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Storage and incubation of *Echinostoma revolutum* eggs recovered from wild *Branta canadensis*, and their infectivity to *Lymnaea tomentosa* snails

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Abstract

Echinostoma revolutum eggs recovered from naturally infected wild Canada geese (*Branta canadensis)* were cold stored (4–6°C) for up to 72 weeks. Successful hatching followed incubation for from 6 to 8 days at an optimum temperature of between 25 and 30°C. A partial life cycle from adult worm to metacercarial encystment in *Lymnaea tomentosa* snails was completed in the laboratory. Snails were infected both by free miracidia and by ingestment of unhatched embryonated eggs. Infection was equally successful in environmental temperature ranges from 10 to 25°C, and at challenge levels of 2, 5 or 10 embryonated eggs per snail. Exposure to 10 eggs was lethal. Ingestion by snails of embryonated eggs with successful infection at 10°C suggests that embryonated eggs may be used to infect wild snails when the environmental water temperature has reached 10°C.

Introduction

Incubation of *Echinostoma revolutum* and *E. trivolvis* eggs from laboratory infected hosts and their infectivity to laboratory snails have been studied. Moravec *et al.* (1974), working with the eggs of *E. revolutum* from white mice, reported development in 8 days at 28°C. Fried & Weaver (1969) reported that eggs of *E. revolutum* (later renamed *Echinostoma trivolvis*, B. Fried, personal communication) hatched in the dark as well as in the light, and Fried (1985) incubated eggs of *E. trivolvis* to hatch in 2 to 3 weeks at 22–23°C. Fried & Scheuermann (1987) incubated eggs of *E. trivolvis*, from laboratory infected hamsters, in tap water at 22–24°C for 2–3 weeks and found that miracidia of the Pennsylvania strain of *E. trivolvis* were specifically infective to the Pennsylvania strain of *Helisoma trivolvis* snails. Kanev (1994) recovered *E. revolutum* eggs from laboratory bred avian hosts and incubated them at 28°C to hatch in 9 days. These eggs were stimulated to hatch by exposure to light and the miracidia were infective to laboratory bred lymnaeid snails.

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The present study determines the effect of cold storage duration ($\leq$ 26 weeks and $>$ 26 weeks) and temperature range on the incubation success of *Echinostoma revolutum* eggs recovered from wild Canada geese, and also determines the infectivity of such fully developed eggs to laboratory *Lymnaea tomentosa* snails originally from Lake Wanaka, New Zealand.

Materials and methods

Echinostome egg harvest and storage

Gravid echinostomes were recovered from the intestines of freshly killed Canada geese during New Zealand Fish and Game waterfowl management culls conducted in two consecutive winter seasons. Eggs were recovered by individual worm dissections and then by homogenizing several whole worms in a blender with 2 l of fresh water followed by sedimentation through a 2-l plastic container into a 250 ml beaker, thence into 40 ml Petri dishes. Eggs were washed several times in fresh water, sorted into lots of 1000 under the dissecting microscope, and then pipetted into plastic tissue flasks containing a 10 ml solution of distilled water and antibiotic/antimycotic. The antibiotic/antimycotic (GIBCO CAT No. 600-5240A9, 10,000 U ml^{-1} penicillin G

sodium - 10,000 μg ml^{-1} streptomycin sulphate - 25 μg ml^{-1} amphotericin B as fungizone in 0.85% saline) was diluted 1/199 by volume. Eggs were then cold stored (4–6°C) in tissue flasks.

Echinostome egg incubation

Samples of 100–260 eggs were pipetted, using a sterile technique, from cold-stored tissue flasks into individual sterile polystyrene Petri dishes (100 × 15 mm style) in 40 ml of incubation solution (antibiotic-antimycotic/distilled water, 1/199 by volume). Eggs were incubated for at least a week beyond the observation of the final hatch but not longer than 59 days at temperatures ranging from 10 to 45°C in 5°C steps using two incubators. Egg development was monitored twice daily and the incubation solution was replaced as it became cloudy. At times it was necessary to wash eggs from their original Petri dishes into new sterile dishes to arrest fungal contamination.

