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Transmission of crayfish plague

Birgit Oidtmann[1,*], Elke Heitz[1], David Rogers[2], Rudolf W. Hoffmann[1]

[1]Institute of Zoology, Fish Biology and Fish Diseases, University of Munich, Kaulbachstr. 37, 80539 Munich, Germany
[2]David Rogers Associates, Aquatic Biological Consultants, 9 The Moat, Castle Donington, Derby DE74 2PD, United Kingdom

ABSTRACT: Two possible means of transmission of crayfish plague were investigated: via fish (as vectors), and via crayfish (as hosts or vectors when dead). The crayfish transmission experiments focussed on both the viability of the fungus in dead crayfish when kept in simulated field conditions, and on the treatments which kill viable forms of *Aphanomyces astaci* within the recently dead host (cadaver). It was found that *A. astaci* remains viable for 5 d, and possibly longer in crayfish kept in water at 21°C after dying of crayfish plague. Heating (boiling for 1 min) was the quickest way of decontaminating crayfish cadavers. Freezing took considerably longer: after 48 h at –20°C, viable stages were still present. It was also found that *A. astaci* is unlikely to survive passage of the gastrointestinal tract of either mammals or birds as no viable stages were found after 12 h at 37°C. Two basic modes of transmission of crayfish plague via fish were investigated: (1) after passage of initially viable forms of *A. astaci* through the digestive tract of fish and (2) via fish skin. If *A. astaci* was fed to fish as infected abdominal cuticle, it was still viable after passage through the gastrointestinal tract. When pure mycelium or spores were fed to fish there was no indication of viable forms of *A. astaci* after passage through the gastrointestinal tract. Transmission via fish skin was not observed under the experimental conditions applied. The results on fish as vectors have practical importance for fish transport and stocking because the present study shows that there is a risk of transmission of crayfish plague via fish faeces. The investigation of crayfish as vectors delivers methods that could be used for treatment of crayfish imported for human consumption into crayfish-plague-free areas. This application is particularly important for the importation of American crayfish, which are in general suspected to carry *A. astaci* in their cuticle.

KEY WORDS: *Aphanomyces astaci* · Crayfish plague · Vectors · Crayfish · Transmission · Fish · Fish transport · Disinfection

INTRODUCTION

Crayfish plague is the most serious disease to affect freshwater crayfish. The fungus *Aphanomyces astaci* causing the disease is highly pathogenic to crayfish species native to Europe, Asia and Australia, but has little or no effect on American crayfish species (Unestam 1969a, 1972, 1975, Unestam & Weiss 1970).

It is presumed that the disease was first brought into Europe from North America, around 1860 (Cornalia 1860, Alderman 1996). Since then, it has spread throughout Europe and has reached the geographically more peripheral areas only in the past 20 yr: England in 1981 (Alderman et al. 1984), Turkey in 1985 (Baran & Soylu 1989, Rahe & Soylu 1989), Ireland in 1987 (Reynolds 1988). The most serious situation is possibly in Sweden, where estimates suggest that 90% of all native crayfish populations have disappeared (Edsman 2000).

Although the existence of crayfish plague has been acknowledged for 140 yr, information regarding pathways of transmission and longevity of the fungus *Aphanomyces astaci* is limited.

In order to be able to understand the possible ways of transmission, it is important to be aware of the life cycle of *Aphanomyces astaci*. The fungus can be present in 3 distinct forms: mycelium, zoospore and cyst. The mycelium is found in the cuticle of either Ameri-

*E-mail: b.oidtmann@zoofisch.vetmed.uni-muenchen.de

can crayfish (carrier crayfish) or susceptible infected crayfish of other origins (European, Asian, or experimentally infected Australian crayfish). Zoospores, the infectious stage of the fungus, are released from the mycelium when the mycelium grows out of the crayfish cuticle. This usually occurs when a susceptible crayfish is severely ill or dead, or, in the case of American species, during moulting or at death. Zoospores are released into the water and a weak chemotaxis helps them to find their way to the next possible host (Unestam 1969b). Zoospores can be transformed into cysts and then again to zoospores, a phenomenon termed 'repeated zoospore emergence' (Cerenius & Söderhäll 1984). Further *A. astaci* life cycle information is presented in Cerenius & Söderhäll (1984, 1985) and Söderhäll & Cerenius (1999).

