Host–parasite interaction of a muscle-infecting didymozoid in the Atlantic mackerel *Scomber scombrus* L.

S. Pascual, E. Abollo, and C. Azevedo


Numerous ribbon-like aggregations of eggs of a trematode didymozoid were found embedded in the connective tissue, mostly between the epaxial skeletal muscle fibres, of mature mackerel *Scomber scombrus* collected from post-recruits of the eastern stock. Egg masses contained numerous developmental stages, including pre-vitellogenous oocytes to fully embryonated eggs. Genomic DNA isolation, PCR amplification, cloning, and sequencing strongly supported the view that the eggs belong to a yet unidentified didymozoid. There was minor muscular damage to the host, namely focal distortion of host fibre architecture to accommodate the parasite, and the mild host response consisted of different stages of encapsulation. Concurrent heavy infection makes the mackerel flesh unappealing to eat, although the quality of the fish is not significantly affected and there is no public health danger.

© 2005 International Council for the Exploration of the Sea. Published by Elsevier Ltd. All rights reserved.

Keywords: didymozoid, infected muscle, mackerel, trematode.

Received 17 June 2005; accepted 28 August 2005.

S. Pascual: Instituto de Investigaciones Marinas (CSIC), ECObiOMAR, Eduardo Cabello 6, 36208, Vigo, Spain. E. Abollo: Centro de Investigaciones Marinas, CIMA, Pedras do Corro, Pontevedra, Spain. C. Azevedo: Department of Cell Biology, Institute of Biomedical Sciences and CIIMAR, University of Oporto, Largo Prof. Abel Salazar, 4099-003 Porto, Portugal. Correspondence to S. Pascual: tel: +34 986 23 1930, ext 183; fax: +34 986 29 2762; e-mail: spascual@iim.csic.es.

Introduction

Flesh quality is a concern to any industry that sells a fresh meat product. Poor muscle quality of fish has been attributed to many factors, including processing problems, high fat feeds, stress, and quality of the fish at harvest (Talbot, 1994). Parasites have also been considered a factor critical in influencing conventional seafood quality and price (Malouf, 1986; Alvarez-Pellitero and Sitja-Bobadilla, 1993). Infections by some microsporidians, especially multivalvulid myxosporidians, have been associated with post-harvest autolysis in several marine fish species (Lom and Dyková, 1992). Among the largest, most numerous, and most diversified assembly of muscle-invading marine parasites are the free or encysted stages of didymozoid digenean trematodes and anisakid nematodes. Both have a long history of infection of large areas of muscle in a variety of fish species worldwide (Lester, 1980; Koie and Lester, 1985; Korotaeva, 1985; Abollo et al., 2001; Karlsbakk, 2001). Deterioration of fish condition and loss of marketability have been related to extensive fish muscle degeneration, parasite invasion, and chronic inflammatory reactions. Further, Thompson (2000) recorded emerging zoonoses and allergies associated with the presence of these helminth parasites in fish muscle, underlining the economic and public health importance of instituting control measures for edible seafood during inspections.

The Atlantic mackerel *Scomber scombrus* is an epipelagic and mesodemersal species that is most abundant in cold and temperate shelf sea areas. Its total catch reported to the FAO for 1995 was 610 947 t (www.fao.org), most of which was traded fresh, frozen, smoked, or canned for human consumption. Despite its importance mostly to local economies, little is known about agent–host interactions involving it. This study provides data on the host–parasite interactions of a muscle-infecting didymozoid in the eastern stock of Atlantic mackerel (Gibson et al., 1981), addressing the molecular characterization of the parasite, analysing prevalence data, and investigating the ultrastructure of host–parasite interactions.
Material and methods

Sampling

During 2002, 200 post-recruiting fish of the eastern stock of Atlantic mackerel were collected by fishers operating on a groundfish spawning area near Vigo on the North Atlantic coast of Spain (ICES Division IXa North). In all, 50 were collected each season, winter (January–March), spring (April–June), summer (July–September), and autumn (October–December). The study was carried out over a full year, the duration of a fish cohort, to explore potential seasonal differences in infection. The entire musculature of each market-size fish was carefully examined ashore for dot-, nodule-, or cyst-like parasites. Each fish was longitudinally filleted into muscle sections of about 4 mm, and each fillet was examined over a candling table with a dissecting microscope.

