Vol. 94: 59–72, 2011
doi: 10.3354/dao02303

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published March 16

OPEN ACCESS

In vitro effect of seven antiparasitics on *Acolpenteron ureteroecetes* (Dactylogyridae) from largemouth bass *Micropterus salmoides* (Centrarchidae)

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ABSTRACT: Few drugs are approved by the United States Food and Drug Administration for treating parasite infections in minor species such as fish, due in part to the high cost of developing such drugs and to a relatively small market share for drug sponsors. Because *in vivo* effectiveness trials for antiparasitic drugs are costly, time consuming, and use many animals, a systematic *in vitro* screening approach to describe parasite motility could help find promising drug candidates. We evaluated the effects of 7 antiparasitics on the activity and survival of the endoparasitic monogenean *Acolpenteron ureteroecetes* (Dactylogyridae) collected from the posterior kidneys of juvenile largemouth bass *Micropterus salmoides* (Lacepede, 1802) (Centrarchidae) held in the laboratory. Tests were conducted in 12 well tissue culture plates; each well had 3 parasites, and we tested 3 concentrations and 1 control for each of the 7 antiparasitics. The parasites were observed immediately after adding the drug, at 1 to 3 h, and 17 to 26 h, and video recordings were made. Drug effects were recorded by documenting morbidity (reduced movement, tremors, contracted body, abnormal morphology) and mortality. *A. ureteroecetes* was strongly affected by the quinoline praziquantel, the imidazothiazide levamisole, and the organophosphates dichlorvos and trichlorfon. The parasites were moderately affected by the macrocyclic lactones ivermectin and emamectin, and generally unaffected by the benzimidazole mebendazole. Our study demonstrates the utility of characterizing *in vitro* responses with video microscopy to document responses of fish parasites for initial screens of drug effects on a fish monogenean.

KEY WORDS: Parasite · Largemouth bass · *In vitro* · Antiparasitic · Bioassay

INTRODUCTION

Aquaculture in the USA has grown into a billion dollar per year industry (USDA 2006). However, despite this growth, domestic aquaculture is still a relatively small segment of US agriculture, and thus is a small market for US Food and Drug Administration (USFDA)-approved drugs. Because of this minimal incentive, and the high cost for the pharmaceutical industry to develop drugs for aquaculture species, there are only 7 drugs approved for farmed fish in the USA. As a result, Congress enacted the Minor Use and Minor Species (MUMS) Animal Health Act in 2004, to establish new regulatory procedures intended to make more medications legally available for the treatment of minor animal species, (meaning species other than major agriculture and companion animals). In response to MUMS, USFDA has conducted a number of studies to

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support the development and availability of drugs to treat bacterial and parasitic infections in minor species.

Parasitic diseases are a significant problem for aquaculture, and can cause morbidity and mortality at all stages of the production cycle. In the USA, only 1 drug is approved by the USFDA (i.e. formalin, with 4 USFDA-approved compounds: Parasite-S, Formalin-F, Formacide-B, and Paracide-F) for control of certain external parasites (ectoparasitic flagellates, ciliates, and monogeneans) on fish, and no drugs are approved for use against internal parasites in fish. This situation is in sharp contrast to that for mammals, for which there are many drugs approved for external and internal parasites. Some antiparasitic drugs approved for mammals may be useful for aquaculture species.

The USFDA, in conjunction with the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products, (VICH), provides guidance documents detailing the methods that should be used to evaluate parasiticides in studies used to support drug approvals in terrestrial animals. However, the USFDA does not currently provide comparable guidance for parasiticides in fish. Our present study is part of a larger effort by USFDA scientists to develop guidelines to evaluate antiparasitic treatments for fish.

We chose to work with the endoparasitic monogenean *Acolpenteron ureteroecetes* Fischthal and Allison, 1940, found in the ureters and kidneys of largemouth bass *Micropterus salmoides* (Fischthal & Allison 1941), because it can easily be propagated in the laboratory (Gieseker et al. 2007). The adult parasite lives in the ureters, passing eggs which can remain in the kidney or be passed out in the urine. The eggs hatch and the larvae can infect new fish or reinfect the original host. Although the infection in wild fish is typically of low density (Fischthal & Allison 1941), in closed aquaculture systems, heavy *A. ureteroecetes* infections lead to losses of juvenile bass (DuPlessis 1948, Petrie-Hanson 2001).

In our previous laboratory experiments, we documented the gradual development of the infection over 3 to 4 mo, and the subsequent plateau of high prevalence and high density infection (Gieseker et al. 2007). Because of its gradual progression, *Acolpenteron ureteroecetes* has the potential to be a suitable *in vivo* model of monogenean infection, since drug effects at different stages of the infection can be investigated. The laboratory system we established also allowed us to develop methods to collect and maintain *A. ureteroecetes in vitro* for up to 30 h, allowing ample time to evaluate treatment effects.

The present *in vitro* study was designed to (1) develop a bioassay for *in vitro* testing of antiparasitics using the endoparastic monogenean *Acolpenteron ureteroecetes*, and (2) use the bioassay to determine the effect of 7 different antiparasitics on activity and survival of this parasite. The compounds represented 1 quinoline, 1 imidazothiazide derivative, 2 organophosphates, 2 macrocyclic lactones and 1 benzimidazole. These compounds were used because they have been reported to be useful for treating fish parasites, and have been listed in veterinary formularies (Stoskopf 1993, Noga 1996, Lewbart 2005). Supplemental materials in the form of video captured motion pictures are available at www.int-res.com/articles/suppl/d094p059_supp/ to demonstrate the effects of the antiparasitic drugs. These videos were evaluated by observation of motion (normal or abnormal/reduced, tremors or contraction) and gross morphology. No morphometric software was used in this study.

