

SHORT COMMUNICATION

## Monostome cercariae induced malformations in amphibians: effect of infection at the pre-limb-bud stage tadpoles of *Polypedates cruciger* Blyth

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**Abstract:** Global biodiversity loss and disease emergence are interrelated and have become a challenging environmental issue. Infection by the digenetic trematode, *Riberioa ondatrae* induces malformations in amphibians and is considered an emerging infection. The effect of infection on amphibians is not only parasite and host species specific, but also depends on the timing of infection. Recent evidence suggests that infection by cercariae of a monostome trematode species induces severe malformations in the common hourglass tree frog, *Polypedates cruciger* Blyth (1852) when exposed at the limb-bud stage of development. The aim of this study was to examine whether infection by this trematode species at the pre-limb-bud stage also induces malformations in *P. cruciger*. Infection at the pre-limb-bud stage resulted in malformations (64.5%) and significantly reduced survival (77.5%). It also affected the growth and lengthened the larval period. Infections acquired at the pre-limb-bud stage caused a higher mortality and induced fewer malformations than infections at the limb-bud stage, as reported in a previous study. Hence, the effect of infection of monostome cercariae seems to depend on the developmental stage at which the tadpoles are exposed. Understanding the stage specific dynamics of malformations and associated mortality might be important to amphibian conservation.

**Keywords:** Amphibians, *Polypedates cruciger*, frog malformations, trematode infection.

### INTRODUCTION

During the past decade, reports on malformed frogs in the wild have increased worldwide<sup>1,3</sup>. Both laboratory and field experiments have confirmed that infection by the digenetic trematode, *Riberioa ondatrae*, induces malformations in amphibians that

are consistent with malformations observed in wild amphibian populations<sup>4,7</sup>. Infection by *R. ondatrae* is considered an emerging disease of amphibians and is closely associated with anthropogenic alterations of the ecological and evolutionary relationships between hosts and pathogens<sup>8-11</sup>. Extensive use of pesticides and eutrophication are associated with the increased transmission and pathology of trematode infections in amphibians<sup>12-15</sup>. There is evidence that infectious diseases lead to amphibian declines which has become a challenging environmental issue<sup>16</sup>.

Timing of infection is an important determinant of the trematode-induced limb malformations<sup>7</sup>. Limb-pattern formation is a time-dependent process, and errors that occur at different times during development may cause different types of limb malformations<sup>17</sup>. Moreover, as the tadpole grows, the ability of cells of the developing limb-bud to produce deformed limbs declines<sup>18</sup> and the likelihood that limb elements will be duplicated may be restricted to specific periods of limb development<sup>7,19</sup>. A previous study<sup>20</sup> showed that amphibian responses to trematode infections depended on host stage. Infections of *R. ondatrae* cercariae acquired at the pre-limb-bud stage of the northern leopard frog, *Rana pipiens* resulted in a high mortality rate and the tadpoles infected at the limb-bud stage displayed a high malformation rate. However, infections acquired at the paddle stage had no effect on limb development or tadpole survival<sup>20</sup>.

Digenetic trematodes have a complex life cycle that includes two or more hosts. Adults live and reproduce in

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the gastrointestinal tract of a bird, reptile or a mammal, which acts as the definitive host. Eggs are deposited in the environment with the faeces of the definitive host. The first free-living larval stage is the miracidia. They hatch out of the egg and enter freshwater snails, the first intermediate host. Within the snail, miracidia undergo several polyembryonic stages and develop into the cercarial stage. Cercariae are released in large numbers from the snail. They penetrate and encyst as metacercariae in the second intermediate host, often a tadpole or a fish<sup>21,22</sup>. Maturation to adulthood resumes when the infected tadpole or fish is eaten by a suitable vertebrate definitive host. The occurrence of cercariae-induced malformations in amphibians is likely a natural phenomenon. Many parasites change the morphology or behaviour of their intermediate hosts in ways that modify the risk of predation by a definitive host, thereby facilitating completion of its life cycle<sup>23,25</sup>. Limb malformations caused by cercariae infections are hypothesized to have adaptive significance for the parasite in that they interfere with the movement of affected individuals, thereby increasing the susceptibility to predation and facilitating completion of the life cycle<sup>7,26</sup>. However, accelerated eutrophication owing to nitrogen and phosphorus enrichment and agrochemicals have changed aquatic ecosystems, increasing the density of infected snail hosts and enhancing per-snail production of infectious parasites<sup>12,13</sup>.

