Vol. 130: 235–240, 2018
https://doi.org/10.3354/dao03272

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published September 27

NOTE

Horizontal transmission of *Endolimax piscium*, causative agent of systemic amoebiasis in Senegalese sole *Solea senegalensis*

M. Constenla[1,*,], F. Padrós[1,**], A. Villanueva-González[2], R. del Pozo[3], O. Palenzuela[3]**

[1]Departament de Biologia Animal, de Biologia Vegetal i d'Ecologia and Servei de Diagnòstic Patològic en Peixos, Universitat Autònoma de Barcelona, 08193 Barcelona, Spain

[2]Estación de Ciencias Mariñas de Toralla (ECIMAT), Illa de Toralla, E-36331 Vigo, Spain

[3]Instituto de Acuicultura de Torre de la Sal (IATS-CSIC), 12595 Castellón, Spain

ABSTRACT: Systemic amoebiasis of Senegalese sole *Solea senegalensis* is caused by *Endolimax piscium* Constenla, Padrós & Palenzuela, 2014 a cryptic parasitic member of the Archamoebae whose epidemiology is yet unknown. To test whether the parasite can be transmitted horizontally, an experimental trial by cohabitation between non-infected and infected fish was designed. Transmission of the parasite from naturally infected to healthy fish was confirmed in the experiment, with the water as the most likely route of infection. Under the conditions of the study, the infection process was remarkably slow, as parasites could be detected by *in situ* hybridization within the intestinal mucosa of recipient fish only after 17 wk of cohabitation, and none of the new hosts displayed clinical signs of disease. Long prepatent period and the need for additional triggering factors for the development of the clinical condition are suggested. The intestinal mucosa is proposed as the tissue where the amoeba can survive as endocommensal, but also as an invasion route from which the parasite would disperse to other organs.

KEY WORDS: *Endolimax* · *Solea senegalensis* · Cohabitation · Transmission · Amoebiasis · Aquaculture

INTRODUCTION

Endolimax piscium Constenla, Padrós & Palenzuela, 2014 is a recently described Archamoebae causing systemic disease in Senegalese sole *Solea senegalensis* Kaup, under culture conditions (Constenla et al. 2014). Although mortality is not high, the disease is mainly characterised by severe and extensive lesions, especially in the skeletal muscle, which make the fish unmarketable and reduce its economic value. Histologically, the condition has been described as an amoebic granulomatous disease (Constenla et al. 2014). In addition to the lesions, presence of the amoebae within the intestinal epithelium of Senegalese sole has been demonstrated using *in situ* hybridization (ISH), not only in clinically diseased fish but also in an unexpectedly high number of asymptomatic animals (Constenla et al. 2016). To date, no treatments have been described to control the disease, and therefore prophylaxis appears as the only option for its management. In this context, it becomes essential to be able to detect the parasite in carrier fish through suitable detection methods (Constenla et al. 2016), but also to investigate mechanisms of transmission of the parasite.

Species in the intestinal amoeba complex (*Entamoeba–Endolimax–Iodamoeba*) present some com-

*Corresponding author: maria.constenla@uab.cat
**These authors contributed equally to this work

mon interesting epidemiological traits. Most of them have a cosmopolitan distribution and some share similar transmission mechanisms, such as the presence of infectious mature cysts in the water or soil and a faecal–oral route. Numerous studies have focused on the transmission of these amoebae involving human stool (Garrido-González et al. 2002, Gomila-Sard et al. 2011, Heredia et al. 2012). The most studied species is *Entamoeba histolytica* Schaudinn, 1903 due to its pathogenicity in humans. *Entamoeba* spp. infections are maintained by transfer of infective cysts from faecally contaminated food or water to non-infected individuals through ingestion (Beaver et al. 1984, Haque et al. 2003). Experimental infection with *Entamoeba histolytica* causing amoebic liver abscess can also be induced in rodents by inoculating virulent trophozoites through various routes (Meerovitch & Chadee 1988), including the portal vein or directly into the liver parenchyma via intraperitoneal injection (Shibayama et al. 1998).

