Pathological and epidemiological observations on Rickettsiosis in cultured sea bass (*Dicentrarchus labrax* L.) from Greece.

F. Athanassopoulou¹*, D. Groman², Th. Prapas³, O. Sabatakou⁴.

1. Laboratory of Ichthyology & Fish Pathology, University of Thessaly, Faculty of Veterinary Medicine, School of Health Sciences, 221 Trikalon str., Karditsa, 43100 Greece. E mail: eathan@vet.uth.gr
2. Aquatic Diagnostics Services, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, Canada, CIA 4P3.
3. Fish Diseases Dept., Veterinary Centre of Athens, Ministry of Agriculture, Agia Paraskevi, Athens, Greece 15310
4. Department of Histology, National Agricultural Research Foundation, Institute of Veterinary Research of Athens, Athens, Greece 15310.

ABSTRACT:

A systemic infection of a *Rickettsia*-like organism in cultured sea bass is described for the first time. In hatcheries, clinical signs were lethargy, inappetence and discolouration.. Twenty days after transfer to sea cages from hatcheries where the disease existed, fish showed erratic swimming, loss of orientation, lethargy and abnormal behaviour. Mortality reached 30 % in colder months of the year in hatcheries and 80% in cages. Surviving fish in cages did not show any clinical signs of RLO infection the second year. Histological lesions in both hatchery and caged infected fish were found in cranial sensory, nervous and integumental systems and alimentary organs, charcterized by a necrotizing, mixed leucocytic dermatitis, osteochondritis and diffuse subcutaneous branchitis. Intra-cranially, there was an ascending perineritis and necrotizing congestive meningoencephalitis Evidence for transfer of infective agents across the blood brain barrier was confirmed by the presence of immunohistochemical positive rickettsia within capillary endothelium and histiocytes in inflamed regions of the optic tectum and the cerebellum. In the most severe cases, infection spread to the statoacoustical (semicircular) canal system and ependymal lining of ventricles, with marked rickettsial-laden histioocyte infiltration of the canal lumen. The organisms were found by immunohistochemistry to be related to *P. salmonis*..

KEY WORDS:


*corresponding author
Rickettsia-like organisms (RLO) infections of fin-fish have been reported in several salmonid and non-salmonid species in both fresh and seawater since 1939 (Mauel and Miller 2002). This organism was not considered of economical importance to the global fin-fish aquaculture industry until Piscickettsia salmonis was confirmed as the aetiology agent of mass mortalities in the Chile during the 1990’s (Fryer et al. 1992). All cultured salmonid species can be affected by this intracellular bacteria, and in diseased fish it provokes a systemic response affecting most internal organs, but preferentially targeting the liver (Almendras et al. 2000). For other RLO’s the pathology may vary depending on both the immunogenicity of the RLO and the species of fish affected; eg, RLO infections of the Hawaiian tilapia result in a systemic granulomatous inflammatory response (Mauel et al. 2003). Initial published reports on RLO’s affecting cultured juvenile sea bass (Dicentrarchus labrax) were described by Comps and colleagues (1996) from sea-cages, at rearing temperatures ranging from 12-15°C, in the Mediterranean off the coast of France. In this outbreak the reported pathology was restricted to the mesencephalic regions of the brain. Subsequently, the organism was identified from cultured sea bass of the coast of Greece (Athanassopoulou et al. 1999), with moribund fish showing similar pathology; eg, brain, olfactory nerve and internal organs inflammation. These samples were preliminarily screened by immuno-histochemistry and found to cross react with antisera to P. salmonis. Recently, Steiropoulos and colleagues (2002) confirmed this finding by demonstrating antigenic similarities between P. salmonis and European sea bass RLO isolates from Greece.

In this paper we document the systemic histopathology of rickettsial infections of cultured juvenile sea bass, and provided epidemiology data on the prevalence of this disease in caged reared sea bass.
**Material and methods.**

*Fish:* During the years 1995-1999 2000 sea bass larvae (0.2-1.5g) from two of the largest production and supply units (hatcheries) and 450 sea bass (weight 2-15g) from five cage farms. Sampling was conducted in each farm at monthly intervals. At each visit fish were randomly selected from cages or tanks showing clinical signs as well as from cages or tanks holding fish exhibiting no clinical signs. From these cages /tanks each time 20 fish were collected and examined. In three cage farms, fish were supplied by the above sampled hatcheries and in two fish came from other hatcheries. Hatcheries were supplied with bore-hole water supplemented with filtered but otherwise untreated sea water. In cages, stocking density of the fish ranged between 12-15Kg/m\(^3\) and fish were fed on commercial feeds.

