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Optimised conditions for the *in vitro* excystment of *Calicophoron daubneyi* metacercariae

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Running Title: *In vitro* excystment of *C. daubneyi*

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16 **Summary**

17 Paramphistomosis, caused by *Calicophoron daubneyi*, is an emerging infection of ruminants
18 throughout Western Europe. Despite its prevalence, many questions remain regarding the basic
19 biology of this parasite and how it interacts with its host. Consequently, there is a need to
20 develop methods to study *C. daubneyi in vitro* to improve our understanding of rumen fluke
21 biology. Towards this, we aimed to identify a suitable protocol for *in vitro* excystment of *C.*
22 *daubneyi* metacercariae. Six methods that have been used to excyst metacercariae from a
23 number of trematode species were tested with *C. daubneyi* metacercariae. Three of these
24 achieved an average of >50% excystment whilst one method, which included an acid-pepsin
25 treatment, incubation in reducing conditions and an alkaline/bile salt solution to activate the
26 larvae, consistently gave >80% excystment. The latter protocol also showed no detrimental
27 effect on the motility of newly excysted juvenile (NEJ) parasites when observed for up to 24
28 hours in RPMI 1640 medium post-excystment. The successful production of *C. daubneyi* NEJs
29 *in vitro* is a significant step forward, and will enable the discovery of infective stage-specific
30 parasite antigens and facilitate drug screening trials, to aid the development of much needed
31 diagnostic and therapeutic options for paramphistomosis.

32

33 **Key Words:** Paramphistome, Metacercariae, Excystment, *Calicophoron daubneyi*

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35

36

37 **Key findings:**

38 • The *in vitro* excystment of *C. daubneyi* metacercariae has not been previously
39 described.
40 • An *in vitro* excystment protocol for *C. daubneyi*, typically producing >80% excystment
41 was identified.
42 • This will enable the study of infective NEJs, and the development of required
43 diagnostics/therapeutics.

44

45 ## Introduction

46 Paramphistomosis is a serious endemic infection of ruminant livestock in tropical and sub-
47 tropical regions (Rojo-Vázquez *et al.* 2012), and in recent years it has been identified as an
48 emerging infection in Western Europe (Huson *et al.* 2017). *Calicophoron daubneyi* has been
49 confirmed in a number of studies as the primary rumen fluke species infecting ruminant
50 livestock across Western Europe (Ferreras *et al.* 2014; Malrait *et al.* 2015) including the UK
51 and Ireland (Gordon *et al.* 2013; Martinez-Ibeas *et al.* 2016; Jones *et al.* 2017). Morbidity and
52 mortality attributed to paramphistome infections is invariably associated with acute disease,
53 where ingested paramphistome metacercariae excyst in the small intestine, and the resulting
54 NEJs cause significant damage to the intestinal tissues as they move from the small intestine
55 lumen to the sub-mucosa (Millar *et al.* 2012; Pavan Kumar *et al.* 2016). Immature
56 paramphistomes are thought to remain in the small intestine for up to 3 months, feeding on host
57 tissue, before they complete their migration to the rumen where they mature and infections
58 become patent (Sanabria and Romero, 2008).

59 Currently, where an active case of paramphistomosis is suspected, there is no diagnostic
60 test available which can confirm pre-patent acute disease in an animal, therefore clinical

61 paramphistomosis can only be confirmed during post-mortem examination. Mature infections
62 may only be diagnosed by faecal egg count tests unless a post-mortem examination is
63 performed by a veterinarian or in the abattoir. The therapeutic treatment and control of
64 paramphistomosis at present relies on a single anthelmintic compound; oxyclozanide (Arias *et*
65 *al.* 2013) but this is often used off-licence as it is only approved for use against fasciolid
66 infection (asides from a single formulation of oxyclozanide to treat paramphistomosis, licensed
67 only in France: Douvistome). Clearly, the lack of an appropriate diagnostic test and approved
68 treatment options are not desirable in the face of this emerging parasitic infection, which has
69 the potential to cause significant clinical disease where large numbers of metacercariae are
70 encountered and ingested by their ruminant hosts.