To determine whether *E. piscium* can be horizontally transmitted, and to assess the likely route of entry of the parasite within the Senegalese sole, an experimental infection trial by cohabitation was designed.

MATERIALS AND METHODS

A total of 275 fish were used for the experimental infection trial, which was carried out in compliance with applicable animal welfare regulations and with approval from the University of Vigo ethics committee (procedure approval reference 14rev/12). Two groups of fish were used in this study: a group of control healthy juvenile Senegalese sole (recipients or R) and a group of fish with clinical symptoms of amoebic granulomatous disease (donors or D), as described in Constenla et al. (2014). Both groups were selected from previous epidemiological surveys at different Senegalese sole on-growing farms (Constenla et al. 2016). R fish were obtained from a farm routinely checked for the presence of the parasite (by histology and molecular methods: conventional histology [CH], *in situ* hybridisation [ISH] and polymerase chain reaction [qPCR]) with no cases recorded. A subsample of these fish (n = 25) was also tested with the same 3 tests prior to the beginning of the experiment to confirm their status (Table 1). D fish were selected from a farm where the disease had been detected and confirmed (same methods as above). A subsample (n = 10) was also analysed (same 3 methods as above) before the start of the cohabitation experiment. Fish were transported and acclimated to the experimental conditions at ECIMAT facilities (Vigo, Spain), and 150 D fish (mean weight = 151 g) were placed in a 1500 l fiberglass cylindroconical tank (D-tank) with continuous open flow (750 l h^{-1}) of seawater (37.5‰ salinity) through a sand filter (40 µm nominal) (Brasil Series BL, Kripsol) and under natural photoperiod and temperature conditions (Table 1). A smaller (150 l), quadrangular floating plastic mesh cage was introduced into the D-tank (fastened 70 cm above the D-tank bottom), harbouring the R fish (n = 75, mean weight = 11 g). Central aeration in the tank ensured resuspension of deposited materials and potentially infectious particles.

The individual R:D ratio was approximately 1:2 (1:9 in biomass at the start of the experiment) and the cohabitation condition remained for 4 mo (April–August 2012). This system allowed cohabitation of fish while preventing direct contact between the 2 stocks. Fish were fed daily ad libitum (one feeding event per day) with commercial pelleted diets (LE

Table 1. Samplings performed during the experimental infections between April and August of 2012 with prevalence (P%) of *E. piscium* within intestine (Int) and muscle (Mu) from conventional histology confirmed by *in situ* hybridisation (CH+ISH) and real-time polymerase chain reaction test (qPCR). dpe: days post-exposure; n: number of fish; TW: total weight (g; mean ± SD); TL: total length (cm; mean ± SD)

Sampling	Time (dpe)	Water temp. (°C)	Fish group	n	TW	TL	CH+ISH		qPCR	
							P%Int	P%Mu	P%Int	P%Mu
0	0	15	Control	25	12.44 ± 4.36	10.40 ± 2.36	0.00	0.00	0.00	0.00
1	15	14–15	Recipient	13	12.75 ± 4.27	10.70 ± 1.04	0.00	0.00	7.69	7.69
2	30	14–18	Recipient	14	13.10 ± 4.82	9.79 ± 1.30	0.00	0.00	21.43	14.29
3	45	16–18	Recipient	15	10.10 ± 2.68	9.63 ± 2.13	0.00	0.00	13.33	0.00
4	60	17–20	Recipient	15	9.11 ± 3.19	10.27 ± 1.16	0.00	0.00	0.00	6.67
5	130	17–21	Recipient	14	9.30 ± 4.41	10.42 ± 1.38	28.57	0.00	42.86	21.43
			Control	13	14.47 ± 6.14	13.39 ± 4.42	0.00	0.00	0.00	0.00

feed, range 2–5 mm, Skretting España) according to their size. In addition, a smaller control tank, kept under the same conditions as the D-tank, was maintained in a different isolated room with 25 fish from the same stock of the recipients.

