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Malformations and mortality in the Asian Common Toad induced by exposure to pleurolophocercous cercariae (Trematoda: Cryptogonimidae)

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ABSTRACT

Malformations and increased mortality due to infection by the digenetic trematode, *Riberioa ondatrae* have been reported for many species of amphibians. Severe malformations have also been reported in the Common Hourglass Tree Frog, *Polypedates cruciger* induced by pleurolophocercous cercariae in Sri Lanka in addition to the changes in the behaviour, development and survival of the host. We exposed pre-limb bud stage tadpoles (Gosner stages 25–26) of the Asian Common Toad, *Duttaphrynus melanostictus* to the same pleurolophocercous type cercariae under laboratory conditions. Molecular and morphological identification showed that these cercariae belonged *Acanthostomum burminis* infecting freshwater snakes as definitive hosts. These cercariae induced malformations (27.8%) and reduced survival to metamorphosis (53.8%). The magnitude of the effects increased with the dose of cercariae. Types of malformations were mainly axial, such as scoliosis and kyphosis. Severe limb malformations such as extra or missing limbs as reported for amphibians exposed to *R. ondatrae* were not observed in the *D. melanostictus*. Same authors reported a higher percentage of malformations previously when *P. cruciger* was exposed to the cercariae *A. burminis* compared to *D. melanostictus*. However, tadpoles of *D. melanostictus*, which are smaller compared to those of *P. cruciger*, experienced higher mortality than *P. cruciger* tadpoles. Trematode induced malformations and mortality in amphibians are highly variable and depend on multiple factors such as host species differences such as resistance to infection and tolerance, life-history characteristics such as size at metamorphosis and length of the metamorphosis period, and other factors such as size of the amphibian at the time of trematode exposure.

1. Introduction

Many species of digenetic trematodes use amphibians as an intermediate host. Among these, *Ribeiroia ondatrae* have been reported to cause limb malformations among amphibian populations across the globe [1–5]. Laboratory exposure to *R. ondatrae* cercariae also causes high levels of malformations and mortality in frogs, toads and salamanders confirming the association between parasite infections and malformations [2,6–8]. These malformations occur primarily in the hind limbs, including missing limbs or limb parts as well as malformed and extra limbs. Frequency of malformations varies among different amphibian species exposed to the same parasite [1,2,9]. Parasites can alter the morphology or/and behaviour of their intermediate hosts in ways that modify the risk of predation by a definitive host, thereby facilitating completion of its life cycle [10]. Limb malformations caused by metacercarial 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 [1,7,10,11].

The influence of trematode infection on survival and malformations of amphibians appears to be both parasite and host species specific. Furthermore, as described by Raberg et al. [12] consequences of parasitic infection may depend on the ability of host to limit the parasitic burdens (resistance to disease) and the ability to limit the damage caused by the parasite (tolerance). Moreover, these differences in host-response to pathogens are related to their life-history characteristics [13]. These authors pointed out that the species which develop quickly and metamorphosed smaller are particularly more prone to infections. Fast living species appear to invest less for defense in concern of growth and

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reproduction while slow living species appear to spend more on defense [13]. Life-history characteristics such as higher growth rate, smaller size at metamorphosis and shorter lifespan were known to report higher parasite (*R. ondatrae*) load and pathology compared to slow-growing species that live longer [13]. However, early growth characteristics such as length of the metamorphosis period and size at metamorphosis are more important in determination of host defense for the larval amphibians which expose to aquatic larvae of trematodes [13]. This is particularly important for anuran species such as *D. melanostictus* which develops quickly in water but still achieving smaller body sizes even though they attain large body sizes in the adult stage.

Recent reports of malformed frogs from a nature reserve in Sri Lanka [14] led to a laboratory study [15] where tadpoles of the Common Hourglass Tree Frog *Polypedates cruciger* were exposed to a pleurolophocercous type of cercariae following a protocol similar to Johnson et al. [6,2]. High frequencies of malformations (up to 92%) including limb malformations such as amelia, ectromelia, ectrodactyly, and also axial malformations, such as kyphosis and scoliosis have been reported in *P. cruciger* [15]. However, in a later study when the same species of tadpoles were exposed at an earlier, pre-limb bud stage (Gosner stages 25 and 26; [16]) they experienced higher mortality and fewer malformations than when exposed at the limb bud stage (Gosner stage 27; [17]). Cercariae-induced malformations and mortality in amphibians are highly variable and depend on multiple factors such as resistance to infection, tolerance and life-history characteristics of the host, etc. In this study we identified this cercaria and tested the hypothesis that cercariae of *Acanthostomum burmini* induce malformations in the Common Asian toad, *Duttaphrynus melanostictus* which is a very common toad species with an island wide distribution associated with human altered habitats. It is known to have expanded its natural ranges and established a higher relative dominance following habitat disturbances. It is also known to be less sensitive to the perturbations in the environment and therefore is an ideal amphibian species to study its susceptibility to infection. We also investigate how resistance, tolerance, and rate of malformations differed between *D. melanostictus* and *P. cruciger* which differ in both taxonomy and life history characteristics.

