

## ALTERNATIVE LIFE CYCLE STRATEGIES OF *MEGALODISCUS TEMPERATUS* IN TADPOLES AND METAMORPHOSED ANURANS

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### Summary:

*Megalodiscus temperatus* (Stafford, 1905) is a common paramphistome trematode of North American amphibians with a two host life cycle and has been reported to infect frogs and rarely tadpoles. In this study we document the alternative life cycle strategy of *M. temperatus* in tadpoles and metamorphosed anurans. We show through field work and experimental infections that *M. temperatus* can establish in both anuran life stages and worms become gravid and release eggs in both tadpoles and metamorphosed frogs. However, worms exhibit differences in route of infection, development, egg production, and diet in tadpoles and metamorphosed anurans. These alternative life history strategies of *M. temperatus* suggest different selective pressures on the development and reproductive success of these worms in tadpoles and metamorphosed anurans, and we discuss the evolutionary avenues for and constraints on amphibian trematode life cycles presented by these two different anuran life stages.

**KEY WORDS :** *Megalodiscus temperatus*, trematoda, alternative life cycle, life cycle evolution, tadpoles, anurans.

Although frogs and toads have long been used as subjects for the study of parasites, most parasite ecology and life history studies have concentrated on metamorphosed anurans and few studies exist on parasites of tadpoles (Adamson, 1981; Bolek & Coggin, 2003; Bolek *et al.*, 2003; Kehr & Hamann, 2003; Bolek & Janovy, 2007a, b). Tadpoles are the ephemeral, feeding, non-reproductive larvae in the life cycle of anurans and differ significantly in their biology from adults. Temperate-zone anuran tadpoles are found in streams, ponds, or ephemeral bodies of water, where they feed on suspended and/or epibenthic algae. Tadpole digestive systems are dramatically different from those of predatory adults. Tadpoles thus are considered vertebrate analogs to larvae of holometabolous insects (McDiarmid & Altig, 1999). However, parasites of tadpoles have not been studied extensively, particularly in terms of trematode life cycle

evolution, and most show abbreviated two host life cycles from the typical three host trematode life cycle (Grabda-Kazubska, 1976; McDiarmid & Altig, 1999). In this paper, we examine the alternative life cycle strategies of *Megalodiscus temperatus*, a trematode that has been reported in both the larval and adult anurans (Krull & Price, 1932) and discuss avenues for and constraints on amphibian trematode life cycle evolution in tadpole and metamorphosed anuran.

### MATERIALS AND METHODS

During April-September 2001-2007, 596 adult anurans of six species were collected by hand, from Pawnee Lake (40° 51.589' N, 96° 53.468' W) and Elk Creek (40° 53.145' N, 96° 50.048' W), and 53 tadpoles of a single species were collected by seining during March and April 2007 from Elk Creek all located in Lancaster County, Nebraska, USA, and examined for *M. temperatus*. These hosts included 110 plains leopard frogs, *Rana blairi*, Mecham, Littlejohn, Oldham, Brown, and Brown, 1973, 112 bullfrogs, *Rana catesbeiana*, Shaw, 1802, 72 Cope's grey treefrogs, *Hyla chrysoscelis*, Cope, 1880, 133 western chorus frogs, *Pseudacris triseriata triseriata* (Wied-Nuweid, 1838) 100 Woodhouse's toads, *Bufo woodhousii*, Girard, 1854, and 66 northern cricket frogs, *Acris crepitans blanchardi* Harper, 1947, from Pawnee Lake, and three bullfrogs and 53 bullfrog tadpoles from Elk Creek. Anurans were killed, and the snout vent length (SVL) was measured for adult anurans, whereas tadpoles were killed and aged according to Gosner (1960) and the SVL and total length was recorded. All anurans and tadpoles were examined for metacercarial infections on the skin and juvenile and adult worms in the digestive track within 72 hr of collection. Trematodes were removed from the digestive tract placed on slides in a drop of aged tap water, covered with a cover slip and examined for maturity, the presence of eyespots and cecal content, and the number of eggs in the uterus was counted. Worms were then allowed to release their eggs in water, and fixed in alcohol-formalin-acetic acid (AFA) or 95 % ethanol; representative specimens were stained with