Groups of 13–15 R fish and 5–10 D fish were sampled at different days post-exposure (dpe) (Table 1). At each sampling point, fish were euthanized with an overdose of anaesthetic (phenoxyethanol), measured and weighed (Table 1). Fish were necropsied and examined for macroscopical lesions, and pieces of muscle, liver, spleen, kidney, digestive tract, heart and gills were fixed in 10% buffered formalin for histopathological studies. Muscle samples were consistently taken from the medial dorsal zone. Skeletal muscle and intestine are the main target tissues for *E. piscium* as previously reported (Constenla et al. 2016) and thus portions (0.125 μm^3) of both organs were also preserved in 90% ethanol for molecular diagnostic tests. In addition, water samples (500 ml) were taken from each tank at 0, 1, 2 and 4 mo. The water was filtered through 5 µm pore cellulose ester filters (Millipore ref. SMWP04700), which were then fixed in 100% acetone and stored at 4°C until use.

The presence of *E. piscium* stages in the different organs within the fish was studied using 3 methods: (1) CH: fixed samples of different organs were processed by routine histology and sections (4 µm) were stained with haematoxylin and eosin (H&E); (2) ISH: paraffin sections (5 µm) of intestines and muscle, and from other internal organs presenting lesions, were processed for ISH as described in Constenla et al. (2016); (3) qPCR: samples of muscle and intestine preserved in ethanol, and water filters, were tested by qPCR as previously described in Constenla et al. (2016). Cellulose ester filters were previously dissolved in acetone using the method of Sanders & Kent (2011), with some modifications. A tissue or sample was considered infected when stages of the parasite were identified by CH and confirmed by ISH, or when DNA amplification was achieved through qPCR.

RESULTS AND DISCUSSION

During the first month of the trial, an unexpected outbreak of *Tenacibaculum* was detected. Approximately 40% R fish presented progressive erosion on the caudal fin, and 3 of them died. Fish were treated accordingly (1 h bath of 50 ppm oxytetracycline [Oxytevall®] in closed circuit for 3 consecutive days) and the episode remitted. No further mortality was registered till the end of the trial.

The condition of R fish at the different sampling points, as determined by ISH and qPCR, are summarised in Table 1. In the first month (15 and 30 dpe), 6 R fish were found positive through qPCR in intestine, muscle or both, and these positives coincidentally were animals affected by *Tenacibaculum*. The intensity was low, with threshold cycle (Ct) values >30 except for 2 fish. Histopathological examination and ISH tests in both samplings were negative for the amoebae (Table 1).

During the second month of the trial (45 and 60 dpe), some cells compatible with amoeboid organisms were observed by light microscopy within the intestinal epithelium of 5 R fish.

However, ISH could not confirm the presence of *E. piscium* (Table 1). Only 1 muscle and 2 intestine samples from 3 different fish were found positive through qPCR, but again with Ct >30.

At the end of the experiment (130 dpe), no macroscopical lesions were found in any of the R fish. However, CH and ISH clearly confirmed relevant numbers of *E. piscium* in 4 of the intestines from the R fish (Fig. 1) (Table 1). Samples from the same animals and 2 additional fish were also found positive by qPCR in the intestines, whereas only 3 fish were positive at the muscle (Table 1). Differing from the previous samplings, Ct values of qPCR in intestine samples were compatible with moderate infection levels in several individuals. No presence of *E. piscium* was detected in any other organ of R fish in any of the samplings.

Control fish sampled at the end of the trial (n = 13) did not present any lesion or clinical sign associated with systemic amoebic disease, and they were negative for the parasite by all diagnostic techniques.

