

# Malabsorption syndrome observed in the common octopus *Octopus vulgaris* infected with *Aggregata octopiana* (Protista: Apicomplexa)

C. Gestal<sup>1,\*</sup>, M. Páez de la Cadena<sup>2</sup>, S. Pascual<sup>1</sup>

<sup>1</sup>Area de Parasitología, FEPMAR- PB2, and <sup>2</sup>Area de Bioquímica y Biología Molecular, Grupo BB1, Facultad de Ciencias, Universidad de Vigo, Apto. 874-36200 Vigo, Spain

**ABSTRACT:** *Octopus vulgaris* infected with *Aggregata octopiana* were collected from an open-water culture system in the Ría of Aldán (NW Spain). Digestive tract infection values were determined with the use of a Neubauer chamber by counting the number of *A. octopiana* sporocysts. After determining enzyme activity values by the colorimetric Api-Zym system Biomerieux®, one representative enzyme of glycosidases, peptid hydrolases and phosphoric hydrolases showing high activity was spectrophotometrically analysed. The enzymes were maltase and leucine-aminopeptidase (LAP) involved in the absorption process, and acid phosphatase, a lysosomal enzyme, respectively. Enzymatic activity of maltase and LAP decreased significantly, with increased sporocyst counts. However, acid phosphatase activity increased with severity of infection, indicating the presence of degradative enzymes from phagocytic cells in the infected area. A detrimental effect on gastrointestinal function may result from a decrease or malfunction of absorption enzymes. The results suggest a malabsorption syndrome resulting from parasitic infection.

**KEY WORDS:** *Octopus vulgaris* · *Aggregata octopiana* · Absorption enzymes · Maltase · Leucine-aminopeptidase · Acid phosphatase · Lysosomal enzyme

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## INTRODUCTION

The common octopus *Octopus vulgaris* (Cephalopoda: Octopodidae) is an important species in world fisheries (Roper et al. 1984, Domain et al. 2000, Josupeit 2000), representing a major protein resource in most fish-eating countries (Guerra 1992, Quetglas et al. 1998). It has great potential for mariculture and it is a valuable experimental animal for biomedical and behavioural research (Hanlon & Forsythe 1985).

Despite the importance of this species, little is known of the epizootiology and physiopathology of even its

most common parasitic diseases. Parasitic infection by the eimerioid coccidian genus *Aggregata* (Protozoa: Apicomplexa) has been noted as the most important infectious agent in wild and cultured cephalopod stocks from Spanish-Atlantic waters (Pascual et al. 1996, Gestal 2000). Gestal et al. (1999b) described the ultrastructure of *A. octopiana* in Galician waters (NW Spain). Gametogony and sporogony stages of the parasite occur in the digestive tract of the common octopus, the definitive host. Asexual stages are present in a crustacean, possibly the shrimp *Palaemon serratus*, the intermediate host (Gestal 2000, Gestal et al. 2002). These authors also described the histopathological effects of *A. octopiana* infections in the digestive tract of *Octopus vulgaris*. Although enteric coccidiosis is not believed to be the primary cause of death, severe

\*Present address: Grupo ECOBIOMAR, Instituto de Investigaciones Marinas (CSIC), Eduardo Cabello 6, 36208 Vigo, Spain. E-mail: cgestal@iim.csic.es

*Aggregata* infection may weaken the octopus, making it more vulnerable to other biotic and abiotic stressors.

The caecum is the target organ for both trophically transmitted *Aggregata* infections and absorption in cephalopods (Boucaud-Camoud & Boucher-Rodoni 1983, Hochberg 1990). Consequently, our paper addresses the impact of this enteric infection on the activities of absorption and lysosome enzymes in octopus caecum.

