

Echinostome infection in green frogs (*Rana clamitans*) is stage and age dependent

M. P. Holland¹, D. K. Skelly^{1,2}, M. Kashgarian³, S. R. Bolden¹, L. M. Harrison⁴ & M. Cappello⁴

¹ School of Forestry and Environmental Studies, Yale University, New Haven, CT, USA

² Department of Ecology and Evolutionary Biology, Yale University, New Haven, CT, USA

³ Pathology and Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT, USA

⁴ Departments of Pediatrics and Epidemiology and Public Health, Child Health Research Center, Yale School of Medicine, New Haven, CT, USA

Keywords

Echinostoma revolutum; *Rana clamitans*; mortality; tadpoles; trematodes; emerging infectious disease.

Correspondence

Manja P. Holland. Current address: Yale University, 370 Prospect Street, New Haven, CT 06801, USA.

Email: manja.holland@yale.edu

Received 18 April 2006; accepted 19 June 2006

doi:10.1111/j.1469-7998.2006.00229.x

Abstract

The increasing threat of emerging infectious diseases in both wildlife and humans has spurred interest in the causes of disease emergence, including the role of anthropogenic change. A prior field study of infection patterns in amphibians suggests that echinostome infection may be an emerging disease of green frogs *Rana clamitans* living in urbanized environments. We examined the impact of echinostome infection on green frog tadpoles at a wide range of developmental stages (Gosner stage 25–39). Echinostome infection was associated with green frog mortality rates of up to 40% in an early developmental stage, and none in later developmental stages. Tadpoles exposed to higher echinostome doses exhibited higher edema rates, a potential sign of compromised renal function. Histopathological analysis further supported the hypothesis that echinostome-induced tadpole mortality resulted from compromised renal function. Given that the timing of highest cercarial shedding can coincide with the most vulnerable stages of green frog tadpole development, echinostomes could significantly impact green frog survival in nature.

Introduction

Environmental change mediated by humans is recognized as a leading cause of both human and wildlife emerging infectious diseases (Gratz, 1999; Patz *et al.*, 2000; Daszak, Cunningham & Hyatt, 2001). Emerging infectious diseases have been defined as diseases that are newly recognized, rapidly increasing in incidence or geographic range, or new to a population (Morse, 1995; Daszak, Cunningham & Hyatt, 2000). Urbanization is one of the forms of anthropogenic change that is being investigated for its role in disease emergence (Morse, 1995; Schrag & Wiener, 1995; Gratz, 1999; Patz *et al.*, 2000; Daszak *et al.*, 2001). A small group of studies have examined the impact of urbanization on macroparasites, but so far the overall impact of urbanization remains inconclusive (Skelly *et al.*, 2006). According to several recent studies, two trematodes seem to emerge in amphibians in urbanized and otherwise anthropogenically modified environments (Johnson *et al.*, 2003; Johnson & Chase, 2004; Beasley *et al.*, 2005; Skelly *et al.*, 2006).

Emerging infectious diseases of amphibians are the focus of growing global concern as they are increasingly linked with population declines, extinctions and developmental deformities (Berger *et al.*, 1998; Daszak *et al.*, 1999; Daszak, Cunningham & Hyatt, 2003; Stuart *et al.*, 2004; Lips *et al.*, 2006; Pounds *et al.*, 2006). A trematode parasite *Ribeiroia ondatrae* is an emerging disease that causes limb deformities

in frogs in North America (Johnson *et al.*, 1999, 2002, 2003). Echinostomes are a group of trematodes that have received much less attention, but are widely distributed and appear to emerge as important disease agents in urbanized and otherwise human-modified environments in a variety of amphibian hosts (Beaver, 1937; Najarian, 1953; Ulmer, 1970; McAlpine & Burt, 1998; Beasley *et al.*, 2005; Skelly *et al.*, 2006). Laboratory and field studies have demonstrated that echinostome infection can result in tadpole mortality (Fried, Pane & Reddy, 1997; Schotthoefer, Cole & Beasley, 2003; Beasley *et al.*, 2005). Skelly *et al.* (2006) suggested that echinostome infection is an emerging disease in green frogs *Rana clamitans* in urbanized environments. Echinostomes are widely distributed and the infection intensity and prevalence in tadpoles is generally low, but this study demonstrated that echinostome infection prevalence in green frog tadpoles in some urban ponds can approach one, with mean infection intensities ranging from hundreds to thousands of echinostomes (Skelly *et al.*, 2006). Moreover, Beasley *et al.* (2005) found that cricket frog *Acris crepitans* populations experiencing higher levels of echinostome infection had lower rates of larval recruitment.

Echinostomes have a complex life cycle requiring them to infect three different hosts. Adult echinostomes reside in the intestinal tract of a wide range of aquatic bird and mammal hosts, while the first intermediate host is generally a snail (Huffman & Fried, 1990; Sapp & Esch, 1994; Kanev *et al.*,

1995; Fried, 2001). Echinostome cercariae infect a broad range of second intermediate hosts, including tadpoles. After entering the tadpole body, they migrate to the host's kidneys where they encyst as metacercariae (Beaver, 1937; Prudhoe & Bray, 1982; Anderson & Fried, 1987; Huffman & Fried, 1990; Martin & Conn, 1990; Fried *et al.*, 1997). Echinostomes slow growth and cause stage-dependent mortality in infected leopard frog *Rana pipiens* tadpoles (Fried *et al.*, 1997; Schotthoefler *et al.*, 2003). The precise mechanism by which echinostome infection leads to tadpole mortality is unknown. Several studies report edema following infection, indicating compromised renal function or failure, which may be responsible for the observed morbidity and mortality (Beaver, 1937; Fried *et al.*, 1997; Schotthoefler *et al.*, 2003).