*Clinical Evaluation:*

Collected fish and larvae underwent necropsy immediately after sampling. Macroscopic examination was carried out in the external surface, the gills and the internal organs by methods described by Roberts (1989). Prevalence was calculated on the number of dying fish showing nervous signs deriving from a stock where the presence of RLO’s was confirmed by histology or immunohistochemistry in nervous tissues. Mortality was calculated on a weekly basis by farm personnel as part of their husbandry procedures.

*Bacteriology/cell culture:*

Kidney and spleen samples were inoculated on Tryptone Soy Agar (TSA) and Thiosulphate Citrate Bile Salt Agar (TCBS) for bacteriology according the methods described by Roberts & Shepherd (1997). Suspected brains from infected sea bass were also sent to USA for isolation in striped snakehead (SSN-1) cell cultures according to standard methods (Winton, pers. Comm.).

*Histology:* Either whole fry or selected tissues from moribund fish were fixed in 10% Neutral Buffered Formalin and processed for histology, with 5µm sections stained routinely with either, Haematoxylin and eosin (H & E), Giemsa or Gram staining methods. Fish tissues were observed by light microscopy and selected photographs taken on a Ziess Photomicroscope III.

*Immunohistochemistry:* An immuno-histochemical procedure adapted from Almendras et al (2000) was applied to selected histologic sections of whole fry or tissues to visualize rickettsial agents. Briefly, de-parafinized tissue sections were incubated overnight in H\(_2\)O\(_2\) to remove endogenous peroxidase, washed in PBS for 10
min., incubated in 5 % normal goat serum for 30 min. to block for non-specific antibody attachment, then incubated for a further 60 min. in a 1:200 dilution of the primary antibody (rabbit anti-\(P.\) \textit{rickettsia}, supplied by Dr. John Fryer, Oregon State University), washed in PBS for 10 min., incubated for another 60 min. in a 1:200 dilution of the secondary antibody (Goat anti-rabbit HRP; Sigma), washed again in PBS for 10 min., and visualized with either DAB (3,3'-diaminobenzidine hydrochloride; brown color) or AEC (3-amino-9-ethylcarbazole; red color) substrate for 4 min. The development of the DAB was stopped using a 5 min dH\textsubscript{2}O bath (or AEC stopped using PBS), and the slide counterstained in 4.7 % Harris Haematoxylin to allow for interpretation.

**Results.**

*Clinical findings and epidemiology:* In hatcheries, clinical signs were lethargy, inappetence and discolouration. Twenty days after transfer to sea cages from hatcheries where the disease existed, fish showed erratic swimming, loss of orientation, lethargy and abnormal behaviour. Mortality reached 30% in colder months (10-16\textdegree C) of the year (December-March) in hatcheries and 80% in cages (Fig. 1). Surviving fish in cages did not show any clinical signs of RLO infection during the second year. However, according to data collected by the vets on the farms, surviving fish were more susceptible to bacterial and parasite infections (\textit{Vibrio} sp. and Isopoda infections). Fish stock from hatcheries where the infection was not present showed either no infection at all or very low prevalence (<10%). RLO infections were more prominent in areas where Isopoda infections were a recurrent problem (Lytra and Bouboulis, pers. comm.).

*Histopathology and immuno-histochemistry:* A representative histopathological collection of tissues was taken from selected moribund individuals, targeting the cranial sensory, nervous and integumental systems, as well as alimentary organs; ie, liver and stomach. Lesions were similar to larvae and caged fish. Specifically, the integument and subcutaneous cranial skeletal systems of affected individual often showed a necrotizing, mixed leucocytic dermatitis, osteochondritis (Fig. 2a) and diffuse subcutaneous branchitis,* predominated by granulocytes and rickettsia- laden histiocytes (Fig. 2b). Similar integumental inflammation was noted along the cranial sensory cannals and nares. Intra-cranially, the optic, olfactory sensory nerve sheaths and retina were infiltrated (Fig. 2c), resulting in an ascending perineritis and necrotizing congestive meningoencephalitis (Figs. 2d, 3a, 3b). Evidence for transfer
of infective agents across the blood-brain barrier was confirmed by the presence of immunohistochemical-positive rickettsia within the capillary endothelium and histiocytes in inflamed regions of the optic tectum (Fig. 3b) and the cerebellum (Fig. 4a). In the most severe cases, infection spread to the statoacoustical (semicircular) canal system and the ependymal lining of the ventricles, with marked rickettsia-laden histiocyte infiltration of the canal lumen (Fig. 4b). Visceral organs were equally affected, showing moderate to marked necrotizing histiocytic gastritis (Fig. 3c) and hepatitis (Fig. 3d). Immunohistochemical confirmation of a rickettsial agent was confirmed (Fig. 4c) with the organism readily identifiable within the gastric submucosa and within sloughed mucosal epithelial debris.

