A protistan endoparasite in embryos and yolk-sac larvae of cod *Gadus morhua* and turbot *Scophthalmus maximus*

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ABSTRACT: A protistan endoparasite has recently been discovered in the yolk of embryos and yolk-sac larvae of cod *Gadus morhua* and turbot *Scophthalmus maximus*. Eight batches of cod embryos were studied, and all batches were infected with minimum prevalences ranging from 14 to 88 %. The endoparasite seemed to be transmitted to the cod embryos via the fish gametes, and the infection occurred in cod from all the major seas surrounding Denmark. Infected cod embryos and yolk-sac larvae have a higher mortality rate than uninfected ones. Several stages of the parasite were identified. The smaller stages of the cod endoparasite invaded the larval tissues and bloodstream. The large, multinucleated stages of the parasite can multiply by budding off small parasites. Other features are associated with endoparasitic infection, i.e. thin-walled and thick-walled spherical structures were observed in the yolk. The thin-walled structures ejected multiple parasite-like bodies of varying sizes into the yolk. The thick-walled, highly refractile hollow spheres in the yolk were of protistan origin. Light microscopy of the turbot endoparasite has previously indicated a strong resemblance with the cod endoparasite. Present transmission electron microscopy reveals that the turbot and cod endoparasites are identical or very closely related.

KEY WORDS: Protistan endoparasite · Prevalences · Geographic distribution · Route of transmission · Larval cod and turbot · Mortality

INTRODUCTION

Previous papers (Pedersen 1993a, Pedersen et al. 1993) have described the discovery of a protistan endoparasite in embryos and yolk-sac larvae of Baltic cod *Gadus morhua* and turbot *Scophthalmus maximus*. In both species of fish, the prevalence of infection was high, often 20 to 40 %. These prevalences are, however, minimum estimates from screenings of embryos and yolk-sac larvae at low magnifications (25 to 50×).

A similar phenomenon was reported by Hollande & Cachon (1952, 1953) who found a lethal dinoflagellate endoparasite, *Ichthyodinium chabellardi*, in the yolk of embryos and larvae of *Sardina pilchardus*.

The aim of the present paper was to assess whether or not the cod infection is limited to cod spawning in the Bornholm Deep in the Baltic sea (Buchmann et al. 1993) and to elucidate the way in which the recently discovered protistan endoparasite (Pedersen 1993a,

Pedersen et al. 1993) is transmitted to the embryo. Further, preliminary studies were made of the consequences of the infection with respect to larval survival potential.

MATERIALS AND METHODS

Studies performed in Helsingør. Mature cod *Gadus morhua* were obtained from local fishermen in Gilleje and Helsingør, both located at the northern part of the Øresund (the strait between Denmark and Sweden) in February and March 1993. Eggs from ca 5 mature female cod were in each case fertilised with sperm from ca 5 males and incubated in running seawater (salinity 31 ppt) at 6 to 8 °C. In one case, however, fertilisation and incubation conditions were different. In order to exclude the possibility of the parasite being transmitted to the embryos via the recirculated seawater in the

laboratory, eggs were fertilised in sterile-filtered (0.22 µm), pasteurised (80 °C for 1 h) seawater in sterile (160 °C for 1.5 h) glass containers, covered with plastic film and incubated at 8 °C in a constant-temperature room. No antibiotics were used, but the sterile-filtered, pasteurised seawater was exchanged 1 to 2 times a day. Whatever the incubation regime, eggs and larvae were screened at intervals for the presence of the parasite at 25 to 50× magnification. For transmission electron microscopy (TEM) of cod, larvae were fixed for 2 to 3 h in 2.5 % cold glutaraldehyde buffered with cacodylate (pH 7.4) and subsequently stained with saturated uranyl acetate in 70 % ethanol for 30 min, dehydrated in ethanol and propylene oxide and embedded in Epon.

Studies performed in Bülker Huk. A study on cod embryos was done at a turbot fish farm adjacent to the Baltic near the mouth of the Kieler Fjord. Fertilised cod eggs were obtained from a cod broodstock on the island of Bornholm in the Baltic. In addition, 2 batches of fertilised cod eggs were obtained from Lysekil at the Swedish west coast. The eggs were incubated at 5.5 to 6.5 °C in running seawater at 22 ppt. For TEM of turbot *Scophthalmus maximus* parasites, 1 batch of fertilised eggs was transferred to Helsingør. Newly hatched infected larvae were fixed in the same way as above with the addition of a postfixation in osmium tetroxide.