## MATERIALS AND METHODS

**Collection of specimens.** A total of 24 common octopuses *Octopus vulgaris* was collected from an open-water culture system in the Ría de Aldán, Galicia, Spain (NE Atlantic Ocean: 42° 15' N, 8° 48' W). The culture system allowed for easy and available sampling, made it possible to standardise feeding parameters, and thus provided a sample of animals of similar size, grown in similar environmental conditions. To minimise stress, all transported animals were maintained at the laboratory in polyethylene culture tanks containing 200 l of aerated and continuously flowing (60 l h<sup>-1</sup>) natural seawater at 18 to 20°C for 24 h before the experiments. All animals were sized (DML = dorsal mantle length) and weighed (TBW = total body weight) to calculate the *K*-Fulton condition index [ $K = (TBW/DML^3) \times 100$ ].

**Preparation of crude extracts.** The biochemical study of the enzymatic activities in the host caecum was conducted with excised tissue. The animals were primarily anaesthetised in a seawater:ethanol 70% ratio of 5:1. The caecum was removed and vigorously washed in 10 mM homogenisation phosphate buffer, pH 7.0, and weighed, after removing the digestive tract. For the sporocyst count, host tissue extracts obtained from a portion of caecum was prepared by homogenisation in an electric tissue grinder (IKA-UltraTurrax T-25) following standard procedure (Gestal et al. 1999a). The number of *Aggregata octopii* sporocysts was estimated in a Neubauer count chamber. Sporocyst concentration was expressed as the total number of sporocysts per g of caecum tissue (SG) and as the total number of sporocysts per whole caecum sample (TS).

Remaining caecal samples were sealed in aluminium foil and stored at -80°C for further enzymatic assays. After thawing, a piece of tissue was weighed for all samples and ground in ice-cold homogenisation buffer with 15 strokes in a Potter Elvehjem homogeniser. The final concentration of each sample was adjusted to 0.05 g ml<sup>-1</sup> by adding homogenisation buffer. Aliquots of total tissue extracts were frozen at -80°C until further processing.

**Protein assay.** Protein content of the crude tissue extracts was measured according to Bradford's method (1976) with bovine serum albumin as standard. Absorbance was measured at 595 nm; values were expressed as mg of protein per mg of tissue.

**Api-Zym system test.** A total of 19 digestive enzymes (see Table 1) in crude extracts were tested semi-quantitatively with the Api-Zym system Biomerieux®:  $\alpha$ -fucosidase,  $\alpha$ -mannosidase,  $\alpha$ - and  $\beta$ -galactosidase,  $\alpha$ - and  $\beta$ -glucosidase, *N*-acetyl- $\beta$ -glucosaminidase,  $\beta$ -glucuronidase, acid phosphatase and phosphatase at pH 5.4, and chymotrypsin, trypsin, valine-amino-peptidase, cystine-aminopeptidase, leucine-amino-peptidase (LAP), lipase (C14), esterase/lipase (C8), esterase (C4), alkaline phosphatase at pH 6.5 to 8.5. The extracts were diluted in distilled water to a protein content of 0.25 mg ml<sup>-1</sup> and 65  $\mu$ l thereof were added to each microcupule on the test trays. The trays were incubated for 4 h at 35 to 38°C following the recommended procedure. Values from 0 to 5 corresponding to 0– $\geq$ 40 nmol of hydrolysed colorimetric substrate were assigned to each reaction, following a colour chart. Assays were run in duplicate.

**Spectrophotometry.** An enzyme representative of those groups which showed high activity in the Api-Zym tests was spectrophotometrically analysed: an  $\alpha$ -D-glucosidase (maltase) and LAP involved in the absorption process, and acid phosphatase, a lysosomal enzyme. Based on the preliminary results of the Api-Zym system, the most important but representative enzymes of glycosidases, peptid hydrolases and phosphoric hydrolases, were spectrophotometrically analysed to account for nutrient absorption and phagocytosis, respectively.