Several pathways of transmission causing the spread of the fungus have been suggested. Briefly, the possible pathways are: crayfish themselves (American crayfish or infected susceptible species), contaminated water, items that have been in contact with contaminated water, and also animals that either have been in contact with spore carrying water or possibly animals that have been feeding on infected crayfish.

Crayfish can cause the spread of crayfish plague in several ways: American crayfish species carrying the crayfish-plague fungus in an unapparent infection can spread fungal spores into new areas by colonising new habitats. Commercial trade of live crayfish for human consumption, accidental co-transport during fish transport and crayfish used as bait for fishing may assist colonisation of new areas. An unpublished study (Oidtmann et al. unpubl.) shows that the majority of American crayfish populations tested to date carry *Aphanomyces astaci* in their cuticle. Therefore every translocation of American crayfish into previously *A. astaci* free areas converts those areas into crayfish-plague endangered areas; usually it is only a matter of time until susceptible crayfish in such areas develop the disease.

Even more obvious risks for the spread of crayfish plague arise during crayfish plague outbreaks (in susceptible crayfish species). In the course of the disease, susceptible crayfish are progressively paralysed and show abnormal behaviour such as daytime activity (normally crayfish are predominantly nocturnal). This makes them easy prey for an increased range of predators, which may eat the crayfish or abduct them to other locations.

Two studies have contributed knowledge concerning fish as potential vehicles for the spread of crayfish plague. Häll & Unestam (1980) as well as Ahne & Halder (1988) demonstrated that *Aphanomyces astaci* spores germinate on fish scales *in vitro* (Häll & Unestam 1980: salmon scales; Ahne & Halder 1988: common carp and rainbow trout scales). The only published *in vivo* study to date is by Ahne & Halder (1988). In their experiments, transmission of *A. astaci* to noble crayfish via the skin of grass carp *Ctenopharyngodon idella* was not observed during an observation time of 3 wk.

The question of survival times of *Aphanomyces astaci* spores or cysts in water has been dealt with in several studies (Rennerfelt 1936, Unestam 1969b, Cerenius & Söderhäll 1984, 1985, Cerenius et al. 1984, Matthews & Reynolds 1990). The latest study on this subject, presented by CEFAS (2000), has shown that the survival of both spores and mycelium is at least 14 d at 0 to 10°C. The study by Cerenius & Söderhäll (1985) suggests that the survival time might be even longer under favourable conditions due to the possibility of repeated zoospore emergence.

Prior to our current study, the following information was available regarding the survival of *Aphanomyces astaci* under certain environmental conditions and on temperature treatment methods to kill viable stages of *A. astaci.* An unpublished study by Persson & Söderhäll (cited in Smith & Söderhäll 1986) investigated the effects of various temperature treatments and drying on *A. astaci* mycelium and spores *in vitro*. This study (although not published) has for many years been the only source of information concerning possible temperature treatments of spores and mycelium for disinfection purposes. While our study was already in progress, another study was presented by CEFAS (2000), reporting the effect of various temperature treatments on spores and pure cultures of mycelium cultured *in vitro* at a temperature range between +10 and –20°C, as well as at +60 and +70°C. The CEFAS study also included *in vivo* experiments with crayfish infected with *A. astaci* mycelium. Those crayfish were either frozen at –20°C or heated in boiling water.

Most of the studies presented to date were *in vitro* studies. The study recently presented by CEFAS was the first to investigate the longevity of *Aphanomyces astaci* in infected crayfish themselves.

The intention of our study was (1) to investigate decontamination methods of infected crayfish, (2) to determine the limit of the period when infectious stages of *A. astaci* can be released from crayfish that have died due to crayfish plague and (3) to assess the potential role of animals as live vectors, in particular, fish.

MATERIALS AND METHODS

Crayfish cadavers as vectors. ***Experimental animals:*** The treatments (below) were applied to 100 recently dead noble crayfish *Astacus astacus* (20 to 100 g), referred to from this point as cadavers, infected with crayfish plague *Aphanomyces astaci*. Different numbers of animals were used in the treatments because

the infected crayfish did not die simultaneously; treatments had to be set up when sufficient numbers of recently dead (max. 12 h dead) animals were available.