The first field observation of deformed amphibians in Sri Lanka was reported recently from two protected nature reserves<sup>27</sup>. Deformed specimens of an endemic monotype genus *Lankanectes corrugatus* and the common frog *Rana temporalis* have been found with missing limbs and short bones and digits in the hind limbs<sup>27</sup>. This was further investigated by exposing the limb-bud stage tadpoles (Gosner stage 27)<sup>28</sup> of the common hourglass tree frog, *P. cruciger* to monostome cercariae in the laboratory<sup>29</sup> following a previously reported protocol<sup>4,5</sup>. This resulted in high frequencies of malformations (up to 92%), such as missing limbs (amelia), incomplete limbs (ectromelia), missing toes (ectrodactyly) and also axial malformations such as abnormally convex thoracic spine (kyphosis) and lateral deviation in the spine (scoliosis)<sup>29</sup>, some of which were identical to those observed at field sites<sup>27</sup>. The authors also reported reductions in survival and growth of the tadpoles<sup>29</sup>.

The common hourglass tree frog, *P. cruciger* (Anura: Ranidae), is a widely distributed, entirely arboreal, endemic frog, distributed predominantly in the wet zone of Sri Lanka<sup>30</sup>. It attaches its unpigmented eggs in a foam nest onto a substrate few inches above the water level and the tadpoles hatch out and fall into the water body.

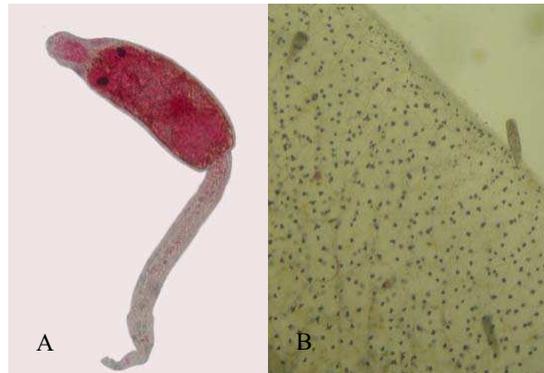
This tree frog is mostly associated with human-modified habitats and is known to have expanded its natural ranges and established a higher relative dominance following habitat disturbances<sup>30</sup>. We tested the hypothesis that the early exposure to the cercariae induces malformations in *P. cruciger* by infecting the tadpoles at pre-limb-bud stage (Gosner stage 25 and 26) to this monostome cercariae.

## METHODS AND MATERIALS

Egg clutches of *P. cruciger* were collected from small ponds and pools in the Peradeniya University park. The eggs were allowed to hatch under laboratory conditions in dechlorinated tap water. Tadpoles were fed with commercial fish feed twice a day (10% body weight). The debris and faeces that collected at the bottom of the tanks were siphoned out and the water levels were topped up daily. The water in the tanks was renewed completely once a week. Five-day-post-hatch tadpoles in the pre-limb-bud stage (Gosner stage 25 and 26)<sup>28</sup> were used in the experiments. Emergent tadpoles from 4 clutches (n=160) were used in the study.

Monostome cercariae were collected from the freshwater snail species *Thiara scabra* as described previously Rajakaruna *et al.*<sup>29</sup>. Snails were placed in individual plastic specimen cups containing 50 mL of dechlorinated tap water and subsequently exposed to intense natural sunlight for 3-4 h allowing them to shed cercariae. The cercariae were identified by making permanent slide preparations after treatment with Gilson's fixative and staining in Borax Carmine.

Live cercariae were siphoned into a pipette, placed on a petri dish and counted using a stereo-dissecting microscope. Cercariae were administered to five-day post-hatch tadpoles which were placed individually in a solid watch glass containing a small amount of water (~ 25 mL). Twenty tadpoles from each clutch (n=20× 4 clutches) were exposed to 48 cercariae following previously reported methods<sup>29</sup>. Each tadpole was placed in the watch glass and 12 cercariae were introduced each day for 4 consecutive days. The containers were inspected through a dissecting microscope at 15 min intervals to ensure that all cercariae had penetrated the tadpoles. The exposed tadpoles were then placed in a glass tank (30x18x20 cm) with a water volume of 200 mL per tadpole. A control group was set up using the tadpoles from each clutch (n=20) without exposing to cercariae. Exposure was repeated using tadpoles from 4 different egg clutches separately. All the tanks were kept at room temperature, with daytime temperature varying between 27- 31°C, under a natural photoperiod of approximately 12:12 h.



