

# Myxozoan transmission via actinospores: new insights into mechanisms and adaptations for host invasion

D. M. KALLERT<sup>1\*</sup>, S. PONADER<sup>2</sup>, E. ESZTERBAUER<sup>3</sup>, M. EL-MATBOULI<sup>1</sup> and W. HAAS<sup>2</sup>

<sup>1</sup> Institute of Zoology, Fish Biology and Fish Diseases, University of Munich, Kaulbachstraße 37, D-80539 Munich, Germany

<sup>2</sup> Institute for Zoology I, University Erlangen, Staudtstraße 5, D-91058, Erlangen, Germany

<sup>3</sup> Veterinary Medical Research Institute, Hungarian Academy of Sciences, Hungária krt 21, H-1143 Budapest, Hungary

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## SUMMARY

Various mechanisms that enable and improve transmission success of myxozoan actinospore stages towards fish hosts are described, based upon a combination of experimental data and functional analysis of morphological characters. For this purpose, laboratory-reared actinospores of *Myxobolus cerebralis*, *Myxobolus parviformis*, *Henneguya nuesslini* and *Myxobolus pseudodispar* were employed to exemplarily investigate aspects of host attachment and invasion. The process of polar filament discharge of *M. cerebralis* actinospores was analysed, showing that full discharge occurs in less than 10 msec. Additionally, a mechanism that rapidly contracts the discharged filament after discharge is described for the first time. Its purpose is most likely to bring the actinospore apex rapidly into intimate contact with the surface of the host. Unlike *M. cerebralis*, *M. parviformis* actinospores did not discharge polar filaments after mechanical and chemical stimulation, suggesting a different mode of triggering. For *H. nuesslini* actinospores, experimental results indicated that polar filament discharge is independent of external calcium-ion concentration but is influenced by osmolality. After attachment of an actinospore and prior to penetration into the host, an ensheathed unit ('endospore'), containing the sporoplasm, was emitted from the valves in a manner which varied from species to species. Experimentally induced sporoplasm emission was time-dependent and was found to be independent of polar filament discharge in *H. nuesslini*. Remarkably, it could be concluded that the sporoplasm is able to recognize host-stimuli while still within the intact spore. An updated summary of the sequential course of events during host recognition and invasion by actinospores is given.

Key words: Myxozoa, *Myxobolus cerebralis*, polar capsule discharge, actinospore, host invasion, sporoplasm.

## INTRODUCTION

Along with the high importance of fish diseases caused by Myxozoa, studies of the life-cycles, parasite-host-relationships and developmental aspects of the phylum have resulted in a better understanding of life-history traits (Kent *et al.* 2001). Most myxozoans probably exhibit a complex life-cycle, developing alternately in vertebrates (mostly teleosts) and invertebrates (mostly annelids). Non-motile, waterborne actinospores, shed by the aquatic invertebrate hosts, comprise the means of conveyance of the infective stages towards fish. Actinospores are assumed to be only viable for several days to few weeks (Markiw, 1992; Yokoyama *et al.* 1993; Xiao and Dessler, 2000). Hence, special adaptations for rapid host recognition and attachment are essential for successful parasite transmission during the brief period of spore viability.

The discharge of their characteristic polar filaments anchors the actinospore to the fish surface.

\* Corresponding author: Institute of Zoology, Fish Biology and Fish Diseases, University of Munich, Kaulbachstraße 37, D-80539 Munich, Germany. Tel: +49 89 2180 3561. Fax: +49 89 2180 3912. E-mail: kallert@zoofisch.vetmed.uni-muenchen.de

Gills, skin and the buccal cavity have been confirmed as portals of entry for *M. cerebralis*, while *Thelohanellus hovorkai* and *Sphaerospora truttae* prefer entry via the gills (Yokoyama and Urawa, 1997; Holzer *et al.* 2003). *Tetracapsuloides bryosalmonae* and *Henneguya ictaluri* enter via the skin, while the former was also reported to infect gills (Morris *et al.* 2000) and the latter was additionally found in the intestine and the buccal cavity (Belem and Pote, 2001; Longshaw *et al.* 2002). Consumption of infected oligochaetes may also aid infection in some species. Attachment to and penetration of hosts by actinospores, and subsequent migration of the amoeboid sporoplasm, should be considered as separate steps, which possibly require different host signals (Kallert *et al.* 2005a). Kallert *et al.* (2005a) demonstrated that *M. cerebralis* actinospores become mechanically excitable after chemo-sensitization by host mucus prior to polar filament discharge. Such a combination of signals inhibits erroneous reactions upon contact with non-host aquatic organisms or any dead matter. However, no mechanoperceptive structure has yet been described in myxozoans.

Many physiological aspects of this evolutionary successful parasite group remain unclear (Yokoyama, 2003) and a better understanding of host-invasion in Myxozoa requires information on

their morphological and behavioural adaptations to parasitism and on the physiological requirements for host-recognition and host-attachment. Elucidation of these mechanisms is valuable for our understanding of the epidemiology of disease outbreaks caused by Myxozoa in fish populations. Moreover, artificial induction of spore responses may be an effective biological control approach to prevent great losses in hatchery and wild fish populations. Presenting preliminary findings with laboratory-cultured myxozoans, the present paper shows by which means myxozoan parasites ensure transmission success during the difficult task to recognize and invade fish hosts.