Other crayfish, needed for investigation of infectivity following treatment, i.e. live crayfish needed for contact experiments to test the results, were also noble crayfish *Astacus astacus*, but from an uninfected source.

Treatments of plague-infected crayfish cadavers: The treatments employed were essentially a range of temperature treatments over different time periods. As *Aphanomyces astaci* is particularly sensitive to desiccation (Persson & Söderhäll unpubl., cited in Smith & Söderhäll 1986), a variation imposed between Treatments 1 and 2 addressed this factor. Cadavers were submitted to the treatments as described in Table 1 and outlined below: For Treatment 1 (dry methods), crayfish cadavers were stored in air either at room temperature (ca. 21°C) or at 37°C in an incubator. For Treatment 2 (wet methods), crayfish cadavers were covered in tap water and stored either at room temperature (ca. 21°C) or at 37°C in an incubator. For Treatment 3 (thermal tests), crayfish cadavers were heated in boiling water for 1 or 6 min. In Treatment 4 (freezing), infected crayfish cadavers were stored at –20°C in a freezer.

Investigation of infectivity following treatment: To investigate whether the treated crayfish cadavers still supported viable plague fungus or were able to release infectious zoospores, 2 methods were employed: firstly cultivation, and secondly contact experiments.

Cultivation (to test for infectivity): Soft abdominal cuticle from the treated cadavers was plated on River-Glucose-Yeast (RGY) agar (Alderman 1982) and the growing fungus was subcultured on Pepton-Glucose-1 (PG1) agar (Unestam 1965) according to the cultivation (necropsy and plating) technique described in Oidtmann et al. (1999). Fungal cultures obtained after plating samples on agar were identified according to Oidtmann et al. (2002).

Contact experiments (second test for infectivity): Five initially healthy noble crayfish were exposed to cadavers treated as described in Treatments 1 to 4 (21 settings) for a period of 5 d in a 20 l aquarium. After 5 d, the treated cadavers were removed. Water temperature was 16 to 18°C.

Fish as vectors. Two basic modes of transmission of crayfish plague by fish were investigated: (1) oral uptake of potentially infectious material and (2) contact of fish skin to *Aphanomyces astaci* zoospores.

Oral uptake of potentially infectious material: *Fish:* Four species of fish were tested: rainbow trout *Oncorhynchus mykiss* (15 cm), common carp *Cyprinus carpio* (15 cm), eel *Anguilla anguilla* (30 cm) and perch *Perca fluviatilis* (10 cm). The fish were procured from fish farms that were not rearing crayfish. The fish were starved for 36 h prior to force feeding.

Crayfish: Infected crayfish *Astacus astacus* used for force feeding (and exposure of fish skin, see Expts 10, 11, 14 and 15 below) were obtained from a naturally occurring crayfish plague outbreak.

Healthy susceptible noble crayfish *Astacus astacus* used for contact experiments were procured from a crayfish farm.

Force feeding was performed with: (1) Cuticle from *Astacus astacus* that had died of crayfish plague the night before force feeding. Segments of the soft abdominal cuticle infected with hyphae and occasionally eyestalks were fed. Two grams of infected material was fed per meal; the cuticle pieces were no larger than 2 × 2 mm; (2) *Aphanomyces astaci* spores: 1.5 ml of spore solution from the German *A. astaci* strain M1/96 (spore production method after Cerenius et al. 1984), at a concentration of 10 000 spores ml^{-1} was administered per animal; (3) *A. astaci* mycelium. Mycelium from *A. astaci* strain M1/96 was grown in PG1 medium (Cerenius et al. 1984) after subculturing it from cultures grown on PG1 agar.