**Figure 1:** (A) monostome cercaria (B) cercariae penetration through the tail fin of the tadpole and penetrated cercariae inside the tail.



**Figure 2:** Different stages of representative malformed tadpoles of *Polypedates cruciger* exposed to monostome cercariae. A to D - tadpoles with kyphosis (hunched back), E & F - tadpoles with edema and G - a tadpole with an ulcer. H - a normal metamorph and I - a malformed metamorph with kyphosis.

**Table 1:** Comparison of mean snout-vent length (SVL), body weight and TE<sub>50</sub> of *P. cruciger* at metamorphosis using Kruskal-Wallis test

	Mean SVL	H	Mean weight	H	TE <sub>50</sub>	H
Treatment	± SD (cm)	(p)	± SD (g)	(p)	(Days)	(p)
Control	1.48 ± 0.96	48.39	1.030 ± 0.285	70.77	45	5.33
Exposed	0.90 ± 0.16	(<0.001)	0.599 ± 0.127	(<0.001)	108	(<0.001)

**Table 2:** Percentage survival and malformations of exposed and control of groups of *P. cruciger* at different life stages

Treatment	Parameter	Percentage		
		10 days	30 days	Metamorphs
Survival	Control	98.8%	97.5%	97.5%
	Exposed	90.0%	81.6%	77.5%
	χ <sup>2</sup>	7.792	15.376	18.939
	P	<0.05	<0.001	<0.001
Malformations	Control	0	0	0
	Exposed	75.8%	71.5%	64.5%

**Table 3:** Types and relative frequencies of malformations in the metamorphs of *P. cruciger*

Type of malformation	Frequency	
	Control	Exposed
Hind limb	0	0
Fore limb	0	0
Axial	0	0.69
Skin	0	0.31
Mean # of malformations per malformed metamorph ± SD	0	1.196 ± 0.243

Tadpoles were raised in the tanks until metamorphosis. Mortality was recorded daily. All the tadpoles in experimental and control set up were observed carefully for malformations at 10 d (Gosner stage 27) and 30 d post-hatch (Gosner stage 31)<sup>28</sup> and at metamorphosis. The percentage of malformations was calculated as the number of malformed individuals at each stage divided by the number of surviving individuals at that particular stage. The dead malformed individuals were also included in these calculations. At metamorphosis all the malformed individuals were anesthetized and preserved in 5% formalin. Laboratory rearing of the tadpoles and anesthetizing (using tricaine methane sulfonate MS-222) and killing of the malformed amphibians were carried out according to the protocols approved by the Canadian Council on Animal Care<sup>31</sup>. Malformations were categorized using the Field Guide to Malformations of Frogs and Toads<sup>32</sup>.

Growth of the tadpoles was monitored by measuring snout-to-vent length (SVL; to the nearest 0.1 cm using a vernier caliper) and body weight (to the nearest 0.01 g) at 10 and 30 days post-hatch and at metamorphosis. Time required for the forelimb emergence of half the number of tadpoles (TE<sub>50</sub>) was also recorded. Data from 4 clutches were pooled and analysed. The differences in mean body weight and SVL of tadpoles and metamorphs in the exposed and the control groups were analysed using a Kruskal-Wallis test for unequal sample sizes. The total number of individuals that survived at 10 and 30 days post-hatch and at metamorphosis in the exposed and control groups were compared using a chi square test. The number of malformations in each tadpole/metamorph was counted. Results were analyzed using MINITAB 14.0 for Windows.

The specimens of monostome cercariae described in this study are deposited in the National Museum, Colombo (Reference number: 2007.24.1).

## RESULTS

Cercariae released from the freshwater snail species *T. scabra* readily aggregated around the tadpoles and penetrated the skin within 5–10 min (Figure 1A and 1B). In three hours, no cercariae were visible in the containers, indicating that all had found their way into the tadpoles. The majority of cercariae were seen penetrating at the tail region (Figure 1B), with few at the head and other parts of the body.

