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Understanding myxozoan infection dynamics in the sea: Seasonality and transmission of *Ceratomyxa puntazzi*

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ABSTRACT

Ceratomyxa puntazzi affects the sharpsnout seabream, *Diplodus puntazzo*, a recently explored aquaculture species in the Mediterranean. Little is known about the transmission and seasonality of marine myxozoans, although this knowledge is of considerable importance for the design of management strategies for aquaculture. In the present study on *C. puntazzi* we investigated the potential pathways of transmission as well as the parasite abundance in fish and its density in environmental water samples, throughout a full year. We performed monthly sentinel fish exposures in a *C. puntazzi* enzootic environment and quantified waterborne stages in seawater. Two novel *C. puntazzi*-specific PCR and quantitative PCR assays were developed to determine infection levels in fish and water samples. *Ceratomyxa puntazzi* presents marked seasonal changes in parasite density, with a double-peaked prevalence of infection in sentinel fish in spring and late summer/autumn, at 16–24 °C, and a covert infection during the winter months. Invasive blood stages were detected all year round by PCR. The combination of sentinel fish exposure with the quantification of waterborne stages allowed us to attribute this pattern in *C. puntazzi* density to higher numbers of actinospores in the water, while myxospores are predominant in summer and winter. We demonstrated that temperature increase triggered actinospore production in the invertebrate host in a benthic habitat and we suggest that the life cycle dynamics of the invertebrate host explain the double-peaked infection prevalence in fish. Experimental transmission of different *C. puntazzi* developmental stages in seawater or by oral and intracoelomic injection was unsuccessful which indicates fish-to-fish transmission is unlikely to occur in aquaculture systems. This is the first model studying seasonality and infection dynamics of a marine myxozoan.

1. Introduction

Myxozoans are a group of metazoan microparasites, mainly known for the diseases they provoke in fisheries and aquaculture (Yokoyama et al., 2012). Myxozoans alternate between a vertebrate host (predominantly fish), and an invertebrate host (annelids or bryozoans). In the vertebrate host, the parasite produces myxospores which are transmitted to the invertebrate. The latter produces actinospores which are transmitted to the fish host. More than 30 life cycles have been described from freshwater myxozoans which predominantly use oligochaete hosts (Lom and Dyková, 2006). However, information about the life cycles and the transmission of marine myxozoans is scarce as finding a specific invertebrate host in a large, extremely diverse and invertebrate-rich environment is difficult (Køie et al., 2004). To date, only five marine life cycles have been described and all were found to use polychaete hosts (Køie et al., 2004, 2007, 2008; Rangel et al., 2009; Karlsbakk and Køie, 2012). In the predominantly marine genus *Ceratomyxa* Thélohan, 1982, two life cycles have been elucidated and both of them involve a polychaete alternate host of the family Sabellidae: (i) *Ceratomyxa shasta* (Noble, 1950) occurs in salmonids in North America and infects *Manayunkia speciosa* Leidy (Bartholomew et al., 1997), and (ii) *Ceratomyxa auerbachi* Kabata, 1962, cycles between the herring *Clupea harengus* L. and *Chone infundibuliformis* Krøyer (Køie et al., 2008).

The actinospores of these *Ceratomyxa* spp. measure less than 10 μm. Due to the miniature size of these infective stages and that of most other marine actinospores (Køie et al., 2004, 2007, 2008; Rangel et al., 2009), their elimination from the incoming water source in aquaculture tank systems is difficult. Fine mesh filters quickly clog up and UV treatment may not be feasible. In sea cages, it is impossible to control waterborne myxozoan infections. Currently the most effective means to control myxozoan diseases is based on the knowledge of their seasonality and transmission

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which allows for the design of management strategies aimed at reduction of the exposure to infective stages. In freshwater habitats, exposing fish late in the season leads to the suppression of disease due to reduced water temperatures (Clifton-Hadley et al., 1986) and it avoids peaks of actinospore densities (Hallett et al., 2012).

In recent years, quantitative PCR (qPCR) has been shown to be a sensitive and specific tool which permits measurement of myxozoan microparasite densities (e.g. Cavender et al., 2004; Hallett and Bartholomew, 2006, 2009; Kelley et al., 2006; Funk et al., 2007; Griffin et al., 2008, 2009; True et al., 2009; Bjork and Bartholomew, 2010; Jørgensen et al., 2011; Hallett et al., 2012; Harada et al., 2012; Seo et al., 2012; Piazzon et al., 2012). However, only a few studies applied qPCR to determine the number of infective spore stages in environmental samples, and none of them focussed on marine habitats (Hallett and Bartholomew, 2006, 2009; Griffin et al., 2009). Only one study includes long-term monitoring of actinospore emission rates throughout the year (Hallett et al., 2012).

Transmission of myxospores to naïve receptor fish has been unsuccessful (McGeorge et al., 1996; Moran et al., 1999), however, different pre-sporogonic stages have been shown to be infective (Grossheider and Körting, 1993; Hedrick et al., 1993; Diamant, 1997; Moran et al., 1999; Redondo et al., 2002; Diamant et al., 2006). In pathogenic species of *Enteromyxum* Palenzuela, Redondo and Álvarez-Pellitero, 2002, direct fish-to-fish transmission of pre-sporogonic stages inside dislodged intestinal cells has been demonstrated to cause infection in naïve receptor fish (Diamant, 1997; Redondo et al., 2002).

