

Use of praziquantel for treatment of flatworm parasites in centrarchid fish

by

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The student author and the program of study committee are solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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ABBREVIATIONS

CTG	CellTiter-Glo
AMDUCA	Animal Medicinal Drug Use Clarification Act
FDA	Food and Drug Administration
PI	Propidium Iodide
IACUC	Institutional Animal Care and Use Committee

DEDICATION

I would like to thank my mentor, Dr. Matthew Brewer and my program of study committee: Dr. Douglas Jones and Dr. Adam Krull for their guidance. I would like to thank current and past lab members especially Dr. Jeba Jesudoss Chelladurai and Kylie Thompson for their support throughout my time at Iowa State. Finally I would like to thank my parents Steve and Laurie Bader, my brother Steven, and my girlfriend Isabelle Gerbatsch for their love and support throughout my time at Iowa State. Without you this would have been a much rougher path.

CHAPTER 1: GENERAL INTRODUCTION

Introduction

Platyhelminth parasites are common parasites of fish, both in the wild and in aquaculture systems. These parasites include cestodes along with monogenean and digenean trematodes. Praziquantel is a drug with anti-flatworm activity and is widely used in human and veterinary medicine. At this time praziquantel is not approved for the use in fish, but use may be justified under the guidance of the Animal Medicinal Drug Use Clarification Act (AMDUCA). While some studies have investigated the use of praziquantel in aquaculture, there are still several gaps in the knowledge needed for the use of praziquantel in fish species.

Thesis organization

This thesis investigates the use of praziquantel in fish for the elimination of platyhelminth parasites. Chapter 2 reviews the literature relevant to the current knowledge base of the usage of praziquantel in aquaculture. Chapter 3 examines biochemical assays for determining death of metacercariae as an alternative to current methods that rely on movement and morphology. Chapter 4 assesses the use of injectable praziquantel for killing of *Posthodiplostomum minimum* metacercariae in bluegill sunfish (*Lepomis macrochirus*). Chapter 5 describes infestation of black crappies (*Pomoxis nigromaculatus*) with the monogenean *Cleidodiscus* and subsequent successful treatment with topical praziquantel. The final chapter summarizes the knowledge gaps filled by this work and identifies needs for future research in the field.

CHAPTER 2: USE OF PRAZIQUANTEL TO CONTROL PLATYHELMINTH PARASITES OF FISH

A paper to be submitted

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Abstract

Fish are common definitive and intermediate hosts for a variety of parasitic flatworms. In unstressed wild populations, parasitic infections often go unnoticed and are perceived to represent a lesser threat to fish health. In contrast, platyhelminth parasitism of captive fish often results in decreased weight gain and increased mortality which often necessitates chemotherapeutic treatment. The presence of platyhelminth parasites in fish tissues is not only unappealing but in some cases also represents a threat to human health. In veterinary medicine, one of the most commonly used agents with anti-flatworm activity is praziquantel, yet, no praziquantel products are labeled for use in fish in the United States. Veterinarians may use praziquantel preparations approved for other vertebrate species under the Animal Medicinal Drug Use Clarification Act (AMDUCA). However, such extra label use should be informed by scientific evidence including efficacy and tissue residue studies. Herein, we review studies testing the efficacy of praziquantel for treatment of platyhelminths along with an assessment of routes of administration, pharmacokinetics, and toxicity information.

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Introduction

Parasitic flatworms of the phylum Platyhelminthes represent a diverse spectrum of organisms that infect (live within) and infest (live upon) vertebrates. The monogeneans are propagated by a direct life cycle whereby adult helminths live on the definitive host, reproduce sexually, and shed ova or larvae which enter the environment and eventually infect a new definitive host. In contrast, cestodes and trematodes employ indirect life cycles whereby an intermediate host is required for larval development and/or asexual multiplication before returning to the definitive host. Fish serve as both definitive and intermediate hosts for a spectrum of platyhelminth parasites.

There is a breadth of platyhelminth parasite taxa that infect fish and the consequences of infection vary according to the location of the parasite in the host. Similarly, the need to intervene and treat infected or infested animals depends on the intensity of parasite load. In production aquaculture, it becomes necessary to treat fish to prevent economic losses. In other cases, fish may contain larval cestodes or trematodes that are infectious for humans, thereby requiring treatment.

Praziquantel is a synthetic drug that was discovered by Bayer in the 1970s (Adam et al., 2005). Remarkably, praziquantel is effective against a broad range of cestodes (tapeworms) and trematodes (flukes) and is a mainstay of anti-platyhelminth parasite therapy in both human and veterinary medicine. Despite being widely studied and used, the precise mechanism of action of praziquantel remains a subject of investigation. Following exposure to the drug, there is disruption of the worm tegument which is characterized by vacuolization and blebbing (Staudt et al., 1992). This process is thought to be related to binding voltage-gated Ca^{2+} channels and disruption of Ca^{2+} homeostasis via altering membrane permeability

(Doenhoff et al., 2008). There is also evidence that praziquantel-induced damage to the tegument renders the parasite more susceptible to immune-mediated clearance (Ribeiro et al., 2004). These effects are specific to platyhelminths, and praziquantel has a wide margin of safety (Frohberg, 1984).

Currently, the only agents labeled for use against parasites in fish are dilute formalin preparations (U. S. Food and Drug Administration, 2016). These preparations are only labeled for monogeneans (discussed below) but not for cestodes or digenean trematodes. Because there are no drugs labeled for digenean trematode and cestode parasites, veterinarians may justify off-label use of praziquantel within the framework of the Animal Medicinal Drug Use Clarification Act (AMDUCA). In addition, AMDUCA may allow for use of praziquantel in situations where formalin cannot be utilized. For example, formalin cannot be added to certain systems due to damaging effects on biofilters thereby indirectly increasing harmful ammonia levels in the water (Keck and Blanc, 2002). When considering off-label use, it is important to evaluate the data available for the particular host and parasite species being addressed. In this manuscript, we review the body of knowledge regarding the use of praziquantel for treatment of monogeneans, digenean trematodes, and cestodes infecting fish.

Efficacy: monogeneans

Monogeneans are larviparous or oviparous ectoparasites of fish skin and gills. They utilize direct life cycles and can be transferred from host to host through direct or indirect contact. In aquaculture systems, dangerous levels of monogenean parasites may accumulate. Damage caused by feeding monogeneans can lead to tissue damage which often leads to

secondary microbial infections. Monogenean parasitism of gills can cause suffocation leading to death and may induce high levels (>80%) of mortality when high parasite burdens are present (Thoney and Hargis Jr, 1991).

Dilute formalin preparations are labeled only for the monogeneans *Cleidodiscus*, *Dactylogyrus*, and *Gyrodactylus* (U. S. Food and Drug Administration, 2016). Formalin is approved for these monogeneans as a bath treatment since the parasites live externally on the fish. In a variety of situations, formalin may not be a feasible treatment. For example, formalin can cause serious insult to gills, eyes, and skin when mixed improperly. In addition, formalin indirectly reduces dissolved oxygen from the water and contains algicidal and bactericidal activity (Neely, 1963). Therefore, in order to prevent suffocation of fish and ensure biofilter survival, increased aeration and the ability to bypass any biofilter is necessary. The temperature of the water also needs to be considered, as high water temperatures have been shown to produce greater toxicologic effects when formalin toxicity occurs (Piper and Smith, 1973). In addition, extremely cold water environments may lead to increased conversion of formaldehyde into paraformaldehyde which is more toxic to fish (Rucker et al., 1963). In terms of route of administration, formalin is only available for use as a bath treatment which is a disadvantage if there is a large volume of fish or water.

Formalin is not approved for the administration via parenteral or oral routes.

Several studies have tested the efficacy of praziquantel versus various monogenean parasites and these studies are summarized in Table 1. In general, bath and dip treatments are effective for removing monogeneans and this is not surprising due to the location of the parasites on the surface of the fish host. Bath treatments ranging from 2-40 mg/mL have been investigated and these treatments are typically applied for 24-48 hours. Interestingly,

several studies have also revealed that oral praziquantel administration was effective for removing monogeneans. Monogenean trematodes have the ability to detach and survive in the environment for a short period of time before reattaching to a new host (Schmahl and Taraschewski, 1987). This gives them the opportunity to avoid the praziquantel for short periods of time during oral treatments. A bath treatment, however, will ensure that even when parasites detach they remain in a treated environment (Sharp et al., 2004). Given the high success of praziquantel treatment for monogeneans and the potential problems with formalin administration, praziquantel bath treatments pose a viable substitution for fish infected with monogenean parasites. Praziquantel does not target bacteria or viruses, and does not have the potential to damage essential microbes in biofilters. In light of the concerns associated with formalin administration, praziquantel would be a suitable substitute for treatment of monogeneans.

Efficacy: digeneans

Digenean trematodes have diverse and complex indirect life cycles. The most common life cycle follows that eggs are voided from the definitive host, a miracidium hatches and penetrates a snail. Following asexual replication in the snail, cercariae are released; this larval stage then encysts on a second intermediate host or on vegetation prior to being ingested by the definitive host. Fish may serve as definitive hosts for digeneans, but they are also common intermediate hosts for flukes that infect other vertebrate definitive hosts. Larval digenean trematodes present in the tissues of fish are termed metacercariae or metacercarial cysts. In the cases of *Heterophyes*, *Echinostoma*, *Clonorchis*, and others, humans can serve as definitive hosts and become infected following ingestion of

metacercariae in undercooked fish (Chai et al., 2005; Hedegaard Clausen et al., 2012; Li et al., 2013). Many other genera of digenean adults parasitize avian and mammalian hosts that consume fish prey. Therefore, metacercariae can be a threat to human and veterinary health or be unappetizing features of fish fillets. Metacercariae do not multiply within the fish and therefore do not typically represent a direct threat to fish health even when they infect sensitive organs such as the brain, or nervous system (Khalil, 1968). However, infection with large numbers of metacercariae can be clinically significant (Hoffman, 1958). Adult trematodes are relatively non-pathogenic in fish unless present in large numbers. For example, blood flukes can cause considerable damage to gills by obstructing the passage of blood, and can cause anemia leading to production losses (Hoffman et al., 1985) (Evans, 1974).

There are currently no drugs labeled for the treatment of digenean trematodes in fish. However, several studies have assessed the efficacy of praziquantel for treatment of both metacercariae and adult digenean trematodes (Table 2). Oral formulations of praziquantel removed 91-100% of adult *Cardicola* blood flukes. Studies investigating metacercariae of *Clinostomum* and *Diplostomum* range from 68-100% efficacy. Different host fish species may require different doses for treatment of the same parasite. Székely and Molnár (1991), found a dramatic decrease in efficacy of a bath treatment in silver carp as opposed to grass carp parasitized with *Diplostomum spathaceum*.

Interestingly, several studies involving elimination of metacercariae were achieved by bath treatment (Lorio, 1989; Plumb and Rogers, 1990; Szekely and Molnar, 1991). This was somewhat surprising given the intramuscular location of the parasites, and the limited knowledge of pharmacokinetics from bath treatments. The only study assessing

pharmacokinetics of bath treatments found a very low maximum concentration (.49 µg/L) when rockfish were subjected to 100 mg/L praziquantel bath treatment for 4 minutes (Kim et al., 2001).

A challenge with studies involving metacercariae is that metacercarial death is often measured as determined by movement and this may be unreliable, as many encysted metacercariae have limited mobility (Asanji and Williams, 1975). In addition, metacercariae that have died may take extended time to be removed by the host response. Therefore, counting the total number of metacercariae immediately following treatment may not reveal differences in treated and untreated fish. Additional techniques for determining death of metacercariae are needed in order to advance research in this area. One useful method for detecting metacercarial death is propidium iodide staining. Propidium iodide stains cells with compromised membranes. Metacercariae extracted from treated fish can be stained by propidium iodide *ex vivo* (Bader et al., 2017b) and the level of staining is proportional to the dose of praziquantel for *Posthodiplostomum* metacercariae (Bader et al., 2017a).. This method is at least as sensitive as motility scoring and provides a quantitative assessment of metacercarial death. Future studies would benefit from using this quantitative method since it could be used to compare the relative level of praziquantel susceptibility or resistance among different parasite isolates.

Efficacy: cestodes

Cestodes can typically be found in the small intestine of the definitive host. Like digeneans, cestodes require an intermediate host for larval (metacestode) development. Fish may be either the definitive host or an intermediate hosts. For some parasites such as

Diphyllbothrium, fish are the intermediate hosts for cestodes that infect humans. Like digeneans, there are no drugs labeled for use against cestodes in fish. Cestodes do not have a free-living stage so praziquantel treatment represents a viable strategy to maintain control over these parasites.

