Working towards optimising oral praziquantel for treating monogenean ectoparasites of captive fishes

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[Slide 1] Introduction: Monogenea

Monogenea are parasitic flatworms parasitic on teleosts, chondrichthyes, certain aquatic reptiles and amphibians, and 1 mammal host, the Hippo. The Hippo is parasitized by the monogenean *Oculatrema hippopotami* which attaches itself to the eye of the Hippo.

There are between 4000-5000 described species of Monogenea (Whittington 2005) and these are highly specialised and can be separated into two subclasses: [Slide 2] Polyopisthocotylea and Monopisthocotylea. Characteristic of all monogeneans is their posterior attachment organ or haptor (opishaptor) which can be separated into multiple attachment units and can be asymmetrical or symmetrical in the Polyopisthocotylea, or it can be a single symmetrical unit often divided into numbers of loculi as in the Monopisthocotylea. The attachment structures characteristic of the Polyopisthocotyleans includes suckers and clamps containing "skeletal" (sclerotized) structures to maintain shape and rigidity. Monopisthocotyleans have hooked structures and/or an often elaborate array of modified haptoral structures for enhanced attachment. Monopisthocotyleans also possess often elaborate anterior adhesion glands which facilitates attachment during locomotion. Polyopisthocotyleans are found in the gill tissue, branchial chamber and buccal cavity of fishes where they fed on blood. They are generally sedentary, so they don't move around much once established on the tissue, and they are oviparous (egg-laying). Monopisthocotyleans are parasites of the skin, the gills, branchial chamber, buccal cavity, fins, nasal fossae, eyes, urogenital system, body cavity, digestive tract, heart muscle and blood vessels. Despite these exceptions, monogeneans are still considered ectoparasitic. Monopisthocotyleans are epithelium and mucus feeders (grazers) and are generally highly motile. Most of them are oviparous but some like members of the genus *Gyrodactylus* are viviparous and produce live well-developed young. [Slide 3] Monogeneans have a direct life-cycle. The name "Monogean" means "one generation." They have the ability to increase rapidly in numbers on captive fishes because they do not require intermediate hosts. What's more is that the increased numbers of fishes (potential hosts) in captivity and the reduced spatial arrangement lends itself to an increased success of larvae finding a host. Monogeneans are generally considered to be species-specific which is a co-evolutionary adaptation including niche-reservation. However, given the changes in infection pressure in a captive environment monogeneans do have the ability to host switch to closely related species of the same host genus and therefore exhibit stenoxenic specificity in some cases. Monogeneans in large enough numbers are known to cause disease. Lesions caused by feeding worms can often be secondarily infected by opportunistic bacteria.

[Slide 4] Introduction: Praziquantel

Praziquantel is an anthelmintic developed by Bayer in Germany in the 1970s (actually 1975). It was originally developed to treat Schistosomiasis and cestode infections in humans but it has also been used for other diseases of veterinary importance. It has been used to treat fishes in public aquaria with varying degrees of success, but its scientific development in public aquaria has stagnated in comparison to aquaculture.

[Slide 5] Now, if we look at the top 20 cited papers on the use of praziquantel to treat fishes on the internet, it is not surprising that only a handful of these originate from or concern public aquaria.

[Slide 6] The reason for this may be three-fold:

- Praziquantel is not registered for use in fish in many countries
- Registered products are often registered for specific finfish species based on efficacy and tissue retention times
- Research in aquaculture is fuelled by the necessity for profit

Empirical data from research into the drug's efficacy in many fish species is lacking, and public aquaria rely nearly exclusively on anecdotal information obtained from our shared experiences.

[Slide 7] Praziquantel usage in public aquaria: important points to consider

We still treat monogeneans in public aquaria as a unit (as "flukes")
With diversity in species come differences in their biology, ecology, susceptibility to treatments, reinfectability (host-switching) and their pathogenicity.
Since 2008, 17 new species representing 8 genera, two of which are new, and 5 monogenean families were encountered at Two Oceans Aquarium and uShaka Sea World (Durban) alone. Eight of these have since been published. Most of these were benign. However, two species were significantly pathogenic, but it was the publication of one species in particular (*Heterocotyle tokoloshei*) which made us challenge the thinking behind our approach to treating monogeneans in public aquaria.