From these results, it is clearly demonstrated that *E. piscium* can be horizontally transmitted to healthy fish by cohabitation with naturally infected animals. This study also demonstrates that no direct contact between fish is necessary for the contagion to occur. Contact with contaminated water seems sufficient for the transmission, although faecal transmission could not be evaluated in this study. In intensive culture conditions, the faecal–oral contagion route is likely to occur readily between bottom-dwelling flatfish reared at high density. Positive qPCR results were obtained from water samples taken from the experimental tank at the end of the experiment, confirming it as the vehicle for the transmission of the parasite. Water samples of the control tank were all negative. Unfortunately, it is not possible to determine the type

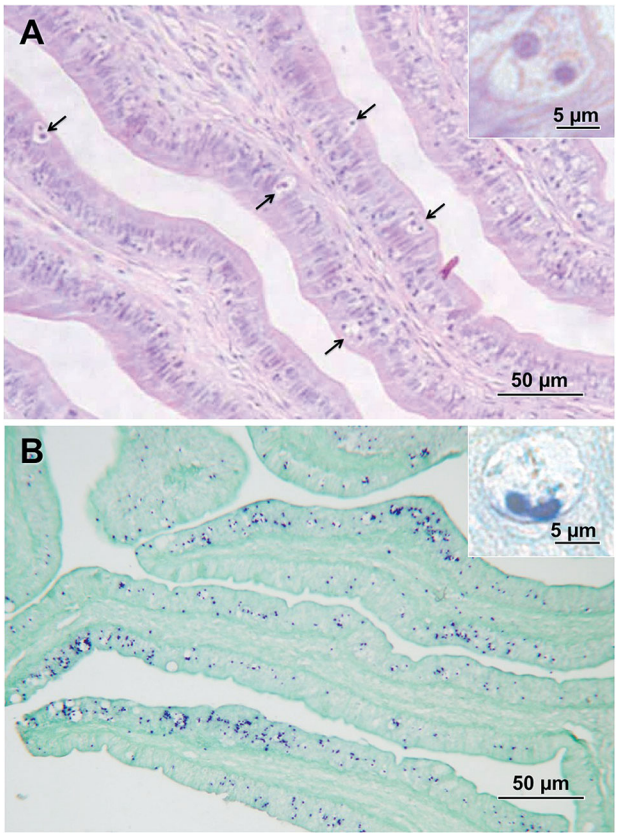

Fig. 1. Histological sections of intestines from Senegalese sole recipient fish sampled at the end of the experimental infections (4 mo post-exposure). (A) Several amoeba-like cells within the intestinal epithelium (H&E stain). Inset: detail of these organisms; (B) *E. piscium* ISH-positive signal (purple cells) in a section of the same intestine. Inset: detail of *E. piscium* ISH-positive

of stages detected through these qPCR tests. Entamoebidae species include parasites with 2 stages in their life cycle: a trophozoite and a cyst (Silberman et al. 1999). The latter is the resistant stage, which allows the parasite to remain in the environment after leaving the host and until reaching new hosts to continue their life cycle. Recently, cyst-like structures ('pseudocysts') were described for *E. histolytica* as a rapid survival response of trophozoites to a stressful condition (Luna-Nácar et al. 2016). In the present study, as in previous thorough analyses of infected fish (Constenla & Padrós 2010, Constenla et al. 2014), *E. piscium* cyst or 'pseudocyst' stages were never identified. This contrasts with the closest related terrestrial Entamoebidae species, and it seems to suggest that the transmission of *E. piscium* between fish can be achieved without cysts.

Other marine fish intestinal parasites like *Enteromyxum* spp. (Myxozoa) are exceptional cases among their group in which a direct transmission can occur without the involvement of spores or physically resistant stages. In these parasites, proliferative cells dwell in the paracellular space in intimate contact with host cells at the intestinal mucosa, and strips of epithelium can be released within mucous casts, offering some protection until they reach a new host (Redondo et al. 2004, Sitjà-Bobadilla & Palenzuela 2012). The niche of *E. piscium* in the intestinal epithelium would allow an analogous mechanism of dissemination from trophic stages in the aquatic environment.

