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Effect of temperature and antibiotics on the hatching of *Microxeromagna armillata* (Mollusca : Hygromiidae) eggs: developing an *in vitro* bioassay for fungal egg parasites

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Abstract

Eggs of *Microxeromagna armillata* were incubated on water agar at different temperatures (5–40°C) and with each of three antibiotics (chloramphenicol, neomycin and streptomycin) at a range of concentrations (50–500 mg L^{-1}), to optimise conditions for a bioassay for egg parasites. Hatching of *M. armillata* progressed unimpaired over a wide temperature range (8–30°C). Delayed hatching occurred at 5°C and eggs died at temperatures of 37°C and above. Streptomycin had the least effect on hatching, with *M. armillata* tolerating concentrations up to 250 mg L^{-1}. Chloramphenicol and neomycin were inhibitory, even at concentrations of 50 mg L^{-1}.

Introduction

Microxeromagna armillata (Lowe, 1852) is a small terrestrial snail of Mediterranean origin (Hausdorf 1990) that has naturalised in Australia (Smith and Kershaw 1979). *Microxeromagna armillata* is of concern because it is considered quarantinable by some countries (Robinson 1999) and it is found as a contaminant of citrus fruit exports (Lush 1999). It is one of several introduced Mediterranean snails (viz. *Cernuella virgata* (daCosta, 1778), *Cochlicella acuta* (Müller, 1774), *Prietocella barbara* (Linnaeus, 1758) and *Theba pisana* (Müller, 1774)) that have become pests of agriculture, particularly in grain-production areas of southern Australia (Butler and Murphy 1977; Baker 1986; Baker *et al*. 1991). Consequently, these species are the subject of considerable research to understand their biology under local conditions and to find suitable methods for control.

Although biological control of pest snails has had some consideration in Australia (Hopkins and Baker 1993; Coupland and Baker 1995; Charwat and Davies 1999), no studies have examined fungal parasites of snail eggs. Fungal parasites of nematode eggs provide effective biocontrol in certain circumstances (Stirling 1991), so it is possible that snails could similarly be controlled by egg parasites. To evaluate the ability of fungi isolated from snail egg clusters to colonise eggs, it is necessary to establish a suitable bioassay. Because a supply of *M. armillata* eggs was available, this species was chosen for initial development of a bioassay. However, there was no information on conditions suitable for hatching *M. armillata* eggs, a necessary first step in developing a bioassay.

In initial attempts, bacterial growth around eggs of *M. armillata* incubated on water agar was inhibitory to fungal growth. Attempts to surface sterilise eggs of *M. armillata* with commonly used sterilants failed. Sodium hypochlorite (1%) rapidly dissolved the eggshell and diluted Hibitane (chlorhexidine gluconate 1% w/v, isopropyl alcohol 0.8%; ICI

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Pharmaceuticals, Melbourne, Australia) was absorbed, killing the snail. An alternative approach may be to control bacteria by inclusion of an antibiotic in the medium, provided it was not inhibitory to hatching. Therefore, the present study examined the effect of incubation temperature and exposure to antibiotics on the hatching of *M. armillata* eggs, with a view to selecting suitable conditions for the bioassay of fungal parasites.

Materials and methods

Source of snail eggs and hatching conditions

Pairs of laboratory reared *M. armillata* were enclosed in vented polycarbonate containers (200 mL, 70 mm diameter) with 150 mL field soil collected from Nangiloc, Victoria (34°28′21″S, 142°20′54″E) and several decaying citrus leaves. Snails were housed under a transparent waterproof cover in a shade house. Additional food (1:1:1 mix of rolled oats (Home Brand, Grocery Wholesalers, Sydney, Australia), skim milk powder (Diploma, Bonlac Food Supplies, Melbourne, Australia) and calcium carbonate (Univar, APS Finechem, Sydney, Australia)) was provided as required. Egg clusters were recovered at weekly intervals by sifting through the entire soil, which was then moistened and returned to the container. Eggs deposited were 0.9–1.2 mm in diameter, in clusters deposited up to 30 mm below the soil surface (A. L. Lush, unpublished observations). Clusters were stored individually on moist filter paper in the dark at 16°C for up to 7 days until used. At the time of collection, the eggs could be up to 1 week old. Therefore, immediately before use in the experiments, the egg clusters were suspended in sterile water and sorted under a dissecting microscope to select young eggs, with no obvious movement or shell development, before washing three times in sterile water.