Genomic DNA isolation, PCR amplification, cloning, and sequencing

The eggs, preserved in 70% ethanol, were lysed in 500 µl Tris—HCl 20 mM pH 8.0, EDTA 5 mM, NaCl 400 mM, and SDS 1% with proteinase K (20 mg ml⁻¹) overnight at 37°C. Subsequently, the DNA was purified with one phenol:chloroform:isoamyl alcohol extraction, followed by one chloroform:isoamyl alcohol extraction. DNA was precipitated with ethanol and sodium acetate 3 M pH 5.2 overnight at −20°C. The precipitated pellet was dried and resuspended in 50 µl of Tris—EDTA (TE) buffer (Sambrook and Russell, 2001).

Two conserved primers, U1 forward (5’ AAC CTG GTT GAT CCT GCC AGT 3’) and U2 reverse (5’ TGA TCC TTC TGC AGG TTC ACC TAC 3’), derived from known conserved regions of the 18S rRNA gene, were used in PCR to amplify the small subunit ribosomal DNA gene (Medlin et al., 1988; Figueras et al., 2000). PCR reactions were performed in a total volume of 25 µl containing PCR buffer at 1× concentration, MgCl₂ at 1.25 mM, nucleotides at 0.4 mM, primers at 0.1 µM, Taq DNA polymerase at 0.025 units µl⁻¹, and 10–100 ng of DNA. The cycling protocol was 94°C for 3 min, 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, then 72°C for 10 min. PCR products were separated on a 1% agarose gel stained with ethidium bromide and analysed using a UV image analysis system.

Fresh PCR products were ligated into cloning vector pCR2.1 (Invitrogen) at 14°C overnight, then transformed into E. coli One Shot Top 10° Chemically Competent (Invitrogen). Transformed cells were screened by PCR using the vector’s M13 forward (5’ GTA AAA CGA CCG CCA G 3’) and reverse (5’ CAG GAA ACA GCT ATG AC 3’) primers. The positive clones with a fragment length of about 2000 bp were directly sequenced. DNA sequencing was performed by the dideoxy-chain termination method using a BigDye terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and an automated DNA sequencer ABI PRISM™ 3100 (Applied Biosystems). Each product was sequenced employing the M13F and M13R primers. All sequences generated were searched for similarity using the basic local alignment search tool (BLAST) at Web servers of the National Centre of Biotechnology Information.

Ultrastructure

Fresh squash preparations of small, infected pieces of muscle were examined under Nomarski differential interference contrast (DIC) optics, and the parasites were photographed. Histological preparations were made from infected muscle fixed in 10% formaldehyde for 24 h, then embedded in paraffin, sectioned at 4 µm, and stained with haematoxylin and eosin, following standard methods (Culling et al., 1985). Semithin sections stained with toluidine blue were also examined by light microscopy. For scanning electron microscopy (SEM), an egg suspension was fixed for 4 h in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3 at 4°C), then washed for 30 min in the same buffer. The sample was finally dehydrated in an ethanol series, critical-point-dried in CO₂ using a Polaron E3000, and sputter-coated in a Polaron SC500, using 60% gold—palladium. The parasites were examined with a Philips XC30 SEM operating at 10–20 kV. For transmission electron microscopy (TEM), small fragments of the infected muscle were fixed in 3.0% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.2, for 12 h at 4°C, washed in the same buffer for 4 h at 4°C, then post-fixed in buffered 2.0% osmium tetroxide for 4 h at the same temperature. After dehydration in a graded ethanol series, the fragments of infected muscle were embedded in Epon, sectioned by diamond knife, double-stained with uranyl acetate and lead citrate, and observed in a JEOL 100CXII TEM operated at 60 kV.