The purpose of this study was to examine the impact of echinostome infection on growth, development, physiology and mortality in green frog tadpoles exposed to several doses of cercariae at a wide range of developmental stages. Given that green frogs spend their first winter as tadpoles, we conducted separate laboratory studies on field-collected first- and second-year tadpoles to determine whether they exhibit different responses to echinostome infection. Previous dissection of field-collected tadpoles from north-eastern Connecticut demonstrated that echinostomes infect both first- and second-year tadpoles in nature (M. P. Holland, unpubl. data). We used histological techniques to understand the potential effect of echinostome infection on renal function and the role of infection in tadpole mortality.

Methods

Laboratory experiments

We conducted a laboratory experiment on first-year green frog larvae to examine the impact of echinostomes on growth, development, edema and mortality. Portions of recently laid egg masses were collected in June 2005 from three ponds (Pretty Beaver Pond, Jen's Pond and Kozey Road Beaver Pond) at Yale Myers Forest (3800 ha) in north-eastern Connecticut, USA. The ponds from which eggs were collected lacked snails capable of hosting echinostomes, and had low overall snail density. In addition, echinostome cysts were absent from kidneys dissected out of green frog tadpoles from each pond. Egg mass portions from each pond were raised separately outdoors in 150 L plastic wading pools, and tadpoles were fed a 3:1 mixture of rabbit chow (Kaytee Inc., Chilton, WI, USA) and fish food (TetraMin Inc., Melle, Germany). We moved the tadpoles to two indoor incubators (Precision Model 818, Precision, Winchester, VA, USA) with full spectrum fluorescent lights (Sunstick, 40 W, Sylvania, Danvers, MA, USA) at early Gosner (1960) stage 25 (mean weight ± 1 SE = 25.0 ± 8.5 mg). Temperature loggers (Hobo Pendant, Onset Computer, Bourne, MA, USA) were kept in each incubator throughout the experiment (mean temperature = 21 °C). A total of 180 tadpoles was divided into 36 groups of five, with each group housed in a plastic

container (32 × 18 × 11 cm). Each container was filled with 3 L aged tap water. Containers were divided into five treatment groups (six containers in each group, except 12 in control): control (no exposure to cercariae); 40 cercariae early Gosner stage 25; 80 cercariae early Gosner stage 25; 80 cercariae late Gosner stage 25; 120 cercariae late Gosner stage 25. Each container was stocked with tadpoles from a single pond, and the pond was distributed evenly across treatment groups. The tadpoles exposed at early Gosner stage 25 were infected 48 h following establishment in incubator containers. Two tadpoles died during the 48-h period before infection and were replaced with alternates from the same clutch and developmental stage. We carried out infections using <8-h-old cercariae shed from field-collected (from pond Toll-25 in Tolland, CT, USA) *Planorbella trivolvis* (= *Helisoma trivolvis*). Cercarial shedding was induced by placing snails *c.* 13 cm from a 60 W light. Tadpoles were infected individually in 120 mL containers with 25 mL aged tap water containing the appropriate number of cercariae for 2 h, and were subsequently returned to their containers. Tadpoles infected late in Gosner stage 25 were infected 13 days after the tadpoles infected early in Gosner stage 25 with the same infection protocol (mean weight ± 1 SE = 75 ± 25.5 mg). We carried out complete water changes with aged tap water every 3 days throughout the experiment, and assessed edema and mortality daily. Tadpoles were fed a finely ground 3:1 mixture of rabbit chow and fish flakes following each water change. The amount of food added to each container was calculated as 10% of the total body weight of the container per day. Tadpoles were weighed every 6 days, and the most recent weight was used for calculating food amounts. The experiment was run for a total of 7 weeks, during which we rotated the containers every 3 days using an algorithm that placed them on each shelf in both incubators over an 8-day period. We replaced tadpoles that died to avoid the potential impact of reduced tadpole density on growth and development. Replacement tadpoles were maintained outside the incubators, but received the same infection treatments as tadpoles in the incubators. Dead tadpoles were replaced with replacement tadpoles that had been exposed to the same dose of cercariae at the same time during the experiment. Photoperiod was maintained at 14 h light/10 h dark throughout the experiment, and was set to mimic the approximate photoperiod that the tadpoles would experience in their ponds at Yale Myers Forest. All tadpoles were euthanized by an MS-222 overdose (1:1000) at 6 weeks. We removed and dissected the kidneys from nine animals from each treatment group under a dissecting microscope to determine the proportion of echinostomes that had encysted.

We conducted a second laboratory experiment to determine the impact of echinostomes on second-year green frog tadpoles. Second-year tadpoles were collected in June 2005 from three ponds (Pretty Beaver Pond, Jen's Pond and Dentist Pond) at Yale Myers Forest. Pond selection criteria were the same as those described above for first-year green frogs. The tadpoles ranged from Gosner stage 27 through Gosner stage 39 at the time of collection. Following

collection, 72 tadpoles were placed in individual plastic (31 × 20 × 11 cm) containers with 3 L aged tap water. The containers were kept on tables in a room with no natural light, and maintained on a 14 h light/10 h dark schedule with 20 W full-spectrum fluorescent lights (GE Chroma 50, General Electric, Fairfield, CT, USA). Tadpoles were divided into three treatment groups, with developmental stages evenly distributed among them: control (no exposure to cercariae); 100 cercariae; 200 cercariae. Containers were rotated to avoid potential positional effects. The method for obtaining cercariae was the same as that described for the first-year experiment. Infections were carried out in individual plastic containers as described above, except that infections took place in a larger volume (75 mL) of aged tap water. Tadpole feeding and water changes were carried out as for the first-year experiment. We weighed the tadpoles every 6 days. Those that died or reached Gosner stage 42 were not replaced. Tadpoles were weighed and euthanized by an MS-222 overdose (1:1000) at Gosner stage 42 upon the emergence of forelimbs. The experiment was run for 6 weeks, at which point 68 of 72 tadpoles had developed forelimbs. We removed and dissected the kidneys from nine preserved animals from each treatment group under a dissecting microscope to determine the proportion of echinostomes that encysted. The proportion of echinostomes that encysted was calculated as the number of echinostomes found encysted divided by the number of cercariae to which the tadpole was exposed.

