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In vitro excystation of the metacercariae of *Ribeiroia ondatrae*

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Abstract In vitro excystation studies were done on the metacercarial cysts of *Ribeiroia ondatrae* obtained from naturally infected leopard frogs, *Rana pipiens*. Studies were done in Petri dish cultures in various media maintained at 41°C. The media consisted of 0.5% trypsin—0.5% bile salts in Earle's balanced salt solution at pH 7.8 (TB), 1% acid pepsin (AP) in 0.85% saline adjusted to pH 2 with HCl; acid saline (AS) adjusted to pH 2 with HCl, and saline (S). The TB medium, used successfully to excyst the metacercariae of numerous species of digeneans, did not induce excystation of *Ribeiroia ondatrae*. Some excystation occurred in AS but not in S. Excystation was 100% in AP with most organisms excysting within 30 s after placement in the medium at 41°C. Excystation in vitro in AP at 41°C is compatible with the fact that this organism establishes itself in the acid-pepsin environment of the proventriculus of avian hosts.

Recent evidence suggests that infection by the metacercarial stage of the digenean *Ribeiroia ondatrae* accounts for a considerable proportion of limb malformations observed in amphibians in North America (Schotthoefer et al. 2003). Trematode-induced limb malformations in amphibians have aroused considerable interest. (e.g., Johnson et al. 1999; Blaustein and Johnson 2003; Johnson and Sutherland 2003). Because of the increased interest in this digenean, workers are attempting to fill in missing gaps on the biology of *R. ondatrae* (Johnson et al. 2004). One area of research interest is concerned

with the in vitro excystation of the encysted metacercariae (cysts). As reviewed by Fried (1994), the ability to obtain chemically excysted metacercariae can lead to new studies on the physiology, biochemistry, immunology and ultrastructure of digeneans. To date, studies on the cysts of *R. ondatrae* have been concerned with mechanical excystation, in which the larvae are released with the aid of needles. Mechanical excystation is tedious and may also result in damage to the larvae during the process. This work was initiated to develop a simple method for the chemical excystation of metacercarial cysts of *R. ondatrae*.

Cysts were removed from various sites in the body of naturally infected leopard frogs, *Rana pipiens*, collected from an amphibian malformation hotspot in Eden Prairie, Minn., in July 2004. The cysts were shipped in Ringer's solution (NaCl, 9.0 g/l; KCl, 0.4 g/l; CaCl₂, 0.2 g/l) by overnight mail from Madison, Wis. to Easton, Pa. Of about 75 cysts sent, 60 were intact and contained viable larvae as determined by the appearance of a live organism in the cyst (Fig. 1). Various excystation procedures were tried using ten cysts per 4 ml of medium maintained at 41°C in 3-cm diameter Petri dishes. The media used were prepared as described in Fried and Roth (1974) and consisted of 0.5% trypsin—0.5% bile salts in Earle's balanced salt solution at pH 7.8 (TB medium), or 1% acid pepsin (AP) in 0.85% saline (NaCl) adjusted to pH 2 with 6 N HCl, or 0.85% saline (S). Observations were made within 10 min of preparing the cultures and then at 4 h. A positive control consisted of placing 100 cysts of *Echinostoma caproni* in the TB medium at 41°C, since this medium is very effective in excysting the metacercariae of this echinostome (Fried and LaTerra 2002).

The results of the experiment showed that after 10 min, none, ten, six, and none of the *Ribeiroia ondatrae* encysted metacercariae had excysted in TB, AP, AS, and S, respectively. Observations were made again at 2 h, at which time two additional organisms had excysted in AS, but none had excysted in the TB or S media. Many of the cysts were dead or partially

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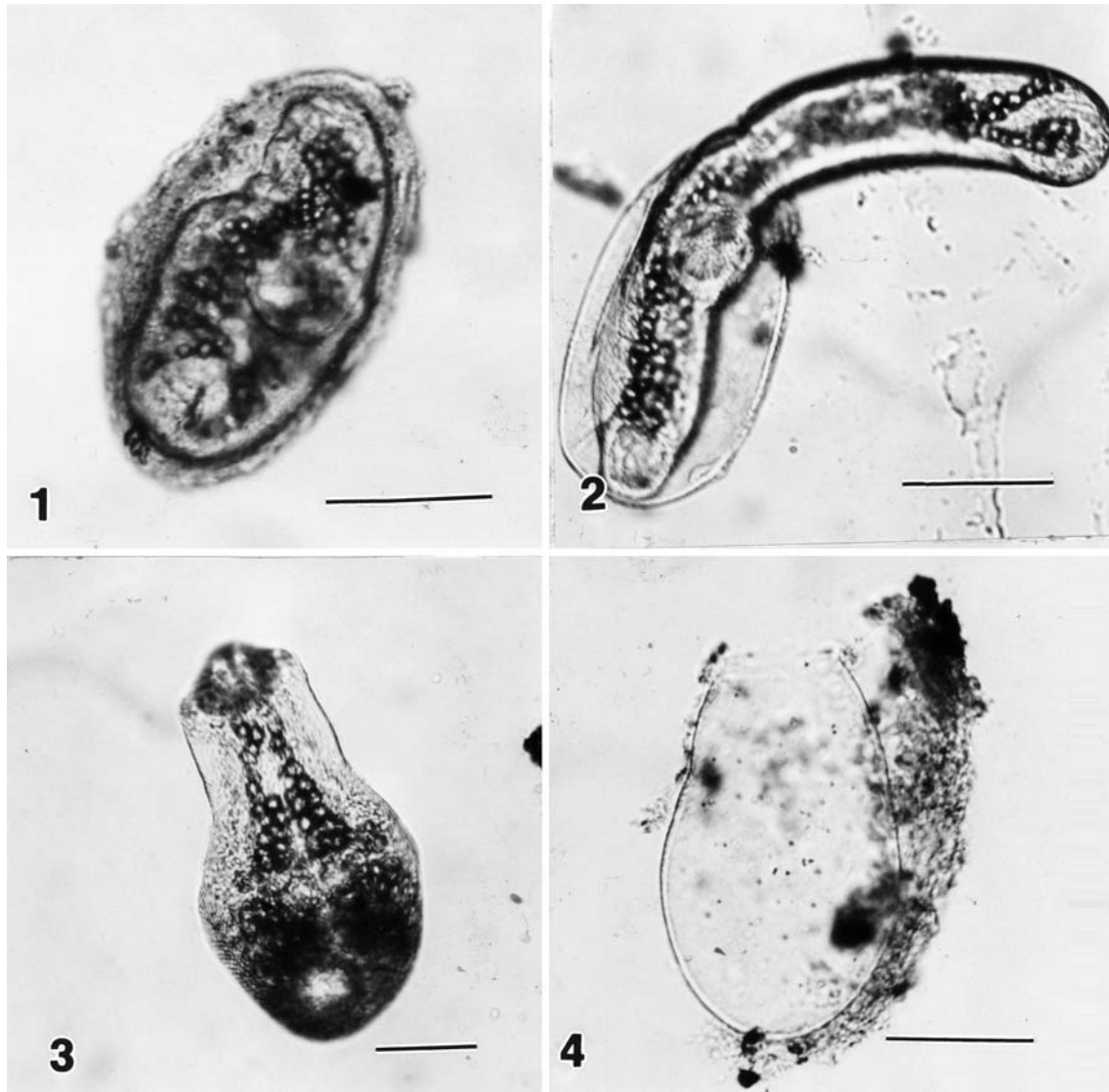