[Slide 8] If we look at the specific case study involving this species, a large female *Dasyatis brevicaudata* exhibited laboured breathing and spasms and had become lethargic on exhibit and was refusing to feed. She was removed from the exhibit to a 4000L quarantine tank where we checked for the presence of monogeneans. This was confirmed with the presence of thousands of tetrahedral monogenean eggs which were collected from the bottom of the quarantine tank. Initially, the ray was treated with 20mg/L praziquantel for 12 hours following the results of the *in vitro* trial of Chisholm and Whittington (2002). 3084 worms and numerous eggs were recovered after this treatment but 24 hours later, we were still recovering thousands off freshly-produced eggs. So, the ray was anaesthetised with 2-Phenoxyethanol and intubated with praziquantel at 150mg/kg following parts of the work done by Hirazawa *et al.* (2004) on spotted halibut and Williams *et al*. (2007) on yellowtail kingfish. 12 hours post-treatment, approximately 392 000 worms and thousands of eggs were recovered. 24 hours post-treatment, 3383 worms but no further eggs were recovered, and 48 hours to 10 days post-treatment no further worms or eggs were recovered.

[Slide 9] Why?

Why had the oral treatment been so successful, but why had the bath treatment failed?

Monogeneans must be in direct contact with the praziquantel concentration for it to be successful. Monogeneans can react by withdrawing between tissue (Chisholm and Whittington 2002) and/or the stereotypic reaction of the gill tissue provides significant protection to the worms from exposure to the drug. Gills generally respond to infection and injury in a limited number of ways. There is often an infiltration of haemocytes into the tissue, causing an inflammatory response. Progenitor cells at the base of the lamellae proliferate epithelium which then migrates up the lamella and in the process many cause fusion of the secondary lamellae or the primary lamellae in severe cases, or even the entire arch in extreme cases. It is this response which can also envelop attached monogeneans which sit between the lamellae. So, in a way, the response of the host itself ironically serves as protection for the parasite.

So, why 150mg/kg then?

Sarah Poynton et al. (1997) used oral prazi at 3, 8, 15, 19mg/kg to treat captive Lemon sharks infested with *Neodermopthirius*, but their treatments were ineffective. Kim et al. (1998) were successful in intubating *Sebastes schlegeli* with 200mg/kg praziquantel to treat *Microcotyle sebastis*. Max Janse and Borgsteede (2003) were unsuccessful in treating *Aetobatus narinari* (spotted eagleray) with between 10 and 40mg/kg oral praziquantel. Hirazawa *et al.* (2004) had some success with treating spotted halibut for *Neobenedenia girellae* with orally administered praziquantel at 40 mg/kg and 150mg/kg, citing palatability issues with the latter, and Rissa Williams *et al*. (2007) used 100 and 150mg/kg oral praziquantel on yellowtail kingfish with palatability issues, but found success when intubating the fish with the same dosage under anaesthetic.

It has previously been considered that the ineffectiveness of orally administered praziquantel was related to monogenean feeding biology (their sub-class) because blood-feeders were more likely to be affected by the drug. It certainly appears that way if you consider the results of Poynton *et al.* (1997) and Janse and Borgsteede (2003). However, it does not explain the relative success had by Hirazawa *et al.* (2004) and Williams *et al.* (2007) who were treating representatives of the Monopisthocotylea.

[Slide 10] Here I would like to propose that the success of orally administered praziquantel is more a function of the relationship between its bioavailability in fish tissue and the susceptibility of the parasite(s) being treated for.

These lesser-known interests (Bioavailability and Susceptibility) we took forward at Two Oceans Aquarium in a project co-funded by Monterey Bay Aquarium and Two Oceans Aquarium in an attempt to gain a better understanding on how best to treat monogeneans in our public aquaria.

The null hypothesis was that uptake and delivery would be the same in teleosts and elasmobranchs and if this were true then a teleost parasite treatment model could be used to generate statistical significance and the results could then be extrapolated to treat elasmobranchs.

To test the bioavailability initially in blood plasma we employed High Performance Liquid Chromatography following the rational and methodology of Kim *et al*. (2001). We selected *Argyrosomus japonicus* as our representative of the teleost and *Rhinobatos annulatus* as our elasmobranch representative. *Argyrosomus japonicus* is a South African and Australian aquaculture species and juveniles were generally easily available from farms in significant quantities. *Rhinobatos annulatus* is a common rhinobatid ray found along our coastline and was relatively easy to collect.