Histology

We performed histological analyses to examine the potential impact of echinostomes on kidney function by assessing the location of echinostome encystment within the kidney structure. We examined histological sections for evidence of altered leukocyte presence or distribution as an assessment of host immune response. The kidney tissue was also examined for signs of inflammation. We performed histological analysis on kidneys from tadpoles collected from a suburban pond with high levels of echinostome infection (Toll-25) in Tolland, CT, USA and an uninfected pond (Kozey Road Beaver Pond) in Yale Myers Forest, CT, USA. Tadpoles were euthanized by an MS-222 overdose (1:1000) and then fixed in 10% neutral buffered formalin. Kidneys were dissected out and placed in 70% ethanol before paraffin embedding. Paraffin-embedded kidneys were sectioned and stained with either hematoxylin–eosin or Wright's stain. Photos were taken through an Olympus AH-3 microscope with an Olympus Vanox AHBS3 camera (Olympus America Inc., Melville, NY, USA).

DNA extraction, amplification and sequencing for parasite identification

Genomic DNA was extracted from 10 metacercarial cysts dissected out of a single first-year green frog tadpole and stored in 70% ethanol. Before DNA extraction, ethanol was evaporated off the metacercariae in a vacuum centrifuge.

DNA was extracted using a Qiagen DNeasy tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The final elution volume was 250 μ L. A portion of the 28S ribosomal DNA gene used by others to examine digenean trematode phylogeny was amplified by polymerase chain reaction (PCR) using a digenean-specific primer Dig12 (5'-AAG CAT ATC ACT AAG CGG) and a reverse primer LSU 1500R (5'-GCT ATC CTG AGG GAA ACT TCG) (Tkach *et al.*, 1999; Tkach, Pawlowski & Mariaux, 2000; Snyder & Tkach, 2001; Olson *et al.*, 2003). The PCR reaction consisted of 5 μ L 10X buffer, 3 μ L 25 mM MgCl₂, 1 μ L (5 U) Taq polymerase (Perkin Elmer, Wellesley, MA, USA), 1 μ L 10 mM dNTP mixture, 100 ng of each primer and genomic DNA. The total reaction volume was brought to 50 μ L with sterile deionized water. The reaction was run on a thermocycler (MJ Research, Hercules, CA, USA) using the following program parameters: initial denaturation (94 °C for 30 s), 40 cycles (94 °C for 15 s, 55 °C for 20 s, 72 °C for 1 min) and a final extension (72 °C for 7 min). Amplified products were ligated into pCR 2.1 (Invitrogen Corp., Carlsbad, CA, USA) following the manufacturer's instructions and used to transform chemically competent INV α F' *Escherichia coli* (Invitrogen). Positive colonies were used to prepare miniprep DNA and the sequence results were analyzed using the BLAST algorithm available through the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov/BLAST/).

Statistical analyses

Weight data were log-transformed to correct non-normality detected with the D'Agostino–Pearson K^2 test for normality (Zar, 1998). We analyzed both first- and second-year log-transformed larval weights by repeated measures ANOVA using PROC GLM in SAS 9.1 (SAS Institute, c2004, Cary, NC, USA). We used pond of origin of the tadpoles as a blocking factor for analysis of both first- and second-year weight responses. The developmental stage of first-year tadpoles at the conclusion of the experiment and the time to metamorphosis for second-year tadpoles were analyzed in separate ANOVAs using PROC GLM in SAS 9.1. We analyzed mortality and edema of first-year tadpoles by logistic regression using PROC GENMOD in SAS 9.1. All encystment rates were arcsine square root transformed before analysis by *t*-test.

Results

Laboratory experiments

Echinostome infection resulted in mortality in some green frog tadpoles exposed early in Gosner stage 25. Tadpoles exposed to 40 and 80 echinostome cercariae experienced mortality rates of 30 and 40%, respectively (Fig. 1). When compared with the uninfected control group, tadpoles in the groups exposed early in development to 40 and 80 cercariae, respectively, each experienced significant rates of mortality (40 cercariae: logistic regression, $\chi^2_1 = 5.32$, $P = 0.021$;

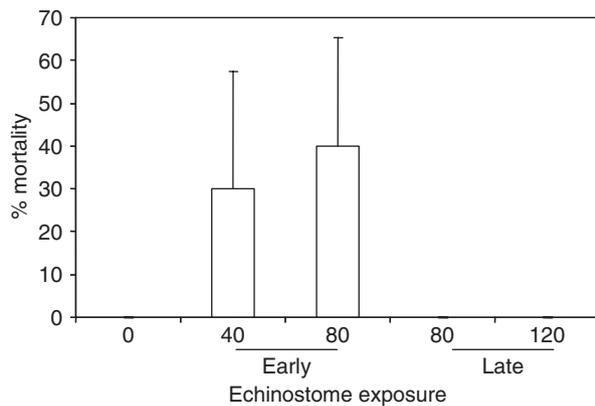


Figure 1 Larval green frog *Rana clamitans* (first year) mortality during a 7-week laboratory experiment. Tadpoles were exposed to echinostomes at early Gosner stage 25 (40 or 80 cercariae) or late Gosner stage 25 (80 or 120 cercariae). Mean mortality rates were calculated as the mean mortality rate across the six containers within each treatment group, with standard error representing variation in mortality rates across the six containers. Error bars represent +1 SE.

