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VERSITA

Heterocotyle tokoloshei sp. nov. (Monogenea, Monocotylidae) from the gills of *Dasyatis brevicaudata* (Dasyatidae) kept in captivity at Two Oceans Aquarium, Cape Town, South Africa: Description and notes on treatment

David B. Vaughan[1]* and Leslie A. Chisholm[2, 3]

[1]Aquatic Animal Health Research, Two Oceans Aquarium, P.O. Box 50603, Victoria and Alfred Waterfront, Cape Town, 8000, South Africa; [2]Monogenean Research Laboratory, Parasitology Section, The South Australian Museum, North Terrace, Adelaide, South Australia, 5000, Australia; [3]Marine Parasitology Laboratory, School of Earth and Environmental Sciences (DX 650 418), The University of Adelaide, North Terrace, Adelaide, South Australia, 5005, Australia

Abstract

Heterocotyle tokoloshei sp. nov. is described from the gills of a single Short-tail stingray, *Dasyatis brevicaudata*, kept in captivity at the Two Oceans Aquarium in Cape Town, South Africa. The stingray exhibited laboured gill ventilation and deteriorating health on exhibit and was removed to the quarantine area for parasitological study and treatment. A 12 h bath treatment of praziquantel at 20mg/l, pre-dissolved in ethanol, removed 3084 parasites from the gills of the ray. However, the presence of a large number of eggs 24 h post-treatment indicated that viable egg laying adults remained on the gills and that the treatment was not 100% effective. Praziquantel was subsequently administered orally by intubation to the same ray at 150 mg/kg under anaesthetic (2-phenoxyethanol at 0.15 ml/l for approximately 1 h), which resulted in the removal of approximately 392 000 parasites from the gills 12 h post-oral treatment. Twenty-four h post-oral treatment, 3383 worms, but no eggs were recovered. No worms or eggs were recovered 48 h to 10 days post-oral treatment. The ray died approximately 30 days after the completion of the treatment. *Heterocotyle tokoloshei* sp. nov. is the first *Heterocotyle* species described from South Africa and represents the first record of a pathogenic *Heterocotyle* species. The new species can be distinguished from the other 16 species in the genus by the distal region of the male copulatory organ which has distinct small spines and by the morphology of the male copulatory organ accessory piece. Eggs of *H. tokoloshei* sp. nov. are laid singly and hatch spontaneously between 5 and 8 days at 18°C.

Keywords

Monogenea, Monocotylidae, *Heterocotyle*, stingrays, praziquantel treatment, public aquarium, South Africa

Introduction

Monocotylid monogeneans are known to cause health problems in stingrays maintained in public aquaria (Chisholm and Whittington 2002, Janse and Borgsteede 2003, Chisholm *et al*. 2004, Vaughan *et al*. 2008a, Vaughan and Chisholm 2009). Monocotylids parasitising the skin surface of stingrays held in captivity have received the most attention because infections are more visible and physical damage associated with feeding worms typically presents itself as conspicuous tissue discolouration. Infection by skin parasites also often results in a notable change in host behaviour. For example, stingrays infected with members of *Dendromonocotyle* Hargis, 1955 become restless and irritated and will flash (Whittington and Chisholm 2008) or can be observed folding their wings over their backs. Populations of monocotylids infecting host gills, however, can potentially increase unnoticed until the host becomes seriously debilitated.

Members of *Heterocotyle* Scott, 1904 are exclusively parasitic on the gills of their stingray hosts. They have 1 central and 8 peripheral haptoral loculi and are distinguished from other members of the Monocotylidae Taschenberg, 1879 by the presence of 4 accessory structures on the dorsal surface of the haptor and by a single or multiple ridges, said to be sclerotised, on the ventral surface of the haptoral septa (Chisholm and Whittington 1996).

*Corresponding author: dvaughan@aquarium.co.za

Reports of monocotylids infecting captive stingrays are increasing, but little information is available on their control and treatment. No *Heterocotyle* species has been described from South Africa. An exploratory investigation into the deteriorating health of a captive Short-tail stingray, *Dasyatis brevicaudata* (Hutton, 1875), at the Two Oceans Aquarium in Cape Town revealed a large number of monocotylids infecting the gills. These monogeneans are proposed here as a new species of *Heterocotyle*. The heavy infection provided the opportunity to investigate the potential use of praziquantel as a non-invasive means of collection and possible treatment of these monogenean parasites.

