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Experimental transmission of *Enteromyxum leei* to freshwater fish

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ABSTRACT: The myxosporean *Enteromyxum leei* is known to infect a wide range of marine fish hosts. The objective of the present study was to determine whether freshwater fish species are also receptive hosts to this parasite. Seventeen species of freshwater fish were experimentally fed *E. leei*-infected gut tissue from donor gilthead sea bream *Sparus aurata* obtained from a commercial sea bream cage farm. Four of the tested species, tiger barb *Puntius tetrazona,* zebra danio *Danio rerio,* oscar *Astronotus ocellatus* and Mozambique tilapia *Oreochromis mossambicus*, were found to be susceptible with prevalences ranging from 53 to 90%. The course of infection and pathology was limited to the gut mucosa epithelium and was similar to that observed in marine hosts. Little is known of the differences in physiological conditions encountered by a parasite in the alimentary tract of freshwater vs. marine teleost hosts, but we assume that a similar osmotic environment is maintained in both. Parasite infectivity may be influenced by differences in the presence or absence of a true stomach, acidic gastric pH and digestive enzyme activity both in the stomach and intestine. Variability in susceptibility among species may also stem from differences in innate immunity. Dimensions of spores produced in the donor sea bream and recipient freshwater species are variable in size, as previously observed in other captive marine host species.

KEY WORDS: Direct transmission · Myxosporea · *Sparus aurata* · *Danio* · *Oreochromis* · *Puntius* · *Astronotus*

INTRODUCTION

Since the emergence of *Enteromyxum leei* in sea bream *Sparus aurata* in the early 1990s, this parasite has gradually spread to culture systems all over the Mediterranean. Its original host(s) is still unknown, but the list of fish species susceptible to this myxosporean is constantly growing and at present exceeds 40 (Diamant et al. 1994, Le Breton & Marques 1995, Diamant 1998, Paperna 1998, Kent 1999, Padrós et al. 2001, Marino et al. 2004, Yasuda et al. 2005). We recently identified *E. leei* infections in *Oreochromis mossambicus* specimens caught in the Red Sea off the coast of Eilat, Red Sea. This cichlid is a freshwater fish, but as a highly euryhaline species is known to inhabit estuarine areas and drainage canals, and individuals swept into the sea may be observed in the shallow coastal waters of Eilat (Golani & Lerner 2006). Although there are a few existing records of *E. leei* infections in wild fish living around enzootic mariculture farms (Paperna 1998, authors' unpubl. data), *E. leei* has not been observed to date in any feral population that was not associated with an adjoining mariculture activity.

Since the natural host(s) of *Enteromyxum leei* is unknown, we cannot rule out the possibility that *E. leei* may have a non-marine (brackish or freshwater) origin. In light of the infection identified in the freshwater *Oreochromis mossambicus*, we experimentally challenged a series of freshwater fish species by feeding them *E. leei*-infected sea bream gut tissue. This was conducted using a similar experimental procedure as previously described for marine species (Diamant 1997, 1998). Our objective was to determine whether freshwater fish species are receptive hosts to this parasite, and to explore their potential as laboratory animals for maintaining *in vivo* infections of this parasite.

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MATERIALS AND METHODS

Seventeen different freshwater species were tested for their susceptibility to *Enteromyxum leei*. These belonged to 6 different families: Callichthyidae, Characidae, Cichlidae, Cyprinidae, Osphronemidae and Poecilidae (see Table 1). All ornamental species were purchased from retail pet shops in Israel. Stocks of *Oreochromis mossambicus* were locally cultured fish at the National Center for Mariculture (NCM), Eilat. All fish were held in 20 l freshwater aquaria with aeration and box filters, at a controlled temperature of 24°C. Infected donor sea bream *Sparus aurata* were obtained from an enzootic sea-cage farm operating off the coast of Eilat, Red Sea. Lethargic, listless individuals found lingering at the water surface were collected with a dip net, placed in a plastic bag in an ice cooler and brought to the lab, where they were dissected within 2 h. The alimentary tract was removed, and specimens found to host stages of *E. leei* in the gut mucosa upon microscopical examination were finely minced.