Prior to force feeding, fish were anaesthetised (rainbow trout, carp and perch with ethyleneglycol-

Table 1. Conditions of treatments of crayfish-plague-infected crayfish cadavers. Cultivation experiments: no. of crayfish cadavers submitted to cultivation per exposure time ÷ experimental setting (total no. crayfish submitted to cultivation in the respective treatment experiment). Contact experiments: no. of crayfish cadavers used for contact experiments per exposure time ÷ experimental setting (total no. crayfish used for contact experiments in the respective treatment experiment)

Treatment	Setting			No. of experimental animals		
	Method	Exposure temperature (°C)	Exposure periods	Per setting (per treatment method)	For cultivation	For contact experiments
1	Crayfish cadavers stored without water	21 37	24, 48 h, 3, 5 d 12, 24, 36 h	4 (28)	1 (7)	3 (21)
2	Crayfish cadavers stored in water	21 37	24, 48 h, 3, 5 d 12, 24, 36 h	4 (28)	1 (7)	3 (21)
3	Boiling	100	1, 6 min	7 (14)	2 (4)	5 (10)
4	Freezing	–20	18, 24, 48 h, 3, 5 d	6 (30)	2 (10)	4 (20)

Table 2. Conditions in transmission experiments, investigating the role of fish as potential transmitters of crayfish plague after oral uptake of infectious material, using noble crayfish as potential receptors (species investigated: rainbow trout, common carp, eel, perch); no. of fish treated per fish species for each experiment: 5; no. of receptor crayfish used in contact experiments for each experiment: 5

Expt	Material force-fed	Amount of material force-fed per meal	Time interval of force feeding (h)	Duration of force-feeding period (d)
1	Infected cuticle	2 g	48	14
2	Spores	1.5 ml (10000 spores ml^{-1})	24	5
3	Mycelium	0.5 g	24	5
4[a]	Infected cuticle	2 g	24	3

[a]Time interval between last force-fed meal and contact experiment with crayfish was 3 d

monoethylether, Merck; eel with MS 222, Sigma). The force feeding was performed with a baby-tracheal tube.

The following experiments were performed: (1) Transmission experiments using noble crayfish as potential receptors (Expts 1 to 4, summarised in Table 2). The fish in Expts 1 to 3 were placed twice consecutively in an 'intermediate tank' for 30 min before returning them to the experimental aquarium, in order to avoid contamination of the water in the final aquarium by infectious material due to regurgitation. Five noble crayfish were placed in each aquarium from the beginning of the experiments onwards. Size of initially healthy crayfish was 3 to 4 cm.

Expt 4 involved force feeding the fish with infected cuticle daily for 3 d followed by a 3 d period of non-feeding. After the period of starvation, the fish were placed in an aquarium with 5 noble crayfish.

(2) Force feeding followed by investigation of digestive-tract contents (Expts 5 to 7, summarised in Table 3). In these experiments, fish were fed only once with infectious material (infected cuticle, *Aphanomyces astaci* spores or *A. astaci* mycelium), and were then sacrificed at certain time intervals after the meal. Their digestive tract contents were investigated macroscopically and microscopically for remains of force-fed material, and additionally by plating digestive tract content on RGY agar.

In Expts 1 to 7 (aquarium size was 60 l) water temperature was 16 to 18°C for perch, rainbow trout and eel, and 18 to 20°C for carp; there was no water flow in Expts 1 to 4, but flowing water (4 exchanges d^{-1}) was used in Expts 5 to 7.

In all experiments where live or dead crayfish were kept in aquaria with fish (Expts 1 to 4 and 8 to 11 below), the crayfish were placed in plastic mesh cages measuring 20 × 15 × 15 cm.

Contact of fish with Aphanomyces astaci spores in the water: A summary of the experimental set-ups is presented in Table 4. Two possible methods of transmission of *A. astaci* via fish skin were tested: Skin injuries were inflicted on rainbow trout *Oncorhynchus mykiss*, average body length 12 cm, before exposing them to *A. astaci* spores, or they were simply exposed to spores without any prior skin injury. Skin injury was applied by wiping off the skin mucus with a sterile swab from 1 cm^2 beside the dorsal fin (according to Svendsen & Bogwald 1997).

Spore solution (500 ml) of *Aphanomyces astaci* strain M1/96 (50000 spores ml^{-1}) was added to the aquarium (30 l; aquarium concentration 833 spore ml^{-1}) immediately after placing the treated fish in it. The rainbow trout were left for 24 h in the static bath-spore suspension; sufficient oxygen was supplied by a diffuser (Expts 8, 9, 12 and 13).