Both mean SVL and weight of the individuals in the groups exposed to cercariae were significantly lower than those not exposed to cercariae (Kruskal-Wallis, SVL- $H=48.39$ ,  $df=1$ ,  $p<0.001$ ; weight- $H=70.77$ ,  $df=1$ ,  $p<0.001$ , Table 1). Exposed tadpoles took more time to metamorphose than control tadpoles. The mean TE<sub>50</sub> value for *P. cruciger* was 45 days for control tadpoles, while the mean TE<sub>50</sub> for tadpoles exposed to cercariae was 108 days (Kruskal-Wallis test,  $H=5.33$ ,  $df=1$ ,  $p<0.001$ ; Table 1).

Exposure to cercariae at the pre-limb-bud stage significantly reduced survivorship of tadpoles (10 days

old tadpoles  $\chi^2 = 7.792$ ;  $P < 0.05$ ; 30 days old tadpoles  $\chi^2 = 15.376$ ;  $P < 0.001$ ) and metamorphs ( $\chi^2 = 18.939$ ;  $P < 0.001$ ; Table 2).

Tadpoles exposed to cercariae also developed malformations while none of the tadpoles in the control group had any malformations. Malformations observed were mainly in the spine, such as hunched back (kyphosis) and curvature (scoliosis) while edema and skin ulcers were also observed (Figure 2). There were no limb malformations. Edema caused shifting of the centre of gravity and twisting the body axis, which in turn affected the swimming behaviour of tadpoles. Tadpoles affected with edema were seen swimming upside down, not being able to balance their body while swimming. Edemas are often fatal and the tadpoles die soon after they rupture. Malformations were observed in 75.8% of tadpoles at 10 day post-hatching (Table 2). However, some malformations such as those in the tail, disappeared during metamorphosis when the tail resorbed. Hence, only 64.5 % of metamorphs were malformed (Table 2). Relative frequencies of the malformations are given in Table 3. The mean number of malformations was 1.19 per malformed metamorph.

## DISCUSSION

The results show that exposure of pre-limb-bud stage tadpoles (Gosner stages 25 and 26) of *P. cruciger* to monostome cercariae significantly affected its survival and induced malformations. A previous study has shown that tadpoles of the same frog species exposed to the same type of cercariae at limb-bud stage (Gosner stages 27 and 28) produce a much higher percentage of malformations (92%) but do not cause a significant reduction in survival (percentage survival 88%)<sup>8</sup>. This shows that the infection by the same monostome cercariae may have two distinct detrimental effects on *P. cruciger* when exposed at different stages of its development. Infections acquired early in development, the pre-limb-bud stage, may decrease tadpole survivorship while infections acquired by tadpoles at later stages of development, on the other hand, may not affect the survivorship, but can disrupt limb development and induce severe malformations especially when infection coincide with the limb-bud stage of development as reported in a previous study<sup>29</sup>. However, a study that simultaneously examines the effects of this trematode on frogs of varying developmental stages will be necessary to definitively conclude that response to infection is stage dependent.

Another study<sup>20</sup> examined the effects of timing of infection on tadpole survival and limb development in *R. pipiens*. They exposed the tadpoles individually to cercariae of *R. ondatrae* at the pre-limb-bud (Gosner

stages 24 and 25), limb-bud (Gosner stages 27 and 28), and paddle (Gosner stages 31-33) stages of development and monitored through metamorphosis. Their results showed that the effects of infection were stage-specific. Infections acquired at the pre-limb-bud stage resulted in a high mortality rate (up to 97.5%), whereas tadpoles infected at the limb-bud stage displayed a higher malformation rate than the pre-limb-bud stage, and the magnitude of effects increased with the level of exposure to cercariae. They also showed that infections acquired at the paddle stage had no effect on limb development or tadpole survival, and suggested that the timing of *R. ondatrae* infection in relation to the age of tadpole populations in the wild was an important determinant of the degree to which populations are affected by *R. ondatrae*<sup>20</sup>. Holland *et al.*<sup>33</sup> also showed that infection of echinostome cercariae was associated with mortality rates of up to 40% in the green frog, *Rana clamitans* when exposed at an early developmental stage, and none in later developmental stages. They also reported that relatively small differences in development, even within a single Gosner developmental stage, appeared to render larvae invulnerable to infection-related mortality<sup>33</sup>.