MATERIALS AND METHODS

Overview of the experiment. To induce *Acolpenteron ureteroecetes* infections, juvenile largemouth bass were housed in a recirculating aquaculture system that shared water with tanks of infected sub-adult and adult largemouth bass. The juvenile bass were held in the infection system for up to 8 mo, for *A. ureteroecetes* prevalence to reach 75 to 80% (Gieseker et al. 2007). The parasites were collected from juvenile bass early in the infection cycle, since the initial infections yielded higher density infection than did the long-established infections.

After collection, *Acolpenteron ureteroecetes* were transferred to culture plates containing filtered tank water. Drugs of varying concentrations were added, and then the observations began. Control parasites did not receive any drug treatment, but received the appropriate diluents. In preliminary experiments, although control *A. ureteroecetes* lived for at least 48 h, they were vigorous for only 30 h; therefore experimental observations were ended after the first day.

Fish. Two batches of juvenile largemouth bass (n = 200 each) were obtained from a commercial fish farm in New York state. Upon arrival at our laboratory, a subsample of 20 fish from Batch A measured 12.3 ± 2.5 cm long (fork length; mean ± SD) and weighed 20.0 ± 6.5 g, 2 of these fish had very light *Acolpenteron ureteroecetes* infections; a subsample of 5 fish from Batch B measured 9.1 ± 0.9 cm long and weighed 8.0 ± 2.4 g, no *A. ureteroecetes* infections were detected in these fish. Finding occasional *A. ureteroecetes* in the farmed fish was not unexpected, since the parasite is endemic in New York state.

The juvenile fish were given a pellet feed (Rangen) with 41% protein, at 3 to 5% body weight (BW) once daily, for 5 d wk^{-1}. The fish were acclimated in flow-

through aquaria (60 l) for 2 mo prior to being moved into the infection system.

Infection system. A recirculating system consisting of 4 tanks was used to infect experimental fish. One tank contained previously infected sub-adults, and 2 tanks contained previously infected adults. Juvenile fish were added to the fourth tank, which shared water with the other tanks in the system. This system fostered continued infection with *Acolpenteron ureteroecetes*, by allowing parasites to spread via the water. The juvenile bass were held in the infection system for 3 to 8 mo prior to sacrifice for harvesting the parasites. In this time, prevalence reached 75 to 80%, with moderate to heavy density (Gieseker et al. 2007). At harvest, the 117 juveniles were 16.4 ± 2.3 cm long (fork length), and weighed 64.0 ± 28.3 g.

Infected carrier fish. The carrier bass consisted of 40 sub-adults and 23 adults infected during our previous study in which we characterized the time course of *Acolpenteron ureteroecetes* infection (Gieseker et al. 2007). Sub-adult fish were given the same feed as the juveniles, at 1 to 2% BW once a day, 5 d wk^{-1}. Adult fish were fed extruded feed with 35% protein, at 0.5 to 1% BW once a day, 3 d wk^{-1}.

Water quality. Temperature, dissolved oxygen, and pH, were monitored continuously with electronic meters (Models 5300 and 9100, Royce Technologies), and maintained at 20.2 ± 1.3°C (mean ± SD), 7.0 ± 1.3 mg l^{-1}, and 7.4 ± 0.3, respectively.

Antiparasitics. Seven antiparasitics were tested: 1 quinoline, praziquantel (Amtech); 1 imidazothiazide derivative, levamisole (Fluka-Sigma); 2 organophosphates, trichlorfon (Sigma) and dichlorvos (Spelco-Sigma); 2 macrocyclic lactones, emamectin (Intervet/Schering-Plough) and ivermection (Sigma); and 1 benzimidazole, mebendazole (Sigma). The concentrations of most of the drugs we used were based on doses recommended in the literature and in fish health formularies (Buchmann & Bjerregaard 1990, Stoskopf 1993, Noga 1996, Kim & Choi 1998, Hirazawa et al. 2000, Lewbart 2005).

Stock solutions of the drugs. Stock solutions of the drugs were prepared the day before testing, or on the day of testing, using sterile, ambient temperature, vacuum-filtered water (0.2 µm membrane filtration unit, Nalgene) from the infection system. The 3 water-insoluble compounds, emamectin, ivermectin, and mebendazole, were first dissolved in 1 ml of methanol or reagent alcohol, then diluted to the stock concentration with water. All subsequent dilutions of the stock solutions were made with filtered water. Serial dilutions were used to make a working solution of the drug at twice the final concentration needed for each dose level. Reagent blanks, for solvent effects, were tested in the bioassay.

Harvesting parasites. Juvenile fish were euthanatized by severing the cervical spine and double pithing. Using sterile technique, the posterior kidney was removed, and placed in 1 ml of ambient-filtered tank water in a single well (6 ml volume) of a 12-well culture plate. Teasing needles were flamed, and used to pull the kidney into small pieces without excess mincing, which can damage the parasites. The kidney fragments were left in the well plate for 1 to 2 h to allow parasites to migrate out of the tissue. Then sterilized forceps were used to remove the shredded tissue, and the remaining parasites were counted using an inverted microscope (Olympus IX70).

The parasites were transferred to another 12-well plate (3 parasites $well^{-1}$) for the dosing trials, using a 10 µl pipetor with 200 µl tips. The 200 µl tip orifice and 10 µl volume were large enough to move the parasites without damage, while limiting the amount of debris transferred into the new well.

Bioassay. Three drug concentrations and one control were tested in triplicate, for all drugs except mebendazole for which a single dose and control were run in triplicate. The amount of filtered water needed to dilute the working solution (1 ml) was first added to each well of a 12-well culture plate, then 3 parasites were placed in each well. After a 15 to 30 min acclimation, 1 ml of the 2× drug solution was added to each well to achieve the final concentration.