71 In order to develop both the diagnostic tools and anthelmintic treatments for
72 paramphistomosis, the identification of suitable diagnostic and anthelmintic targets is required.
73 To facilitate this, researchers require access to the infective (and most pathogenic) stages,
74 namely the *C. daubneyi* NEJs and immature small intestine-dwelling flukes. These specimens
75 are impractical to obtain from naturally-infected animals in the abattoir, as is common for the
76 collection of mature rumen fluke. Therefore, a reliable protocol is required to excyst *C.*
77 *daubneyi* metacercariae *in vitro.* When successfully excysted and maintained *in vitro*, the
78 resulting *C. daubneyi* NEJs will facilitate the study of infective stage-specific parasite
79 molecules to support diagnostic development through proteomic or transcriptomic experiments
80 (Robinson *et al.* 2009), as well as providing a source of infective stage parasites for *in vitro*
81 studies such as the screening of existing/novel anthelmintics (Panic *et al.* 2013). However,
82 anecdotal evidence from the research community suggested that *C. daubneyi* metacercariae
83 were difficult to excyst using protocols largely developed for the liver fluke, *Fasciola hepatica*.
84 Here, six previously-published methods, which had been developed for the *in vitro* excystment
85 of other trematode species, were modified and tested against *C. daubneyi* metacercariae. An

optimal protocol consistently giving >80% parasite excystment under *in vitro* conditions is described.

Materials and Methods

Parasites

C. daubneyi metacercariae (Miskin isolate) were obtained from Ridgeway Research (Gloucestershire, UK). Metacercariae were harvested from *Galba truncatula* snails which had been previously infected with *C. daubneyi* miracidia. Metacercariae were washed briefly in water before use.

In vitro excystment of C. daubneyi metacercariae

Six methods which have been previously described for the *in vitro* excystment of various trematode parasite species; *Fasciola hepatica* (McGonigle *et al.* 2008)*, Fasciola gigantica* (Nagar *et al.* 2010)*, Zygocotyle lunata* (Fried *et al.* 1978)*, Paramphistomum spp* (Huesca-guillén *et al.* 2007)*, Acanthoparyphium spinulosum* (Bass and LeFlore, 1984) *and Neascus pyriformis* (Schroeder *et al.* 1981) were selected to test with *C. daubneyi* metacercariae. Whilst other methods were available, the selected methods were chosen to avoid testing highly similar protocols. Some modifications, based on preliminary observations and the availability of reagents, were made to the published methods. These are detailed in Table 1. All excystment experiments performed here included incubations at 39˚C (the approximate body temperature of the major definitive hosts of *C. daubneyi*, namely cattle, sheep and goats) with gentle agitation at 60 rpm in a shaking incubator.

108 Initially all 6 protocols were tested in parallel, with 20 metacercariae per treatment.
109 With the exception of method 1 (incubation in 0.5% sodium hypochlorite) all groups of
110 metacercariae were incubated at 39˚C in dH_2O for 10 mins then washed twice in dH_2O with a
111 2 min, 500 x g centrifugation applied between washes. Protocols were then followed as detailed
112 in Table 2, with 2 washes in dH_2O performed between all media changes, but with no
113 centrifugation of the metacercariae after incubation in the activation media. Excystment of
114 NEJs was monitored after 2, 4 and 6 hours incubation in the excystment media and after an
115 overnight incubation (Fig. 2). Where the excystment protocol called for a salt solution to be
116 used, Locke's solution (LS) (0.9% NaCl, 0.042% KCl, 0.02% $NaHCO_3$, 0.024% $CaCl_2$) at pH
117 7.4 was used in all cases.

118

119 *Post-excystment maintenance of NEJs*

120 Excysted NEJs were collected from the respective excystment media by pipette under a
121 dissecting microscope, and transferred to a 2 ml microcentrifuge tube containing 1 ml of warm
122 (39 ˚C) RPMI 1640 culture media, supplemented with 100 IU/ml penicillin and 100 mg/ml
123 streptomycin. One change of the RPMI 1640 media was performed after collection of the last
124 NEJs into each tube and parasites were then maintained for a 24 hour period at 39 ˚C in an
125 incubator. NEJs were observed for activity at 4, 8 and 24 hours of incubation. All chemicals
126 were purchased from Sigma-Aldrich unless otherwise stated.