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Seminchon's acetocarmine. Rectal flukes were identified based on the description by Stafford (1905) and redescriptions by Krull & Price (1932), Brooks (1976) and Prudhoe & Bray (1982). Prevalence (percentage of infected organisms in a sample); mean intensity (MI, mean number of worms per infected host); and/or mean abundance (MA, mean number of individuals of a particular parasite species per host including uninfected hosts) were calculated for the amphibians examined. The chi-square test for independence was used to compare differences in prevalence of gravid and non-gravid worms, whereas Student's *t*-test was used to compare differences in mean abundance among these stages in field collected tadpoles during different times during the year.

For snail infections, colonies of *Planorbella* (*Helisoma*) *trivolvis* (Say, 1817) were established in the laboratory from wild strains collected in August 2006 from Elk Creek. Snails were maintained in 3.75 L jars with aged tap water on a diet of frozen mustard greens, maple leaves and Tetra Min® fish food. Adult *M. temperatus* flukes were obtained from wild-caught plains leopard frogs and western chorus frogs from Pawnee Lake. For infections adult worms removed from frogs, or infected frogs were placed in 110 × 35 mm standard dishes partially filled with aged tap water and worms were allowed to release eggs. Twenty-five 3-day-old laboratory reared *P. trivolvis* were added to a standard dish with hatched miracidia. Observations were taken on miracidia interactions with individual snails for 2 hr and then 12 hr later. Worms used in the infections were then fixed in AFA, stained, and identified to species. Exposed snails were maintained in 3.75 L jars and all survivors were isolated in 70-ml plastic containers filled with aged tap water and observed weekly for shedding cercariae.

For tadpole infections, eggs of western chorus frogs and plains leopard frogs were collected from Pawnee Lake during April 2007, and young tadpoles (Gosner stage 30-35) of bullfrogs were collected during July-September 2007 from Nevens Pond (41° 12.459' N, 101° 25.081' W) Keith County, NE. Eggs of chorus frogs and plains leopard frogs were allowed to hatch in the laboratory. Tadpoles of the three different species were maintained on a diet of frozen mustard greens and Tetra Min® fish food in individual 45.5-L tanks filled with aged tap water before exposure to cercariae of *M. temperatus*.

For infections, both naturally infected *P. trivolvis* snails from Elk Creek and laboratory reared and infected snails were used. Cercariae from naturally infected snails were identified to species based on descriptions of the cercariae by Krull & Price (1932) and recovering adult worms from infected anurans (see below). Because bullfrog tadpoles were not laboratory reared these were divided into three equal groups and assigned

to time-0 controls, experimentals, or time-T controls. Time-0 controls were dissected at the beginning of the experimental trial, whereas time-T controls were maintained throughout the experiment and examined for *M. temperatus* infections along with the experimental group.

To examine cercarial behavior with tadpoles, 18 Gosner stage 28-30 western chorus frog tadpoles, five Gosner stage 25-27 plains leopard frog tadpoles, and five Gosner stage 34-35 bullfrog tadpoles were individually isolated. Western chorus frog and plains leopard frog tadpoles were placed in 5 ml well plates filled with aged tap water, whereas the larger bullfrog tadpoles were placed in 110 × 35 mm standard dishes partially filled with aged tap water and to each was added 10 *M. temperatus* cercariae. After 24 hr, each well plate or standard dish was then examined for dead cercariae or formed metacercariae on the bottom of the containers or on tadpole. After exposure tadpoles were maintained in individual 70 ml plastic containers or standard dishes, and fed Tetra Min® fish food or laboratory reared algae. To document when worms become sexually mature, tadpoles of western chorus frogs were dissected daily starting on the fourth day post exposure (DPE) and on 4, 15, and 21 DPE in plains leopard frog tadpoles; and 10 or 20 DPE in bullfrog tadpoles. To determine if and when worms became gravid in tadpoles, 10 bullfrog tadpoles were maintained in a 110 L tank with an infected snail and examined 1, 5, and 16 weeks post exposure (WPE). All worms were removed from the digestive tract of tadpoles placed on microscope slides in a drop of aged tap water covered with a cover slip and examined for the presence of testis, ovary, uterus, vitellaria, and eggs, with a Wild M20 Phase-contrast microscope.