#### MATERIALS AND METHODS

##### *Parasite cultivation*

Myxospores were isolated by tissue homogenisation for 2–5 min at 11 500 rpm using an Ultra Turrax (IKA Labortechnik, Staufen, Germany). Tissue remnants were then removed by passing the homogenate through 100  $\mu\text{m}$  mesh filters. The homogenate was then topped up to 500 ml with tap water with the suspension settling overnight at 4 °C. All fish were kept at 16–18 °C under a constant flow of well water and fed on conventional fish flakes. Oligochaete cultures were maintained in 5 litre plastic containers containing a 5–6 cm layer of autoclaved mud and coarsely grained sand (1:1 v/v) and were held at 12–15 °C in the dark. Oligochaetes were fed weekly using a mixture of frozen *Artemia*, spray-dried *Spirulina* (MaBitech) and frozen lettuce. Autoclaved mud from the settling pond of a salmonid hatchery was added weekly. Only well water free of copper and chlorine was used for oligochaete cultures.

*Henneguya nuesslini* and host fish were obtained from a local hatchery (Bavaria, Germany). Myxospores were either obtained from muscle or pieces of the tail of adult, naturally infected brown trout. Cultivation of the parasite was conducted according to Kallert *et al.* (2005*b*). Four to seven-month-old (3–6 cm) parasite-free brook trout (*Salvelinus fontinalis*) and brown trout (*Salmo trutta*) were used for infection with actinospores from infected oligochaete cultures. *Myxobolus parviformis* actinospores originated from an established parasite culture described by Kallert *et al.* (2005*c*), using myxospores obtained from cysts isolated from gill filaments of laboratory-infected bream. For *M. cerebralis* cultivation, infected cultures of *Tubifex tubifex* were obtained from the long-term established laboratory cultures at the Institute of Zoology, Fish Biology and Fish Diseases of the University of Munich (Germany). Additionally, fresh oligochaetes (>200 000 individuals) from the salmonid hatchery were transferred to aerated plastic beakers (4 litre) and used for

infection with *M. cerebralis* myxospores after a monitoring period (including control cultures without myxospore addition) as described by Kallert *et al.* (2005*a*). *Myxobolus pseudodispar* originated from naturally infected oligochaetes from the salmonid hatchery. Actinospores from single oligochaetes were used for infection of laboratory-reared myxozoan-free roach (*Rutilus rutilus*) at a dose of  $\sim 2000$  actinospores per fish. The 18S rDNA sequence of this parasite isolate was submitted to GenBank (Accession no. EF466088) to enable future identification, as different isolates of spores morphologically identified as *M. pseudodispar* can show high variability (up to 4% difference) in these sequences. The isolate examined was most similar to *M. pseudodispar* ex *Blicca bjoerkna* (GenBank Accession no. AF466654).

Unless otherwise noted above, oligochaetes from a laboratory stock were infected by addition of  $\sim 0.5\text{--}2 \times 10^6$  myxospores after incubating the freshly prepared oligochaete cultures for at least 1 week. Non-exposed control cultures did not produce notable amounts of actinospores. All species form Triactinomyxon-type actinospores (TAMs). They were harvested from oligochaete cultures by filtration using 20  $\mu\text{m}$  nylon meshes or from single oligochaetes isolated in cell well plates according to the method of Yokoyama *et al.* (1991).

##### *Test substrates*

The mucus substrates for experimental use were prepared as described previously (Kallert *et al.* 2005*c*). Adult rainbow trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio*) were obtained from a local distributor. Common bream (*Abramis brama*) and roach were caught in the river Aisch, Germany. Where necessary, mucus was concentrated by removing water from the solution by partial lyophilization in a SpeedVac, deionized water was used for dilution. Artificial incubation media (standard fresh water 'SFW 100' according to Meier-Brook (1978), containing 100 ppm  $\text{Ca}^{2+}$ ) were prepared by dissolving the respective salts in deionized water. In 1 volume of  $\text{Ca}^{2+}$ -free water (7 mOsm/kg), the osmolality was restored according to the value of the  $\text{Ca}^{2+}$ -containing mixture (12 mOsm/kg) by addition of NaCl. Osmolality was measured in triplicate using 150  $\mu\text{l}$  samples by freezing-point-depression using an Osmometer Automatic (Knauer, Berlin), calibrated with deionized water and a 400 mOsm NaCl solution (12.687 g/kg). All osmolality values given refer to unbuffered substrates.

##### *Polar filament discharge*

*Discharge rates.* Polar filament discharge rates were determined as described by Kallert *et al.* (2005*a*). Briefly, 9  $\mu\text{l}$  of buffered test substrate were added to

21  $\mu\text{l}$  of buffered spore suspension on slides and covered with a cover-slip (20  $\times$  20 mm), followed by mechanical stimulation for 3 s at 50 Hz using a Mini Shaker Type 4810 (Brüel & Kjær, Copenhagen). Buffered deionized water served as control in all experiments. All media containing parasites or test substrates were adjusted to pH 7.5 (5 mM sodium phosphate buffer). Spore suspensions were stored at 12 °C and test substrates were kept on ice during use. Whenever possible, a blind protocol was used by coding the test substrates.

**Discharge process.** To visualize the process of polar filament discharge, video sequences were recorded using an Axiophot (Zeiss) and a video camera (Canovision EX1 8 mm, Canon). *M. cerebralis* actinospores were placed on a glass slide in 30–40  $\mu\text{l}$  of water covered with a cover-slip and 4–12  $\mu\text{l}$  of a 30% aqueous  $\text{NH}_3$  solution were added slowly from the side to induce filament firing. Still frames were taken as screenshots from the Video using Ulead Video Studio 7.0 SE software.