Infection of snails

Laboratory maintained *L. tomentosa* snails were placed singly in individual flat-bottomed tubes (SAMCO 25 mm) one-third full of stream water and capped with polythene covers that had been pierced to facilitate gas exchange and to prevent escape. Snails were individually exposed to either 2, 5 or 10 fully developed echinostome eggs that had been incubated at 28°C (Kanev, 1994) and the tubes were then placed in incubators set at 10, 15, 20 or 25°C. Thirteen to 18 snails were used for each challenge exposure at each temperature. The snails were examined 24 h post-exposure to determine if all eggs had either hatched or been ingested. Snails were then maintained at room temperature (17–22°C), fed oven-dried lettuce and the water was changed morning and night until they died or released cercariae. Infection was confirmed in live snails by close observation of developing parasites through the shell. Snails that died were dissected.

Statistical analysis

Eggs harvested over two separate seasonal wildlife management culls were grouped by cold storage durations of 26 weeks or less (CS-1) and more than 26 weeks (CS-2) for analysis. Multifactorial analyses of variance (arc/sine transformation, general linear model, MINITAB™, 1991) were used to determine the effects of cold storage duration and incubation temperature on maximum hatch proportions (the highest cumulative hatch expressed as a proportion of the total number of eggs in each sample incubated) and hatch duration (the time in days it took for all eggs in each sample to complete hatching), and the effects of cold storage duration, exposure temperature and challenge levels on the proportion (the final number per sample expressed as a proportion of the total number challenged) of snails becoming infected.

Results

Echinostome egg incubation

Eggs did not develop at 10°C. Attempts to incubate eggs at 15°C were terminated after 59 days by which time the eggs were still alive but developing slowly. To verify the viability of these eggs the temperature was reset at 30° and hatching commenced 5 days later (fig. 1). In the range 20–40°C (fig. 2), eggs started and completed hatching earlier as the temperature increased. The longer period of cold storage prior to incubation delayed the start of hatching but had a variable effect on hatch duration. Eggs did not require light stimulation to hatch. Several were found, on morning inspection, to have hatched during the previous night. All eggs died within 6 h at 45°C incubation.

Hatch duration decreased significantly with increasing temperature (Fdf 4,26 = 8.23, $P < 0.0001$). Cold storage prior to incubation had no significant effect on hatch duration (Fdf 1,26 = 0.91, $P = 0.348$). Effects of cold storage (Fdf 1,26 = 26.22, $P < 0.0001$) and temperature (Fdf 4,26 = 4.67, $P = 0.006$) on hatching percentage were significant (fig. 3). The highest hatch percentage occurred at 25°C for eggs cold stored ≤ 26 weeks and at 30°C for eggs cold stored > 26 weeks.

Infection of snails

Lymnaea tomentosa snails were similarly infected (fig. 4) at challenge levels of 2, 5 and 10 eggs each (Fdf 2,9 = 0.41, $P = 0.675$). Evidence for snail infection by free swimming miracidia was implied by the finding of empty worm egg casings. Some snails were observed to crawl over embryonated eggs, following which eggs were not left behind. These snails became infected and no empty egg casings were found. Snails exposed to 2 and 5 eggs each survived with parasites developing through all stages to encyst as metacercarial cysts (fig. 5). In most cases the cercariae encysted without emerging from the snails. Snails exposed to 10 eggs each became infected and died before the parasites could develop to the metacercarial stage. Different exposure temperatures (fig. 6) produced similar infection levels (Fdf 3,9 = 0.79, $P = 0.532$). The longer period of cold storage significantly reduced infection levels (Fdf 2,9 = 11.94, $P = 0.007$).

Discussion

Cold storage, incubation and hatching

Changes in environmental temperature influence the survival, development, and hatching of eggs harvested from *Echinostoma revolutum* worms found in wild Canada geese. Christensen *et al.* (1980) reported that unembryonated *E. liei* eggs (later named *Echinostoma caproni*, B. Fried, personal communication) could be maintained at 4°C with an unchanged hatchability following incubation for up to at least 20 weeks. The present research shows that, for *E. revolutum*, eggs (which were grouped into ≤ 26 weeks and > 26 weeks for statistical analysis) can be cold stored for up to 72 weeks, be incubated and still be capable of hatching and infecting host snails. Lack of development at 10°C in the laboratory suggests that eggs

