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In vitro transition of *Schistocephalus solidus* (Cestoda) from coracidium to procercoid and from procercoid to plerocercoid

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ABSTRACT

With the present study, a culture system for successive life-cycle stages of the tapeworm *Schistocephalus solidus* was developed and this report documents for the first time, cultivation of the procercoid stage of *S. solidus* from eggs. Additionally we have transformed procercoids dissected from experimentally infected copepods and cultured procercoids into the early plerocercoid stage *in vitro*. Observations in the culture suggest that the coracidia can interact with their external environment and need no host specific stimuli, except for the components in the culture medium, for activation and hatching from the embryophore. Increasing the culture medium pH from 7.3 to 8.0 improved escape rates and frequencies of hook contractions, suggesting that the oncosphere may recognize and respond to environmental conditions along the host intestine. Procercoids in the culture did not stop growing indicating that conditions within the copepod may be important to limit growth and to induce transformation to plerocercoids. When procercoids are dissected from copepods and transferred to the culture, the outer tegument layers and cercomer starts to loosen. Comparison of the lectin staining of the loosened outer tegument layers and cercomer in procercoids dissected from copepods confirms that transitions of both, the oncosphere to procercoid and procercoid to plerocercoids, has taken place in the *in vitro* cultures.

1. Introduction

The tapeworm *Schistocephalus solidus* is a hermaphrodite with a complex life-cycle involving a cyclopoid copepod as first host, followed by the three-spined stickleback, *Gasterosteus aculeatus*, and finally a homeothermic host, most often a bird (Smyth, 1946). The parasites' eggs are expelled into water with the final host's faeces, where embryos start to develop, if the temperature is high enough. After 3 weeks at 18 °C, coracidia, the first larval stage, hatch. To hatch, the egg needs to be illuminated (Dubinina, 1966), probably because the coracidia produce a light-released enzyme to open the egg operculum through which the ciliated coracidium creeps out (Smyth and McManus, 1989). The coracidium has a brief life span, and has to be ingested by the first intermediate host within 96–120 h at 5–8 °C (Dubinina, 1966) or 24–36 h at 18 °C (Wedekind, 1997). In the copepod's gut the oncosphere (*the larva contained within the embryophore*) is thought to escape from the inner and outer cavity of the inner egg envelope, the embryophore, before it penetrates the copepod's gut and enters its body cavity. Here, the larva grows and develops into the procercoid. When fully developed the infective procercoid is characterized by a cercomer, a structure of unknown function. Just after ingestion of the infected copepod by the stickleback host, the procercoid "switches coats" by losing its outer surface coat and the cercomer, most often in the frontal part of the stickleback intestine (Hammerschmidt and Kurtz, 2007). The third larval stage, the plerocercoid, develops and grows to become infective to the definitive host after several months (Hopkins and Smyth, 1951; Clarke, 1954). *Schistocephalus* sp. are characterized by a long-lasting plerocercoid stage in the fish intermediate host (Dubinina, 1966; Chervy, 2002). In terms of host involvement, the plerocercoid has been the most studied phase in the life-cycle, as the parasite has long-term effects on the host, often with detrimental impact on the health, fecundity, and behaviour of infected fish (Dubinina, 1966; Arme and Owen, 1967; Milinski, 1985, 2006; Smyth and McManus, 1989; Barber and Huntingford, 1995; Barber and Arnott, 2000; Barber and Scharsack, 2010). Although life-cycle stages are usually studied in isolation, recent research indicates that state of the tapeworms in their

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copepod host, seems to have a significant impact on the life history traits of the parasites in their second host (Hammerschmidt and Kurtz, 2005a; Hammerschmidt et al., 2009). Fitness of the parasite is hence determined by a cascading effect of its transition through its host spectrum (Parker et al., 2009).

S. solidus has developed into a natural model to study the ecology and evolutionary questions of host–parasite interactions (Hammerschmidt and Kurtz, 2007, 2009 and references therein) and complex life-cycles (Parker et al., 2003, 2009; Hammerschmidt et al., 2009). Many questions address the life-cycle as a whole, and hence all stages of the parasite come into focus. The parasite is characterized by taking most of its energy from its fish host. Maturation is triggered by increasing temperature to 40 °C and the tapeworm will reach sexual maturity after 36 h (Smyth, 1954). Because the plerocercoid can easily be removed sterile from the coelom of the stickleback, it was the first cestode successfully matured *in vitro* to produce fertile eggs (Smyth, 1946). This early *in vitro* breeding of *S. solidus* gave rise to a large number of studies on the basic biology, biochemistry, and physiology of this parasite (Smyth, 1990). This was due to the fact that culturing techniques allow experiments to be carried out, under controlled reproducible conditions, free from interference by host-related factors (Smyth, 1990). Cultures are also important for general parasitology studies because it may be possible to track different developmental stages or transition processes of parasites within their natural hosts. However, younger stages of *S. solidus* have only been investigated in their natural hosts. Extensions of *in vitro* cultures to *S. solidus's* complete life-cycle would therefore provide a powerful tool, as it would allow investigation of its different stages under rigorous experimental conditions.

