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VIABILITY OF *NOTOCOTYLUS ATTENUATUS* (TREMATODA: NOTOCOTYLIDAE) METACERCARIAE UNDER ADVERSE CONDITIONS

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ABSTRACT: The viability of *Notocotylus attenuatus* metacercariae was 80% at 20 wk post-encystment (PE) and decreased to 10% at 24 wk PE. Cyst viability was influenced by the duration of cercarial swimming activity prior to encystment, by the occurrence of cyst associations, and by the type of cyst storage. This is the first report on cyst associations formed by Notocotylidae cercariae. Cyst associations were formed only by cercariae encysting shortly after their emergence from snails. In cyst associations metacercariae did not overlap, but were separated by regular distances ranged from 20.4 to 24.5 μm $\bar{x}$ = 22.3 μm, SE = 0.41. The mucoid materials which formed the external cyst wall covered the areas between parasites seven if distances were comparable with the cyst size. In cyst associations, metacercariae in the water and those located close to the water were viable up to 24 wk PE. Cercariae with extended swimming activity did not form associations even when present in numbers, ≤50 cercariae per cm^3. The cysts established by these cercariae had a thin external cyst wall, and were not viable by 12 wk PE.

Key words: *Notocotylus attenuatus*, metacercariae, metacercarial cyst.

INTRODUCTION

Successful strategies for the dispersion and survival of many digenetic trematodes depend on the viability of various stages in the life cycle, particularly the infective metacercarial stage. Determining the pattern of survival of such cysts in adverse conditions should shed light on the mechanisms of transmission of a species which is an important parasite of migrating waterfowl.

Cysts of *Notocotylus attenuatus* (Rudolphi, 1809) Kossack, 1911, are abundant on snails and plants in a complex of ponds located in eastern Maryland (USA). The ponds on this site are inhabited by a single species of pulmonate snail (*Physa acuta*), which is heavily infected with *N. attenuatus*. Cercariae of this species emerge from April to October; thus later in the year, cysts must spend ≤5 mo under adverse winter conditions to be infective during the spring migration of wildfowl. No studies have been carried out on the viability of *N. attenuatus* cysts under various conditions of stress; however, the method of Graczyk and Shiff (1993a) facilitates the study of cyst viability under different conditions of storage. Our objective was to estimate viability of *N. attenuatus* cysts under conditions simulating natural conditions.

MATERIALS AND METHODS

Physa acuta snails were collected from Duckhead Pond (38°58′N, 76°12′E) at Horsehead Sanctuary, Grasonville, Maryland. Infected snails were placed in aquaria with small, floating pieces of polyethylene sheeting to provide a convenient surface for encystment of emerging cercariae (Fried, 1970). Cysts were scraped off and fed to five, 3-wk-old domestic ducks (Peking strain) from which adult flukes were recovered for identification. Parasite fixation and identification were done by the protocols of Graczyk and Shiff (1993a).

The study was organized into three experiments. In Experiment I, we examined the viability of cysts in conditions simulating natural desiccation. Fifty shedding cercariae snails (7 to 9 mm) were placed for 3 days in a molded glass aquarium (15 × 25 × 20 cm) with rounded corners and filled to a depth of 5 cm with filtered pond water (FPW). Metacercariae appeared to coalesce on the walls of the aquarium and formed associations approximately 5 cm long and 3 cm wide in the rounded corners of the vessel. The snails were then removed and the water level was reduced to 1 cm above the bottom, thus exposing part of the cyst association and leaving the other part under water. The aquarium was covered and the water level was maintained at that level for 6 mo. Cyst associations were di-

vided by marking the glass surface outside into four equal parts. Every 2 wk a cyst sample from each of the four parts was scraped gently with a scalpel blade and excysted in vitro. The viability of metacercariae was determined by the percent of parasites excysted in vitro. Three replicates were done at each time point with a $\bar{x}$ ($\pm$SE) of 100 ($\pm$1.2) cysts per replicate.