Eggs were transferred aseptically to sterile water agar (1.5%; Sigma, St Louis, MO, USA) in 55-mm polystyrene Petri dishes and sealed in Parafilm (Pechiney Plastic Packaging, Menasha, WI, USA). Dishes contained five to eight equally spaced eggs. Eggs were examined daily for hatching and any hatchlings were removed to avoid consumption of unhatched eggs.

Effect of temperature and antibiotics on hatching

Three dishes of five eggs each were incubated at 5, 8, 16, 20, 26, 30, 37 and 42°C and examined daily for up to 5 weeks or until all eggs had hatched. From the data collected percent total hatch and mean days to hatch were calculated.

Filter (0.45-m)-sterilised solutions of streptomycin sulphate (Sigma), chloramphenicol (Sigma) and neomycin (Sigma) were added to molten water agar immediately before pouring plates to give final concentrations of 50, 125, 250 and 500 p.p.m. Fifteen eggs (five on each of three plates) were exposed to each concentration of streptomycin and sixteen eggs (eight on each of two plates) were exposed to each concentration of chloramphenicol and neomycin. Thirty-two eggs (eight on each of four plates) were included on unamended plates as controls. Plates were incubated at 16°C and observed daily for 8 days.

Statistical analysis

Analysis of variance was applied to the data using GENSTAT 5 (Lawes Agricultural Trust, Rothamsted Experimental Station, Harpenden, Hertfordshire, UK).

Results

Hatching occurred at temperatures of 30°C and below, with over 90% of eggs hatching, except at 5°C, at which temperature only 80% of eggs hatched. No hatching occurred at 37 and 42°C, although some embryo development was observed at 37°C in the first few days. The mean number of days to hatching (for those eggs that hatched) is presented in Fig. 1. There was no significant effect of temperature on the hatching rate between 16 and 30°C, but temperatures below 16°C reduced the hatching rate ($P < 0.001$).

The effects of antibiotics on hatching are presented in Fig. 2. Streptomycin had the least effect (Fig. 2*a*). At 500 mg L^{-1}, streptomycin delayed hatching (mean hatching time of 4.2 days per egg *v.* 2.1 days for control; $P = 0.05$) but, at this concentration, the reduction in percentage hatch from 80% to 60% was not significant. All concentrations of neomycin (Fig. 2*b*) delayed hatching (overall mean hatching time of 3.9 days; $P = 0.05$) and, at 500

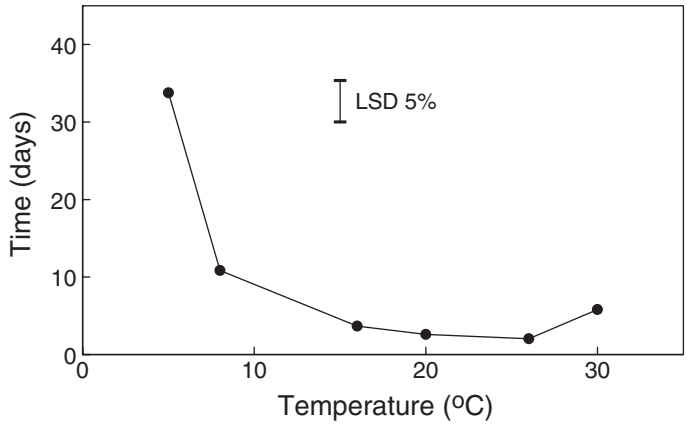

Fig. 1. Mean days to hatching for eggs of *Microxeromagna armillata* incubated on water agar over a range of temperatures (no hatch observed at temperatures of 37°C and above).

mg L^{-1} neomycin, the hatch was reduced to 14% ($P < 0.01$). Chloramphenicol (Fig. 2*c*), at concentrations of 125 and 250 mg L^{-1}, delayed hatching (mean hatching times of 6.3 and 4.6 days, respectively; $P = 0.05$). The mean percentage hatch was reduced to approximately 30% by 50–250 mg L^{-1} chloramphenicol, but 500 mg L^{-1} chloramphenicol had no effect (a significant quadratic effect; $P = 0.02$). All antibiotic treatments eliminated visible bacterial growth. Bacterial growth around eggs on control plates had no apparent effect on hatching.