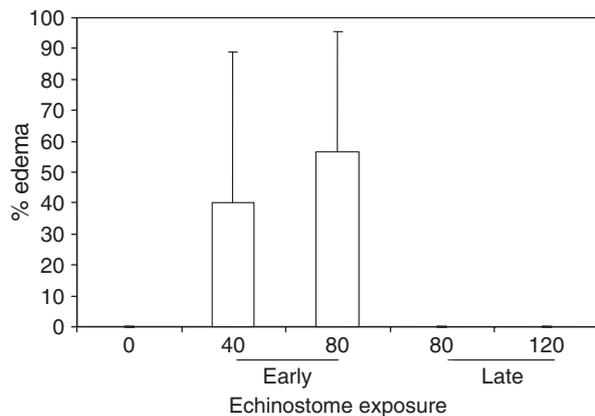


Figure 2 Larval green frog *Rana clamitans* (first year) edema 24 h post-echinostome infection. Tadpoles were exposed to echinostomes at early Gosner stage 25 (40 or 80 cercariae) or late Gosner stage 25 (80 or 120 cercariae). Edema rates peaked 24 h post-infection and decreased gradually to zero within 1 week post-infection. Mean edema rates were calculated as the mean edema rate across the six containers within each treatment group, with standard error representing variation in edema rates across the six containers. Error bars represent +1 SE.

80 cercariae: logistic regression, $\chi^2_1 = 7.48$, $P = 0.006$). In addition, tadpoles exposed to 40 or 80 echinostome cercariae experienced edema at rates of 40 and 57.7%, respectively (Figs 2 and 3). The edema rates experienced by groups of tadpoles exposed to 40 and 80 cercariae were each significantly greater than the zero rate of edema experienced by the unexposed control tadpoles (40 cercariae: logistic regression, $\chi^2_1 = 7.48$, $P = 0.006$; 80 cercariae: logistic regression, $\chi^2_1 = 11.29$, $P < 0.001$). First-year larvae exposed to echinostomes later in Gosner stage 25 experienced neither edema nor mortality (Figs 1 and 2). First-year larvae exposed to

echinostomes did not exhibit altered growth (repeated measures ANOVA, $F_{5,30} = 0.92$, $P = 0.48$) or rate of development (ANOVA, $F_{5,30} = 1.7$, $P = 0.17$) when compared with tadpoles that were not exposed to cercariae.

Second-year green frog larvae exposed to echinostomes did not experience edema, and mortality (six deaths total) did not differ across treatment groups. The growth of second-year larvae was not altered by echinostome exposure (ANOVA of log-transformed final weight, $F_{2,66} = 0.06$, $P = 0.94$). Neither was time to metamorphosis for second-year larvae affected by echinostomes (ANOVA, $F_{2,66} = 0.17$, $P = 0.84$).

The echinostome encystment rate was higher for first-year tadpoles exposed to echinostomes early (includes both cercarial doses) in Gosner stage 25 (mean encystment rate ± 1 SE = $29.8 \pm 13.2\%$) as compared with larvae exposed later (includes both cercarial doses) in Gosner stage 25 (mean encystment rate ± 1 SE = $10.3 \pm 8.3\%$; $t_{34} = 5.66$, $P < 0.0001$; Fig. 4). The encystment rate was also higher for second-year larvae exposed earlier (includes both cercarial doses) in development during Gosner stage 30–33 (mean encystment rate ± 1 SE = $47.0 \pm 17.6\%$) versus later (includes both cercarial doses) during Gosner stage 34–39 (mean encystment rate ± 1 SE = $30.0 \pm 12.8\%$; $t_{16} = 2.38$, $P = 0.03$; Fig. 4). The ranges of rates of encystment for first-year tadpoles exposed early versus late in development were as follows: early = 7.5–57.5%, late = 1.3–25.0%. The ranges of rates of encystment for second-year tadpoles exposed early versus late in development were as follows: early = 19.5–79.0%, late = 17.0–56.0%. The number of echinostomes that encysted in the right kidney was greater than the number in the left kidney in both first- and second-year tadpoles (first year: $t_{70} = 3.85$, $P = 0.0002$; second year: $t_{34} = 2.69$, $P = 0.011$). The overall left:right encystment ratio for first-year tadpoles was 155:324 (left: mean encystment rate ± 1 SE = 4.3 ± 3.6 ; right: 9.0 ± 6.2) and 314:666 (left: mean encystment rate ± 1 SE = 17.4 ± 20.0 ; right: 37 ± 23.5) for second-year tadpoles. A greater number of echinostomes encysted in the pronephros versus the mesonephros of first-year larvae ($t_{70} = 3.21$, $P = 0.002$). The overall ratio of cysts in the pronephros:mesonephros of first-year tadpoles was 299:161 (pronephros: mean encystment rate ± 1 SE = 8.3 ± 5.4 ; mesonephros: 4.5 ± 4.7).

Histology

A comparison of histological sections of renal tissue from infected and uninfected green frog tadpoles revealed that echinostome-infected tissue often had fewer glomeruli. Histological sections from uninfected Gosner stage 33 tadpoles contained 10–20 glomeruli, while glomeruli were not apparent in similar sections from infected Gosner stage 33 tadpoles (Fig. 5). Some metacercariae encysted within renal ducts (Fig. 5a). The walls of ducts within infected renal tissue, especially those containing metacercarial cysts, were comprised of a thick secretory columnar epithelium that was not observed in uninfected renal tissue. As observed by Martin & Conn (1990) in adult green frogs, an inflammatory

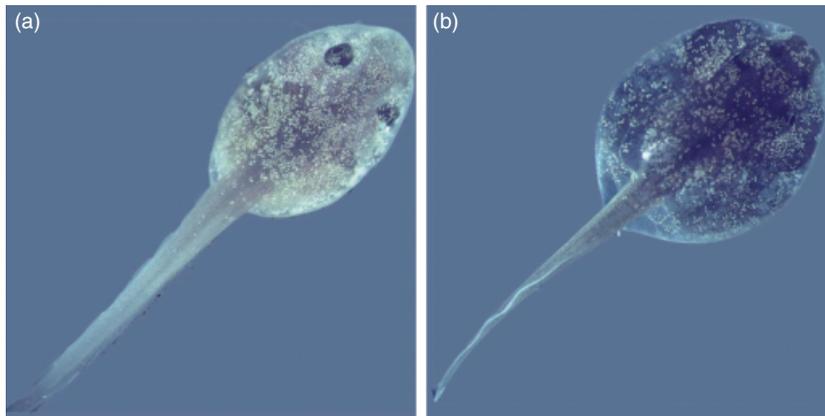


Figure 3 Larval green frog *Rana clamitans* pre- and post-infection. (a) Gosner stage 25 larval green frog pre-echinostome infection. (b) Gosner stage 25 larval green frog 24 h post-echinostome infection, exhibiting generalized edema.