For adult anuran infections, adult frogs and toads of six species were collected from Pawnee Lake (*A. crepitans*, *P. triseriata*, *H. chrysoscelis*, and *B. woodhousii*), Nevens Pond (*R. catesbeiana*), and Cedar Creek (*R. pipiens*, Schreber, 1782) (41° 11.194' N, 101° 21.820' W) Keith County, Nebraska, USA. Anurans of each species were divided into three equal groups of 4-6 individuals and assigned to time-0 controls, experimentals, or time-T controls. Experimental design was the same as used for wild caught tadpoles. All exposed anurans along with time-T controls, were maintained individually in plastic shoe boxes (35 cm × 25 cm × 15 cm), and fed commercial crickets three times a week. Three to 93 DPE all exposed anurans along with the time-T controls were euthanized and examined for rectal flukes. Maturity status and cecal content of each worm was recorded. Length, width, number of eggs in the uterus, and cecal contents were compared for field and laboratory obtained worms from tadpoles and adult anurans. Student's *t*-test for unequal variance was used to compare differences in mean length and width of gravid worms

and average number of eggs in worms recovered from tadpoles and metamorphosed anurans. Voucher specimens of adult gravid worms from tadpole and adult anurans, along with cercariae and rediae stages have been deposited in the H.W. Manter Parasitology Collection, University of Nebraska, Lincoln, Nebraska, USA, 68588-0540.

## RESULTS

Of the 596 field collected adult anurans examined from Pawnee Lake and Elk Creek, only six frogs (1 %) from Pawnee Lake were infected with *M. temperatus*. Five of 110 plains leopard frogs (4.5 %; MA =  $0.05 \pm 0.26$ , range = 0-2; MI =  $1.2 \pm 0.4$ ) and one of 133 western chorus frogs (0.75 %; MA =  $0.008 \pm 0.09$ ) were infected with a total of seven worms. All worms were located in the large intestine, were gravid and contained on average  $156 \pm 86$  (50-250) eggs. Worms were  $2.16 \pm 0.83$  mm in length (1.43-3.98) and  $0.99 \pm 0.48$  mm in width (0.42-1.52), with no eyespots; and contained blood in the cecae. No *M. temperatus* metacercariae were found on the skin of any of the frogs examined.

Of the 53 field collected bullfrog tadpoles from Elk Creek, 23 of 53 tadpoles (43 %; MA  $4.5 \pm 8.6$ , 0,37; MI  $10.3 \pm 10.7$ ) were infected with *M. temperatus*. A total of 237 worms infected these tadpoles 25 (10.5 %) of which were gravid. All worms possessed eyespots and algae in the cecae, and most were located in the large intestine, with a few worms being found in the small intestine. Gravid worms in tadpoles were smaller  $0.84 \pm 0.15$  mm (0.60-1.18) in length and  $0.28 \pm 0.06$  mm (0.20-0.40) width than worms recovered from adult frogs (Fig. 1A, B;  $t = -4.44$ ,  $P = 0.004$ ;  $t = -3.88$ ,  $P = 0.008$ ) and contained fewer eggs  $20 \pm 17$  (1-75;  $t = -4.21$ ,  $P = 0.006$ ). Eggs in gravid worms were in different stages of development, however most worms contained some eggs with fully formed miracidiae that hatched when eggs were released (Fig. 1C). Statistically significant differences existed in prevalence for total and gravid worms and mean abundance for total worms recovered from tadpoles in March and April 2007 (total prevalence March = 9 %; April = 100 %;  $\chi^2 = 41.89$ ,  $P < 0.001$ ; prevalence of gravid worms March = 9 %; April = 40 %;  $\chi^2 = 7.23$ ,  $P < 0.05$ ; MA of total worms March =  $0.27 \pm 0.97$ ; April =  $11.4 \pm 11.0$ ;  $t = -4.49$ ,  $P = 0.0002$ ; MA of gravid worms March =  $0.24 \pm 0.83$ ; April =  $0.85 \pm 1.63$ ;  $t = 2.05$ ,  $P = 0.13$ ), indicating that some worms became gravid within 4-5 weeks.

