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Influence of adult *Anguillicoloides crassus* load in European eels swimbladder on macrophage response

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ABSTRACT

Anguillicoloides crassus has become one of the most important threats to the European eel (*Anguilla anguilla*). Adult parasites colonize the swimbladder leading to an impaired functioning of this organ. The infection is also responsible for an increased in the stress level of infected eels, that could produce an altered immune response as well. Differences in parasite loads and effects in the European and Japanese eel (*Anguilla japonica*) have been described. We have studied the influence of the number of adult parasites present in the swimbladder of wild eels on the macrophage response (phagocytosis and respiratory burst) as part of the first immune response to pathogens. Our results show an increased phagocytozed bacterial survival 24 h post-infection in macrophages of eels infected with more than ten adult parasites compared to macrophages from eels infected with less than those ten adult parasites. Respiratory burst results also showed a less efficient response in macrophages from eels infected with more than ten adult parasites, although in this case results were not found to be significant.

1. Introduction

Eels are catadromic fish that swim to specific oceanic locations for spawning, the Sargasso Sea in the case of the European eel *Anguilla anguilla*. Young eels then swim back to Europe. But in the last decades, the number of these juveniles has significantly decreased [1–3]. One of the reasons involved in this decline has been the impairment of the swimbladder in the adults that have to reach the Sargasso Sea. Eels are benthic when living in continental waters, but they need a functional hydrostatic organ for their spawning migration through the Atlantic Ocean, where they perform vertical migrations ranging from 40 to 600 m. Thus, eels with a damaged swimbladder are unlikely to reach the Sargasso Sea [1,3,4].

Probably the most important cause for the impairment of the swimbladder is the infection by a parasitic nematode *Anguillicoloides crassus* [2,3]. *A. crassus* is a non-indigenous nematode parasite of the European and American eels. It was introduced from the Far East in the early 1980's by the global eel trade, and now it has become the most abundant parasite of the European eel [1,3–6]. Eels become infected with the parasite by eating the intermediate host (copepods and ostracods) or paratenic hosts (other fish species, molluscs, amphibians or insect larvae) infected with the third stage larvae (L3). The L3 migrates through the digestive tract wall to the swimbladder, where it develops into the L4, and finally in adults that suck the eel's blood [3,4].

No external signs are usually evident. Occasionally, there is an impairment of health and an increased susceptibility to other diseases, with an increase in the mortality in the worst cases. Internal signs include thickening of the swimbladder wall, dilated blood vessels, infiltration of white blood cells, fibrosis and changes in the epithelial cells [1,5–9].

Host's response includes a macrophage reaction against larvae. Epithelioid macrophages aggregate around the larvae before they reach the swimbladder lumen, initiating the formation of a connective capsule around it. Macrophages and granulocytes have also been found in the swimbladder wall, but they seem not to attack the L3 and L4 [7]. Antibodies against the L3 have also been detected, but they seem not to be enough to control the infection in the European eel [1,3–5].

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The purpose of this study was to see if the higher susceptibility to other pathogens could be related with an impaired functioning of macrophages, and if this impaired functioning could be parasite load dependent.

2. Material and methods

2.1. Fish

Twenty five eels (25–38 cm long) were caught in the Albufera, a freshwater lake located in the East of Spain. Eels were euthanised and classified attending to the number of adult parasites present in the swimbladder into three groups:

- Group 1 (G1): eels with no adult parasites. This group included 12 eels.
- Group 2 (G2): eels with between 1 and 10 adult parasites. Ten eels.
- Group 3 (G3): eels with more than 10 adult parasites. Three eels.

2.2. Macrophages

Renal macrophages were collected from every two eels in the groups G1 and G2 and pooled together into ice chilled separation medium consisting of L-15 medium supplemented with 2% fetal calf serum (FCS), heparin 10 U ml^{-1}, 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. The kidney was passed through a 100 μm nylon mesh and left on ice for 10 min. The cell suspension was then centrifuged at 1000 g for 15 min, the pellet was resuspended in low serum medium (L-15, 0.1% FCS, 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin), 0.1 mL was pipetted into 96-well tissue culture plates, and cells were left to attach overnight at 20 °C. Then cells were washed with L-15 supplemented with 2% FCS, 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin, to remove non-adherent cells, and new medium (L-15 supplemented with 2% FCS, 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin) was added. For G3 macrophages were isolated from every individual fish as described above.