However, we cannot discount the possibility of cystic or 'pseudocystic' stages in *E. piscium*. These could be quite scarce, have exogenous development, or be morphologically indistinguishable and overlooked in our studies. This possibility is important for fish farm prophylaxis because amoeba cysts can be very resistant to temperature, desiccation and disinfection treatments (Coulon et al. 2010).

Another relevant conclusion of this study is that under these conditions, the infection process seems remarkably slow. Although they were scattered, weak positive samples were recovered by qPCR from intestine and muscle samples during the first month, but it was only after several months that several fish yielded positive results in different organs and through different detection methods and stages of *E. piscium* could be directly detected by ISH in the intestine. This observation does not seem accidental, since incubation periods by free-living amoeba diseases in humans are usually considered to be of long duration and several weeks or months may elapse (Visvesvara et al. 2007).

Invasive intestinal disease by *E. histolytica* can be developed months or years after an initial colon infection (Stanley 2003), suggesting that these long incubation periods could be a common feature in this entire group of amoebae.

In 90% of cases amoebic infections are asymptomatic and self-limited (Haque et al. 2003), however many amoebae are opportunistic pathogens that can spread throughout the organism resulting in disease outbreaks affecting different organs. Most of the D fish in this study (83%) showed *E. piscium* in the intestine with a low to high intensity (Fig. 2A) (based on the score established by Constenla et al. 2016). Although some of these only contained intestinal stages without other alterations, most of them (77%) also presented lesions in other organs, especially muscle lesions (bumps) (Fig. 2B) containing liquefied material under the skin (Fig. 2C) and nodules in the heart (Fig. 2D). The histopathological and molecular

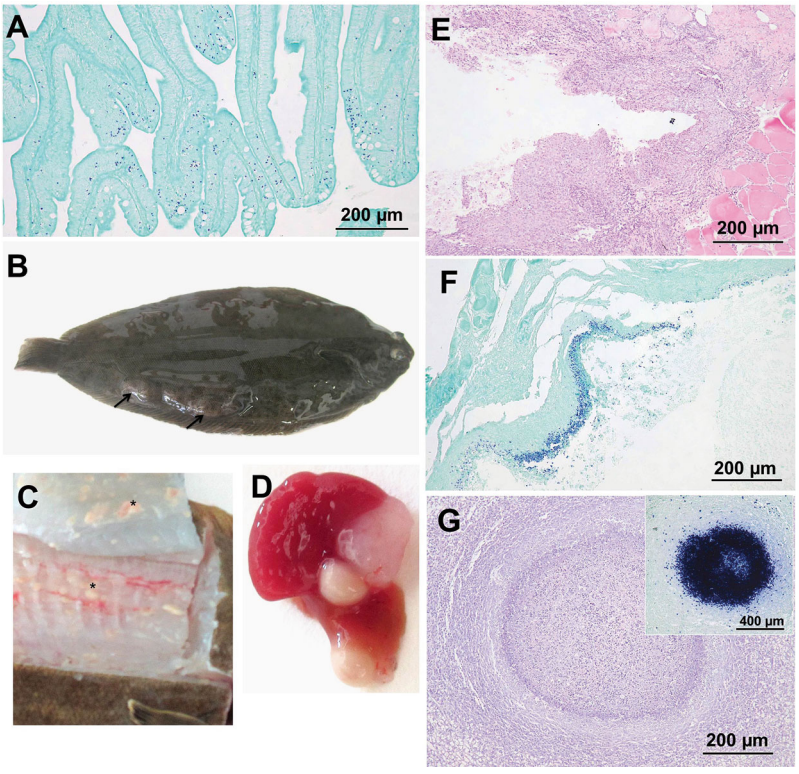

Fig. 2. Macroscopic and histological lesions in different organs from Senegalese sole donor fish. (A) Histological section of intestine infected by *E. piscium* at high degree of intensity (>25 parasite cells observed per field at 100× magnification); (B) ocular side of fish where lumps (arrows) along the base of ventral fin are observed; (C) small lesions (marked with *) with liquefactive aspect within the muscle; (D) 2 circumscribed nodules with abscess-like aspect in the heart; (E) inflammatory reaction progressing among muscle fibres (H&E stain); (F) same inflammatory reaction in muscle with *E. piscium* ISH-positive signal in blue; (G) granulomatous inflammatory reaction (composed by fibroblast and macrophages) with a large core of homogeneous necrotic tissue in liver (H&E). Inset: detail of *in situ* hybridisation-positive signal of *E. piscium* associated to the same lesion in liver