127

128 **Results**

129 Three of the six methods tested (methods 4, 5 and 6) showed no or minimal parasite excystment
130 and were not carried forward for further trials. The remaining three methods were tested in
131 triplicate with 20 metacercariae/treatment, with an average excystment rate of 11/20 (53%) for

132 method 1, 14/20 (70%) for method 2 and 18/20 (90%) for method 3, respectively. These
133 excystment levels were compared using a one-way ANOVA with Tukey's pairwise comparison
134 using PAST (Hammer *et al.* 2001) and the level of excystment seen for method 3 was shown
135 to be significantly higher than the excystment achieved with both method 1 ($p<0.01$) and
136 method 2 ($p<0.05$), as detailed in Fig. 1. To obtain optimal levels of excystment of
137 metacercariae it was necessary to incubate the activated metacercariae overnight and collect
138 NEJ parasites the following day after approximately 20 hours incubation in excystment media
139 (Fig. 2).

140 It was observed that excystment of *C. daubneyi* NEJs occurred after much activity of
141 the parasite within the cyst, with an aperture appearing at a single point in the cyst wall through
142 which the NEJ could escape, as shown in Fig. 3. Following excystment, NEJs were successfully
143 maintained for 24 hours in RPMI1640 medium supplemented with 100 IU/ml penicillin and
144 100 mg/ml streptomycin, and showed constant movement when observed.

145 Method 3, modified from the protocol described by Fried *et al*. (1978) for excystment
146 of *Zygocotyle lunata*, was the most successful. This protocol was further tested with groups of
147 100, 500 and 1,000 metacercariae. Here the alkaline excystment medium was filter sterilised
148 (0.22 μm, Millipore Ltd, Hertfordshire, UK) before the addition of 100 IU/ml penicillin, 100
149 μg/ml streptomycin, and 2 μg/ml amphotericin B to remove any undissolved bile salts and
150 possible microbial contaminants. These further excystment trials demonstrated that the
151 protocol is still highly successful when applied to larger numbers of metacercariae. These tests
152 yielded 84%, 86% and 80% excystment rates, respectively. By the time the final 1000
153 metacercariae excystment test was performed, metacercariae had been stored post harvesting
154 for up to 10 weeks at 4 ˚C, and >80% excystment of active, viable NEJs was still observed.

155

156 **Discussion**

157 Paramphistomosis, caused by *C. daubneyi*, is on the increase throughout Europe and is thought
158 to be more prevalent than the liver fluke, *F. hepatica*, in some parts of the UK and Ireland
159 (Toolan *et al.* 2015; Jones *et al.* 2017). Whilst the impact of chronic rumen fluke infection on
160 animal health and production remains largely unknown, clinical disease and mortality linked
161 to significant immature parasite burdens in the small intestine, although rare, have been
162 reported in both sheep and cattle (Foster *et al.* 2008; Mason *et al.* 2012; Millar *et al.* 2012). To
163 begin to understand how NEJ and immature *C. daubneyi* parasites contribute to the pathology
164 of infected animals, and to aid the development of diagnostic tools and treatment options, we
165 must first be able to study these life cycle stages *in vitro.* Towards this goal, we describe for
166 the first time a successful method for the *in vitro* excystment of *C. daubneyi* metacercariae.

167 Previously, treatments including exposure to CO_2 (Dixon, 1966), reducing conditions
168 (Bass and LeFlore, 1984), acid-pepsin treatment and the presence of both bile salts and trypsin
169 (Fried *et al.* 1978) have all been suggested to be necessary for the *in vitro* excystment of
170 trematode parasites. Here, the two protocols which included a 15 min acid-pepsin treatment
171 (methods 2 and 3) produced the highest levels of excystment, although this step does not appear
172 to be an absolute requirement for the emergence of NEJs given the 54% average excystment
173 seen in method 1 where no acid-pepsin treatment was included. Furthermore, no excystment
174 was seen with method 5 which included an hour long acid-pepsin treatment, perhaps indicating
175 that prolonged exposure to such conditions may be detrimental to the excystment process.
176 Greater levels of excystment were also seen with the two protocols that included a sodium
177 dithionite treatment (methods 2 and 3), whereas a lower average excystment was seen in
178 method 1 where L-cysteine was used to create reducing conditions. The removal of the 1%
179 trypsin from the alkaline/bile salt medium in method 4 was necessary as during initial trials it
180 was seen that, although up to 90% excystment was achieved, the NEJs that emerged were