[Slide 11] Methods

A total of 81 *A. japonicus* were separated into 3 tanks of equal size and volume containing 27 fish each, representing 3 separate replicates. They were anaesthetised with 2-Phenoxyethanol and weighed and given 150mg/kg praziquantel orally via autopipette. Three fish were randomly selected from each tank at 8 time intervals for blood extraction from the caudal vein. Sodium heparin-charged needles and syringes were used to prevent clotting and approximately 500µl was extracted from each fish. The blood was the centrifuged for 10 minutes at high speed to separate out the plasma, which was removed and immediately frozen. We were restricted to 18 individual rhinobatids so we tagged these with subcutaneous pit tags inserted into the dorsal saddle to facilitate repeated measures. All rhinobatids were pre-weighed. Rhinobatids were anaesthetised with 2-Phenoxyethanol and force-fed the 100% praziquantel which was compressed into tablets. We followed the same blood extraction and processing protocol as for *A. japonicus*.

[Slide 12] Once all the samples had been collected, they were flown to my colleague (Sandy Bye) in KwaZulu Natal to determine plasma praziquantel levels using High Performance Liquid Chromatography. For the mobile phase, 3.4g of monopotassium phosphate was added to 450ml of distilled water to make up the buffer which was then adjusted to a pH of 3.0 sing phosphoric acid. Thereafter, 500ml of the buffer was added to 500ml of acetonitrile.

[Slide 13] For the HPLC sample preparation 1ml of 100% acetonitrile was added to the vlume of the plasma sample, and the sample was then allowed to rest for 10 minutes at 4°C. The sample was then centrifuged at 10 000 x g for 10 minutes and the supernatant was evaporated to dryness. The resultant residue was dissolved in 1ml of mobile phase and 100µl was injected at 1ml/min at 217nm on a C18 column. For the standard preparation, 15.9mg of praziquantl was added to 100ml of mobile phase and was diluted 10:100

[Slide 14] Results

If we look at the results for *A. japonicus* we noted an initial spike of praziquantel in the blood plasma followed by a gradual decline to 96 hours which is generally comparable to other studies. However, there was a large variation (a large standard deviation) in the data from the initial group which we considered to be a combination of sampling time error (we could not sample all the fishes simultaneously) and the possibility of individual regurgitation. We were also concerned whether we were getting any interference from the anaesthetic in the reading of praziquantel in the HPLC so we had three anaesthetics tested using HPLC (2-Phenoxyethanol, Tricaine methanesulfonate (MS-222) and Isoeugenol), all of which were shown to have non-interference and completely different retention times.

[Slide 15] The results of the praziquantel blood plasma concentration in *R. annulatus* indicate a slow increase in concentration of praziquantel over time with a possible peak around or after 96 hours (?) – although further testing for a longer period of time would provide a more precise determination of the bioavailability curve.

[Slide16] However, if we overlay the results for both species it becomes immediately apparent that the delivery in *A. japonicus* is a lot higher than that of *R. annulatus*. If we assume that we are getting a peak praziquantel blood concentration in *R. annulatus* at 96 hours, then the delivery of praziquantel for *R. annulatus* is approximately 25% that of *A. japonicus*. The difference in delivery can likely be explained as a function of the first-pass metabolism which is expected to be different in teleosts and elasmobranchs given the differences in physiology of their livers and elasmobranchs are

generally known to have a slow metabolic rate. If we make a further assumption that there is relative similarity between *R. annulatus* and our large female *D. bevicaudata* mentioned previously, then at 12 hours post-treatment we may have had a significant result before praziquantel peaked in the blood plasma.

[Slide 17] So what does this all mean?

Well, to begin with we need to reject our hypothesis that uptake and delivery of praziquantel is the same in teleosts and elasmobranchs!

Elasmobranchs may metabolise praziquantel more efficiently through the first pass metabolism (FPM), therefore it is not surprising that previous workers had no success with comparatively low dosages. It also suggests that praziquantel is either delivered to muscle and skin in elasmobranchs relatively quickly, or that Monopisthocotylean monogeneans could also be feeding on blood(?).

What we still need to know:

- What is the lethal concentration of praziquantel in plasma, skin and mucus for monogeneans? – with this we can optimise oral dosages based on delivery
- If delivery is higher in teleosts, are we using too much praziquantel? Certainly, a reduction would alleviate palatability issue
- The effect of Cimetidine on FPM in elsmobranchs needs to be investigated!
- Cimetidine is a histamine receptor antagonist which inhibits gastric acid secretion and is known to reduce FPM in teleosts (Kim *et al.* 2001b)

[Slide 18] In addition to the work which we were doing on praziquantel, we had the opportunity to identify and describe two new species of monogeneans from *R. annulatus*, the first monogeneans described from this host species. These include *Pseudoleptobothrium christisoni*, a microbothriid found on the skin and *Neoheterocotyle robii* found between the secondary gill lamellae.

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