The viability of *Sphaeridiotrema pseudoglobulus* (Digenea) eggs following cold water storage as a possible overwintering strategy

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SUMMARY

Sphaeridiotrema pseudoglobulus is a digenean parasite believed to be involved in a yearly fall die-off of ducks in Québec, Canada. Hatching characteristics of eggs stored at 7 °C for 0–28 weeks in the lab and following maintenance overwinter in a lake are described. The hatching success of eggs stored for 4–28 weeks remained constant (71–81 %) but slightly less than that observed in fresh eggs (90 %). The hatching success of eggs kept overwinter under natural conditions did not differ from that of eggs stored an equivalent length of time in the lab at 7 °C (74·7 and 75·8 %, respectively). With the exception of fresh eggs (17·7 days), the mean hatch time of eggs steadily decreased with increased storage time (18·9 days following 4 weeks storage to 11·4 days at 28 weeks storage) due to a slow embryonation of the eggs at 7 °C. Hatching characteristics of a subsample of eggs incubated at 10, 15 and 20 °C were compared and the embryonation rate was found to increase with incubation temperature. The majority of eggs stored at 10 °C embryonated but failed to hatch. When their incubation temperature was raised to 15 °C, a further 46 % hatched within the following week. The survivorship functions of miracidia hatching from eggs stored for 8, 12, 16 and 20 weeks differed but the mean expected life-span of the miracidia did not decline with increasing storage time as expected. The results of these experiments are discussed in relation to the potential importance of overwintered eggs in the development of the infective pool of metacercariae.

Key words: *Sphaeridiotrema pseudoglobulus*, overwintering, trematode eggs, dabbling ducks.

INTRODUCTION

Late summer mortality of dabbling ducks (*Anas* spp.) is an annual event in the marshes and tributaries of the St Lawrence River in Southern Québec, Canada (Gibson, Broughton & Choquette, 1972; Hoeve & Scott, 1988). Early studies implicated the digenean *Cyathocotyle bushiensis* Khan, 1962 as the aetiological agent (Gibson *et al.* 1972). More recently, Hoeve & Scott (1988) reported large numbers of a second digenean, which they identified as *Sphaeridiotrema globulus* (Rudolphi, 1814), in addition to *C. bushiensis*, in ducks found dead near Montreal. Subsequent work has shown that the *Sphaeridiotrema* specimens from Québec represent a previously unrecognized species, *Spaeridiotrema pseudoglobulus* (McLaughlin, Scott & Huffman, 1993). *S. globulus* is a known pathogen of waterfowl (Price, 1934; Huffman & Roscoe, 1989) and Hoeve & Scott (1988) have suggested that *S. pseudoglobulus* may similarly contribute to the waterfowl mortality reported in Québec, either directly or synergistically with *C. bushiensis*.

In Québec, the prosobranch snail *Bithynia tentaculata* serves as the first intermediate host and is also an important second intermediate host (Ménard & Scott, 1987*a*). Seasonal studies using sentinel blue-winged teal suggest that the prevalence and intensity of metacercariae remains low until mid July (Hoeve & Scott, 1988).

Given the low numbers of metacercariae available to adult ducks in the spring, together with the lag time between the deposition of unembryonated eggs and the subsequent production of cercariae, it is unlikely that these infections contribute greatly to the metacercarial pool that exists in early August when waterfowl mortality begins. We suggest that the metacercarial pool more likely results from infections overwintered in the snail or from infections acquired by snails earlier that spring from overwintered eggs deposited the preceding fall.

This study examines the survival and hatching dynamics of eggs kept in cold storage under laboratory conditions and overwinter under natural conditions, as well as the survival characteristics of the miracidia, in an attempt to determine the potential role of overwintered eggs in the ecology of *S. pseudoglobulus*.

MATERIALS AND METHODS

Metacercariae of *S. pseudoglobulus* were obtained from naturally infected *B. tentaculata* collected from Rivière du Sud, Québec, Canada. These were fed to eight, 2-week-old, Pekin ducklings, purchased from Brome Lake Duck Farms, Knowlton, Québec.