Materials and methods

Host and treatment using praziquantel

A female *D. brevicaudata* kept on exhibit at Two Oceans Aquarium showed signs of laboured gill ventilation and refused food offered via SCUBA during routine public-exhibition feeding. The 140 kg ray was removed from the exhibit in August 2008 and isolated in a 4000 l quarantine holding tank supplied with fresh, filtered seawater maintained at 18°C. The stingray was examined for any external parasites on both the dorsal and ventral skin surfaces, but none was observed. Twelve h post-isolation the bottom surface of the tank was siphoned and the water was filtered through a 23 µm sieve. The contents of the filter were examined using an Olympus SZ60 stereo zoom microscope to check for the presence of monogenean eggs. An unquantifiable mass of tetrahedral eggs was observed, confirming a large infection. Some of these eggs were kept for egg hatching experiments. The supply of incoming fresh seawater to the holding pool was closed and the pool was treated for 12 h with 20 mg/l of pure praziquantel powder dissolved in 200 ml ethanol, after which time the siphoning procedure was repeated. In addition to a large number of eggs, monogeneans were recovered and were fixed in absolute ethanol. The supply of incoming fresh seawater was resumed for 24 h to allow a complete turnover of the treated water volume. The bottom of the tank was siphoned again, and a large mass of eggs but no further worms were recovered, thus it was clear that the efficacy of the bath treatment was not 100%. Immediately thereafter, the stingray was anaesthetised using 0.15 ml/l of 2-phenoxyethanol (Vaughan *et al*. 2008b) for approximately 1 h and intubated with praziquantel at 150 mg/kg before being revived. The praziquantel was mixed with seawater and administered with a 60 ml syringe and a length of 0.5 mm plastic tubing inserted into the stomach. At 12 h post-oral treatment, the bottom surface of the tank was siphoned again and filtered. The presence of eggs was noted and worms recovered were counted under the dissection microscope using a grid consisting of 870 equal squares. The mean number of monogeneans counted in 50 randomly selected blocks was calculated and then multiplied by the total number of blocks to determine an approximation of the total number of worms. Siphoning of the bottom of the tank was repeated every 24 h for a further 10 days but no further treatments (bathing or intubation) were administered.

Processing of monogeneans

Approximately half the worms recovered during the oral treatment were fixed unflattened in absolute ethanol and the other half in 10% buffered neutral formalin (BNF). Sixteen specimens used for the species description were flattened under coverslip pressure and fixed separately in BNF. These were returned to freshwater before being stained with alum carmine, dehydrated in a graded ethanol series, cleared in cedarwood oil and individually mounted in Canada balsam. Some specimens preserved in absolute ethanol were subjected to proteolytic digestion (Vaughan *et al*. 2008a) to provide clear definition of the sclerotised structures of the haptor and of the sclerotised male copulatory organ. This method was also used to provide further information on whether or not the sinuous ridges on the haptoral septa are sclerotised.

Permanently mounted monogeneans, digests and eggs were examined using an Olympus CX 41 compound light microscope fitted with phase-contrast and dark-field optics. Photomicrographs were taken with an Olympus Altra20 digital microscope camera and drawings were made with the aid of a drawing tube. All measurements were done using Olympus AnalySIS5® software and are given in micrometres as the mean ± standard deviation, followed in parenthesis by the range and number measured. The slightly curved male copulatory organ was measured along the curve.

Taxonomic terminology follows that of Chisholm and Whittington (1996). The type series of the new species is deposited in the Iziko South African Museum (SAMCTA) in Cape Town, 8000, South Africa, and the Australian Helminthological Collection (AHC) at the South Australian Museum (SAMA), North Terrace, Adelaide, South Australia, 5000, Australia. The voucher material, consisting of all remaining worms collected in this study, is deposited in SAMCTA.

Egg collection and hatching

Two replicate sets of 50 eggs each were collected for hatching from the bottom of the quarantine tank 12 h after the ray was first isolated. The eggs were cleaned of debris and 1 egg was placed into each well (volume = 4 ml) of 10, 12-well tissue plates with lids. The plates were allowed to float at the surface of the water, ensuring that lids remained in place, in glass incubation tanks containing fresh filtered seawater. Incubation tanks were maintained at 18°C using thermostatically controlled glass immersion aquarium heaters. Lighting (2 non-filtered standard white-light 30 watt fluorescent tubes) was controlled using an automated timer set to a 12 h light-dark (LD) regime (i.e., light on 06:30, light off 18:30). Half the volume of each well was replaced daily with fresh filtered seawater from the incubation tanks using a syringe to deliver the water slowly. The eggs were checked daily and the development of the larvae was noted.