The majority of the research investigating use of praziquantel to treat cestodes involves fish that are definitive hosts of the invasive Asian tapeworm *Bothriocephalus acheilognathi* (Table 3). Bath treatments were effective for this gastrointestinal parasite, removing 66-100% of adult parasites from the intestinal lumen (Kline et al., 2009; Mitchell and Darwish, 2009; Mitchell, 2004; Pool et al., 1984; Ward, 2007). Adults of *Khawia sinensis*, *Atractolytocestus cestus*, and *Bothriocephalus* sp. were also removed following oral treatment with praziquantel (Pool et al., 1984). These experiments suggest that the praziquantel bath is ingested thereby killing adult cestodes within the lumen of the intestine. It appears that cestodes are susceptible to praziquantel with doses as low as .25 mg/L for 24 hours which was 100% efficacious for *Bothriocephalus acheilognathi* in sunshine bass (Mitchell, 2004). In summary, it appears that adult cestodes can be removed from fish by praziquantel. Going forward, there is a need to determine if praziquantel can be used to remove larval cestodes such as *Diphyllbothrium* from fish tissues.

Route of administration

In terrestrial vertebrates, praziquantel is typically administered orally or parenterally. In aquaculture systems, there are often thousands of individual fish in a single facility and this poses a challenge in terms of administering medication. Potential routes of administration include oral, injectable, and topical (baths and dips) which each have

advantages and disadvantages. Relatively little research has involved head to head comparison of different administration routes for a particular parasite. The host, the environment, and the target parasite should be taken into account when selecting route of administration.

Oral administration of praziquantel is especially desirable when treating platyhelminths that reside in the gastrointestinal tract, such as cestodes. For large populations of fish, the most convenient form of oral treatment is in-feed medication. However, there are several disadvantages to this approach. For example, food may be consumed disproportionately therefore providing individuals with different doses of medication. This is especially problematic since parasitized animals can have a decreased food intake, leading to sub therapeutic doses being administered (Crompton, 1984). Additionally, fish will establish feeding hierarchies which can lead to an uneven distribution of treatment and potential sub-therapeutic dosing of individual fish (Samaee, 2015). Such dosing has the potential to lead to promotion of parasite drug resistance (Shalaby, 2013). It can take extensive time for fish to become acclimated to just artificial feed; it may take several weeks to acclimate fish to such a new diet (Moura et al., 2000). Therefore, it may not be practical to implement in-feed treatment if the fish are not acclimated to a pelleted diet.

Another challenge for feed-based administration of praziquantel is its bitter taste which is known to lead to decreased consumption (Partridge et al., 2014). To combat problems with palatability, preparations consisting of the (R)- enantiomer may be used instead of racemic mixtures since the (S)- enantiomer is responsible the bitter taste, and only has limited anthelmintic properties (Meyer et al., 2009). However this may not be the ultimate solution since the (R)- enantiomer has a stronger smell which decreased feed

consumption at levels of 10 mg/Kg (Partridge et al., 2016). Other methods have been investigated for masking the taste, including mixing with fish oil, krill extracts, sugar or commercial fish attractants (Yamamoto et al., 2011). Another strategy for stimulating consumption is to withhold feed briefly prior to introduction of medicated pellets (Pool et al., 1984). Gastric intubation has also been performed (Tubbs and Tingle, 2006), which is more stressful to the fish but allows for the direct control of the dosing of each fish. Gavage feeding may represent an option when a small number of individuals require treatment.

Although there are challenges associated with in-feed treatment, this route of administration is particularly attractive due to the possibility of treating an entire population of fish without handling individual animals. Unfortunately, there are no formulations of praziquantel available for use in feed. In the research literature, a variety of methods have been employed in order to prepare praziquantel for feeding. For example, praziquantel has been suspended in cooking oil or added to carboxymethyl cellulose sodium (CMC) as a sticking agent for distribution onto commercial feed (Ishimaru et al., 2013; Pool et al., 1984). Alternatively, a praziquantel paste has been prepared and mixed with feed prior to pelleting to create a medicated feed (Kim et al., 2003). Although these methods have been demonstrated experimentally, it is unclear if this strategy could be implemented in a commercial aquaculture facility.

Relatively little research has assessed injectable praziquantel for fish, with only two studies examining the effects on digeneans in channel catfish (Lorio, 1989) and bluegill (Bader et al., 2017b). There has also been a single study on the pharmacokinetics of intravenously injected praziquantel in yellow amberjack (Tubbs and Tingle, 2006). This is probably related to the fact that increased handling of the fish become time intensive and

expensive. Another concern is that increased handling of fish can lead to stress and mortality events (Midtlyng, 1997). Injectable forms of praziquantel are available for other species and this could be attractive in certain situations where only a small number of individuals need to be treated. Parenteral praziquantel may also be desirable for treating tissue-dwelling platyhelminths such as larval digeneans and cestodes.

A third possible route of administration is topical via bath or dip treatment whereby praziquantel is added directly to the water for a defined amount of time. Topical administration is particularly desirable when targeting monogenean ectoparasites. By convention, a bath treatment typically involves a low concentration of drug for an extended period of time whereas a dip utilizes a high concentration of drug for an abbreviated timeframe. Both methods provide a uniform treatment to each fish (Samuelsen and Lunestad, 1996). Stocking density should be taken into account when administering bath treatments. For instance, Mitchel and Darwish (2009) found a significant decrease in efficacy when providing identical bath treatments to Grass Carp at a stocking density of 120 grams of fish per liter instead of 60 grams of fish per liter. Future studies should assess calculation of topical doses on a weight per weight basis in addition to weight per volume of water. The observation that treatments administered in the water are effective for some tissue-dwelling digeneans and cestodes is thought to occur through praziquantel being absorbed mainly through the gills, with small amounts being absorbed through the skin (Kim et al., 2001).

Praziquantel may be administered in oral, injectable, or topical forms and the most appropriate route of administration should be determined in the context of the target species, parasites, and laws regarding extra label administration. While ornamental fish may be

treated at any time, it is important to establish withdrawal periods for each route of administration so that fish intended for human consumption avoid drug residue violations.

Pharmacokinetics and tissue residues

When administering antiparasiticides, it is important to consider tissue distribution and elimination of the proposed treatment. Platyhelminths can occur in a variety of locations and one must ensure that the anthelmintic is reaching the desired target tissue. Following oral administration in fish, praziquantel is distributed throughout the animal, including muscle, kidney, plasma, and liver. Praziquantel is metabolized into *cis*- and *trans*-hydroxypraziquantel (*cis*-4-OH-PZQ and *trans*-4-OH-PZQ) which have some antiparasitic properties (Meister et al., 2014; Tubbs et al., 2008). A proportion of the drug is excreted in the native form while other metabolites are excreted via the kidneys (Björklund and Bylund, 1987). Relatively few studies have addressed the pharmacokinetics of topical praziquantel, however, it appears that the drug is also distributed in the muscle and plasma following administration. There is evidence that praziquantel administered in the water is mainly absorbed through the gills, with small amounts absorbed through the skin before being spread throughout the rest of the body (Kim et al., 2001).

In the case of using praziquantel in fish intended for human food, an understanding of pharmacokinetics is also important for establishment of proper withdrawal times. Currently there is no residue limit specified for praziquantel in fish marketed for food and therefore no detectable quantity of praziquantel would be tolerated. Since there is no recommended treatment regimen for using praziquantel in fish, there has not been consistency in the dosing and methods of administration when testing for tissue residues in fish (Table 4).

The majority of residue studies focusing on oral delivery administered doses ranging from 10-500 mg/kg. There is evidence that the rate of metabolism and prevalence in fish may be dependent on host species and environmental conditions (ex. stocking density/salt concentrations). For instance, for Pacific bluefin tuna that were fed 15 mg/kg praziquantel in treated feed, praziquantel was below detectable levels (.2 µg/g) within 24 hours (Ishimaru et al., 2013) while grass carp that were kept either in brackish water or fresh water, still had detectable levels (.05 µg/ml) of praziquantel at 96 hours post treatment when they were only given 10 mg/kg (Xie et al., 2015). Grass carp that were kept in freshwater also achieved higher praziquantel concentrations in the plasma, muscle, liver and kidney when compared to those treated in brackish water. While many studies did not establish the timepoint for complete praziquantel elimination in hosts, a 400 mg/kg oral treatment given for 3 days to rockfish, which is a much larger treatment needed for most parasites removal was completely eliminated within 168 hours (7 days). This indicates that even large oral treatments need only a short withdrawal period.

Only a single praziquantel dip treatment has been examined for pharmacokinetics. In this study rockfish were placed into a 100 mg/L bath treatment for 4 minutes, plasma and muscle samples were examined for the presence of praziquantel. Maximum concentrations were achieved in the plasma at 12 hours and was undetectable by 96 hours post treatment. Maximum concentrations in the muscle were achieved at 3 hours and was undetectable by 48 hours post treatment.

The pharmacokinetics and tissue residues associated with injectable praziquantel are understudied in fish. There is evidence that an injectable treatment can eliminate digenean trematodes (Bader et al., 2017b; Lorio, 1989), however, no studies have utilized injectable

treatments for monogenean or cestodes parasites. While the assumption is that injectable praziquantel is a rational choice for plathyhelminthes found in tissue, only a single study examined the effects of a 40 mg/kg intravenously administered praziquantel injection in yellowtail amberjack (Tubbs and Tingle, 2006). In this study, praziquantel was still present in the skin and plasma after 24 hours.

Future residue studies should examine maximal doses that have been shown to be effective against parasites. Treatment for monogeneans may require bath treatments up to 10 mg/L for 48 hours (Forwood et al., 2013a), while injectable and oral treatments require up to 25 mg/kg and 200 mg/kg single dose, respectively (Kim et al., 1998; Lorio, 1989). It appears that praziquantel is quickly eliminated from the host tissues. When rockfish were administered 400 mg/kg orally for 3 days, there were no detectable limits of praziquantel after 168 hours (7 days) (Kim et al., 2003). However, studies examining individual species of fish should be consulted when determining withdrawal times.

Toxicity

In general, praziquantel has a wide margin of safety with relatively few side effects. A few studies have demonstrated adverse physical reactions to treatment in fish. For example, silver carp exposed to a 100 mg/L bath treatment for 18 minutes swam upside-down and erratically. These fish were then moved to fresh water and were able to recover after 30 minutes without any lasting detrimental effects (Szekely and Molnar, 1991). Bath treatments have varying levels of toxicity based on the species being treated. When golden shiners and grass carp were treated with high doses of praziquantel, non-lethal doses were established at 45 mg/L and 60 mg/L respectively for a 24 hour period (Mitchell and Hobbs,

2007). There have not been any toxic effects reported in fish that have been fed or intubated with large doses of praziquantel. The largest dose that has been studied was a single 500 mg/kg dose administered to rainbow trout orally and in this case no adverse effects were observed (Björklund and Bylund, 1987). Efficacy studies have revealed that the highest doses needed to kill relatively resilient monogeneans was 40 mg/L, but this concentration can be reduced by extending the treatment time (Forwood et al., 2013a; Forwood et al., 2013b). Therefore, it appears that the doses relevant to treatment are much lower than those needed to cause side effects. It is unknown if there are any fish species susceptible to adverse reactions caused by praziquantel.

Environmental concerns

There have been relatively few studies addressing the fate of praziquantel in the environment (Bártíková et al., 2016). However, there is a general concern that anthelmintics present in small quantities can lead to resistance, (Chaiworaporn et al., 2005; Köhler, 2001; Yoshimura and Endoh, 2005) or destruction of other organisms in aquatic environments (Morley, 2009). The main concern of environmental praziquantel contamination is unintentional killing of free-living non-parasitic flatworms. Free-living flatworms have also shown to indirectly influence macro- and meiofauna (Majdi et al., 2013), potentially changing foodwebs if removed. When *Dugesia japonica* was subjected to 70 µM praziquantel for 48 hours it was discovered that praziquantel caused bipolar regeneration, creating a second head on the opposite end of the body when damaged. Even though orally treated fish can excrete active praziquantel (Björklund and Bylund, 1987), it is unclear if concentrations relevant to environmental disturbance are produced. While the environmental

concentrations are likely dependent upon dose and stocking density, there is no evidence that high concentrations of praziquantel is being discharged into the environment after a treatment regimen.

A limited number of trials have been performed to observe the effects of praziquantel on arthropods and plants. Dung beetles exposed to praziquantel may experience changes in coloration, however, the LD50 for this species is greater than 1000 mg/kg (Hempel et al., 2006). Interestingly, doses as low as 25 mg/kg are adequate for eliminating metacestodes inside dung beetles (Dhakal et al., 2015). The common reed (*Phragmites australis*) has not only been shown to have no adverse reactions to all doses studied, but has also shown to have the ability to metabolize praziquantel, providing a means of phytoremediation (Marsik et al., 2016).

Selection for drug resistant parasites is a concern, however, there are relatively few reports of praziquantel resistant parasites. In the case of *Schistosoma*, there have been reports of praziquantel resistance associated with mass drug administration in human patients (Ismail et al., 1999; King et al., 2000). In aquaculture systems, disposal of praziquantel could be problematic if resistance can arise as a result of exposure to sub-therapeutic doses (Köhler, 2001). Residual praziquantel may be present in water used for dip or bath treatments, and fish given oral or parenteral praziquantel may excrete the drug into the water for up to 36 hours post treatment (Björklund and Bylund, 1987). Operators may be able to use UV photocatalytic degradation to eliminate praziquantel, however, it is not clear if this is necessary or practical. (Havlíková et al., 2016). In marine environments, bacteria may metabolize praziquantel thereby eliminating it from a system (Thomas et al., 2016). Going

forward, there is a need to investigate the fate of praziquantel in aquaculture systems in order to more accurately assess concerns about resistance or environmental contamination.