Parasites were examined at 3 time periods: (1) 0 h immediately after adding the antiparasitic to the well (immediate), (2) 1–3 h (early), and (3) 17–26 h (late). In the control wells, at 17–26 h, most parasites were alive, and of normal behavior and appearance, therefore this time period was a suitable cutoff for our observations.

At each time period, each parasite was scored as being normal (not affected), abnormal (affected) or dead. If abnormal, we noted the type of behavior and morphology according to specific criteria we adopted (Table 1). Death was defined as either a lack of movement or presence of obvious autolysis. Short videos were taken of the parasites to document normal movement, and the effect of the antiparasitics. The images were captured with StreamPix 3 digital video recording software (version 3.24.1, Norpix) using an inverted microscope fitted with a digital camera (Olympus 750).

RESULTS

Overview

The exposure effects criteria allowed us to document and quantify the effects of the 7 antiparasitics, and the different doses, at 3 time periods over a 26 h period. We used Time when either abnormal behavior or death

Table 1. Exposure effect criteria for *in vitro* testing of anthelminthics against *Acolpenteron ureteroecetes*

Response	Criteria
No apparent effect	
Normal behavior	Normal movement: Relaxation and contraction with twisting along longitudinal axis Twisting at the anterior and posterior ends of the body Hooklets moving in opisthaptor
Adverse effects	
Abnormal behavior	(1) Reduced movement: Reduced extent and/or speed of relaxation and contraction of body Reduced extent and/or speed of twisting at anterior and posterior ends Reduced movement of hooklets in opisthaptor (2) Tremors (3) Contracted
Abnormal morphology	(1) Indentations or swellings (2) Ringed constrictions (3) Breaks in surface of body
Death	(1) No movement (2) Autolysis

Table 2. Overview of video captures of normal and abnormal behavior and morphology of *Acolpenteron ureteroecetes* during *in vitro* exposures to antiparasitics. Videos are available in the supplement at www.int-res.com/articles/suppl/d094p059_supp/

Subject	Video ID	Duration (s)
Control		
Adult	CONT-1-attaching	0.08
	CONT-2-stretching-rotating	0.18
	CONT-3-rotating-sucker	0.03
	CONT-4-rotating-haptor	0.09
	CONT-5-haptor-hooklets	0.16
Egg	CONT-6-egg-repro tract	0.23
	CONT-7-egg-embryo	0.06
Larva	CONT-8-hatchling	0.31
Response: abnormal behavior		
Slowed motion	RX-1-reduced motion-less stretching	0.25
	RX-2-reduced motion-ends bend	0.09
Contracted	RX-3-contracted-minor body stretch	0.09
	RX-4-contracted-minimal hooklet motion	0.06
Tremors	RX-5-contracted-tremors-2-near dead	0.38
Response: abnormal morphology		
Segmental contractions	RX-6-reduced motion-segmented	0.26

were observed, coupled with Number of parasites affected, to characterize the antiparasitic effectiveness. The susceptibility of *Acolpenteron ureteroecetes* to the 7 antihelminthics was summarized as (1) intense effect: praziquantel, levamisole, dichlorvos, trichlorfon; (2) moderate effect: emamectin, ivermectin; or (3) no observable effect: mebendazole.

Bioassay

No apparent effect

We assumed that normal parasite behavior and morphology indicated no response to the drug, that is to say, that the parasite was not susceptible to the drug. Normal behavior of the control monogeneans was documented using video microscopy (Table 1 and videos 'CONT-1 to CONT-8' listed in Table 2; all videos are available in the supplement at www.int-res.com/articles/suppl/d094p059_supp/). Normal behavior consisted of moderate to vigorous activity, including attaching at anterior and posterior ends of the body, relaxation and contraction with twisting along the longitudinal axis of the body (Videos 'CONT-1-attaching' and 'CONT-2-stretching-rotating'), twisting at the ends of the body (Videos 'CONT-3-rotating-sucker' and 'CONT-4-rotating-haptor'), and hooklets actively moving in the opisthaptor (Video 'CONT-5-haptor-hooklets') (Tables 1 & 2).

Additional videos of the control parasites show the position of eggs within the parasite's uterus, motion of embryos within eggs, and swimming of the hatchlings (oncomiracidia) (Table 2; videos 'CONT-6-egg-repro tract', 'CONT-7-egg-embryo' and 'CONT-8-hatchling').

Adverse effects on behavior, morphology and survival

Abnormal behavior included reduced extent and speed of the normal relaxation, contraction, and twisting; and unusual movements namely contractions and tremors (Table 1, videos 'RX-1' to 'RX-5' in Table 2).

An additional manifestation of response was abnormal morphology, with indentations, swellings, ringed constrictions, and breaks in the surface of the body, or surface blebs (Tables 1 & 2, Video 'RX-6-reduced motion-segmented').

The parasite was assumed to be dead when there was no visible movement, and/or it was autolysed or had some other major physical damage (Table 1).

Response to the antiparasitics

Controls

Most of the controls had normal behavior at all 3 time periods. A few control parasites (11 of 90) had slowed motion during the late observation periods. Deaths were very rare (6 of 90), and if seen, they usually occurred at the late time period. Control parasites never developed contracted morphology or tremors.

Some of the worms we used laid eggs *in vitro* during the observation period. In a few cases, the embryos could be seen moving within the eggs (Video 'CONT-7-egg-embryo') Some of the control eggs hatched after the 26 h point. The hatching oncomiracidia typically swam vigorously throughout the water column (Video 'CONT-8-hatchling'). Since only few oncomiracidia were observed, no conclusions could be made regarding expected survival time.

