

Severe mortality in mesocosm-reared sharpsnout sea bream *Diplodus puntazzo* larvae due to epitheliocystis infection

Pantelis Katharios*, Maria Papadaki, Nikos Papandroulakis, Pascal Divanach

Institute of Aquaculture, Hellenic Centre for Marine Research, PO Box 2214, Heraklion 71003, Crete, Greece

ABSTRACT: This paper describes severe mortalities recorded in sharpsnout sea bream *Diplodus puntazzo* larvae reared in mesocosms. The mortalities were attributed to epitheliocystis infection. The pathology associated with the disease is described using histological techniques. Microscopical examination showed a massive infection of the skin, fins, and oral cavity, with impaired feeding, respiration, and osmoregulation being the most likely cause of death. This is the first report of epitheliocystis disease in sharpsnout sea bream and in fish at such an early developmental stage.

KEY WORDS: Epitheliocystis · *Chlamydia* · Sea bream · Fish larva · Disease · Mortality

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INTRODUCTION

Epitheliocystis is an infectious disease affecting the gills and the integument of many marine and freshwater fish species (Nowak & LaPatra 2006). It is caused by Gram-negative bacteria, recently characterized as *Chlamydia* (Order: Chlamydiales), forming intracellular inclusions (Draghi et al. 2007). These inclusions progressively enlarge, resulting in hypertrophied cells that resemble spherical cysts. Although the pathogen cannot be cultured, and therefore Koch's postulate with experimental challenges has yet to be demonstrated, mortalities have been associated with the presence of the bacterium (Paperna 1977, Bradley et al. 1988, Venizelos & Benetti 1996, Crespo et al. 2001, Goodwin et al. 2005). Mortalities (4 to 100%) and prevalence (20 to 100%) of the disease varies depending on fish species, developmental stage of the host, and intensity of infection. Mortality has been reported only in cultured fish, with early life stages being more susceptible (Nowak & LaPatra 2006). The pathology associated with epitheliocystis includes epithelial hyperplasia, inflammation of the affected tissue, proliferation of the mucous cells, and necrosis; however, in many cases, the cysts provoke no cellular response (Paperna 1977, Grau & Crespo 1991, Crespo et al. 1999, 2001, Nowak & Clark 1999).

Sharpsnout sea bream *Diplodus puntazzo* (Cetti, 1777) has been successfully introduced to Mediterranean aquaculture, and it is a species with high market value (Divanach et al. 1993, Sara et al. 1999, Papandroulakis et al. 2004). It is a species with high growth rates that, while easily reared in early life stages, has high mortality rates in on-growing facilities mainly due to diseases (Athanassopoulou et al. 1999, Munoz et al. 2007). In this paper we describe a case of severe mortalities of sharpsnout sea bream larvae reared in the semi-intensive hatchery of the Institute of Aquaculture, HCMR, Crete, attributed to epitheliocystis hyperinfection. This is the first report of epitheliocystis in this species and the first report of the disease in such early fish larvae (pre-flexion stage).

MATERIALS AND METHODS

Rearing. Sharpsnout sea bream were reared in the facilities of the Institute of Aquaculture using semi-intensive mesocosm technology (Papandroulakis et al. 2004). Five 40 m³ tanks were stocked consecutively with sharpsnout sea bream eggs at a density of approximately 8 l⁻¹. The tanks were filled with natural sea water (salinity 40 ppt), which was renewed from a lit-

*Email: katharios@her.hcmr.gr

toral well (salinity 32 ppt) at a rate that progressively increased from 5% daily at the beginning to 50% daily by 25 d post hatching (dph). The fish were fed on enriched rotifers *Brachionus plicatilis* and enriched *Artemia* sp. nauplii, while microalgae *Chlorella minutissima* were added daily in the rearing water. Temperature ranged between 21 and 23°C.

Epizootiology of infection. The earliest mortalities among sharpnose sea bream larvae were recorded at 7 dph while the latest (in terms of mortality onset) were recorded at 20 dph. All 5 tanks were severely affected by the disease with final mortalities reaching 100% within 25 d. Moribund fish were lethargic and cachectic, swimming at the surface of the water. Sampling to assess the intensity and prevalence of infection was performed every second day, and 15 to 20 fish from each population were examined fresh under a stereoscope.

Histology. Twenty larvae (15 dph) with signs of the disease (spherical cysts in the integument) were killed with a high dose of the anesthetic 2-phenoxyethanol and preserved for histology.