Fig. 1 Intact metacercarial cyst in saline

Fig. 2 Breaching of metacercarial cyst in the acid pepsin (AP) medium

Fig. 3 An excysted metacercaria free in the AP medium and in the contracted state

Fig. 4 An empty cyst in the AP medium

digested in TB by 2 h. The excysted metacercariae in both the AP and AS media were alive and active at 4 h in these media at 41°C, at which time the experiment was terminated. All of the *E. caproni* encysted metacercariae excysted within 2 h in the TB medium.

Because optimal excystation was seen after 10 min in the AP medium, additional studies were done on cysts placed in prewarmed (41°C) AP medium. Of ten cysts tested this way, all had excysted within 30 s after placement into the prewarmed AP. Some of these cysts excysted within 5 s. Excystation as observed under the dissecting scope showed activation of the organism (rapid rotation within the cyst), and its release through an aperture at the narrow end of the pear-shaped cyst. It was not possible to determine if a mucoid-like plug,

characteristic of cysts in the genus *Philophthalmus*, was present (Fried 1994). During excystation, some larvae became breached in the cysts (Fig. 2), prior to their complete emergence. Excystation was scored as complete only when an organism emerged from the cyst and was free in the medium (Fig. 3). An empty cyst following the emergence of the larva is shown in Fig. 4.

A review of the literature indicates that at least six species of digeneans, mainly from various microhabitats in the avian or mammalian small intestine, have been excysted successfully in the TB medium (see review in Fried 1994). The TB medium was used successfully in the only other psilostome studied, i.e., *Sphaeridiotrema globulus* by Fried and Huffman (1982). The TB medium killed cysts of the psilostome *R. ondatrae* within 2 h. Of interest is the fact that species of *Clinostomum* (esophageal and buccal cavity digeneans in avian hosts) have been excysted successfully in AP and AS media (Fried 1994). It is not surprising that *R. ondatrae* metacercariae can be excysted successfully in AP medium, since the larvae become established in the proventriculus of avian

hosts where the internal environment is similar to that of AP medium at 41°C.

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References

- Blaustein AR, Johnson PTJ (2003) Explaining frog deformities. *Sci Am* 288:60–65
- Fried B (1994) Metacercarial excystment of trematodes. *Adv Parasitol* 33:91–144
- Fried B, Huffman JE (1982) Excystation and development in the chick and on the chick chorioallantois of the metacercariae of *Sphaeridiotrema globulus* (Trematoda). *Int J Parasitol* 12:427–431
- Fried B, LaTerra R (2002) In vitro and in vivo encystment of the cercariae of *Echinostoma caproni*. *J Parasitol* 86:1124–1129
- Fried B, Roth RM (1974) In vitro excystment of the metacercariae of *Parorchis acanthus*. *J Parasitol* 60:465
- Johnson PTJ, Sutherland DR (2003) Amphibian deformities and *Ribeiroia* infection: an emerging helminthiasis. *Trends Parasitol* 19:332–335
- Johnson PTJ, Lunde KB, Ritchie EG, Launer AE (1999) The effect of trematode infection on amphibian limb development and survivorship. *Science* 284:802–804
- Johnson PTJ, Sutherland DR, Kinsella JM, Lunde KB (2004) Review of the trematode genus *Ribeiroia* (Psilostomidae): ecology, life history, and pathogenesis with special emphasis on the amphibian malformation problem. *Adv Parasitol* 57:191–253
- Schotthoefer AM, Koehler AV, Meteyer CU, Cole R (2003) Influence of *Ribeiroia ondatrae* (Trematoda: Digenea) infection on limb development and survival of northern leopard frogs (*Rana pipiens*): effects of host stage and parasite-exposure level. *Can J Zool* 81:1144–1153