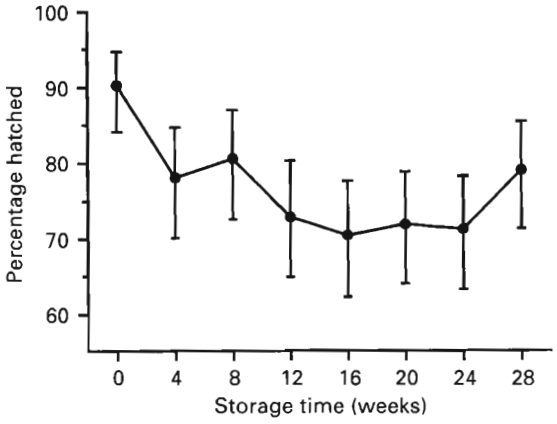

Fig. 1. Hatching success ($\bar{x} \pm 95\%$ CL) of *Sphaeridiotrema pseudoglobulus* eggs stored for varying lengths of time at 7 °C.

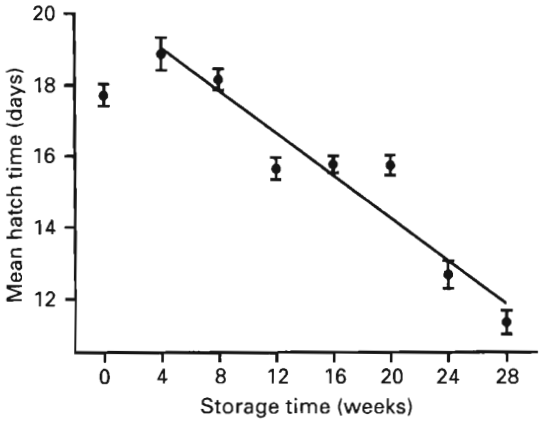

Fig. 2. Mean hatching times ($\pm 95\%$ CL) of *Sphaeridiotrema pseudoglobulus* eggs stored for varying lengths of time at 7 °C. (●) Observed values; (——) best fit linear regression through observed values from eggs stored for 4 through 28 weeks at 7 °C: $y = a - bt$, where $a = 20{\cdot}231 \pm 0{\cdot}761$ (S.E.) and $b = 0{\cdot}298 \pm 0.043$ (S.E.).

Droppings were collected 6 days post-infection, pooled, diluted with water and passed through three screens (150, 95 and 37 μm). The eggs were collected from the 37 μm screen.

Some eggs were used immediately. The remainder were divided into 17 samples consisting of several hundred eggs each. Each sample was placed in a plastic tube made from a *Drosphila* vial screened at each end with 37 μm nylon mesh (Lee *et al.* 1992). Eight containers were kept at 7 °C in an aerated aquarium in the laboratory; nine were kept overwinter under natural conditions in a local artificial lake with a controlled water depth.

Experiment 1

Hatching rate and success of fresh eggs ($t = 0$) and of those stored for 4, 8, 12, 16, 20, 24 and 28 weeks ($t = 4$–28) at 7 °C were tested as follows. At each interval, one container was selected at random and six 24-well culture plates, each containing 1 egg/well, were set up. These were incubated at 20 °C under constant incandescent illumination and examined daily. The remaining eggs were incubated in a Petri dish under identical conditions (for use in Exp. 3 below). Each experiment lasted 35 days and hatching dates were recorded for individual eggs in the culture plates. Eggs that failed to hatch by day 35 were considered dead.

Nine containers were kept overwinter under natural conditions in a small local lake. Three containers were placed in each of three mesh bags that were placed on the bottom at depths of 0·5, 1·0 and 1·5 metres in mid-October. They were retrieved 29 weeks later in early May when surface temperatures had reached 10 °C. These were treated in the same manner as outlined above, except that eggs from the three containers at each depth were pooled prior to use. Eggs held at each depth were treated separately.

Experiment 2

It became evident that there was little difference in the hatching times of eggs stored at 7 °C for increasing lengths of time. Accordingly, on week 13, a small subsample of eggs was removed from two randomly selected containers. The eggs were pooled and twelve 24-well plates set up as described above. Six were incubated at 10 °C and six at 15 °C. Although many of the eggs held at 10 °C contained miracidia by day 53, few had hatched. On day 60 (7 days after the last hatching was observed at 10 °C), three of the six plates held at 10 °C were removed and incubated at 15 °C. Monitoring of all plates continued for another 21 days.

