

Biased distribution of trematode metacercariae in the nephric system of *Rana* tadpoles

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Abstract

Echinostoma sp. use larval anurans as intermediate hosts. The cercariae enter the tadpoles via the cloacal opening and form metacercarial cysts in the kidneys, pronephroi, and Wolffian ducts. To examine the distribution of *Echinostoma* metacercariae in *Rana sylvatica* and *Rana clamitans* tadpoles, 200 individuals of each species were exposed to free-swimming cercariae. There was a significant left:right bias in the distribution of metacercariae within both *R. sylvatica* and *R. clamitans* tadpole hosts, with trematodes preferentially encysting in nephric structures on the right side. In *R. sylvatica* and *R. clamitans*, respectively, 56.7% and 96.8% of the metacercariae were on the tadpoles' right side. Asymmetry in the distribution of parasites followed the direction of the asymmetry in tadpole kidney size, but was much greater. Trematodes preferentially encysted in the head kidneys of *R. clamitans*, which regress at metamorphosis. The right head kidney was the most commonly infected structure in *R. clamitans* tadpoles, containing 72.7% of all cysts in that species. Despite the preference of trematodes to encyst in the head kidney, there was no correlation between the number of cysts in the right kidney and the number in the right head kidney. This suggests that limited space in the head kidney does not influence metacercarial formation in the kidney proper. The high frequency of unilateral encystment in both anurans, and in the head kidneys of *R. clamitans*, may be the result of a co-evolved relationship that ultimately benefits both the host and parasite by ensuring host survival.

Key words: trematodes, metacercariae, *Rana*, tadpoles, *Echinostoma*

INTRODUCTION

Several trematode species need an intermediate tadpole host to complete their life cycle (e.g. Haseeb & Fried, 1997). The metamorphosing tadpole serves as a particularly effective link between aquatic and terrestrial ecosystems by transporting larval trematodes from aquatic snails to terrestrial vertebrates. Although tadpoles have commonly been used to study various aspects of trematode physiology and behaviour (e.g. Beaver, 1937; Fried, Pane & Reddy, 1997), the distribution of metacercariae within the bodies of tadpoles has received little attention. Previous studies on the localization of metacercariae within amphibian hosts have mostly focused on the adult frog (e.g. Martin & Conn, 1990).

Echinostomatid cercariae enter tadpoles through their cloaca and encyst within the tissues of the nephric system (e.g. Beaver, 1937; Fried *et al.*, 1997). This organ system undergoes substantial change when the tadpoles metamorphose, and primary renal function is transferred from the larval (head) kidneys to the more caudal adult kidneys. The distribution of echinostomatid

metacercariae in the larval nephric system has not previously been examined.

The factors that might influence metacercarial localization within tadpoles remain largely undetermined (Sukhdeo & Sukhdeo, 1994), but both the host and the parasite could be greatly affected by cyst distribution. Cercariae, in general, may be expected to encyst in locations where they can maximize their chances of reaching the definitive host. However, as large vertebrate predators typically completely consume tadpole bodies, distribution of metacercariae within tadpoles may not be a crucial factor in whether they are ingested by the definitive host. Alternatively, since the survival of the host is in the best interest of both the host and the parasite, metacercarial distribution may follow a pattern that reduces the impact of parasite infection on the intermediate host. Furthermore, as the chances of the amphibian encountering the definitive (i.e. non-aquatic) vertebrate host may be greater after metamorphosis, it may be in the best interest of the parasite to avoid lethally injuring the tadpole.

In this study, *Rana sylvatica* and *Rana clamitans* tadpoles were exposed to echinostomatid cercariae and the distributions of metacercariae within the nephric systems of the hosts observed. We report an unusual

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asymmetry in metacercarial distribution, which may be co-adaptive for both tadpoles and trematodes.

METHODS

Animal subjects

Amphibians

The amphibian species *R. sylvatica* and *R. clamitans* are common in Canada (Conant & Collins, 1991) and are found throughout Nova Scotia (Gilhen, 1984). In April 1998, 12 clutches of *R. sylvatica* eggs were collected from 12 separate ponds around Halifax, Nova Scotia. In early August, 3 clutches of *R. clamitans* eggs were collected from 3 separate ponds in Harrietsfield, Nova Scotia. All eggs were hatched in the laboratory under the same conditions, and the tadpoles were fed boiled lettuce *ad libitum* throughout their development. The 2 amphibian species were used in 2 separate, but identical, experiments during summer 1998. To eliminate sibship effects, groups of tadpoles were assembled by combining equal numbers of tadpoles from each egg clutch into a single group, and then haphazardly dividing this large group into individual groups of 10.