Table 3. Conditions in transmission experiments, investigating the role of fish as potential transmitters of crayfish plague after oral uptake of infectious material, by investigating gut contents of fish after force feeding of infectious material. Species investigated: rainbow trout, common carp, eel, perch

Expt	Material force fed	Amount of material force fed per meal	Time of sacrifice after force feeding	No. of fish per fish species sacrificed for each time delay (from column 4)	Total no. of fish per fish species sacrificed and investigated (total no. of fish investigated)
5	Infected cuticle	2 g	8, 16, 24 h	3	9 (36)
6	Spores	1.5 ml (10000 spores ml^{-1})	2, 4, 10, 14, 24 h	1	5 (20)
7	Mycelium	0.5 g	8, 16, 24, 32, 40, 48 h	1	6 (24)

Table 4. Experimental conditions in transmission of crayfish plague via fish skin. Method: method used to determine whether infectious stages of crayfish plague were released from the fish or were attached to them

Expt	Skin injury	No. of fish used for each experiment	Time of sacrifice after investigation of skin samples	Mode of exposure of fish to *Aphanomyces astaci* spores	Method
8	+	5	Does not apply	Spore solution in aquarium water	Contact experiment with 5 initially healthy crayfish
9	–	5			
10	+	5		Contact with crayfish carrying crayfish plague	
11	–	5			
12	+	10	2, 3, 4, 5, 7, 10, 14 and 21 d[a]	Spore solution in aquarium water	Investigation of skin samples for presence of *Aphanomyces astaci* by light microscopy (fresh preparations) and plating samples of skin on RGY agar
13	–	10			
14	+	10		Contact with crayfish carrying crayfish plague	
15	–	10			

[a]After the start of experiments

In a similar experimental set-up, instead of exposing the rainbow trout to spores alone, the fish were reared with 10 crayfish exhibiting clinical symptoms of an advanced stage of crayfish plague. The crayfish were removed after 5 d (Expts 10, 11, 14 and 15).

After exposure of the fish to spores or infected crayfish, the fish were washed to minimise the risk of a carryover of spores via water. The fish were transferred 3 times to spore-free water tanks and remained for 8 h in each tank.

Two procedures were employed to determine whether infectious stages of crayfish plague were released from the fish or were attached to them: contact with 5 healthy noble crayfish after the washing procedure, or skin was investigated for the presence of fungus by light microscopy (fresh pre-paration) and by plating samples of skin on RGY agar.

Crayfish that died during contact experiments to test for remains of infectivity after oral uptake of potentially infectious material by fish (Expts 1 to 4) or transmission via fish skin (Expts 8 to 11) were investigated for the presence of crayfish-plague infection by standard methods: for the presence of fungal hyphae in the cuticle followed by plating fungus carrying cutide on agar (Oidtmann et al. 1999). The observation period was 6 mo for the contact experiments. Fungal cultures obtained after plating samples on agar were identified according to Oidtmann et al. (2002).

RESULTS

Crayfish cadavers as vectors

The results summarised in Table 5 show: (1) Infected cadavers kept at room temperature (approximately 21°C) in air were still infectious after 48 h. (2) Infected cadavers stored in water at 21°C were still able to

Table 5. Infectivity of cadavers following Treatments 1, 2 and 4. Method: method used to investigate presence of infectious stages. +: live *Aphanomyces astaci*; –: no live *A. astaci*; blank spaces: not tested. Numbers in brackets (Dxx–yy) indicate the first and last day after the start of the experiment when the contact crayfish died. Shaded areas indicate presence of viable *A. astaci* following treatment

Treatment	Exposure conditions	Method	12 h	18 h	24 h	36 h	48 h	3 d	5 d
1	21°C, dry	Contact experiments			+(D27–41)		+(D27–41)	–	–
		Cultivation			+		+	–	–
	37°C, dry	Contact experiments	–		–	–			
		Cultivation	–		–	–			
2	21°C, wet	Contact experiments			+(D12–19)		+(D14–25)	+(D14–27)	–
		Cultivation			+		+	+	+
	37°C, wet	Contact experiments	–		–	–			
		Cultivation	–		–	–			
4	–20°C	Contact experiments		+(D31–42)	+(D34–39)		+(D35–51)	–	–
		Cultivation		+	+		+	–	–

infect initially healthy noble crayfish after 3 d and the fungus could be isolated from the infected cadavers after 5 d. (3) Infected cadavers stored at –20°C were still infectious after 48 h. (4) Exposing infected cadavers to 37°C, either in water or air, killed the fungus within 12 h.