Receptor fish were maintained with dry food for several days before the experiments were initiated, and feeding was ceased 24 h prior to initiation. The parasitic status of the fish prior to treatment (t_0) was evaluated by microscopic examination of fresh intestinal smears from 2 to 3 individuals per group. Fish were then fed a single meal of freshly prepared, finely-minced infected gut tissue. Although some of the tested species are known to be herbivorous, all were observed to pick at the tissue bits and appeared to ingest the particles.

The sampling schedule was based on preliminary observations indicating that initial infections at 24°C appeared no earlier than Day 12 post-feeding. Thus, fish were examined on the following days post-infection (p.i.): 12, 15, 20, 25, 30, 35, 40, 43. In all cases, at least 2 individual fish were taken at random with a dip net, killed with an overdose of clove oil and immediately dissected. In some species, 3 to 10 individuals per sampling event were sacrificed and examined.

Samples of visceral squashes and wet smears of gut mucosa and gall bladder were examined under a light microscope. The viscera, including most parts of the alimentary tract, were fixed in Buffferred Neutral Formalin (BNF) and processed for paraffin histology to further determine the presence of *Enteromyxum leei* stages.

RESULTS

None of the fish examined prior to the initiation of feeding experiments (t_0) displayed presence of any gut myxosporean infection. The results of experimental feeding trials with contaminated guts are given in Table 1. Four species were clearly susceptible to the parasite. The earliest sign of infection was observed on Day 13 p.i.: a tiger barb *Puntius tetrazona* was found dead in the tank, and sporoblasts and spores were clearly identifiable in a wet smear of the gut mucosa (Fig. 1). Spores were first detected in the oscar *Astronotus ocellatus* and Mozambique tilapia *Oreochromis mossambicus* on Day 15 p.i.. Fresh spores from *O. mossambicus* are shown in Fig. 2. In all 3 species, spores continued to be observed in wet smears of fish gut sampled on subsequent days. In zebra danio *Danio rerio*, suspected *Enteromyxum leei* vegetative stages

Table 1. Freshwater fish species fed with infected *Enteromyxum leei* sea bream gut tissue. Prevalence of *E. leei* was based on detection of parasitic vegetative stages and spores in wet smears and/or histological sections

Family	Species	Common name	N	No. infected	Prevalence (%)
Callichthyidae	*Corydoras aeneus*	Bronze corydoras	5	0	0
Characidae	*Hyphessobrycon megalopterus*	Black phantom tetra	15	0	0
	Hyphessobrycon anisitsi	Buenos Aires tetra	15	0	0
Cichlidae	*Astronotus ocellatus*	Oscar	13	11	85
	Mikrogeophagus ramirezi	Ram cichlid	15	0	0
	Oreochromis mossambicus	Mozambique tilapia	30	27	90
	Pterophyllum scalare	Freshwater angelfish	50	0	0
Cyprinidae	*Carassius auratus*	Goldfish	25	0	0
	Cyprinus carpio	Koi	25	0	0
	Danio rerio	Zebra danio	15	8	53
	Puntius conchonius	Rosy barb	15	0	0
	Puntius tetrazona	Tiger barb	15	12	80
Osphronemidae	*Trichogaster trichopterus*	Threespot gourami	5	0	0
Poeciliidae	*Poecilia latipinnia*	Sailfin molly	30	0	0
	Poecilia reticulata	Guppy	30	0	0
	Xiphophorus hellerii	Swordtail	30	0	0
	Xiphophorus maculatus	Platyfish	30	0	0