Treatments

We observed that *Acolpenteron ureteroecetes* was immediately affected by some doses of praziquantel and levamisole *in vitro* (Table 3). When exposed to praziquantel at 5, 10, and 100 ppm, all parasites immediately showed abnormal behavior. The parasite's movements were slowed, they would contract to approximately half of their normal body length, and often developed full body tremors as they relaxed during the immediate and early observation periods (Video 'RX-5-contracted-tremors-2-near dead'). At the late time period, parasites no longer contracted, but only moved the anterior and posterior (opisthaptor) ends (Video 'RX-2-reduced motion-ends bend'). When exposed to praziquantel at 500 and 1000 ppm, the parasites developed immediate tremors with moderate contraction, and all were dead at 1–3 h.

Parasites exposed to levamisole had no observable response at the low con-

Table 3. *Acolpenteron ureteroecetes*. *In vitro* effects of 9 antiparasitics at 3 time periods. Nine parasites were tested for each treatment dose. The number of parasites affected at each time period are listed. If mortalities occurred, then the numbers are listed as no. of affected parasites/no. of remaining parasites. AD = all dead

Drug	Conc. (ppm)	Immediate (0 h)		Early (1–3 h)		Late (17–26 h)	
		Abnormal behavior	Dead	Abnormal behavior	Dead	Abnormal behavior	Dead
Praziquantel							
Trial a	0	0	0	0	0	1	0
	5	9	0	9	0	8/8	1
	10	9	0	9	0	9	0
	100	9	0	9	0	9	0
Trial b	0	0	0	0	0	1/8	1
	100	9	0	9	0	3/8[a]	1
	500	9	0	AD	AD	AD	AD
	1000	9	0	AD	AD	AD	AD
Levamisole							
Trial a	0	0	0	0	0	0	1
	0.1	0	0	1	0	0	0
	1	0	0	0	0	0	0
	10	5	0	1	0	3	0
Trial b	0	0	0	0	0	2/6	3
	10	9	0	4/7	2	6/6	3
	50	9	0	7	0	4/4	5
	100	9	0	5	0	2/2	7
Trichlorofon							
	0	0	0	0	0	0	0
	0.25	0	0	3	0	2/2	7
	0.5	0	0	5	0	4/4	5
	1	0	0	9	0	2/2	7
Dichlorovos							
	0	0	0	0	0	0	0
	0.25	0	0	0[b]	3[b]	AD	AD
	0.5	0	0	6	0	AD	AD
	1	0	0	2/2	7	AD	AD
Ivermectin							
	0	0	1	2/8	1	4/8	1
	10	0	0	1	0	8/8	1
	50	0	0	1	0	4/4	5
	100	3	0	1/6	3	2/2	7
Emamectin							
Trial a	0	0	0	0	0	0	0
	0.1	1	0	1	0	0	0
	1	0	0	0	0	0	0
	10	2	0	5	0	5/5	4
Trial b	0	0	0	0	0	0	0
	10	0	0	1/1	8	1/1	8
	50	1	0	2/2	7	AD	AD
	100	2	0	2/2	7	AD	AD
Mebendazole							
	0	0	0	0	0	3	0
	100	8	0	0	0	3	0

[a] At 17–26 h, parasites had surface blebs
[b] Three parasites impaired at 1 h but dead by 2 h

centrations of 0.1 and 1.0 ppm. However, doses greater than 10 ppm caused all parasites to immediately contract fully and stop moving (0 h). Some worms in the 10 ppm dose group were less contracted and moving slowly again by 1–3 h, compared to the worms exposed to 50 and 100 ppm which were barely moving. By the late observation period, all the treatment dose worms were either moribund with minimal intermittent motion in the opisthaptor or dead.

The organophosphates, trichlorfon and dichlorvos, also caused significant morbidity and mortality at dosage ranges of 0.25 to 1.0 ppm. No immediate response was noted to trichlorfon or dichlorvos at any of the doses. During the early observation period, the response to trichlorfon was a dose-dependent reduction in motion, with less stretching (Videos 'RX-1-reduced motion-less stretching' and 'RX-2-reduced motion-ends bend'). By the later observation period, some of the parasites were still alive, but their motion was severely impaired. These parasites had very contracted bodies, subtle body movement and occasional hooklet motion (Video 'RX-3-contracted-minor body stretch'). Some unusual peristaltic-like motion was observed with trichlorfon (Video 'RX-6-reduced motion-segmented').

Parasites treated with dichlorvos showed a dose response at the early time period, with the worms exposed to 1 ppm extremely contracted with minimal motion (Video 'RX-4-contracted-minimal hook movement'). By the late time period, all worms were dead, and contracted.

Acolpenteron ureteroecetes was also affected by the avermectins, emamectin and ivermectin. Responses to these drugs were less intense than the responses to praziquantel, levamisole, and the organophosphates. Parasites exposed to emamectin had no apparent response at 0.1 ppm and 1.0 ppm doses. Some parasites exposed to 50 and 100 ppm slowed immediately, and only slight movements were seen. By the end of the early observation period, most of the 10, 50 and 100 ppm parasites were dead, and the survivors had very limited movement. All emamectin treated parasites were dead by the late observation period.

There was generally no immediate response to ivermectin. A few of the parasites exposed to 100 ppm ivermectin were dead at the early time period, and all parasites exposed to ivermectin at 10, 50, and 100 ppm had little movement by the late time period, with over half dead in the 50 and 100 ppm concentration.

Some of the *Acolpenteron ureteroecetes* exposed to 100 ppm mebendazole contracted initially and slowed their movement. However, these parasites had normal motion by the early observation period of 1–3 h, and continued to move normally at the later observation period, 17–26 h.