Conclusion

Culture of fish for food and ornamental purposes continues to grow in popularity and it is important for veterinarians to be involved in the medical care of these species. Many therapeutic techniques and treatments developed and used in mammals require validation before they can be considered safe and effective in aquatic species.

Fish, like other vertebrates, suffer production issues of health, welfare, and feed efficiency when infected with helminth parasites. Both larval and adult forms of cestodes and trematodes parasitize fish and in some instances pose a threat to human health. However, relatively few therapeutic options are available for removal of platyhelminths in fish. Praziquantel is an anthelmintic effective for treating flatworm infections and has broad applications in human and veterinary medicine. In general, praziquantel is effective for treatment of cestodes and trematodes in fish. Both ectoparasitic monogeneans and endoparasites infecting fish are effectively removed with treatment, but the amount of time necessary for total elimination of praziquantel from the host is unknown for most treatment regimens. Praziquantel has shown to have very low levels of toxicity in fish, requiring a 100 mg/L bath treatment or oral doses above 500 mg/kg before negative effects are noted. Although disposal of praziquantel does not appear to pose dangerous environmental concerns, additional studies are needed to determine preferred methods for disposal of water containing the drug. Future studies should address the elimination of larval cestodes and trematodes, especially in the context of human health. Additionally there are still multiple

treatment methods that have not been attempted. There is currently no data available on the effects of injectable praziquantel on monogeneans, dip or injectable treatments on cestodes, or the pharmacokinetics of bath treatments in the host fish (Table 5).

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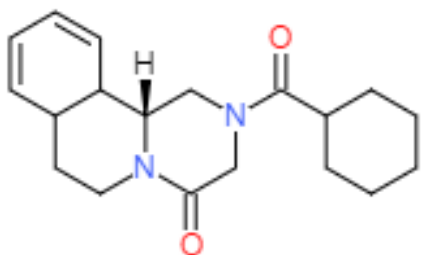


Figure 2.1: Structure of (R)-praziquantel, the enantiomeric active form of praziquantel

Table 2.1: Effects of praziquantel treatments on adult monogeneans. When multiple treatments reached 100% efficacy against the given monogenean only the lowest dose is reported.

Parasite	Host	Route	Dose	Efficacy	Source
<i>Ancylo discoides vistulensis</i>	European catfish	Bath	10 mg/L for 5 hours	15% 3-4 days post treatment	(Szekely and Molnar, 1990)
<i>Benedenia seriolae</i>	Yellowtail amberjack	Feed	50 mg/kg for 6 days	58.1	(Williams et al., 2007)
<i>Benedenia. seriolae</i>	Yellowtail amberjack	Bath	2.5 mg/L for 24 hours	>99%	(Sharp et al., 2004)
<i>Benedeniella posterocolpa</i>	Cownose rays	Dip	20 mg/L for 90 minutes	100%	(Thoney, 1990)
<i>Cleidodiscus sp.</i>	Black Crappie	Bath	1.5 mg/L for 24 hours	>80%	(Bader et al., 2017)
<i>Clemacotyle australis</i>	White-spotted eagle rays	Bath	25 mg/L for 45 minutes	100%	(Janse and Borgsteede, 2003)
<i>Dactylogyrus intermedius</i>	Goldfish	Bath	13.5 mg/L for 48 hours	93% 6 days post treatment	(Zhang et al., 2013)
<i>Dactylogyrus sp.</i>	Guppy	Bath	3 mg/L for 24 hours	100%	(Fridman et al., 2014)
<i>Gyrodactylus aculeati</i>	Stickleback	Bath	10 mg/L for 16 hours	100%	(Schmahl and Taraschewski, 1987)
<i>Gyrodactylus aculeati</i>	Stickleback	Bath	20 mg/L for 2 hours	100%	(Schmahl and Taraschewski, 1987)

Table 2.1 continued

<i>Gyrodactylus sp.</i>	Rainbow trout	Dip	10 mg/ml for 3 hours	97.7%	(Santamarina et al., 1991)
<i>Gyrodactylus sp.</i>	Rainbow trout	Dip	100 mg/ml for 60 minutes	100%	(Santamarina et al., 1991)
<i>Gyrodactylus turnbulli</i>	Guppy	Bath	3 mg/L for 24 hours	71.1%	(Fridman et al., 2014)
<i>Haliotrema abaddon</i>	West Australian dhufish	Bath	2 mg/L for 30 hours	~100%	(Fajer-Ávila et al., 2007)
<i>Heterobothrium okamotoi</i>	Takifugu rubripes	Feed	40 mg/kg for 20 days	100%	(Hirazawa et al., 2000)
<i>Lepidotrema bidyana</i>	Silver perch	Bath	10 mg/L for 48 hours	99%	(Forwood et al., 2013a)
<i>Lepidotrema bidyana</i>	Silver perch	Bath	40 mg/L for 24 hours	77%	(Forwood et al., 2013b)
<i>Lepidotrema bidyana</i>	Silver Perch	Feed	75 mg/kg daily for 6 days	79%	(Forwood et al., 2013a)
<i>Microcotyle sebastis</i>	Rockfish	Feed	100 mg/kg single treatment	33.3%	(Kim et al., 1998)
<i>Microcotyle sebastis</i>	Rockfish	Feed	200 mg/kg single treatment	100%	(Kim et al., 1998)
<i>Neobenedenia girellae</i>	Chub mackerel	Feed	150 mg/kg for 3 days	>80%	(Yamamoto et al., 2011)
<i>Zeuxapta seriolae</i>	Yellowtail amberjack	Bath	2.5 mg/L for 24 hours	100%	(Sharp et al., 2004)
<i>Zeuxapta seriolae</i>	Yellowtail amberjack	Bath	2.5 mg/L for 48 hours	100%	(Sharp et al., 2004)
<i>Zeuxapta seriolae</i>	Yellowtail amberjack	Feed	50 mg/kg for 6 days	81.4%	(Williams et al., 2007)

Table 2.2: Effects of praziquantel treatments on adult digeneans. When multiple treatments reached 100% efficacy against the given digeneans only the lowest effective dose is reported. When a higher concentration reported a lower efficacy both treatments were included.

Parasite	Definitive/ Intermediate host	Host	Route	Dose	Efficacy	Source
<i>Cardicola forsteri</i>	Definitve	Pacific Bluefin Tuna	Feed	150 mg/kg single dose	91% 24 days post treatment	(Hardy-Smith et al., 2012)
<i>Cardicola forsteri</i>	Definitve	Pacific Bluefin Tuna	Feed	75 mg/kg single dose	95% 24 days post treatment	(Hardy-Smith et al., 2012)

Table 2.2 continued

<i>Cardicola forsteri</i>	Definitve	Pacific Bluefin Tuna	Feed	15 mg/kg for 3 days	100%, eggs still viable	(Shirakashi et al., 2012)
<i>Cardicola opisthorchis</i>	Definitve	Pacific Bluefin Tuna	Feed	7.5 mg/kg for 3 days	100%	(Ishimaru et al., 2013)
<i>Clinostomum complanatum</i>	Intermediate	Sunshine bass	Bath	.25 mg/L for 24 hours	100%	(Mitchell, 1995)
<i>Clinostomum marginatum</i>	Intermediate	Channel catfish	bath	.65 mg/L + 15 mg/kg for 24 hours	80.2% 5.5 months post treatment	(Lorio, 1989)
<i>Clinostomum marginatum</i>	Intermediate	Channel catfish	Bath	2 mg/L for 2 hours	100% 21 days post treatment	(Plumb and Rogers, 1990)
<i>Clinostomum marginatum</i>	Intermediate	Channel catfish	Bath	2 mg/L for 4 hours	50% 21 days post treatment	(Plumb and Rogers, 1990)
<i>Clinostomum marginatum</i>	Intermediate	Channel catfish	injection	25 mg/kg	73.6% 5.5 months post treatment	(Lorio, 1989)
<i>Diplostomum spathaceum</i>	Intermediate	Channel catfish	Bath	2 mg/L for 4 hours	90.1% 21 days post treatment	(Plumb and Rogers, 1990)
<i>Diplostomum spathaceum</i>	Intermediate	Grass carp	Bath	1 mg/L for 24 hours	80.2%	(Szekely and Molnar, 1991)
<i>Diplostomum spathaceum</i>	Intermediate	Grass carp	Bath	1 mg/L for 90 hours	100%	(Szekely and Molnar, 1991)
<i>Diplostomum spathaceum</i>	Intermediate	Grass carp	Dip	50 mg/L for 20 minutes	94.4%	(Szekely and Molnar, 1991)
<i>Diplostomum spathaceum</i>	Intermediate	Grass carp	Dip	100 mg/L for 20 minutes	75.3%	(Szekely and Molnar, 1991)
<i>Diplostomum spathaceum</i>	Intermediate	Grass carp	feed	330 mg/kg	100%	(Szekely and Molnar, 1991)
<i>Diplostomum spathaceum</i>	Intermediate	Silver carp	Bath	1 mg/L for 24 hours	68.6%	(Szekely and Molnar, 1991)
<i>Diplostomum spathaceum</i>	Intermediate	Silver carp	Dip	50 mg/L for 20 minutes	96.8%	(Szekely and Molnar, 1991)
<i>Posthodiplostomum minimum</i>	Intermediate	Bluegill	Intramuscular injection	5 mg/kg	~100%	(Bader et al., 2017)

Table 2.3: Effects of praziquantel treatments on cestode parasites. When multiple treatments reached 100% efficacy against the given cestode only the lowest effective dose is reported. When a higher concentration reported a lower efficacy both treatments were included.

Parasite	Definitive/ Intermediate host	Host	Route	Dose	Efficacy	source
<i>Atractolytocestus huronensis</i>	Definitive	Common carp	Intubation	50 mg/kg single dose	100% 4 days post treatment	(Sudová et al., 2010)
<i>Bothriocephalus acheilognathi</i>	Definitive	Bonytail chub	Bath	1.5 mg/L for 24 hours	100%	(Ward, 2007)
<i>Bothriocephalus acheilognathi</i>	Definitive	Grass Carp	Bath	.25 mg/L for 24 hours	100%	(Mitchell, 2004)
<i>Bothriocephalus acheilognathi</i>	Definitive	Grass Carp	Bath	.75 mg/L for 24 hours	100%	(Mitchell and Darwish, 2009)
<i>Bothriocephalus acheilognathi</i>	Definitive	Grass Carp	Bath	1.5 mg/L for 12 hours	86.70%	(Mitchell and Darwish, 2009)
<i>Bothriocephalus acheilognathi</i>	Definitive	Grass Carp	Bath	12 mg/L for 6 hours	90%	(Mitchell and Darwish, 2009)
<i>Bothriocephalus acheilognathi</i>	Definitive	Grass Carp	Bath	2.8 mg/L for 12 hours	100%	(Mitchell, 2004)
<i>Bothriocephalus acheilognathi</i>	Definitive	Grass Carp	Bath	9 mg/L for 12 hours	66.7%	(Mitchell and Darwish, 2009)
<i>Bothriocephalus acheilognathi</i>	Definitive	Grass Carp	Feed	35 mg/kg for 3 days	100%	(Pool et al., 1984)
<i>Bothriocephalus acheilognathi</i>	Definitive	Red Shiner	Bath	2.5 mg/L, for 19 days	100% 2.5 post treatment	(Mitchell and Darwish, 2009)
<i>Bothriocephalus scorpii</i>	Definitive	Red snapper	Bath	6 mg/L for 24 hours	100%	(Kline et al., 2009)
<i>Bothriocephalus scorpii</i>	Definitive	Turbot	Intubation	5 mg/kg for 3 days	100%	(Sanmartín Durán et al., 1989)
<i>Khawia sinensis</i>	Intermediate	Common carp	Intubation	50 mg/kg single dose	100% 6 days post treatment	(Sudová et al., 2010)

Table 2.4: Studies on the pharmacokinetics of praziquantel in fish. C_{max} is the maximum concentration found in the given tissue.

T_{max} is the amount of time it took for the maximum concentration to be reached in the host tissue. T_{1/2} represents the elimination

half-life of praziquantel in the given tissue. Elimination time (h) represents the total amount of time from the administration of

praziquantel until levels were no longer detectable in the host tissue. Blank boxes represent parameters that were not examined in the

given study.