In each experiment, 20 groups of 10 tadpoles were exposed to free-swimming trematode cercariae (see below), with each group being housed in a separate Rubbermaid[®] RoughTote[®] plastic container (20 (height) × 26 × 33 cm). Each tank contained *c.* 7 l of aged tap water and an airline with bubbling stone. Every week, 25% of the water in the tanks was replaced with clean, aged tap water on a day when the tanks were not receiving any parasites. All tadpoles were fed boiled lettuce *ad libitum* throughout the experiments. At the end of the experiments, the tadpoles were measured from the tip of the snout to the tip of the tail, and staged according to Gosner (1960).

Parasites

The trematode cercariae used in this study were released from the snail *Helisoma trivolvis trivolvis*. Six of these snails were collected from a small pond in Pictou, Pictou County, Nova Scotia. This species commonly serves as the intermediate host for *Echinostoma* sp. (Beaver, 1937; Fried, 1985; Schmidt & Fried, 1996a,b, 1997; Schmidt, Fried & Reddy, 1998) and can be found throughout the boreal and deciduous forest regions of eastern Canada – from Nova Scotia to south-eastern Manitoba (Clarke, 1981).

The trematodes were identified as members of the genus *Echinostoma* by the presence of a cephalic collar of spines around the anterior end of the cercariae (Beaver, 1937; Schell, 1970). They were further assigned to *Echinostoma trivolvis* because of their life history; i.e. they use *Helisoma trivolvis* snails as their first intermediate host (Beaver, 1937; Schmidt & Fried, 1996a,b,

1997), they readily infect and encyst in the kidneys of *Rana* tadpoles (Beaver, 1937; Fried, 1985; Martin & Conn, 1990; Fried *et al.*, 1997), and they form cysts of a size and structure consistent with those of *E. trivolvis* (Gulka & Fried, 1979; Martin & Conn, 1990). Voucher specimens of the cercariae were placed in the Zoology Collection of the Nova Scotia Museum of Natural History, Halifax, Nova Scotia.

We stimulated cercarial emergence from the molluscan hosts by placing the snails in a 9 cm petri dish filled with dechlorinated, room temperature tap water. The dish was then placed under a 60 W light bulb, positioned *c.* 15 cm away from the light source, and left there for 2 h. The combination of light and heat stimulated the emergence of cercariae. The cercariae were easily visible under a dissecting microscope and were collected in groups of 20 with a pipette and placed in 7 ml plastic vials, which were then emptied into the tadpole tanks. Trematodes were collected from the snails every 2–3 days during the experiments, and the 6 snails provided 800 cercariae.

By the time the experiments were complete, 9200 cercariae had been collected from the 6 snails. The *R. sylvatica* experiment ran for 7 days before the animals began to metamorphose, and these tadpoles were exposed to 120 cercariae per tank. The *R. clamitans* study ran for 28 days and these tadpoles received 340 cercariae per tank. At the conclusion of the experiments, all tadpoles were promptly killed in dilute MS222 (Sigma-Aldrich Canada Ltd, Oakville, Ontario) and preserved in 10% neutral buffered formalin for subsequent dissection.

Metacercarial load and distribution

All 400 tadpoles used in the experiments were dissected by GWT to determine the location of parasite infection. The fixed tadpole was placed ventral side up in a small wax dish filled with enough water to cover the entire specimen. The tadpole was stabilized with pins through the mouth and the tail and a sagittal incision was made along the midline from the cloaca to the margin of the branchial baskets. Four lateral, transverse incisions were then made around the abdomen of the tadpole, terminating near the spine. These incisions produced a flap of skin on each side of the tadpole that, when pinned back, exposed the entire abdominal cavity. The intestines and digestive organs were removed along with any fat bodies that were present. This procedure gave an unobstructed view of the entire urogenital system of the tadpole.