**$\text{Ca}^{2+}$ -dependency.** To investigate the role of  $\text{Ca}^{2+}$  in the surrounding medium on polar filament discharge, *H. nuesslini* actinospores were transferred from their original medium to SFW ('Standard fresh water' according to Meier-Brook, 1978) with and without  $\text{Ca}^{2+}$ . The original water of the actinospore suspension was replaced by transferring separate filtration units (10 ml plastic tubes with 20  $\mu\text{m}$  mesh as bottom) in 5 ml cups with SFW for 30 min and repeated 4 times, changing the medium each time. Polar filament discharge rates were measured using carp mucus homogenate (1 mg/ml final concentration). Individual controls were included showing the discharge rates for actinospores incubated in each SFW mixture separately.

**Discharge stimuli.** The possible combination of chemical and mechanical stimuli for polar filament discharge in *M. parviformis* actinospores was tested as described by Kallert *et al.* (2005c). Briefly, immobilized single actinospores were exposed to a sequence of stimuli comprising 2.5  $\mu\text{l}$  of control (buffered) water, mechanical stimulation (3-fold apical tipping), 2.5  $\mu\text{l}$  of host mucus and repeated mechanical stimulation with 10 sec observation intervals between all steps. To test whether the actinospores were able to discharge at all, a saturated solution of urea was applied.

#### *Sporoplasm release*

To determine if actinospores had an intrinsic mechanism to cause their apical sutures to open upon polar filament discharge to enable sporoplasm release, *H. nuesslini* actinospores were microscopically observed after addition of rainbow trout mucus

(1 mg/ml final concentration). The spore suspension (40  $\mu\text{l}$ ) was placed on a slide and plastilin spacers were used to ensure that pressure from the cover-slip did not affect sporoplasm release. Discharged and undischarged spores were observed for 15 min using a stereomicroscope. Sporoplasm release (emergence of at least 1/3 of the primary cell mass) with and without preceding polar filament discharge was recorded. The period from substrate mixing until sporoplasm release was recorded.

To confirm the cellular integrity of *M. parviformis* sporoplasms after emergence from the surrounding sheath, fluorescein-diacetate (FDA) staining was used as described by Yokoyama and Urawa (1997) and Markiw (1992) on spore isolates incubated in carp mucus. Sporoplasm emission from *M. pseudo-dispar* actinospores was recorded using a digital camera (Moticam 2000, Motic) under a stereomicroscope, after a 1 : 3 (v/v) addition of carp mucus.

#### *Statistical methods*

Statistical analysis was performed using SPSS for Windows. Where necessary, data were arcsine square root transformed to obtain approximately normally distributed data; homogeneity was controlled using the Levene test. Mean values and standard errors were calculated from the transformed data and were retransformed thereafter. Differences between means were tested for statistical significance by a multivariate comparison procedure (Tukey HSD multiple *t*-test after one-way-ANOVA). Correlation between binominal variables was tested by cross-tabulation followed by calculation of Yates' continuity correction as well as the Phi-value and the contingency coefficient as symmetric measures. Mantel-Haenszel statistics (homogeneity of the odds ratio) was used to test for independence in response between the binary variables.

## RESULTS

### *Polar filament discharge*

**Discharge process.** Video analysis of polar filament discharge in *M. cerebralis* actinospores showed that complete extrusion requires less than 10 msec (Fig. 1A–C). Most notably, the polar filament first rapidly elongates, then contracts shortly after discharge to about half its length (Fig. 1D and E). This 'dragging mechanism' could be observed to pull the whole spore body about 35  $\mu\text{m}$  along the slide by means of the sticky filament (Fig. 2A–C).

**$\text{Ca}^{2+}$ -dependency.** *H. nuesslini* actinospores showed a significant discharge rate in SFW with  $\text{Ca}^{2+}$  ( $P < 0.001$  vs control), but discharged their polar filaments less frequently when the SFW was  $\text{Ca}^{2+}$ -deficient ( $P < 0.05$ , Table 1). When the osmolality of