For the histological examination, the larvae were fixed in 4:1 formaldehyde:glutaraldehyde (McDowell & Trump 1976), dehydrated in a 70 to 95% ethanol series, and embedded in glycol methacrylate resin (Technovit 7100, Heraeus Kulzer). Serial sections were obtained at a thickness of 3 to 5 µm on a microtome (Leica RM 2245) using disposable blades. After drying, slides were stained with methylene blue/azure II/basic fuchsin (Bennett et al. 1976), Gram stain, and Giemsa and examined under a light microscope (Leica DMLB). Microphotographs were taken with an ALTRA 20 digital camera attached to the microscope and analyzed using the ImagePro 6.1 (MediaCybernetics) image analysis system.

RESULTS

All fresh specimens grossly examined under the stereoscope had numerous cysts in the skin and the oral cavity (100% prevalence), with the majority of the fish being hyper-infected. Most of the live larvae that were grossly examined had more than 30 cysts in the integument. The cysts were circular and ellipsoid with yellowish and brownish coloration (Fig. 1).

Light microscopy observations under high magnification (×1000) of both fresh and sectioned specimens revealed that the cysts were actually intracellular granular inclusions containing particles resembling the elementary and reticulate bodies described in chlamydial infections and specifically epitheliocysts (Fig. 2). The cysts were stained Gram-negative and Giemsa-positive (Fig. 3).

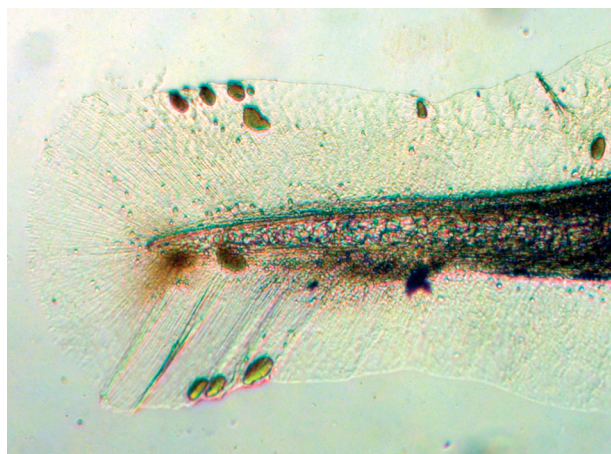


Fig. 1. *Diplodus puntazzo*. Caudal primordial fin of a larva at the flexion stage with numerous yellow and brown ellipsoid cysts

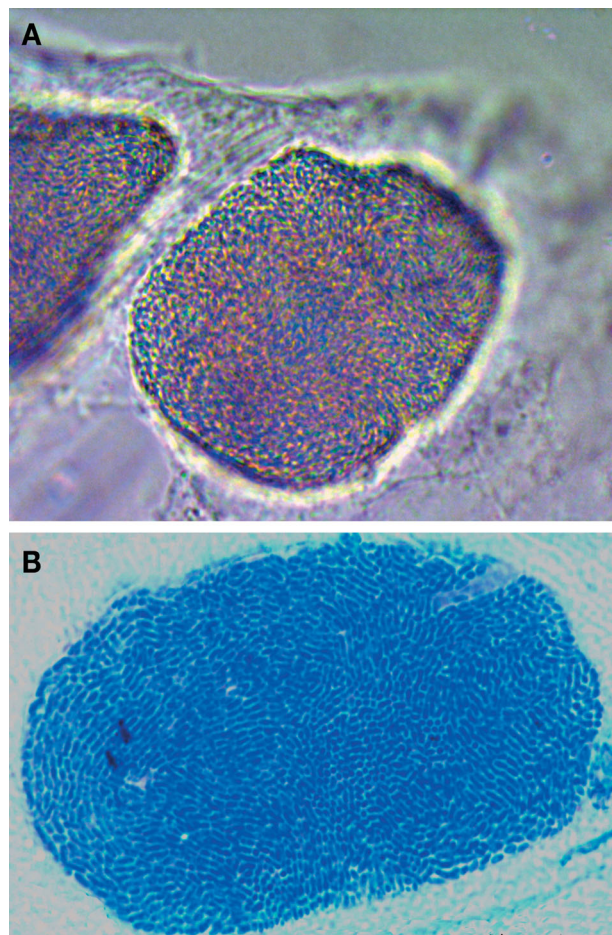


Fig. 2. *Diplodus puntazzo*. High power magnification (×1000) micro-photographs of epitheliocyst inclusions from larvae. (A) Fresh mount of the inclusion, (B) cross section of tissue containing the inclusion. Note the various shaped particles within the cyst resembling the elementary and reticulate bodies characteristic of the *Chlamydia* developmental cycle