Discussion.

Strains of rickettsia isolated from non-salmonid fishes are not well studied and their relationship with *P. salmonis* has not being studied. In Mediterranean fish RLO’s were reported for the first time in cultured 30-70g sea bass in France in 1996 (Comps et al. 1996). At that time, the disease was characterized by meningeal inflammation, nervous signs, blindness which resulted in coordination problems, the inability to find food and high mortalities especially in cold months of the year. Lesions concerned the mesencephalon only; these were characterized by local necrosis and an inflammatory reaction with infiltration of basophilic cells. RLO’s were identified inside macrophage cells present in the inflammatory lesions or were dispersed in the nervous tissue of the brain. Under electron microscopy RLO had a similar morphology to *P. salmonis*; however, there were some differences such as the presence of polymorphic dense particles (PEDB), making the identification difficult. Thus, the disease did not occur as the systemic type usually found in other fish (Comps et al. 1996). In Greece a similar disease was observed for the first time in cultured sea bass (0.5-10g) in 1997, a few days after transfer to sea cages. The disease was characterized by nervous signs, especially in cold water temperatures (<16ºC) and resulted in high mortalities (Athanassopoulou et al. 1999). Since then, the disease has been detected in several farms around Greece with increasing mortality in both larvae and caged fish. The pathology of this organism observed in sea bass in the present study suggests that the infection can become systemic, but it tends to target the sensory system of the cranium, the brain and multiple points in the cranial integument (ie, jaw, scale pockets, nares). These findings are similar to those
reported by Comps et al. (1996). The brain pathology, seen in our case (meningitis), was also similar to that seen in British Columbia for Atlantic salmon that were found to be infected with a RLO. (Brocklebank, Speare, Amstrong & Evelyn 1992; Brocklebank Evelyn, Speare & Amstrong 1993). The two other recent reports on RLO pathology in other species of fin-fish involved the Hawaiian tilapia (Mauel et al., 2003) and the white sea bass (Chen et al., 2000). Our pathology differs from that noted in the tilapia where the inflammation was more chronic and primarily granulomatous and was distributed primarily in organs like the retinal rete, spleen, kidney and liver. In addition, the agent affecting the tilapia did not react with antibody raised against *P. salmonis*. In white sea bass, the pathology was similar to that we observed in the present study. Skin lesions were common and these were noted in the jaw and cranial integument as in our fish. The liver was less of a target in sea bass when compared to salmon (Almendras et al. 1997), however, the pathology was similar to that seen for salmon in Chile. In addition this paper reports similar lesions to those seen in salmon in other organs: eg, the pancreas, retina, brain stem, meninges and the lamina propria of intestine. Smith et al. (1999) suggested that the skin and gills are routes of entry of *P. salmonis*. The presence of the organism in head epidermis of sea bass infected with the *P. salmonis*-like organism in our study agrees with this suggestion.