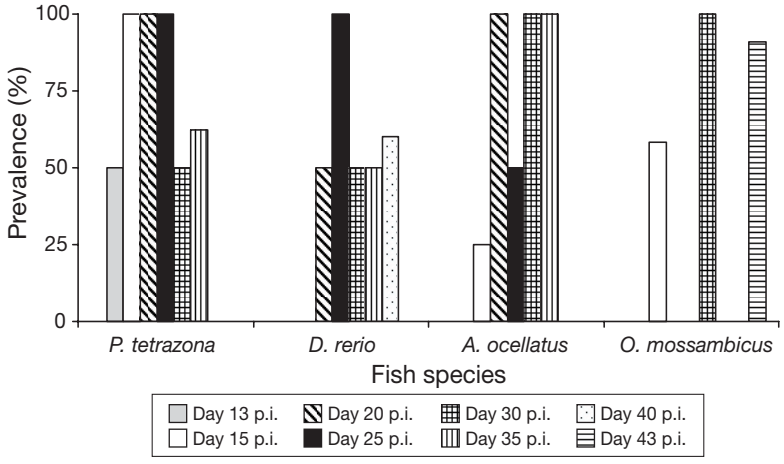

Fig. 1. Prevalence of *Enteromyxum leei* in 4 susceptible freshwater fish species (*Astronotus ocellatus*, *Oreochromis mossambicus*, *Danio rerio* and *Puntius tetrazona*) experimentally fed donor sea bream gut. p.i.: post-infection

were first observed in gut smears on Day 20 p.i. Confirmation of infection was obtained by histological section of the same fish, after detection of the characteristically shaped spores and elongated polar capsules. Measurements of spores as well as comparisons with those from other host species are given in Tables 2 & 3.

Histologically, the earliest manifestation of parasite presence observed in all 4 susceptible species were discrete patches of infection with trophozoites, dispersed in the gut mucosa epithelium. In the infection foci, proliferative stages were mostly located in the deep layers of the mucosa epithelium, near the basal membrane adjoining the lamina propria. In the advanced phase of infection, sporogenic stages were observed near the apical region of the columnar mucosa epithelium cells, often just below the brush border (Figs. 2, 3 & 4).

Damage to the host tissue included disruption of the mucosa structure, degradation, desquamation, and separation from the underlying connective tissue. The lumen typically contained cellular debris and mucous casts in which sporoblasts and spores could be identified (Figs. 2d & 4b).