**Maltase assay ( $\alpha$ -D-glucose glucohydrolase EC 3.2.1.20).** Maltase activity was measured in crude tissue extracts following the Messer & Dahlquist (1966) method. Maltose standard was used as the enzymatic substrate at a concentration of 190 mg ml<sup>-1</sup>. A total of 40  $\mu$ l of enzymatic sample (dilution 1:4 in homogenisation buffer from the initial concentration) was incubated for 20 min at 37°C in the presence of 40  $\mu$ l of substrate dissolved in 0.1 M maleate buffer, pH 6.0. The released glucose was determined using the enzymatic colorimetric test Spinreact using a Tris/glucose oxidase reagent. A total of 1.2 ml of the reagent was added to the reaction mixture and incubated for 10 min at 37°C. After incubation and before reading, samples were centrifuged for 2 min at 600  $\times$  *g*. One unit (U) of maltase enzyme is defined as the amount required to hydrolyse 1  $\mu$ mol of maltose min<sup>-1</sup> under optimal conditions of pH and temperature.

**Acid phosphatase assay (EC 3.1.3.2).** Acid phosphatase activity was measured using *p*-nitrophenylphosphate 16 mM as standard substrate dissolved in

sodium acetate buffer, 180 mM, pH 5.0. A mixture containing 0.2 ml of the substrate and 50  $\mu$ l of enzymatic sample (1:3 dilution in homogenisation buffer of total extract tissue) was incubated for 25 min at 37°C. The reaction was stopped by addition of 0.6 ml of 0.25 M NaOH and tubes were centrifuged at  $700 \times g$  for 5 min. Released *p*-nitrophenol in the resulting supernatant was measured at 410 nm and the amount was calculated using a standard curve. One U of acid phosphatase is defined as the amount required to hydrolyse 1  $\mu$ mol of *p*-nitrophenol  $\text{min}^{-1}$  under optimal conditions of pH and temperature.

**LAP assay (EC 3.4.11.1).** The assay was conducted with samples diluted 1:4 in homogenisation buffer. Aminopeptidase activity was assayed with L-leucine nitroanilide using nitroanilide as standard, following the procedures of Wachsmuth et al. (1966) and Boetius & Felbeck (1995). L-leucine nitroanilide was dissolved in a 0.14 M NaCl solution with distilled water at 1 mg  $\text{ml}^{-1}$ . A final substrate concentration of 1 mM was obtained by mixing 50  $\mu$ l L-leucine nitroanilide with 25  $\mu$ l of a 10 mM  $\text{MnCl}_2$  solution, 25  $\mu$ l phosphate buffer (pH 7.5, 0.006 M) and 25  $\mu$ l diluted tissue extract. After 10 min of incubation at 37°C, 1 ml of cold water was added to stop the reaction. Samples were centrifuged for 3 min at  $600 \times g$  at 4°C, and the release of nitroanilide was measured spectrophotometrically at 405 nm. One U of LAP is defined as the amount required to hydrolyse 1  $\mu$ mol of *p*-nitrophenol  $\text{min}^{-1}$  under optimal conditions of pH and temperature.

Values of the enzyme activities were expressed as total activity (TA, expressed as U) per mg of caecum tissue (TA  $\text{mg}^{-1}$  tissue).

**Statistical analyses.** Values of SG were separated into 2 groups, the first with low (2000 to <100 000) infection values, and the second with high (100 000 to 3 000 000) infection values. The enzymatic activities expressed as TA  $\text{mg}^{-1}$  tissue are given as mean + SD. Enzymatic activity data were compared with infection values measured at the infrapopulation level (Bush et al. 1997). The significance of the relationships between the enzymatic activities and sporocyst counts, and between the latter and the condition index, were determined by using regression models and analysis of variance (1-way ANOVA). The variables' enzymatic activity and sporocyst counts were logarithmically transformed [ $\log_{10}(n+1)$ ] to reduce the variance, which helped to normalise the data (Elliot 1979). We have followed Weldon & Slauson (1986) in recognising one important aspect of regression, the relative importance of the relationship (the proportion of variation associated with the relationship, as measured by  $R^2$ ). The limit of statistical significance was set at  $p < 0.05$ . Data analysis was performed using the SPSSWIN 10.0 statistical software.