For snail infections, as soon as snails were placed with *M. temperatus* miracidiae, individual miracidiae changed their behavior from randomly swimming to following individual snails. No miracidiae were observed penetrating snails, however 12 hr PE snails were observed

with their apertures closed against the substrate with 1-6 miracidiae sticking out from under their shells (Fig. 1D). Of the 25 snails exposed 12 survived and 8 began shedding cercariae 36-40 DPE. Rediae developed in the hepatopancreas (Fig. 1E and F); they were sluggish when freed from snail tissue and were 0.40-0.80 mm in length, with a round pharynx (Fig. 1G). Cercariae were pharyngeate, monercous amphistomes with pigmented eyespots (Fig. 1H) morphologically identical to the description by Krull & Price (1932).

No cercariae formed metacercariae directly on any tadpoles. Tadpoles were observed sucking in most cercariae with their mouths through respiratory currents. Some metacercariae were formed on the substrate of the containers, and occasionally these would stick to the surface of tadpoles (Fig. 1I), however all were brushed off; some tadpoles were observed ingesting these metacercariae. All infected tadpoles contained worms in the large intestine (Fig. 1J). In general tadpoles lost worms over time; *P. triseriata* tadpoles examined 4, 5, 6, 6, 6, 7, 8, 9, 10, 11, 12, 12, 12, 13, 14, 15, 15, and 15, DPE contained 9, 8, 1, 3, 3, 9, 9, 3, 7, 3, 0, 1, 2, 5, 2, 0, 0, and one worms respectively; *R. blairi* tadpoles examined 4, 15, 15, 21, and 21 DPE contained 6, 1, 1, 1, and one worms respectively, whereas *R. catesbeiana* tadpoles examined 10, 20, 20, 20, and 20 DPE contained 1, 9, 7, 6, and one worms respectively. All worms contained eyespots, and had developed testes, ovary, uterus, and vitellaria 9 DPE (Fig. 1K) and began producing granules in the uterus 12 DPE, however, none of these worms became gravid. Of the 10 bullfrog tadpoles left in a 110 L tank with an infected snail 90 % became infected. Three tadpoles examined 1 WPE contained 0, 2, and 15 worms, five tadpoles examined five WPE contained 1, 14, 17, 25, and 36 worms; whereas two tadpoles examined 16 WPE contained 200 and 250 worms. All worms, contained eyespots, alga in the cecae (Fig. 1L and M). Most worms were sexually mature and contained sperm in the seminal vesicle when examined five and 16 WPE, however, none of the worms were gravid. None of the time-0 or time-T control bullfrog tadpoles was infected. In all tadpoles that were fed alga 100 % of worms ( $n = 619$ ) contained alga in the cecae, however, when tadpoles were fed Tetra Min® fish food 18 % ( $n = 11/61$ ) of worms contained tadpole blood in the cecae, whereas the remaining 82 % ( $n = 50$ ) worms had empty cecae.

Within 24 hr of exposing adult anurans to cercariae, all formed metacercariae on the skin of the six species of frogs and toads exposed (Fig. 1N). After anurans ingested their skin, worms developed in all six species, although not all exposed individuals became infected. Prevalence ranged from a high of 80 % in cricket frogs to a low of 20 % in bullfrogs (Table I). None of the time-0 or time-T control anurans was infected. Worms began losing their eyespots 35 DPE,