Prevalence and mean intensity of infection were determined according to Bush et al. (1997). A non-parametric test was used to generate statistical confidence in analysing seasonal and host—parasite relationships.

Results

Parasite

During necropsy, examination by eye of some filleted muscle sections revealed a total of four to eight parallel yellowish threads about 8 mm long by 200–290 µm wide. They conformed to dorsoventrally flattened filamentous worm fragments plus numerous ribbon-like egg masses of an unidentified tetrametadoe coiled inside the fish muscle in a zigzag fashion. Loop masses contained large numbers of eggs, with developmental stages ranging from the untanned smaller pre-vitellogenous oocytes in the proximal region to fully embryonated eggs in the distal part of the uterus (Figures 1−5). These larger reniform eggs characteristically showed internal masses of undifferentiated cellular material or germ
balls of the miracidium (Figure 5). A large operculum is located 1.8–4 mm from the top of the generally bean-shaped eggs. The eggs measured 25–30 μm long and had a smooth, thick (0.25 μm), scleratin-like shell (Figures 4–6).

The universal U1 and U2 primers successfully amplified over 1900 bp from every sample examined. The DNA sequence of PCR was registered in GenBank, with accession number DQ087251. BLAST searches using the obtained sequence confirmed that it belonged to 18S rDNA and bore close similarity to the family Didymozoidae. The highest nucleotide identity values were obtained with Didymozoon scombri (AJ287500) and Didymozoid sp. (AY222102, AY222103, AY222104), at 95% sequence identity.
Host–parasite interaction

Histological and semithin sections revealed egg masses both superficially and in deep connective tissue layers between muscular fibres, through the epaxial and hypaxial musculature in the cranio-dorsal (50%), medio-lateral (25%), and caudal (25%) regions of mackerel 244–335 mm long and 115–394 g in weight. Single egg masses were common in the fish muscle, but multiple infections (up to eight residual aggregations of eggs in a 335-mm, 303-g mackerel) were found in 14.3% of the infected fish. Overall prevalence of infection reached 18.5% (11.6–25.4 CI). No statistically significant seasonal changes in infection values were evident in a year-long fish cohort ($U = 362$; $p = 0.5696$), but prevalence was greater in late spring (20%) and summer (24%) than in autumn (14%) or winter (16%). No significant relationships were found between prevalence of infection and fish body length ($U = 435$; $p = 0.7265$) or weight ($U = 362.5$; $p = 0.0625$).

The parasites always followed the orientation of the muscle fibres and did not traverse the myosepta. Focal damage to the constricted muscle fibres surrounding the parasite could be seen in some histological and semithin sections by
increasing the mechanical pressure, which locally distorted the fish muscular architecture. Nevertheless, myofibres remote from the parasite always retained their structural integrity.

Ultrastructure examination of host–parasite interaction revealed early and intermediate stages of parasite encapsulation. An early host reaction consisted of a 1.13–1.32-μm-wide capsule with pockets of amorphous material and cell debris, which in intermediate stages of encapsulation resulted in an inner, closely applied capsule of infiltrated leukocytes surrounding the egg masses (Figures 6 and 7). Numerous membranes of variable size were seen in the integument of the adult didymozoid, in direct contact with the inner capsule. The parasite integument was intact, and the...
sub-integumental cells were normal. Accumulation of floc-culent electron-dense material not normally present on the integument and its surface was observed near the basement membrane and the surface of the integument (Figure 8). Late stages of encapsulation were associated with residual aggregations of embryonated eggs, consisting of numerous fibroblasts and large amounts of collagen (Figures 9 and 10). In this case, the residual integument covering the egg masses was degraded and not easily identifiable.