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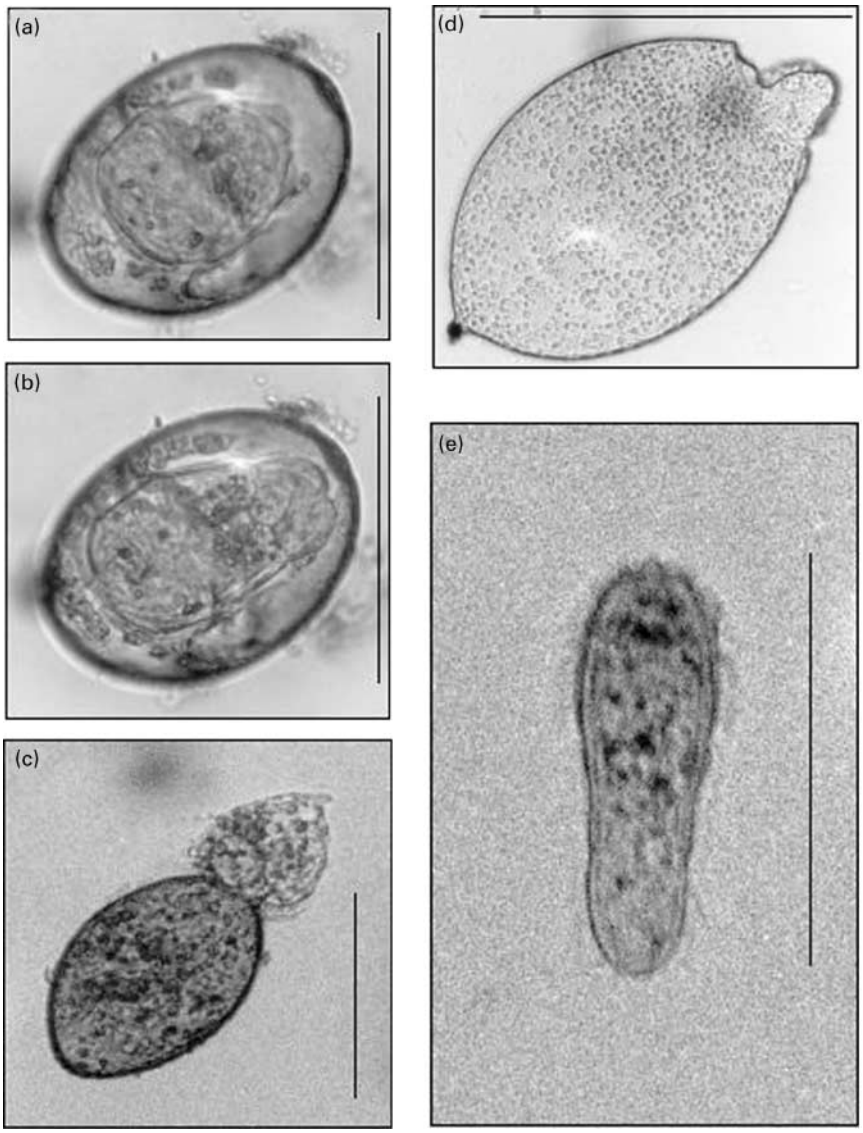

Fig. 1. Fully developed incubated eggs of *Echinostoma revolutum*. (a and b) Live miracidium rapidly moving within eggshell; (c) hatching; (d) after hatch; (e) rapidly swimming miracidium. Bars are all 100 μm. Measurement of swimming miracidium (e) is approximate.

may accumulate with no development in the lake when the winter water temperature is at or below 10°C, then commence development when temperatures rise in the spring. The slow development of eggs in the incubator at 15°C followed by hatching within 5 days when the temperature was raised to 30°C (compared with a hatch duration of 7–13 days when eggs were incubated strictly at 30°C) may indicate a synchronization of development at or below 15°C. McKindsey & McLaughlin (1993), suggested that long term exposure of accumulating trematode eggs (*Sphaeridiotrema pseudoglobulus*) to temperatures below 10°C may loosely synchronize their development through the winter and lead to a surge in development and hatching when lake water temperatures rise. As a consequence of synchronization, eggs deposited in the late autumn or early winter may develop and hatch in a shorter time as water temperatures rise than would eggs deposited in late winter or early spring. Miracidia may then be available in the late spring to infect overwintering uninfected adult snails. The duration of development and hatching observed at 20°C (3 weeks and 2 weeks, respectively) and at 25°C (2 weeks and 1 week, respectively) may serve to spread the hatch from spring through late summer. In late summer, when temperatures in shallow water have been observed to rise above 25°C, the even shorter development time and hatching duration (e.g. 1 week and <1 week, respectively, at 30°C) might ensure that a maximum number of miracidia are available to infect juvenile snails before the onset of winter. In Lake Wanaka, this would result in an availability of