DISCUSSION

In vitro tests for antiparasitics

There are currently no drugs approved by USFDA to treat internal fish parasites and only 1 drug approved to treat external parasites. This is partially due to the fact that there are relatively few resources available for developing and testing drugs for minor and aquaculture species. One approach to reduce costs and numbers of experimental animals when evaluating potential drugs for use in fish is to conduct *in vitro* screens of approved terrestrial drugs against fish pathogens to find out which compounds are worthy of further study *in vivo*.

Evaluating the effectiveness of drugs to rid fish of parasites is a challenging task, especially if the parasites are internal. External parasites can be enumerated using non-lethal techniques such as skin smears and gill biopsies. Determining internal parasite load, however, usually requires sampling vital organs, thus sacrificing the fish. The study of the effects of a variety of different antiparasitics against internal parasites *in vivo* requires using an adequate number of fish to be certain that a high percentage of animals are infected. In addition, enough animals need to be tested to ensure that significant differences in parasite load can be demonstrated between treated and untreated fish. An *in vitro* study design can help reduce the number of experimental animals since the parasites can be obtained from relatively few fish. The *in vitro* study design allowed us to characterize changes in parasite behavior indicating a drug effect, the time course to abnormal behavior and time to parasite death. Such parameters would be very hard to determine *in vivo*.

In our study, *Acolpenteron ureteroecetes* survived *in vitro* with relatively normal activity up to about 30 h, which provided adequate time to assess the effects of antiparasitics. Freshly collected *A. ureteroecetes* are very active, rapidly extending and contracting their bodies. Alterations of their normal movement provided a good marker of drug effect.

In vitro tests have previously been used to evaluate the resistance of parasites to various therapeutic agents. *In vitro* egg hatch assays have been used to measure resistance both experimentally (Martin et al. 1989) and as a field diagnostic aid (Le Jambre 1976, Ihler & Bjørn 1996, Geerts & Gryseels 2000). An *in vitro* larval development test (LDT) has been used to evaluate anthelmintic resistance in multiple mammalian species (Coles et al. 1988, Praslicka et al. 1997, Königová et al. 2003, Várady et al. 2006, 2009) and has been included in the guidelines of the World Association for the Advancement of Veterinary Parasitology (WAAVP) (Coles et al. 1992).

Motility as an endpoint has been used to characterize effects of drugs on adult trematodes (Saowakon et al. 2009), nematodes (Bowen & Vitayavirasak 2005, Buckingham & Sattelle 2009, Ardelli et al. 2009) and copepods (Sevatdal et al. 2005, Westcott et al. 2008). Although sophisticated programs have been used to analyze helminth motility (Tsibidis & Tavernarakis 2007, Restif & Metaxas 2008, Tsechpenakis et al. 2008, Buckingham & Sattelle 2009), such an approach may not be feasible for screening antihelminthics for aquaculture, due to limited resources. Basic observational techniques, such as those we used, can still provide valuable data on susceptibility of a parasite to a drug.

Overview of drug effects

We tested 7 different drugs, from 5 different classes, in our *in vitro* screen. The quinoline, imidazothiazide, and organophosphate compounds had the most deleterious effect on motility and survival. The macrocyclic lactones had a moderate effect, while the benzimidazole generally had no effect.

Praziquantel

Acolpenteron ureteroecetes was sensitive to praziquantel in our assay. We observed immediate contraction with slowed movement and whole body tremors at all of our doses (5 to 1000 ppm); mortality only occurred at the 500 and 1000 ppm. In previous studies on the use of praziquantel with fish parasites, the most commonly reported gross effects were on motility via contractions and/or tremors (Bylund et al. 1977, Hirazawa et al. 2000). In the monogenean *Heterobothrium okamotoi*, there was immediate contraction upon *in vitro* exposure to 20 ppm praziquantel, although tremors were not reported (Hirazawa et al. 2000). In plerocercoid cestode larvae from fish, Bylund et al. (1977) observed peristaltic muscle contractions at 10 to 300 ppm praziquantel, and mortality at the highest doses (600 to 700 ppm). Tegument damage (vacuolization) has only been observed with electron microscopy (Schmahl & Mehlhorn 1985, Schmahl & Taraschewski 1987).

Praziquantel, a pyrazinoisoquinoline drug, is widely used to treat parasitic infections in fish, animals and humans. For many species of bony and cartilaginous fish, praziquantel is used to treat monogenean and cestode infections (Bylund et al. 1977, Schmahl & Mehlhorn 1985, Sanmartín Durán et al. 1989, Kim et al. 1998, Hirazawa et al. 2000, 2004, Kim & Cho 2000, Chisholm & Whittington 2002, Janse & Borgsteede 2003, Onaka et al. 2003, Mitchell 2004, Sitjà-Bobadilla et al. 2006). In dogs, cats, horses, and ruminants, this drug is used to treat trematodes, cestodes, and nematodes (Johansen et al. 1996, Jenkins & Romig 2000, Genchi et al. 2004, Ghazaei 2007, Grandemange et al. 2007, Slocombe et al. 2007), and in humans, praziquantel is a primary therapy for schistosomiasis (Tracy & Webster 2001).

Praziquantel causes paralytic muscle contractions and tegument damage in parasites from fish and mammals, via an influx of calcium, which disrupts calcium homeostasis (Bylund et al. 1977, Schmahl & Mehlhorn 1985, Schmahl & Taraschewski 1987, Day et al. 1992, Redman et al. 1996, Hirazawa et al. 2000). However, the influx of calcium is not correlated with death of the worms in the case of schistosomes (Pica-Mattoccia et al. 2008).