Host	Route	Dose	Tissue	C _{max}	T _{max} (h)	T _{1/2} (h)	Elimination time (h)	paper
Grass carp – brackish water	Intubation	10 mg/kg	Plasma	.76 µg/g	.5	1.85	Present at 96	(Xie et al., 2015)
Grass carp – brackish water	Intubation	10 mg/kg	Muscle	.51 µg/g	1	1.12	Present at 96	(Xie et al., 2015)
Grass carp – brackish water	Intubation	10 mg/kg	Liver	2.70 µg/g	.5	2.69	Present at 96	(Xie et al., 2015)
Grass carp – brackish water	Intubation	10 mg/kg	Kidney	2.99 µg/g	1	2.87	Present at 96	(Xie et al., 2015)
Grass carp – fresh water	intubation	10 mg/kg	Plasma	.91 µg/g	.5	3.25	Present at 96	(Xie et al., 2015)
Grass carp – fresh water	intubation	10 mg/kg	Muscle	.62 µg/g	.5	.31	Present at 96	(Xie et al., 2015)
Grass carp – fresh water	intubation	10 mg/kg	Liver	3.87 µg/g	.5	.35	Present at 96	(Xie et al., 2015)
Grass carp – fresh water	intubation	10 mg/kg	Kidney	3.39 µg/g	1	.76	Present at 96	(Xie et al., 2015)
Pacific Bluefin Tuna	Feed	15 mg/kg single dose	Serum	2.0 µg/ml	1.5		24	(Ishimaru et al., 2013)
Pacific Bluefin Tuna	Feed	15 mg/kg single dose	Muscle	1.6 µg/ml	0.5		24	(Ishimaru et al., 2013)
Pacific Bluefin Tuna	Feed	15 mg/kg single dose	Liver	10.2 µg/ml	0.5		24	(Ishimaru et al., 2013)
Pacific Bluefin Tuna	Feed	15 mg/kg single dose	Kidney	3.8 µg/ml	1.5		24	(Ishimaru et al., 2013)

Table 2.4 continued

Rainbow trout	Intubation	500 mg/kg	serum	10.6 ug/g	4		Present at 32	(Björklund and Bylund, 1987)
Rainbow trout	Intubation	500 mg/kg	Bile fluid	16.1 ug/g	8		Present at 32	(Björklund and Bylund, 1987)
Rainbow trout	Intubation	500 mg/kg	liver	31.8 ug/g	4		Present at 32	(Björklund and Bylund, 1987)
Rainbow trout	Intubation	500 mg/kg	muscle	10.2 ug/g	8		Present at 32	(Björklund and Bylund, 1987)
Rockfish	dip	100 mg/L for 4 minutes	Plasma	5.96 ug/L	12		96	(Kim et al., 2001)
Rockfish	dip	100 mg/L for 4 minutes	Muscle	.49 ug/L	3		48	(Kim et al., 2001)
Rockfish	Feed	200 mg/kg for 3 days	muscle				48	(Kim et al., 2003)
Rockfish	Feed	200 mg/kg for 3 days	skin				96	(Kim et al., 2003)
Rockfish	Feed	400 mg/kg for 3 days	muscle				144	(Kim et al., 2003)
Rockfish	Feed	400 mg/kg for 3 days	skin				168	(Kim et al., 2003)
Rockfish	Intubation	100 mg/kg	plasma	At 24 hours ~3.2 ug/ml			Present at 24	(Kim and Kim, 2002)
Rockfish	Intubation	200 mg/kg	Plasma	At 24 hours ~7.2 ug/ml			Present at 24	(Kim and Kim, 2002)
Rockfish	Intubation	400 mg/kg	Plasma	8.59 ug/ml	9		120	(Kim et al., 2001)
Rockfish	intubation	400 mg/kg	muscle	4.20 ug/g	9		120	(Kim et al., 2001)
Yellowtail amberjack	Intravenously	40 mg/kg	Plasma	20.30 µg/ml	0.5	8.57	Present at 24	(Tubbs and Tingle, 2006)
Yellowtail amberjack	Intravenously	40 mg/kg	Skin	8.70 µg/ml	1.5	7.23	Present at 24	(Tubbs and Tingle, 2006)
Yellowtail amberjack	intubation	40 mg/kg	Plasma	12.73 µg/ml	1.0	6.57	Present at 24	(Tubbs and Tingle, 2006)
Yellowtail amberjack	Intubation	40 mg/kg	Plasma	10.62 µg/ml	6	7.91	Present at 24	(Tubbs and Tingle, 2006)
Yellowtail amberjack	Intubation	40 mg/kg	Skin	5.26 µg/ml	1.5	5.78	Present at 24	(Tubbs and Tingle, 2006)
Yellowtail amberjack	Intubation	40 mg/kg	Skin	3.96 µg/ml	6	4.72	Present at 24	(Tubbs and Tingle, 2006)

Table 1.5: Summary of parasitic species and hosts that praziquantel has been tested on in aquaculture. Dip treatments are considered any bath treatment lasting 3 hours or less. Boxes that are left blank represent treatment strategies that currently have no data present.

	Monogenean	Digenean	Cestode	Pharmacokinetics
Bath	9 species (<i>Ancylo-discoides vistulensis</i> , <i>Benedenia seriolae</i> , <i>Cleidodiscus sp.</i> , <i>Clema-coty-le australis</i> , <i>Dactylogyrus sp.</i> , <i>Gyrodactylus aculeati</i> , <i>Gyrodactylus turnbulli</i> , <i>Haliotrema abaddon</i> , <i>Lepidotrema bidyanain</i>) 9 hosts (European catfish, Yellowtail amberjack, Black crappie, White-spotted eagle rays, goldfish, guppy, stickleback, West Australian dhufish, silver perch)	3 species (<i>Clinostomum complanatum</i> , <i>Clinostomum marginatum</i> , <i>Diplostomum spathaceum</i>) in 4 hosts (sunshine bass, channel catfish, grass carp, silver carp)	1 species (<i>Bothriocephalus acheilognathi</i>) in 4 hosts (Bonytail chub, grass carp, red shiner, red snapper)	
Dip	2 species (<i>Benedenia seriolae</i> , <i>Gyrodactylus sp.</i>) in 2 hosts (Cownose rays, Rainbow Trout)	1 species (<i>Diplostomum spathaceum</i>) in 2 hosts (grass carp, silver carp)		1 host at 1 dosage (Rockfish, 100 mg/L for 4 min)
Inject		2 species (<i>Clinostomum marginatum</i> , <i>Posthodiplostomum minimum</i>) in 2 hosts (channel catfish, bluegill)		1 host at 1 dosage (yellowtail amberjack, 40 mg/kg)
Oral	6 species (<i>Benedenia seriolae</i> , <i>Heterobothrium okamotoi</i> , <i>Lepidotrema bidyana</i> , <i>Microcotyle sebastis</i> , <i>Neobenedenia girellae</i> , <i>Zeuxapta seriolae</i>) in 5 hosts (Yellowtail amberjack, Takifugu rubripes, silver perch, chub mackerel)	3 species (<i>Cardicola forsteri</i> , <i>Cardicola opisthorchis</i> , <i>Diplostomum spathaceum</i>) in 2 hosts (Pacific Bluefin Tuna, Grass Carp)	3 species (<i>Atractolytocestus huronensis</i> , <i>Bothriocephalus acheilognathi</i> , <i>Khawia sinensis</i>) in 3 hosts (Common carp, grass carp, turbot)	5 hosts at 9 doses (grass carp 10 mg/kg, pacific Bluefin tuna 15 mg/kg, rainbow trout 500 mg/kg, rockfish 200 mg/kg for 3 days 400 mg/kg for 3 days 100 mg/kg 200 mg/kg 400 mg/kg, yellowtail amberjack 40 mg/kg)

**CHAPTER 3: ASSESSMENT OF *IN VITRO* KILLING ASSAYS FOR
DETECTING PRAZIQUANTEL-INDUCED DEATH IN
POSTHODIPILOSTOMUM MINIMUM METACERCARIAE**

A paper submitted for publication in Parasitology Research

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Abstract

Control of parasitic infections may be achieved by eliminating developmental stages present within intermediate hosts, thereby disrupting the parasite life cycle. For several trematodes relevant to human and veterinary medicine, this involves targeting the metacercarial stage found in fish intermediate hosts. Treatment of fish with praziquantel is one potential approach for targeting metacercariae. To date, studies investigating praziquantel-induced metacercarial death in fish rely on counting parasites and visually assessing morphology or movement. In this study, we investigate quantitative methods for detecting praziquantel-induced death using a *Posthodiplostomum minimum* model. Our results revealed that propidium iodide staining accurately identified praziquantel-induced death and the level of staining was proportional to the concentration of praziquantel. In

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contrast, detection of ATP, resazurin metabolism, and trypan blue staining were poor indicators of metacercariae death. The propidium iodide method offers an advantage over simple visualization of parasite movement and could be used to determine EC₅₀ values relevant for comparison of praziquantel sensitivity or resistance.

1. Introduction

Trematodes are parasitic flatworms with clinical importance in both human and veterinary medicine. Digenean trematodes have an indirect life cycle involving intermediate hosts. Adult trematodes live and mate within the definitive host while a mollusk serves as the first intermediate host. For many trematode species, cercariae leave the snail and penetrate a second intermediate host in order to form metacercariae that are infectious for the definitive host. The metacercarial cyst can reside in the tissues of the second intermediate host for extended periods of time until they are consumed in order to complete the life cycle. Various fish species frequently serve as second intermediate host since they are in close proximity to snails and are likely to be eaten by humans and other vertebrates.

Historically, trematode control has mainly focused on treatment of infected definitive hosts in addition to snail control. Overall, the fish second intermediate host is not commonly treated in order to remove metacercariae. Yet, targeting second intermediate hosts has value in terms of breaking the life cycle. For example, proper cooking of fish prevents transmission of *Clonorchis sinensis* and *Opisthorchi viverrini* (Keiser and Utzinger, 2005). In veterinary medicine and in aquaculture production systems, it becomes desirable to eliminate metacercariae, due to the unsightly appearance of metacercariae in fillets and potential health consequences of metacercariae in fish. This is true for *Posthodiplostomum*

minimum, an intestinal trematode that infects Ciconiiformes and Charadriiformes (herons, gulls, terns, etc.), and uses Centrarchid fish as their second intermediate host. *P. minimum* metacercariae are often found in fish species such as bluegills (*Lepomis macrochirus*) and black crappies (*Pomoxis nigromaculatus*) raised for recreational stocking efforts.

Praziquantel is one of the few agents with anti-trematode activity and is used widely in both humans and animals. In fish, praziquantel has been used to kill adult monogeneans and digenean trematodes (Ishimaru et al, 2013; Bader et al, 2017). Because there is also a need to eliminate metacercarial stages, numerous studies have assessed the use of praziquantel in fish including oral (Hardy-Smith et al., 2012), topical (Mitchell, 1995), and injectable treatments (Lorio, 1989). However, such studies are difficult to conduct since assessment of efficacy is based on counting metacercariae (Hardy-Smith et al., 2012; Plumb and Rogers, 1990). Counts alone are problematic since dead metacercariae can persist in fish tissues post-treatment. Movement is sometimes used to assess parasite viability, however, some encysted metacercariae have limited mobility (Asanji and Williams, 1975). Another disadvantage of such studies is that they do not allow fine scale detection of the minimum drug concentrations needed to kill the parasites and therefore cannot detect differences in praziquantel sensitivity among isolates or treatment regimens.

Assays have been developed for estimating viability of various cell types. For example, ability to metabolize resazurin (Alamar Blue) has been used as a surrogate measurement of viability (Rampersad, 2012). Similarly, ATP production can be used to detect and quantify cell death (Cree and Andreotti, 1997). In addition to measures of metabolism, membrane integrity can also be used to differentiate live and dead cells. Trypan blue is commonly used to detect dead cultured cells (Bhuyan et al., 1976). Similarly,

propidium iodide enters cells with compromised membranes and intercalates into DNA (Gould et al., 2008). Thus, there are numerous methods for conducting killing assays that involve indirect measurements of cellular metabolism or membrane integrity. Yet, these methods have not been adequately exploited for the study of trematode metacercariae.

P. minimum metacercariae are commonly found in wild-caught or extensive-culture-reared Centrarchid fish, with numerous metacercariae commonly recovered from the liver and kidneys (Lane et al., 2015). In the present study, we evaluated *in vitro* methods for evaluating praziquantel-induced death of metacercariae using *P. minimum* as a model.

2. Materials and methods

2.1 Parasites

Lepomis macrochirus were obtained from anglers that obtained the fish from ponds near Ames, Iowa, by legal methods. Liver and posterior kidneys were removed and *P. minimum* metacercariae were extracted with the aid of a dissection microscope. Metacercariae were placed in 24 well plates with each well containing 300 μ L RPMI, 10% bovine serum, 60 μ g/mL penicillin, and 100 μ g/mL streptomycin. For parasite killing assays, metacercariae were co-incubated with dilutions of praziquantel (Bimeda, Le Sueur, MN) or propylene glycol which served as a vehicle control. Dead (control) metacercariae were generated by killing parasites in a heat block for 15 minutes at 60 °C. Assays were conducted both for metacercariae within cysts as well as metacercariae removed from cysts. Following addition of praziquantel, plates were incubated overnight at room temperature prior to assessment. One way analysis of variance was used to compare viability assay results among multiple groups using GraphPad Prism statistical software.

2.2 Assays for detecting praziquantel-induced death

Propidium iodide assays were conducted by co-incubating metacercariae with 50 $\mu\text{g/ml}$ propidium iodide (Sigma) in the dark for 15 minutes. Metacercariae were removed from the well and imaged on glass microscope slides using a fluorescence microscope. Exposure time and camera sensitivity were calibrated with control parasites. Percent of parasite tissue area fluorescing was determined with image analysis software (Halo FL v1.0, area quantification module). In this approach, the analysis software identifies the percentage of total parasite area that is stained. At least 5 metacercariae were used for each experimental condition and experiments were conducted a minimum of two times. Dose response curves were constructed by applying a four parameter logistic regression assuming a variable slope model.