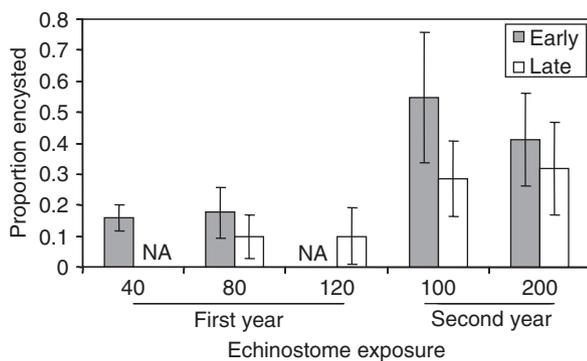


Figure 4 Proportion of echinostomes encysted in green frog *Rana clamitans* larvae following exposure to a range of cercarial doses at different developmental stages. The proportion of echinostomes that encysted was calculated by dividing the number of echinostome cysts found during dissection by the number of cercariae to which the tadpole was exposed. First-year green frog larvae were exposed to echinostomes early in Gosner stage 25 or late in Gosner stage 25. Second-year green frog larvae were exposed to echinostomes during Gosner stages 30–33 (early) or stages 34–49 (late). Nine tadpoles were dissected from each treatment group (cercarial dose) to determine the proportion of echinostomes that encysted. NA indicates that tadpoles were not exposed to parasites at that particular dose and developmental stage. Error bars represent ± 1 SE.

response comprised mostly of eosinophils and macrophages developed around some of the metacercariae in infected tissue. We found no overall difference in the leukocyte population in infected versus uninfected histological sections.

Parasite identification

On the basis of alignments to existing sequences in the NCBI database, the echinostome investigated in this study is *Echinostoma revolutum*. The sequence obtained in this study (GenBank accession number DQ471888) was aligned to *E. revolutum* 28s ribosomal DNA (accession number AY222246) and was found to be 99% identical with five mismatches out of 1001 bases. An *Echinoparyphium cinctum* 28s ribosomal DNA sequence (GenBank accession number AF184260) is the second closest match to our sequence, with

96% identity and 29 mismatches in 943 bases. There was no similarity between our sequence and a partial sequence of *Echinostoma trivolvis* 28s ribosomal DNA (GenBank accession number U58097).

Discussion

Infection by echinostomes was associated with green frog mortality rates of up to 40%, but this effect was strongly dependent on the developmental stage of host tadpoles. Relatively small differences in development, even within a single Gosner developmental stage, appeared to render larvae invulnerable to infection-related mortality. In fact, the age difference between tadpoles that experienced mortality and those that did not was only 13 days. While it is possible that differences in mortality between tadpoles exposed early versus those exposed 13 days later could have been due to differences in cercariae rather than tadpole age, this seems unlikely given that the cercariae were collected by the same protocol and were from several snails. Tadpoles exposed to echinostomes early in Gosner stage 25 experienced higher rates of mortality when exposed to higher cercarial doses. However, first-year tadpoles infected later in development and infected second-year tadpoles experienced neither edema nor mortality. This finding is consistent with the observation that live, field-collected second-year tadpoles in some host populations consistently exhibit infection intensities of over 1000 parasites without visible consequences (Skelly *et al.*, 2006). Therefore, exposure to higher doses of echinostomes later in development may not cause mortality, while exposure to lower doses early in development can result in significant mortality. The host's developmental stage appears to be a key determinant of the outcome of echinostome exposure.

The strong dependence of infection response on development may be critical in natural amphibian host populations. Surveys of field-collected host snails infected with echinostomes from ponds in the north-eastern United States indicate that shedding of echinostome cercariae by naturally infected snails peaks in mid-summer (Sapp & Esch, 1994; Schmidt & Fried, 1997). Most green frog breeding occurs in June and July in north-eastern Connecticut, implying that

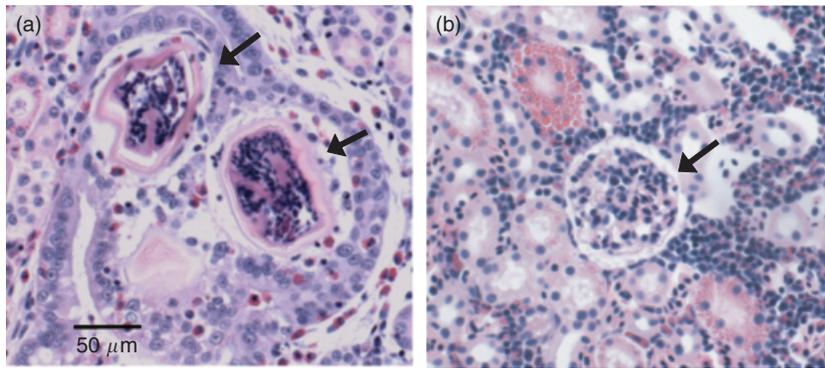


Figure 5 Light micrographs of green frog *Rana clamitans* tadpole kidney sections. (a) Histological section of an echinostome-infected Gosner stage 33 tadpole kidney. The arrows each point to a metacercarial cyst. Note that the two cysts are within the lumen of a duct, which is comprised of secretory columnar epithelium. (b) Histological section of an uninfected Gosner stage 35 tadpole kidney. The arrow points to a healthy glomerulus. Note: The scale is the same in both photomicrographs.

