FOLIA PARASITOLOGICA 51: 188–198, 2004

# Studies on transmission and life cycle of *Enteromyxum scophthalmi* (Myxozoa), an enteric parasite of turbot *Scophthalmus maximus*

**María J. Redondo**, **Oswaldo Palenzuela** and **Pilar Alvarez-Pellitero**

Instituto de Acuicultura de Torre de la Sal (CSIC), Ribera de Cabanes, 12595 Castellón, Spain

Key words: Myxozoa, Myxosporea, *Enteromyxum*, life cycle, transmission, turbot, intestinal explants, *in vitro*

**Abstract.** In order to elucidate the transmission and dispersion routes used by the myxozoan parasite *Enteromyxum scophthalmi* Palenzuela, Redondo et Alvarez-Pellitero, 2002 within its host (*Scophthalmus maximus* L.), a detailed study of the course of natural and experimental infections was carried out. Purified stages obtained from infected fish were also used in *in vitro* assays with explants of uninfected intestinal epithelium. The parasites can contact and penetrate loci in the intestinal epithelium very quickly. From there, they proliferate and spread to the rest of the digestive system, generally in an antero-posterior pattern. The dispersion routes include both the detachment of epithelium containing proliferative stages to the intestinal lumen and the breaching of the subepithelial connective system and local capillary networks. The former mechanism is also responsible for the release of viable proliferative stages to the water, where they can reach new fish hosts. The finding of parasite stages in blood smears, haematopoietic organs, muscular tissue, heart and, less frequently, skin and gills, suggests the existence of additional infection routes in transmission, especially in spontaneous infections, and indicates the role of vascular system in parasite dispersion within the fish. The very high virulence of this species in turbot and the rare development of mature spores in this fish may suggest it is an accidental host for this parasite. This may also question the existence of a two-host life cycle involving an actinosporean stage in this species. Further studies are needed to clarify this open point of the life cycle.

*Enteromyxum scophthalmi* Palenzuela, Redondo et Alvarez-Pellitero, 2002 is a marine histozoic myxozoan parasite that causes fatal emaciative disease in farmed turbot (*Scophthalmus maximus* L.) (Palenzuela et al. 2002). The parasite invades the fish intestine and produces acute enteritis, starvation and, eventually, death. Mortality can reach 100% of affected stocks (Branson et al. 1999).

Myxozoan prevention and control are impaired by the lack of effective treatments and scarce knowledge on different aspects of life cycles and transmission, mainly in the case of marine species. The complete life cycle involving both actinosporean and myxosporean phases is known for several freshwater species (Kent et al. 2001), but evidence of the heteroxenous cycle in the marine environment has only recently been found for the estuarine *Ellipsomyxa gobii* (Køie et al. 2004). However, spontaneous direct fish-to-fish transmission has been demonstrated for both *Enteromyxum leei* (syn. *Myxidium leei*) (Diamant 1997) and *E. scophthalmi* (Redondo et al. 2002). Yasuda et al. (2002) reported the direct transmission of *Myxidium fugu* and *Myxidium* sp. in the tiger puffer, *Takifugu rubripes*. Interestingly, recent molecular studies have demonstrated that both *Myxidium* species should be included in the genus *Enteromyxum* (Yanagida et al. 2003). In addition, a case of possible direct transmission by ingestion of eggs was reported in *Kudoa ovivora* in labrid fishes (Swearer and Robertson 1999).

Although we have demonstrated the fish-to-fish transmission of *E. scophthalmi* (Redondo et al. 2002), little is known about the routes of invasion and dispersion of the parasite within the fish. In order to elucidate these aspects of the life cycle, a detailed study of the course of spontaneous and experimental infections was carried out using histological and ultrastructural techniques. In addition, the parasite-host interactions were studied *in vitro*, using intestinal explants.

## MATERIALS AND METHODS

### Spontaneous infections

Two fish stocks were sampled monthly after their introduction to the growing facilities of a turbot farm in Galicia, north-west Spain (stock A introduced in October 1997, stock B in April 1998). All fish received a pump-ashore, flow-through water supply and were kept under natural temperature and photoperiod. Details of the sampling schedule for both stocks are presented in Table 1.

### Experimental infections

Donor fish were initially obtained from an *E. scophthalmi*-affected farm located in north-west Spain. These stocks were suffering high mortality due to the parasite. Infection was confirmed by the occurrence of disease symptoms and the presence of parasite spores in intestinal scrapings of some fish. Uninfected receptor fish were reared with a flow-through

This paper was presented at the 6th International Symposium on Fish Parasites in Bloemfontein, South Africa, 22–26 September 2003.

Address for correspondence: P. Alvarez-Pellitero, Instituto de Acuicultura de Torre de la Sal (CSIC), Ribera de Cabanes, 12595 Castellón, Spain. Phone: ++34 964 319 500; Fax: ++34 964 319 509; E-mail: alvarezp@iats.csic.es

**Table 1.** Studied spontaneous infections, with details on turbot stocks and sampling scheme. B – blood smears; H – histology.

| Fish group (introduction date) | Sampling dates | No. fish | Studies | Temperature (°C) |
|---|---|---|---|---|
| Group A (October 97) | 6-11-97 | 9 | B,H | 16.5 |
| | 1-12-97 | 10 | H | 14.8 |
| | 28-1-98 | 20 | H | 13.5 |
| | 4-3-98 | 10 | B,H | 14.3 |
| | 26-3-98 | 10 | H | 15.6 |
| | 23-4-98 | 10 | H | 14 |
| | 20-5-98 | 16 | B,H | 15.3 |
| Group B (April 98) | 22-5-98 | 10 | H | 14.3 |
| | 25-6-98 | 10 | H | 13.8 |
| | 27-7-98 | 10 | H | 14 |
| | 28-8-98 | 10 | H | 14.5 |
| | 28-9-98 | 20 | H | 16.5 |
| | 28-10-98 | 5 | B,H | 15.3 |
| | 12-11-98 | 12 | B,H | 18 |
| | 20-01-99 | 10 | B,H | 10.7 |
| | 02-02-99 | 8 | B,H | 11.4 |

supply of cartridge-filtered (1 µm mesh) and UV-irradiated seawater. This pathogen-free water was used for the transmission experiments when required.

Three experimental transmission trials were conducted: via effluent, using water from tanks containing diseased fish; *per os* by feeding parasite-containing intestinal tissue; and via cohabitation of infected and uninfected fish. In each experiment, a group of control fish from the same stock as the test fish was maintained under equivalent conditions, but without exposure to parasites. Details of the experimental design can be found in a previous work (Redondo et al. 2002). Information on the sampling schedule for each experiment is presented in Table 2.

Since the experimental infection model was obtained, the life cycle has been maintained *in vivo* at the Instituto de Acuicultura Torre de la Sal (IATS) facilities by several series of cohabitation of infected and uninfected fish. Additional material was collected and studied from these groups.

**Sampling procedure**

Fish were killed by overexposure to MS222 and bled from the caudal vein before the necropsy. Blood smears were prepared and stained with May-Grünwald-Giemsa staining for assessment of the presence of parasite stages. Samples of the digestive tract (oesophagus, stomach, anterior, medial and posterior parts of the intestine), spleen, kidney, gills, skin/muscle, liver and gall bladder were fixed in 10% neutral buffered formalin. In addition, fresh intestinal mucosa scrapings, liquid from the intestinal lumen (if present) and bile were examined with a light microscope.