Experiment 3

Survivorship of miracidia hatching from eggs stored for 8, 12, 16 and 20 weeks (trials 1–4) at 7 °C was studied as follows. On the day after hatching was first observed, the eggs in the Petri dish were transferred to a conical centrifuge tube, allowed to settle and then transferred to a new dish. The dish was then carefully scanned using a dissecting microscope and any miracidia found were discarded. Miracidia hatching subsequently were collected every 30 min and placed individually in separate wells of a 9-spot depression plate. They were kept at 20 °C under constant illumination and examined at 30 min intervals until they died. Five to eight plates were used for each trial.

Statistical analysis

Hatching times of eggs stored for varying lengths of time at 7 °C were compared using the regression approach to analysis of variance (ANOVA), followed

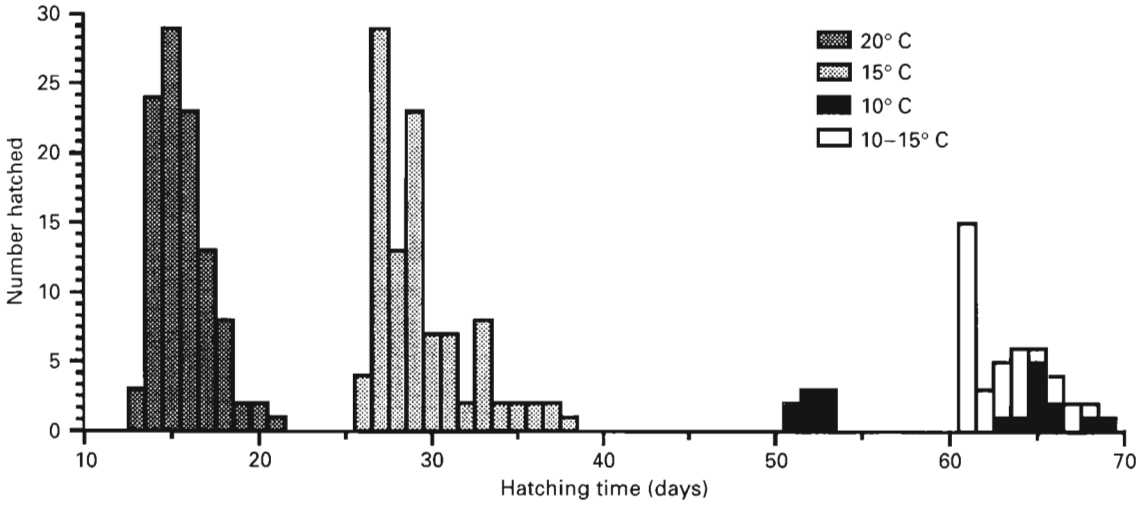

Fig. 3. Hatching rates and success of *Sphaeridiotrema pseudoglobulus* eggs stored at varying temperatures. See text for details.

by a Tukey test. A linear regression of the mean hatching times was then carried out to determine if any trends existed. A Mann–Whitney U-test was used to compare the hatching times of eggs at 15 and 20 °C. Hatching success was compared using the Kruskal–Wallis test or a Chi-square contingency test if only two trials or groups of trials were to be considered. Miracidial survivorship functions were obtained through non-linear regression and the resulting slopes compared by the Lee-Desu statistic. A linear regression of the average expected life-spans of the miracidia was then performed. All analyses were carried out using the Statistical Package for Social Sciences (SPSS) (Norušis & SPSS, 1990). The level of significance was set at $P \leqslant 0{\cdot}05$. A Bonferonni adjustment ($P \leqslant 0{\cdot}05/2(3) = 0{\cdot}008$) was used for the pairwise comparisons of the miracidial survivorship functions.

RESULTS

Experiment 1

The hatching success of eggs stored for varying lengths of time at 7 °C is shown in Fig. 1. The hatching success of fresh eggs was significantly greater than that of stored eggs ($H = 18{\cdot}52$, $P < 0{\cdot}01$) but there was no significant difference in the hatching success of eggs kept in cold storage for 4–28 weeks ($H = 7{\cdot}97$, $0{\cdot}10 < P < 0{\cdot}25$).