Although there have been attempts (Smyth, 1959), transformation from eggs to fully developed procercoids, have, to our knowledge (see also Smyth and McManus, 1989) never been successfully conducted in Diphyllobothridea nor Bothriocephalidea. Transmission to the plerocercoids has however, earlier been successfully conducted in *Spirometra mansoni* (Berntzen and Mueller, 1964), but not in *S. solidus*.

2. Materials and methods

2.1. Transition of coracidia to procercoids: the artificial copepod

Infected sticklebacks, were caught during autumn 2006 from the lake Skogseidvatnet 60°13′N5°53′E, Western Norway, and brought to the Max Planck Institute for Evolutionary Biology in Plön, Germany. Here the fish were kept in large tanks and fed *ad libitum*. The tapeworms were dissected aseptically from the sticklebacks under sterile conditions and bred in an *in vitro* system that replaces the bird's gut (Smyth, 1946; Wedekind, 1997). Tapeworms were bred in size-matched pairs to avoid selfing (Lüscher and Milinski, 2003). Eggs were then allowed to develop in sterile filtered tap water for 3 weeks at 18 °C in the dark. In the evening before use, they were exposed to light for hatching (Dubinina, 1966).

Newly hatched coracidia in water were diluted 1:10 with 18 °C culture medium (for medium composition, see Table 1), or 18 °C sterile tap water and 200 µl aliquots were transferred to wells of 96-well flat bottom microtitre plates. The cultures were incubated in a moist chamber in dark, with 2% CO_2 at 18 °C. Three times per week, 50% (v/v) of the culture supernatant was replaced by fresh medium (Table 1). Larvae were monitored, using an inverted microscope at 200× magnification. The frequency of coracidia transformed to oncospheres and surviving untransformed coracidia (number out of 20 counted per well) were recorded in culture after 48 h in culture media with pH 7.3 and 8.0. Transformation rates were compared to coracidia incubated in sterile tap water.

Table 1
Additives to Minimum Essential Medium (MEM, Sigma M2279) used for cultivation of procercoid and plerocercoid stages (culture medium).

Ingredients	Catalogue No.	Final concentration
L-Glutamin	Sigma G7513	10 mmol l^{-1}
Antibiotic, antimycotic	Sigma A 5955	10 ml l^{-1}
Glucose	Roth X997.2	1.5 g l^{-1}
Yeast extract	Becton Dickinson 212750	5 g l^{-1}
Sodium bicarbonate	Merck 1.06329.05000	5.04 g l^{-1} (pH 7.3)
Fetal bovine serum[a]	Sigma F4135	10% v/v
Chicken serum[a]	Sigma C5405	10% v/v

For cultures with higher pH, the medium was titrated to pH 8.0 with 1 mol l^{-1} NaOH solution. Note that the pH referred to is based on fresh medium.
[a] Heat inactivated.

The number of hook contractions per minute in transformed oncospheres, was counted in the two pH regimes 2 h after the coracidia were introduced to the culture medium.

2.2. Transformation of procercoids to plerocercoids: the artificial stickleback

The first intermediate hosts, *Macrocyclops albidus* copepods, were cultured and exposed to one coracidium each in the laboratory as described previously (van der Veen and Kurtz, 2002). The culture-population originated from 20 individuals from lake Skogseidsvatnet, Norway, the habitat of origin of the parasites. Instars ranging from the C5 copepodite stage and older were used as hosts for the procercoids. Copepods were kept at 20 °C and 16:8 light: dark cycle. For infection, they were kept singly in wells of a 24-well microtitre plate and starved for 2 days before exposure to the parasite. Coracidia from three different parasite pairs were used for infection. Fourteen days post-infection, procercoids were dissected out of the copepods. Infected copepods were rinsed in sterile filtered water, placed under the microscope, and anaesthetized with carbonated water with added antibiotics (200 µg/ml, Sigma A 5977). After 2 min, the carbonated water was replaced by sterile water. The abdomen of the copepod was removed to release the procercoids into water, which were then transferred into the culture medium with a pipette. To evaluate the effect of the anaesthization, we also conducted 10 dissections using sterile filtered instead of carbonated water. Cultured procercoids were regularly inspected with an inverted microscope.

2.3. Lectin labelling of surface layers

To evaluate the development of the parasites in culture, we analyzed their surface carbohydrate composition by using lectin labelling. In *S. solidus*, the different developmental stages can easily be distinguished by the composition of their surface carbohydrates: the surface of procercoids is dominated by PNA (*Arachis hypogaea*) binding sugars (ß-galactose-1,3 *N*-acetylgalactosamine (GalNAc) and D-galactose), whereas plerocercoids exhibit relatively more WGA (*Triticum vulgaris*) binding sugars (*N*-acetylglucosamine (GlcNAc) and sialic acid residues) on their surface (Hammerschmidt and Kurtz, 2005b).

We compared the relative lectin binding intensity from procercoids that developed in culture (after 14 days: $N = 5$ and after 33 days: $N = 33$) with the ones that were directly dissected out of the copepod at 14 days post-infection ($N = 20$) and with those, which were dissected out of the copepod at 14 days post-infection, and put into culture (for 7–14 days; $N = 19$). As further reference, we used data on relative lectin binding intensity from 7-day-old plerocercoids ($N = 31$), published by Hammerschmidt and Kurtz