For histological processing, formalin-fixed tissue samples were embedded in Technovit-7100 resin (Kulzer, Heraeus, Germany). Sections (1–3 µm) were stained with toluidine blue or Giemsa staining. Infection intensity was evaluated in the histological sections following a scale of 1+ to 6+, according to the number of parasite stages present in microscope fields at 300× magnification. The presence and severity of pathological lesions typical of the disease were also evaluated in the sections and classified as minor, moderate, or severe. For transmission electron microscopy (TEM) studies, pieces of tissues were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). Samples were washed several times with the same buffer, postfixed in 1% (w/v) osmium tetroxide in the same buffer, dehydrated and embedded in Spurr's resin. Ultrathin sections were double-stained with aqueous uranyl acetate and lead citrate.

***In vitro* studies**

**Parasite material**. Live parasites were obtained from experimentally infected fish and processed as explained previously (Redondo et al. 2003a). Briefly, the intestinal fluid was collected from infected fish using a syringe and deposited in 15-ml centrifuge tubes containing Hanks' Balanced Salt Solution (HBSS) supplemented with a PSA antibiotic/antimycotic mixture (1 × PSA = 100 U·ml$^{-1}$ penicillin, 100 µg·ml$^{-1}$ streptomycin, and 0.25 µg·ml$^{-1}$ amphotericin B). Infection intensity was evaluated using a microscope at 300× magnification. Samples rich in parasite stages were centrifuged 10 min at 365 × *g* and the pellet was washed twice in fresh HBSS containing 2 × PSA. Stages in the pellet were counted and their viability estimated using eosin dye-exclusion methods.

**Intestinal explants.** After necropsy of healthy turbot, portions of anterior or medial parts of intestine were collected, placed in sterile HBSS containing 2 × PSA, and cut into small pieces up to 7 × 3 mm. One or two small intestine pieces were placed (epithelial layer facing upwards) in each well of 24-well tissue culture plates, containing 800 µl of Leibovitz's L-15 medium supplemented with 10% heat-inactivated foetal bovine serum (FBS), 1 × PSA and 20 mM HEPES. Medium osmolarity was adjusted to 350 mOsm·kg$^{-1}$ by the addition of sodium chloride.

**Table 2.** Studied experimental infections, with details on the experimental fish groups and sampling schemes. B – blood smears; H – histology.

| Experimental fish group | Experimental period | Samplings (days p.e.) | No. fish (per sampling) | Studies | Temperature (°C) |
|---|---|---|---|---|---|
| Effluent infection | 6-99/8-99 | 12, 20, 32, 55 | 10 | H | 18 ± 1 |
| *Per os* infection | 10-99/12-99 | 2, 4, 8, 15, 22, 29*, 36 | 10 | B*, H | 18 ± 1 |
| Cohabitation infection | 1-00/4-00 | 3, 6, 13, 20, 27, 34, 41 48, 55, 62, 76 | 10<br>5 | B, H<br>B, H | 10.6–14.2<br>14.3–15.5 |

*No blood smears available for sampling point 29 days p.e.

**Parasite culture with intestinal explants**. Purified parasite stages were added to well plates containing the explants. Control wells contained intestine pieces with no parasites added. Intestine portions were recovered at different times post-exposure (p.e.) (see below), fixed and embedded in resin as described above. Different experiments were carried out, using 150,000 or 300,000 parasites per well and incubation times of 15 min, 30 min, 2 h, 6 h, 24 h and 48 h p.e. In order to test the possible role of epithelial mucus on the parasite invasion process, the epithelial mucus of intestine pieces was thoroughly washed away with HBSS containing 2 × PSA, in one experiment at 4ºC. Parallel intestine pieces were used unwashed. Explants were recovered at the same times and processed as described above.

## RESULTS

### Spontaneous infections Fig. 1

**Group A.** Parasites were first detected in the intestine of 10% of fish sampled in January 1998, about four months after their introduction (p.i.) in the growing tanks. Both prevalence and intensity remained low at the next sampling, in early March. A dramatic rise in infection levels was observed at the fifth sampling (about six months p.i.), and during the last two samplings (approximately seven and eight months p.i) prevalence reached 90–100% and intensity was high (5+). In blood smears, however, parasite stages were detected earlier in all fish examined five weeks p.i. (first sampling). Prevalence in blood decreased slightly at the next time point when blood smears were taken (sampling 4), and returned to 100% at the last sampling. In addition, parasite stages were occasionally found in other organs such as skin/muscle, gills, or renal blood vessels, at the fifth and later samplings. Parasite maturation was observed along the sequential samplings, from exclusively young stages detected in the first findings to a mixture of assorted stages observed as the infection progressed, including some developing sporoblasts present in the final samplings.

**Group B.** In the initial samplings, the infection pattern in the intestine was quite similar to that of group A, with the first parasite detection occurring 5 months p.i. (10% prevalence). A subsequent increase in infection levels was also observed, though maximum prevalence (100%) was attained earlier (six months p.i.). Intensity of infection reached medium values in the last 4 samplings, in which blood stages were also detected in smears from every fish. The parasite was seldom found in other organs, as only the spleen from one fish was found parasitized at the fourth sampling (5 months p.i.). In this group, parasite maturation occurred somewhat earlier than in group A, as sporoblasts and spores appeared at the fifth and subsequent samplings.

### Experimental infections Fig. 2

**Effluent transmission.** Parasite stages were first detected at day 20 p.e., in light infections of oesophagus and anterior intestine of 30% of fish examined. Prevalence and intensity increased in further samplings. At the same time, a progressive invasion of the remaining parts of the digestive tract was observed. The parasite was detected in other organs (spleen, kidney, pancreas, gills, muscular tissue) only in the final samplings, when infection was already severe in the intestine.

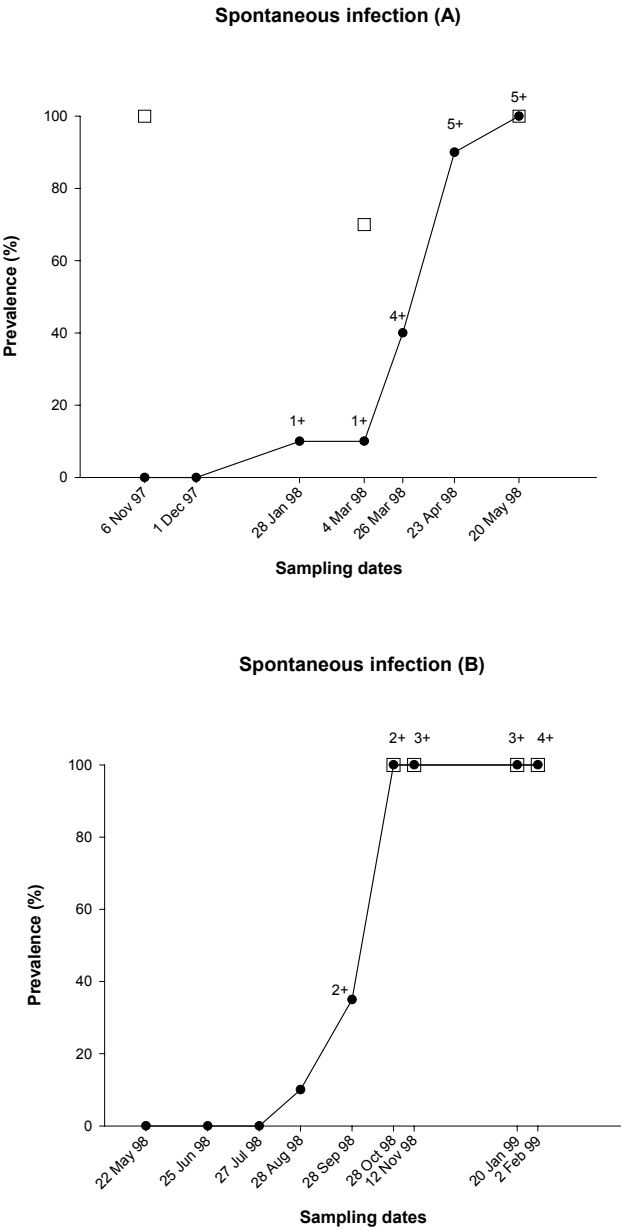



**Fig. 1.** Prevalence of *Enteromyxum scophthalmi* in spontaneous infections of turbot (Group A, top; Group B, bottom), determined at each sampling point along the period studied. Infection intensity (1+ to 6+) is represented at each sampling point as determined by histology. Data from histological sections (●) and blood smears (□) when available.

