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Single-dose Metronidazole Clears *Opalina* sp. from Juvenile *Bufo woodhousii*

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ABSTRACT: Protozoans of the family Opalinidae are intestinal commensals in amphibians. To test the hypothesis that these organisms are susceptible to the antiprotozoal antibiotic metronidazole, we randomly assigned 60 juvenile Woodhouse's toads (*Bufo woodhousii*) to receive a single oral dose of metronidazole or water. In pilot trials, the prevalence of opalinids in untreated members of this population was over 70%. One-third of the study population was dissected at each of 3 time points: 18 hr, 1 wk, and 2 wk post-treatment. An examiner blinded to the toad's treatment history determined the presence or absence of opalinids using a dissecting microscope. Opalinids were found in 3/10 toads in the treatment group and 9/10 in the control group after 18 hr ($P < 0.02$), in none of the treatment group and 8/10 in the control group after 1 wk ($P < 0.001$), and in none of the treatment group and 10/10 in the control group after 2 wk ($P < 0.0001$). These results suggest that a single-dose of metronidazole quickly and reliably clears opalinids from juvenile Woodhouse's toads with no evidence of short-term recurrence. The treatment was well tolerated, with no apparent morbidity and no mortality in either group. Future exploration of opalinid-related host fitness consequences may be facilitated by this simple method of developing a protozoan-free host population.

Organisms of the genus *Opalina* are commensal protozoans of reptiles, amphibians, fish (marine and fresh water), mollusks, and termites; however, they are most commonly found in anurans and have a wide geographic distribution (Metcalf, 1929; Sandon, 1976). Opalinids are multinucleated, mouth-less, and covered with rows of flagella (Wenrich, 1935; Mignot, 1994; Mitchell, 2007). Opalinids were first discovered by Leeuwenhoek in 1683; approximately 200 species of opalines comprising 5 genera are currently recognized (Delvinquier and Patterson, 1993; *Cepedea* (Metcalf, 1920), *Opalina* (Purkinje and Valentin, 1835), *Protoopalina* (Metcalf, 1918), *Protozelleriella* (Delvinquier et al., 1991), and *Zelleriella* (Metcalf, 1920)). Most hosts harbor 2–5 different species of these protozoans; however, specific combinations are more prevalent (Nathan and James, 1972; Wilbert and Schmeier, 1982; Schorr et al., 1990). The opaline protozoans generally occur in the large intestine and cloaca of their hosts (Kudo, 1922; Wenrich, 1935; Schorr et al., 1990). Amphibians are known to host a wide variety of other micro- and macroparasites in addition to opalinids and have been model systems for field and laboratory research in the biomedical, teaching, animal behavior, life history, toxicology, physiology, evolutionary, and reproductive fields (Goater and Goater, 2001; Mitchell, 2007). In amphibians infection occurs during the tadpole stage of development. In their aquatic environment tadpoles ingest opaline cysts released into the water with the feces from infected adults or other tadpoles (Brumpt, 1915; Metcalf, 1928, 1940; Mofty and Smyth, 1960; Delvinquier and Freeland, 1988). The Woodhouse's toad (*Bufo woodhousii*) from North America is known to harbor many different types of parasites (Goldberg et al., 1996; Bolek and Janovy, 2007). Individuals from our study area were most commonly infected with protozoans (*Opalina* and *Nyctotherus cordiformis*) and cestodes (*Distoichometra bufonis*) (D. Nickol and D. Tufts, pers. obs.; Hardin and Janovy, 1988).

Although opalinids are usually described as commensal protozoans that do not harm the host, some studies suggest that high infection intensity

may interfere with normal host behavior, growth, and development (Hegner, 1923; Nathan and James, 1972). Little is known about the role opalinids play within fish hosts, but Foissner and colleagues (1979) showed increased mortality of infected *Symphysodon aequifasciata* individuals. Additionally, under certain experimental conditions, some protozoans may compete with their hosts for various resources (Nathan and James, 1972). For example, Hegner (1923) found that tadpoles heavily infected with a euglenoid flagellate did not reach the same body size as non-infected tadpoles, and heavily infected tadpoles never completed metamorphosis, implying that there may be some type of interspecific competition for food or resources between the anuran host and the protozoan, especially in malnourished individuals. In settings where these types of interactions may confound experiments performed with infected individuals, a parasite-free population would be beneficial (Kessel, 1930; Cairns, 1953).