Hatching times for eggs stored for varying lengths of time at 7 °C (Fig. 2) differed significantly ($F = 242.67$, $P < 0{\cdot}0001$). The mean hatching time rose significantly from 17·7 days for fresh eggs to 18·9 days following 4 weeks cold storage (Tukey test, $P < 0{\cdot}05$), then showed a significant decline with increasing storage time ($F = 31{\cdot}38$; $P < 0{\cdot}005$).

Hatching success of eggs kept under natural conditions was similar at all depths (0·5 m, 72·9 %; 1·0 m, 82·7 %; 1·5 m, 80·0 %) ($H = 3{\cdot}91$; $0{\cdot}10 < P < 0{\cdot}25$) but hatching times of eggs kept at 0·5 and 1·5 m (12·3 days) were significantly longer than those kept at 1·0 m (11·4 days) ($F = 14{\cdot}94$; $P < 0{\cdot}0001$). As the hatching success of eggs stored under natural conditions did not vary significantly, the data were pooled and compared to those kept at 7 °C in the lab for 28 weeks. There was no significant difference in the hatching success of eggs stored under natural conditions (74·4 %) and those stored under laboratory conditions (75·8 %) ($\chi^2 = 0{\cdot}02$; $0{\cdot}75 < P < 0{\cdot}90$).

Experiment 2

Only 8 eggs (5 %) from the six plates incubated at 10 °C hatched within the first 60 days (before half of the plates were raised to 15 °C). Only a further 11 eggs (15 %) from the three plates maintained at 10 °C hatched during the following 21 days. Significantly more eggs hatched from the three plates that were raised to 15 °C during that same period (33 eggs, 46 %) ($\chi^2 = 15{\cdot}74$; $P < 0{\cdot}0001$). Hatching occurred quickly upon warming; 15 (21 %) of the eggs hatched within the first 24 h, the remaining 18 (25 %) hatched over the course of the following week (Fig. 3).

The hatching rate of eggs stored at 15 °C was compared to that of eggs stored at 20 °C for 12 weeks in Exp. 1. There was no significant difference in the overall hatching success of eggs incubated at 15 °C (70 %) and 20 °C (73 %) ($\chi^2 = 0{\cdot}331$; $0{\cdot}50 < P < 0{\cdot}75$). However, highly significant differences were observed in the hatching times of eggs incubated at the two temperatures ($U = 10710$, $P < 0{\cdot}0001$). The mean hatching time for eggs stored at 15 °C was nearly twice as long (29·4 days) as that for eggs stored at 20 °C (15·6 days) (Fig. 3).

Experiment 3

The survivorship of miracidia hatching from eggs stored for 8, 12, 16 and 20 weeks is shown in Fig. 4.