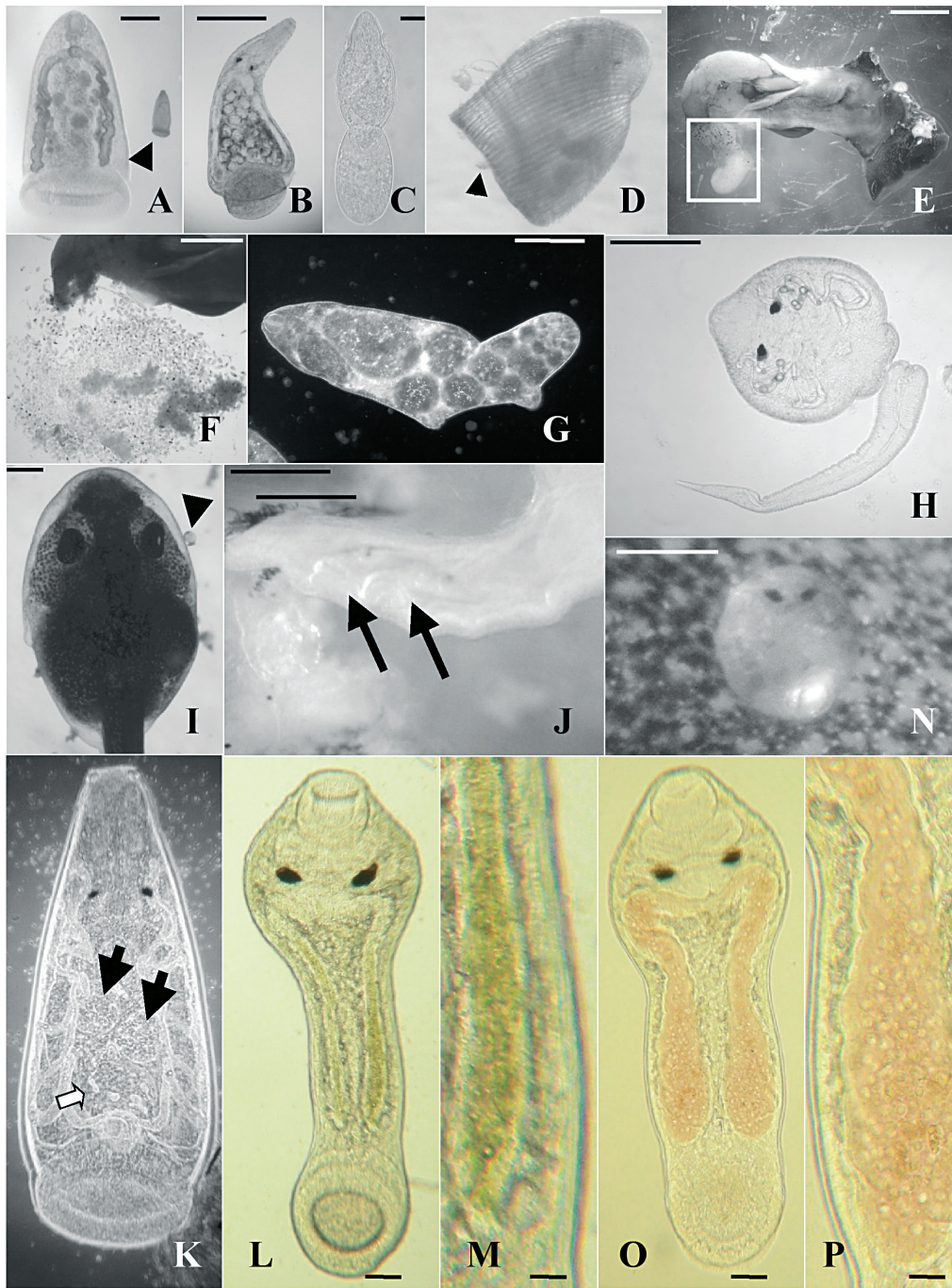


Fig. 1. – Life cycle stages of *Megalodiscus temperatus* in snails, tadpoles and adult anurans. A. Comparison of a typical adult gravid worm recovered from an adult anuran and tadpole (black arrow). Scale bar = 0.8 mm. B. Adult gravid worm recovered from a tadpole, note the eggs and eyespots. Scale bar = 0.25 mm. C. Miracidia hatching from an egg released by a gravid worm from a tadpole. Scale bar = 25  $\mu$ m. D. A three day old *Planorbella trivolvis* with a *M. temperatus* miracidium trying to penetrate under the shell (arrow). Scale bar = 0.5 mm. E. A three month old laboratory reared and infected *Planorbella trivolvis* with shell removed, showing the infected hepatopancreas (Box) with *Megalodiscus temperatus* rediae. Scale bar = 75 mm. F. Close up of E. Scale bar = 2 mm. G. *Megalodiscus temperatus* redia removed from the hepatopancreas of an infected snail. Note the round pharynx and developing cercariae. Scale bar = 80  $\mu$ m. H. *Megalodiscus temperatus* cercaria from a laboratory infected snail. Scale bar = 180  $\mu$ m. I. A tadpole of *Rana blairi*, with a *Megalodiscus temperatus* metacercaria attached to the skin (arrow). Scale bar = 1 mm. J. Large intestine of a tadpole of *Pseudacris triseriata* with two attached *Megalodiscus temperatus* adult worms 10 DPE (arrows). Note the eyespots. Scale bar = 100  $\mu$ m. K. Sexually mature worm from J. Note the presence of eyespots, and the developed testes (black arrows) and ovary (white arrow). Scale bar = 30  $\mu$ m. L. Two weeks old worm from an experimentally infected tadpole of *Pseudacris triseriata* fed algae. Note the green cecae full of algae. Scale bar = 30  $\mu$ m. M. Enlarged cecum of L. Scale bar = 5  $\mu$ m. N. *Megalodiscus temperatus* metacercaria on the skin of an experimentally infected adult *Pseudacris triseriata* frog. Scale bar = 150  $\mu$ m. O. Two weeks old worm from an experimentally infected adult *Pseudacris triseriata* fed crickets. Note the red cecae full of frog blood. Scale bar = 30  $\mu$ m. P. Enlarged cecum of O. Scale bar = 5  $\mu$ m.

	Prevalence (No. infected/ No. exposed)	Mean intensity + 1 SD	Mean abundance + 1 SD (range)
<i>Acris crepitans</i>	80 (4/5)	2 ± 1.15	1.6 ± 1.3 (0-3)
<i>Pseudacris triseriata</i>	67 (4/6)	3.8 ± 2.2	2.5 ± 2.6 (0-6)
<i>Hyla chrysoscelis</i>	20 (1/5)	1	0.2 ± 0.4 (0-1)
