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An intestinal *Eimeria* infection in juvenile Asian seabass (*Lates calcarifer*) cultured in Vietnam - a first report

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ABSTRACT

This is the first report of an intestinal *Eimeria* infection in Asian seabass (*Lates calcarifer*) at the histopathological and ultrastructural levels. The *Eimeria* infection was often associated with severe pathology and significant mortality in the absence of other pathogens. This showed that it is an important disease of juvenile *L. calcarifer* in small scale nurseries in Vietnam. Heavy infection and high prevalence levels of the *Eimeria* infection are suspected to be linked to the low daily water exchange rates practised in these nurseries. Although systemic iridovirus infection was concurrently observed in some of the fish examined, it was not as consistently present in diseased fish as the *Eimeria* infection.

Keywords: *Lates calcarifer, Eimeria*, systemic iridovirus, pathology
1. Introduction

Asian seabass or barramundi (*Lates calcarifer*) is an aquaculture food fish of rapidly growing importance in Australia and Asia. The culture of *L. calcarifer* is typically divided into specialized operations in hatcheries, nurseries and grow-out farms. Farms are generally small to medium scale though some larger grow out farms recently reported an annual production of 300-400 tonnes (http://www.marineproduce.com/annual_reports.html; http://www.seafoodsource.com/newsarticledetail.aspx?id=4294990500). Thailand and Indonesia are currently the largest producers of cultured *L. calcarifer* at 15,700 and 4,417 tonnes, respectively (http://library.enaca.org/AquacultureAsia/Articles/april-june-2010/8-cage-culture-asia.pdf). The emerging *L. calcarifer* industry in Vietnam depends on the grow-out of juvenile fish from small scale nurseries, from which the samples in this study were taken.


Piscine apicomplexan parasites exhibiting epicytoplasmic development on host cells may belong to the genera *Cryptosporidium*, *Eimeria*, *Epieimeria* or *Goussia* (Paperna 1995). These parasitic infections have not been reported in *L. calcarifer* except for a brief mention of *Cryptosporidium* by Glazebrook and Campbell (1987). *Cryptosporidium* is typically epicytoplasmic while *Eimeria* and *Goussia* may be either epicytoplasmic or intracytoplasmic parasites (Molnar and Baska, 1986; Landsberg and Paperna, 1987; Molnar 1989; Lukes and Dykova, 1990; Szekely and Molnar, 1992; Landsberg 1993; Benajiba *et al.*, 1994; Paperna
and Vilenkin, 1996; Alvarez-Pellitero et al., 1997; Baska 1997; Alvarez-Pellitero and Sitja-Boadilla, 2002; Alvarez-Pellitero et al., 2004; Ryan et al., 2004; Murphy et al., 2009). These apicomplexan parasites are distinguished by the morphology of their oocysts. Oocysts of *Eimeria* and *Epieimeria* have four dizoic sporocysts each with a Stieda body or polar plug. In *Goussia*, oocysts are characterized by four dizoic sporocysts each with a suture line. *Cryptosporidium* oocysts have four naked sporozoites. (Davies and Ball, 1993; Molnar 2006)

Meronts, gamonts and oocysts of piscine *Cryptosporidium* with a size range of 3-5µm are much smaller than corresponding stages of *Eimeria* and *Goussia*, in the size range of 5-20 µm. The presence of invaginating feeder organelles at the attachment juncture of *Cryptosporidium* distinguishes it from the other genera (Valigurova et al., 2008). The attachment organelles of epicytoplasmic *Eimeria* and *Goussia* vary ultrastructurally from monopodial to multiple finger-like attachment organelles (Paperna 1991; Benajiba et al. 1994; Alvarez-Pellitero et al. 1997; Lukes 1992; Lukes and Stary, 1992).

This is the first report of an intestinal *Eimeria* infection in juveniles of *L. calcarifer*, at the histopathological and ultrastructural levels. This infection was often associated with severe pathology even in the absence of other significant pathogens, and is therefore a significant disease of *L. calcarifer* in small scale nurseries in Ca Mau, Vietnam.