Preliminary infection experiments indicated that the parasites entered the tadpoles through the cloaca and encysted in the kidneys, pronephroi (head kidneys), and Wolffian ducts (Fig. 1). The metacercarial cysts were *c.* 0.1 mm in diameter and could be seen relatively easily through a dissecting microscope. Cysts present on the surfaces of the kidneys and head kidneys were easily seen and those cysts that were deeper inside the organs

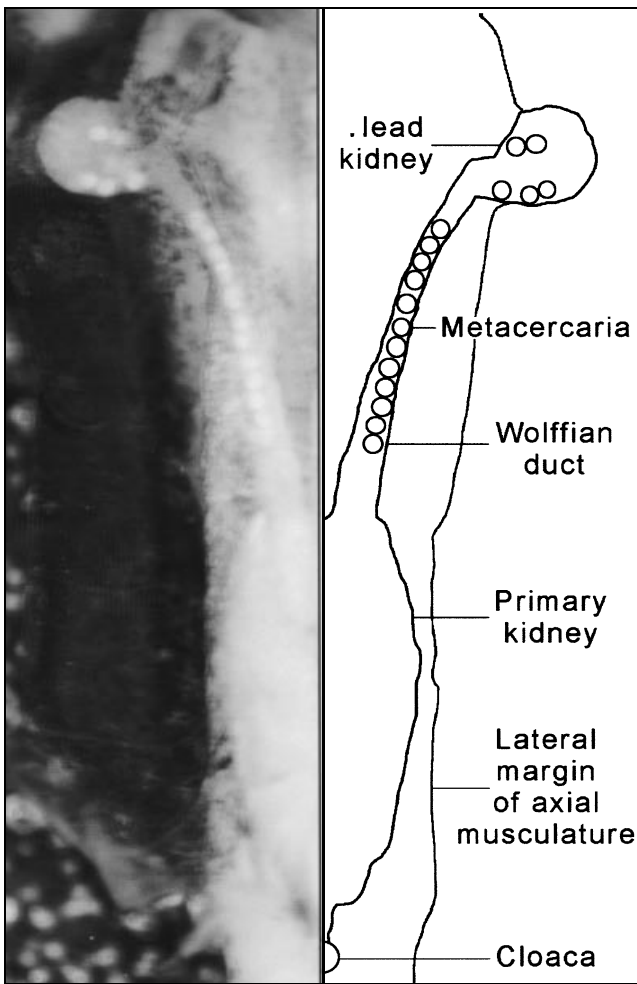


Fig. 1. Dissected *R. sylvatica* tadpole. Photograph: right, opisthonephric kidney, head kidney, and Wolffian duct; whitish circles, metacercariae. Line drawing: mirror images of these structures.

could be found by teasing the tissue apart. The number of cysts present, as well as their locations within the nephric system, were recorded for each tadpole. Metacercarial cysts were recorded as being in 1 of 6 possible locations: (1) right kidney; (2) left kidney; (3) right Wolffian duct; (4) left Wolffian duct; (5) right head kidney; (6) left head kidney. Chi-square tests were used to compare the observed distributions of cysts with those expected given no lateral bias.

RESULTS

Of the 2400 trematode cercariae used in the *R. sylvatica* experiment, 1616 (67.33%) eventually encysted in a tadpole. Trematode cysts were not randomly distributed within the tadpoles' nephric systems (Fig. 2a). There was a statistically significant bias of the parasites to encyst on the right side of the tadpole (915 right:701 left; $\chi^2 = 28.34$, $P < 0.001$). Of the six recorded locations, the right kidney was the most commonly infected area, containing 25.9% of the total cysts.