In Experiments II and III, we determined the viability of metacercariae in relation to the duration of swimming activity prior to encystment. For Experiment II, 50 snails shedding cercariae were placed in an aquarium for 3 days. The tank was filled with FPW to a depth of 2 cm with small pieces of polyethylene added. In Experiment III, five aquaria were filled with FPW to a depth of 2 cm with small pieces of polyethylene added, 50 shedding snails were placed in each aquarium for 2 hr, and water was gently stirred with a magnetic stirrer. Water was stirred for 4 hr to prevent encystment of the cercariae during 2 hr after removing the snails. During the experiment the polyethylene pieces were partially submerged in the water. Polyethylene pieces taken from Experiments II and III were cut into a smaller parts and divided into four equal groups (A through D) and stored for 6 mo. Group A cysts were refrigerated at 4 C in sterile Locke's (1:1) solution (Ash and Orihel, 1987). Group B cysts were kept at 26 C in FPW. In Group C cysts were stored covered and folded with metal foil. Group C cysts were stored at 4 C in a petri dish containing wet cotton wool to prevent cyst desiccation. Group D cysts were exposed to the open air. Metacercariae were sampled every 2 wk for $\leq$6 mo from groups A through C, and every 12 hr from group D and excysted in vitro. Three replicates were carried out at each time point with a mean $\bar{x}$ ($\pm$SD) of 100 ($\pm$2.5) cysts per replicate.

For in vitro excystation, cysts were incubated for 1 hr in an aqueous solution (1:2) of cationic detergent EDTA-20 (N,N′,N′-polyoxyethylene(10)-N-tallow-1,3-diaminopropane) (Sigma Chemical Co., St. Louis, Missouri), pH 7.5 (Graczyk and Shiff, 1993a). Then cysts were pretreated for 15 min in 1% hydrochloric acid-1% pepsin medium, pH 2.0 (Fried and Ramundo, 1987), and subsequently treated for 2 hr in a medium containing 0.5% trypsin and 0.5% bile salts in Earle's balanced salt solution (BSS), adjusted to pH 7.8 with 7.5% $NaHCO_3$ (Fried and Roth, 1974). The rate of excystation was determined after 2 hr treatment in an alkaline bile salts-trypsin medium (Fried and Roth, 1974). Larvae completely free from the cyst wall were considered excysted.

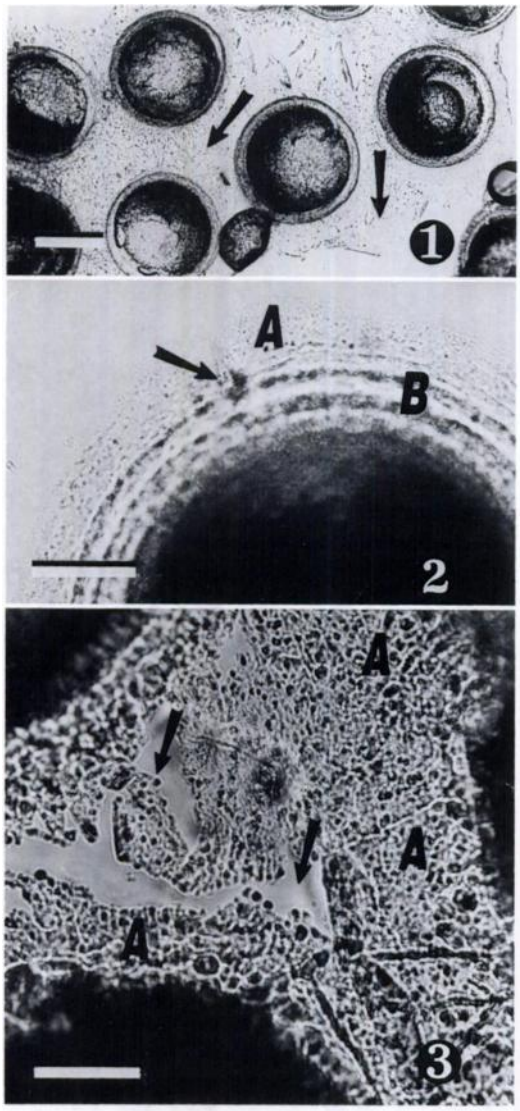
An analysis of variance (ANOVA) and two-sample *t*-test were performed on the rates of excystation using the Analytical Software Statistix 3.5 (Analytical Software, St. Paul, Minnesota, USA). All other statistical treatments followed procedures in Sokal and Rohlf (1981).