Discussion

The present study has demonstrated that eggs of *M. armillata* will hatch on agar over a remarkably wide range of temperatures (8–30°C), with only minimal effect on development rate. This wide temperature tolerance of *M. armillata* eggs differs from *Helix aspersa* Müller, 1774 (Guéméne and Daguzan 1983) and many other invertebrates, where hatching has a relatively narrow optimal temperature range (in *H. aspera*, hatching occurred only at 20–25°C at 100% relative humidity). Because the eggs used were stored at 16°C for over 1 week before exposure to the various temperatures, this finding cannot be assumed to apply to eggs exposed immediately after deposition. Nevertheless, these data provide the flexibility to run a bioassay either at temperatures consistent with those experienced by eggs in the soil or at temperatures optimal for the fungi being assayed.

The three antibiotics assessed inhibit bacterial growth by binding to prokaryote rRNA, inhibiting protein synthesis (Corcoran and Hahn 1975), and successfully controlled bacterial contamination in our experiments. *Microxeromagna armillata* was least affected by streptomycin, but neomycin and chloramphenicol had clear detrimental effects. Chloramphenicol also causes mammalian toxicity through its inhibition of mitochondrial protein synthesis (Hardman and Limbird 2001), a phenomenon supporting the prokaryotic origin of mitochondria (Margulis 1981). Therefore, the effect of antibiotics that bind to prokaryotic ribosomes on molluscs is more likely to be direct toxicity, rather than by control of an essential symbiotic bacterial endophyte. Yao *et al*. (1993) also reported a molluscicidal effect of bacteriostatic antibiotics.

Incorporation of streptomycin in the medium at concentrations of 250 mg L^{-1} or less provides a suitable option for a bioassay. However, it may be possible to use more toxic

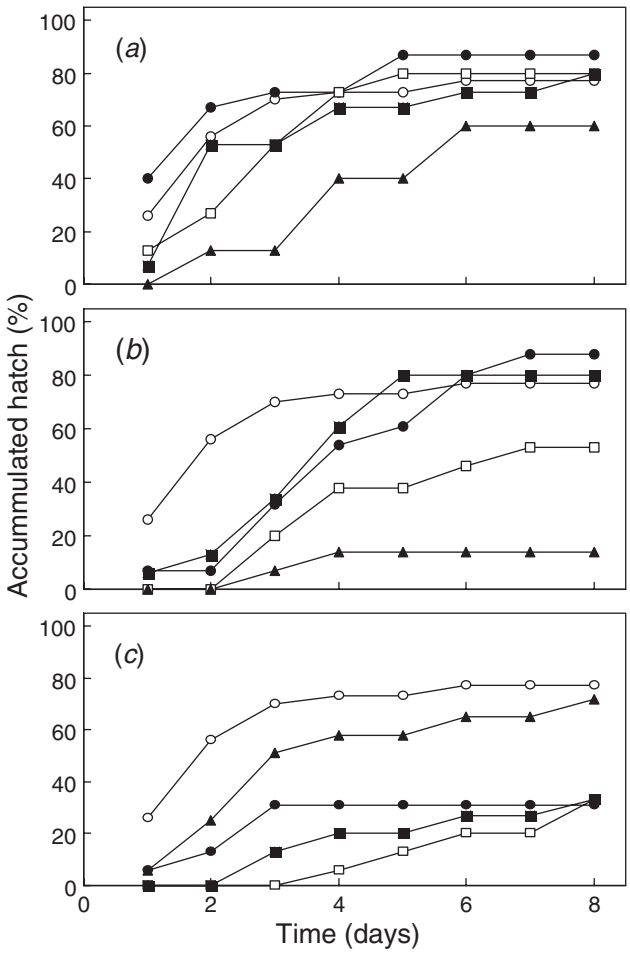

Fig. 2. Effect of (*a*) streptomycin sulphate, (*b*) neomycin and (*c*) chloramphenicol at concentrations of 0 (○), 50 (●), 125 (□), 250 (■) and 500 p.p.m. (▲) on the hatch of *Microxeromagna armillata* eggs.

antibiotics (such as neomycin, chloramphenicol or others) by only exposing the eggs briefly to these antibiotics. It is likely that streptomycin would be non-toxic to other snail species, but this would need confirmation.

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