***Per os* transmission.** Parasites were first detected in blood smears as early as day 2 p.e., whereas they did not appear until day 8 p.e. in sections of the anterior intestine. Prevalence in the digestive tract was 100% in the

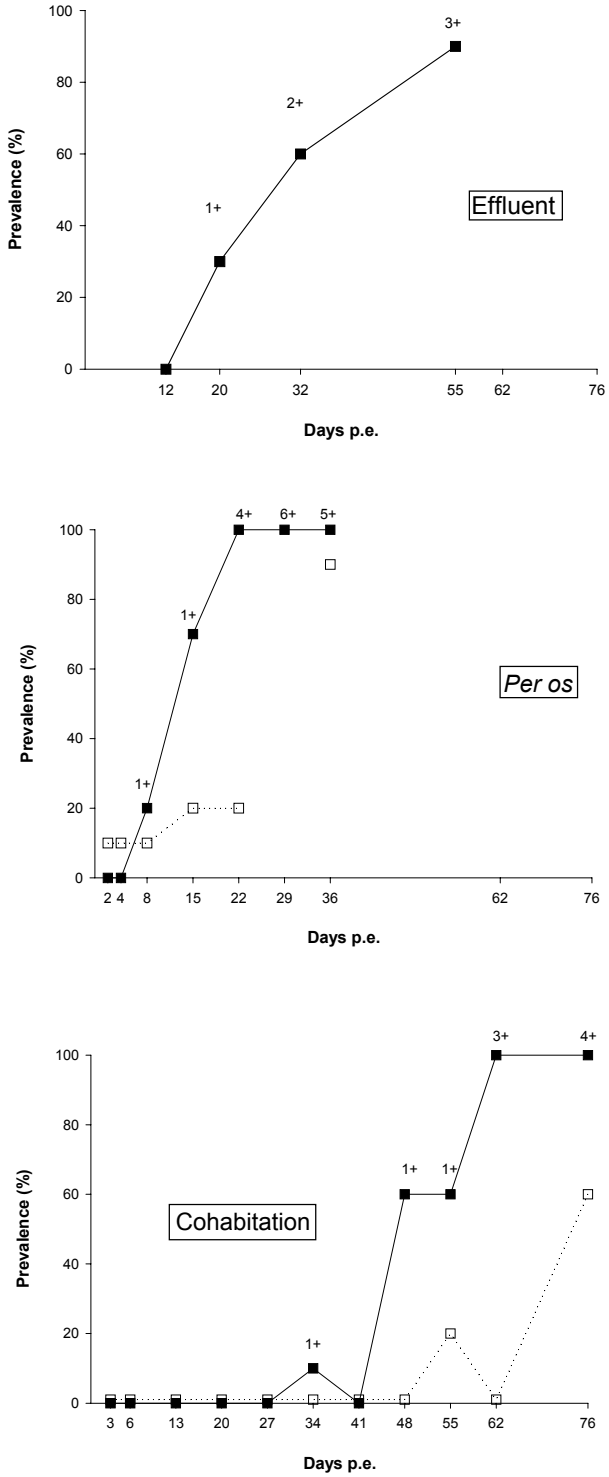


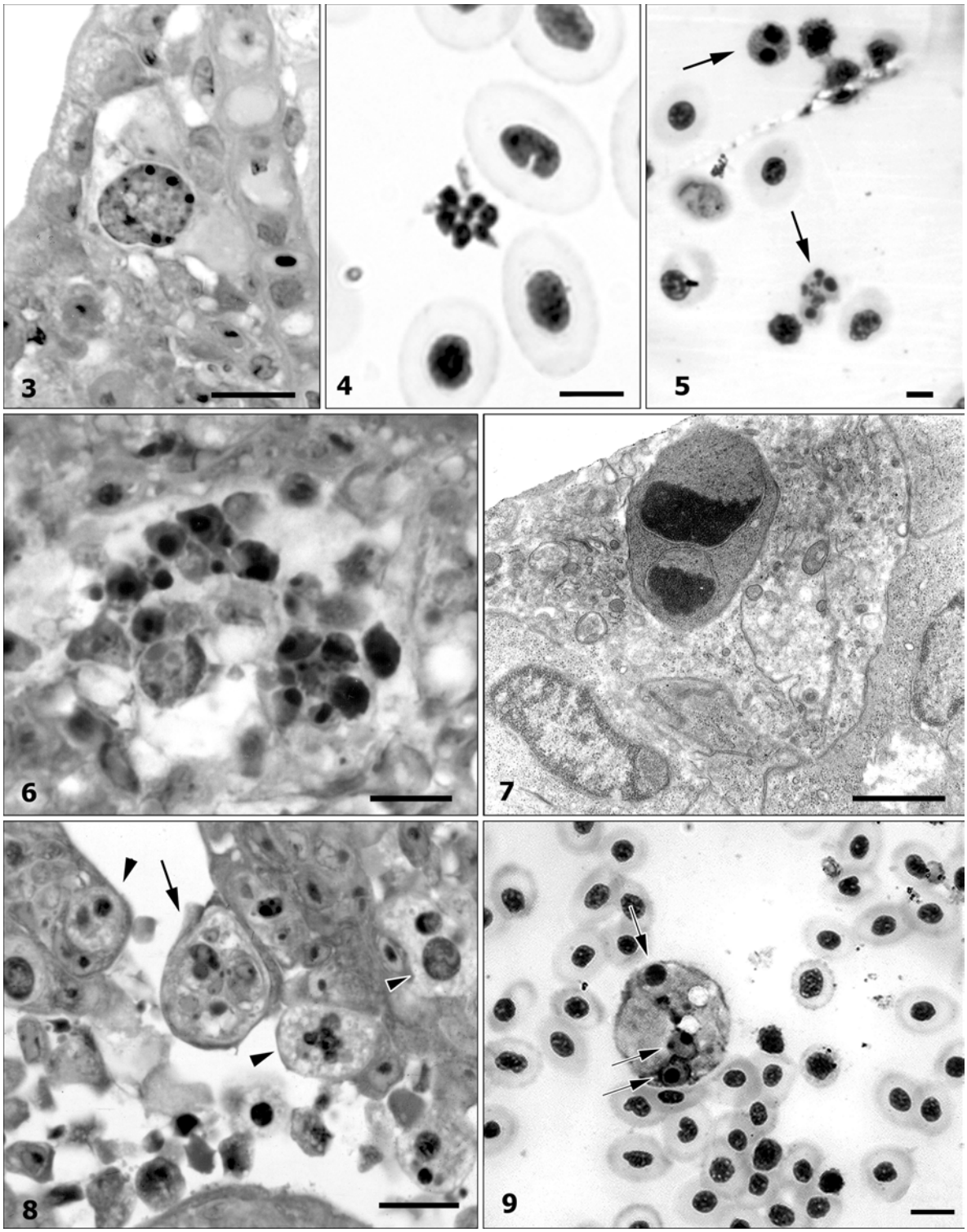

**Fig. 2.** Prevalence of *Enteromyxum scophthalmi* in experimental infections of turbot (top: effluent transmission; middle: *per os* transmission; bottom: cohabitation), determined at each sampling point along the period studied. Infection intensity (1+ to 6+) is shown at each sampling point, as determined by histology. Data from histological sections (■) and blood smears (□) when available.

final three samplings (from day 22 p.e. on), with a moderate to high infection intensity. The presence of blood stages was moderate until day 22 p.e., but increased dramatically in the final sampling, reaching 90% prevalence and high intensity. Few parasites were found in other tissues (muscle, pancreas, kidney, liver, heart, or gall bladder), on the fifth (day 22 p.e.) and subsequent samplings.