Table 1. Enzyme activities determined with the Api-Zym system. Units are standards used for the commercial test. Values 0–5 correspond to 0– $\geq 40$  nmol nitrophenyl released by each sample. -AP: -aminopeptidase

Enzyme	Range of values
<b>Glycosidases</b>	
$\alpha$ -fucosidase	0–2
$\alpha$ -mannosidase	0
$\beta$ -galactosidase	0–0.5
$\alpha$ -galactosidase	0
$\beta$ -glucosidase	0
$\alpha$ -D-glucosidase	0–0.5
<i>N</i> -acetyl- $\beta$ -glucosaminidase	2–>5
glucuronidase	0–0.5
<b>Peptid hydrolases</b>	
Chymiotrypsin	0.5–>5
Trypsin	0
Valine-AP	1–>5
Cystine-AP	0.5–4
Leucine-AP	>5
<b>Ester hydrolases</b>	
Lipase	0–0.5
Esterase/lipase	0.5–1.5
Esterase	0.5–2
<b>Phosphoric hydrolases</b>	
Alkaline phosphatase	1.5–>5
Acid phosphatase	>5
Naphthol phosphohydrolase	>5

## RESULTS

### Api-Zym system test

Results from the Api-Zym system test provided an overview of the enzyme hydrolytic potentials of the common octopus (Table 1). The highest activities were found in LAP, acid phosphatase and naphthol phosphohydrolase. In all cases,  $>40$  nmol of hydrolysed substrate was observed. Relatively high enzymatic activity was also observed for the alkaline phosphatase, peptide hydrolases (30 and 40 nmol), and glycosidases (between 5 and 20 nmol for  $\alpha$ -fucosidase and *N*-acetyl- $\beta$ -glucosaminidase).

### Spectrophotometry

When the population was divided into 2 groups based on sporocyst concentration (Table 2), we observed that hosts with severe infection showed diminished activity in maltase and LAP, compared with that of hosts with a low number of sporocysts, whereas acid phosphatase showed increased activity. When comparing maltase and LAP activities in relation to the severity of the infection, the regression pattern indicated a significant statistical decrease in enzyme activities as

Table 2. Mean and standard deviation (SD) of the enzymatic activities (expressed as total activity per mg of caecum tissue [TA mg<sup>-1</sup>]) for low infected (Group 1) and heavy infected (Group 2) host samples. SG: total number of sporocysts per g of caecum tissue; LAP: leucine-aminopeptidase

Enzyme	Group 1 (2000–<100 000 SG) (TA mg <sup>-1</sup> ) Mean ± SD	Group 2 (100 000–3 000 000 SG) (TA mg <sup>-1</sup> ) Mean ± SD
Maltase	0.552 ± 0.298	0.414 ± 0.077
LAP	13.577 ± 7.182	8.157 ± 4.144
Acid phosphatase	3.061 ± 1.353	4.282 ± 0.541

Table 3. Regression analyses for the relationship between enzymatic activities (expressed as total activity per mg of caecum tissue [TA mg<sup>-1</sup>]), and number of sporocyst per whole caecum sample (TS)

Enzyme	a	b	F	R <sup>2</sup>	p
Maltase	0.447100	-0.162002	4.7391	0.1841	0.0410
LAP	1.725233	-0.120938	6.6685	0.2410	0.0174
Acid phosphatase	0.107594	0.094239	14.2593	0.4044	0.0011

Table 4. Regression analyses for the relationship between the K-Fulton condition index (CI) and the number of sporocysts per g of caecum tissue (SG) and the total number of sporocysts per whole caecum sample (TS)

CI	a	b	F	R <sup>2</sup>	p
SG	0.000640	-8.80827 × 10 <sup>-5</sup>	14.15000	0.4337	0.0012
TS	0.000472	-4.38984 × 10 <sup>-5</sup>	12.75794	0.2330	0.0009

parasite count increased, whereas acid phosphatase showed an increasing pattern (Table 3). Moreover, a significant statistical relationship was found between the condition index and sporocyst counts. The condition index decreased as the variables SG and TS increased (Table 4).