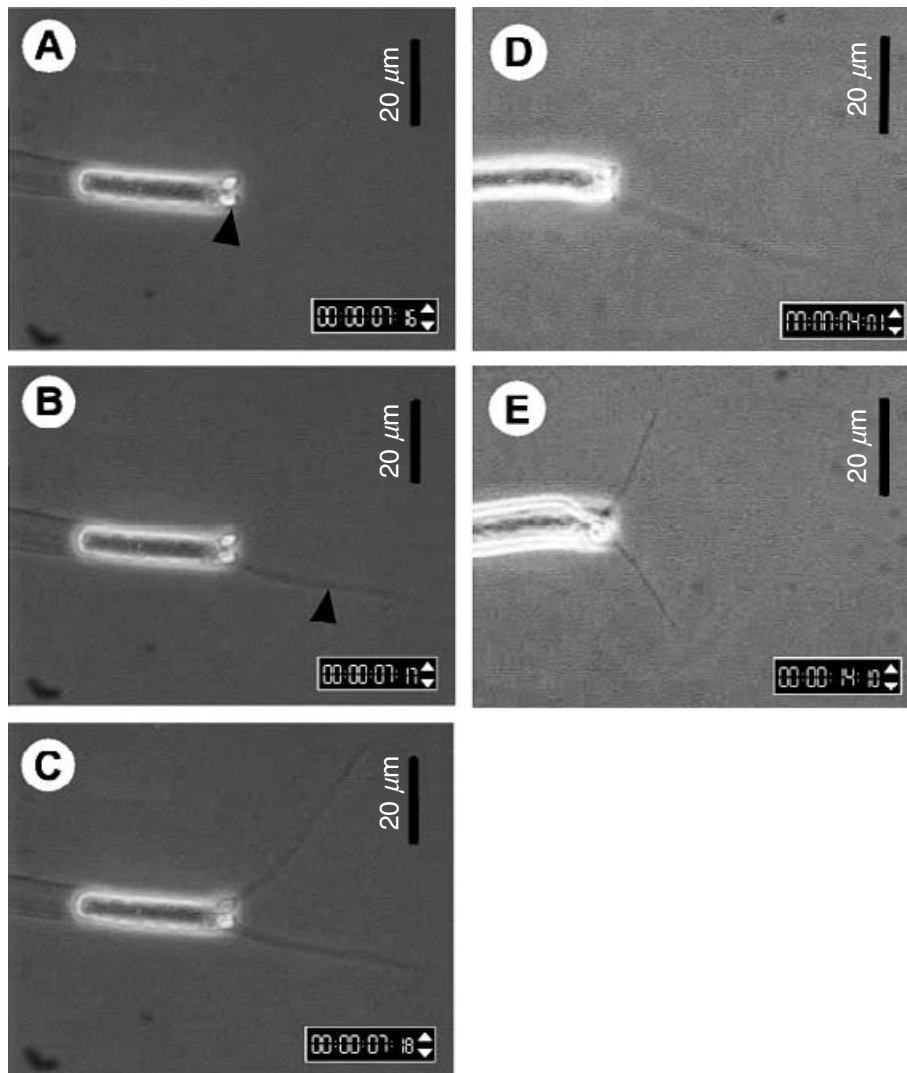


Fig. 1. Polar filament discharge of *Myxobolus cerebralis* actinospores induced by addition of 30% ammonia (phase-contrast optics). (A) Actinospore apex with polar capsules (arrowhead). (B) The same spore having discharged one filament (arrowhead) 0.01 s later. (C) Discharge of a second filament 0.01 s later, both still elongated. (D) Different actinospore during polar filament discharge showing elongation of the first filament. (E) Both abridged discharged filaments of the same spore 10 s later. Counter included from the original video footage shows centiseconds (small scale) and sec (large scale).

the  $\text{Ca}^{2+}$ -deficient SFW (7 mOsm/kg) was adjusted to the value of  $\text{Ca}^{2+}$ -containing water (12 mOsm/kg), the discharge rate was equal to that measured in  $\text{Ca}^{2+}$ -containing water ( $P=0.57$ , Table 1). However, the responses in  $\text{Ca}^{2+}$ -deficient water (with and without adjusted osmolality) were not significantly different from the respective controls (without mucus addition) ( $P=0.3$  and  $0.08$  respectively). The responses to controls (without mucus) differed among the media (Table 1). Therefore, the discharge rates obtained in controls could be subtracted from the responses caused by mucus addition in the respective media for equalization. As a consequence, the discharge rate in  $\text{Ca}^{2+}$ -containing water was significantly higher than that in  $\text{Ca}^{2+}$ -deficient water with adjusted osmolality ( $P<0.05$ ). The responses in both  $\text{Ca}^{2+}$ -free media were not significantly different from each other ( $P=0.7$ ).

**Discharge stimuli.** Of 29 single *M. parviformis* actinospores, only 3 (10.3%) did react after the first mechanical stimulation, and 1 further specimen (3.8%) of the remaining reacted after mucus addition. None of the 25 remaining spores reacted after the ensuing mechanical stimulation within the observation period of 10 sec. General discharge ability of the actinospores was tested by addition of a saturated urea solution, showing that all spores had a functioning attachment apparatus.

#### *Sporoplasm release*

The period the amoeboid sporoplasm required to leave the valve structure after stimulation by mucus was determined for *H. muesslini* actinospores. The majority of sporoplasms left the valves within 6 min, whereas most were emitted around 5 min (Fig. 3,