RESULTS

Prevalences and geographical distribution

Eight batches of cod *Gadus morhua* embryos were studied (Table 1), and all batches were infected. The recorded prevalences of infection, as observed at 25 to 50×, varied from 14 to 88 %. Fig. 1A shows a typical, medium sized (45 µm) endoparasite located in the yolk

sac of a young cod larva. Most embryos and larvae contained 1 to 2 large parasites, but up to 73 large and medium sized parasites have been observed in individual embryos (50×). Table 1 shows the areas from which the parent fish originated. As in a previous study (Pedersen et al. 1993), infected embryos and larval offspring were found from Baltic cod. In addition, embryos from cod from the Øresund, Kattegat and North Sea were infected.

Screenings for the presence of the parasite (at 25 to 50×) were done at the egg stage (Table 1). The optimum time for screening was just after gastrulation, at the stage when the body of the embryo surrounds half the periphery of the yolk. At this stage, there is no free tail, which may otherwise obscure the parasite. Also, the parasite seems to increase its diameter with time, young parasites being small with a smooth surface, older parasites large (70 to 80 µm) with a highly irregular surface.

Transmission of the endoparasite to the embryo

Batch 4 H (Table 1) was kept apart from contact with unsterilised seawater during fertilisation and incubation. The embryos were screened at the optimum stage and had a high rate of infection (Table 1). The embryos contained the typical parasites as shown in Fig. 1A, some also contained transparent, refractile spheres in the yolk at the caudal end of the embryo, and 1 embryo had such spheres with inclusions in it (see below).

Survival potential of infected embryos

Two experiments were performed under different abiotic conditions, one in Helsingør and one in Bülker

Table 1 *Gadus morhua*. Prevalence and geographical distribution of the endoparasite. Date of fertilisation, date of screening for parasites, infection prevalence in % and date of hatching for the different batches of cod eggs. Area: area of origin of the mature parent fish. H and B designate batches studied at Helsingør and Bülker Huk, respectively. nm: not measured. All batches were infected

Batch	Fertilised	Screened	Prevalence		Hatched	Area
			%	n		
1 H	8/2	19/2	14	72	23/2	Øresund
2 H	11/2	24/2	22	46	26/2	Øresund
3 H	14/2	25/2	20	15	1/3	North Sea
4 H	24/2	2/3	33	42	8/3	Øresund
5 H ^a	7/3	12/3	88	57	22/3	Øresund
6 B	12/5	17/5		nm	nm	Kattegat
7 B	13/5	17/5		nm	27/5	Kattegat
8 B	28/5	1/6		nm	9/6	Baltic

^a In contrast to the other prevalences, the prevalence for this batch includes embryos with refractile spheres in the yolk

Fig. 1. Light microscopy of parasites in live *Gadus morhua*. (A) A medium-sized spherical parasite in a yolk-sac larva. Note the irregular surface. Scale bar = 20 μ m. (B) Two parasites in the heart of a yolk-sac larva (arrow). Scale bar = 20 μ m. (C) A large (70 μ m), very irregular parasite in the interstice between the yolk-sac membrane and the surface epithelium of an embryo (arrow). A small (12 μ m) parasite is located between the eggshell and the surface of the embryo (arrowhead). e: eggshell, s: surface epithelium of embryo; y: yolk-sac membrane. Scale bar = 30 μ m. (D) A thin-walled structure ejects multiple parasite-like bodies of varying sizes into the yolk. Scale bar = 30 μ m. (E) A thin-walled structure with a disintegrating membrane (right) releases parasite-like bodies into the yolk. Scale bar = 20 μ m. (F) A yolk-sac larva with highly refractile spheres in the caudal end of the yolk sac (arrow). Scale bar = 300 μ m. (G) Thick-walled, highly refractile spheres in the yolk sac of the larva in (F). One sphere filled with unidentified contents (arrow) is adjacent to a group of 3 empty spheres (arrowhead). Scale bar = 30 μ m.