The most sensitive method of detecting RLO’s is by their growth in cell cultures, therefore, our efforts were initially concentrated on culturing the organism in striped snakehead (SSN-1) cells, but, this was unsuccessful, possibly due to unsuitability of this cell line for sea bass RLO’s. The same outcome has happened in previous work (Comps et al. 1996). Up to now, the study of RLO in fish has been limited to salmonids where the organism has been isolated in embryonated eggs and specific cell cultures. Thus, despite the fact that some cell cultures have been developed for warm water species, there are still problems to overcome (Comps et al. 1996). However, there must always be confirmation by immunological or molecular means. Immunofluorescence and immunochemistry tests have also been developed for *P. salmonis* detection (Lannan et al. 1991; Alday – Sanz et al. 1994) as well as an ELISA (Cassiggoli 1994). Recently, molecular methods have proved very useful for quick diagnosis in salmonids (Mauel et al. 1996). In contrast, for mediterranean fish, despite the increase of production and the severity of RLO disease in sea bass, no similar diagnostic tools have been developed. No isolation of the responsible organisms has
been made, nor have techniques of early detection been developed. Diagnosis is usually made in Giemsa-stained histological sections from fish stocks where mortality is high and as a result, treatment is unsuccessful and the disease eventually stops after the temperature increases. In our case, immunohistochemistry staining of brain sections with labelled anti *P. salmonis* antibodies was positive (Athanasopoulou et al. 1999) and this is the first step for identification and diagnostic purposes. A reliable and quick diagnostic method as these already developed for salmonids, would facilitate the early diagnosis and successful treatment in view of the fact that, even in salmonids, although the organisms are sensitive to antibiotics *in vitro*, treatments in the field are not always successful (Fyer et al. 1992). Wild fish around cages can be a source of primary infection of sea bass, but, the highest prevalence observed in caged stock originating from infected hatcheries needs further extensive investigation and may require broodstock tests. Because of all these reasons, as well as because of the worldwide transfers of fish, it is of primary importance to develop reliable and quick diagnostic methods (Fryer et al. 1992; Almendras & Fuentealba 1997).

For preventive purposes, therefore, it is necessary to understand how the infection is transmitted and its pathogenesis as no vaccines available for commercial use to date.

The present work demonstrates the need of further research in order to understand the infectivity ability of different strains and to compare strains isolated in different areas (i.e. in freshwater vs marine water, in Northern and Mediterranean areas).

REFERENCES


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Figure Legends:

**Fig.1.** Overall prevalence of *Rickettsia*-like infections of sea bass in Greece (Year 1996).

**Figs. 2a-d.** *Dicentrarchus labrax* lesions from infection with a RLO:

Fig. 2a. Transverse section through the rickettsial infected upper jaw and maxillary acellular bone (j) showing the epidermis (e) and marked inflammation of the underlying dermis, hypodermis and periosteum. Haematoxylin & Eosin. Bar = 83 µ.

Fig. 2b. Higher magnification of jaw bone (j) and periosteum showing histiocytic inflammation with intracellular rickettsial agent (arrow). Giemsa. Bar = 21 µ.

Fig. 2c. Oblique section through the olfactory nerve showing inflammation of perineurium and associated blood vessel. Haematoxylin & Eosin. Bar = 21 µ.

Fig. 2d. Transverse section through the olfactory lobe (ol) of the mid-telencephalon. Arrows indicate regions of congestion and meningeal inflammation. Haematoxylin & Eosin. Bar = 83 µ.

**Figs. 3a-d.** *Dicentrarchus labrax* lesions from infection with a RLO:

Fig. 3a. Transverse section through the ventral lateral aspect of the optic tectum (ot) in the anterior-mesencephalon, surrounding arachnoidea, dorsal-cranial cartilage, acellular bone and overlying epidermis (e). Arrows denote congested meningeal and arachnoidal vasculature. Haematoxylin & Eosin. Bar = 83 µ.

Fig. 3b. Higher magnification of an inflamed region in the outer optic tectum showing a rickettsia-laden histiocytic inflammation (arrow). Haematoxylin & Eosin. Bar = 21 µ.

Fig. 3c. Transverse section through a rickettsial infection of the stomach showing a diffuse inflammatory infiltrate within the submucosa (sm), location of the gastric glands and mucosa (arrow) and sloughed cellular debris in the lumen (l). Haematoxylin & Eosin. Bar = 83 µ.

Fig. 3d. Transverse section of a rickettsial infected liver parenchyma detailing sinusoidal congestion and foci of histiocytic inflammation (arrow). Haematoxylin & Eosin. Bar = 33 µ.
**Figs. 4a-c.** *Dicentrarchus labrax* immunohistochemical staining of lesions containing a RLO:

Fig. 4a. Immuno-histochemical staining of rickettsial agents within histiocytes (arrow) infiltrating the outer cerebellum in the metencephalon. AEC & Haematoxylin. Bar = 13 µ.

Fig. 4b. Immuno-histochemical staining of rickettsial agents within histiocytes (arrow) infiltrating the lumen of the posterior semicircular canal in the cranium. AEC & Haematoxylin. Bar = 13 µ.

Fig. 4c. Immuno-histochemical staining of rickettsial agents within histiocytes (arrow) and mucosal epithelium of the stomach. AEC & Haematoxylin. Bar = 13 µ.