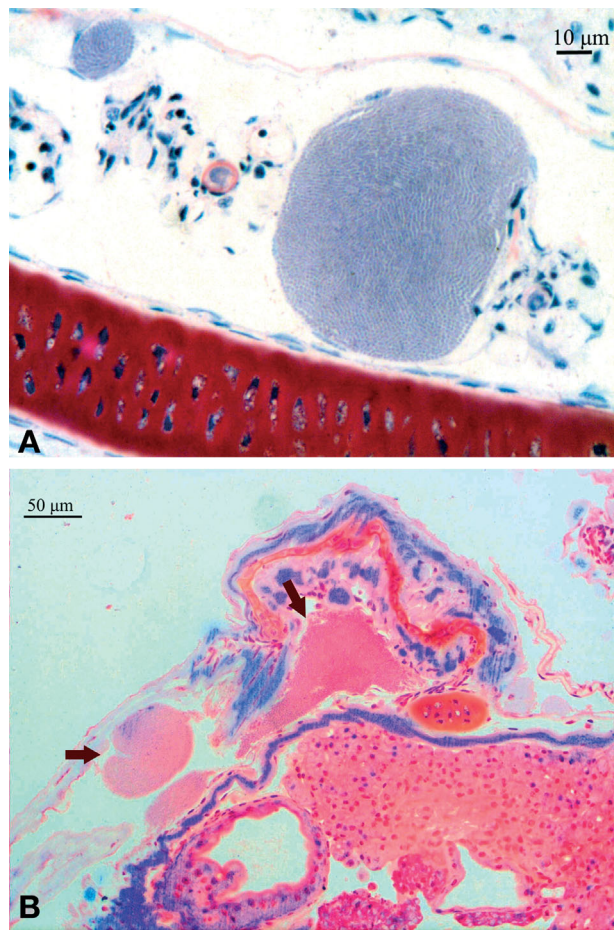


Fig. 3. *Diplodus puntazzo*. (A) Cross section of the head area of a larva with 2 inclusions positively stained with Giemsa. (B) Gram-negative stained epitheliocystis (arrows)

The ellipsoid epitheliocystis inclusions varied in size ranging between $16 \times 21 \mu\text{m}$ (minor \times major axis) and $89 \times 115 \mu\text{m}$ ($n = 25$). The inclusions were located mainly on the primordial fins, in the skin epithelium, and in the oral cavity (Fig. 4). All cysts identified in the histological sections had a granular texture and were never observed to provoke any cellular response of the host such as hyperplasia or increased cellularity. The only host response recorded was the encapsulation of the cyst within a thin layer of squamous epithelial cells. In heavily infected fish, the encapsulated cysts embedded in the epithelium of the oral cavity physically blocked the entrance of the mouth (Fig. 5) and the esophagus (Fig. 4B).

DISCUSSION

To our knowledge, this is the first report of epitheliocystis disease in sharpsnout sea bream *Diplodus pun-*