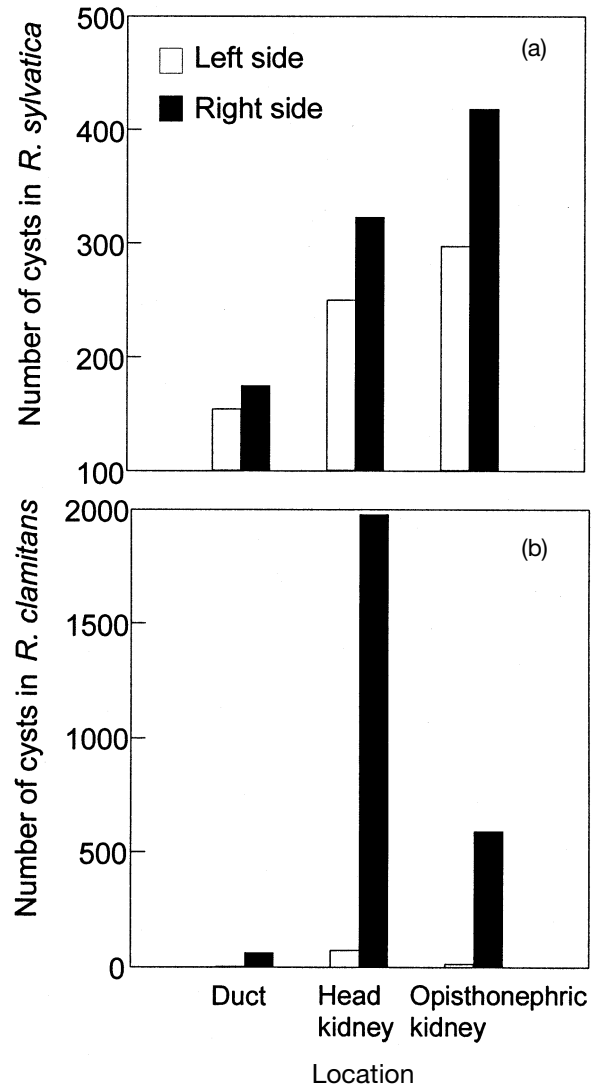


Fig. 2. Distribution of metacercariae within the nephric system of: (a) 200 *R. sylvatica* tadpoles; (b) 200 *R. clamitans*.

Of the 6800 larval trematodes used in the *R. clamitans* experiment, 2717 (39.96%) eventually encysted in a tadpole. Even though these tadpoles were exposed to 2.8 times as many parasites as *R. sylvatica*, their infection rate was 40% less (Table 1). As with *R. sylvatica*, trematodes preferentially encysted on the right side of the tadpoles' bodies (Fig. 2b; 2629 right:88 left; $\chi^2 = 2376.4$, $P < 0.001$). Specifically, 96.8% of all cysts counted were in the right kidneys, right head kidneys and right nephric ducts. Furthermore, the right head kidney was the most commonly infected structure, containing 72.7% of all observed cysts. Of the 2717 trematode cysts counted in 200 *R. clamitans* tadpoles, only one was found in a tadpole's left nephric duct.

Since the *R. sylvatica* experiment used tadpoles that were relatively close to metamorphosis, these tadpoles were significantly longer ($t = 7.49$; d.f. = 398; $P < 0.0001$) and developmentally more advanced ($t = 38.32$; d.f. = 398; $P < 0.0001$) than the *R. clamitans* tadpoles (Table 1). However, the absolute mean difference in

Table 1. Summary of the results of two infection experiments using *Rana* tadpoles and *Echinostoma* cercariae. *Rana sylvatica* and *R. clamitans* were used in two separate experiments that differed only in their duration and in the number of cercariae used

	<i>R. sylvatica</i>	<i>R. clamitans</i>
Sample size (<i>n</i>)	200	200
Average tadpole stage \pm SE	36.54 \pm 0.25	27.98 \pm 0.11
Average tadpole length \pm SE	36.73 \pm 0.26	33.46 \pm 0.41
Length of experiment (days)	7	28
No. of cercariae used	2400	6800
No. of metacercariae formed	1616	2717
Infection rate (%)	67.33	39.96

total length between the species was only 3.26 ± 0.55 mm ($\bar{x} \pm$ SE).

To see if there was a correlation between the number of cysts in the right head kidneys and the number in the right kidneys of tadpoles, these variables were plotted against each other and a simple linear regression performed. If trematodes encyst in the right kidney only when the right head kidney is full, the number of cysts in these two structures should be positively correlated. The regression yielded a correlation coefficient (*r*) of 0.10 and a *P*-value of 0.16.

DISCUSSION

The fact that trematodes encyst in nephric structures on the right side of *R. sylvatica* significantly more frequently than the left (Fig. 2a, $P < 0.001$) parallels a natural size asymmetry of the kidneys in this species. The right kidney is *c.* 20% longer and wider than the left in *R. sylvatica* tadpoles (pers. obs.), an asymmetry documented by Nodzinski, Wassersug & Inger (1989) for pelobatid tadpoles. Hypothetically, trematodes may encyst in the right kidney more frequently than the left simply because there is more available space on the right side. Nephric structures with no obvious size asymmetry (the head kidneys and nephric ducts themselves) display a smaller degree of asymmetry in the number of cysts they contain (see Fig. 2a). However, trematodes must 'decide' to enter the right or left mesonephric duct shortly after entering the cloaca, and it is not obvious how they could discern relative kidney size at that point.