Huk, respectively. In Helsingør, 13 infected and 21 uninfected embryos from batch 4 H were sorted out on March 3 (Table 2) and strict isolation of these embryos from unsterile seawater was terminated. To avoid

growth on the eggshells, incubation was done in the dark, but the eggshells were nonetheless covered with a brownish overgrowth from March 9. The number of live or dead eggs and larvae was recorded (Table 2).

Table 2. *Gadus morhua*. Survival of infected embryos. Time to hatching and death rates in infected and uninfected eggs and larvae from batch 4 H. Incubation at 8 °C was done in glass jars, each holding 10 to 13 eggs and 200 ml filtered (0.22 µm) seawater. Mortality is recorded relative to the previous observation date. The yolk supply was exhausted in the larvae but not in the embryos between March 16 and 19. The remaining embryo on March 19 hatched the following day. e: embryos, l: larvae

Date (March)	No. uninfected		No. infected	
	Live	Dead	Live	Dead
3	21 e	0	13 e	0
5	21 e	0	13 e	0
8	16 l + 4 e	1 l	3 l + 10 e	0
9	14 l + 1 e	5 l	5 l + 5 e	2 l + 1 e
10	13 l	1 l + 1 e	5 l + 4 e	1 e
11	13 l	0	5 l + 3 e	1 l
12	11 l	2 l	5 l + 2 e	1 l
13	11 l	0	5 l + 2 e	0
14	9 l	2 l	6 l + 1 e	0
15	9 l	0	6 l + 1 e	0
16	9 l	0	6 l + 1 e	0
17	9 l	0	5 l + 1 e	1 l
18	9 l	0	5 l + 1 e	0
19	9 l	0	4 l + 1 e	1 l

The uninfected group had finished hatching on March 10, whereas the infected group had not finished hatching 9 d later. The larvae hatching later than March 12 swam in tight spirals and were incapable of normal swimming (more infected larvae than reported here were studied, i.e. a total of 21 infected embryos. The missing 8 embryos are excluded from Table 2 because the diagnosis of infection in these specimens was difficult. In all, 5 spiral swimmers were observed). The infected embryos were lying on the bottom of the jars from March 10 onwards. Until then like the uninfected group the infected embryos had remained at the surface. From March 12, the eggshells of infected embryos were seen to be irregular in shape and the shells vibrated due to embryonic movements.

The overall mortality rate of the uninfected embryos was 57 % and that of the infected group was 62 %. As 2 of the survivors in the infected group were spiral swimmers, the mortality estimate for this group is 77 %.

The main portion of eggs from batch 4 H was transferred to a 114 l tank with water flow 4 d before hatching. Newly hatched larvae were screened for the presence of the parasite, and the incidence had decreased from 33 % (Table 1) to 2.5 % (n = 80). The tank bottom contained numerous dead larvae and unhatched eggs.

From batch 7 B (Table 1) infected and uninfected cod embryos were isolated in 2 glass jars, each containing 250 ml seawater, on May 17. The embryos surrounded half the circumference of the yolk. The 2 groups of em-

bryos were studied at infrequent intervals (Table 3). The experiment had to be terminated when the uninfected group had finished hatching. At this time 1 unhatched egg remained in the infected group. The mortality rate of the uninfected group was 0 % and that of the infected group was 82 %. Normal eggs and larvae were found at the surface, dead and dying eggs at the bottom of the jars.

Other features associated with endoparasitic infection

When infected larvae – screened at 25 to 50× – were observed in light microscopy at 250×, several smaller parasites (ca 10 µm) were seen in addition to the large ones (ca 80 µm) used to diagnose infected from uninfected animals. The smaller parasites were often located in the yolk adjacent to a large parasite, but they were also encountered in the loose connective tissue surrounding the heart. Also, a typical parasite was seen in the lumen of the beating heart of a larva (Fig. 1B). Even large parasites were occasionally found outside the yolk sac. In embryos and yolk-sac larvae large parasites have been observed in the interstice between the membrane surrounding the yolk and the surface epithelium covering the yolk sac (Fig. 1C).

In some cases the large parasites seemingly disappeared with time. In embryos screened on May 24 (Table 3) the large parasites were clearly visible in some specimens whereas they could no longer be observed in others (50×). Large parasites have a highly irregular surface structure (Fig. 1C) and can apparently break up into smaller organisms.

