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# Gastric cryptosporidiosis in farmed Australian Murray cod, *Maccullochella peelii peelii*

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ABSTRACT

Protozoa morphologically consistent with *Cryptosporidium* sp. were histologically-evident in the stomach of three cohorts of Murray cod (*Maccullochella peelii peelii*) from a single aquaculture facility in Australia. One cohort was asymptomatic. A second cohort had shown low-level mortalities, reduced appetite, and slowed growth, and fluid distension of the stomach and small intestine. Fish of the third cohort, sampled a year following diagnosis of the second cohort, were of stunted size and had displayed abnormal swimming behaviour. Microscopically, infection was associated with mild to moderate gastric mucosal mononuclear leukocyte infiltrate and occasional gastric mucosal epithelial cell necrosis and sloughing. *Cryptosporidium molnari* was confirmed in one cohort using SSU rDNA phylogenetic reconstruction with all available *Cryptosporidium* genotypes in fish. This study is the first to report *Cryptosporidium* sp. infection in Murray cod. Associated pathology and clinical signs highlight the possibility of production losses for the affected industry.


## 1. Introduction

Cryptosporidia are apicomplexan parasites that infect gastrointestinal epithelial cells of mammals, birds, reptiles, amphibians, and fish (Chalmers and Davies 2010; Ryan 2010; Šlapeta 2009). Cryptosporidiosis of intensively produced livestock has been extensively studied. It is considered a serious condition, as it is difficult to treat, results in important economic losses and is potentially zoonotic (De Graaf et al., 1999). In contrast, there is scant literature regarding cryptosporidiosis in fish, despite infection being increasingly reported in dozens of species of marine and freshwater fish within the last decade (Alvarez-Pellitero and Sitjà-Bobadilla 2002; Murphy et al., 2009; Gabor et al., in press). Direct transmission and environmental stability are characteristics of *Cryptosporidium* spp. that promote their establishment in cultured fish populations (Sitjà-Bobadilla and Alvarez-Pellitero 2003).

Murray cod, *Maccullochella peelii peelii*, is an iconic Australian freshwater temperate fish that is currently listed as a vulnerable species due to dramatic population declines related to ecological stress (Department of the Environment and Heritage 2003). Aquaculture of Murray cod is a rapidly growing industry that originated to produce fingerlings for restocking depleted wild populations. More recently an industry has developed growing fish for sale into the high quality seafood restaurant trade. Fish are typically grown on commercial extruded aquaculture diets over 12–24 months to 0.8–1.5 kg in a variety of indoor recirculation systems, outdoor earthen ponds, and within cages suspended in irrigation dams. Fish are sold live and chilled and are highly regarded by domestic and export markets (valued at $15–30 AUD/kg) (Department of Primary Industries 2008). The objective of this study was to describe the clinical and pathological features of gastric cryptosporidiosis in Murray cod from a single grow out facility in Australia caused by *Cryptosporidium molnari*.

## 2. Materials and methods

### 2.1. Sampling site and procedures

Diagnostic materials and medical records that pertained to three diagnoses of gastric cryptosporidiosis in Murray cod were reviewed. All cases were from a single aquaculture facility in Australia which has a hatchery, a nursery and grow-out system. Murray cod broodfish spawn annually in ponds. The eggs are collected from spawning boxes and are hatched within tanks in the hatchery building on flow through water. The larvae are stocked into fertilised plankton ponds for extensive larval rearing for around 3 months where they grow to approximately 1 g in size. Fish are then either sold, or moved into the nursery system for weaning onto extruded aquaculture diets. The nursery recirculation system comprises four small 1200 L tanks, and utilises a swirl separator and shell grit bed filter for solids filtration. Stocking densities range from 40 to 120 kg of biomass per 1000 L. A trickle filter is utilised for degassing, and biofiltration. A downflow oxygen contactor utilises a bulk liquid oxygen supply to maintain oxygen saturation, with monitoring and alarming for oxygen in place.

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The nursery system is maintained at 5 g/L of salt to help control ectoparasites that may be introduced when fingerlings are brought indoors from the earthen larval rearing ponds. The source water for the nursery is from a bore. Dissolved oxygen is maintained between 90–110% saturation. Temperatures range from 17–25 °C depending on the season. Total ammonia nitrogen is maintained under 0.5 ppm. System pH is maintained around 7.0. Fish are held in the nursery up to 6 months. The grow-out system comprises a recirculation system composed of two large 20,000 L tanks, effluent flows to a swirl separator then to a drum filter and through a shell grit bed. Depending on the sale size, fish are held in the grow-out system for 3–12 months. The system has a capacity for heating, and boosted oxygen through the use of a liquid oxygen supply within a contact vessel. A trickle filter is utilised for degassing and biofiltration. Top-up water is sourced from an onsite bore. The system operates without addition of salt. Tanks were routinely treated with formalin at 20 ppm every 2 weeks to control ectoparasitism and reduce bacterial loads in the system. Winter water temperatures are maintained above 20 °C. The fish are fed a high quality commercial extruded aquaculture diet prepared by Skretting Australia by hand and automatic feeders. Many fish are grown to 100–300 g in the system during the winter before being stocked into earthen ponds or on-sold to other aquaculture ventures for further grow-out. Murray cod are the only species grown out, however the facility produces a range of other freshwater native species fingerlings including silver perch (*Bidyanus bidyanus*) and golden perch (*Macquaria ambigua*).