Although the distribution of cysts in *R. sylvatica* tadpoles suggests that the size of nephric structures may influence encystment sites, the results of the *R. clamitans* experiment suggest that other factors are more important in determining cyst location. The overwhelming difference in the number of cysts found in the various nephric structures of *R. clamitans* (Fig. 1b; $P \ll 0.001$) suggests that: (1) trematodes preferentially encyst in structures on only one side of the tadpole; (2) tadpoles have some control over the location of trematode encystment; (3) both organisms have coevolved to minimize the impact of trematode infection on tadpole health. The biased distribution of cysts within the

nephric systems of *R. clamitans* cannot be accounted for simply by the asymmetry in the size of the various nephric structures. This point is clear because although 96.8% of all cysts were on the right sides of tadpoles, the only visible nephric asymmetry in *R. clamitans* was a right kidney that was approximately 20% larger than the left (pers. obs.). Nor does the distribution reflect any difference in the relative sizes of the nephric structures, as 72.7% of all the cysts were present in the right head kidney, a relatively small structure.

The biased distribution of cysts on the right side of *R. clamitans* tadpoles may be solely the result of trematodes somehow distinguishing the right side from the left side, and then selecting the right side only, but the advantage to the trematode of such a dextral distribution is unclear. Presumably, metacercariae can reach the next host equally well from either the left or right side of the tadpole, since no predators on anuran larvae are known to eat one side only of tadpoles!

The distribution of cysts seen in the *R. clamitans* experiment may suggest that the tadpoles themselves have some influence on the location of trematode encystment. By sequestering trematodes in structures on one side only, tadpoles may be able to avoid total (bilateral) blockage of their renal system, and thus, renal failure. This idea is supported by the fact that of the 2717 cysts found in 200 *R. clamitans* tadpoles, only 14 (0.5%) were encountered in the left kidney (Fig. 2b). Furthermore, by preferentially encysting in the head kidneys, the trematodes are targeting tissues that will degenerate as the tadpole matures (Duellman & Trueb, 1986). If the tadpole were somehow able to destroy or isolate cysts during the degradation of the head kidneys, this would further reduce the impact of the trematode infection. However, the ultimate fate of cysts in the head kidneys following degradation is unknown.

The preference of trematodes to encyst in the head kidneys also suggests that tadpoles may be at an advantage in delaying the degradation of the head kidneys until metamorphosis. We suggest that a potential adaptive value for tadpoles in retaining these 'vestigial' organs may be that they serve as 'sinks' for the trematodes and spare the primary kidneys from heavy infection.

Considering the overwhelming propensity of the trematodes to encyst in the right head kidney of *R. clamitans*, we speculated that trematodes may encyst in the right primary kidney only when space in the head kidney becomes limited. The lack of a significant correlation between the number of cysts in the right head kidney and the number in the right primary kidney, indicates that trematodes encyst in the right primary kidney even when space is available in the head kidney.

Rana sylvatica commonly breed in temporary ponds (Kats, Petranka & Sih, 1988; Hopey & Petranka, 1994) that lack the freshwater snail hosts required by *Echinostoma* sp. (pers. obs.). Thus, these tadpoles may rarely encounter trematodes in nature, and may not have evolved the defensive mechanism that shunts parasites away from the primary kidneys and into the head

kidneys. *Rana clamitans*, because of their longer larval period, on the other hand, require a relatively permanent breeding pond (Kats *et al.*, 1988) and probably co-occur more frequently with freshwater snails. Exposure to and infection by trematode parasites may have allowed *R. clamitans* to develop an effective means of reducing metacercarial formation in the primary kidneys.

Rana clamitans were exposed for four times as long to almost three times as many cercariae as *R. sylvatica*, yet had an infection rate 40% less than *R. sylvatica* (Table 1). This difference in parasitic load is probably not the result of differences in absolute size between the two species. Although tadpole size may affect the ability of cercariae to enter the cloaca (i.e. the larger the tadpole, the larger the cloacal opening), the relatively small size difference between the two species used here would translate into a negligible difference in cloacal size. We suggest that *R. clamitans* may simply be more resistant to infection by *Echinostoma*.

In conclusion, the factors determining lateralized encystment of *Echinostoma* remain unknown. However, the left:right bias in the distribution of cysts documented here could benefit both the host and the parasite. Restricting the anatomical distribution of parasites may benefit tadpoles by reducing the physiological cost of infection. This may ultimately benefit the parasite as well by ensuring that the tadpoles live long enough to undergo metamorphosis, making them accessible to the next host in the parasite's life cycle.

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