<i>Bufo woodhousii</i>	40 (2/5)	1*	0.4 ± 0.5 (0-1)
<i>Rana catesbeiana</i>	20 (1/5)	4	0.8 ± 1.8 (0-4)
<i>Rana pipiens</i>	50 (2/4)	2*	1 ± 1.2 (0-2)

\* Gravid worms.

Table I. – Prevalence, mean intensity, and mean abundance of *Megalodiscus temperatus* in six species of laboratory exposed adult frogs and toads.

and all worms contained anuran intestinal content 65 % (n = 22/34) or blood 35 % (12/34; Fig. 1O and P) in the cecae. Only five worms became gravid. Worms in anurans began producing eggs 42 DPE when they were 1.43 mm in length and 0.60 mm in width and all gravid 100 % worms (n = 5/5) contained blood in the cecae. Worms produced more eggs as they aged: one egg 42 DPE, two and 15 eggs 64 DPE, and 100 and 200 eggs 93 DPE.

## DISCUSSION

Although a number of amphibian trematodes are known to infect tadpoles and/or metamorphosed amphibians in their abbreviated two host life cycles, most species become gravid in metamorphosed anuran (see Prudhoe & Bray, 1982). Studies on life cycles of anuran plagiurchiids in Europe and North America indicate that most species utilize either a tadpole or metamorphosed anuran in their life cycles, indicating host stadial specificity. Additionally, these plagiurchiids show narrow host specificity, infecting closely related anurans and rarely cross host family boundaries. Studies on four closely related species of *Glyptelminis* and *Haplometrana* in North America showed that cercariae of *G. hylareus*, Martin, 1969, and *G. pennsylvaniensis*, Cheng, 1961, can only infect the tadpole stages of treefrogs where they form metacercariae in the musculature and body cavity and migrate to the intestine as tadpoles metamorphose into froglets, whereas cercariae of *G. quieta* (Stafford, 1900) and *H. utahensis* Olsen, 1937, only infect the skin of metamorphosed true frogs (Ranidae); when frogs ingest their skin, worms mature in the small intestine (Olsen, 1937; Leight, 1946; Martin, 1969; Sullivan & Byrd, 1970). Studies on two related European plagiurchiids *Opisthioglype ranae* (Frölich, 1791) and *O. (Dolichosaccus) rastellus* (Olsson, 1876) show variations on this pattern of host stage specificity (Grabda-Kazubaska, 1969). Cercariae of *O. rastellus* can only infect tadpoles of true frogs when the cercariae are ingested through respiratory currents and form metacercariae in the buccal

