5.379e ⁻⁶	3.726	0.00035	<i>P</i> <0.001
	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	<i>P</i> <0.01
	Weight (g)	-5.518e ⁻⁹	3.118e ⁻⁹	-1.770	0.0805	<i>P</i> <0.1
	Wild	-1.409e ⁻⁵	4.473e ⁻⁶	-3.150	0.00228	<i>P</i> <0.01
	Interaction (total infection: low infection)	-4.959e ⁻¹¹	2.618e ⁻¹¹	-1.895	0.06170	<i>P</i> <0.1

Highlights

- *Aggregata octopiana* triggers phagocytic ability of hemocytes, but reduces ROS and NO.
- Season of collection and infection affects the phagocytic ability of hemocytes.
- ROS and NO production were lower in wild than reared specimens.
- NO production decreased in heaviest specimens.

Figure 1

Figure 2