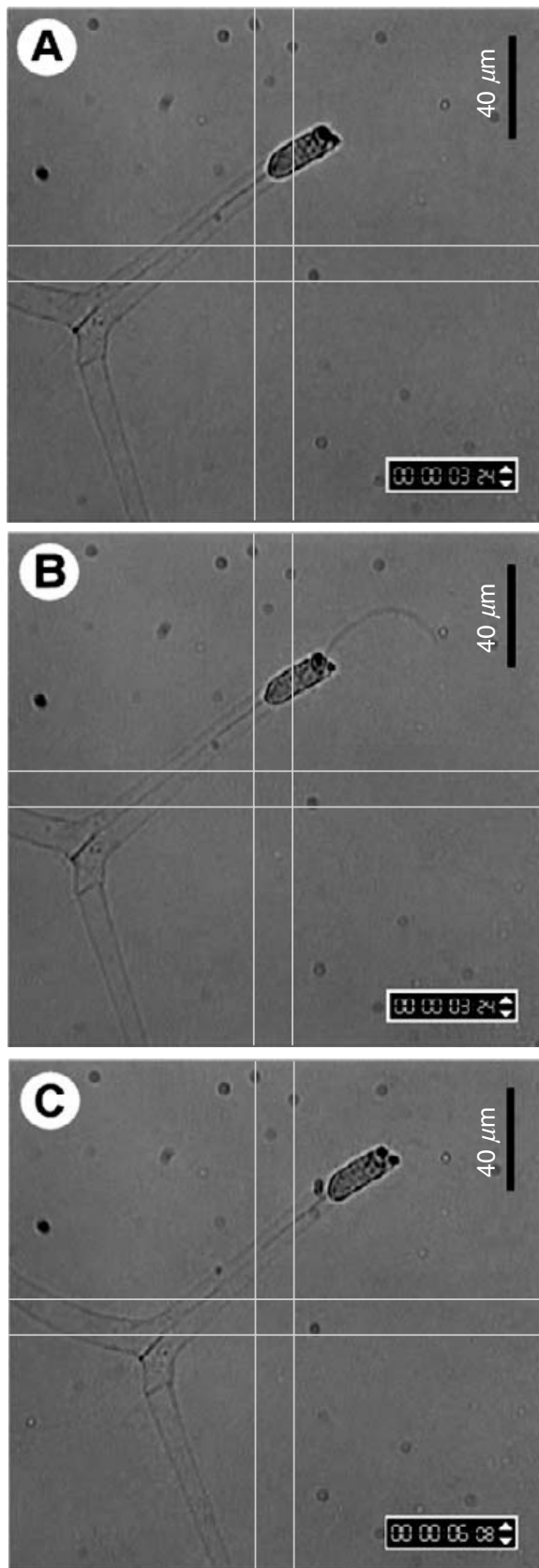


Fig. 2. Polar filament discharge of *Myxobolus cerebralis* actinospore induced by addition of 30% ammonia (bright-field optics, grid shows actinospore movement). (A) Actinospore prior to discharge. (B) The same spore with fully discharged filament. (C) The same spore with abridged filament less than 3 s later; note different

with the mean time of emission around 5.4 min, s.d. = 2.8). The observation of discharged and undischarged *H. nuesslini* actinospores for 15 min after stimulation by mucus homogenate revealed that among actinospores with discharged polar filaments, 60.6% kept their sporoplasm inside the spore valve (109 individuals counted), while for non-discharged actinospores, 28.4% released their sporoplasms (95 individuals counted). In most undischarged actinospores in which the sporoplasm did not emerge, the amoeboid mass exhibited characteristic movements within the style of the spore (also observed in all other species) that were absent without mucus incubation. There was very weak correlation between the responses, as shown by the Phi-value of 0.114 and the contingency coefficient of 0.113 (with a significance of 0.104). Additionally, according to Mantel-Haenszel's chi-square analysis, there is no strong relationship between the two reactions ( $P=0.14$ ). Therefore, the sporoplasm emission reaction is *per se* independent from polar filament discharge (no linear correlation) in *H. nuesslini* actinospores. Nevertheless, 39.4% more sporoplasms were released when polar filaments were discharged, but release was not an imperative consequence after filament discharge.

Observing actinospores reacting to mucus, we could discern 2 different modes of sporoplasm emission in *M. parviformis* and *M. pseudodispar*. In one mode, the sporoplasm emerged as an amoeboid mass from the valve cells as typically seen on slides after incubation in fish mucus. Alternatively, a sheathed unit (corresponding to the 'endospore' described by Janiszewska, 1955) was emitted, out of which subsequently the sporoplasm emerged. The cellular integrity of the actively moving amoeboid primary cell could be confirmed by FDA-staining of *M. parviformis* 'endospores' (Fig. 4). Unlike *M. parviformis* (see Kallert *et al.* 2005c), the *M. pseudodispar* valve cell did not open in a splayed finger-like manner to release the sheath unit, and it was rather released from the valve cells as a 'plug' (Fig. 5).

#### DISCUSSION

The experimental outcome along with a step-by-step analysis of morpho-physiological aspects and the reactions actuated by actinospores during host invasion provide valuable information on myxozoan transmission. An understanding of how actinospores recognize and enter the fish host is not only of importance concerning epidemiology of myxozoans,

location after movement along the slide. Counter included from the original video footage shows milliseconds (small scale) and sec (large scale).

Table 1. Effect of  $\text{Ca}^{2+}$ -ions on polar filament discharge of *Henneguya muesslini* actinospores during response to carp mucus homogenate (1 mg/ml)

(The actinospores were transferred to artificial medium ('Standard fresh water' (SFW) according to Meier-Brook, 1978). NaCl was used for osmolality equilibration of  $\text{Ca}^{2+}$ -deficient water. Actinospores and substrates were mixed on a slide (21 + 9  $\mu\text{l}$ ) with application of vibrations (3 s, 50 Hz, Amplitude 0.5 mm). Letters indicate a significant difference (ab,  $P \leq 0.001$ ; ABCD,  $P \leq 0.05$ ; Tukey HSD). Total number of actinospores counted per substrate 286–398 (8 replicates).)

Substrate	Discharge rate [%]	S.E.
SFW + $\text{Ca}^{2+}$	21.3 <sup>a</sup>	+3.5/–3.3
SFW + $\text{Ca}^{2+}$ + mucus	42.1 <sup>abAB</sup>	+4.4/–4.3
SFW – $\text{Ca}^{2+}$	15.0 <sup>bCD</sup>	+2.4/–2.3
SFW – $\text{Ca}^{2+}$ + mucus	26.8 <sup>A</sup>	+3.3/–3.1
SFW – $\text{Ca}^{2+}$ + NaCl	23.9 <sup>B</sup>	+2.7/–2.6
SFW – $\text{Ca}^{2+}$ + NaCl + mucus	33.5 <sup>CD</sup>	+3.5/–3.4

the information is useful also to researchers performing laboratory work with specimens of this phylum. For this study, we chose suitable species to investigate each of the mechanisms and tried to give comparative information about differences between certain species.