2. Materials and methods

2.1. Test animals

Eggs of *D. melanostictus* were collected from small ponds and pools in the Peradeniya University Park (7°15′15′′N 80°35′48′′E/7.25417°N 80.59667°E). Eggs were allowed to hatch under laboratory conditions in dechlorinated tap water at room temperature. Tadpoles were fed with fish pellets twice a day (10% body mass per day). The debris and feces collected at the bottom of the tank were siphoned out and water levels were replenished daily. Water in the tanks was replaced completely once a week. Daytime temperature varied between 27 and 31 °C and the tanks were kept under a natural photoperiod of approximately 12:12 h.

2.2. Collection of cercariae from snails

Pleurolophocercous cercariae released from the freshwater snail species *Thiara scabra* (Family: Thiaridae) were used in the study. The snails were collected from muddy bottoms of Mahaweli river (7°15′15″N 80°35′48″E/7.25417°N 80.59667°E) and were kept in plastic vials containing 10–15 ml of water to receive sunlight, inducing cercarial shedding. The infected snails with pleurolophocercous cercariae (~80% of *T. scabra*) were separated and the cercariae were identified morphologically [18,19] by making slide mounts using Gilson's fixative and Grenacher's Borax Carmine alcoholic stain. Out of four types of cercariae released by *T. scabra* (gymnocephalous 13%, gymnophallus 4%, furcocercous 9% and pleurolophocercous 75%), only the pleurolophocercous cercariae type which were identified as *Acanthostomum* sp. were used in the study. These positive snails were kept in separate vials to receive a continuous supply of pleurolophocercous cercariae. These cercariae were used to infect the pre-limb bud stage tadpoles (stages 25–26; [16]) of *D. melanostictus* within two hours of releasing from the snail host.

2.3. Molecular identification of cercariae

Of the nine species of cryptogonimids that infect snakes, six species from the genus *Acanthostomum* have been described from the region [20–22]. Cercariae from a single snail were collected and fixed in 95% ethanol. Genomic DNA for comparative molecular analysis was isolated according to Tkach and Pawlowski [23]. DNA fragments of approximately 1350 base pairs at the 5′ end of the 28S gene (including variable domains D1–D3) were amplified by PCR on an Eppendorf Master Gradient thermal cycler using forward primer digl2 (5′-AAGCATATCACTAAGCGG-3′) and reverse primer 1500R (5′-GCTATCCTGAGGGAAACTTCG-3′). PCR reactions were performed in a total volume of 25 μl using PuRe Taq Ready-To-Go™ PCR Beads (GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, HP7 9NA, England), 1 μl of genomic DNA extract and 10 mM of each PCR primer. The thermocycling profile was as follows: initial denaturation for 5 min at 95 °C; 40 cycles of 30 s at 95 °C, 30 s at 53 ° C, 2 min at 72 °C; and final extension for 7 min at 72 °C. PCR products were purified using Qiagen Qiaquick™ (Valencia, California) columns, and cycle-sequenced directly using ABI BigDye™ (Foster City, California) chemistry, alcohol-precipitated, and run on an ABI Prism 3100™ automated capillary sequencer. PCR primers and four internal primers were used in sequencing reactions. Internal forward primers: 300F (5′-CAAGTACCGTGAGGGAAAGTTG-3′), 900F (5′-CCGTCTTGAAACACGGACCAAG-3′); internal reverse primers: 300R (5′-CAACTTTCCCTCACGGTACTTG-3′), ECD2 (5′-CTTGGTCCGTGTTTCAAGACGGG-3′). For comparative purposes, we used a sequence of the same DNA region obtained from *Acanthostomum burminis* [20,21] found in the snake *Xenochrophis piscator* Schneider, Thailand. The same DNA isolation, PCR and sequencing protocols were used in the latter case as in the processing of the cercariae from Sri Lanka. Contiguous sequences were assembled using Sequencher™ (GeneCodes Corp., ver. 4.1.4), and submitted to GenBank under accession numbers KC489791 (*Acanthostomum burminis*) and KC489792 (*Acanthostomum* sp.). For pairwise comparisons, sequences obtained from cercariae were aligned together with sequences of multiple taxa of opisthorchiid and cryptogonimid digeneans using Clustal W as implemented in the BioEdit program, version 7.0.1 [24]. GenBank BLAST tool as well as local BLAST tool as implemented in the BioEdit program, version 7.0.1 [24] was used to compare the sequences of cercariae with sequences currently available in the GenBank and an extensive collection of yet unpublished sequences available at the laboratory of one of the co-authors (VT).

2.4. Exposure of tadpoles to cercariae

Live cercariae were counted using a dissecting microscope. Four different doses of cercariae (control = 0, low = 16, medium = 32, high = 48) were used as described in Johnson et al. [2]. Each tadpole (5 days post-hatch, Gosner stages 25–26) was placed in a separate specimen cup containing 15–20 ml of dechlorinated tap water and cercariae were released in the cup allowing them to penetrate the tadpole body. The penetration was observed under a dissecting microscope and the containers were examined every half hour to ensure that no free swimming cercariae remained. In all the experiments, exposure was carried out over four consecutive days, with one-fourth the dose being given per day per tadpole. In this manner, 20 tadpoles from a clutch were exposed to each dose, and a total of 320 tadpoles belonging to four separate clutches were tested in the study.