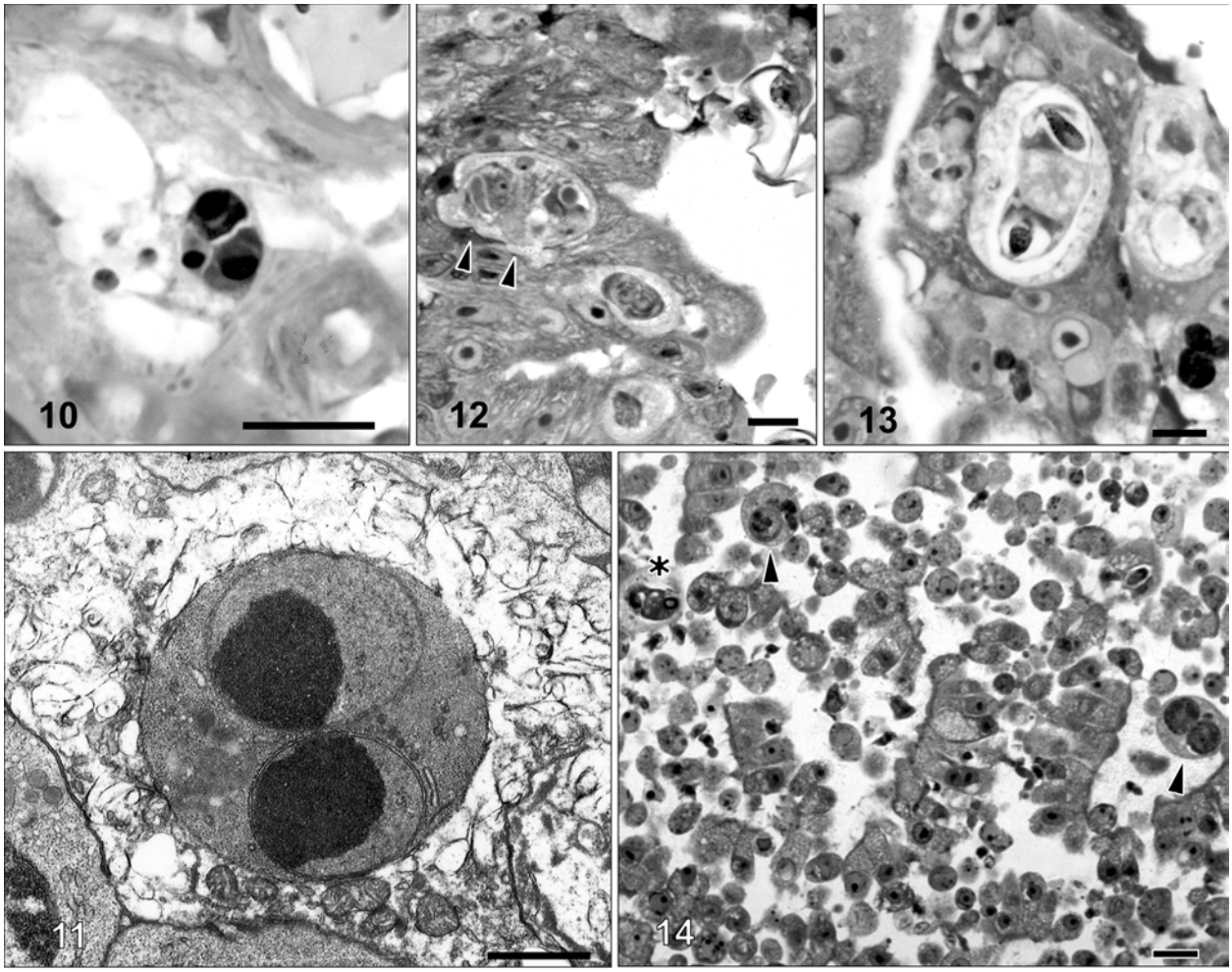
**Cohabitation transmission.** The onset of infection was clearly delayed when compared with effluent and *per os* transmission experiments. The first stage was observed in a kidney vessel of a single fish at day 34 p.e., but parasites did not appear in blood smears until day 55 p.e. Intestinal stages were first detected on day 48 p.e., although degraded parasites were found earlier in the basal epithelium, starting on day 13 p.e. Once the intestine infection was triggered, it followed the usual pattern of increasing severity. Prevalence reached 100% in the two final samplings.

## Parasite stages observed during the course of infections

In both spontaneous and experimental infections, different parasite developmental stages were observed. We have designated them as stages 1 to 5. **Stage 1** (ST1) is a trophozoite with one to several nuclei. It was detected in the gills during spontaneous infections (Fig. 3), blood (both in spontaneous and in *per os* infections, Figs. 4–5), haematopoietic organs, or clustered in subepithelial connective tissue (Fig. 6). Transmission electron micrographs of early stages were also obtained from the spleen of cohabitation-infected fish (Fig. 7). **Stage 2** (ST2) is a trophozoite in which the primary (P) cell harbours one secondary (S) cell, and eventually one or more nuclei. In contrast, in **Stage 3** (ST3) a P cell harbours one or more S cells, which in turn also harbour tertiary (T) cells. Stages 1 and 2 occasionally seemed to appear in an intracellular position (Fig. 7), although they often were embedded in a withered matrix (Fig. 11), making it difficult to interpret their precise localisation. Stages 2 and 3 were the most frequently observed stages in both spontaneous and experimental infections. They were mainly located in the intestinal epithelium (Fig. 8), but also occasionally in other tissues such as blood (sometimes phagocytosed by macrophages, Fig. 9), haematopoietic organs, gills, the pancreatic zone (Fig. 10) or subepithelial connective tissue of the digestive tract (Fig. 11). **Stage 4** (ST4) and **Stage 5** (ST5) correspond to sporogenesis and they were only found in the intestine. ST4 is a plasmodium harbouring a differentiating sporoblast with maturing spores, and occasionally S cells or their remnants (Figs. 8, 12). ST5 refers to nearly or fully mature spores (Fig. 13), sometimes still contained in the sporoblast. In advanced infections, different parasite stages are released to the intestinal lumen together with detached epithelial remnants (Fig. 14).




**Figs. 3–9.** Different developmental stages (ST) of *Enteromyxum scophthalmi* observed in spontaneous and experimental infections. **Fig. 3.** ST1 within the gill tissue in a spontaneous infection. **Figs. 4–5.** Initial stages (ST1) in blood smears from spontaneous (Fig. 4) and *per os* experimental infections (day 36 p.e., Fig. 5, arrows). **Fig. 6.** Cluster of ST1 in the intestine subepithelial connective tissue (spontaneous infection). **Fig. 7.** ST1, probably intracellular in the spleen of a cohabitation-infected fish. **Fig. 8.** Several stages in the intestine epithelium (spontaneous infection), including ST2 and ST3 (arrowheads) and a plasmodium harbouring an early sporoblast (ST4, arrow). **Fig. 9.** Macrophage harbouring several stages (ST2, arrows) in a blood smear of a *per os*-infected fish (day 36 p.e.). Figures are toluidine blue-stained resin sections (Figs. 3, 6, 8), May-Grünwald-Giemsa-stained blood smears (Figs. 4, 5, 9) and a transmission electron micrograph (Fig. 7). Scale bars: Figs. 3, 6, 8, 9 = 10 μm; Figs. 4, 5 = 5 μm; Fig. 7 = 1 μm.