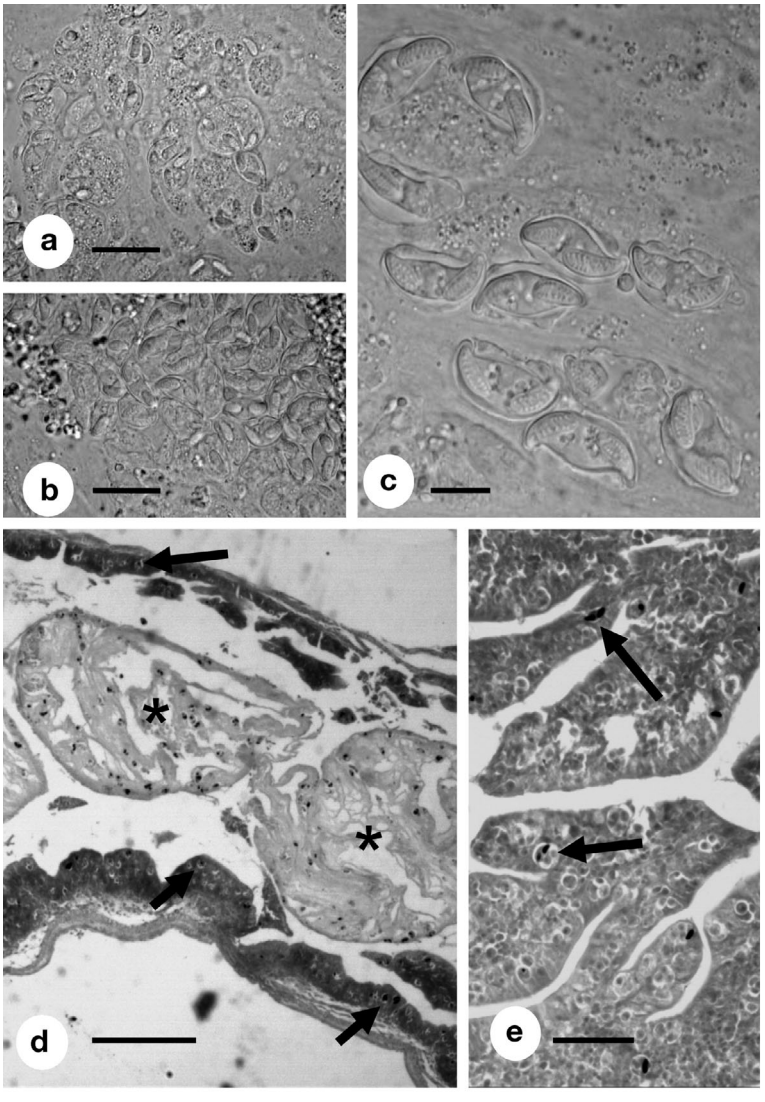

Fig. 2. *Oreochromis mossambicus* (Mozambique tilapia). Gut mucosa infected with spores and sporoblasts of *Enteromyxum leei*. (a) Wet mount showing spores and sporoblasts (scale bar = 20 µm); (b) wet mount showing spores (scale bar = 20 µm); (c) high-powered magnification of spores (wet mount; scale bar = 10 µm); (d) gut section showing spores in mucosa epithelium (arrows) and mucus casts in lumen (*) (Gram stain; scale bar = 100 µm); (e) high-powered magnification of spores in mucosa epithelium (Gram stain; scale bars = 50 µm)

DISCUSSION

In the present study, we show for the first time that *Enteromyxum leei* — hitherto regarded a parasite of marine fish — is capable of infecting, developing and producing spores in freshwater fish. Four out of 17 species experimentally fed gut tissue from donor sea bream *Sparus aurata* were infected. In all 4 species, spore formation indicated that the parasite developed to

Table 2. Fresh spore measurements (N = 25 per host species) in fresh feces from experimentally infected *Oreochromis mossambicus* and fresh bile from sea-cage cultured sea bream *Sparus aurata* from Eilat, Israel

Species	Spore measurement Mean ± SD	Range
Oreochromis mossambicus		
Spore length	17.8 ± 0.7	16.5–18.9
Spore width	9.0 ± 0.7	8.0–10.6
Polar capsule length	8.9 ± 0.5	8.3–10.0
Polar capsule width	3.3 ± 0.3	3.0–3.5
Sparus aurata		
Spore length	15.7 ± 0.6	14.75–17.1
Spore width	9.0 ± 0.6	8.26–10.26
Polar capsule length	8.65 ± 0.3	7.67–2.95
Polar capsule	3.22 ± 0.2	3.0–3.54

maturity. Moreover, in 3 species, *Oreochromis mossambicus*, *Astronotus ocellatus* (Cichlidae) and *Puntius tetrazona* (Cyprinidae), the infection prevalence exceeded 80%. These values are comparable to those observed in infections of highly susceptible species such as *Diplodus puntazzo* (Athanassopolou et al. 1999), *Takifugu rubripes* (Yasuda et al. 2002), *Paralichthys olivaceus* (Yasuda et al. 2005) and *Istiblennius edentulus* (authors' unpubl. data). In sea bream, cases of such high prevalence have been only exceptionally observed in experimentally infected fish (e.g. Sitjà-Bobadilla et al. unpubl.).

In our experiments, which were carried out at 24°C, the first signs of *Enteromyxum leei* infection were discernible about 2 wk after ingestion of contaminated infected tissue, which is the fastest reported to date (see Sitjà-Bobadilla et al. unpubl.). The pathogenesis of *E. leei* in marine hosts is characterized by invasion of the epithelial mucosa of the gut, initially with little or no inflammatory response. In advanced, chronic infections, disruption of the integrity of the mucosa, desquamation and detachment of the epithelium occurs, and host tissue fragments, mucus and parasite stages may then be seen to accumulate in the gut lumen. The same pathological features were observed in the hind-gut of the infected freshwater fish, and the course of infection in all 4 species was indistinguishable from that observed in previously studied marine fish (Diamant et al. 1994, Padrós et al. 2001, Sitjà-Bobadilla et al. unpubl.). However, it should be noted that in contrast to some marine hosts, e.g. *Diplodus puntazzo* (Le Breton & Marques 1995) and *Sparus aurata* (authors' unpubl. data), no involvement of the gall bladder was seen in any of the studied freshwater species.