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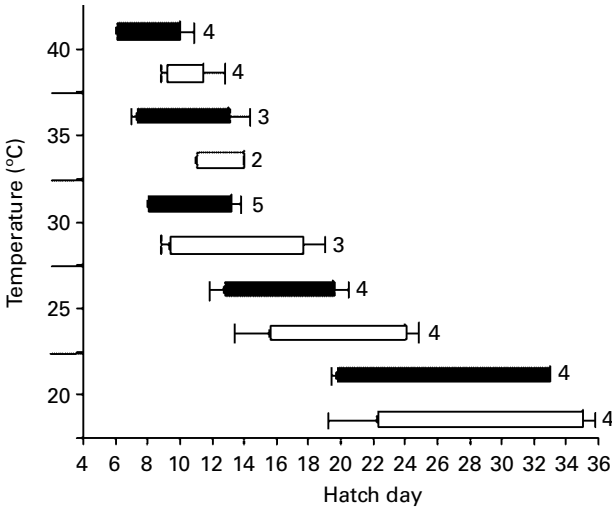

Fig. 2. *Echinostoma revolutum* egg incubation. Initial hatch day (left side bar) and final hatch day (right side bar). Eggs cold stored ≤ 26 weeks (closed bar) and > 26 weeks (open bar). Bar lengths indicate hatch duration. T-bars indicate standard errors of the mean (initial and final hatch days). Temperatures are discrete. Numbers are replicates of 100–260 eggs each.

echinostome miracidia to infect not one, but two generations of host snails in one season – overwintering uninfected adult snails, and the snail cohort hatching in the springtime.

The optimum temperature for embryonation and hatching of these eggs in the laboratory appears to be between 25°C and 30°C, in agreement with other studies (Fried & Weaver, 1969; Moravec *et al.*, 1974; Lee *et al.*, 1990; Behrens & Nollen, 1993; Kanev, 1994; Idris & Fried, 1995; Lo, 1995). Water temperatures in Lake Wanaka have been observed during snail recoveries to vary from 8°C in the

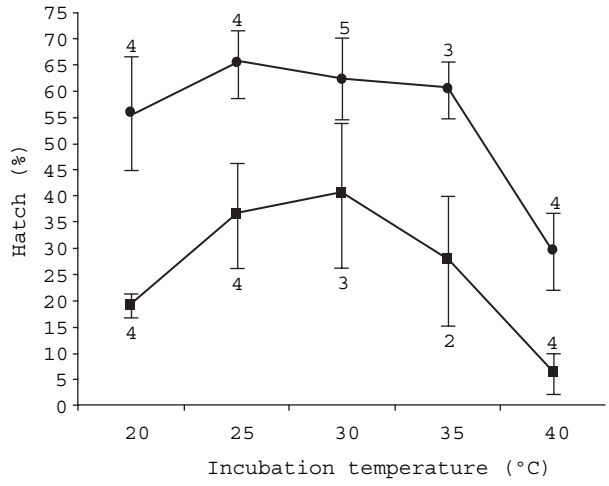

Fig. 3. *Echinostoma revolutum* egg incubation. Comparison of percent hatch of eggs cold stored ≤ 26 weeks (•) and > 26 weeks (■) and then incubated at 20, 25, 30, 35 and 40°C. T-bars indicate standard error. Numbers are replicates of 100–260 eggs each.

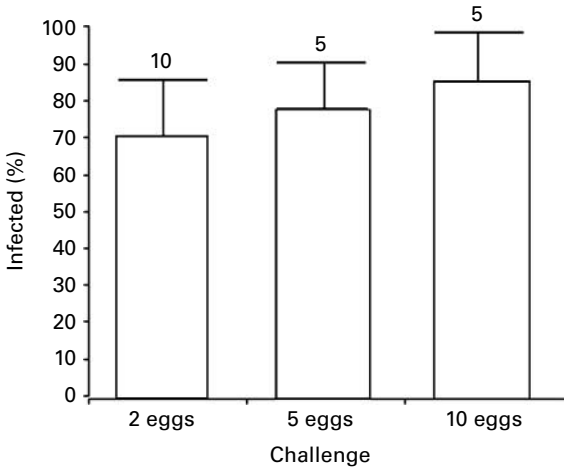

Fig. 4. Infection of individual *Lymnaea tomentosa* snails with fully developed incubated *Echinostoma revolutum* eggs. T bars indicate standard error. Numbers are replicates of 13–18 snails each.

winter to a maximum of 26°C in the summer (personal observation). The maximum hatching percentage (55–65%) observed in the laboratory occurs over the temperature range of 20 to 35°C.