**Figs. 10–14.** Different developmental stages (ST) of *Enteromyxum scophthalmi* observed in spontaneous infections. **Fig. 10.** ST2 in the pancreatic zone. **Fig. 11.** ST2 in a withered area of intestine subepithelial connective tissue. **Figs. 12, 13.** Sporogonic stages in the intestinal epithelium. **Fig. 12.** ST4 (arrowheads). **Fig. 13.** Sporoblast containing a nearly mature spore (ST5) still within the primary cell. **Fig. 14.** Parasite stages detached to the intestinal lumen together with epithelial remnants (ST3 – arrowheads; ST5 – asterisk). Figures are toluidine blue-stained resin sections (Figs. 10, 12–14) and transmission electron micrograph (Fig.11). Scale bars: Figs. 10, 12–14 = 10 μm; Fig. 11 = 1 μm.

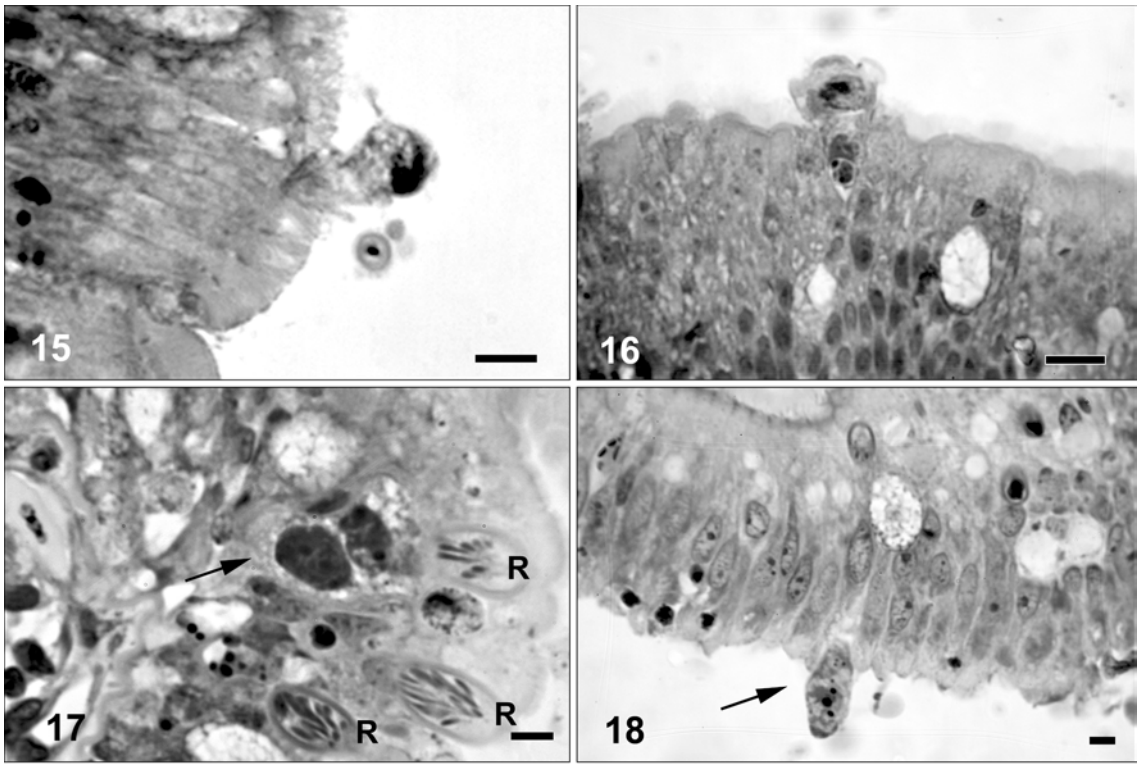
The increasing severity of the intestine lesions observed during the infections was in general positively correlated to the number and degree of maturation of the parasite stages present. However, the oedema and detachment of the epithelial layer sometimes occurred in rather early infections, with few detectable parasites.

***In vitro* studies**

Parasite stages (mainly ST3) were observed contacting or penetrating through the epithelial surface of intestinal explants, or even inside the epithelial layer, as soon as 15 min p.e. and at subsequent exposure times (Figs. 15–17). At 2 h p.e., or even sooner, parasites were found mainly in the basal and middle parts of the epithelial layer. Detachment of the epithelial layer from the basal membrane started at 30 min p.e., and some parasites seemed to breach in through the basal side (Fig. 18). Epithelial cells were completely detached or degraded in explants sampled at 24 and 48 h p.e. and some parasites could be observed in the debris (not shown). No clear differences could be observed between the epithelia that had been washed to eliminate the mucous layer versus unwashed epithelia.

## DISCUSSION

The current study on the dynamics of spontaneous and induced *Enteromyxum scophthalmi* infections in turbot and the data from the *in vitro* exposure experiments allowed us to obtain new insight into the parasite life cycle and transmission. These data complement previous studies focused on aspects of the experimental transmission (Redondo et al. 2002), *in vitro* cultivation (Redondo et al. 2003a), and ultrastructure of this parasite (Redondo et al. 2003b). Using the information ob-




**Figs. 15–18.** Toluidine blue-stained resin sections of intestinal explants sampled at different times after exposure (p.e.) to *Enteromyxum scophthalmi* stages *in vitro*. Parasite stages (ST3) at different degrees of penetration or already inside the epithelium. **Fig. 15.** Sampled at 15 min p.e. **Fig. 16.** Sampled at 30 min p.e. **Fig. 17.** Sampled at 6 h p.e. (ST3 – arrow; R – rodlet cells). **Fig. 18.** ST3 (arrow) penetrating through the basal part of detached epithelium, sampled at 30 min p.e. Scale bars = 5 μm.

tained up to now, a tentative model life cycle of this parasite is suggested (Fig. 19).

**Onset of infections**

In spontaneous infections, parasites were first detected in fish blood (group A) as soon as one month after their introduction to growing facilities. However, almost five months were required for parasite detection in the digestive tract. After the final samplings of groups A and B, fish started to suffer the characteristic disease symptoms and severe mortality (data not shown) typical of *E. scophthalmi* epizootics occurring in turbot farms (Branson et al. 1999). Thus, a relatively long prepatent period occurred between the first detection of the parasite in the stocks and disease symptoms. The progress of infection was faster in group B, which was probably due to seasonal variations of temperature, as group B was introduced in spring (April) while group A was introduced in autumn (October). The extended prepatent period and significant influence of temperature on the onset of myxozoan infections after exposure to enzootic waters have been widely reported (Bartholomew et al. 1989a, McGeorge et al. 1996, Moran and Kent 1999), and may be related to several ecological and physiological factors on both sides of the host-parasite relationship.

In our experiments, the three assayed routes successfully infected the fish. The *per os* route was the fastest and most effective, suggesting that ingestion of developmental stages from donor fish is also the main route used by the parasite in the cohabitation and effluent trials, which to some extent reproduce the situation of spontaneous infections among cultured fish (Redondo et al. 2002). However, the early occurrence of blood stages in spontaneous infections may suggest that parasite penetration could also follow other routes. More or less detailed sequential studies of myxozoans infections have suggested gills, buccal cavity, and skin as main portals of entry to the fish (El-Matbouli et al. 1999, Morris et al. 2000, Belem and Pote 2001, Holzer et al. 2003). However, these models involve heteroxenous cycles and invasive stages that appear to be originated from actinospores.