study confirmed that these lesions were caused by *E. piscium* (Fig. 2E,F) and also revealed lesions in the liver (Fig. 2G), kidney and gonad associated with the parasite. Surprisingly, only 15% of the D fish had systemic lesions without parasites being detected in the intestine.

Therefore, results from this and other studies of sole systemic amoebiasis (Constenla & Padrós 2010, Constenla et al. 2014) point to the intestinal mucosa as the main invasion route in the pathogenesis of these organisms. These observations suggest that *E. piscium* can remain endocommensal within the intestinal epithelium with little or no pathogenic role.

At a certain point, the dispersion of the parasite throughout other organs may arise, becoming systemic and causing overt infections and extensive lesions. In some advanced cases, the amoeba could even be undetectable in the intestine, as in some D fish. In our study, systemic infections, causing damage to other organs, could not be attained in the R fish, despite the fact that parasites were detected by qPCR in the skeletal muscle of some fish together with intestinal stages, and as early as 15 dpe. This suggests the need of additional triggering factors or specific conditions for the development of overt clinical infections. Many amoeba outbreaks can be favoured by immunodepression or suboptimal environmental conditions (Nash et al. 1988, Noble et al. 1997). Brain lesions in humans caused by *Acanthamoeba* species, *Balamuthia* or even *Entamoeba*, among others, are usually seen in individuals suffering from other chronic diseases, having compromised immune systems, or during outbreaks of bacterial infections such as *Legionella* and *Mycobacterium* (Visvesvara et al. 2007). Coincidentally, in our study, the first positive cases detected (in the first and second sampling), were fish affected by *Tenacibaculum*.

These filamentous bacteria are known to cause damage in the epidermis, inner layers of dermis and even muscle, and they could provide a gateway to the parasite. With the exception of the few, weak qPCR-positive samples, infections could not be consistently diagnosed by different methods until the last sampling, at 130 dpe. At this point, the increased numbers of positive R fish could partly result from increasing temperatures as the study progressed, approaching the discomfort range for sole (Morais et al. 2014). In any case, even if these factors favoured the transmission to some extent, they did not trigger systemic clinical infections during the >4 mo length of this trial. In our previous surveys in sole farms we found sole with 12 cm size (i.e. roughly 4 mo old) as the youngest age class with confirmed granulomatous lesions due to *E. piscium*, although most cases affected older fish (M. Constenla unpubl. data). Thus, it seems that the development of clinical infections in our experimental fish would have required a longer period and/or additional triggering factors.

Acknowledgements. This work was partially supported by the research grants PROMETEO 2010/006 and ISIC 2012/003 (Generalitat Valenciana). Experiments in ECIMAT were supporting by the Programme for Consolidation of Competitive Research Units (Xunta de Galicia), cofounded by the European Regional Development Fund. M.C. was supported by a PhD student grant from the Universitat Autònoma de Barcelona ('P.I.F. Programme').

LITERATURE CITED

Beaver PC, Jung RC, Cupp EW (1984) Clinical parasitology. Lea & Febiger, Philadelphia, PA

Constenla M, Padrós F (2010) Histopathological and ultrastructural studies on a novel pathological condition in *Solea senegalensis*. Dis Aquat Org 90:191–196

Constenla M, Padrós F, Palenzuela O (2014) *Endolimax piscium* sp. nov. (Amoebozoa), causative agent of systemic granulomatous disease of cultured sole, *Solea senegalensis* Kaup. J Fish Dis 37:229–240