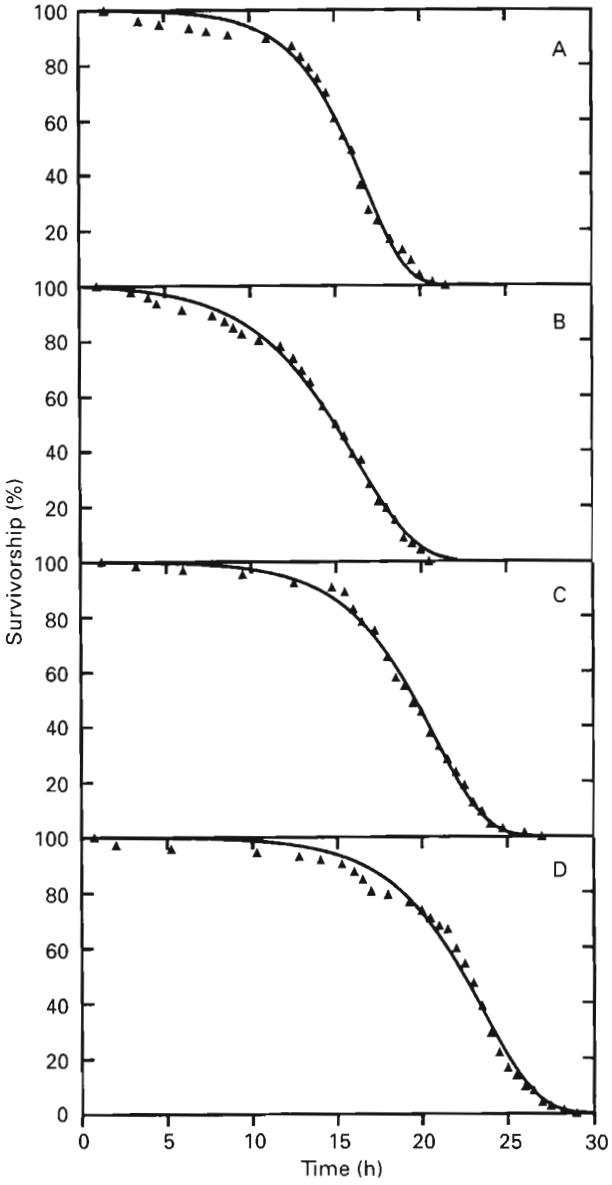

Fig. 4. Survivorship of *Spaeridiotrema pseudoglobulus* miracidia hatching from eggs stored for (A) 8, (B) 12, (C) 16 and (D) 20 weeks at 7 °C. (▲) Observed values; (——) best fit curves from the model (equation (1)) described in the text. See Table 1 for parameter estimates and fit of the model.

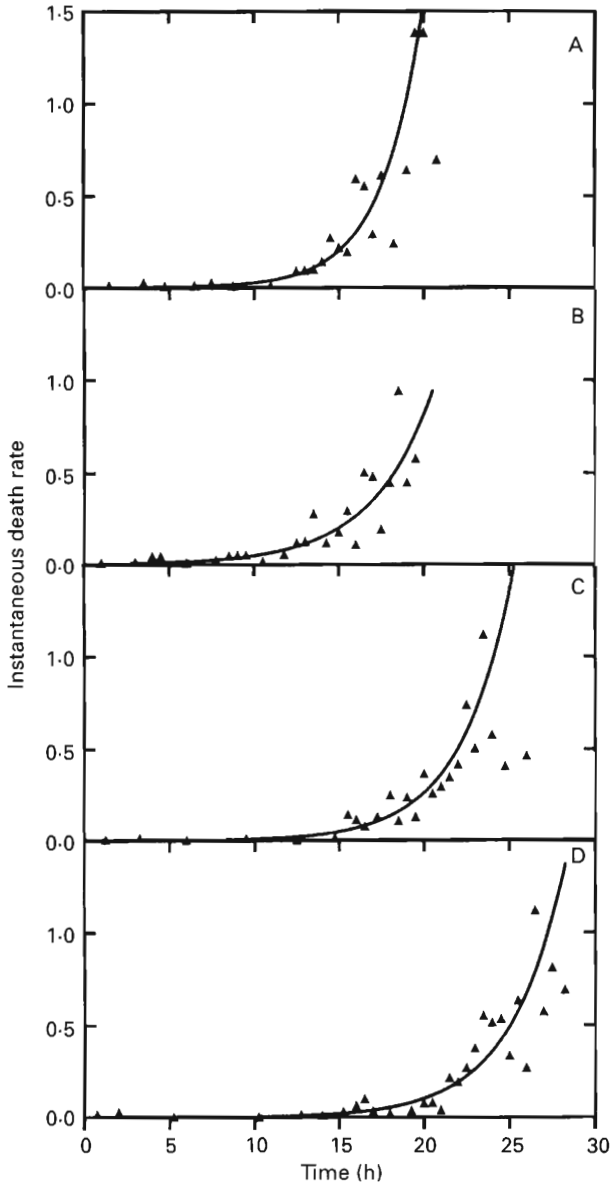

Fig. 5. Age-dependent instantaneous death rates of *Sphaeridiotrema pseudoglobulus* miracidia hatching from eggs stored for (A) 8, (B) 12, (C) 16 and (D) 20 weeks at 7 °C. (▲) Observed values calculated from equation (3) in the text; (——) best fit curves from the model (equation (2)) described in the text. See Table 1 for parameter estimates.