Ceratomyxa puntazzi Alama-Bermejo, Raga and Holzer, 2011 inhabits the bile of the sharpsnout seabream, *Diplodus puntazzo* (Walbaum, 1792), a commercially important fish in the Mediterranean whose aquaculture has recently been initiated. This parasite is an opportunistic parasite that affects the epithelial cells in the gall bladder and provokes mild pericholangitis in the liver (Alama-Bermejo et al., 2011). *Ceratomyxa puntazzi* produces motile, proliferative as well as spore-forming stages in the bile of sharpsnout seabream (Alama-Bermejo et al., 2012). The invertebrate host involved in its life cycle is not known. In order to better understand the seasonality and dynamics of *C. puntazzi* transmission and to determine whether parasite stages can be transmitted from fish to fish, the present study focussed on the following goals: (i) to determine whether proliferative or spore-forming piscine stages of *C. puntazzi* can be transmitted to, and are capable of resuming their development in, naïve receptor fish; (ii) to investigate the seasonal infectivity of *C. puntazzi* actinospores by monthly exposures of sharpsnout seabream to a *C. puntazzi*-enzootic environment, throughout a whole year; (iii) to detect and understand the dynamics of the parasite in the environment by quantification of *C. puntazzi* transmission stages in seawater samples taken at different depths, using qPCR. We aimed to provide the first known long-term study on transmission of myxozoans in the marine environment and on the effects of dilution in a large water body. Last but not least, we hoped to suggest management strategies for *C. puntazzi* in sharpsnout seabream cultures in the Mediterranean.

2. Materials and methods

2.1. Origin of fish and infection prevalences in donor fish

Parasite stages for experimental transmission were obtained from wild specimens of sharpsnout seabream ($n = 77$; 8.15–29.2 cm total length; 8.5–448.6 g) from San Pedro del Pinatar by local shore fishing (Murcia, Spanish Mediterranean coast) in 2008. Donor fish presented a prevalence of 27.5% in the bile ($n = 67$; see Section 2.2.1). The infection in the bile included pre-sporogonic proliferative and sporogonic stages as well as mature spores. Spores from these fish were used as a control for qPCR ($n = 2$; see Section 2.5.4). Blood stages were obtained from first year sharpsnout seabream ($n = 8$; see Section 2.2.2), showing an infection prevalence of 87.5% in the bile. Blood stages were not numerous enough to be visible in Diff Quick® stained blood smears but prevalence by PCR (see Section 2.4) was 50% and four fish were used for intracoelomic blood transmission (see Section 2.2.2).

Specific pathogen free (SPF) sharpsnout seabream from a Greek hatchery ($n = 323$; 9.1–24 cm total length; 9.5–277.3 g; age 3+ years) obtained in 2006 were used for experimental transmission and as sentinel fish. Control fish ($n = 10$ for experimental transmission and $n = 24$ for sentinel fish exposure) were kept unexposed in tanks. All fish were maintained in separate tanks with UV filtered and ozone treated seawater.

All fish handling protocols in this study were approved by the ethics committee for animal welfare of University of Valencia, Valencia, Spain (license number: A1312449905843). Fish were anesthetized or euthanized using 60 ppm of clove oil for 2–10 min. In order to avoid DNA cross contamination, 10% hydrogen peroxide was used routinely to clean scissors and tweezers during sampling.

2.2. Fish-to-fish: experimental transmission of *C. puntazzi* blood and bile stages

2.2.1. *Ceratomyxa puntazzi* bile stages

The gall bladder of wild sharpsnout seabream was isolated, held with forceps directly above the opening of an autoclaved 1.5 ml eppendorf tube and ruptured. From the collected bile, 4 µl were placed on a microscopic slide, covered with a cover-slip and examined for the presence of *C. puntazzi*, using light microscopy at 400× magnification. Infected bile samples were pooled and 3 µl of the pool were used for counting the *C. puntazzi* stages in a haemocytometer. The number of stages in the total volume of bile was then calculated and initially 10 µl of bile (316 stages per µl) were used for oral transmission. High mortalities were observed in the fish, probably due to the bile composition; the protocol was then changed to include PBS. The mixture of bile and parasite stages from different fish was centrifuged for 3 min at 1064*g*, the supernatant was discarded and the pellet of *C. puntazzi* stages was resuspended in 0.1 M PBS at a concentration of 212 stages per µl. Parasite stages were viable, motile and dividing in PBS as determined by neutral red uptake. The mixture was kept cool (10–12 °C) until further used within a maximum time of 1 h.

2.2.2. *Ceratomyxa puntazzi* blood stages

Sharpsnout seabream in their first year were obtained from the wild (see Section 2.1). The fish were sedated, 50 µl of blood were taken from the caudal vein of all fish, using a heparinised syringe with a fine needle (0.3 mm diameter) and fish recovered. The blood samples were checked for the presence of *C. puntazzi* stages in blood smears and by PCR using DNA extracted from 4 µl of blood (see Section 2.4). Four fish with positive blood PCR results were sedated, the maximum volume of blood was extracted, samples were pooled (1.5 ml) and kept cold until used for infection (maximum 1 h).

2.2.3. Experimental design and fish sampling

Experimental transmission was examined using bile stages and blood stages. The transmission methods via bile stages were (i) oral (intraesophageal) transmission (OT): thirty SPF fish were intubated with 50 µl of PBS-parasite mixture (10,600 parasite stages) by using a micropipette. The opening of the tip was placed into the oesophagus past the gill region. (ii) Intracoelomic transmission (IT): a syringe with a 0.3 mm diameter needle was used for intraco-