Heat treatment of *Aphanomyces astaci*-carrying cadavers in boiling water killed the fungus within 1 min.

Fish as vectors

Force-feeding experiments

Crayfish-plague transmission could not be effected by force feeding of fish with spore suspension (Expt 2) or pure *in vitro* cultured mycelium of *Aphanomyces astaci* (Expt 3), nor could viable stages of *A. astaci* be observed in the gastrointestinal tract in either case (Expts 6 and 7).

Fish force fed soft abdominal cuticle infected with *Aphanomyces astaci* transmitted crayfish plague to cohabitated crayfish (Expt 1). Pieces of cuticle carrying viable stages of *A. astaci* were also found in the gastrointestinal tract of such fish (Expt 5). Only when such fish had been starved for 3 d following force feeding with crayfish-plague infected cuticle was transmission to cohabitated crayfish not observed (Expt 4).

In Expt 1 (force feeding of fish with *Aphanomyces astaci*-infected cuticle), the mortalities among the crayfish put into contact with the force fed fish were observed on Days 31 to 39 in perch, Days 33 to 47 in carp, Days 37 to 50 in rainbow trout and Days 58 to 64 in eels.

In Expt 5, the crayfish cuticle in the gastrointestinal tracts of fish was found to have been only slightly digested (and remained infective).

Transmission via skin

Transmission to target crayfish via fish skin could not be demonstrated during these experiments (Expts 8 to 11). Also there was no indication of *Aphanomyces astaci* on the fish skin upon viewing fresh preparations of skin samples or by cultivation of skin samples on agar (Expts 12 to 15).

DISCUSSION

This study has focussed on the investigation of 2 possible means of crayfish-plague transmission: via crayfish and via fish.

Crayfish cadavers as vectors

The following questions were addressed in this study: (1) Would fish, mammals or birds be able to release infectious material after eating crayfish carrying *Aphanomyces astaci*? (2) How long do the crayfish cadavers stay infectious? And (3) Which treatments decontaminate diseased crayfish or kill viable stages of *A. astaci* in crayfish, e.g. imported American crayfish?

Our study contributes the following answers to Questions (1) to (3), respectively: (1) Mammals and birds are unlikely to serve in spreading crayfish plague after eating infected crayfish. This assumption is based on our finding that after 12 h at 37°C, no viable stages of *Aphanomyces astaci* were found in such treated crayfish. The question of fish as vectors is addressed below. (2) Crayfish cadavers held in water at 21°C remain infectious for at least 5 d. Crayfish cadavers left at the site of a plague outbreak are therefore a considerable source for further spread of plague. Our experiments did not cover the time period beyond 5 d; therefore, further studies are needed to determine how long the crayfish-plague fungus lives in crayfish cadavers at temperature ranges which can be found in crayfish habitats. The results on the survival of *A. astaci* mycelium at 10°C in the CEFAS study (see Table 6) suggest that this may be considerably longer than 5 d. (3) It was shown that various easy-to-perform treatments can be used in order to decontaminate infected crayfish. This applies not only to crayfish from crayfish-plague outbreak sites, but also to crayfish which are imported for human consumption, or crayfish used as bait for angling purposes. The easiest way to decontaminate crayfish was boiling at 100°C for 1 min. This is in accordance with the results presented by CEFAS. A major difference appears, however, in results concerning treatment at –20°C. Whereas the CEFAS study did not detect viable forms of *A. astaci* 3 h after exposure to –20°C, we were able to cultivate *A. astaci* from crayfish placed at –20°C after 48 h. We suspect that the following factors may be responsible for these differences: In our study, crayfish which had developed crayfish plague in a natural infection were used, whereas in the CEFAS study, crayfish were artificially infected with *A. astaci* mycelium by inserting mycelium into the abdominal musculature through a small cut made with a sterile cataract knife in the ventral intersegmental soft cuticle. In a natural infection, often an extended mycelium develops in the cuticle before the fungus breaks through the basal lamina of the epithelium and penetrates into the body. In the cuticle, the mycelium is to some extent protected against direct aggression by antifungal reaction of the crayfish host. Penetration of the fungal hyphae into the crayfish body usually occurs in several locations, which