Trypan blue staining was performed by adding 300 μg of trypan blue (Fisher Scientific) to each well, followed by incubation for 3 minutes at room temperature. Parasites were washed three times with PBS followed by imaging on glass microscope slides using a light microscope. Percent of parasite tissue staining was determined with image analysis software (Halo FL v1.0, area quantification module). At least 5 metacercariae were used for each experimental condition and experiments were conducted a minimum of two times.

For resazurin metabolism assessment, 30 μL of 10x Alamar Blue (Fisher Scientific) was added to each well and incubated for 1 or 24 hours at room temperature. Following incubation, 50 μl of culture media was transferred to a 96 well plate and fluorescence (570 nm excitation, 590 nm emission) and absorbance values (570 nm and 600 nm) were recorded with a plate reader (Spectramax M2, Molecular Devices) at 1 and 24 hours. A minimum of 5 metacercariae were used for each experimental condition.

ATP production was assessed with a commercial recombinant luciferase (Celltiter Glo, Promega) that produces light in the presence of ATP. 300 μ L of Celltiter Glo reagent was added to each well, followed by incubation at room temperature for 10 minutes on an orbital shaker. Following incubation, 100 μ L was transferred to a 96 well plate and luminescence was recorded with a luminometer (Spectramax M2, Molecular Devices). A minimum of 4 metacercariae were used for each experimental condition.

3. Results

3.1 Metacercariae killed by heat or praziquantel are selectively stained by propidium iodide

Parasites were co-incubated with propidium iodide and fluorescence was measured using a fluorometric plate reader as well as image analysis software. Although differences in fluorescence could not be detected by a plate reader (data not shown), staining of dead parasites could be visualized by fluorescence microscopy (Figure 1). When metacercariae were contained within a cyst, we observed an increased level of background fluorescence (Figure 1). For excysted parasites, quantification of staining by image analysis software revealed that the level of staining was proportional to the concentration of praziquantel ($r^2=0.99$, Figure 2). In our hands, propidium iodide staining was more sensitive than motility scoring for detecting dead parasites (data not shown).

3.2 Resazurin metabolism and ATP luminescence assays fail to detect praziquantel-induced death.

Parasites were co-incubated with resazurin (Alamar Blue) followed by spectrophotometric quantitation of the reduced form. In the present study, there was no

difference in fluorescence or absorbance measurements among live and dead parasites.

Attempts to optimize the concentration of resazurin and incubation conditions did not resolve this problem. Experiments attempting to correlate absorbance or fluorescence signal with the number of metacercariae in each well were conducted and these experiments also failed to detect differences in resazurin metabolism.

Metacercariae were co-incubated with a commercially available recombinant luciferase, followed by luminescence detection. In this assay, ATP produced by cells leads to luminescence that can be detected with a plate reader. For excysted metacercariae, the luminescence signal was proportional to the number of parasites in each well for the range tested (1-4). However, the ATP assay was unable to detect differences among ATP production of live, heat-killed, or praziquantel-killed metacercariae (data not shown).

3.3 Trypan blue penetrates metacercarial cysts but is poorly correlated with praziquantel-induced death

Metacercariae were co-incubated with trypan blue and the percentage of parasite area stained was quantified using image analysis software. These experiments revealed that trypan blue penetrates the cyst wall thereby obscuring staining of metacercariae (Figure 3). In excysted parasites, trypan blue staining was very weak and computer-aided detection of staining was not sensitive enough to discriminate live and dead parasites ($p=0.71$, Figure 4).

4. Discussion

Trematodes remain significant pathogens in both human and veterinary medicine. Treatment of fish intermediate hosts with praziquantel may be desirable in order to break the life cycle or improve the health of fish in aquaculture settings. However, few assays are

available to assess metacercariae death other than counting parasites, observing movement, or morphological changes (Keiser, 2010). In the present study, we examined several methods for assessing praziquantel-induced parasite death using *Posthodiplostomum minimum* metacercariae as a model since they can be conveniently retrieved from Centrarchid fish.

Propidium iodide penetrates cells with compromised membranes and intercalates into DNA. Propidium iodide staining has been used to quantify cell death in both bacteria and various eukaryotic cells (Williams et al., 1998). Staining can be measured by fluorimetry or image quantification software. In this present study, both heat and praziquantel-killed metacercariae were stained by propidium iodide. While this staining could not be detected in a plate reader, image analysis software could readily be used to detect live and dead parasites. The amount of signal was proportional to the dose of praziquantel, suggesting that this method could be used to measure the relative praziquantel sensitivity or resistance in different populations of metacercariae. In addition, this method could potentially be used to determine effective drug concentrations *in vitro*, thereby creating a reference point for designing *in vivo* praziquantel trials.

Trypan blue stains cells with damaged or compromised membranes and is regularly used to differentiate live and dead cultured cells. In the present study we found that trypan blue penetrated the metacercarial cyst wall. This obscured the view of the parasites and made it difficult to determine if the parasite was actually stained. When *P. minimum* metacercariae were removed from the cyst, differences in live and dead parasites could not be observed despite the use of sensitive computer-aided detection software. Therefore, trypan blue was not suitable for whole organism staining.

Resazurin is metabolized by living cells into a reduced form; the reduced form can be quantitated by spectrophotometry or fluorimetry (Czekanska, 2011). In this study, neither method was suitable for differentiating live and killed metacercariae at 1 or 24 hour time points. In addition, resazurin metabolism was not correlated with the number of metacercariae present in a particular well. These results are similar to those observed for another trematode, *Echinostoma caproni* (Panic et al., 2013).

ATP production has been used as a measure of viability for *Schistosoma mansoni* (Panic et al., 2015), but had been found to be unsuccessful with metacercariae (Panic et al., 2013). In the present study, we utilized a commercial recombinant luciferase that produces light in the presence of ATP. Our results demonstrated that this method could not discriminate live and dead metacercariae. We did find that ATP levels were correlated with the number of metacercariae in each well. However, praziquantel and propylene glycol (vehicle control) both falsely elevated the amount of luminescence signal detected. Thus, ATP detection by recombinant luciferase was inadequate for determining metacercariae death.

Eliminating metacercariae in fish intermediate hosts represents a tool for parasite control. However, relatively few methods have been available to assess the efficacy of drugs used to treat intermediate hosts other than parasite counts. Studies entailing treatment of fish may be difficult due to a limited ability to detect dead and dying metacercariae. Using *P. minimum*, we found that praziquantel-mediated death could be accurately quantified using propidium iodide staining coupled with image analysis software. Our data revealed that propidium iodide staining was proportional to the concentration of praziquantel, suggesting that this method could be used to compare praziquantel sensitivity among different

populations. Going forward, studies are needed to assess whether propidium iodide-based assays can be used to assess parasite killing in metacercariae recovered from a treated host.

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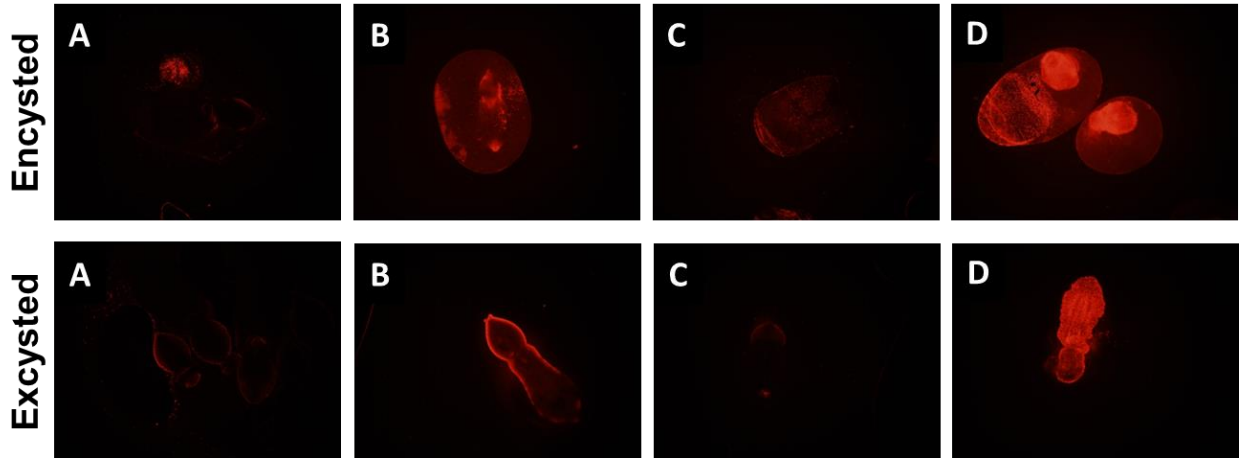


Figure 3.1: Propidium iodide staining of *P. minimum* metacercariae. A) Untreated Control, B) Heat-killed (positive control), C) Propylene glycol (vehicle control), and D: 500 µg/ml praziquantel. Top row: encysted metacercariae. Bottom row: mechanically excysted metacercariae.

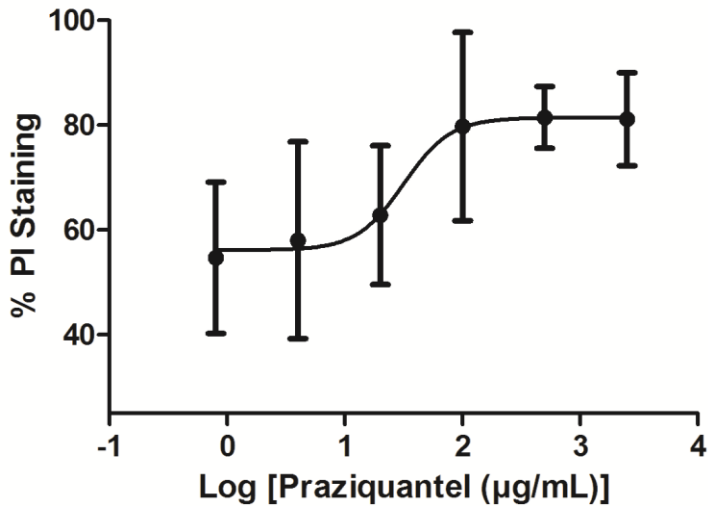


Figure 3.2: Propidium iodide staining of excysted *P. minimum* metacercariae is proportional to the dose of praziquantel ($r^2=0.99$). Each data point represents the percentage of positively stained pixels \pm SEM, (n=minimum of 5 parasites for each dose).

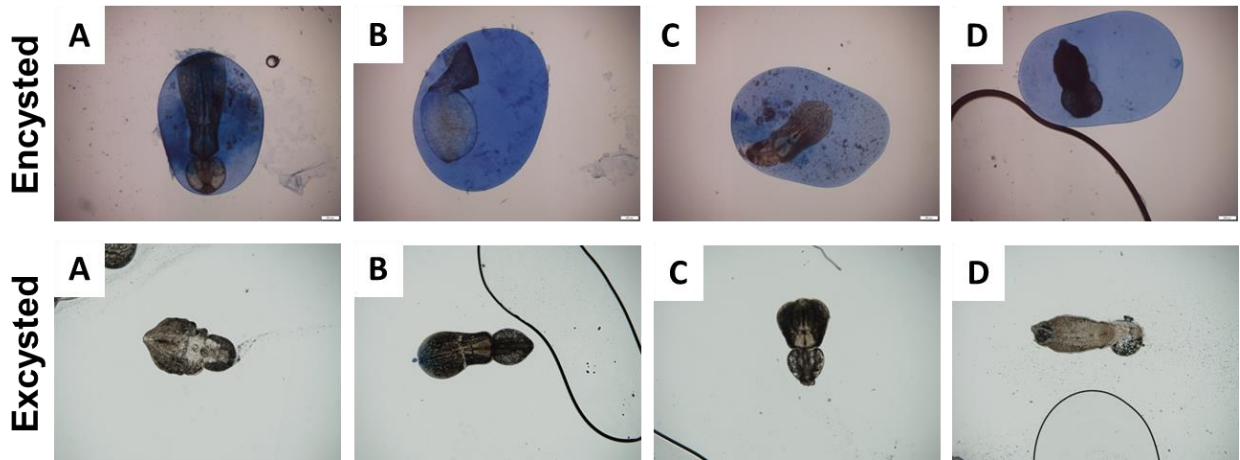


Figure 3.3: Trypan blue staining of *P. minimum* metacercariae. A) Untreated, B) Heat-killed (positive control), C) Propylene glycol (vehicle control), D: 500 µg/ml praziquantel. Top row: encysted metacercariae. Bottom row: mechanically excysted metacercariae.