Levamisole

In our study, *Acolpenteron ureteroecetes* contracted immediately upon contact with levamisole at doses of 10, 50, and 100 ppm; some parasites died within 3 h, and all were dead by 17–26 h. Levamisole had a similar *in vitro* effect on 3 ectoparasitic monogeneans from fish: in H*eterobothrium okamotoi*, 20 ppm caused the parasite to contract and fall off gill arches (Hirazawa et al. 2000), in *Diplozoon paradoxum*, 10 to 50 ppm reduced the movement and damaged the tegument (Schmahl & Taraschewski 1987), and in *Gyrodactylus aculeati*, exposure to 10 to 50 ppm reduced motility (Schmahl & Taraschewski 1987).

Levamisole, a synthetic imidazothiazide, is used in fish and in a wide variety of animals to control parasitic nematodes (Thienpont et al. 1966, Jacobs 1987, Williams et al. 1991, Williams & Broussard 1995). It has been investigated as a possible treatment for monogeneans (Schmahl & Taraschewski 1987, Hirazawa et al. 2000) and is also used in pet aquarium medicine to treat nematodes (Harms 1996). Levamisole may be effective as a bath treatment but there is a rather small margin of safety. At 100 ppm, levamisole caused toxicity in sticklebacks *Gasterosteus aculeatus* (Schmahl & Taraschewski 1987). Adult fish died after a 25 min bath exposure, but juvenile fish were able to recover. Although it is effective *in vitro* and in baths, levamisole administered in feed has not proven effective to control monogeneans (Hirazawa et al. 2000, Schalch et al. 2009).

Levamisole acts on nematode muscles by stimulating nicotinic acetylcholine receptors on the muscle membrane, causing prolonged membrane depolarization resulting in sustained muscle contractions (Harrow & Gration 1985, Martin 1997). Levamisole (and related compounds pryantel and morantel) acts on the same

ion channels as acetylcholine (Harrow & Gration 1985, Pinnock et al. 1988, Levandoski et al. 2003).

Organophosphates

Although *Acolpenteron ureteroecetes* did not immediately respond to the organophosphates, a dose response was observed within 3 h at concentrations between 0.25 and 1 ppm. Affected parasites had slowed movement; at lower doses parasites were still able to move their entire bodies, but at higher doses the severely affected parasites were only moving their haptors. By 24 h, *A. ureteroecetes* was either severely impaired (only moving individual haptor hooklets) or dead at all doses. All of the parasites exposed to dichlorvos were dead by 24 h, and most of those exposed to trichlorfon had also died.

Acolpenteron ureteroecetes was sensitive to both dichlorvos and trichlorfon at levels similar to those recommended (0.25 to 1 ppm) for prolonged immersion (Imada & Muroga 1979, Noga 1996). Imada & Muroga (1979) found that 0.5 to 1.0 ppm trichlorfon (24 h) significantly decreased the prevalence of *Pseudodactylogyrus microchis* on eels *Anguilla anguilla*, although other researchers could not reproduce this effect (Buchmann et al. 1987). In addition, several *Gyrodactylus* species (*G. elegans, G. aculeati*) have also been found to tolerate therapeutic levels of trichlorfon, either from intrinsic or developed tolerance (Goven et al. 1980, Schmahl & Taraschewski 1987). An *in vitro* exposure to trichlorfon (Neguvon®) or dichlorvos (Nuvan®) affected cholinesterase activity in *Pseudodactylogyrus anguillae*. Trichlorfon did not immobilize the parasites completely at 100 ppm, but dichlorvos did at concentrations as low as 1.5 ppm (Buchmann & Mellergaard 1988). In the present study, *A. ureteroecetes* was sensitive to much lower dosages of organophosphates (0.25 to 1 ppm) compared with the higher dosages of praziquantel and levamasole (10 to 100 ppm). However, the toxicity of the drug to the host must be considered when evaluating the response of parasites to drugs *in vitro* and *in vivo*. For example, eels developed spasms when exposed to 5 ppm trichlorfon, suggesting a tolerance limit (Buchman et al. 1987).

Both trichlorfon and dichlorvos have been recommended for treating external monogenean infections in fish (Stoskopf 1993, Noga 1996), but researchers have found varied sensitivity to these drugs (Imada & Muroga 1979, Goven et al. 1980, Schmahl & Taraschewski 1987, Buchmann et al. 1987). Both drugs have also been used to control sea lice on salmon farms (Brandal & Egidius 1979), and to eradicate anchor worm *Lernaea* sp. larvae (Noga 1996, Tonguthai 1997).

In addition, trichlorfon and dichlorvos are used in a variety of animals to control parasitic nematodes. Dichlorvos is also used in swine and dogs for intestinal and lung nematodes (Robinson 1979), and both of these organophosphates are used in horses against strongyle nematodes and bot fly larvae (Drudge et al. 1984).

Organophosphates inhibit acetylcholinesterase thereby blocking degradation of the neurotransmitter, acetylcholine, disrupting motor neurons (Martin 1997). The mode of action is not specific and therefore can have toxic effects to the host and other non-target organisms (Egidius & Møster 1987, Le Bris et al. 1995).

Avermectins

In our study, ivermectin and emamectin caused abnormal behavior at 10, 50 and 100 ppm in the first hour. Activity was slowed, and the worms did not relax to a fully extended length, but remaining approximately half of their fully extended length, and movement was restricted to only the prohaptor and haptor regions. Worms exposed to 50 or 100 ppm were severely compromised or dead by the late time period.