With respect to polar filament discharge, the observed speed of discharge and the length of the polar filament indicate that actinospores need close contact between their apical region and the host surface for successful attachment. In *M. cerebralis* actinospores, there is a putative chemically triggered thigmo-perceptive structure (Kallert *et al.* 2005a), suggested by the observation that the actinospores only discharged their filament when a chemical (mucus-derived) signal was present prior to physical contact. An intrinsic retraction mechanism was always observed immediately after discharge of the filament in several species. Its purpose could be to mediate ultimately close contact with the viscous fish surface or to pull the 'endospore'-construct out of the valve envelope. The actinospores thereby have developed an excellent mechanism to pull their apical region tightly towards the host surface, enabling the sporoplasm to directly enter the epidermal layer. The artificially induced discharge without mechanical stimulation for visualization was only possible using ammonia as an effector for practical reasons. The use of ammonia instead of a natural trigger did not influence the characteristic course of the reaction. It remains unknown how the initial elongation and subsequent contraction of the polar filament actually occurs, but it is likely that either proteinaceous conformation changes upon hydration or the stored energy due to the well-known filament twist play a role.

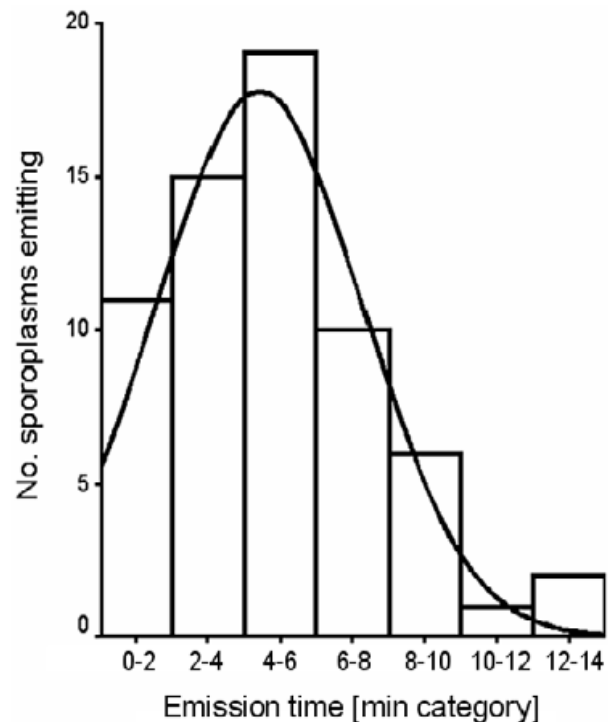


Fig. 3. Duration until onset of the emission response of *Henneguya muesslini* sporoplasms after stimulation with mucus in time categories representing the period (2 min per category) after stimulation in which the emission movement was observed (64 actinospores counted). A curve has been plotted showing Poisson-distribution.

Uspenskaya (1982) assumed  $\text{Ca}^{2+}$ -ions to be involved in the polar filament discharge mechanism and suggested that it is an active process due to contractile proteins. The role of calcium as an effector or even the driving force behind the filament extrusion is also implicated in cnidarians: removal of calcium from the medium inhibited discharge of nematocysts in some species (Santoro and Salleo, 1991; Kawai *et al.* 1997; Russell and Watson, 1995). Additionally, calcium plays a role in cell-signalling in cnidae-bearing complexes (Cannon and Wagner, 2003). By  $\text{Ca}^{2+}$ -replacement experiments, we showed that external  $\text{Ca}^{2+}$  was not a major effector for the discharge mechanism in *H. muesslini* actinospores, but it appeared that the osmolality of the surrounding medium had to exceed a certain value to enable normal discharge rates. When the effect of osmolality differences was considered and the different responses in control media were subtracted from the discharge rates with mucus,  $\text{Ca}^{2+}$ -ions indeed seemed to play a role. Discharge rates in  $\text{Ca}^{2+}$ -containing water were doubled compared to those in  $\text{Ca}^{2+}$ -deficient water at the same osmolality. However, in  $\text{Ca}^{2+}$ -free medium with restored osmolality by NaCl-addition, filament discharge rates were higher than in media with  $\text{Ca}^{2+}$ , indicating that eventually only an artificial effect of NaCl was observed. This may play a role in membrane

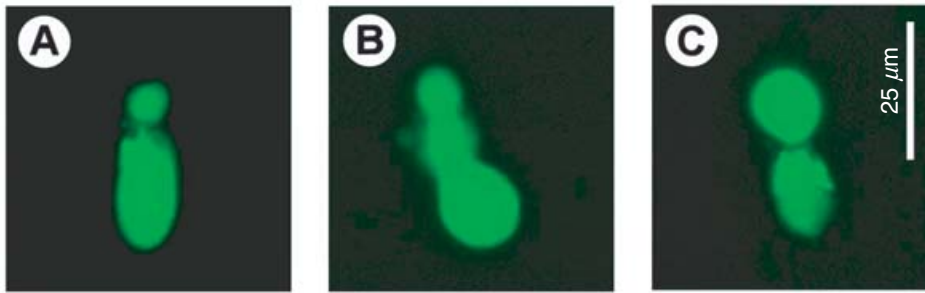


Fig. 4. Sporoplasm emission of *Myxobolus parviformis* sheath unit ('endospore') induced by addition of bream mucus after fluorescein-diacetate-staining. (A) Ball-like pseudopodium emerging first. (B) Apical opening and active emergence of the sporoplasm. (C) Fully emerged sporoplasm with sheath bag attached; note membrane staining of actively emerged amoeboid primary cell.