cavity, or when metacercariae form on the substrate in ponds and are ingested as tadpoles feed on algae. Metacercariae hatch within days and migrate to the intestine where they become gravid. Most of these worms are lost when tadpoles metamorphose and their intestine changes drastically from an herbivorous to a carnivorous diet. Young frogs can only become infected with *O. rastellus* when they feed on infected tadpoles, but most worms are lost as froglets mature. In contrast, cercariae of *O. ranae* can penetrate both tadpole and metamorphosed stages of true frogs and metacercariae form in the tissue but only migrate to the intestine in metamorphosed frogs where they become gravid.

In contrast, our field and laboratory studies on *M. temperatus* show that this paramphistome, distantly related to the plagiurchiids, infects a wide range of anurans in three different families and worms become gravid in tadpoles and adult anurans. *Megalodiscus temperatus* has been reported from 25 species of amphibians and reptiles including representatives from three families of anurans, four families of caudatans and one species of snake (Brooks, 1976; Prudhoe & Bray, 1982). We hypothesize that this lack of host specificity has apparently allowed *M. temperatus* to reproduce in anuran tadpoles. However, the dramatically different anuran life stages have favored alternative life cycle strategies in this trematode. The similarities in the route of infection of tadpoles by the inhalation of cercariae or ingestion of metacercariae from the substrate of *O. rastellus* and the formation of metacercariae on the skin of adult frogs by *G. quieta* and *H. utahensis* clearly suggests that tadpoles and adult anurans present different avenues for and constraints on transmission of trematodes, and these have evolved multiple times in unrelated trematode families.

Our observations also reveal that once established in tadpoles, the aquatic herbivorous life style of tadpoles, compared to the less aquatic carnivorous life style of adult anurans imposes different selection pressures on reproduction in *M. temperatus*. Worms in tadpoles are smaller, reproduce within 4-5 weeks and produce fewer eggs, and when given the opportunity feed on algae, whereas worms in metamorphosed anurans are larger,



reproduce within 7-9 weeks and produce more eggs, and feed on anuran blood. Laboratory and field studies on *M. temperatus* by Krull & Price (1932) and Brooks (1976) indicate that gravid worms in metamorphosed anurans are 2.1-6.0 mm long by 0.9-2.5 mm wide and commonly feed on frog blood. In our frog and toad experimental infections, all gravid worms that contained one or two eggs were larger (1.75 mm) than the largest gravid worm from tadpoles (1.2 mm) that contained 75 eggs. It is unclear why gravid worms from tadpoles are smaller and predominantly feed on algae and not blood as they do in adult frogs. However, we suggest that a low nutrient diet of algae compared to a high nutrient diet of blood in frogs may prevent worms in tadpoles from growing as large as in frogs and indirectly affect their reproductive output. Our observations on the ecology of tadpoles and adult anurans, along with the generalist nature of *M. temperatus* suggest that these reproductive differences and life cycle strategies are well suited in different life stages of anurans. We hypothesize that in order to overcome the overwhelming probability of infecting a snail first intermediate host, a worm in an metamorphosed anuran which can vary in its habitat (aquatic, semi-terrestrial, terrestrial, or arboreal) depending on the species of frog infected must produce more eggs compared to an aquatic tadpole that is always found in water, in order to complete the life cycle. In order to test some of our hypotheses, we urge other parasitologists to examine the larval stages of anurans for their parasites and examine life cycles of other unrelated amphibian trematodes from a phylogenetic perspective because few such studies exist. Only then will we have a better understanding of the selective pressures on the avenues for and constraints on trematode life cycle transmission in anuran hosts.

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