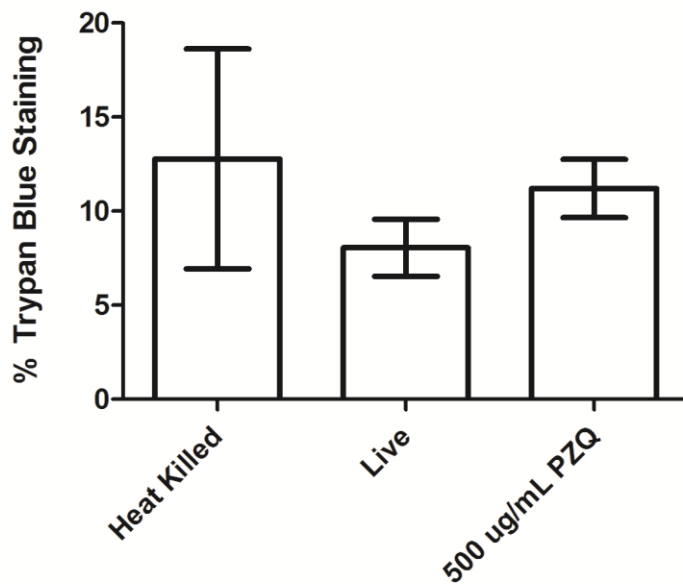


Figure 3.4: Trypan blue staining is unsuitable for detecting live and praziquantel-killed metacercariae. The proportion of staining did not significantly differ among live, heat-killed, or praziquantel-killed parasites ($p=0.71$).

**CHAPTER 4: EFFICACY OF INJECTABLE PRAZIQUANTEL FOR
POSTHODIPILOSTOMUM MINIMUM TREMATODE METACERCARIAE
INFECTING *LEPOMIS MACROCHIRUS***

A paper to be submitted

Chris Bader, Jeba Jesudoss Chelladurai, David E. Starling, Douglas E. Jones, and Matthew T.

Brewer

Abstract

Digenean trematodes have complex life cycles and control of these flatworms can be accomplished by eliminating immature parasite stages from intermediate hosts. In aquaculture systems, presence of trematode metacercariae can negatively impact fish health and can lead to economic losses. *Posthodiplostomum minimum* is a parasite of birds that uses bluegill sunfish (*Lepomis macrochirus*) as the intermediate host and is commonly found in fish used to stock waterways for recreational purposes. In this study, we evaluated killing of *P. minimum* metacercariae by injectable praziquantel in naturally infected bluegills. Using propidium iodide staining and motility assessment, we found that 5 mg/kg administered intramuscularly was effective for parasite killing. However, metacercarial death was not

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apparent until day 7 post-treatment. Our results demonstrated that propidium iodide staining is an effective method for detecting death in metacercariae *ex vivo*. This method was at least as sensitive as objective motility scoring and provided quantitative assessment of parasite death. Future studies involving treatment of metacercariae in fish with praziquantel may need to be carried out over a period of weeks in order to accurately assess parasite killing and would benefit from using the propidium iodide method.

1. Introduction

Aquaculture is rapidly growing throughout the world, with some estimates showing that production for species such as salmon, carp and tilapia will increase over 65% by the year 2030 (Kobayashi et al., 2015). In addition to raising fish that are directly marketed for human consumption, many aquaculture facilities produce fish that are used to support stocking efforts in public and private fisheries. In 2004, the US government had stocked 1.75 billion fish of 104 species throughout the waters of the US (Halverson, 2008). In aquaculture facilities, increased stocking density can lead to amplification of parasite life cycles (Born-Torrijos et al., 2016). In some cases, parasites impact fish health by impairing weight gain or inducing mortality events (Barber and Svensson, 2003; Thoney and Hargis Jr, 1991). On the other hand, parasitism can lead to economic losses due to unsightly appearance of parasites in filets or viscera. This is true of *Posthodiplostomum minimum*, a digenean trematode that infects fish eating birds and uses Centrarchid fish as intermediate hosts. Hundreds of *P. minimum* metacercariae can be found in the liver and kidney, but can also encyst in the heart, spleen, muscle, mesenteries, and ovaries (Grizzle and Goldsby, 1996; Hoffman, 1958). In

some fish, metacercariae can be observed replacing over 50% of liver and kidney parenchymal tissue.

Despite the problems caused by metacercariae invading fish tissues, there are no products labeled for the treatment of digenean trematodes in fish in the United States. A logical choice for treatment of these platyhelminthes is praziquantel. Praziquantel has been used to treat cestodes and trematodes in a variety of veterinary species and has a wide margin of safety (Bader et al., 2017b; Dayan, 2003; Frohberg, 1984). While the mechanism of action of praziquantel is not entirely understood, there is evidence that the drug disrupts calcium homeostasis. This disruption is associated with vacuolation and blebbing of the tegument which leads to increased immune-mediated clearance of parasites (Greenberg, 2005; Ribeiro et al., 2004). Veterinarians can justify the use of praziquantel for trematode parasites in fish through the framework of the Animal Medicinal Drug Use Clarification Act (AMDUCA) (Davis et al., 2009). However, the efficacy of praziquantel for *P. minimum* has not been assessed and therefore it is difficult to make evidence-based treatment decisions for this parasite in Centrarchid fish.

Several routes of administration have been proposed for praziquantel in fish. For ectoparasitic monogeneans, topical bath treatments may be used (Bader et al., 2017b), but bath treatments may not achieve high tissue concentrations as compared to oral treatments (Kim et al., 2001). Other studies have examined oral administration by coating pelleted food (Kim et al., 1998); while this method seems easy in theory, praziquantel has a bitter taste and feeding may decrease compliance (Partridge et al., 2014). It is also difficult to achieve uniform consumption with this strategy and it can possibly lead to under-dosing of fish. Although it has the disadvantage of being time consuming, oral gavage of praziquantel has

been successfully used in some species, (Hirazawa et al., 2000). Intramuscular injection has also been successful in killing trematode metacercariae in channel catfish (Lorio, 1989). It has also been shown that praziquantel has the potential to be more effective at preventing the development of *Nanophyetus salmincola* in the definitive host when chinook salmon are treated before being fed to coyote pups (Foreyt and Gorham, 1988). In addition to having better control over individual dosages, intramuscular injections have been shown to achieve high concentrations in the kidney and liver (Olliaro et al., 2014), areas that metacercariae are often found. Despite this, intramuscular injections have not been widely studied in fish.

Efficacy studies rely on accurately determining death in metacercariae. One difficulty that exists for assessing treatment efficacy for metacercarial parasite stages is that the organisms remain within the tissues of the host for an extended period of time, even if the treatment is successful. Accordingly, counting of parasites is unsuitable for determining treatment efficacy. Observation of motility can be used to assess killing of metacercariae, however, this method is subjective and dependent on the observer. In addition, it is difficult to quantitate motility beyond its presence or absence. In a previous *in vitro* study, we determined that propidium iodide (PI) stains metacercariae that are killed by praziquantel and the staining is proportional to the dose of praziquantel administered (Bader et al., 2017a). The present study was undertaken to test the hypothesis that injectable praziquantel could be used to treat *P. minimum* in bluegills and that parasite death could be determined by PI staining of metacercariae removed from the tissues of treated fish.

2. Materials and Methods

2.1 Infected fish

Lepomis macrochirus, naturally infected with *P. minimum*, were acquired from licensed anglers who obtained the fish near Ames, Iowa by legal methods. Animals were acclimated to laboratory conditions in a 150 gallon tank supplied with a commercial aerator, which was maintained at room temperature with high aeration. Water quality parameters (ammonia, nitrate, nitrite, chlorine, pH, water hardness, and alkalinity) were monitored daily. Fish were acclimated and fed commercial pellets for a minimum of two weeks prior to experimentation. All procedures were in accordance with applicable laws and were approved by the Institutional Animal Care and Use Committee (IACUC).

2.2 Treatment

Fish were randomly assigned to a treatment (n=8) or negative control (n=4) group. Fish were weighed and the treatment group received 5mg/kg praziquantel (Bimeda, Le Sueur, MN) intramuscularly in the epaxial muscle just lateral to the dorsal fin. Treated and control fish were housed in separate tanks following treatment. On days 4, 7, 14, and 23 post-treatment, two treated and one control fish were sacrificed and examined. *P. minimum* were removed from the liver and kidney with the aid of a dissection microscope and parasites were removed from the metacercarial cyst by gentle mechanical manipulation with pipettes.

2.3 Analysis

Mechanically excysted parasites were co-incubated with 50 µg/mL PI in RPMI for 15 min at room temperature and placed on a glass microscope slide for fluorescence

microscopy. A minimum of 10 metacercariae were evaluated from both the liver and kidney of each fish. Any parasite that was visibly mechanically damaged during manipulation was not included in the analysis. Movement of metacercariae was assessed by observing each organism for 30 seconds and determining if parasite movement was present or absent.

Parasites were imaged with light and fluorescence microscopy on an Olympus BX60/DP70 microscope and camera using identical camera settings (sensitivity, exposure time, black balance) for both treated and control parasites. Images of metacercariae were analyzed with HALO image analysis software FL v1.0 (Indica Labs, Corrales, NM). The area quantification module was used to determine total parasite area and total area positive for propidium iodide staining. Using this information, the percentage of positively stained area was calculated. Statistical comparisons among groups were made using one way ANOVA and Tukey test for multiple comparisons using Graphpad Prism 5.

3. Results

3.1 Propidium iodide staining reveals killing of metacercariae in praziquantel-treated fish and is highly correlated with motility analysis

PI enters cells with damaged membranes and intercalates into DNA, dead cells can be subsequently visualized by fluorescence microscopy. Previous studies demonstrated that *in vitro* killing of *P. minimum* by praziquantel leads to positive PI staining (Bader et al., 2017a). In the present study, we removed metacercariae from praziquantel-treated and negative control fish to test the hypothesis that parasites killed *in vivo* could be detected by PI staining when removed from the animal. Our experiments revealed that propidium iodide staining was markedly visible in metacercariae from treated fish as compared to control fish (Figure

1). Motility of metacercariae was highly correlated with PI staining ($r^2=.88$, Figure 2). Therefore, PI staining was a good predictor of death in metacercariae.

3.2 Injectable praziquantel results in killing of *P. minimum* metacercariae.

We assessed the efficacy of injectable praziquantel by staining metacercariae with PI *ex vivo*. In parasites recovered from both the kidney and liver, we observed that PI staining was significantly higher in parasites recovered from praziquantel treated fish compared to untreated controls on days 7, 14, and 23 post-treatment ($P<0.05$, Figures 3,4). For metacercariae recovered from the liver and kidney of praziquantel treated fish, PI staining was significantly higher on day 7 compared to day 4, and was significantly higher on days 14 and 23 compared to day 7 ($P<0.05$, Figures 3, 4). In contrast, PI staining of parasites was not statistically different in the treatment and control groups at day 4 post-treatment. Thus, metacercarial death was not evident until approximately 7 days post-treatment.

4. Discussion

Fish can be definitive or intermediate hosts for digenean trematode parasites. Excessive parasite burdens have negative consequences on fish health and can cause undesirable lesions. In addition, fish can be hosts for metacercariae that are infectious for humans and animals. *P. minimum* occurs in viscera and occasionally in muscle tissue of Centrarchid fish; high parasite burdens can lead to decreased growth rates (Gizzle and Goldsby Jr., 1996; Wilson et al., 1996). *P. minimum* metacercariae are undesirable in fish stocked for recreational efforts or harvested for consumption even though *P. minimum* is not a zoonotic threat as it is a host adapted parasite of piscivorous birds.

In the present study, we evaluated the efficacy of a single intramuscular dose (5mg/kg) of praziquantel for killing *P. minimum* metacercariae. Although the pharmacokinetics of injectable praziquantel have not been studied in fish, studies involving oral administration found that the drug is rapidly metabolized in the liver and kidney, leading to low or even non-detectable levels of the drug present in these tissues within 24-96 hrs (Björklund and Bylund, 1987; Ishimaru et al., 2013; Xie et al., 2015). We focused our assessment on parasites recovered from the liver and kidney to determine if metacercariae in these organs would be killed despite a presumed rapid depletion of the drug in these tissues.

Our results indicated that the single intramuscular 5 mg/kg dose killed *P. minimum* metacercariae even though they were still visible in host tissues. We assessed parasite killing by observation of motility as well as propidium iodide staining. In past studies, we demonstrated that the level of propidium iodide staining is proportional to the concentration of praziquantel when parasites are killed *in vitro* (Bader et al., 2017a). This is the first study to demonstrate that killed parasites from a treated animal can also be identified by propidium iodide staining *ex vivo*.

The propidium iodide method has the advantage of being quantitative and objective. Therefore, we prefer this method over observation of motility for determining praziquantel-induced death. This method is quantitative and could be used to compare competing treatment regimens as well as relative drug sensitivity and resistance status among different parasite isolates. Interestingly, there was a strong correlation between the level of propidium iodide staining and observation of motility by trained observers (Figure 4).

For both motility and PI staining, death of metacercariae was not evident until 7 days post-treatment. PI staining was intense at days 14 and 23 post-treatment. PI staining was

slightly decreased in treated parasites on day 23, although this difference was not statistically significant. This reduction in staining may be due to degradation of parasite tissues. Our results suggest that assessment of death in metacercariae should not be conducted immediately following praziquantel treatment. Instead, a week or more may be required for accurately detecting parasite killing.

There are no FDA-approved products for treatment of digenean trematodes in fish, although veterinarians could prescribe praziquantel under the framework of AMDUCA. In order to use praziquantel under AMDUCA, a suitable withdrawal period must be determined (Davis et al., 2009). Therefore, the next step toward evidence-based use of praziquantel in food fish is to conduct tissue residue studies.