green frog tadpoles in early developmental stages coincide with periods of high echinostome cercariae shedding. On the basis of dissection of field-collected tadpoles, green frog tadpoles in ponds with moderate rates of echinostome infection are likely to be exposed by Gosner stage 25 to at least 80 cercariae, an exposure level that resulted in 40% mortality in this study (M. P. Holland, pers. obs.). Thus, the coincident natural timing of susceptible development stages and cercarial shedding could indicate that echinostomes have a substantial effect on tadpole survival in nature (Schotthoefer *et al.*, 2003). In addition, relatively minor changes in either the timing of tadpole development or cercarial shedding resulting from anthropogenic change could drastically alter the impact of disease emergence.

While existing evidence suggests that kidney failure may result from echinostome infection, the ultimate mechanism for echinostome-induced mortality of tadpoles is unclear. This study demonstrated strong concordance between the rates of generalized edema following echinostome exposure and rates of mortality; tadpoles exposed to higher echinostome doses exhibited higher edema rates. Generalized edema has been noted in leopard frog tadpoles following echinostome exposure, and can indicate compromised kidney function (McClure, 1919; Fried *et al.*, 1997; Schotthoefer *et al.*, 2003). Edema does not appear to be a perfect predictor of mortality. Edema rates were higher than mortality rates in this study. Some tadpoles experiencing relatively severe edema survive, suggesting that physiological resilience may be an important part of the response of tadpoles to echinostome infection. The survival rate of tadpoles with edema in the realistic context of natural predators and competitors remains an intriguing question for further study. In addition, severe edema could be a useful indicator of potential echinostome presence and mortality risk in nature as it is relatively easy to observe during appropriate developmental stages.

Histopathological analysis provides evidence, beyond association with edema, that kidney failure is a likely cause of mortality following echinostome infection. The development of granulomas of fibrotic tissue and leukocytes around echinostome cysts in tadpole kidneys are similar to the response observed in adult green frog and leopard frog kidneys reported by Martin & Conn (1990). The lack of visible glomeruli, the kidneys' main filtration unit, in histo-

logical sections of renal tissue from infected tadpoles suggests that the metacercarial cysts disrupt the glomeruli either by directly obliterating them or by blocking downstream ducts and causing the Bowman's capsules to collapse. Regardless of the mechanism, disruption of the glomeruli could have a grave impact on renal function.

Tadpoles rely on two separate pairs of kidney structures through the process of development. Initially, tadpoles are dependent on two small head kidneys, the pronephroi, for renal function. The pronephroi have limited function as they each contain only one glomerulus (Fox, 1963). Renal capacity increases dramatically as tadpoles begin to develop the larger paired mesonephroi, which function through adulthood (Viertel & Richter, 1999). The complete reliance of tadpoles at early developmental stages (pre-Gosner stage 25) on their pronephroi for renal function likely makes them more susceptible to adverse effects of echinostome infection (Viertel & Richter, 1999; Schotthoefer *et al.*, 2003). The blockage of the two glomeruli in the pronephroi could have devastating consequences for renal function in early-stage tadpoles that depend entirely on their pronephros (Fox, 1963). The mesonephroi develop around the time that the hind-limb buds appear, approximately late Gosner stage 25 through Gosner stage 26 (Fox, 1963; Gealekman & Warburg, 2000). The number of glomeruli in tadpole mesonephros increases throughout development to the thousands that are present in adult animals (Gealekman & Warburg, 2000). The lower number of glomeruli and reliance on the pronephroi, combined with possible blockage of glomeruli by echinostome cysts early in development, may contribute to the higher susceptibility of tadpoles to adverse effects of echinostome infection at early developmental stages (Schotthoefer *et al.*, 2003). The decrease in impact of echinostome infection on mortality in late Gosner stage 25 may correspond to the early stages of mesonephros development. Existing evidence suggests that physical blockage of kidney ducts and glomeruli by echinostome cysts and host encapsulation is the likely cause of mortality.

Finally, the echinostome from at least one tadpole in this study appears to be *E. revolutum* based on the comparison of our sequence to sequences in the GenBank database. There has been much confusion in the literature about the identification of echinostomes belonging to the 'revolutum' group, which currently includes nine species of the genus

Echinostoma (Fried & Graczyk, 2004; Fried & Toledo, 2004). Of the members of the revolutum group, both *E. revolutum* and *E. trivolvis* encyst as metacercariae within tadpole kidneys (Kanev, 1994; Kanev *et al.*, 1995). While *E. trivolvis* was originally identified in North America, *E. revolutum* is widely distributed on several continents but has only recently been identified in North American snails (Sorensen *et al.*, 1997; Fried & Graczyk, 2004). *Echinostoma revolutum* has only been reported to use snails of the genus *Lymnaea* as a first intermediate host. The parasite examined in this study came from *P. trivolvis* snails, which can be infected by *E. trivolvis*, but have not been reported to be infected by *E. revolutum*. The DNA sequence of the parasite in this study does not show any similarity to the 28S ribosomal DNA sequence of *E. trivolvis* in the GenBank database (accession number U58097). We conclude that the echinostome examined in this study is likely to be *E. revolutum*, and we believe that this represents the first report of the presence of *E. revolutum* in north-eastern United States.

The significant adverse effect of echinostomes on tadpole mortality rates in laboratory studies combined with their ability to affect many anurans warrants further study. While echinostome infection emergence and high infection intensities in larval amphibians appear to occur under a limited set of conditions, the wide distribution of echinostomes combined with ever-increasing landscape alteration and urbanization make emergence increasingly likely in expanding geographic areas. Future studies should examine more precisely the risks that tadpoles face in nature as a result of echinostome exposure. A more detailed characterization of factors related to anthropogenic change that lead to increases in echinostome infection will improve our understanding of echinostome infection specifically, and contribute to a broader knowledge of factors that lead to wildlife disease emergence.