No significant relationship ( $F = 1.09228$ ,  $p = 0.3077$ ) was found between protein concentration of tissue extracts and sporocyst counts for any sample.

## DISCUSSION

In Galician waters, histopathological studies on infected caecum of the common octopus showed a high degree of invasiveness and pathogenicity of *Aggregata octopiana* (Gestal 2000). This intracellular parasite produces hypertrophy and degradation of infected cells with distension of the infected tissue area, causing rupture of the basal membrane and a breakdown of junctions among epithelial cells. Severe loss of epithelial cells and atrophy of mucosal folds occurred

in affected intestine and caecum. In heavy infections, most infected host tissue was replaced by parasites. Loss of intestinal and caecum epithelium and destruction of the organ architecture was observed (Gestal 2000). Severe cellular change should result in detrimental effects on the gastrointestinal function, which could affect the individual and population fitness (Ferron & Legget 1994).

We have applied biochemical analysis as a method for measuring fitness and to demonstrate the impact of *Aggregata octopiana* infection in the common octopus. We determined the levels of some enzymes using a colorimetric and semi-quantitative method. However, no differential patterns were easily discerned between subsamples related to the severity of infection. Subsequently we selected 2 enzymes involved in the intestinal absorption process and 1 lysosomal enzyme for quantitative analysis, comparing the possible relationship between infection severity (by counting the number of sporocysts) and the level of activity of these enzymes.

Using 2 different approaches we obtained similar results. A decrease in the absorption involved enzymes maltase and LAP, and an increase in the lysosomal-enzyme acid phosphatase related to the increased number of sporocysts. Since no variations were observed in the protein concentration related to the severity of infection, the observed differences in the activity of the enzymes are likely not due to a general decrease of total proteins.

Firstly, our results are interpreted as an overincrease of lysosomal activity, probably due to the massive presence of hemocytes and phagocytic cells attracted to the infected area. After phagocytosis, enzymes in phagocytic cells degrade foreign material according to the classical pathway of lysosomal digestion (Oubella et al. 1994).

Secondly, the obtained results also suggest that a malfunctioning of absorption enzymes is likely to occur in heavily infected octopus. Coccidians' metabolism produces considerable quantities of lactic acid, which may cause markedly lowered intestinal pH (Long 1973). Lowered intestinal pH causes denaturation of proteins, one of the factors responsible for disturbance in absorption of nutrients, and leakage of materials from the tissues by hampering deactivating-absorption

enzymes (Long 1973). Maltase and LAP, which are active at pH  $\geq$  7.0, may be affected by the acidification of heavily infected caecum lumen, and consequently glucose and amino acid absorption could be reduced. Important substrates in the diet of cephalopods, such as glucose and free amino acids (Lee 1994), could not be digested and completely absorbed by caecal epithelial cells. Therefore, although substrates are available within the caecum lumen, their transport through the membrane is hindered by a failure in enzyme activities. This could result in a decrease of nutrient absorption and consequently in the deterioration of fitness of the common octopus. This argument is supported by a decrease in the *K*-Fulton condition index in more heavily infected octopus.

Similar results were described by Preston-Mafham & Sykes (1970) and Long (1973) in birds and chickens infected with intestinal coccidia of the genus *Eimeria*. These authors described changes in the infected epithelium of absorption, producing a significant absorption decrease, a reduction of serum protein levels and body weight loss.

The results herein showed that although organ architecture may not be heavily histologically altered by parasitic infection, a detrimental effect on the gastrointestinal function may be produced because of decreased or malfunctioning absorption enzymes, which is designated as malabsorption syndrome.

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