Table 3. *Enteromyxum leei* spore measurements from freshwater fish species, compared with data from previous studies from the marine environment. F: formalin fixed; EH: ethanol fixed and rehydrated in Hanks' balanced salt solution (HBSS); G: ethanol-fixed, Giemsa-stained air-dried smear; L: live; P: paraffin embedded

Myxosporean host	Polar capsule Width	Length	Spore Width	Length	Remarks	Source
Sparus aurata	3	7	7–8	15–18	F	Diamant (1992)
Sparus aurata	3.2 (2.8–3.8)	7.4 (6.2–8.8)	6.9 (5.6–7.8)	14.7 (13.2–15.2)	F	Diamant et al. (1994)
Sparus aurata	3.6 (3.1–4.3)	8.2 (6.9–9.8)	7.8 (7.5–8.6)	16.2 (15.2–17.7)	L	Diamant (1998)
Sparus aurata	2.8 (2.5–4)	8.6 (8–9)	9.7 (8–11)	16.7 (15–18)	L	Sakiti et al. (1996)
Sparus aurata	2.9 (2.3–3.5)	7.7 (7.5–8.1)	8.2 (7.5–8.1)	17.7 (16.7–18.4)	EH	Padrós et al. (2001)
Sciaenops ocellatus	3.8 (3.3–4.5)	8.4 (7.0–9.8)	7.4 (7.0–8.7)	17.5 (15–5–19.5)	L	Diamant (1998)
Sparus aurata	2.8 (2.5–3.8)	7 (5.5–8.1)	6.5 (5.4–7.2)	14.9 (13.5–14.9)	G	Present study
Astronotus ocellatus	2.8 (2.5–3.7)	6.9 (5.3–7.9)	6.7 (5–6.8)	14. 0 (12.7–15)	G	Present study
Astronotus ocellatus	2.8 (2.6–3.7)	6.5 (5–7.5)	5.4 (5–6.8)	13.8 (12.5–15)	P	Present study
Danio rerio	3.2 (2.5–3.7)	6.7 (6.2–7.5)	5.5 (5–6.2)	14.5 (13.7–15)	P	Present study
Oreochromis mossambicus	2.7 (2.5–3.7)	6.6 (5–7.5)	5.1 (5–6.2)	14.1 (13.7–15)	P	Present study
Puntius tetrazona	3.4 (2.5–5)	6.7 (6–7.5)	5.4 (5–6.2)	14.3 (13.7–15)	P	Present study

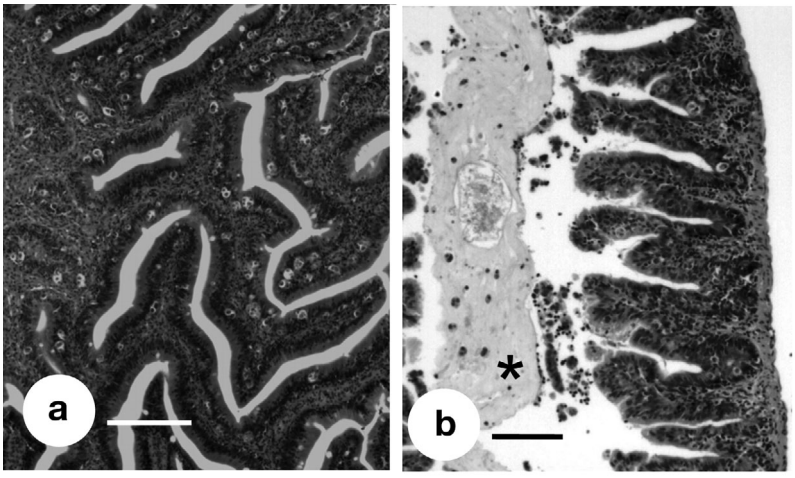

Fig. 3. Sections of gut infected with *Enteromyxum leei*. (a) *Barbus tetrazona* (tiger barb); (b) *Danio rerio* (zebra danio); *: mucus cast. Both sections stained with hematoxylin and eosin (H&E) (scale bars = 100 µm)