Acknowledgements

Thanks to I. Tattersall for providing invaluable assistance in the field and with laboratory experiments. V. Tkach provided assistance with identifying the echinostome DNA sequence. Thanks also to M. Urban and J. Reuning-Scherer for providing expert statistical advice. Comments from M. Urban, T. Langkilde, E. Lee, T. Halliday and two anonymous reviewers greatly improved this paper. This work was supported by funds from the Yale Institute for Biospheric Studies Center for Field Ecology and the Yale Hixon Center for Urban Ecology. M.P.H. was supported by a fellowship from the National Center for Environmental Research (NCER) STAR Program, US EPA (Grant FP-91656201-0). M.C. is a recipient of a Hellman Family Fellowship from the Office of the President, Yale University.

References

Anderson, J.W. & Fried, B. (1987). Experimental-infection of *Physa heterostropha*, *Helisoma trivolvis*, and

Biomphalaria glabrata (Gastropoda) with *Echinostoma revolutum* (Trematoda) cercariae. *J. Parasitol.* **73**, 49–54.

- Beasley, V.R., Faeh, S.A., Wikoff, B., Eisold, J., Nichols, D., Cole, R., Schotthoefer, A.M., Staehle, C., Greenwell, M. & Brown, L.E. (2005). Risk factors and the decline of the cricket frog, *Acris crepitans*: evidence for involvement of herbicides, parasitism, and habitat modifications. In *Amphibian declines: the conservation status of United States species: 75–87*. Lannoo, M.J. (Ed.). Chicago: University of Chicago Press.
- Beaver, P.C. (1937). Experimental studies on *Echinostoma revolutum* (Froelich): a fluke from birds and mammals. *Ill. Biol. Monogr.* **15**, 1–96.
- Berger, L., Speare, R., Daszak, P., Green, D.E., Cunningham, A.A., Goggin, C.L., Slocombe, R., Ragan, M.A., Hyatt, A.D., McDonald, K.R., Hines, H.B., Lips, K.R., Marantelli, G. & Parkes, H. (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proc. Natl. Acad. Sci. USA* **95**, 9031–9036.
- Daszak, P., Berger, L., Cunningham, A.A., Hyatt, A.D., Green, D.E. & Speare, R. (1999). Emerging infectious diseases and amphibian population declines. *Emerg. Infect. Dis.* **5**, 735–748.
- Daszak, P., Cunningham, A.A. & Hyatt, A.D. (2000). Emerging infectious diseases of wildlife – threats to biodiversity and human health. *Science* **287**, 443–449.
- Daszak, P., Cunningham, A.A. & Hyatt, A.D. (2001). Anthropogenic environmental change and the emergence of infectious diseases in wildlife. *Acta Trop.* **78**, 103–116.
- Daszak, P., Cunningham, A.A. & Hyatt, A.D. (2003). Infectious disease and amphibian population declines. *Divers. Distrib.* **9**, 141–150.
- Fox, H. (1963). The amphibian *Pronephros*. *Q. Rev. Biol.* **38**, 1–25.
- Fried, B. (2001). Biology of echinostomes except *Echinostoma*. *Adv. Parasit.* **49**, 163–210.
- Fried, B. & Graczyk, T.K. (2004). Recent advances in the biology of *Echinostoma* species in the ‘revolutum’ group. *Adv. Parasit.* **58**, 139–195.
- Fried, B., Pane, P.L. & Reddy, A. (1997). Experimental infection of *Rana pipiens* tadpoles with *Echinostoma trivolvis* cercariae. *Parasitol. Res.* **83**, 666–669.
- Fried, B. & Toledo, R. (2004). Criteria for species determination in the ‘revolutum’ group of *Echinostoma*. *J. Parasitol.* **90**, 917–917.
- Gealekman, O. & Warburg, M.R. (2000). Changes in numbers and dimensions of glomeruli during metamorphosis of *Pelobates syriacus* (Anura; Pelodadidae). *Eur. J. Morphol.* **38**, 80–87.
- Gosner, K.L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* **16**, 183–190.
- Gratz, N.G. (1999). Emerging and resurging vector-borne diseases. *Annu. Rev. Entomol.* **44**, 51–75.