In this study, we found that a 5 mg/kg intramuscular injection was effecting for killing *P. minimum* metacercariae. Interestingly, PI staining was an accurate and quantitative method for determining death of metacercariae *ex vivo*. Future studies are needed to assess tissue residues in Centrarchid fish following praziquantel treatment. Going forward, metacercarial killing should be assessed no-earlier than one week post treatment in these species.

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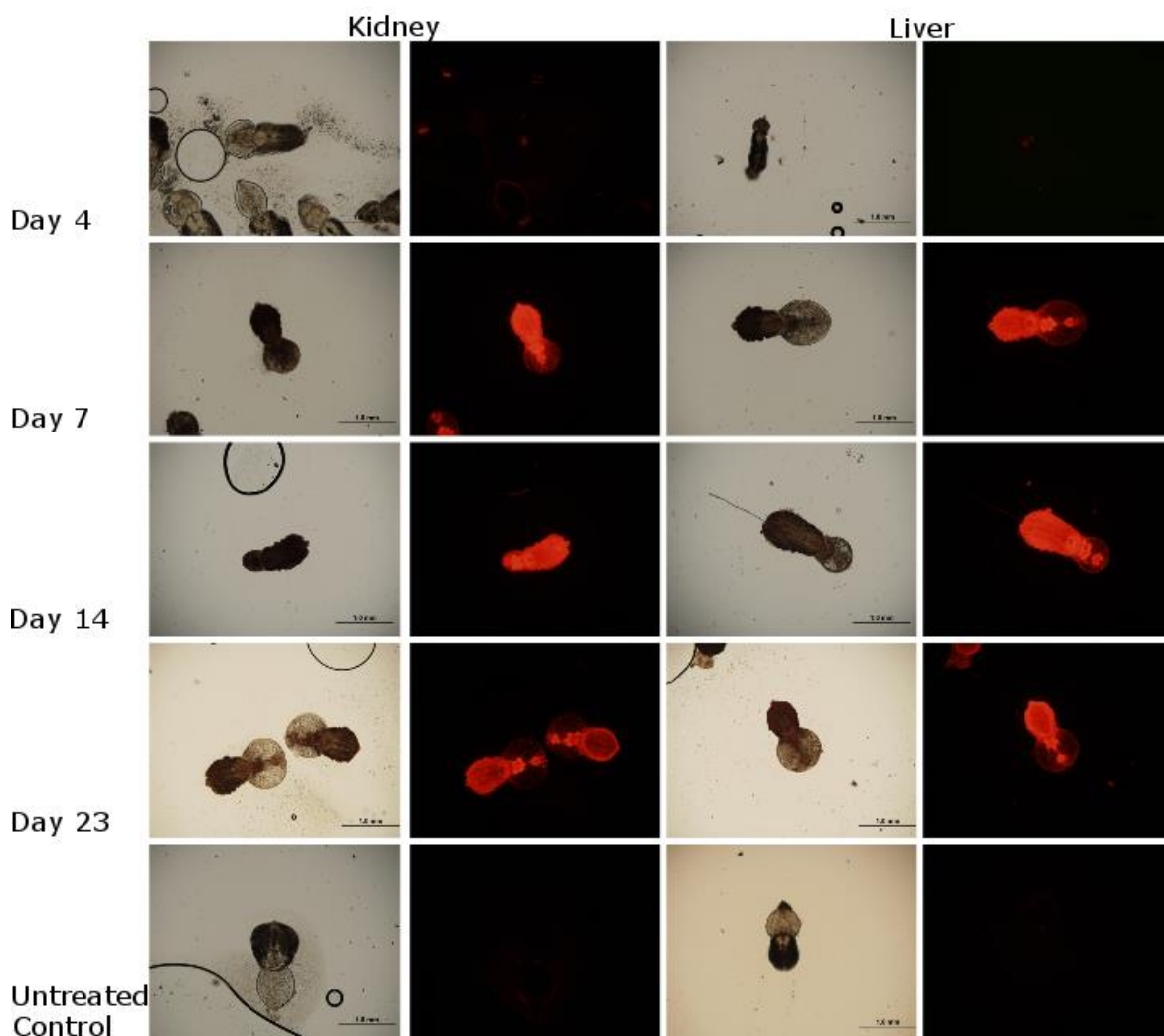


Figure 4.1: Propidium iodide staining of *P. minimum* metacercariae from bluegill treated with 5 mg/kg IM praziquantel. Positive staining is not evident until day 7 post-treatment. Fluorescence signal is quantified in figures 3 and 4.

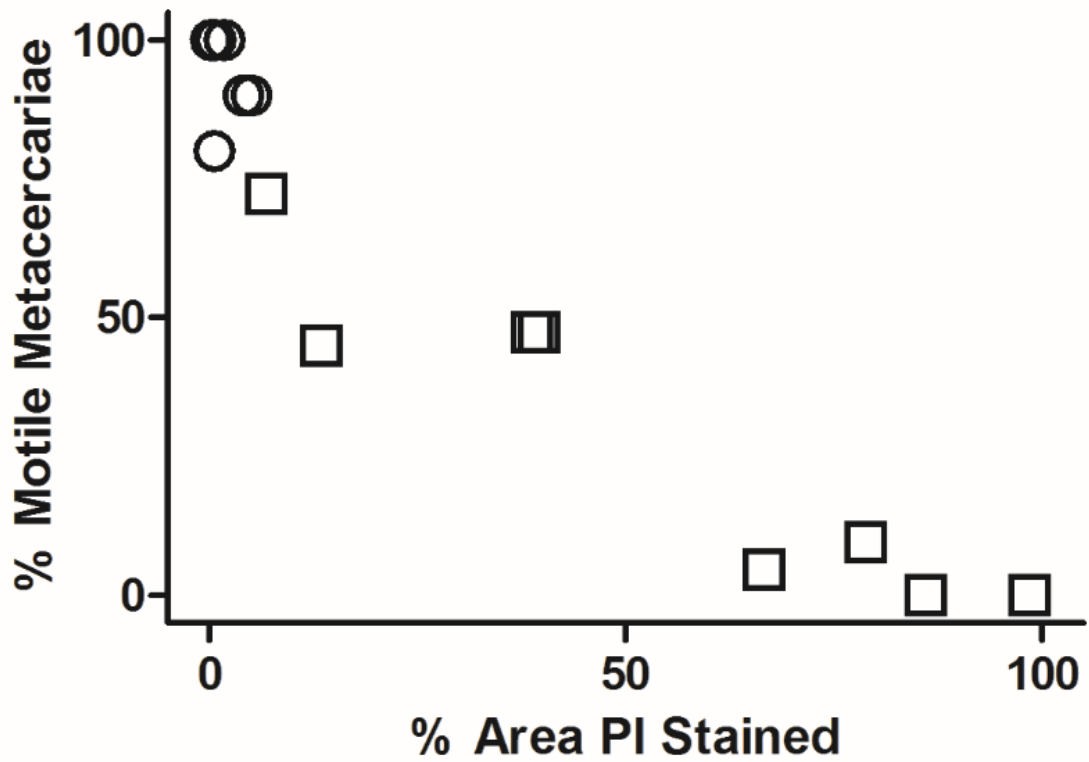


Figure 4.2: PI staining is inversely proportional to motility for *Posthodiplostomum minimum* metacercariae ($r^2=0.88$). Each data point represents the average of 9 or more metacercariae. Squares represent praziquantel-treated groups and circles represent untreated control groups.

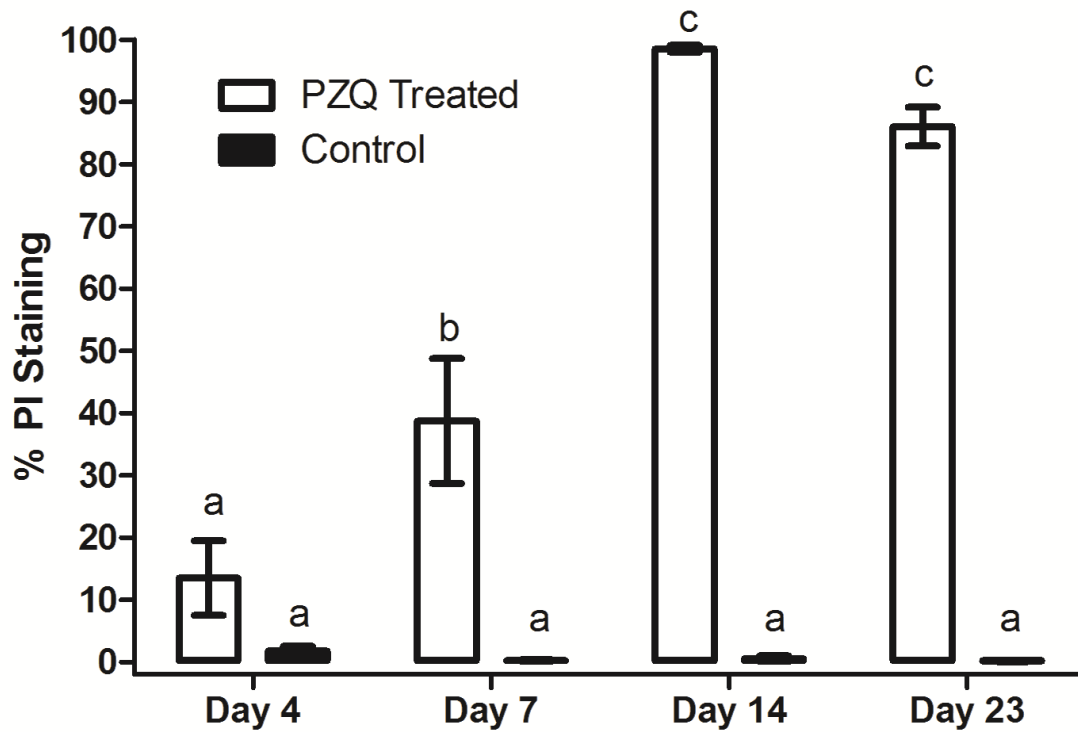


Figure 4.3: Killing of metacercariae recovered from the kidney of bluegill as determined by propidium iodide staining. Each bar represents the average area \pm SEM of the parasite positively stained. Treatment bars represent the average of 20 metacercariae, control bars represent 10 metacercariae. Different letters indicate that groups are significantly different from one another ($p < 0.05$)

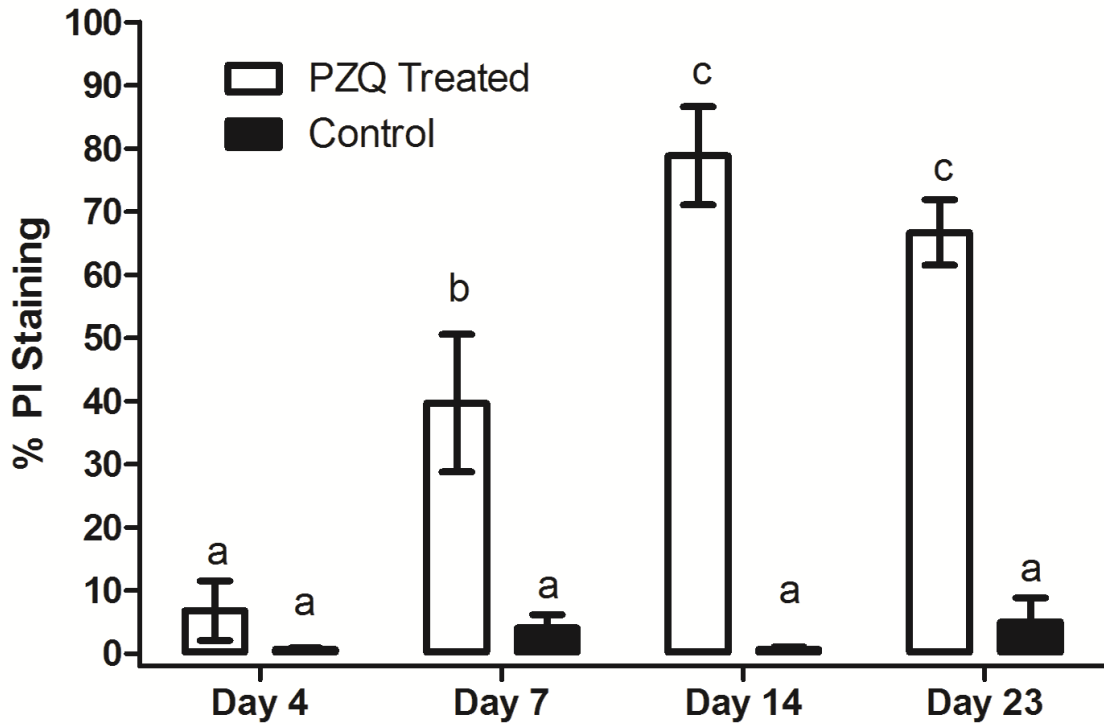


Figure 4.4: Killing of metacercariae recovered from the liver of bluegill as determined by propidium iodide staining. Each bar represents the average area \pm SEM of the parasite positively stained. Treatment bars represent the average of 20 metacercariae, control bars represent 10 metacercariae. Different letters indicate that groups are significantly different from one another ($p < 0.05$)

**CHAPTER 5: OUTBREAK OF *CLEIDODISCUS* IN JUVENILE BLACK CRAPPIES
(*POMOXIS NIGROMACULATUS*) AND BATH TREATMENT WITH
PRAZIQUANTEL**

Modification of a paper published in the Journal of Fish Diseases

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Many species of the Centrarchidae, the sunfish family, are popular for fish stocking efforts in the Midwestern United States. The increase in recreational fishing in public and private waters has forced several commercial aquaculture facilities that stock these waterbodies to raise large populations of Centrarchid species such as bluegills (*Lepomis macrochirus*) and crappies (*Pomoxis* spp.). Unfortunately, the high stocking density of these aquaculture environments has the potential to lead to increased transmission of infectious diseases such as parasitic helminths. Such pathogens are of particular concern because aquaculture conditions can lead to build up of parasite burdens that are not experienced in wild populations, therefore leading to negative consequences on fish health. This is particularly true for parasites with direct life cycles since they can accumulate in large numbers that eventually lead to clinical signs and mortality events.