There seem to be at least 2 ways in which the parasites can multiply in the yolk. One is the budding off of smaller parasites from the surface of a large specimen as indicated in Fig. 1C (also cf. Pedersen et al. 1993). The other is the ejection of numerous parasite-like bodies from thin-walled structures (Fig. 1D, E). Part of the thin membrane disintegrates (the larvae were not pressed by the cover slip) and the content is liberated into the yolk.

Table 3. *Gadus morhua*. Mortality in infected and uninfected embryos and larvae from batch 7 B. Mortality is recorded relative to the previous observation date. e: embryos, l: larvae

Date (May)	No. uninfected		No. infected	
	Live	Dead	Live	Dead
17	16 e	0	17 e	0
24	2 l + 14 e	0	2 l + 14 e	1 e
26	7 l + 9 e	0	1 l + 4 e	7 l + 4 e
27	16 l	0	2 l + 1 e	2 l

Finally, another phenomenon is associated with endoparasitic infection. Many embryos and larvae of both cod and turbot contain highly refractile, thick-walled spheres in the yolk sac. The spheres are always adjacent to fish tissue. Frequently, these spheres are found at the caudal end of the embryo (Fig. 1F), but they can be located anywhere along the axis of the body. Some of the spheres are seemingly empty (Fig. 1G) and others have contents (Fig. 1G).

TEM of cod parasites

In cod, an empty refractile sphere and a sphere containing a large multinucleated parasite were studied using TEM. The nuclei of the wall of the sphere were often very flat, elongated and branched (not shown), but they can also have an oval appearance (Fig. 2A). The mitochondria in the cells of both the empty sphere and the sphere containing the large parasite had tubular cristae (Fig. 2B). The inside of the sphere wall exhibited many delicate extensions (Fig. 2A to C). With 1 exception (Table 1, batch 5 H) embryos containing these protistan spheres were not included in the prevalences of infection. Further, selections of embryos for the mortality studies were based on the presence of a large parasite as depicted in Fig. 1A, C, so embryos containing refractile spheres only were excluded.

TEM of turbot parasites

Fig. 3A shows mitochondria with tubular cristae from a turbot parasite. The cytoplasm is scarce, and the endoparasite is densely packed with organelles necessary for protein synthesis (mitochondria and ribosomes). Fig. 3B shows a typical endoparasite nucleus which exhibits dense chromatin near the periphery. The parasite contained numerous nuclei, and cell membranes were evident (Fig. 3C). A flagellum protruding into the intercellular space was observed (Fig. 3C).

DISCUSSION

The present endoparasitic infection in cod *Gadus morhua* is geographically widespread as it occurs in all the major seas surrounding Denmark (Table 1). Further, the endoparasitic infection has been observed in turbot *Scophthalmus maximus* (Pedersen 1993a) and – assuming the 2 parasites are identical (see below) – the parasite seems to be nonspecific with regard to the host. Although mature cod and turbot are very different they share features such as having pelagic eggs and preying on benthos. In contrast, the parasite has

never been observed during previous experiments on larval herring *Clupea harengus* (Pedersen, 1984, 1993b, Pedersen et al. 1987, 1990, Hjelmeland et al. 1988, Pedersen & Hjelmeland 1988, Pedersen & Andersen 1992, Ueberschär et al. 1992) the parents of which have benthic eggs and pelagic feeding. The infective stage of the parasite could predominantly be pelagic and thus infect the embryos of cod and turbot. The present paper shows, however, that the embryos were not infected from the seawater in the laboratory so the infective agent seems to be transmitted via the fish gametes. This finding is supported by the recorded prevalences of 0 to 21.5 % in offspring from different females belonging to the same turbot broodstock (Pedersen 1993a). At present we do not know how the mature fish were infected, but it is possible that an infective stage of the parasite is present in the benthic prey of cod and turbot.