- Huffman, J.E. & Fried, B. (1990). *Echinostoma* and Echinostomiasis. *Adv. Parasit.* **29**, 215–269.
- Johnson, P.T.J. & Chase, J.M. (2004). Parasites in the food web: linking amphibian malformations and aquatic eutrophication. *Ecol. Lett.* **7**, 521–526.
- Johnson, P.T.J., Lunde, K.B., Ritchie, E.G. & Launer, A.E. (1999). The effect of trematode infection on amphibian limb development and survivorship. *Science* **284**, 802–804.
- Johnson, P.T.J., Lunde, K.B., Thurman, E.M., Ritchie, E.G., Wray, S.N., Sutherland, D.R., Kapfer, J.M., Frest, T.J., Bowerman, J. & Blaustein, A.R. (2002). Parasite (*Ribeiroia ondatrae*) infection linked to amphibian malformations in the western United States. *Ecol. Monogr.* **72**, 151–168.
- Johnson, P.T.J., Lunde, K.B., Zelmer, D.A. & Werner, J.K. (2003). Limb deformities as an emerging parasitic disease in amphibians: evidence from museum specimens and resurvey data. *Conserv. Biol.* **17**, 1724–1737.
- Kanev, I. (1994). Life-cycle, delimitation and redescription of *Echinostoma revolutum* (Froelich, 1802) (Trematoda, Echinostomatidae). *Syst. Parasitol.* **28**, 125–144.
- Kanev, I., Fried, B., Dimitrov, V. & Radev, V. (1995). Redescription of *Echinostoma trivolvis* (Cort, 1914) (Trematoda, Echinostomatidae) with a discussion on its identity. *Syst. Parasitol.* **32**, 61–70.
- Lips, K.R., Brem, F., Brenes, R., Reeve, J.D., Alford, R.A., Voyles, J., Carey, C., Livo, L., Pessier, A.P. & Collins, J.P. (2006). Emerging infectious disease and the loss of biodiversity in a neotropical amphibian community. *Proc. Natl. Acad. Sci. USA* **103**, 3165–3170.
- Martin, T.R. & Conn, D.B. (1990). The pathogenicity, localization, and cyst structure of Echinostomatid metacercariae (Trematoda) infecting the kidneys of the frogs *Rana clamitans* and *Rana pipiens*. *J. Parasitol.* **76**, 414–419.
- McAlpine, D.F. & Burt, M.D.B. (1998). Helminths of bullfrogs, *Rana catesbeiana*, green frogs, *R. clamitans*, and leopard frogs, *R. pipiens* in New Brunswick. *Can. Field Nat.* **112**, 50–68.
- McClure, C.F.W. (1919). On the experimental production of edema in larval and adult Anura. *J. Gen. Physiol.* **1**, 261–267.
- Morse, S.S. (1995). Factors in the emergence of infectious diseases. *Emerging Infect. Dis.* **1**, 7–15.
- Najarian, H.H. (1953). The life history of *Echinoparyphium flexum* (Linton 1892) (Dietz 1910) (Trematoda, Echinostomidae). *Science* **117**, 564–565.
- Olson, P.D., Cribb, T.H., Tkach, V., Bray, R.A. & Littlewood, D.T.J. (2003). Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). *Int. J. Parasitol.* **33**, 733–755.
- Patz, J.A., Graczyk, T.K., Geller, N. & Vittor, A.Y. (2000). Effects of environmental change on emerging parasitic diseases. *Int. J. Parasitol.* **30**, 1395–1405.
- Pounds, J.A., Bustamante, M.R., Coloma, L.A., Consuegra, J.A., Fogden, M.P.L., Foster, P.N., La Marca, E., Masters, K.L., Merino-Viteri, A., Puschendorf, R., Ron, S.R., Sanchez-Azofeifa, G.A., Still, C.J. & Young, B.E. (2006). Widespread amphibian extinctions from epidemic disease driven by global warming. *Nature* **439**, 161–167.
- Prudhoe, S. & Bray, R.A. (1982). *Platyhelminth parasites of the Amphibia*. Oxford: Oxford University Press.
- Sapp, K.K. & Esch, G.W. (1994). The effects of spatial and temporal heterogeneity as structuring forces for parasite communities in *Helisoma anceps* and *Physa gyrina*. *Am. Midl. Nat.* **132**, 91–103.
- Schmidt, K.A. & Fried, B. (1997). Prevalence of larval trematodes in *Helisoma trivolvis* (Gastropoda) from a farm pond in Northampton County, Pennsylvania with special emphasis on *Echinostoma trivolvis* (Trematoda) cercariae. *J. Helminthol. Soc. Wash.* **64**, 157–159.
- Schotthoefler, A.M., Cole, R.A. & Beasley, V.R. (2003). Relationship of tadpole stage to location of echinostome cercariae encystment and the consequences for tadpole survival. *J. Parasitol.* **89**, 475–482.
- Schrag, S.J. & Wiener, P. (1995). Emerging infectious-disease – what are the relative roles of ecology and evolution. *Trends Ecol. Evol.* **10**, 319–324.
- Skelly, D.K., Bolden, S.R., Holland, M.P., Freidenburg, L.K., Friedenfelds, N.A. & Malcolm, T.R. (2006). Urbanization and disease in amphibians. In *Disease ecology: community structure and pathogen dynamics*: 153–167. Colinge, S.K. & Ray, C. (Eds). Cary, NC: Oxford University Press.
- Snyder, S.D. & Tkach, V. (2001). Phylogenetic and biogeographical relationships among some holarctic frog lung flukes (Digenea: Haematolechidae). *J. Parasitol.* **87**, 1433–1440.
- Sorensen, R.E., Kanev, I., Fried, B. & Minchella, D.J. (1997). The occurrence and identification of *Echinostoma revolutum* from North American *Lymnaea elodes* snails. *J. Parasitol.* **83**, 169–170.
- Stuart, S.N., Chanson, J.S., Cox, N.A., Young, B.E., Rodrigues, A.S.L., Fischman, D.L. & Waller, R.W. (2004). Status and trends of amphibian declines and extinctions worldwide. *Science* **306**, 1783–1786.
- Tkach, V., Grabda-Kazubska, B., Pawlowski, J. & Swiderski, Z. (1999). Molecular and morphological evidence for close phylogenetic affinities of the genera *Macrodera*, *Leptophallus*, *Metaleptophallus* and *Paralepoderma* (Digenea, Plagiorchiata). *Acta Parasitol.* **44**, 170–179.
- Tkach, V., Pawlowski, J. & Mariaux, J. (2000). Phylogenetic analysis of the suborder Plagiorchiata (Platyhelminthes, Digenea) based on partial lsrDNA sequences. *Int. J. Parasitol.* **30**, 83–93.
- Ulmer, M.J. (1970). Studies on the helminth fauna of Iowa I. Trematodes of amphibians. *Am. Midl. Nat.* **83**, 38–64.
- Viertel, B. & Richter, S. (1999). Anatomy: viscera and endocrines. In *Tadpoles: the biology of anuran larvae*: 92–148. McDiarmid, W. & Altig, R. (Eds). Chicago, IL: University of Chicago Press.
- Zar, J.H. (1998). *Biostatistical analysis*. 4th edn. Upper Saddle River, NJ: Prentice-Hall, Inc.