Metronidazole (Flagyl®) is a commercially available antibiotic used to eliminate anaerobic bacteria and protozoans from human and animal hosts. We hypothesized that administration of metronidazole to an amphibian host would result in the clearance of its opalinid population. The objectives of this study were to (1) determine if metronidazole could clear all opalinid protozoans from *B. woodhousii*, (2) determine the approximate amount of time needed for clearance, and (3) confirm the short-term durability of treatment. Although metronidazole has been used to treat a variety of infections in livestock, companion animals, and captive reptiles and amphibians, to our knowledge this is the first evaluation of its effectiveness against opalinids.

In July 2010 we collected 60 juvenile *B. woodhousii* from the North Platte River near Paxton, Nebraska (41°10'52"N, 101°21'56"W). Toads were immediately transported to the laboratory in 5-gal plastic buckets and randomized by coin flip into treatment and control groups of 30 individuals each. Pilot studies supported the effectiveness of a single, 10-mg dose of metronidazole. We prepared a suspension by finely crushing two 500-mg tablets of metronidazole (USP) and suspended the powder in 40 ml of bottled spring water. Each toad in the treatment group received 400 µl of the suspension orally by micropipette for an approximate dose of 10 mg of metronidazole. The flask containing the suspension was stirred prior to each dosing because of the poor water solubility of the antibiotic. We attempted to inject the suspension orally by micropipette into each toad's posterior oropharynx to minimize the possibility of the suspension being regurgitated, but some variability in dosing almost certainly occurred. Toads in the control group received an equivalent oral volume of spring water by micropipette.

Throughout the experiment, all toads were housed in 10-gal aquaria lined with coconut fiber substrate (Zoo Med Eco Earth, San Luis Obispo, California) and covered by a mesh screen. Treatment and control groups were housed separately. Each aquarium housed 10 toads, included rock and plant cover, and a clean, standard-size glass petri dish (100 mm × 15 mm) filled with bottled spring water each day. The aquaria were warmed during the day by incandescent light from a desk lamp. Ambient room temperatures ranged from 22 to 24 C. The toads were fed daily with a variety of commercially raised crickets (Fluker Farms, Port Allen, Louisiana; Ghann's, Augusta, Georgia; Petco, San Diego, California).

One-third of the study population was dissected at each of 3 time points: 18 hr, 1 wk, and 2 wk post-treatment. At each time point, 10 treatment and 10 control toads were selected in random order by coin flip. After selection each toad was passed to the primary author, who remained

unaware of its treatment status. After the toad's length was measured it was pithed, the abdomen was opened with scissors, and the gastrointestinal tract removed en bloc from just proximal to the stomach to just proximal to the anus. This section was subjected to blunt dissection with forceps under the dissecting microscope. A small amount of water was added to the dissection dish, and the presence or absence of opalinids was determined by microscopic examination. Incidental notice was also made of the presence or absence of *Nyctotherus cordiformis* and adults or proglottids of the cestode *Distoichometra bufonis* (Dickey, 1921). The findings were relayed verbally to the investigator responsible for selecting the toads who recorded the body length, dissection results, and treatment status for each toad.

We used Fisher's exact test (2-tailed) to calculate *P*-values for the difference in opalinid prevalence (proportion of the population infected) between treatment and control groups at each time point. We used a Student's *t*-test (unpaired, 2-tailed) to calculate the *P*-value for the difference in toad body length between the groups. Treatment efficacy was calculated as:

$$\frac{(\text{prevalence in controls} - \text{prevalence in treated})}{\text{prevalence in controls}}$$

The number needed to treat (NNT) to clear one toad of opalinids was calculated as:

$$\frac{1}{(\text{prevalence in controls} - \text{prevalence in treated})}$$

All toads in both study groups appeared healthy throughout the study period, and there were no deaths prior to dissection. Average toad length at the time of dissection was 3.16 cm in the treatment group (2.4–4.2 cm) and 3.15 cm in the control group (2.4–4.0 cm) ($df = 58$, $P = 0.95$). Opalinids were present in 3/10 toads in the treatment group and 9/10 in the control group after 18 hr ($P < 0.02$), in none of the treatment group and 8/10 in the control group after 1 wk ($P < 0.001$), and in none of the treatment group and 10/10 in the control group after 2 wk ($P < 0.0001$). The NNT was 1.7 at 18 hr, 1.3 at 1 wk, and 1.0 at 2 wk (Table I). Unless otherwise noted, when opalinids were present they were motile and too numerous to count.