Anderson, Wilson & Carter (1982) and Anderson & Whitfield (1975) have developed a pair of models that describe, respectively, the age-dependent survivorship and instantaneous death rates of miracidia from experimental data such that

$$P_t = \exp[(a/b)(1-\exp(bt))] \quad (1)$$

and

$$\mu_t = a\exp(bt). \quad (2)$$

Here, P_t is the proportion of miracidia alive at age t, μ_t the age-dependent instantaneous death rate of the miracidia, and a and b are constants representing, respectively, the instantaneous death rates of miracidia at hatching and the magnitude of the increase in mortality as the miracidia age. The observed age-dependent instantaneous death rate of the miracidia (Fig. 5) was calculated

$$\mu_t = \ln N_t - \ln N_{t+1}, \quad (3)$$

where N_t is the number of miracidia alive at time t and N_{t+1} the number of miracidia alive at time $t+1$ (Anderson & Whitfield, 1975). The models provide a good fit to the observed data for each experimental trial (Table 1 and Figs 4 and 5).

The survivorship functions derived for the four trials differed significantly (Lee–Desu statistic = 95·27; $P < 0{\cdot}0001$). However, when all possible

Table 1. Constant values and fit of the model (equation (2)) to the experimental miracidial survivorship data for eggs stored for 8, 12, 16 and 20 weeks at 7 °C

Trial number	a (±95% CL)	b (±95% CL)	r^2
1	0·00043 (0.00024)	0·40862 (0·04080)	0·991
2	0·00300 (0.00067)	0·28016 (0·01769)	0·995
3	0·00036 (0.00011)	0·32898 (0·01787)	0·996
4	0·00018 (0.00010)	0·31568 (0·02805)	0·989

pairwise comparisons were made, no significant differences were found in the survivorship of miracidia hatching from eggs stored for 8 and 12 weeks at 7 °C (Lee–Desu statistic = 1·35; $0·25 < P < 0·50$). This was reflected in the average expected life-span, L, for miracidia in each trial, calculated as

$$L = \int_{t=0}^{T_{max}} P_t dt, \quad (4)$$

where T_{max} is the maximum life-span of the miracidia observed under experimental conditions (Bundy, 1981). Using this method, the mean expected life-spans for miracidia hatching from eggs stored at 7 °C for 8, 12, 16 and 20 weeks were estimated at 15·4, 14·3, 19·0 and 21·8 h, respectively. Although the average expected life-span of miracidia from eggs held at 7 °C increased with increasing storage time, the trend was not significant ($F = 8·50$; $0·10 < P < 0·25$).

DISCUSSION

Factors that affect the seasonal infection levels of *S. pseudoglobulus* in *B. tentaculata* are poorly understood. The life-cycle of *S. pseudoglobulus* has not been completed experimentally so developmental data are not available to assist in the interpretation of field studies.

Johnson (1920) suggested four ways in which digenean life-cycle stages, other than the adult, might survive winter; as eggs, as mother or daughter rediae or as metacercariae. Sentinel snails set out in the spring acquire metacercariae, indicating that some rediae of *S. pseudoglobulus* do overwinter (Ménard & Scott, 1987*a*). However, the extent of their contribution to the metacercarial pool is unknown. As the prevalence of metacercariae in snails is low in the spring and early summer (Hoeve & Scott, 1988; unpublished data), it is unlikely that overwintering rediae contribute greatly to the metacercarial pool. Natural mortality of *B. tentaculata* following early spring reproduction (Pinel-Alloul & Magnin, 1971), overwinter mortality of rediae-infected snails (Reader, 1971; Ménard & Scott, 1987*a*) or of the redia itself (Goater *et al.* 1989; Fernandez & Esch, 1991), could all act to limit the number of rediae present in snails in the spring, reducing intramolluscan transmission. Mortality of snails would also reduce the availability of metacercariae and could be a significant factor in the reduced prevalence evident in the spring.

Studies on other species of *Sphaeridiotrema* indicate that ducks are resistant to re-infection (Macy, 1973; Huffman & Roscoe, 1986). The low prevalence of metacercariae, a reduced number of susceptible waterfowl and the comparatively short life-span of the fluke (unpublished data) suggests that returning waterfowl play a limited role in the contamination of wetlands each spring.