Constenla M, Padrós F, del Pozo R, Palenzuela O (2016) Development of different diagnostic techniques for *Endolimax piscium* (Archamoebae) and their applicability in *Solea senegalensis* clinical samples. J Fish Dis 39: 1433–1443

Coulon C, Collignon A, McDonnell G, Thomas V (2010) Resistance of *Acanthamoeba* cysts to disinfection treatments used in health care settings. J Clin Microbiol 48: 2689–2697

Garrido-Gonzalez E, Zurabian R, Acuna-Soto R (2002) Cyst production and transmission of *Entamoeba* and *Endolimax*. Trans R Soc Trop Med Hyg 96:119–123

Gomila-Sard B, Toledo Navarro R, Esteban Sanchis JG (2011) Amebas intestinales no patógenas: una visión clinicoanalítica. Enferm Infec Microb Clin 29:20–28

Haque R, Huston CD, Hughes M, Houpt E, Petri WA Jr (2003) Current concepts: amebiasis. N Engl J Med 348: 1565–1573

Heredia RD, Fonseca JA, López MC (2012) *Entamoeba moshkovskii* perspectives of a new agent to be considered in the diagnosis of amebiasis. Acta Trop 123: 139–145

Luna-Nácar M, Navarrete-Perea J, Moguel B, Bobes RJ, Laclette JP, Carrero JC (2016) Proteomic study of *Entamoeba histolytica* trophozoits, cysts and cysts like structures. PLOS ONE 11:e0156018

Meerovitch E, Chadee K (1988) *In vivo* models for pathogenicity in amebiasis. In: Ravdin JI (ed) Amebiasis: Human infection by *Entamoeba histolytica*. Wiley, New York, NY, p 177–190

Morais S, Aragão C, Cabrita E, Conceição LEC and others (2014) New developments and biological insights into the farming of *Solea senegalensis* reinforcing its aquaculture potential. Rev Aquacult 8:227–263

Nash G, Nash M, Schlotfeldt HJ (1988) Systemic amoebiasis in cultured European catfish, *Silurus glanis* L. J Fish Dis 11:57–71

Noble AC, Herman RL, Noga EJ, Bullock GL (1997) Recurrent amoebic gill infestation in rainbow trout cultured in a semiclosed water recirculation system. J Aquat Anim Health 9:64–69

Redondo MJ, Palenzuela O, Alvarez-Pellitero P (2004) Studies on transmission and life cycle of *Enteromyxum scophthalmi* (Myxozoa), an enteric parasite of turbot *Scophthalmus maximus*. Folia Parasitol 51:188–198

Sanders JL, Kent ML (2011) Development of a sensitive assay for the detection of *Pseudoloma neurophilia* in laboratory populations of the zebrafish *Danio rerio*. Dis Aquat Org 96:145–156

Shibayama M, Campos-Rodríguez R, Ramírez-Rosales A, Flores-Romo L, Espinosa-Cantellano M, Martínez-Palomo A, Tsutsumi V (1998) *Entamoeba histolytica*: liver invasion and abscess production by intraperitoneal inoculation of trophozoites in hamsters, *Mesocricetus auratus*. Exp Parasitol 88:20–27

Silberman JD, Clark CG, Diamond LS, Sogin ML (1999) Phylogeny of the genera *Entamoeba* and *Endolimax* as deduced from small-subunit ribosomal RNA sequences. Mol Biol Evol 16:1740–1751

Sitjà-Bobadilla A, Palenzuela O (2012) *Enteromyxum* species. In: Woo PTK, Buchmann K (eds) fish parasites: pathobiology and protection. CABI, London, p 163–176

Stanley SL (2003) Amoebiasis. Lancet 361:1025–1034

Visvesvara GS, Moura H, Schuster FL (2007) Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris, Naegleria fowleri*, and *Sappinia diploidea*. FEMS Immunol Med Microbiol 50:1–26

Editorial responsibility: Dieter Steinhagen, Hannover, Germany

Submitted: April 4, 2018; Accepted: July 25, 2018
Proofs received from author(s): September 7, 2018