**Proliferation and dissemination in the fish**

Whatever the penetration route is, the parasite must reach the target organs in order to establish the infection. From the results obtained, we deduce that the parasite can reach intestinal loci directly *per os*, but also via blood, which would act as one of the dispersion routes to new loci in the digestive tract.

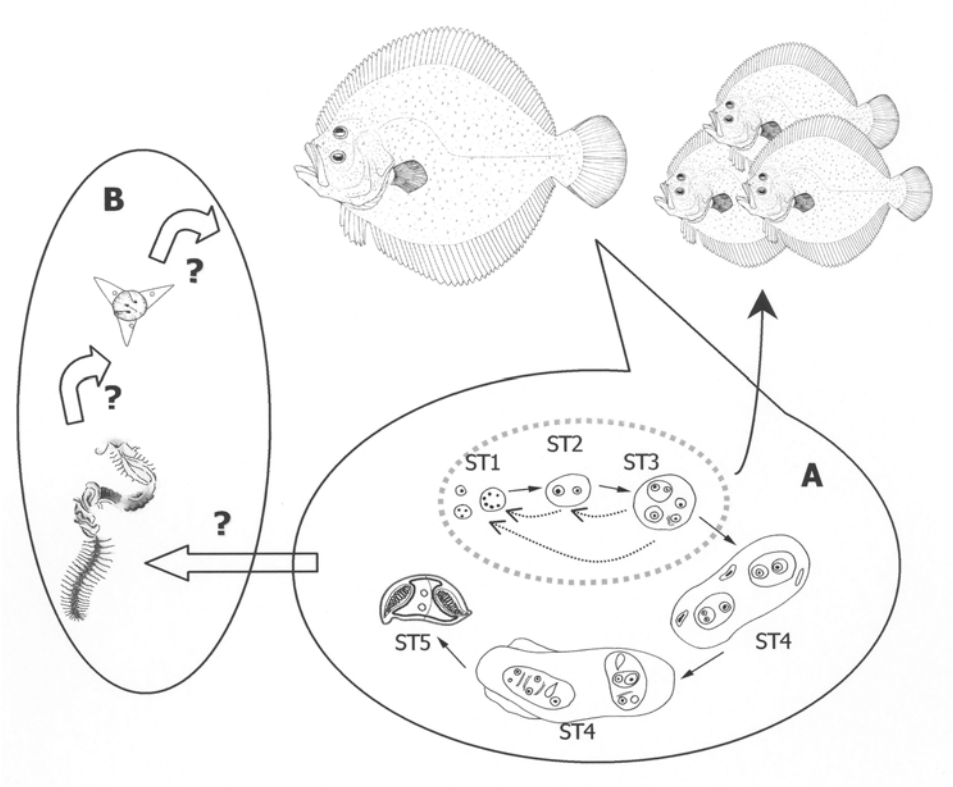



**Fig. 19.** Diagram of the hypothetical life cycle of *Enteromyxum scophthalmi*. In turbot (A), the proliferative and sporogonic cycles include the stages (ST) defined above as ST1 to ST5. ST1 to ST3 are responsible for invasion and dispersion within the fish. ST2 to ST3 are the main infective stages for transmission to other fish (when released with detached epithelial strips) through the faeces. ST4 to ST5 follow the usual pattern of myxozoans sporogony. Scarce spores (ST5) develop in the fish. Whether or not they can start an alternate cycle involving an invertebrate host (B) is currently unknown.

Blood stages have been found in other myxosporean infections, and are mainly involved in their extrasporogonic proliferative development (Lom and Dyková 1992, Paperna and Di Cave 2001). Proliferative blood stages were suggested in *Sphaerospora truttae* infections by McGeorge et al. (1996) and have recently been demonstrated by Holzer et al. (2003) using *in situ* hybridisation. Such proliferative stages are probably implicated in the successful fish-to-fish transmission of some myxosporeans by experimental inoculation of infected material, as described for *Sphaerospora renicola* (Molnár and Kovács-Gayer 1986, Odening et al. 1989), PKX (Hedrick et al. 1993), or *Kudoa thyrsites* (Moran et al. 1999). A proliferative, not merely disseminative phase occurring in blood, should not be disregarded for *E. scophthalmi* in addition to the main proliferative phase in the intestine. However, we have not found obvious stages of this kind in infected turbot.

Once *E. scophthalmi* has reached a locus in the intestine, it appears to be able to multiply while it spreads and colonises the digestive tract, either by breaching the subepithelial connective tissue and vascular network and also by penetration of luminal, detached stages through the epithelial surface. Usually, the earliest parasite foci appeared in the pyloric caeca or anterior intestine and then spread to the rest of the intestine. Infection in the oesophagus and stomach was intense only when intestinal intensity was also high, suggesting reinfection from the anterior intestine. This pattern might be related with the intense vascularisation of the anterior intestine, or in response to an undetermined physiological basis. Interestingly, compared to other enteric myxosporoses, both *Ceratomyxa shasta* and *Enteromyxum leei* tend to follow an inverse, posterior-anterior gradient (Bartholomew et al. 1989b and authors' unpublished observations).

The ability of parasites to breach and penetrate the epithelial layer was observed in previous *in vivo* studies (Redondo et al. 2002), but also in the current *in vitro* experiments using intestine explants, in which this penetration occurred quite fast. Tissue or organ explants are useful experimental models to study physiological (Berge et al. 1999) and pathological aspects in fish and mammals (Mothersill et al. 1988, Stadtländer and Kirchhoff 1989, Dowling and Mothersill 1999, Vonlaufen et al. 2002), when controlled conditions are required. Using this model, we demonstrated the invasion of proliferative stages (ST2 and ST3) both through the mucous epithelial surface and through the base of detached epithelium. The elucidation of the mechanisms

involved in adhesion and penetration requires further studies. In our *in vitro* experiments, no clear differences regarding parasite invasion were observed with explants devoid of mucous layer versus intact epithelium. However, the role of some components of the epithelial mucus in the establishment of enteric pathogenic organisms and in the host-parasite interaction is well known. Host mucins may have roles in preventing parasite establishment by acting as a barrier against pathogen adhesion and invasion, or in parasite expulsion. In turn, mucins might be exploited by the parasites as a source of fuel, binding sites, or host detection targets (Tse and Chadee 1991, Hicks et al. 2000, Theodoropoulos et al. 2001). In fish, mucosal tissues may also have components involved in different physiological and immunological functions (Nakamura et al. 2001) and also in susceptibility to infections (Buchmann 1998). Different carbohydrate terminals may be involved in these interactions (e.g., Basseri et al. 2002, Maruyama et al. 2002). Further studies are needed to understand turbot-*E. scophthalmi* relationships at the molecular level.

Within the intestinal epithelium, *E. scophthalmi* matures through its proliferative and sporogonic development in a temperature-dependent fashion. The spreading to other tissues is sporadic and occurs mainly in advanced infections. However, occasional findings of some degraded stages in the spleen and intestinal epithelium, occurring in early infections, are remarkable. This observation is probably a consequence the host immune response, as parasite stages and debris engulfed in macrophages were found at the epithelial base of the intestine, the subepithelial connective tissue, and circulating blood. In addition, the presence of degraded stages was especially common in the intestines from the cohabitation experiment, when low temperature and light infective pressure caused a more gradual onset of infection, facilitating a more effective immune response by turbot. The involvement of innate and adaptive mechanisms on turbot immune response against *E. scophthalmi* has been demonstrated in preliminary studies (Sitjà-Bobadilla et al. 2003, authors' unpublished observations).