Similar results have been found in other parasites. The copepod, *Lepeophtheirus salmonis,* exhibited uncoordinated movement and inability to attach to the substrate when exposed to emamectin *in vitro* (Westcott et al. 2008). Two main morphologic or behavioral effects have been observed when avermectins are applied to nematodes *in vitro*: (1) inhibited pharyngeal pumping, followed by a generalized, rapid paralysis (Geary et al. 1993, Gill et al. 1995, Keane & Avery 2003). Once the parasites are unable to move or feed, they are eliminated from the host (Geary et al. 1993, Gill & Lacey 1998). Mid-body paralysis following administration of ivermectin to adult *Haemonchus contortus* nematodes *in vitro* was reported by Geary et al. (1993). Reduction in ingestion or pharyngeal pumping, however, occurred at a much lower dose than that which inhibited motility. Gill et al. (1995) exposed *H. contortus* larvae to high and low doses of ivermectin. High doses of ivermectin caused jerky angular motions in larvae soon after exposure. Larvae exposed to low doses had normal activity for up to 36 h of exposure, when motility became sluggish. Development to the next stage, however, was inhibited at the low dose of ivermectin even if motility was normal. Rapson et al. (1985) also found larval development was affected at a much lower dose than that affecting motility in *Trichostrongylus columbriformis*. In contrast, Ardelli et al. (2009) noted that, *in vitro*, the non-parasitic nematode *Caenorhabditis elegans* exhibited a period of hyperactivity between 1 and 2 h after treatment with 2.5 nM

or 5 nM, but not 10 nM (4.3, 8.5, and 17 ppb, respectively) ivermectin. After 2.5 h, however, 67% of worms stop all activity.

Avermectins are currently used in fish medicine. Emamectin benzoate is a semi-synthetic avermectin approved by the EU and Canada to control sea louse *Lepeophtheirus salmonis* affecting salmonids (EMEA 2003, Lees et al. 2008; see also Health Canada — www.hc.sc.gc/dhp-mps/consultation/vet/consultations/past-anterieures/residu/2009-mrl-lmr-vdd-dmv/lett-eng.php). Emamectin has also been shown effective against another fish ectoparasite, the lernaeapodid copepod *Salmincola californiensis* (Roberts et al. 2004), and may be useful for *Lernanthropus kroyeri* infections of sea bass (Athanassopoulou et al. 2009).

Avermectins are effective against a variety of parasites in domestic animals and humans (Lasota & Dybas 1991). Ivermectin is widely used in veterinary medicine against heart worms (*Dirofilaria immitis*) in dogs and cats (Bowen & Vitayavirasak 2005), and is also used to treat many internal and external parasites of cattle, pigs, horses, (Williams & Plue 1992, Geurden et al. 2003, Santarém et al. 2005, López-Olvera et al. 2006, Lyndal-Murphy et al. 2010), and even ostriches (Geurden et al. 2009).

Avermectins are macrocyclic lactones which increase the membrane permeability of invertebrate nerves and muscles to the chloride anion (Cl^-) (Fritz el al. 1979, Martin 1997). The action appears to be on a glutamate-gated Cl^- channel (Cully et al. 1994, Ardelli et al. 2009), which is composed of several subunits (Wolstenholme & Rogers 2005, McCavera et al. 2009). This mechanism is well documented in adult parasites, but the affect on microfilaria has not been elucidated as they appear unaffected *in vitro*, yet disappear rapidly from the blood or skin of treated animals (Geary et al. 2010).

Benzimidazoles

In our study, *Acolpenteron ureteroecetes* was generally not susceptible to mebendazole. Although this drug caused some contraction immediately after administration, the parasites returned to normal activity within the first hour. We also noted this lack of responsiveness in a preliminary evaluation of the benzimidazoles albendazole and ricobendazole (data not shown). Those drugs were tested at much lower dosages, 0.025 to 1.0 ppm, based on residue levels of albendazole and its metabolites found in fish tissues (Shaikh et al. 2007). No adverse response was observed to either of these benzimidazoles at those doses. Based on our *in vitro* findings with mebendazole at the higher dose, we expect benzimidazoles to be relatively ineffective *in vivo*. However, benzimidazole *in vitro* responses do not always reflect *in vivo* responses (Santamarina et al. 1991, Tojo et al. 1992). There may be effects on reproduction of the parasite that are not identified *in vitro*, which could explain the clinical response seen in some studies.

In fish, benzimidazoles have been tested primarily against the platyhelminths. For example, bath treatment with mebendazole was effective on the gill monogenean *Gyrodactylus elegans* but not on *Dactylogyrus vastator* (Goven & Amend 1982). Mebendazole was ineffective treating *Microcotyle* sp. in red porgy *Pagrus pagrus* (Katharios et al. 2006), but showed some efficacy on *Gyrodactylus* sp. of trout *Oncorhynchus mykiss* (Tojo et al. 1992) with prolonged (12 h) but not short bath treatments (1 to 3 h). Benzimidazoles have also been tested against protozoan, microsporidian, and nematode parasites of fish. Triclabendazole caused reduction in the infection degree and trophont size of the ciliate *Ichthyophthiriosis* sp. in rainbow trout when administered orally (Rodriguez & Fernandez 2001, Luzardo-Álvareza et al. 2003). Several benzimidazoles markedly reduce spore infectivity in the microsporidian *Glugea anomala* of sticklebacks *Gasterosteus aculeatus* (Schmahl & Benini 1998). Mebendazole and other benzimidazoles were ineffective against *Anguillicola crassus*, the air bladder nematode of eels (Taraschewski et al. 1988).