Although *Enteromyxum leei* is considered to be a marine parasite, euryhaline host species such as grey mullet have been reported to be susceptible (Paperna 1998). Development in a truly freshwater fish host had not been documented until now. Fish-to-fish transmission through predation or necrophagy is thought to occur by means of extrasporogenic, proliferative stages (Diamant 1997, Redondo et al. 2002). Such transmission probably causes minor exposure of the parasite to the external environment, enabling this myxopsorean to persist, protected in host tissue. Once ingested, the parasite encounters its host's alimentary system, which in teleosts varies considerably depending on feeding habit. Inside, the parasite is confronted with a coordinated sequence of physical, chemical and enzymatic activities that include crushing, mixing, gastric hydrochloric acid secretion and hydrolytic compound breakdown. Then, secretion and activation of intestinal enzymes and peristalsis further act to mix, process and transport digesta towards the anus (Rust 2002). The capacity of the parasite to survive these conditions is of fundamental importance. To maintain their osmotic balance, marine fish are known to actively ingest large amounts of water, unlike freshwater fish. Drinking seawater is very likely to influence salinity in the gut, and freshwater fish that move into seawater are known to increase their water intake through the mouth by 10- to 50-fold (Marshall & Grosell 2005). Nevertheless, a relatively steady osmotic pressure is maintained in the telost intestine, controlled by both neural and hormonal regulatory mechanisms (Buddington & Krogdahl 2004).

When inside their host, some Myxosporea are impervious to water salinity, whereas others are not. *Myxobolus neurobius* and *Myxobolus arcticus*, both freshwater-transmitted histozoic myxosporeans infecting the brain of anadromous salmonids, are capable of survival in both freshwater and marine phases of the fish's life (Margolis 1982). The parasites persist as fully developed spores in brain tissue, which most likely is not greatly affected physiologically by salinity changes in the external environment. On the other hand, *Myxidium salvelini* inhabits the kidney of its host, sockeye salmon *Onchorhynchus nerka*, where it can sporulate in freshwater year-round. Spore development is arrested when *O. nerka* is transferred to seawater, apparently as a result of physiological changes occurring in the kidney, which actively participates in osmoregulation (Higgins et al. 1993). Our observations of *Enteromyxum leei* indicate that transmission from a marine host to freshwater fish hosts is possible, and that this parasite is capable of successfully invading the gut mucosa of the ingesting host despite variable ambient salinity.

A parasite entering a host is met not only by physiological barriers, but also by macrophages and lymphocytes, complement activity and various humoral immune factors (Chevassus & Dorson 1990). If *Enteromyxum leei* has evolved a wide histocompatibility (or antigenic tolerance), then we could expect its low host specificity would apply to any host. Still, many of the freshwater fish tested in our study were not receptive. Indeed, species differences in susceptibility to *E. leei* have also been observed among marine fish species (Sitjà-Bobadilla et al. unpubl., author's unpubl. data). Variation in susceptibility to myxosporean infection owing to genetic factors may be significant even at the strain level, as has been observed for the receptiveness of rainbow trout to the myxosporeans *Ceratomyxa shasta* (Ibarra et al. 1991, 1994) and *Myxobolus cerebralis* (Densmore et al. 2001). Why freshwater species belonging to particular families (Cyprinidae and Cichlidae) become infected with *E. leei*, whereas others (Poecilidae and Characidae) do not, has yet to be determined. We suggest it may be related to differing anatomical and physiological gastric conditions. The intake of parasite spores and cysts via the host stomach represents a universal mechanism of infection. Myxosporean spore valves contain chitin and are highly resistant (Lukes et al. 2001). Nevertheless, *M. cerebralis* spores passing through mouse gastrointestinal tract have been shown to lose their infectivity or to be digested (El Matbouli et al. 2005). In mammals, the presence of gastric acidic chitinase may be responsible for such spore deactivation and destruction (El Mat-

bouli et al. 2005). Interestingly, the occurrence of myxosporean infections in the biliary system of water fowl (Anseriformes) suggest that certain life stages of these parasites are capable of surviving the gastric enzymes of avian hosts (Lowenstine et al. 2002, 2006).