Very scarce mature spores develop in the intestine, never in other parts of the digestive tract, and only in terminally sick fish with severe infections. The presence of parasites in the epithelium triggers an acute response causing the epithelial layer containing parasites to detach. The histopathological alterations of the intestine are usually severe, sometimes from early stages of infection, and detachment of the epithelium could be observed even with few parasites present. Thus, parasite-containing debris can easily reach new intestinal loci from the lumen (see above), but it can also be discharged with the faeces. The survival of some purified *E. scophthalmi* stages after 24 h in seawater has previously been demonstrated *in vitro* (Redondo et al. 2003a). It could be extended by the additional protection of epithelial remnants and faecal mucous casts, allowing viable parasites to reach a new fish. This same pattern is exploited by the close species *Enteromyxum leei* of Mediterranean fish to disseminate and reach new hosts (Diamant 1997, authors' unpublished observations). However, unlike *E. scophthalmi* in turbot, *E. leei* develops a large number of spores and high parasite loads can be present with little histopathological alterations, manifested as chronic or subclinical infections (authors' unpublished observations). The factors behind these differences are unknown, but in the case of *E. leei* the severity of infections appears rather variable depending on the fish host species (Padrós et al. 2001). Similarly, in *Ceratomyxa shasta* infections, the data available suggest the pathogenicity of this species to be mainly modulated by host-related factors (Bartholomew 1998).

The proliferative stages, mainly ST2 and ST3, present in the detached epithelial strips, are the main infective stages involved in experimental transmission to other fish and, probably, also spontaneous infections under intensive culture conditions due to cohabitation with infected fish in the same tank. From a management point of view, a theoretical risk of waterborne contagion should also be considered, i.e., penetration of the farm water supply by proliferative stages originating from wild fish stocks.

**An alternate host?**

In some myxosporean infection experiments using enzootic waters, the involvement of actinosporean stages originating from a different host was suggested. In several cases, this has been demonstrated under experimental conditions (Kent et al. 2001). In *E. scophthalmi*, we have demonstrated the infectivity of stages directly detached from fish intestines, in experiments that allowed us to disregard the participation of alternate hosts. However, in the spontaneous infections, the presence of initial stages in the gills and skin, and especially the high prevalence of parasite stages in fish blood, long before their detection in the target tissue, points to a somewhat different scenario than the cohabitation experimental infections in which blood stages appeared much later. Further studies involving *in situ* hybridisation are underway to identify some unclear, putative early parasite stages (difficult to distinguish from some host apoptotic cells) that may give a more definitive insight into the initial steps of the infection. However, this technique is not stage-specific, and the detection of the earliest infective stages penetrating fish after exposure to infective water does not clarify where such stages originated, as in the case of *Sphaerospora truttae* (Holzer et al. 2003). It is conceivable that those initial stages found in spontaneous infections could proceed from actinosporean stages produced in invertebrate hosts inhabiting the farms or their surroundings. However, such stages currently can not be identified unambiguously. Ongoing research in our

laboratory, using PCR screening of invertebrates, is aimed to clarify the possibility of an alternate cycle. Nonetheless, the low number of spores produced in turbot apparently indicates that this putative heteroxenous life cycle should occur quite rarely compared to the direct fish-to-fish cycle. It is also tempting to hypothesise that low spore production and very high virulence of *E. scophthalmi* in turbot could indicate that it is not the natural host of the myxozoan.

**Acknowledgements.** Funding for this study was provided by the EU and the Spanish *Ministerio de Ciencia y Tecnología* through research grants FEDER 1FD97-0679-C02-01 and AGL2001-2241-C02-01. Additional support was obtained from Stolt Sea farm S.A. We are grateful to J. Monfort for histology processing, and to research support services at the Universities of Barcelona (UB) and Valencia (UPV) for assistance in TEM studies.

## REFERENCES

BARTHOLOMEW J.L. 1998: Host resistance to infection by the myxosporean parasite *Ceratomyxa shasta*: a review. J. Aquat. Anim. Health 10: 112–120.

BARTHOLOMEW J.L., ROHOVEC J.S., FRYER J.L. 1989a: *Ceratomyxa shasta*, a myxosporean parasite of salmonids. Fish Disease Leaflet 80. U.S. Fish and Wildlife Service, National Fisheries Research Center, Kearneysville WV, USA, 8 pp.

BARTHOLOMEW J.L., SMITH C.E., ROHOVEC J.S., FRYER J.L. 1989b: Characterization of a host response to the myxosporean parasite, *Ceratomyxa shasta* (Noble), by histology, scanning electron microscopy and immunological techniques. J. Fish Dis. 12: 509–522.

BASSERI H.R., TEW I.F., RATCLIFFE N.A. 2002: Identification and distribution of carbohydrate moieties on the salivary glands of *Rhodnius prolixus* and their possible involvement in attachment/invasion by *Trypanosoma rangeli*. Exp. Parasitol. 100: 226–234.

BELEM A.M.G., POTE L.M. 2001: Portals of entry and systemic localization of proliferative gill disease organisms in channel catfish *Ictalurus punctatus*. Dis. Aquat. Org. 48: 37–42.

BERGE G.E., BAKKE-McKELLEP A.M., LIED E. 1999: *In vitro* uptake and interaction between arginine and lysine in the intestine of Atlantic salmon (*Salmo salar*). Aquaculture 179: 181–193.

BRANSON E., RIAZA A., ALVAREZ-PELLITERO P. 1999: Myxosporean infection causing intestinal disease in farmed turbot, *Scophthalmus maximus* (L.), (Teleostei: Scophthalmidae). J. Fish Dis. 22: 395–399.

BUCHMANN K. 1998: Some histochemical characteristics of the mucous microenvironment in four salmonids with different susceptibilities to gyrodactylid infections. J. Helminthol. 72: 101–107.

DIAMANT A. 1997: Fish to fish transmission of a marine myxosporean. Dis. Aquat. Org. 30: 99–105.

DOWLING K., MOTHERSILL C. 1999: Use of rainbow trout primary epidermal cell cultures as an alternative to immortalized cell lines in toxicity assessment: a study with nonoxynol. Environ. Toxicol. Chem. 18: 2846–2850.

El-MATBOULI M., HOFFMANN R.W., SCHOEL H., McDOWELL T.S., HEDRICK R.P. 1999: Whirling disease: host specificity and interaction between the actinosporean stage of *Myxobolus cerebralis* and rainbow trout *Oncorhynchus mykiss*. Dis. Aquat. Org. 35: 1–12.

HEDRICK R.P., MacCONNELL E., DE KINKELIN P. 1993: Proliferative kidney disease of salmonid fish. In: M. Faisal and F.M. Hetrick (Eds.), Annual Review of Fish Diseases. Pergamon Press, New York, pp. 277–290.

HICKS S.J., THEODOROPOULOS G., CARRINGTON S.D., CORFIELD A.P. 2000: The role of mucins in host-parasite interactions. Part I – Protozoan parasites. Parasitol. Today 16: 476–481.

HOLZER A.S., SOMMERVILLE C., WOOTTEN R. 2003: Tracing the route of *Sphaerospora truttae* from the entry locus to the target organ of the host, *Salmo salar* L., using an optimized and specific *in situ* hybridization technique. J. Fish Dis. 26: 647–655.