The mechanism by which trematode infection leads to tadpole mortality is unknown. Edema following infections leads to compromised renal function or failure which may be responsible for the mortality<sup>20,33</sup>. Edemas were common in infected *P. cruciger* and often the individuals died when they ruptured. It also hampered the swimming behaviour of *P. cruciger*. The mortality rate of tadpoles with edema is expected to be higher in their natural habitat because of the presence of predators. Edema has also been observed in green frogs infected with echinostome cercariae, with a strong concordance between the rates of edema following echinostome exposure and rates of mortality<sup>33</sup>. It has been suggested that mortality may also be due the encystment of cercariae causing hemorrhage and damage to the skin and the tissue surrounding the cloaca and degeneration of the tail musculature<sup>8,16</sup>. This tissue damage covers a fairly large portion of the tadpole's body when they are small in size<sup>16</sup> and hence might be responsible for the high mortality at early developmental stages. In contrast, tadpoles at the limb-bud stage are larger, and tissue damage caused by encystment of cercariae, although present, does not affect a large proportion of the tadpole's body.

Malformations in *P. cruciger* exposed at the pre-limb-bud stage were predominantly axial (69%). However, exposed individuals of the limb-bud stage reported severe limb malformations including amelia, ectromelia, ectrodactyly, apody, hemimely and micromely in *P. cruciger*<sup>29</sup>. As explained in a previous study<sup>20</sup>, the

limb-bud stage marks a period in limb development when the damage of limb-bud tissue can cause intercalation, a process whereby limb-bud cells proliferate and replace lost cells or lost cells in the case of damage associated with cercariae infection. This explains the occurrence of limb malformations in tadpoles exposed to the parasite at the limb-bud stage, as predicted by in another study<sup>7</sup>. Pathogenicity of the parasite and the type of malformation also depends on the site of encystment<sup>26</sup>. Encystment of cercariae is known to occur primarily in the limb-bud regions and along the base of the tail<sup>7</sup>. Most of the described malformations in amphibians due to *R. ondatrae* exposure are associated with limbs, such as ectrodactyly, polydactyly, amelia, polypodi, taumely<sup>5</sup>, polymely, hemimely and ectromely<sup>4</sup>. It has been<sup>34</sup> reported that the inguinal region (next to the base of the tail- “dead-water zone”) of the tadpole is preferentially penetrated by cercariae so that encystations occur in growing limb-buds of the hind limbs. This is because the cercariae cannot be easily removed by the undulating movements of the tadpoles, unlike the cercariae attached to other areas<sup>34</sup>.

A significant elongation of the growth period was common to all the exposed tadpoles of *P. cruciger* and the metamorphs were significantly smaller in size compared to those in the controls. The longer periods of metamorphosis and smaller size of adults can have many adverse impacts on their biology and ecology. Smaller size at metamorphosis makes them less competitive in foraging and other resource utilization, leading to reduced fitness than the larger individuals<sup>35</sup>. Nonetheless, delaying metamorphosis has a crucial impact on amphibian survival, especially for *P. cruciger*, as they often breed in temporary water bodies, particularly in rice fields and human altered habitats<sup>36</sup>, which may dry up before completion of metamorphosis. Rapid colonization, reproduction and growth are important for the survival of organisms living in rapidly changing ecosystems, such as rice fields<sup>36, 37</sup>.

Malformations can lead to indirect mortality in the tadpoles. In most of the parasitic infections, infected animals are susceptible to predators and provide a route to find the definitive host in completion of its parasitic life cycle<sup>38</sup>. Sessions and Ruth<sup>26</sup> explain the importance of indirect mortality resulting from increased predation of frogs and tadpoles with deformed or missing limbs under field conditions. Parasite can complete its life cycle easily when the infection induces malformation at a high rate making the amphibian host an easy prey for the definitive host. The definitive host or the life cycle of this monostome cercaria is unknown. However, it has a high prevalence in freshwater aquatic ecosystems<sup>39</sup> and

is also known to induce malformations in the common toad, *Bufo melanostictus*<sup>40</sup> under laboratory conditions.

Trematode infections could significantly affect amphibian survival in nature if the timing of cercarial shedding coincides with the most vulnerable stages of tadpole development. Emerging diseases are a growing threat to humans and wildlife alike. They can threaten or eliminate sensitive populations or species, thereby contributing to declines in biodiversity<sup>41-43</sup>.

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