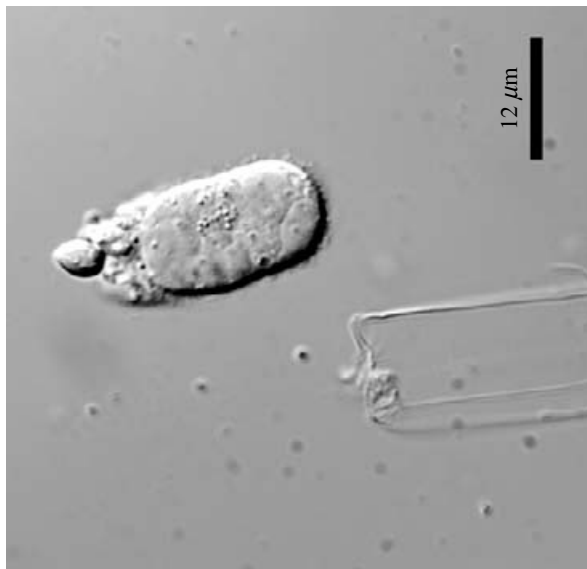


Fig. 5. Expulsion of *Myxobolus pseudodispar* sheath unit ('endospore') induced by addition of roach mucus; note the plug-like apical architecture with no polar capsules left in the valve cells.

conductivity or osmotic balance of the parasite primary cell and thus influence polar filament discharge. However, we assume that exterior  $\text{Ca}^{2+}$ -ion-concentration does not play a significant role, considering that discharge was never fully abolished in  $\text{Ca}^{2+}$ -deficient media. Whether actinospores are able to adjust to media with lower osmolality after prolonged incubation, should be further investigated.

In sporoplasm emission experiments, most *H. nuesslini* sporoplasms left their valve shell after about 5 min after little or no mechanical stimulation and with or without prior polar filament discharge – a result comparable with the data of Yokoyama *et al.* (2006). These authors further observed 'slow' and 'fast' reactions of 2 myxozoan species infecting different fish hosts. In *Thelohanellus hovorkai* actinospores, sporoplasms were released over a 30 min exposure time to host mucus, while *Myxobolus arcticus* actinospores reacted similarly to *M. cerebralis*

in the current study. The aurantiactinomyxon type of *T. hovorkai* has a similar apical ultrastructure as *M. parviformis* triactinomyxon spores with rather counter-sunk polar capsules located below the apical valve cell margin (Kallert *et al.* 2005c), in contrast to fast-reacting species such as *M. cerebralis* and *H. nuesslini* showing polar capsules with protruding tips. This may be an ecological adaptation in response to a need for rapid host recognition. Similarly, the low discharge rates and negative reactivity of *M. parviformis* actinospores in this work may be explained if this parasite requires an extended time to react, a feature which could reflect a specialization towards a certain host invasion route (e.g. via gills or oral uptake) or an environment that bears a higher risk of unintentional mechanical stimulation, for instance by large amounts of plankton. A more rigid apical structure may be an adaptation for prolonged viability, as these actinospores may be better protected or less sensitive than, for example, *H. nuesslini* actinospores. A difference in host preference is apparent between 'quickly' and 'slowly'-reacting parasites; as the former infect stream inhabitants, while the latter seem to prefer bottom feeders in standing waters. However, *M. pseudodispar* is a fast-reacting species predominantly found in slow waters, which would represent an exception to the rule. However, the slow mode of sporoplasm emission observed by Yokoyama *et al.* (2006) may also be the result of increased mechanical sensitivity of the triactinomyxon-type actinospores, as no further mechanical stimulus was applied. This may have extended the period required for reaction of *T. hovorkai* specimens, as fewer polar filaments would have discharged. Surprisingly, *M. parviformis* actinospores did not elicit a similar reaction to a combination of chemical and mechanical stimulation compared to *M. cerebralis*. It is not known whether the incubation time of the chemical stimulus was too short for this species.

Although the experimental conditions of the sporoplasm emission experiments with *H. nuesslini* certainly do not completely mimic contact with a real