KENT M.L., ANDREE K.B., BARTHOLOMEW J.L. El-MATBOULI M.L., DESSER S.S., DEVLIN R.H., FEIST S.W., HEDRICK R.P., HOFFMANN R.W., KHATTRA J., HALLETT S.L., LESTER R.J.G., LONGSHAW M., PALENZUELA O., SIDDALL M.E., XIAO C. 2001: Recent advances in our knowledge of the Myxozoa. J. Eukaryot. Microbiol. 48: 395–413.

KØIE M., WHIPPS C.M., KENT M.L. 2004: *Ellipsomyxa gobii* (Myxozoa: Ceratomyxidae) in the common goby *Pomatoschistus microps* (Teleostei: Gobiidae) uses *Nereis* spp. (Annelida: Polychaeta) as invertebrate hosts. Folia Parasitol. 51: 14–18.

LOM J., DYKOVÁ I. 1992: Protozoan Parasites of Fishes. Developments in Aquaculture and Fisheries Sciences. Vol. 26. Elsevier, Amsterdam, 315 pp.

MARUYAMA H., HIRABAYASHI Y., El-MALKY M., OKAMURA S., AOKI M., ITAGAKI T., NAKAMURA-UCHIYAMA F., NAWA Y., SHIMADA S., OHTA N. 2002: *Strongyloides venezuelensis*: longitudinal distribution of adult worms in the host intestine is influenced by mucosal sulfated carbohydrates. Exp. Parasitol. 100: 179–185.

McGEORGE J., SOMMERVILLE C., WOOTTEN R. 1996: Epizootiology of *Sphaerospora truttae* (Myxozoa: Myxosporea) infections of Atlantic salmon *Salmo salar* at freshwater smolt producing hatcheries in Scotland. Dis. Aquat. Org. 26: 33–41.

MOLNÁR K., KOVÁCS-GAYER E. 1986: Experimental induction of *Sphaerospora renicola* (Myxosporea) infection in common carp (*Cyprinus carpio*) by transmission of SB-protozoans. J. Appl. Ichthyol. 2: 86–94.

MORAN J.D.W., KENT M.L. 1999: *Kudoa thyrsites* (Myxozoa: Myxosporea) infections in pen-reared Atlantic salmon in the Northeast Pacific Ocean with a survey of potential nonsalmonid reservoir hosts. J. Aquat. Anim. Health 11: 101–109.

MORAN J.D.W., WHITAKER D.J., KENT M.L. 1999: Natural and laboratory transmission of the marine myxozoan parasite *Kudoa thyrsites* to Atlantic salmon. J. Aquat. Anim. Health 11: 110–115.

MORRIS D.J., ADAMS A., RICHARDS R.H. 2000: *In situ* hybridisation identifies the gill as a portal of entry for PKX (Phylum Myxozoa), the causative agent of proliferative kidney disease in salmonids. Parasitol. Res. 86: 950–956.

MOTHERSILL C., CUSACK A., MacDONNELL M., HENNESSY T.P., SEYMOUR C.B. 1988: Differential response of normal and tumor esophageal explant cultures to radiation. Acta Oncol. 27: 275–280.

NAKAMURA O., WATANABE T., KAMIYA H., MURAMOTO K. 2001: Galectin containing cells in the skin and mucosal tissues in Japanese conger eel, *Conger myriaster*: an immunohistochemical study. Dev. Comp. Immunol. 25: 431–437.

ODENING K., WALTER G., BOCKHARDT I. 1989: The infection process in *Sphaerospora renicola* (Myxosporidia). Angew. Parasitol. 30: 131–140.

PADRÓS F., PALENZUELA O., HISPANO C., TOSAS O., ZARZA C., CRESPO S., ALVAREZ-PELLITERO P. 2001: *Myxidium leei* (Myxozoa) infections in aquarium-reared Mediterranean fish species. Dis. Aquat. Org. 47: 57–62.

PALENZUELA O., REDONDO M.J., ALVAREZ-PELLITERO P. 2002: Description of *Enteromyxum scophthalmi* gen. nov., sp. nov. (Myxozoa), an intestinal parasite of turbot (*Scophthalmus maximus* L.) using morphological and ribosomal RNA sequence data. Parasitology 124: 369–379.

PAPERNA I., DI CAVE D. 2001: Kidney sphaerosporosis with extrasporogonic stages in the blood in *Baryancistrus* sp. (Loricaridae) from Amazonian Brazil. Parasite 8: 71–74.

REDONDO M.J., PALENZUELA O., RIAZA A., MACÍAS Á., ALVAREZ-PELLITERO P. 2002: Experimental transmission of *Enteromyxum scophthalmi* (Myxozoa), a parasite of turbot (*Scophthalmus maximus*). J. Parasitol. 88: 482–488.

REDONDO M.J., PALENZUELA O., ALVAREZ-PELLITERO P. 2003a: *In vitro* studies on viability and proliferation of *Enteromyxum scophthalmi* (Myxozoa), an enteric parasite of cultured turbot *Scophthalmus maximus*. Dis. Aquat. Org. 55: 133–144.

REDONDO M.J., QUIROGA M.I., PALENZUELA O., NIETO J.M., ALVAREZ-PELLITERO P. 2003b: Ultrastructural studies on the development of *Enteromyxum scophthalmi* (Myxozoa), an enteric parasite of turbot (*Scophthalmus maximus* L.). Parasitol. Res. 90: 192–202.

SITJÀ-BOBADILLA A., REDONDO M.J., PALENZUELA O., LEYVA M.O., MACÍAS M.A., RIAZA A., QUIROGA I., NIETO J.M., ALVAREZ-PELLITERO P. 2003: Immune response of turbot (*Scophthalmus maximus* L.) after experimental infection with *Enteromyxum scophthalmi* (Myxozoa). 11th International Conference of the European Association of Fish Pathologists, St Julians, Malta, 21–26 September 2003. Book of abstracts, P-096.

STADTLÄNDER C., KIRCHHOFF H. 1989: Gill organ culture of rainbow trout, *Salmo gairdneri* Richardson: an experimental model for the study of pathogenicity of *Mycoplasma mobile* 163 K. J. Fish Dis. 12: 79–86.

SWEARER S.E., ROBERTSON R. 1999: Life history, pathology, and description of *Kudoa ovivora* n. sp. (Myxozoa, Myxosporea): an ovarian parasite of Caribbean labroid fishes. J. Parasitol. 85: 337–353.

THEODOROPOULOS G., HICKS S.J., CORFIELD A.P., MILLER B.G., CARRINGTON S.D. 2001: The role of mucins in host-parasite interactions. Part II – Helminth parasites. Trends Parasitol. 17: 130–135.

TSE S.-K., CHADEE K. 1991: The interaction between intestinal mucus glycoproteins and enteric infections. Parasitol. Today 7: 163–172.

VONLAUFEN N., GIANINAZZI C., MULLER N., SIMON F., BJORKMAN C., JUNGI T.W., LEIB S.L., HEMPHILL A. 2002: Infection of organotypic slice cultures from rat central nervous tissue with *Neospora caninum*: an alternative approach to study host-parasite interactions. Int. J. Parasitol. 32: 533–542.

YANAGIDA T., NOMURA Y., KIMURA T., FUKUDA Y., YOKOYAMA H., OGAWA K. 2003: *Enteromyxum* myxozoans from the intestine of cultured tiger puffer in Japan. Sixth International Symposium on Fish Parasites, Bloemfontein, South Africa, 22–26 September 2003, Abstracts book, p. 46.

YASUDA H., OOYAMA T., IWATA K., TUN T., YOKOYAMA H., OGAWA K. 2002: Fish-to-fish transmission of *Myxidium* spp. (Myxozoa) in cultured tiger puffer suffering from emaciation disease. Fish Pathol. 37: 29–33.

Received 19 December 2003 Accepted 12 May 2004