In the case of external parasites, *in vitro* testing might be considered to be very similar to *in vivo* treatment since the parasite is exposed to the drug primarily via a surface route (bath). An *in vivo* response, however, does not always correspond to *in vitro* results. For example, in eels, *Pseudodactylogyrus* spp. responded to *in vivo* treatment with mebendazole (Szekely & Molnar 1987, Mellergaard 1990, Buchmann 1993), however *in vitro* experiments showed different drug-susceptibility of *P. bini* and *P. anguillae* (Buchmann & Bjerregaard 1990). Some inhibition of parasite egg development was also observed *in vitro*. Similarly, different responses were noted between *in vivo* and *in vitro* results for benzimidazoles tested on *Gyrodactylus* sp. from trout (Tojo et al. 1992, Tojo & Santamarina 1998).

Benzimidazoles have a broad spectrum of activity against various parasites (Sharma & Abuzar 1983, McKellar & Scott 1990, Williams & Broussard 1995). Used throughout the world against gastro-intestinal and respiratory nematodes of livestock, some benzimidazoles are also effective against trematodes and cestodes (Schmidt 1998, Robinson et al. 2002, Keiser et al. 2005). Benzimidazoles are also used to treat parasites of companion animals, including protozoan infections such as *Giardia* sp. (Barr et al. 1993, Xiao et al. 1996) and nematodes such as *Toxocara* species (Parsons

1987, Fisher et al. 1993). Microsporidial infections in domestic animals and humans have also been treated using this class of drugs (Dore et al. 1995, Didier et al. 2005).

Although the mechanisms of action of these heterocyclic compounds are not fully understood, benzimidazoles bind the β-tubulin molecule, similar to the 'capping' process of colchicine and vincristine (McKellar & Scott 1990, Lubega & Prichard 1991a,b, Martin 1997), thereby disrupting cell functions such as cell division and transport. Subsequent effects on the rate and turnover of cellular process are thought to inhibit egg hatching (Lacey et al. 1987). Furthermore, interference with apical secretory vesicle transport causes release of digestive enzymes into the intestinal wall of nematodes such as *Haemonchus contortus* (Shompole et al. 2002). In cestodes, the disruption of microtubule formation reduces glucose uptake, followed by reduced glycogen content and tegument surface lesions (Schmidt 1998, Cumino et al. 2009).

The importance of tubulin binding by benzimidazoles has been demonstrated by the finding that parasites that are resistant to these drugs have mutations in the β-tubulin gene (Kwa et al. 1995, Höglund et al. 2009, Lake et al. 2009, Rufener et al. 2009). *In vitro* studies of benzimidazole resistance have used a tubulin-binding assay as well as egg hatch assays (Johansen & Waller 1989, Martin et al. 1989).

Critique of our study

Our study, using 3 concentrations and one control per drug, and 3 time periods, allowed us to screen 7 antiparasitics, and determine the susceptibility of *Acolpenteron ureteroecetes* to them. By using a combination of criteria (abnormal behavior and death) at the different time periods, we divided the effects of the drugs on the parasites into 3 categories: large effect, moderate effect, and no observable effect.

Future refinements to similar studies could include more time points and more parasites to obtain a dataset large enough for statistical analysis. The exact number of parasites per concentration per time point would depend on the variability of the response to treatment to different parasiticides. This could be determined with preliminary studies. We also recommend an additional time point in between the 1–3 h and the 17–26 h time periods, with a consistent narrow time window for each time point, for example 2 h, for a more definitive study.

We suggest that some of the variation in our dataset may have reflected differences in the ages of the worms. This phenomenon has been documented in previous trails of the effects of praziquantel on schistosomes, in which exposure causes immediate contraction, but death depends on the maturity (and sex) of the worms (Pica-Mattoccia & Cioli 2004).

CONCLUSION

This is the first report of avermectin parasiticides having an effect on a monogenean parasite. The responsiveness to emamectin may develop into an additional use for this drug in aquatic species, since it is widely used in salmonids to treat sea lice. *Acolpenteron ureteroecetes* was also sensitive to praziquantel, levamisole, dichlorvos, and trichlorfon at dose levels similar to those seen in other monogeneans that have no intrinsic or developed tolerance. These drugs, therefore, are potential candidates to test *in vivo*. The potential dosages used *in vivo*, however, would depend on several factors such as route of administration, absorption, and the ability of drug to get to the site of infection at an effective concentration.

In addition to evaluating potential treatments for *Acolpenteron ureteroecetes*, this study provides an example of how observations can be systematically used to screen for drug effects *in vitro*. Such an approach can evaluate subtle changes that cannot be observed *in vivo*, particularly for internal parasites. Motility is an important parameter to include in the analysis because parasites must migrate on or within the host, or between hosts to continue their life cycle. Activity, along with other physiological or reproductive parameters, is commonly used to determine potentially successful parasiticide candidates, or to monitor the development of resistance to treatments. A clearly defined set of observational characteristics can help reduce inter-observer error (Westcott et al. 2008). Capturing video images of normal and abnormal motility can also help reduce such errors, since the videos can be used both to train observers, and to record findings so that multiple observers can evaluate the same results. Videos can also be used to document unusual responses. Documenting such findings can help define a wider range of responses that may be observed less frequently, and thus may have been under-reported in past studies.

Some researchers studying parasites of major public health concern such as schistosomes have used elaborate video and computer measurement systems to analyze the effect drugs have on parasite activity. Because such systems may not be readily available to fish researchers, a more classic, observational approach with simple video capture to document findings, as used in this study, can be useful for generating data on the response of parasites to candidate drugs.

Acknowledgements. We thank C.-S. Cheely and N. Hasbrouck for assisting in harvesting parasites and conducting the bioassays.

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Editorial responsibility: Dieter Steinhagen, Hannover, Germany

Submitted: July 23, 2010; Accepted: October 15, 2010
Proofs received from author(s): February 18, 2011