poses difficulties for the immune system of susceptible species. The resources of their immune system are finally exhausted and fail. If mycelium is inserted into the crayfish muscle, the fungus lies unprotected and the immune system of the host may be more successful compared to natural infections in fighting against the pathogen. This theory is supported by the fact that the recovery rate from crayfish in the control group of the CEFAS study was only 40% (8 out of 20 animals), whereas the isolation method used in this study achieves a recovery rate of 70% (Oidtmann et al. 1999). However, this explains only part of the differences, as in 40% of the animals investigated by CEFAS, cultivation from frozen animals should still have been possible. Another reason for the lack of cultivation by CEFAS may be based on the different effects of freezing, depending on whether the fungus is in the cuticle or muscle. As muscle tissue consists mostly of water, freezing would be expected to have stronger effects on this tissue compared to cuticle which is mostly free of water.

The results demonstrate that *in vivo* studies are very important in addition to *in vitro* experiments, as the results may be considerably different. In the example of temperature treatments of crayfish cadavers, *Aphanomyces astaci* in the *in vivo* experiments is to some extent insulated by the crayfish cuticle and body and may therefore survive longer, but the fungus may also—depending on the temperature—suffer from toxic substances released due to degradation of crayfish tissue.

A summary of the data reviewed above on the survival times of various forms of *Aphanomyces astaci* when submitted to temperature treatments and of the study presented is shown in Table 6.

Fish as vectors

Besides crayfish, fish are a natural candidate for the transmission of water-borne diseases. Particularly the fact that fish are frequently transported and stocked

Table 6. Viability of *Aphanomyces astaci* after temperature treatments; comparison of present study with scientific literature. Data for spores by CEFAS, according to raw data in report P0056 (CEFAS 2000)

Treatment		*A. astaci* life stage and medium	Duration of treatment after which *A. astaci* was still viable	Duration of treatment after which no viable *A. astaci* was found	Source
Freezing	−20°C	Spores	20 min	12 h	CEFAS (2002)
		Spores		2 h	Persson & Söderhäll (unpubl.) cited in Smith & Söderhäll (1986)
		Mycelium[a]	10 min	20 min	CEFAS (2002)
		Mycelium		2 h	Persson & Söderhäll (unpubl.) cited in Smith & Söderhäll (1986)
	−20°C	Mycelium in crayfish		3 h	CEFAS (2002)
	−20°C, dry	Mycelium in crayfish	48 h	72 h	Present study
	−15°C	Spores	20 min	12 h	CEFAS (2000)
		Mycelium[a]	10 min	20 min	CEFAS (2000)
	−10°C	Spores	24 h	72 h	CEFAS (2000)
		Mycelium[a]	20 min	12 h	CEFAS (2000)
	−5°C	Spores	24 h	72 h	CEFAS (2000)
		Mycelium[a]	7 d		CEFAS (2000)
	0, 5, 10°C	Spores	14 d		CEFAS (2000)
		Mycelium[a]	14 d		CEFAS (2000)
Room temp.	21°C	Mycelium in crayfish	5 d		Present study
	21°C, dry	Mycelium in crayfish	48 h	72 h	Present study
	30°C	Spores		30 h	Persson & Söderhäll (unpubl.)
		Mycelium		30 h	cited in Smith & Söderhäll (1986)
	37°C	Mycelium in crayfish		12 h	Present study
	37°C, dry	Mycelium in crayfish		12 h	Present study
	60°C	Spores		5 min	CEFAS (2000)
	70°C	Mycelium[a]		5 min	CEFAS (2000)
Boiling	100°C	Mycelium in crayfish		1 min	Present study, CEFAS (2000)

[a]Data for mycelium by CEFAS taken from agar plug method, see CEFAS (2000), control after 48 h, data according to document P0047F, disk summary data sheet)

into new water courses afterwards make it an important issue to determine the risks for the spread of plague due to transport of fish.