A 25% reduction in maximum hatch percentage for eggs cold stored > 26 weeks, compared with those stored < 26 weeks, suggests an age dependent mortality, possibly due to depletion of stored glycogen energy resources (Anderson, 1978). In natural conditions, echinostome eggs deposited in the lake may remain dormant for as long as the water temperature is below 10°C, slowly develop as the temperature approaches 15°C and then develop more rapidly as the temperature rises above 15°C, with the hatching duration reducing as the temperature increases. This may ensure the hatching of as many eggs as possible in the summer before the water temperature declines. As winter approaches, further egg development may cease. These eggs may not survive to hatch, but may have developed enough to be infective to snails which ingest them. In the lake there should be no echinostome eggs older than 26 weeks and therefore the effect of depleted energy resources on hatchability of such eggs should not be an issue.

Infection of snails

It was not necessary to capture miracidia to challenge snails. The placement of fully embryonated eggs in vials with individual snails resulted in infection, whether by ingestion of whole eggs or by the hatching of eggs in the snail's vicinity. Infectivity of fully embryonated *E. revolutum* eggs to *L. tomentosa* was neither significantly affected by changes in enviromental temperatures in the range from 10 to 25°C, nor by experimental challenge levels of 2, 5 or 10 embryonated eggs per snail. Echinostomes have two transmission stages, miracidia and cercariae. Both of these forms are non-feeding and dependent upon endogenous energy resources. They are age- and temperature-dependent (Anderson, 1978;

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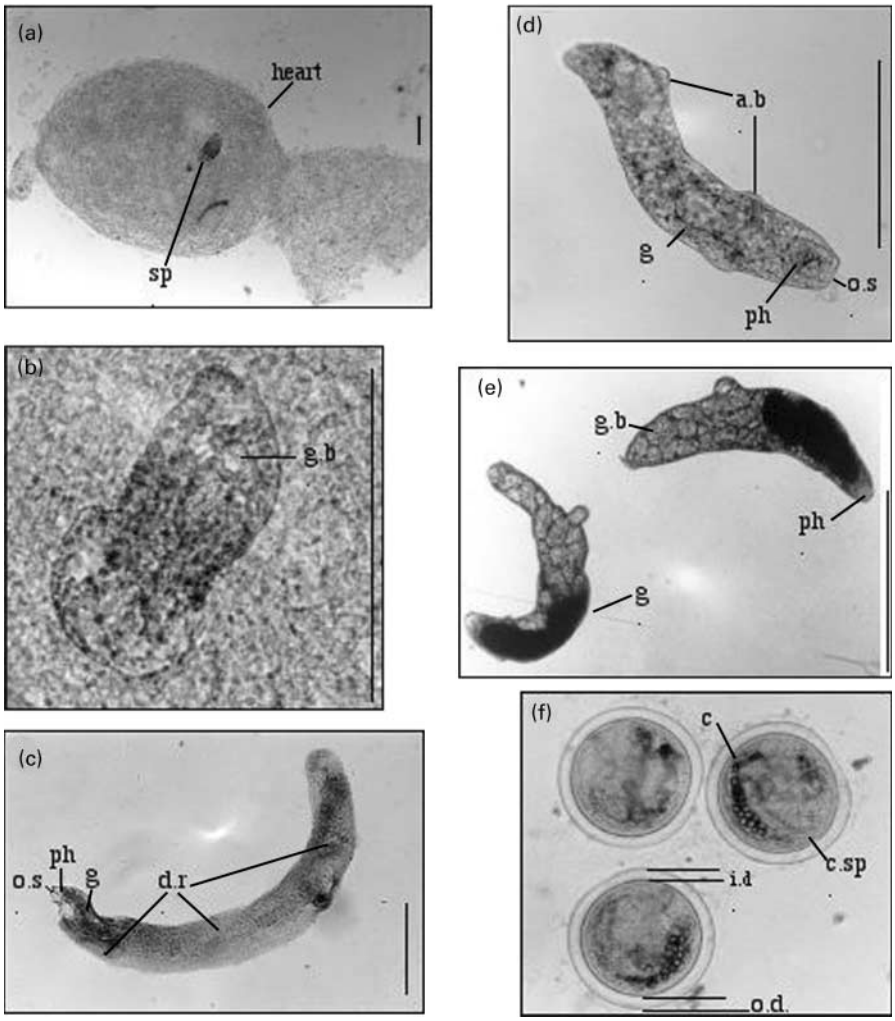