Eggs of *S. pseudoglobulus* remain viable for at least 28 weeks at 7 °C in the laboratory and for 29 weeks at depths of 0·5 to 1·5 m under natural winter conditions. A slight but significant decline in hatching success was observed between fresh eggs and eggs stored for 4 weeks at 7 °C; however, no differences were evident thereafter. Thus, after an initial decline in survivorship, 70–80% of eggs deposited in the fall may survive and hatch the following spring. While eggs of some digeneans (e.g. *Fasciola hepatica*, *Fascioloides magna*, *Hysteromorpha triloba*, *Echinostomum liei*, and *Neodiplostomum intermedium*) may survive overwinter (Rowcliffe & Ollerenshaw, 1960; Hope-Cawdery, Gettinby & Grainger, 1978; Campbell, 1961; Hugghins, 1954; Christensen, Frandsen & Roushdy, 1980; Pearson, 1961, respectively), overwinter survival of digenean eggs is not universal. For example, the eggs of *Apatemon gracilis* and *C. bushiensis*, both species that infect waterfowl, apparently do not survive long enough to overwinter (Raišyté, 1973; Ménard & Scott, 1987*b*, respectively).

Embryonation rates of digenean eggs increase with increasing temperature (Smyth & Halton, 1983). Rowcliffe & Ollerenshaw (1960) reported that embryonation rates of *F. hepatica* eggs increased exponentionally with increasing temperature. Eggs of *S. pseudoglobulus* seem to behave in a similar way; those incubated at 10 °C took about four times as long to hatch as those at 20 °C whereas those incubated at 15 °C took about half as long.

Hatching times were initially retarded following exposure to cold. This is not unusual and has been

reported for a number of digeneans, most recently by Ménard & Scott (1987*b*) for *C. bushiensis*. However, while statistically significant, the actual difference in mean hatching times was less than 2 days and is likely to be of little consequence in the overall ecology of the fluke. What is significant is that embryonation continued, albeit slowly, under experimental conditions at 7 °C and under natural conditions, as evidenced by the decline in mean hatching times.

The eggs in a particular wetland are subject to similar temperature cycles, and a loose synchronization of development should occur over the winter period. Our results demonstrated that eggs will develop and some may hatch at 10 °C after 60 days. An increase to 15 °C resulted in a surge of hatching over the following week. Water temperatures in the littoral zones of southern Québec reach 15 °C in mid-May (Vincent & Vaillancourt, 1980). This coincides with the hatching of early spring cohorts of *B. tentaculata* (Pinel-Alloul & Magnin, 1971; Vincent, Vaillancourt & Harvey, 1981). As the net rate of infection of the snail host is proportional to the density of miracidia and snail hosts present in a system (Anderson, 1978; Wilson & Taylor, 1978), even a loosely synchronized hatch at this time could result in the infection of a large number of snails.

Usinené & Kiseliené (1973) reported that *S. globulus* requires 80 days for cercarial production. Assuming a similar developmental time for *S. pseudoglobulus*, snails infected in mid-May could shed cercariae by late July and contribute to the metacercarial pool available to ducks in August. In contrast, eggs deposited by waterfowl infected on return, would hatch later in the spring and their eventual contribution to the metacercarial pools would be delayed.

There is some disagreement on the viability and infectivity of miracidia hatching from stored eggs. Wilson, Smith & Thomas (1982) suggested that the effective life-span of *F. hepatica* eggs was about 8 weeks under winter field conditions. Krull (1934) found that miracidia hatching from eggs maintained for up to 1·5 years at 2–10 °C were still capable of infecting snails. We have no data on infectivity of miracidia from stored eggs.

To estimate the effect of prolonged storage on the viability of the miracidia, we looked at survivorship of miracidia hatching from eggs stored in cold water for varying lengths of time. Survivorship functions revealed that miracidia from eggs stored for longer periods also survived longer. As eggs stored under cold conditions continue to consume some of their limited energy supplies (Wilson *et al.* 1982) this was not expected. The importance of the observed differences in mean life-spans of the miracidia to the overall transmission dynamics of the miracidia to the snail is unknown.

Given the ability of a large proportion of eggs to overwinter, it appears that eggs deposited by ducks in late autumn may play an important role in the development of the infections in the snail population and ultimately in the size of the metacercarial pool of *S. pseudoglobulus* available to ducks the following summer.

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