The ways crayfish plague could be transmitted during fish transport are: (1) spores and/or cysts in the transport water; (2) spores, cysts and/or mycelium on or in the skin of fish; (3) mycelium, cysts and/or spores in the gastrointestinal tract of fish; and (4) crayfish accidentally transported with the fish.

Our study aimed at investigating the possibility of transferring crayfish plague by fish via skin *in vivo*—(2) above—as well as on investigating the possibility of transfer of crayfish plague after passage through the gastrointestinal tract of fish—(3) above. Points (1) and (4) above are outside the scope of this study.

In relation to (2) above, the studies presented by Häll & Unestam (1980) and Ahne & Halder (1988) demonstrated that *Aphanomyces astaci* spores germinate on fish scales *in vitro.* However, in the *in vivo* experiments performed with grass carp, the authors did not observe transmission of crayfish plague (Ahne & Halder 1988). In those experiments, no skin wounds were applied to the fish. Transmission to noble crayfish was not observed during the observation time, which was up to 3 wk.

In our study, minor skin wounds were applied to rainbow trout in order to increase the chance of transmission. However, transmission of crayfish plague was not observed (observation time: 6 mo). The contrasting results between the *in vitro* studies by Häll & Unestam (1980) and Ahne & Halder (1988), and the *in vivo* study presented by Ahne & Halder (1988) and our study may be explained as follows:

In the *in vitro* experiments, fish scales were devoid of the protective mucous layer and other anti-infectious properties of the fish skin. It is well established that fish skin has efficient antifungal properties (Pickering & Willoughby 1982, Wood et al. 1986, 1988, Lopez-Doriga & Martinez 1998). We suspect that the lack of mucus, and other anti-infectious functions that would be delivered by intact fish skin, permit the germination of *Aphanomyces astaci* on fish scales *in vitro*.

In this study, we applied a skin wound by wiping off the mucus from 1 cm^2 of the skin with a sterile swab. This treatment was not sufficient to allow *Aphanomyces astaci* spores to germinate on the skin. The mucus was probably replaced quickly by newly produced mucus. Besides its anti-infectious properties, simply the continuous production of fish mucus results in a shedding effect (Lopez-Doriga & Martinez 1998). Pickering & Willoughby (1982) investigated how quickly spores of *Saprolegnia diclina* Type 1, a fungal species pathogenic to salmonids, were removed from the skin of brown trout after being exposed to a spore suspension. They found that after 2 h, 95% of all spores had been removed. In conclusion, future studies are needed to investigate whether *A. astaci* spores will settle on fish skin, if the fish skin was more severely damaged.

With reference to (3) above, no studies have so far dealt with the question of whether viable stages of *Aphanomyces astaci* would survive the passage of the gastrointestinal tract of fish.

The experiments performed in this study were designed to discover whether the various forms of *Aphanomyces astaci* (mycelium or spore/cyst) would survive passage through the digestive tract of a variety of fish species and would be infective to crayfish afterwards. The results show that neither pure mycelium nor pure spores fed to the fish survived the passage, but after feeding the fish with infected crayfish cuticle, it was possible to both cultivate the fungus from the contents of the gastrointestinal tract, as well as to induce an infection in crayfish kept in the same aquarium as the fish that were force fed.

It was shown that the variation in physiology and chemistry of the digestive tract of the fish species tested (e.g. rainbow trout with a HCl stomach and carp without) did not influence the result. In all 4 fish species tested, the fungus was isolated from contents of the gastrointestinal tract and from infected crayfish reared with the force-fed fish. These results show that fish which had been feeding on infected crayfish cuticle are able to serve as a vector for crayfish plague. The most obvious way to get the fish 'clean' before stocking into new water courses is to rear the fish for a few days without access to crayfish, so they can empty their gastrointestinal tract of possible remains of crayfish, and to decontaminate the transport water. According to our experiments, fish are 'clean' after 3 d. Possibly this time span could be shortened, but this will be the subject of further studies.

In summary, our study has identified several risks for the spread of crayfish plague and also delivers suitable methods for the prevention of certain ways of spreading crayfish plague.

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