For practical reasons, the prevalences were recorded at 25 to 50× magnification, and such prevalences are minimum estimates. There are several reasons for this. First, the higher the magnification, the more parasites are found; and second, the parasites are difficult to observe, as they are frequently covered by the tail of the embryo or, in case of turbot, by the oil droplet in the yolk. Further, the prevalence declines with time due either to the breaking up of larger specimens into numerous very small parasites (Pedersen et al. 1993) or to the dying off of infected animals. Finally, at the time of recording the prevalences, we were unaware that the thick-walled refractile spheres were of protistan origin (tubular mitochondrial cristae). The frequency of these spheres was high, and if included these spheres would add considerably to the rates of infection (cf. batch 5 H, Table 1).

The survival potential of infected cod embryos was reduced compared to that of the uninfected embryos. The experiment with animals from batch 4 H (Table 2) suggests that infected embryos lose their buoyancy and tend to hatch late. The late hatchers were incapable of normal swimming and would not have been able to catch any prey. Although the estimated mortality of the infected specimens was higher than that of the uninfected group, there was a high background mortality in the latter group. The background mortality may have been due to the organism causing the overgrowth of the eggshells.

In the experiment with cod embryos from batch 7 B (Table 3) 1 infected egg hatched late. A comparison of the background mortality of the uninfected group (0 %) with that of the infected group (82 %) indicates that the parasite is associated with mortality in cod embryos and larvae.

Until now controlled survival tests on infected turbot embryos have not been done. Prior to such tests meth-

ods have to be developed for testing the validity of the diagnosis of uninfected embryos. The rate of development in turbot embryos and parasites is very accelerated compared to that of cod, and large turbot parasites can apparently disappear in 1 d, which makes the diagnosis of controls (50 $\times$) problematic.

The endoparasite described by Hollande & Cachon (1952, 1953) multiplied in the yolk and finally burst the yolk sac of the larvae. In contrast, the present parasite does not rupture the yolk sac, it is invasive and has been observed in the heart, in connective tissue and beneath the surface epithelium of the embryos (Fig. 1B,

Fig. 2. TEM of thick-walled refractile spheres in the yolk of *Gadus morhua*. (A) Section of the wall of an empty refractile sphere showing part of a nucleus. Note the delicate extensions towards the lumen (L). Scale bar = 0.5 μ m. (B) Detail of a thick-walled, refractile sphere showing mitochondria with tubular cristae. L: lumen. Scale bar = 0.5 μ m. (C) A refractile sphere (arrowhead) enclosing a multinucleated endoparasite (bottom half). Scale bar = 1 μ m

C). With access to the blood, it is assumed that the parasite can be found in all larval tissues and organs.

Once inside the embryo, the parasite seems to go through several stages. As the parasite was not observed until gastrulation had begun, the infection probably starts with a small mononucleated cell which grows, presumably nourished by the yolk, and multiplies its nuclei. The larger parasites have been shown to be multinucleated in TEM and appeared to be

breaking up into numerous mononucleated cells (Pedersen et al. 1993). In addition to the budding off of small cells, the present study also shows the expulsion of many large and small parasite-like bodies from thin-walled structures (Fig. 1D, E). The 2 modes of propagation may represent different life stages of the parasite.

At present we cannot interpret the significance of the thick-walled, highly refractile spheres in the yolk of cod and turbot. The spheres are of protistan nature,

Fig. 3. TEM of endoparasite in the yolk of larval *Scophthalmus maximus*. (A) Parasite mitochondria with tubular cristae (arrows). Scale bar = 0.2 μ m. (B) A parasite nucleus with condensed chromatin. Scale bar = 1 μ m. (C) Membrane separating adjoining cells in the parasite (arrowheads). A flagellum protudes from one cell into the intercellular space (arrow). n: nucleus. Scale bar = 0.2 μ m

but the processes which take place inside them are unknown.

TEM of the parasite in turbot showed it to be a protist (tubular mitochondrial cristae) and bases of 2 flagella were found in the multicellular stage of the parasite. The nuclei, with chromatin condensations at the periphery, are similar to those of the cod parasite (cf. Pedersen et al. 1993). Also, the appearance of the cytoplasm, densely packed with mitochondria, ribosomes and vacuoles, and – apart from the flagellum – containing no other organelles, is the same as found in the cod parasite. We believe that the cod and turbot parasites are either identical or extremely closely related.

Considering the high prevalences, the apparently reduced viability of infected cod embryos and larvae and the invasive nature of the infection, we think that part of the high mortality encountered in the early life stages of cod may be caused by the present endoparasite.

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