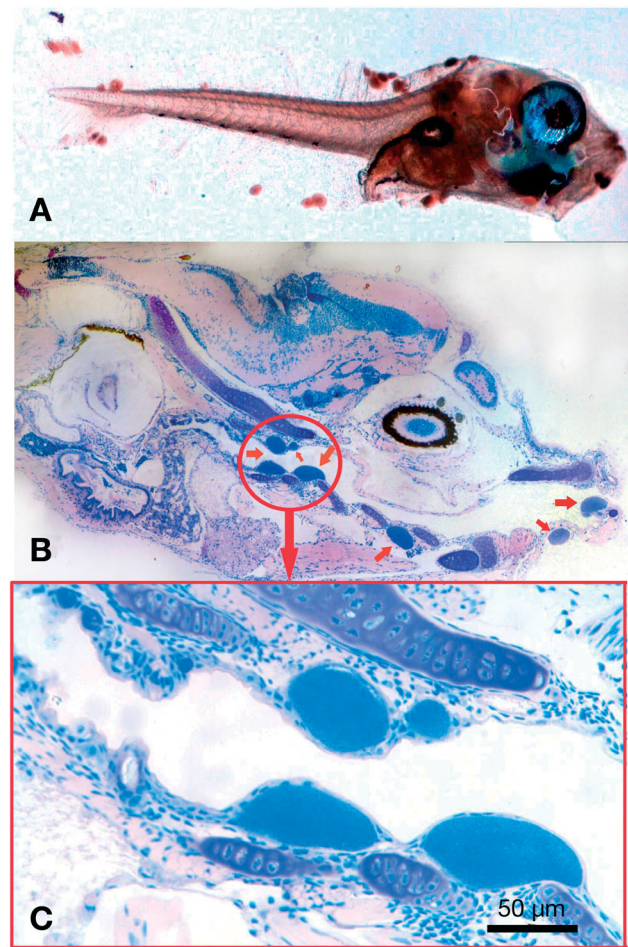


Fig. 4. *Diplodus puntazzo*. (A) Larva bearing numerous cysts in the primordial fins. (B) Cross section of an infected larva showing cysts within the oral cavity (arrows). (C) Detail of the cysts within the oral cavity rostral to the esophagus. Note that the cysts are surrounded by a limiting membrane and/or a thin layer of squamous epithelial cells without eliciting any significant host response

tazzo and the first report of the disease affecting fish at such an early developmental stage (pre-flexion/flexion). The earliest reported stage of fish affected by epitheliocystis infection was the metamorphosis stage (28 to 42 dph) of the Pacific yellowtail *Seriola mazatlanensis* (Venizelos & Benetti 1996). The prevalence of epitheliocystis has been positively correlated with age in wild winter flounder *Pleuronectes americanus* (MacLean 1993), a supposition questioned by Nowak & Clark (1999), who in contrast observed a negative correlation of epitheliocystis prevalence and age in Atlantic salmon *Salmo salar*. Instead, those authors proposed temperature as the main factor affecting the prevalence of epitheliocystis, with higher temperatures enhancing the progress and the development of the disease. On the other hand, Crespo et al. (1990)

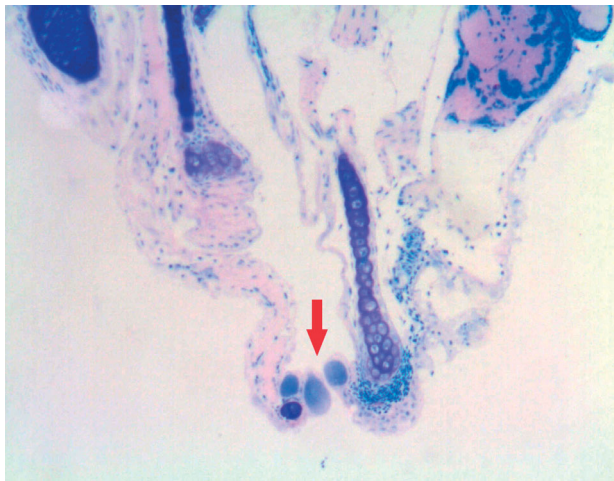


Fig. 5. *Diplodus puntazzo*. Cross section of a larval head area showing 3 inclusions (arrow) mechanically blocking the opening of the oral cavity

related the high mortalities due to epitheliocystis in greater amberjack *Seriola dumerili* to low temperatures, with the caveat that the fish were heavily parasitized by trichodinids and blood flukes, which could also have contributed to high mortalities. Our observations are in agreement with Nowak & Clark (1999), since a massive infection of epitheliocystis was observed in early life stages of sharpsnout sea bream. Furthermore, the onset of the disease was recorded in September when the water temperature in Crete is very high (21 to 23°C) and epitheliocystis was the only pathological finding.

Aquaculture conditions have been claimed to favor the prevalence of the disease. Several authors have indicated that farmed fish are much more susceptible to epitheliocystis than wild fish (Paperna 1977, Grau & Crespo 1991, Nowak & LaPatra 2006). This is probably due to increased fish densities, presence of nutrients, and stress. In our case, the fish were reared using a mesocosm protocol, which uses low fish densities, but requires increased nutrient concentrations in the water. This suggests that low fish densities alone cannot protect the fish from the disease in aquaculture, and nutrient concentration may well be a critical risk factor.