### 2. Materials and methods

#### 2.1 Background information on samples examined

Diseased juvenile *L. calcarifer* 2.5-7cm in body length were sampled from a total of five nurseries in Ca Mau, Vietnam in Jan to Mar 2008, Mar and Dec 2009, and Nov to Dec 2010. Fixed tissue samples were sent to Murdoch University, Australia where they were processed for histopathology (181 fish) and transmission electron microscopy (10 fish).
Alcohol fixed oocysts obtained from discharged waste water from culture tanks were also examined.

2.2 Light microscopy (LM)

Tissues were fixed in 10% phosphate buffered formalin for at least 24 h, dehydrated in an ethanol-xylene series and embedded in paraffin wax. Formalin fixed bony tissues were decalcified in 5% nitric acid overnight prior to dehydration and embedded in paraffin wax. 5µm tissue sections were dewaxed in xylene, rehydrated in an ethanol series and stained by haematoxylin & eosin (H&E) or Giemsa.

2.3 Transmission electron microscopy (TEM)

Tissues were fixed in 5% glutaraldehyde in phosphate-buffered saline (PBS) at 4°C overnight, washed in several changes of PBS and post-fixed in Dalton’s chrome osmic acid for 1 h at 4°C. Fixed tissues were dehydrated through a graded ethanol series to propylene oxide followed by 1 h in 60:40 solution of propylene oxide/epoxy resin, overnight in pure epoxy resin on a rotator and baked in an oven at 60°C for 24 h. Ultra-thin sections were stained with uranyl acetate and lead citrate for viewing on a Philips CM100 Bio TEM.

3. Results

3.1 Field observations made on L. calcarifer nurseries sampled in this study

The L. calcarifer nurseries in Ca Mau, Vietnam were mainly small scale with less than five ½- to 1-tonne tanks. These nurseries obtained their fry from hatcheries in Vung Tau or Khanh Hoa Province in Vietnam, or as imported fry from Thailand. Fiber glass or cement tanks were mainly used as holding facilities with static or closed recirculation systems. Less
than 20-30% partial daily water exchange rates were practised. In earthen ponds which were less commonly used, the fry were kept in nets suspended in the water column. Salinity of rearing water ranged from 15 to 25 parts per thousand (ppt). Stocking density varied from 280 to 350 fish/m³ water. Fish were fed commercial feed pellets supplemented with coarsely chopped trash fish. The trash fish fed consisted of wild caught fish from the sea.

Nurseries stocked 1-3 cm *L. calcarifer* fry obtained from hatcheries, and grow them on to 7-9 cm body length fish to sell to grow-out farmers. Nursery reared 2.5 to 7.0 cm body length *L. calcarifer* juveniles were reported to suffer low grade clinical disease soon after stocking, with a cumulative mortality of up to 30% of stocked fish. Clinical signs included fish preferentially hanging at water surface, inappetance, lethargy, darkened bodies, tail rot and scales loss.

3.2 Histopathology

An *Eimeria* infection was observed in greater than 60% of diseased *L. calcarifer* sampled from the five nurseries, often as early as the first week post stocking. Fish kept in cement or fiber glass tanks or ponds in salinities of both 15 and 25 ppt were found to be infected with this parasite. Concurrent systemic iridoviral disease was observed in approximately 20% of diseased *L. calcarifer* examined. Low grade to heavy gill trichodinid infection was sometimes observed but not associated with any significant pathology.

The primary infection site of the *L. calcarifer Eimeria* was the small intestine. Both merogony and gamogony were mainly epicytoplasmic and occurred simultaneously (Figure 1). Infection levels varied from light to heavy, often with obliteration of the microvillus brush border. Meronts were much smaller in size than gamonts, and had merozoites arranged in rosettes or in parallel (Figures 1 and 2a). Intracytoplasmic meronts or unusually large meronts with at least 18 merozoites were occasionally observed (Figure 2b). Macrogamonts
with foamy cytoplasm due to the presence of amylopectin granules often outnumbered microgamonts. Microgamonts were smaller than macrogamonts and had peripherally arranged nuclei (Figures 1 and 2c). Mature microgamonts had numerous flagellated microgametes (Figure 2c). Meronts measured 4.8 x 3.5 µm (n=5), macrogamonts 13.1 x 7.6 µm (n=10) and microgamonts 8.1 x 6.0 µm (n=5).