Fig. 5. Development of *Echinostoma revolutum* within the intermediate host snail, *Lymnaea tomentosa*. (a) sporocyst in snail heart tissue (bar = 50 μm); (b) sporocyst close up to show developing rediae ((bar = 50 μm); (c) mother redia with developing daughter rediae (bar = 500 μm); (d) daughter redia (bar = 20 μm); (e) daughter rediae with developing cercariae, note gut filled with haematin (bar = 500 μm); (f) live 37 collar spined metacercarial cysts, i.d. (inner diameter) = 137.5 μm, o.d.(outer diameter) = 163.5 μm, n = 10. a.b, ambulatory bud; c, concretion; c.sp, collar spine; d.r. daughter redia; g, gut; g.b, germinal ball; ph, pharynx.

McCarthy, 1999). As environmental temperatures rise, miracidial and cercarial activity increase and endogenous reserves are exhausted more rapidly. They are also less likely to infect their host as their age (in hours) increases.

The methods used during the present study may have artificially improved the probability of snail infection and influenced the results by eliminating the need for hatched miracidia to search, find, and penetrate the host. Miracidia from eggs hatching within the snail may have been at or near peak infectivity and may not have had to use much of their energy reserves. There may also have been some favourable influence of snail metabolism on the parasite's environmental temperature, especially at the experimental water temperature of 10°C. Free-swimming miracidia may be capable of infecting their snail hosts at 10°C. Idris & Fried (1995) did not investigate the infectivity of miracidia of *Echinostoma caproni* at different environmental temperatures; however, they found that miracidia survived the longest at 12°C and the shortest at 38°C. Blankespoor (personal communication) reported that when fully developed eggs of an avian schistosome were placed in vials with individual snails at 6°C, snails became infected, presumably by free-swimming miracidia. He did not observe if snails ingested the eggs, and this possibility needs to be considered in light of this research.

The apparent equal infection success of 2, 5 or 10 eggs may reflect the fact that echinostome miracidia are particularly efficient at finding and infecting their host snails. Kuris (1980) reported that the searching efficiency of *Echinostoma liei* miracidia was robust in volumes of at least 1 litre and in a heterogeneous habitat. The infection

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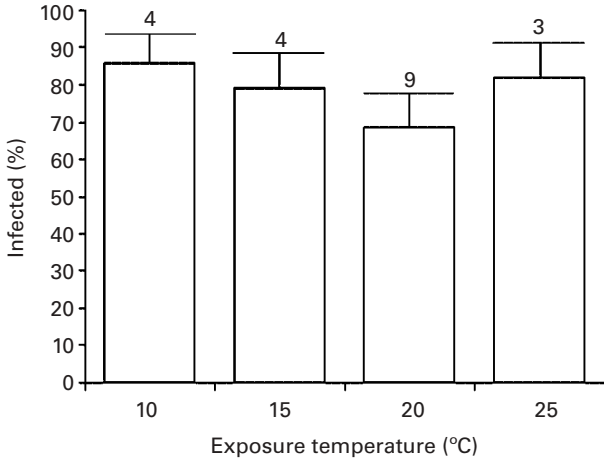

Fig. 6. Infection of *Lymnaea tomentosa* snails with eggs of *Echinostoma revolutum* at different exposure temperatures. T bars indicate standard error. Numbers are replicates of 13–18 snails each.

success experienced during the present study may also be an artefact of the methods employed, and may not necessarily be extrapolated from the laboratory to the field.

The encystation of metacercariae in primary infected snails without the release of cercariae into the environment may also be an artefact of the experimental procedure. Since all experimentally infected snails were maintained singly in their individual vials, there were no other hosts available for the cercariae to find.

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