181 rapidly digested by the trypsin. Hence, it is possible that the trypsin used in the previously
182 described protocol from which method 4 was adapted, and other protocols where trypsin has
183 been included at a similar concentration (LeFlore and Bass, 1983), was only minimally active
184 when included at 1% w/v. Li *et al.* (2004) also included trypsin in their excystment protocol
185 for *F. gigantica*, but at a much lower final concentration of 0.01%. The percentage trypsin used
186 by Li *et al.* (2004) is likely much closer to the *in vivo* concentration of trypsin in the host
187 intestine, with an average of 143 µg/ml trypsin (=0.0143%) reported in human intestinal fluid
188 (Metheny *et al.*, 1997). Our results, however, indicate that the presence of trypsin is not
189 required for the *in vitro* excystment of *C. daubneyi* metacercariae given the success of methods
190 1-3 which all lacked this supplement.

191 For all treatment groups in which NEJs successfully emerged, whilst a
192 considerable number of NEJs appeared after 6 hours incubation in excystment media, maximal
193 excystment was achieved following prolonged incubation, typically overnight (up to 20 hours).
194 This is similar to the excystment time required by Nagar *et al.* (2010) to obtain the maximum
195 number of *F. gigantica* NEJs. Although shorter incubation times have been reported to achieve
196 excystment in other trematode species, the success of the overnight incubation period, with no
197 impact on the motility of the NEJs recovered after this time, makes this an efficient and
198 convenient protocol for excystment of *C. daubneyi* metacercariae *in vitro*. It has recently been
199 shown that *F. hepatica* NEJs can be excysted and maintained *in vitro* for long-term studies of
200 their growth and development (McCusker *et al.*, 2016). Our development of a successful
201 method for *in vitro* excystment of *C. daubneyi* metacercariae now allows similar refinement of
202 culture conditions that permit long-term studies of rumen fluke.

203 The development of the current method for the excystment of *C. daubneyi*
204 metacercariae opens the door for a wide range of *in vitro* experiments using the infective stage
205 of this emerging parasite. One research priority is the study of transcriptome and proteome

206 profiles relating specifically to this infective stage (Huson *et al.* 2017). This would not only
207 inform our knowledge of how these parasites establish and maintain infections in the definitive
208 ruminant host but would also facilitate the discovery of potential diagnostic antigens and
209 vaccine candidates. In addition, the successful development of a method to produce *C.*
210 *daubneyi* NEJs paves the way for further *in vitro* studies to improve our understanding of the
211 developmental and molecular biology of these parasites, along with the development of *in vitro*
212 culture tools for drug susceptibility studies.

213

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217

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Table 1: Details of any modifications made to the original excystment methods tested against *C. daubneyi* metacercariae. [1] Trematode species the excystment protocol was originally designed for.

Method	Species[1]	Notes/modification of the original method	Reference
1	*Fasciola hepatica*	500 x g centrifugation used during sodium hypochlorite wash step.	McGonigle *et al.* (2008)
2	*Fasciola gigantica*	1% acidified pepsin pre-treatment included as for method 4. Taurocholic acid excluded from second incubation stage, and 10% (v/v) bovine bile was included in the final RPMI 1640 incubation instead.	Nagar *et al.* (2010)
3	*Zygocotyle lunata*	After an initial test (data not shown) trypsin was removed from the original protocol as NEJs were killed and digested soon after excystment, although 90% excystment was seen. Locke’s solution used in place of Earle's Balanced salt solution in final excystment media. Sodium tauroglucocholate used in place of sodium glycocholate.	Fried *et al.* (1978)
4	*Paramphistomum* spp	CO_2 bubbling step replaced and CO_2 generated in solution by placing metacercariae in 5 ml 0.45% NaCl, 0.6% NaHCO3, 0.15M sodium dithionite with the addition of 20 µl conc. (37%) HCL immediately before sealing and incubating tubes.	Huesca-guillén and Ibarra-velarde, (2007)
5	*Acanthoparyphium spinulosum*	Locke's solution used in place of Hank’s balanced salt solution in initial 1% acidified pepsin treatment. Sodium tauroglycocholate used in place of sodium taurocholate.	Bass and LeFlore, (1984)
6	*Neascus pyriformis*	Only the described optimised protocol was tested. Sodium tauroglycocholate was used instead of sodium cholate.	Schroeder *et al.* (1981)

Table 2. Details of the six *in vitro* excystment methods tested against *C. daubneyi* metacercariae. LS, Locke's solution.