Significant differences in opalinid prevalence between the treatment and control groups were present at all time points measured. There was no significant difference in average toad size between the treatment and control groups at the time of dissection. All toads in the trial survived to the time of dissection and appeared healthy. *Nyctotherus cordiformis* and *D. bufonis* were incidentally noted in both treatment and control animals.

Our findings indicate that treating juvenile *B. woodhousii* with a single oral dose of metronidazole results in rapid, reliable, and well-tolerated clearance of opalinids from the gastrointestinal tract. Anecdotal observations at the time of dissection suggested that the prevalence and intensity of *Nyctotherus cordiformis* and *D. bufonis* were not obviously decreased in the treated toads as compared to the control population. Future research could assess the effectiveness of agents other than metronidazole in the clearance of these organisms.

Limitations of our study include the relatively short 2-wk follow-up interval after treatment, which might raise questions about the durability of the observed effect, and the remaining uncertainty about optimal dosing and minimum time to opalinid clearance. However, our results imply that 2 wk is sufficient ($P < 0.0001$) for complete *Opalina* removal in *B. woodhousii*. Our shortest post-treatment time interval (18 hr) produced a 65% efficacy, suggesting that metronidazole is quite effective at removing opalinids quickly. Other unresolved questions may include the generalizability of our results to other species of opalinids and to other hosts, as well as the potential effects of the antibiotic on other parasite populations within the host and on the host's overall fitness. Although we did not include other anurans or reptiles in this study, this method might prove effective for other laboratory or captive animals.

TABLE I. Prevalence of opalinids in *Bufo woodhousii*, treatment efficacy, and number needed to treat (NNT) at each study time point.

Time	Prevalence			Efficacy	NNT
	Treatment	Control	<i>P</i>		
18 hr	3/10*	9/10	<0.02	67%	1.7
1 wk	0/10	8/10	<0.001	100%	1.3
2 wk	0/10	10/10	<0.0001	100%	1.0

* Two of these 3 toads had a small number (<10) of immobile, dead-appearing opalinids.

The treatment and control solutions were well tolerated by our study population; however, some mortality had been observed during earlier pilot trials. The observation that deaths occurred no sooner than 5–7 days after treatment, and were evenly distributed between the treatment and control groups, led us to suspect that some factor other than antibiotic toxicity was most likely responsible (i.e., water quality, temperature, enclosure environment, etc.). This conclusion was reinforced by the lack of mortality once modifications were made to the study animal habitat, including the change from plastic terraria to larger glass aquaria for the formal trial. Because no changes were made from pilot studies to the dose or delivery of the treatment and control solutions, we concluded that earlier observed mortality was unlikely to be a result of the study medication.

We were initially concerned that the poor water solubility of the antibiotic and the challenges of oral administration would result in a lack of dosing precision that might compromise treatment efficacy. Our results suggest that, while some variability in dosing almost certainly occurred, the overall effectiveness of the antibiotic was sufficient to overcome this variation and led to reliable clearance of the target organism.

The effectiveness of our intervention reached 100% by 1 wk and remained 100% at 2 wk. The effectiveness at 18 hr would have been higher (89%) had we included the 2 treated toads found to have only a few, immobile opalinids into the "no opalinids" group. The high opaline prevalence in our study population, combined with the high efficacy of our treatment, resulted in a low NNT of <2 toads at all time points. Multiplying this low NNT by the low cost of metronidazole per treated toad (approximately US\$0.02) suggests that creation of sizable opalinid-free research populations should be cost-effective.

The results of our study suggest several areas for further research. It would be interesting to study this method of protozoan clearance in other host species that harbor opalinids or other protozoan species. Within *B. woodhousii*, the effect of metronidazole on *Nyctotherus cordiformis* and *D. bufonis* remains unclear, as our anecdotal observations did not suggest obvious reductions in prevalence. If these organisms are not susceptible to metronidazole but are susceptible to other agents, selective manipulation to the various protozoan and cestode populations within the host might be possible. The relative impact of each parasite on overall host fitness might then be clarified.

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