Sporulated oocysts were very rarely observed in histological tissue sections, in fact in only 1 out of 181 fish examined, and measured 18.5 x 12.3 µm (n = 5). These oocysts in faecal materials within the intestinal lumen had four pairs of sporozoites held loosely within a thin membranous oocyst wall (Figure 3a). Both unsporulated and sporulated oocysts were readily observed in faeces collected from tank bottom and in waste water from rearing tanks by wet mount microscopic examinations (Figures 3b and 3c). Nomarski interference microscopy on alcohol fixed sporulated oocysts showed the absence of Stieda bodies and suture lines. Alcohol fixed sporulated oocysts measured 36.6 x 22.8 µm (n=5). A residual body was present in oocysts, and each pair of sporozoites was held together by a thin sporocyst membrane (Figure 3c).

Squamous to cuboidal intestinal epithelium and low grade to severe mononuclear inflammatory infiltrates in the lamina propria were frequently observed in association with the *Eimeria* infection. The inflammation was sometimes extended into the mucosal epithelium. There were often focal to extensive areas of intestinal mucosal degeneration, necrosis and sloughed necrotic cells in intestinal lumen (Figure 4). Extra-intestinal parasite stages were not commonly observed, except for two macrogamonts in renal tubules from 1 fish. Other abnormalities observed included dermatitis in caudal peduncle, renal glomerular degeneration, moderately reactive spleens with white pulps depleted of leucocytes, and reduced levels of hepatic glycogen stores.
3.3 Ultrastructural observations by TEM

Parasitic stages were observed within complete parasitophorous envelopes at extracytoplasmic positions on the microvillous brush border of intestinal epithelium. Shortening to loss of microvilli and necrosis of affected intestinal epithelium were often observed. Both meronts and gamonts had finger-like attachment organelles that extended into host cells but were limited to the extracytoplasmic cellular boundaries (Figures 5, 7a, 7b and 8). A large number of rodlet cells were often seen in association with these parasitic infections, and sometimes within blood vessels in the intestine (Figure 6).

Meronts had merozoites with apical complexes in various stages of formation and up to eight merozoites (Figures 7a and 7b). Macrogamonts had abundant amylopectin granules (Figure 8) while microgamonts had flagellated microgametes with a large residual body (Figure 5).

4. Discussions

This is the first report of a natural *Eimeria* infection in *L. calcarifer*. While it does not supply all the answers, the high prevalence of the *Eimeria* infection in association with severe pathology showed that it is a significant disease under nursery culture conditions in Ca Mau, one which will need to be managed. Significant pathology was frequently reported in fish with apicomplexan infections (Benajiba et al. 1994; Molnar 2006; Gjurcevic et al., 2008; Morrison et al., 1993; Jendrysek et al., 1994; Hemmer et al., 1998). Although systemic iridoviral disease was also observed, it was not as consistently observed as the *Eimeria* infection in diseased fish. Nonetheless, iridovirus is a serious pathogen which can co-contribute to losses during the culture cycle (Gibson-Kueh et al. 2003).

The *L. calcarifer* *Eimeria* did not possess the feeder organelles typical of *Cryptosporidium* but had finger-like attachment organelles very similar to epicytoplasmic
species of Eimeria and Goussia. Although the sporocysts in oocysts examined in this study lack Steida bodies, this was also the case in some previously described piscine Eimeria (Upton et al., 1984; Landsberg and Paperna 1987; Paperna 1995; Molnar 2006). Therefore, we will refer to this parasite as Eimeria until molecular analysis can be conducted. Since sporulated oocysts were rarely observed in tissue sections of Eimeria infected L. calcarifer, it is presumed that sporulation was mainly exogenous. Both unsporulated and sporulated oocysts were readily observed in faecal materials collected from tank bottoms. The L. calcarifer Eimeria oocysts in histological tissue sections were almost half the size of alcohol fixed oocysts obtained from waste water, likely due to the dehydration process used in histology. There is also the possibility that more than one species of Eimeria were involved.

A study on Goussia carpelli in common carp suggested the correlation of infection rates to stress and immunosupression (Steinhagen et al., 1998). Depletion of splenic white pulp of leucocytes in diseased L. calcarifer examined in this study is expected to have an impact on their immunity, and may explain the heavy Eimeria infection often observed. The diseased fish examined in this study were sampled during the initial post-stocking period when the fish would be recovering from transport and acclimatization stress.