Method	Pre-treatment	Activation, excystment and incubation media
1	20 min in 0.5% sodium hypochlorite	▪ Overnight incubation in 0.5% $NaHCO_3$, 0.4% NaCl, 0.2% sodium tauroglycocholate, 0.07% conc. (37%) HCl, 0.06% L-cysteine
2	15 min in 1% pepsin in LS (pH 2)	▪ 1.5 hrs in 1% $NaHCO_3$, 0.8% NaCl, 0.02 M sodium dithionite (activation of larvae) ▪ RPMI 1640 with 10% (v/v) bovine bile (excystment and overnight incubation)
3	15 min in 1% pepsin in LS (pH 2)	▪ 5 min in 0.02 M sodium dithionite in LS (activation of larvae) ▪ 4 hrs LS with 1% sodium tauroglycocholate (pH 8.8) (initial excystment) ▪ RPMI 1640, 0.01% sodium tauroglycocholate (overnight incubation and further excystment)
4	-	▪ 1 hr in 0.6% $NaHCO_3$, 0.4% NaCl, 0.08% L-cysteine and 0.07% conc. (37%) HCl ▪ 10% v/v bovine bile in LS (excystment and overnight incubation)
5	1 hr in 0.5% pepsin in LS (pH 2)	▪ 10 min in 0.02 M sodium dithionite in LS (activation of larvae) ▪ 0.2% sodium tauroglycocholate (pH 7.8) (excystment and overnight incubation)
6	15 min in acidified LS (pH 2) with 0.03% sodium dithionite	▪ Incubate overnight in 0.5% trypsin, 0.5% sodium cholate (pH 7.4)

Fig. 1. Excystment rates obtained from the 3 methods which showed promising initial results (>50% excystment) against *C. daubneyi* metacercariae. **= $p<0.01$, * = $p<0.05$.