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Monogeneans are flatworm parasites of fish skin and gills. Their life cycle is unique in that they do not have an obligatory intermediate host, and can be transmitted from one fish to another through direct or indirect contact via fomites or the environment. Monogeneans are among the most commonly reported parasitic pathogens of fish (Woo Bruno and Lim 2002). In the wild, monogenean species are limited by a low host population density, but in fisheries where host population densities are especially high, parasite populations can quickly escalate and become detrimental to the host. In low intensity infections, monogeneans can cause tissue damage, leading to secondary bacterial or fungal infections. As parasite concentrations increase, infections lead to increased mucus production and a subsequent decrease in oxygen uptake in the gills leading to suffocation of the fish host. It has been estimated that intense monogenean infections can cause >80% mortality, making them one of the most significant infectious disease concerns in aquaculture (Thoney and Hargis Jr 1991). While some monogenean genera (eg *Dactylogyrus*, *Gyrodactylus*, *Neobenedenia*) are frequently associated with outbreaks of clinical disease (Weber and Govett 2009), relatively little information is available regarding the pathogenic potential of other genera.

Despite the chance for such a massive loss of fish due to monogenean parasitism, treatment options are limited. In the United States, only dilute formalin baths are FDA approved for treatment of monogeneans in fish intended for human consumption (U. S. Food and Drug Administration 2016). Formalin, however, is not an ideal treatment for many aquatic systems as it can damage biofilters, indirectly increasing ammonia levels to toxic levels (Keck and Blanc 2002). Veterinarians may justify off-label use of praziquantel under the framework of the Animal Medicinal Drug Use Clarification Act (AMDUCA). However,

recommended withdrawal times for praziquantel in fish are not specified in the food animal residue avoidance database (Davis Smith Baynes Tell Webb and Riviere 2009) . In other species praziquantel has been administered by parenteral, oral, and topical routes (Forwood Harris and Deveney 2013). In this study we selected a topical bath route in order to achieve uniform distribution of treatment.

Praziquantel is one of the most important anthelmintics used to target trematodes and cestodes in both human and veterinary medicine. The mechanism of action of praziquantel is not completely resolved, however, it appears to disrupt parasite calcium homeostasis by altering membrane permeability and binding voltage-gated calcium channels (Doenhoff Cioli and Utzinger 2008). Praziquantel is safe in a variety of fish species but there is relatively little data available for veterinarians to make evidence-based treatment decisions in Centrarchid hosts. In the present report, we outline the discovery of *Cleidodiscus* monogenean trematodes associated with clinical disease in black crappies (*Pomoxis nigromaculatus*) and the utility of a praziquantel bath treatment for removal of these organisms.

Juvenile black crappies (4-6 cm fork length) reared in a commercial facility presented for routine veterinary examination. In the past, this facility reported fish with increased surface gasping and increased mortality. Several animals were euthanized by spinal severance and necropsies were performed. The principal finding of the necropsies was that microscopic examination of gill clips revealed the presence of numerous motile monogenean trematodes. The trematodes were immobilized on glass slides and submitted to the Iowa State University Department of Veterinary Pathology for parasite identification. Parasite

identification was established by morphological features described previously (Hoffman 1999).

A random sample of 40 fish were obtained and placed in an aerated 150 gallon quarantine tank and allowed to acclimate for 14 days. Fish were then assigned to either a 20 gallon treatment or untreated control tank (n=20 fish per tank). Filtration systems were disabled during the treatment, to prevent loss of praziquantel in the biofilter. The treatment tank contained 1.5 mg/L praziquantel, which was administered by directly adding a commercial soluble praziquantel product (Bimeda, Le Sueur, MN) to water in the tank. Fish were euthanized by spinal severance 24 hours post-treatment. Parasite assessment was conducted by excising gill arches; wet mounts were prepared and examined by light microscopy. Tissues from each fish were examined by two different trained observers. Statistical comparisons of parasite intensity were made using GraphPad Prism statistical software; we tested the hypothesis that parasite intensity was different in the treated fish compared to the control fish using a Mann-Whitney test.

Examination of the recovered trematodes revealed that they were monopisthocotylean monogeneans, bearing four eyespots, a cylindrical body, a lateral vagina, and a haptor with two pairs of hamuli (Figure 1). The mouth was not surrounded by an oral sucker, the organisms contained eggs (were oviparous) and the haptor lacked a suction disc. Each pair of hamuli was joined by a transverse bar; but the transverse bars did not articulate with each other (Figure 2). Taken together, these morphological features indicate this parasite belongs to the family Ancyrocephalidae. The haptor was approximately the same width as the body, and the anchors were large and lacked roots. In combination with the fact that the organisms were recovered from centrarchids, the morphological features of these parasites indicate that

they are *Cleidodiscus* (Hoffman 1999). Identification at the species level was beyond the scope of the current study and is complicated by taxonomic synonyms (Seamster 1938).

Cleidodiscus vancleavi is the only species of the genus reported in *P. nigromaculatus*, and is thought to be synonymous with *Onchocleidus formosus* (Hoffman 1999).

Following praziquantel bath treatment, there was a significant decrease in the intensity of *Cleidodiscus* in the praziquantel-treated group compared to the controls ($p=0.05$, Figure 3). Unfortunately, the treatment did not remove 100% of parasites. An increased dose of praziquantel may have resolved this. The rationale for our chosen dose (1.5 mg/L) is that 1 mL/10 gallons of the 56.8 mg/mL solution of praziquantel facilitated easy administration and provided a dose similar to that reported previously for internal trematode parasites (Plumb and Rogers 1990). A 10mg/mL praziquantel bath was used to treat the monogenean *Lepidotrema bidyana*, resulting in removal of 84% and 99% of juvenile and adult parasites, respectively (Forwood *et al.* 2013). In the present study, we were unable to evaluate multiple drug doses given the small sample size present. Although praziquantel treatment significantly reduced the number of parasites in our study, a higher dose may be needed for complete removal of monogeneans. Future studies should address differences in praziquantel sensitivity among different monogenean taxa. There are other possibilities that could have contributed to our observation that praziquantel did not totally eliminate the *Cleidodiscus* parasites. Since the crappies were naturally infected prior to submission, they were probably not infected with the same number of parasites prior to treatment, and it is possible that those that were severely infected were not able to clear infection despite treatment. In addition, anthelmintic treatments often induce parasite damage that is followed by host immune-mediated parasite clearance (Buchmann 1999). In this case, the 24 hour

period allowed post-treatment may not have been long enough for us to observe removal of all parasites. The carrier for praziquantel is propylene glycol. Experimental conditions did not allow for a vehicle control treatment group. However, we have observed that propylene glycol does not kill platyhelminthes at the concentrations used in this study (unpublished observations).

The consequences of parasitic disease often depend on the location and the intensity of damage caused by the parasite on the host. Monogeneans trematodes may lead to clinical disease, however, there are many potential monogenean pathogens that have not been identified. For example, it has been estimated that only a fraction of the estimated 20,000 gyrodactylid species have even been described (Bakke Harris and Cable 2002). The present case documents *Cleidodiscus* spp. as a possible cause of clinical respiratory disease in *Pomoxis nigromaculatus*.

There is relatively little information available describing the pathological consequences of *Cleidodiscus* infection in Centrarchids. Investigators have reported asymptomatic gill hyperplasia in bluegills infected with the parasite (Thune and Rogers 1981), however, there are no reports of clinical disease associated with this parasite in Centrarchidae. It is possible that clinical signs are only seen in cases of high parasite intensity. In this particular case, clinical signs of surface gasping resolved when the facility tanks and plumbing were cleaned, and in fish placed in the quarantine tank for the present study, which indicates that lower density stocking may also decrease monogenean parasite burdens.

Several studies have described the use of praziquantel in fish species of high economic value. However, there is still a wide range of fish species in which praziquantel

has not been tested. It is essential to study the use of this drug against different parasites in different fish hosts, thereby using an evidence-based approach to using praziquantel in aquaculture systems. Going forward, there is a need for large scale field studies to determine the usefulness of praziquantel for monogeneans under field conditions. In addition, there is a need to examine potential differences in praziquantel sensitivity among different monogenean taxa.

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Figure 5.1: *Cleidodiscus* recovered from a black crappie. Note the presence of head organs on the anterior portion of the parasite, eye spots (narrow arrow, only 2 of 4 eyespots are visible in this plane of focus), a narrow cylindrical body, and lateral vaginal opening (thick arrow).



Figure 5.2: Squash preparation of a representative haptor from *Cleidodiscus* recovered from a black crappie. Note the two pairs of hamuli that each articulate with a transverse bar.

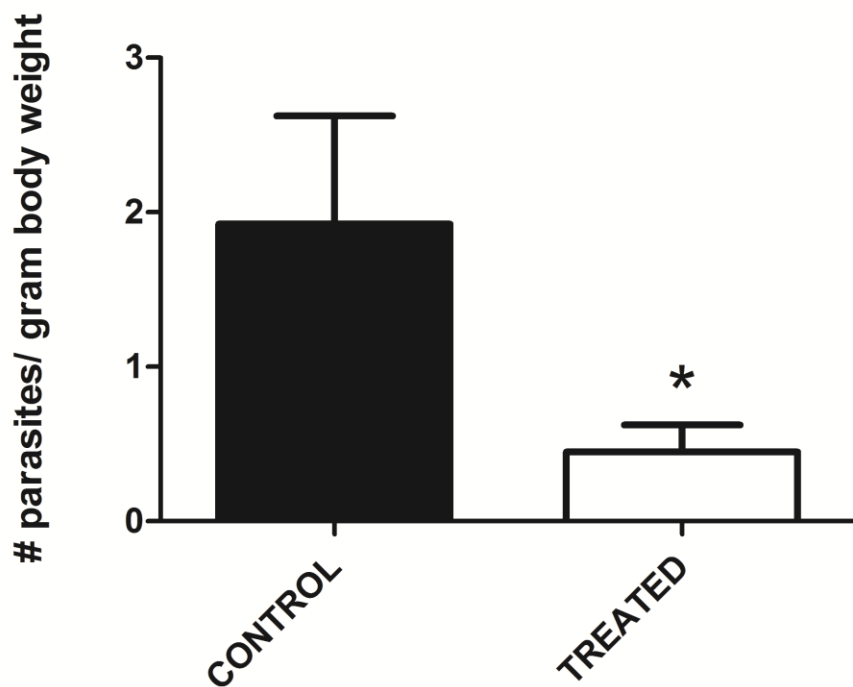


Figure 5.3: Bath treatment of praziquantel results in a significant decrease in the number of *Cleidodiscus* parasites (Bars represent mean parasites counts \pm S.E., *P=0.05).

CHAPTER 6: CONCLUSION

Summary

Platyhelminth parasites are a significant problem in fish and there is a need identify effective treatments for these pathogens. Praziquantel is widely used in both veterinary and human medicine. Several studies have examined the use of praziquantel in fish, however, additional data is needed to fill knowledge gaps regarding the use of this agent for specific parasites and fish hosts. In the studies presented herein, we evaluate the use of praziquantel for monogenean and digenean parasites infecting Centrarchid fish. Our studies revealed that propidium iodide staining is an effective biochemical assay for determining praziquantel-mediated killing of *Posthodiplostomum metacercariae* and that intramuscular administration of praziquantel was an effective treatment for these parasites in bluegill sunfish. These studies also demonstrated that assessment of praziquantel should be conducted at least one week post-treatment in bluegills. We also documented an infestation of *Cleidodiscus* in black crappies and demonstrated how praziquantel can be used to eliminate this parasite.

Recommendations for future research

While many studies have been conducted using praziquantel in fish, there are still several knowledge gaps that still need to be addressed. First, while several treatment regimens have been examined, only a relatively small number of parasites and fish host species have been studied. Going forward, an increased number of fish families should be studied for the pharmacokinetic properties or unintended side effects of praziquantel. In addition, pharmacokinetic and tissue residue studies of praziquantel are necessary to properly determine drug withdrawal times needed for fish intended for human consumption. While

praziquantel does not appear to pose any environmental concerns, additional studies are required to determine the preferred method for the disposal of water containing praziquantel.