The origin of the Eimeria infection in L. calcarifer from nurseries in Ca Mau is unknown, and warrants further study. The feeding of trash fish is a possible source of infection. The stocking of fish in static or closed recirculation aquaculture systems with relatively low daily water exchange rates (20-30%) would encourage the level of Eimeria infection to build up to the high prevalence observed. Large scale L. calcarifer hatcheries and nurseries in Indonesia, Singapore and Australia practised much higher water exchange rates of 100 to 300% an hour (Schipp et al., 2007; personal observations). Whether the Eimeria infection will persist in older fish as a chronic infection or were present in fish before being stocked in nurseries in Vietnam remains to be elucidated and is vital information for its
effective management. Experimental trials will complement what has been learnt from examination of the naturally infected fish in this study.

There are currently no treatment options. The sequestering of these ecytoplasmic parasites in parasitophorus envelopes away from the intestinal lumen and host cell cytoplasm makes it resistant to currently available therapeutic drugs (Sterling 2000). Recent research revealed that addition of proteins produced by Cryptosporidium competitively inhibited their attachment to intestinal epithelium (Tzipori and Ward 2002). A similar approach could be applied for this ecytoplasmic *Eimeria*.

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References


Legends for Figures

Figure 1. *Eimeria* infection on the brush border of intestinal mucosa in *L. calcarifer* from Vietnam showed increased mononuclear infiltrate in lamina propria (Inf). Meronts (Me) were smaller than gamonts (Ma, Mi), and commonly occurred simultaneously. Meronts often had merozoites arranged in rosettes (Me). Macrogamonts (Ma) with foamy cytoplasm, microgamonts (Mi) with peripherally arranged nuclei and darker basophilic stained trophozoites (T). (H&E)

Figure 2a-c. Different developmental forms of meronts and microgamonts. (2a) Meronts with merozoites arranged in parallel (Me) and merozoites apparently still within parasitophorus envelopes (z). (Giemsa). (2b) An unusually large meront with at least 18 merozoites (arrows). (H&E) (2c) Microgamonts with peripherally arranged nuclei (*) and microgametes (arrows). (Giemsa)

Figures 3a-c. (3a) Sporulated oocysts were very rarely seen in histological tissue sections. Sporulating oocysts (*) had four sporocysts. Sporulated oocysts (arrows) in faecal materials within intestinal lumen had four pairs of sporozoites and a thin membranous wall. (H&E) (3b) Unsporulated oocyst observed in discharged tank waste water. (3c) Sporulated oocyst from waste water with oocyst residual body (R) and sporozoites in pairs, bounded by thin sporocyst membrane. Sporocysts (S) had no stieda bodies or suture lines. *(Nomarski interference microscopy)*

Figure 4. The intestinal epithelium was denuded in some areas (arrows) with corresponding intense inflammatory response (Inf) in the lamina propria, and a significant amount of sloughed cellular debri (D) in intestinal lumen. (Giemsa)
Figure 5. Macrogamonts (Ma) with amylopectin granules and microgamonts with microgametes (MiG) at the microvillous brush border of intestines. ‘X’ was presumably a microgamont from which microgametes had been released, thus giving a crenate appearance. Parasitophorous envelopes (PE), residual body (Re) in microgamont. Figure 6. Rodlet cells (Ro) often associated with response to parasitism in fish were observed within blood vessels (bv) in intestines. One of the rodlet cells appeared to be in the process of exiting the blood vessel (*). Vascular endothelial cells (E), fibroblast cells (F) that produced the collagen (C) of blood vessel wall.

Figure 7a. Meront (Me) with finger-like attachment organelles (fao) and residual body (Re). Merozoites (z) had apical complexes (A) at various stages of formation. Trophozoites (T) and developing meront (dMe) in epicytoplasmic position. Figure 7b. Meront with at least 8 merozoites (z) and finger-like attachment organelles (fao).

Figure 8. Macrogamont with abundant amylopectin (A) granules and finger-like attachment organelles (fao) extended into host cell but limited to the epicytoplasmic boundary. Parasitophorus envelope (PE).
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