Sampled fish were euthanized with an overdose of the anaesthetic benzocaine. Fish from all cohorts were fixed in 10% neutral-buffered formalin for over 24 h. Additionally, a portion of fish sampled from the cohort of Case 3 were frozen at −20 °C. Fish shorter than 10 cm were placed into formalin whole, with an incision to open the abdominal cavity, and for those longer than 10 cm, dissected viscera (including gills, heart, liver, kidney, stomach, and intestine) were individually placed into formalin. Fixed tissues were placed into histology cassettes for standard paraffin wax embedding. Fish less than 2 cm in length were longitudinally sectioned into whole body halves before being placed into cassettes. Fish between 2 and 5 cm were serially transversely sectioned into 5 mm thick fillets before being placed into cassettes. For fish longer than 5 cm, portions of sampled organs were placed into cassettes. Tissues containing bony elements, such as gill, were immersed in EDTA solution for decalcification for 24 h prior to processing. The tissues were then routinely processed for histology.

Sections 3–5 mm in thickness were prepared from formalin fixed paraffin-embedded tissue samples from each case. Sections were stained with hematoxylin and eosin (HE); periodic acid-Schiff (PAS); Geimsa; Toluidine blue; Gomori Methenamine silver; Ziehl–Neelsen acid fast, and modified Ziehl–Neelsen acid fast (heat step omitted).

### 2.2. Sequencing of SSU rDNA from Cryptosporidium and subsequent phylogeny

Gastric mucosal scrapings from frozen fish (sampled from the same batch at the same time as the fish from Case 2) were used to obtain a partial SSU rDNA (GenBank™ accession number HQ585890) according to previously published PCR protocol (Ryan et al., 2003). The obtained sequence was used to query available sequences in GenBank™. Subsequently, all fish *Cryptosporidium* sequences available in GenBank™ were retrieved and appended to the reference *Cryptosporidium* alignment (Šlapeta 2009). We appended representative sequences of Apicomplexa; haematozoan (*Theileria parva* L02366), coccidian (*Eimeria tenella* AF026388) and gregarines (*Mattesia geminata* AY334568, *Selenidium terebellae* AY196709) SSU rDNA sequences. The multiple sequence alignment was optimised and phylogenetic reconstruction conducted using Minimum Evolution (ME) in MEGA4.1 (Tamura et al., 2007). The ME tree was reconstructed using the Maximum Composite Likelihood method and a bootstrap branch support was calculated using 1000 replicates. Maximum Likelihood (ML) analysis was conducted using PhyML 2.4.4 (Guindon and Gascuel 2003). The ML tree was reconstructed using the General Time Reversible nucleotide model and a bootstrap branch support was calculated using 100 replicates.

## 3. Results

### 3.1. Cases

**Case 1.** A cohort of 9 month old fish had been moved from the nursery to the grow-out system six weeks earlier. Fish were growing strongly according to industry standards with negligible losses and were 30–50 g in bodyweight. One convenience sample from this apparently healthy cohort was collected on June 6, 2009. It was sampled for histopathological examination to assess health status of fish trialling a diet different to those fish being concurrently held in the nursery system. This same cohort had suffered an outbreak of mortality attributed to gill parasitism three months prior, while being held in the nursery system. No gross lesions were evident on the sampled fish, and no ectoparasites were observed under light microscopy. Gastric cryptosporidiosis was incidentally diagnosed with histopathological examination. Moderate chronic *Epitheliocystis*-branchitis was also present.

**Case 2.** A small proportion of fish from another cohort of ~60–110 mm fish, held in the nursery system, were showing a wasting syndrome consisting of a slowed growth rate, reduced appetite, and low level mortalities around 0.05% of population per day. Clinical examination of skin mucous scrapings and gill biopsies under 400× light microscopy revealed *Ichthyobodo* sp. infestation and indications of mild gas bubble disease. Four thin fish (tank 1) were sampled in June, 2009, for histopathological examination. Grossly, stomach and intestine were distended with fluid (Fig. 1). Histopathological exam revealed gastric cryptosporidiosis in two out of four fish. The fish also exhibited severe chronic branchitis presumptively due to ectoparasitism.

**Case 3.** Following the diagnosis of gastric cryptosporidiosis, additional fish were prospectively sampled to re-assess cryptosporidiosis status a year after Case 2, in June, 2010. Twenty-two six month old, 70–100 mm in length fish were sampled from a single tank of the nursery system. Fish were purposely selected for being smaller than usual and reportedly displaying abnormal swimming behaviour. No gross lesions were seen. Histopathological examination revealed 95% (21/22) of fish having gastric cryptosporidiosis.

**Fig. 1.** Murray cod. The size of the fish is unusually small compared to others in its year class, and the intestine is distended with fluid.