RESULTS

Cercariae encysting shortly after emerging from the snails occurred either individually on polyethylene sheeting, in associations in the rounded corners of the aquaria (Fig. 1), or inside ruffles formed in the polyethylene sheeting. Cyst associations never were observed when cercariae were stimulated to continue swimming before encystment (Experiment III), even when the cercariae were abundant ($\leq$50 cercariae per cm^3). If swimming activity prior to encystment was extended, as in Experiment III, individual cysts formed with a thin mucoid envelope (Fig. 2); when exposed to the open air, they lost their viability 24 hr post-encystment (PE) (Experiment III, Group D). However, cysts in associations, were viable <72 hr PE (Experiment II, Group D) when exposed to the open air. When cysts were allowed to form without disturbance following emergence and the parasites were exposed to the open air (Experiment II, Group D), differences in viability between cysts occurring singly and those in associations were not significant, based on a two-sample *t*-test ($P = 0.315$).

In the cyst associations, metacercariae did not overlap, but were separated by regular distances (Fig. 1). The smallest distance between the cysts ranged from 20.4 to 24.5 μm ($\bar{x}$ $\pm$SE = 22.3 $\pm$0.41 μm) and was approximately equal to double the width of the external cyst wall (mucoid envelope). In these associations, the mucoid materials were spread over the area between cysts (Fig. 1). The ventral layer of cysts became progressively depressed during dessication but was limited to the internal cyst wall. We did not observe cyst rupture; however, the mucoid material between the metacercariae became cracked and distorted (Fig. 3).

Normally developed metacercariae which occurred in groups and were kept



FIGURES 1 to 3. Photomicrographs of *Notocotylus attenuatus* metacercarial cysts. Fig. 1. An association of 24-wk-old metacercarial cysts; note the area between parasites covered by mucoid material (arrows). Fig. 2. Metacercarial cyst (4-wk-old) with a thin mucoid envelope (A) formed by cercaria with extended swimming activity prior to encystment; internal cyst wall (B). Note the narrow tube in the mucoid envelope left by cercarial tail (arrow). Fig. 3. Fragments of mucoid envelope (A) of viable, 24-wk-old metacercariae located 1 cm above the water; note the cracking of mucoid material laid between the cysts (arrows). Bars = 150 μm in Figure 1, and 50 μm in Figures 2 and 3.

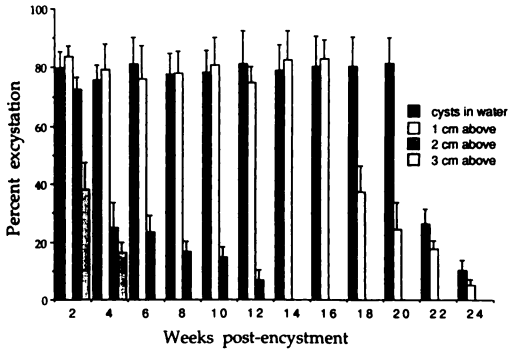


FIGURE 4. Mean percent of in vitro excysted metacercariae of *Notocotylus attenuatus* over time versus cyst location relative to the water level. Each symbol is based on the mean (±SD) of three replicates with 100 (±2.5) cysts per replicate.

in water (Experiment I) had an 80% viability at 20 wk PE. The number of viable parasites decreased significantly (two-sample t-test: df = 31, $P < 0.001$) at 24 wk PE; at 24 wk PE, only 10.3% of the cysts were viable (Fig. 4). Most single metacercariae were dead by week 18 PE, but the differences in numbers of single viable cysts (Fig. 5) and those in associations (Fig. 4) were not significant ≤12 wk PE with a two-sample t-test ($P = 0.363$). This was similar for cysts immersed and those 1 cm above the water level for 16 wk (Fig. 4) when compared with a two-sample t-test ($P = 0.404$).

Normal metacercariae had little difference in survival between types of storage for 12 wk PE (Fig. 5). Cysts established by cercariae with prolonged swimming activity before encystment lost viability by 10 wk PE (Fig. 6). Using an ANOVA test, differences in excystation rates of the cysts sampled from the different type of storage were not significant for normally formed cysts ($F = 2.07$, $P = 0.142$), or for cysts with prolonged swimming activity prior to encystment ($F = 0.35$, $P = 0.079$). Thus the viability of the cysts was not determined by the type of storage. However, there were significant differences in excystation rates between normally formed cysts (Experiment I) with various location