The significance of *Enteromyxum* spp. spores in the life cycle of the parasite is still uncertain, and at least in *E. leei* infection has been shown to depend on vegetative stages (authors' unpubl. data). Indeed, in all 3 known *Enteromyxum* species, transmission between fish effectively occurs through waterborne contamination (Diamant 1997, Redondo et al. 2002, Yasuda et al. 2002). Oligochaetes are the chief intermediate hosts that spread infections of freshwater myxosporeans, although a few are transmitted via polychaetes (Bartholomew et al. 1997, in press). In the marine environment, oligochaete diversity is far lower than in freshwater habitats, and marine myxosporea may be more likely to develop a 'short-cut' horizontal transmission route through ingestion of vegetative stages (predation or necrophagy) (Diamant 1997). Indeed, additional examples of horizontal transmission via ingestion of vegetative stages among myxosporeans apparently exist, e.g. transmission of *Kudoa ovivora*, an ovarian parasite of Caribbean labroid fishes (Swearer & Robertson 1999), and more are likely to be discovered. Horizontal parasite transmission via vegetative (e.g. merogonic) stages through predation occur among the Apicomplexa of terrestrial hosts, such as *Toxoplasma gondii* (see Dubey & Beattie 1988), *Neurospora* spp. (see Dubey 1999) and *Hepatozoon domerguei* (see Landau et al. 1972). In marine fish, coccidian merozoites have been found in both muscles and viscera (Paperna 1979, Paperna & Sabnai 1982), and merogonic stages (presumably of haemogregarinids [*Hepatozoon* spp.]) can be horizontally transmitted. A noteable example of parasite transmission via predation between marine and freshwater hosts occured in rainbow trout in freshwater ponds, which contracted *Ichthyophonus hoferi* when fed contaminated marine fish (Rucker & Gustafson 1953, Alderman 1982). Here too, variable susceptibility to the marine fungus was displayed by different freshwater trout populations, and was attributed to differences in efficacy of host immune cellular response (McVicar 1982).