**Discussion**

The molecular analyses presented here support the view that the eggs found in the musculature of Atlantic mackerel belong to a yet unidentified didymozoid. According to Gibson et al. (1981), live adult didymozoids of Halvorsenius exilis commonly found in eastern Atlantic mackerel occur only in young fish, whereas egg aggregations (which are retained *in utero* until the death of the worm) are found in older fish (3-group) from the western English Channel. Eggs seem to remain in the host tissue and are released into the water only when the fish is eaten and digested, or dies and decomposes. Death of the fish is therefore apparently necessary to make larvae available to the first intermediate host. Our finding is in full agreement with this thesis, because we only found worm fragments and residual aggregations of eggs in the musculature of post-recruit market-size mackerel. Based on cohort infection analysis, Gibson et al. (1981) suggested that most infections in 2-group and older mackerel could be identified by the occurrence of eggs rather than live worms. We also agree with this statement, because in our material, 80% of the residual egg-containing mackerel were ≥ 30 cm long (i.e. age ≥ 2-group; FAO-FIGIS —www.fao.org). Moreover, as the parasite recruits to the reproductive stock all year round at about 20% prevalence, and no significant relationship was found between parasite prevalence and fish length or weight, it is likely that residual egg aggregations really mark the earlier position of dead worms. If so, then it is also likely that metacercariae and adult stages would be common in fish from the pre-recruiting young stock, whereas residual egg aggregates would be predominant in mature fish from the target reproductive stock. Overall, unfortunately, no sequences of PCR products of *H. exilis* are registered in GenBank.

Although trematodes are the most prevalent and conscipuous helminth parasites that infect mackerel (Arandas-Rego and Santos, 1983; Arandas-Rego *et al.*, 1985), neither histopathological nor host tissue reactions have been studied. Tissue infected by didymozoids usually reveals little or even no tissue reaction (Lester, 1980; Karlsbakk, 2001). Gibson et al. (1981) documented no sign of host reaction associated with live *H. exilis* in very close contact with fish tissues. Although our findings were sometimes similar, in most cases we did see a host cellular encapsulation response. It is likely that primarily non-specific mackerel responses react against invading metacercariae but that these responses do not reach the adults. Our ultrastructure data have proved that exposed intengumentary surfaces that are common in early encapsulated adult worms were always

---

**Figures 9—10. Histopathology.** (9) Longitudinal histological section showing a focal parting of skeletal muscular fibres to accommodate a residual aggregation of eggs. Note that the parasite follows the orientation of the muscle fibres (×100). (10) Higher magnification than in Figure 9, showing marked fibrosis (arrow) (×1000).
undamaged. It is likely that an evasion mechanism of host defence may be associated with the release of lyric or toxic substances to host cells enclosed in the numerous electron-dense structures secreted by the parasite. This ultrastructural observation corresponds with early and intermediate phases of encapsulation. Once the adult parasite leaves the fish muscle tissue or dies, late stages of encapsulation are common, in association with worm fragments and residual eggs. Such a strategy developed to avoid destruction by the host response has been suggested for other helminths (Ubelaker et al., 1970; Bogitsh and Wilkel, 1974), and for trematode infections of long duration in fish hosts (Mathews and Mathews, 1993; Buchmann et al., 2001).

Clearly, more research on cytochemical identification and biochemical characterization of such toxic secretions from our material is required.

Numerous egg masses of a muscle-infecting didymozoid are certainly consumed by man, but this does not seem to pose a public health problem. However, cases of concurrent heavy infection in the edible part can reduce the commercial value of mackerel, mostly because of the unappetizing "yellowish presence" of numerous residual ribbon-like eggs along with quinone-staining shell components that are visible to the naked eye.

Acknowledgements
This work was supported by Marie Curie Fellowship contract No. QLK5-CT2000-52101 under the Fifth Framework Programme of the European Community. S. D’Amelio and an anonymous reviewer are thanked for their constructive comments on the draft submission.

References