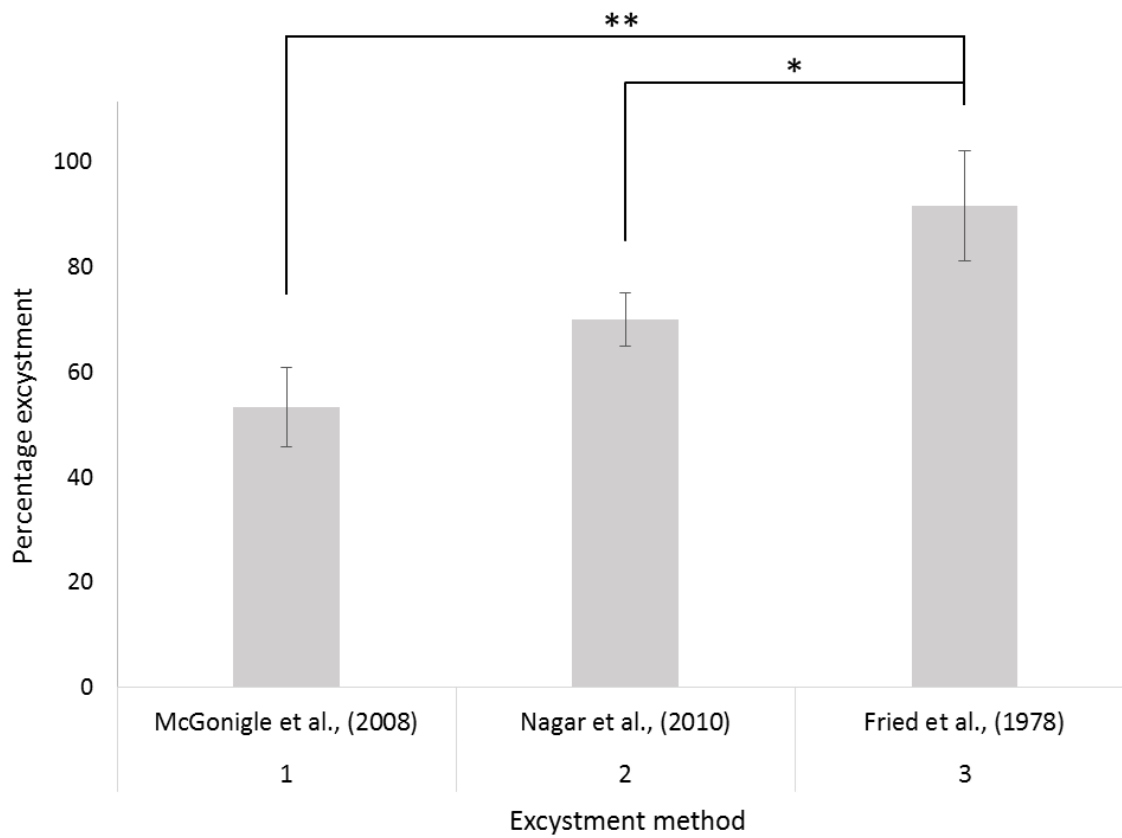

Fig. 2. Percentage excystment of *C. daubneyi* metacercariae after incubation in excystment media over a 20 hour period. Mean values are shown for those methods (1-3) which typically gave >50% excystment.

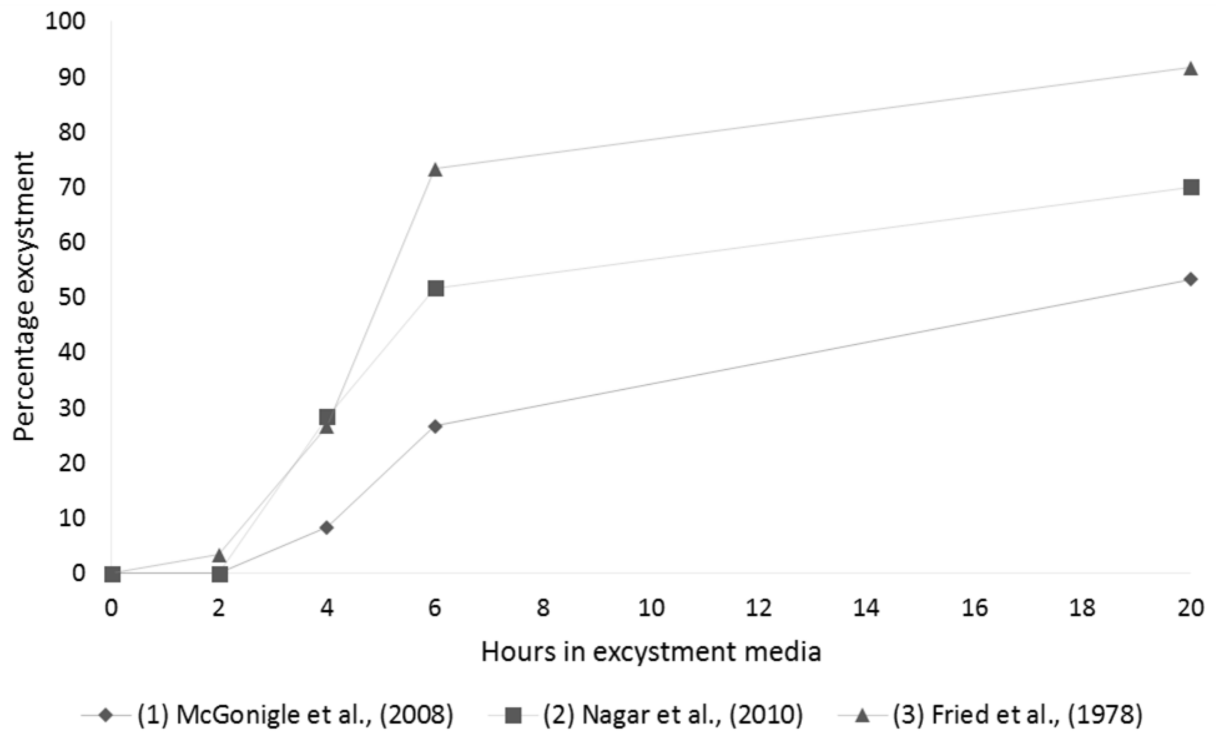

Fig. 3. Activated *C. daubneyi* metacercaria (pre-excystment).