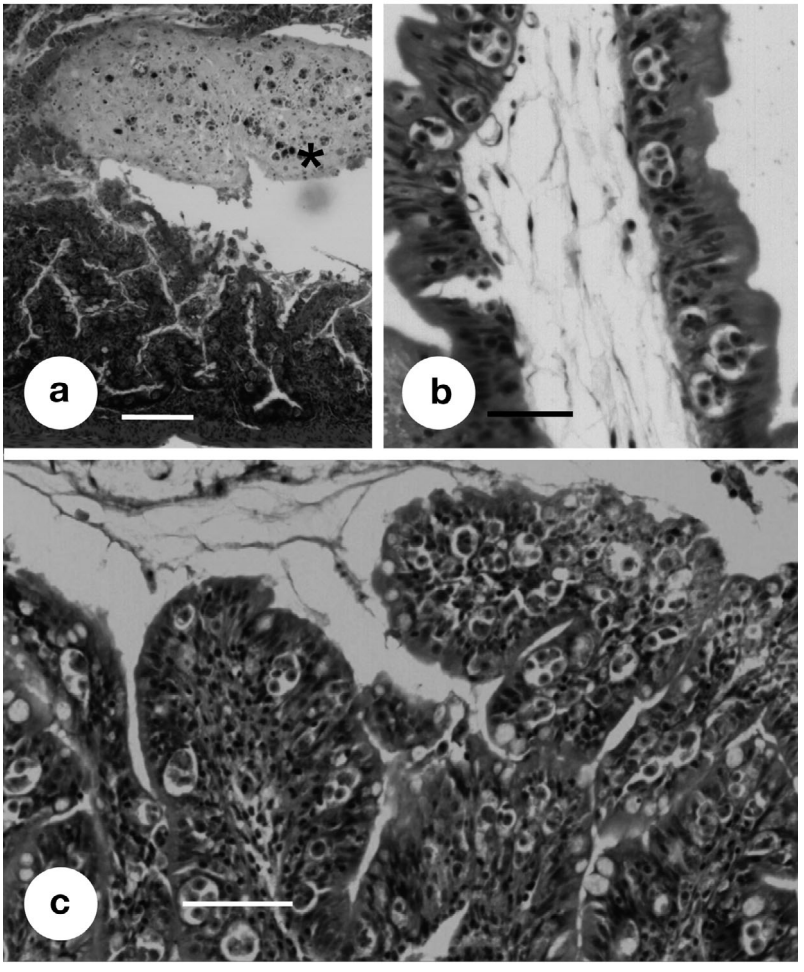

Fig. 4. *Astronotus ocellatus* (oscar). Paraffin sections of gut infected with *Enteromyxum leei*. (a) Mucus cast (*) with spores and sporoblasts in the gut lumen (H&E; scale bar = 100 µm); (b) developing *E. leei* sporoblasts aligned along basal membrane underlying gut mucosa epithelium; H&E; scale bar = 100 µm; (c) heavily infected gut mucosa epithelium with numerous *E. leei* sporoblasts; H&E; scale bar = 25 µm

We must consider yet another possibility, namely that *Enteromyxum leei* now develops via direct fish-to-fish transmission after relinquishing the need for an intermediate host. The phenomenon of eliminating 1 or even 2 hosts from a heteroxenous life cycle (either permanently or temporarily) has recently been observed in several parasitic taxa (Poulin & Cribb 2002, Levsen & Jakobsen 2002). The advantages (and disadvantages) for such plasticity with respect to parasite transmission are intriguing and have yet to be duly investigated. 'Opportunistic direct transmission' may have great significance regarding parasitic survival strategies and could be more common that previously imagined. Spore production is not arrested; rather, the parasite simply diversifies its propagation to a clonal dissemination that is effective even if the putative annelid necessary for its 'natural' development is

absent. Indeed, fish-to-fish transmission was once considered the major mode of infection in Myxosporidia (Polyanski 1970), but once the fish-annelid life cycle connection was discovered (Wolf & Markiw 1984), the putative direct route was rejected as implausible. Since then, fish-to-fish was found to occur in all 3 known species of *Enteromyxum* (Diamant 1997, Redondo et al. 2002, Yasuda et al. 2002).

The spore dimensions produced in freshwater hosts in this study are comparable with those previously reported from sea bream, red drum and various other aquarium-held host species (Diamant et al. 1994, Diamant 1998, Padrós et al. 2001). Some morphometric variability has been reported for *Enteromyxum leei*, assumed to be a result not only of spore fixation method but also of differences in host, a phenomenon addressed by several authors (Kovaleva et al. 1979, Diamant 1998, Padrós et al. 2001, Diamant et al. 2005). Thus, for standardization purposes, measurement of fresh spores should be the preferred method. Although methodological differences in specimen preparation may account for some of variability, spore dimensions in freshwater hosts appear to be smaller than those in locally cultured marine species (*Sparus aurata* and *Sciaenops ocellatus*; see Table 3) or 8 additional marine aquarium species (Padrós et al. 2001). The only documented case of a marine fish with even smaller spores is that of *Amphiprion frenatus*; however, although these myxosporean spores were similar to those of *E. leei*, they could not be positively identified (Kent et al. 1999).

Further studies will need to examine in more detail the susceptibility of freshwater fish to *Enteromyxum leei*, and at what stage the infected fish begin to succumb to the disease. The potential for horizontal transmission of infection between freshwater fish via cohabitation or exposure to contaminated effluent also needs to be assessed. Indeed, the possibility of using freshwater ornamental fish as experimental hosts of this parasite opens new avenues for the study of the biology of this unique parasite. At the same time, the capability of *E. leei* to enter freshwater environments is of concern.

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