Controversial reports on the effect of epitheliocystis in the host have been published. Several authors have described significant proliferative reactions, with severe epithelial cell hyperplasia being the most common observation focused mainly in the lamellae of the gills and pseudobranch (Paperna 1977, Bradley et al. 1988, Goodwin et al. 2005). In other cases, the host response is benign, expressed as limited hyperplasia and encapsulation of the cyst with one or more concentric squamous epithelial layers (Turnbull 1993). Host

response to epitheliocystis is related to the developmental stage of the pathogen, which according to Crespo et al. (1999) can be differentiated by the texture of the cyst. Granular inclusions that are assumed to contain the immature stages of the pathogenic agent are usually found in younger fish and cause little or no host response. On the other hand, amorphous inclusions contain mature stages of the pathogen and in most cases provoke hyperplastic cellular response. These inclusions are mostly found in older fish. In our case, sharpsnout sea bream larvae exhibited only granular cysts that provoked no host response apart from the encapsulation of the cysts within a thin layer of epithelial cells. Despite the fact that no electron microscopy was performed in our samples, it is interesting that the cysts observed under high magnification ($\times 1000$) showed a particle pattern that is more similar to the elongated and small cells described by Crespo et al. (1999) in the amorphous inclusions observed with transmission electron microscopy (Fig. 2). This could be explained by the diversity of the morphology of epitheliocystis in different host species as indicated in a review by Nowak & LaPatra (2006).

Mortalities due to epitheliocystis infection have been associated mainly with the proliferative host response in older fish resulting in hyperplasia of the gill lamellar epithelium, distortion of the tissue structure, inflammation, necrosis, and therefore impairment of the gas transfer and osmoregulation processes (Crespo et al. 1990, Nowak & LaPatra 2006). In younger fish, although the predominant form of the cyst is granular, which, as mentioned earlier, provokes little or no host response, the mortalities, surprisingly, are much higher. In early developmental stages, the morphological and functional changes influence organism performance. Specifically, at the larval stage, fish undergo ontogenetic changes necessary for establishing exogenous feeding such as the opening of the mouth, the completion of the functionality of the oral cavity, the onset of digestive enzyme production, and the alimentary canal formation. Sharpsnout sea bream develop a perfectly functioning mouth by 6 dph (Faranda et al. 1985). Moreover, during the larval stage, sharpsnout sea bream are equipped with free neuromast and olfactory ciliated receptor cells, making the rostral area of the head the most sensitive region of the body (Boglione et al. 2003). In our case, the most affected areas of the examined fish were the skin, the fins, and the head area (outer and buccal and gill cavity). Many fish had numerous cysts in the mouth opening and alongside the oral cavity up to the opening of the esophagus. This could affect fish either mechanically by physically blocking the entrance of the food items leading to death due to reduced food intake at a very sensitive developmental stage or by obstructing the

development or the function of sensory cells that are implicated in larval food detection (Boglione et al. 2003).

Furthermore, in the early developmental stages of teleosts, the gills are not fully developed; instead, the skin, apart from its protective role, has vital physiological functions such as osmoregulation, respiration, and excretion and therefore is much more important (Varsamos et al. 2005). It should be noted that fully developed gills were not observed in any of the sharp-snout sea bream larvae examined by histology. However, in many cases, epitheliocystis inclusions were found near the area where gill filaments would develop (data not shown). On the other hand, skin epithelium was the tissue affected most. It can be therefore logically assumed that mortalities in epitheliocystis-infected sharp-snout sea bream could be also attributed to damage and/or coverage of the skin by the cysts. In the first report of epitheliocystis in the literature (Hoffman et al. 1969), gill lesions were mostly characterized by hyperplasia of the epithelium while skin lesions by a large number of dividing cells in the basal layer adjacent to the infected area. In our case, dividing cells could be occasionally seen near the infected area, though not to the extent described by Hoffman et al. (1969). The hypothesis of mechanical occlusion of respiration and osmoregulation in the gill interlamellar space has also been suggested in sea bass fingerlings in order to explain the mortalities experienced in an epitheliocystis-affected fish farm (Crespo et al. 2001).

The importance of epitheliocystis disease has greatly increased in the last years mainly due to the development of aquaculture and the introduction of new species. New molecular and immunological diagnostic techniques have also contributed significantly to the identification of the disease. Recently, epitheliocystis has begun to be considered a serious disease that could have a great impact on aquaculture production systems. This report enforces this argument, as it provides information about the effect of the disease in the early developmental stages for the first time. Furthermore, our observations strengthen the conclusion of Paperna (1977) that the disease is highly contagious in aquaculture systems, since all consecutively stocked mesocosm tanks of our facilities were eventually affected, with devastating results. Thus, proper hygiene and disinfection of the culture environment is highly advised.

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