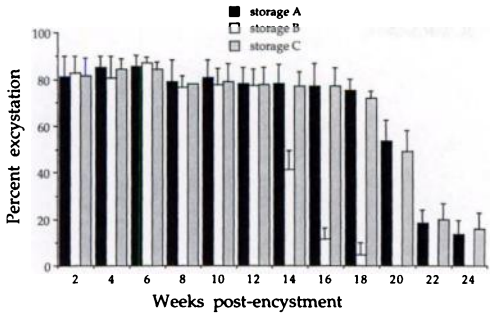


FIGURE 5. Mean percent of in vitro excysted *Notocotylus attenuatus* cysts established by cercariae encysting shortly after emergence from *Physa acuta* snails. Cyst storage: (A) Locke's (1:1) solution, (B) water at 26 C, (C) humidified atmosphere at 4 C. Each symbol is based on the mean (±SD) of three replicates with 100 (±2.5) cysts per replicate.

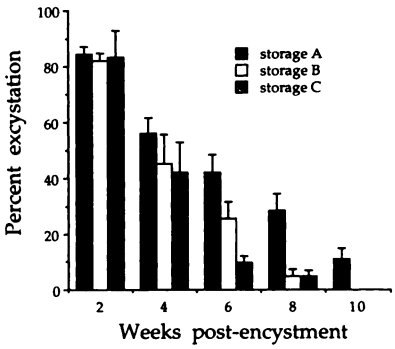


FIGURE 6. Mean percent of in vitro excysted *Notocotylus attenuatus* cysts established by cercariae with extended swimming activity prior to the encystment. Cyst storage: (A) Locke's (1:1) solution, (B) water at 26 C, (C) humidified atmosphere at 4 C. Each symbol based on the mean (±SD) of three replicates with 100 (±2.5) cysts per replicate.

to the water with an ANOVA test ($F = 25.01$, $P < 0.001$).

DISCUSSION

Notocotylus attenuatus is transmitted by pulmonate snails which occur in close proximity to the water surface. Cercariae shed from these snails are likely to encyst near the water surface. The potential exposure to the open air, when water levels drop, must be considered as an environmental factor which may limit cyst viability and consequently affect parasite transmission. In this study we found that cysts located close to the water surface or stored under cool moist conditions can remain viable for ≤6 mo. Such conditions frequently occur in the wild where cercariae encyst on the leaves or on other green plant parts, partially submerged in water or which enfold the edges during drying. Therefore, we conclude that *N. attenuatus* metacercariae are well adapted to overwinter.

Southgate (1971) suggested that the main function of the mucoid envelope was to provide an elastic "skeleton" on which the tough inner cyst wall was formed. Singh and Lewert (1959) noted that the outer cyst wall of *Notocotylus urbanensis* metacercariae disappeared the first few days after formation in water due to bacterial action, and if cysts were exposed to air the outer layer shrank upon drying. We did not observe shrinking of the external wall when cysts were desiccated. The mucoid envelope progressively tightened on the internal cyst wall; thus it played an important role in survival. This protection may have been a function of the chemical nature of the envelope. The external wall of *N. attenuatus* cysts contains mainly acid and neutral mucopolysaccharides as well as muco- or glycoprotein (Pike and Erasmus, 1967). The mucoid envelope also contains a water-soluble glycoprotein which remains on the cysts when exposed to air and may provide protection for the cysts in a humidified atmosphere (Southgate, 1971). The mucopolysaccharide coating persists on the outer surface of the cysts, especially if these are formed immediately after the monostome cercariae emerge from the snail (Kruidenier, 1953). Based on Experiment II, we believe that the integrity of this envelope is an important factor in the long-term survival of the metacercariae.

The existence of associations of *N. attenuatus* cysts has not been reported previously. The occurrence of such associations may indicate that cercariae have preferences for particular surfaces. Based

on the regular distances observed between parasites in these associations and absence of cyst overlap, we believe that cercariae recognize an appropriate surface prior to encystment. Cyst associations may enhance parasite transmission because of improved cyst viability, and because large numbers of parasites may be ingested by a bird at one time.

We noted several parasite adaptations to enhance transmission. These include encystation shortly after host emergence on available surfaces (often on the snail), formation of the cyst associations on suitable surfaces, long-term survival of cysts under normal environmental conditions and resistance of the cysts to desiccation. These factors, taken together with the high infectivity of *N. attenuatus* cysts to the avian hosts (Graczyk and Shiff, 1993b), as well as the absence of a second intermediate host in the life cycle, enhance the transmission of *N. attenuatus* in any aquatic habitat supporting pulmonate snails visited by waterfowl.

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