2.3. Bacteria

Macrophages were tested using a strain of *Yersinia ruckeri* isolated from an outbreak of Enteric Redmouth Disease in rainbow trout in our laboratory. Bacteria were cultured on TSA plates, and stored at −40 °C with 10% sterile glycerol.

2.4. Phagocytosis

Phagocytosis was performed as described by Garcia and Villarroel [10]. Briefly, the number of adherent cells in two wells per pool was estimated by counting in a haemocytometer, and the number of infecting bacteria was adjusted to 100 bacteria per macrophage. Previously, several multiplicity of infections (moi) were tested (1:1, 10:1, 50:1, 100:1, and 1000:1 bacteria:macrophage) and 100:1 was chosen as it was the easiest to work with (data not shown). With the first and second moi (1:1, and 10:1) we were not able to detect any bacteria, the third and fourth (50:1, 100:1) had comparable results, and with the last (1000:1) there was a severe decrease in the macrophage number.

Y. ruckeri was used to infect wells per pool in duplicate for G1 and G2, and triplicate for G3. A bacterial suspension was prepared by centrifuging 1 mL of a broth culture and resuspending the pellet in 1 mL of saline. Prior to inoculating the bacteria at a moi of 100:1, macrophages were washed three times and medium without antibiotic was added. At different times post-infection (0, 4 and 24 h) macrophages were washed with sterile PBS to remove non-adherent bacteria and lysed for 20 min with 100 μL of trypsin-ethylenediaminotetraacetic acid (1×) (trypsin-EDTA) (Sigma). For macrophages that were lysed 24 h after infection, medium was replaced by medium with antibiotic (L-15 supplemented with 2% FCS, 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin) at 4 h post-infection (hpi). After the macrophage lysis, the well content was then diluted in sterile saline (serial 1/10 dilutions), spread onto TSA plates, and the number of colonies was counted after incubating at 22 °C for 24–48 h.

2.5. Respiratory burst

The respiratory burst was estimated by measuring the intracellular O^{2-} using the NBT reduction test. A solution of NBT (1 mg ml^{-1} in PBS) was added to adherent cells in two wells per pool of G1 and G2, and three for G3. As for the phagocytosis assay, *Y. ruckeri* was adjusted to a final concentration of 100 bacteria per macrophage, and added with the NBT solution. Macrophages were incubated for one hour, then fixed in methanol, washed twice with 70% methanol, left to air dry, and finally, formazan was dissolved in 120 μL 2M KOH and 140 μL DMSO. The turquoise blue colour was read in a spectrophotometer at 620 nm, using KOH/DMSO as blank.

2.6. Data analysis

Results from each group were analyzed together. Data were analyzed using ANOVA test. Significance was accepted at probabilities of 0.05 or less.

3. Results

3.1. Phagocytosis (Fig. 1)

As it was expected, the number of bacteria isolated at 0 hpi from the macrophages in the tree groups was very low (ratio 1:1), probably because time was not enough for a higher entrance into the cells. But at 4 hpi, the number had increased to a ratio of 10 bacteria:macrophage. The increase was the same in all the groups, showing a similar capacity of macrophages to phagocytose the bacteria. At 24 hpi macrophages showed their capacity to limit and

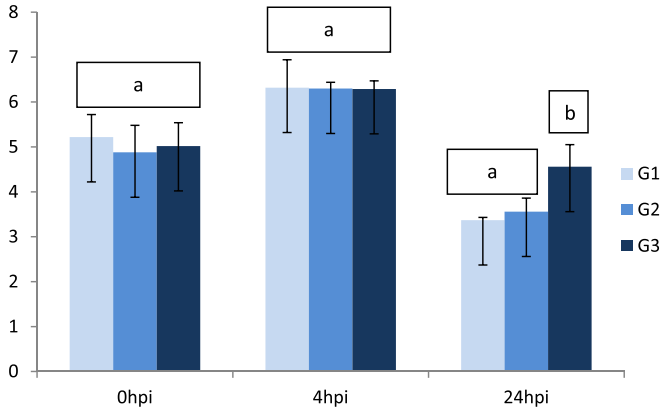

Fig. 1. Logarithm of the number of bacteria isolated from macrophages at 0, 4 and 24 h post-infection (hpi) from the three groups of eels (G1 eels with no adult parasites in the swimbladder, G2 eels with between 1 and 10 adult parasites in the swimbladder, and G3 eels with more than 10 adult parasites in the swimbladder). Data represent mean of 12 replicate wells for G1, 10 for G2 and 9 for G3. Line in each bar represents the standard deviation. Different lower case letters denote significant differences ($p < 0.05$).