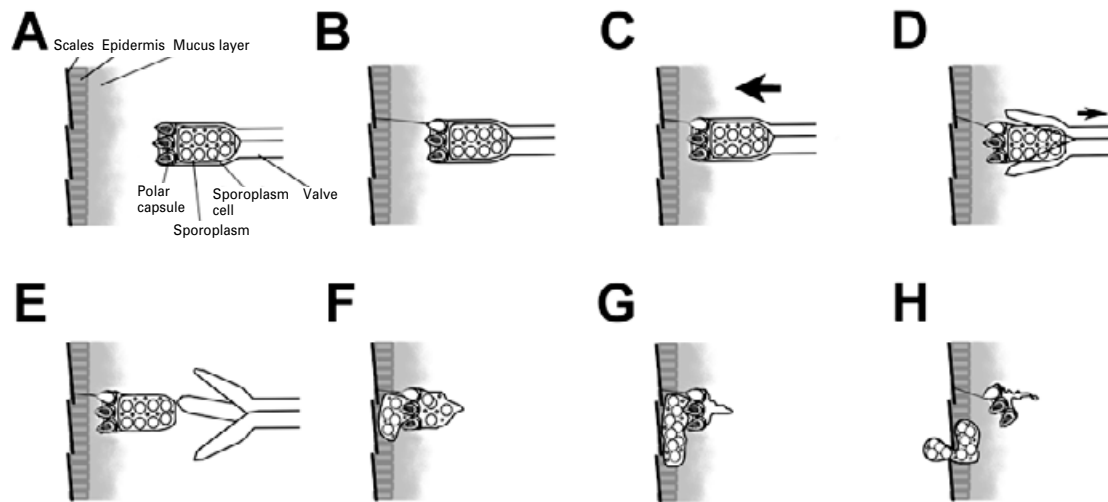


Fig. 6. Schematic course of the events during host invasion by triactinomyxon-type actinospores. (A) Actinospore apical region encountering fish host surface, chemical sensitization. (B) Polar filament discharge after mechanical stimulus upon contact. (C) Adduction (arrow) of the actinospore apex for close contact to the host surface by means of filament constriction. (D) Opening of the apical valves among specific sutures by means of water current traction (arrow). (E) Release of the attached 'endospore', discarding of the valve shell. (F) Active emergence of the sporoplasm. (G) Fully emerged sporoplasm leaving the 'endospore' sheath on the host surface. (H) Penetration of the sporoplasm through the host integument and movement towards deeper layers.

host, we nevertheless demonstrated a suitable method to explore a putative built-in mechanism utilized by the parasite to escape from its covering transmission 'vehicle'. Thus, it could be concluded, that the apical opening does not occur passively by an intrinsic mechanism, as many spores with discharged polar filaments did not open and sporoplasm release did not occur. Although polar filament discharge surely facilitates sporoplasm emission, we could show that it is not an imperative prerequisite. Enhanced movement of the sporoplasms and even a rearrangement of the polar capsules inside the valves were frequently observed during incubation of actinospores in fish mucus for several minutes. Thus, the sporoplasms apparently were activated without being able to emerge. Hence, this suggests that even within an intact actinospore, the sporoplasm is able to recognize mucus components. It is possible that the sporoplasm may be capable of actively triggering polar filament discharge disposition and thereby affect their infectivity status.

A striking feature of many triactinomyxon-type actinospores is the 'endospore' structure which comprises a membrane-sheathed sporoplasm primary cell with polar capsules attached apically. Its purpose is probably the protection of the sporoplasm after attachment and prior to penetration of the host. El-Matbouli *et al.* (1999) documented the 'endospore' structure by SEM on the fish host surface after exposure to *M. cerebralis* actinospores and described it as a "fibrous structure", left after the sporoplasm had penetrated. It has also been observed in *M. parviformis* (Kallert *et al.* 2005c) by light microscopy, and we observed it in the current study in *M. pseudodispar* and occasionally in *M. cerebralis*

after activation by host mucus. The 'endospore' of *M. pseudodispar* often appears ball-like and is easily emitted by mechanical agitation. Its emission seems to be the result of host anchorage via the polar filament immediately after discharge and its characteristics (shape and mode of emission) differ between the species. As an example, *M. cerebralis* 'endospores' are rarely seen set free, whereas in *M. pseudodispar* this is very common after incubation in mucus and application of vibrations. Additionally, the amount of force needed for valve opening prior to sporoplasm release may be directly influenced by the sporoplasm itself, as we have shown the sporoplasm can recognize host cues even before polar filament discharge. In addition, sporoplasm emergence directly from the valvular apex directly would be rather difficult due to the forces actuated by water current on the whole valve shell, which is thus avoided in a very elegant manner. Morphological division of actinospores into 'epi-' and 'endospore', formerly suggested by Janiszewska (1955), should be re-established, as it is not referred to in the current myxozoan taxonomy, although their presence and taxonomical impact are clearly visible. We suggest that the functional differences are taxonomically important and suggest them to be included among established parameters such as morphometrics and secondary cell count. Observations on the presence, composition, shape and function of the 'endospore' should be referred to in future actinospore descriptions and transmission studies.

Our findings with the myxozoan species in this study allowed us to compose a refined picture of the steps actinospores take during host invasion (Fig. 6). When in proximity to a host, the waterborne



actinospores receive chemical cues from host mucus and become mechanically excitable (Fig. 6A). Upon apical contact, a not yet known mechanoreceptor induces fast discharge of the polar filament, which presumably depends on medium osmolality (Fig. 6B). Subsequent retraction of the filament pulls the apical region of the actinospore tightly into the mucous surface (Fig. 6C). Forces generated by a parachute-like mechanism caused by hydrodynamic forces on the floating appendices open the apical valve shell along their sutures (Fig. 6D) and the actinosporean shell is eventually discarded (Fig. 6E). For this purpose, actinospores possess an apical, species-specific suture lining along which the valves open. The sporoplasm (inside a sheath or 'endospore') is released being tightly attached (thereby physically and osmotically protected) to the host surface. The amoeboid mass leaves the membranous sheath (Fig. 6(F)) and moves into the host (Fig. 6G) by penetration of the epidermal tissue (Fig. 6H) and finally follows a path towards its species-specific target cell type or organ. These mechanisms comprise an excellent set of adaptations for successful passive host recognition and invasion in an aquatic environment without energy-intensive active host-finding behaviour.

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