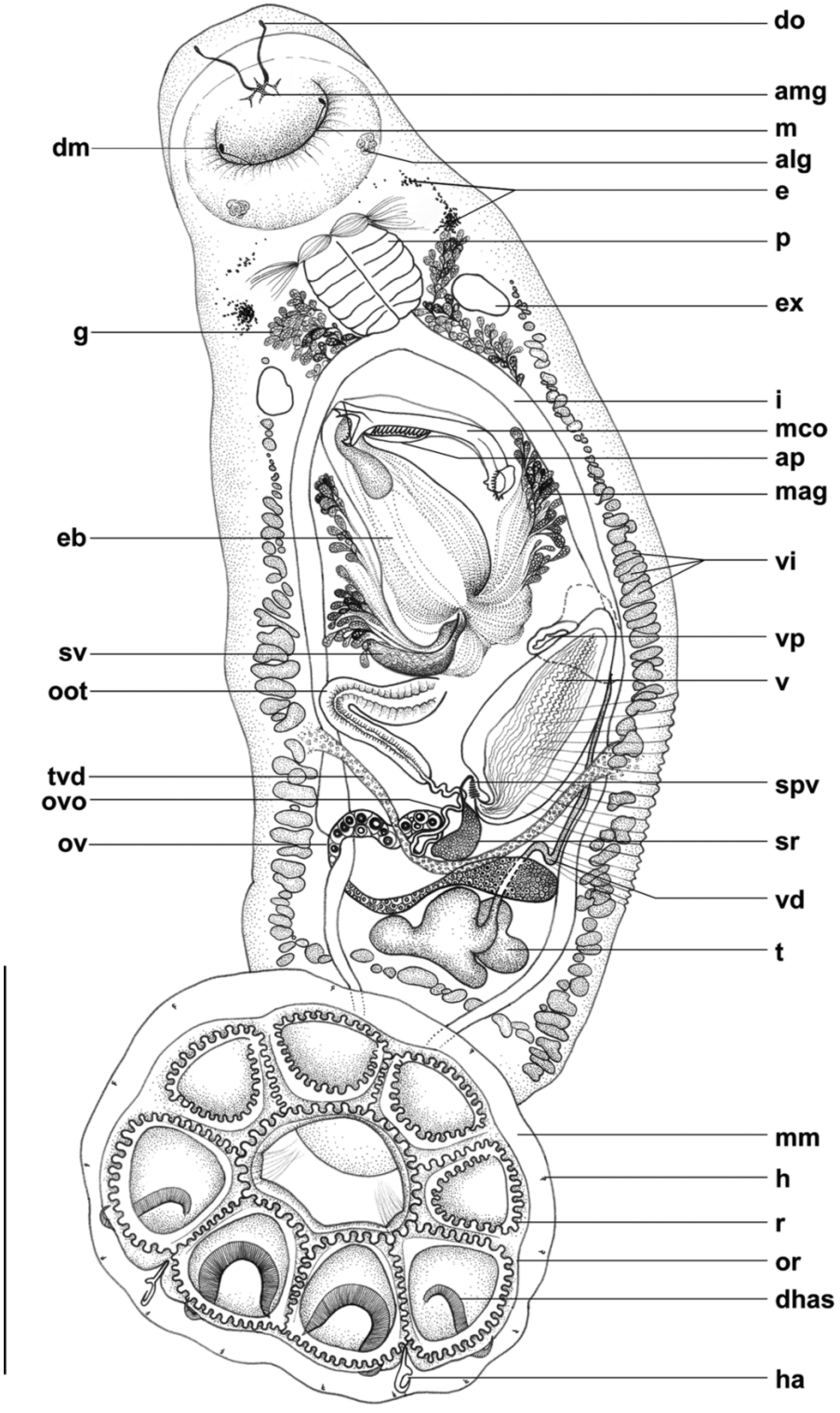

Fig. 1. *Heterocotyle tokoloshei* sp. nov.: Whole worm ventral view composite. **Abbreviations:** alg – anterolateral gland; amg – anteromedian gland; ap – accessory piece; dhas – dorsal haptoral accessory structure; dm – dark mass of unknown function; do – gland-duct opening; e – eyespots as dispersed pigment granules; eb – ejaculatory bulb; ex – excretory bladder; g – glands associated with either side of pharynx; h – marginal hooklet; ha – hamulus; i – intestinal caecum; m – mouth; mm – marginal membrane; mag – male accessory glands; mco – male copulatory organ; oot – ootype; or – outer-ring septum; ov –ovary; ovo – ovovitelline duct; p – pharynx; r – sinuous ridge; spv – sclerotised region of proximal vagina; sr – seminal receptacle; sv – seminal vesicle; t – testis; tvd – transverse vitelline duct; v – vagina; vd – vas deferens; vi – vitellarium; vp – vaginal pore. Scale bar = 400 μm

Results

Monocotylidae Taschenberg, 1879
Heterocotylinae Chisholm, Wheeler et Beverley-Burton, 1995
Heterocotyle Scott, 1904

***Heterocotyle tokoloshei* sp. nov.** (Figs 1 and 2)

Type-host: *Dasyatis brevicaudata* (Hutton, 1875).

Type-locality: Originally collected from Struisbaai, South Africa and held at Two Oceans Aquarium, Dock Road, Victoria and Alfred Waterfront, Cape Town, 8000, South Africa.

Location on host: Gills.

Etymology: This species is named after the Tokoloshe, the small and troublesome mythical creature of the traditional South African Zulu and Xhosa cultures.

Date of specimen collection: 26 August 2008.

Holotype: SAMCTA 29477.

Paratypes: SAMCTA 29478 (10 whole mounts), SAMCTA 29479 (1 sclerotised male copulatory organ and haptor digest), SAMA AHC 29876–29880 (5 whole mounts).

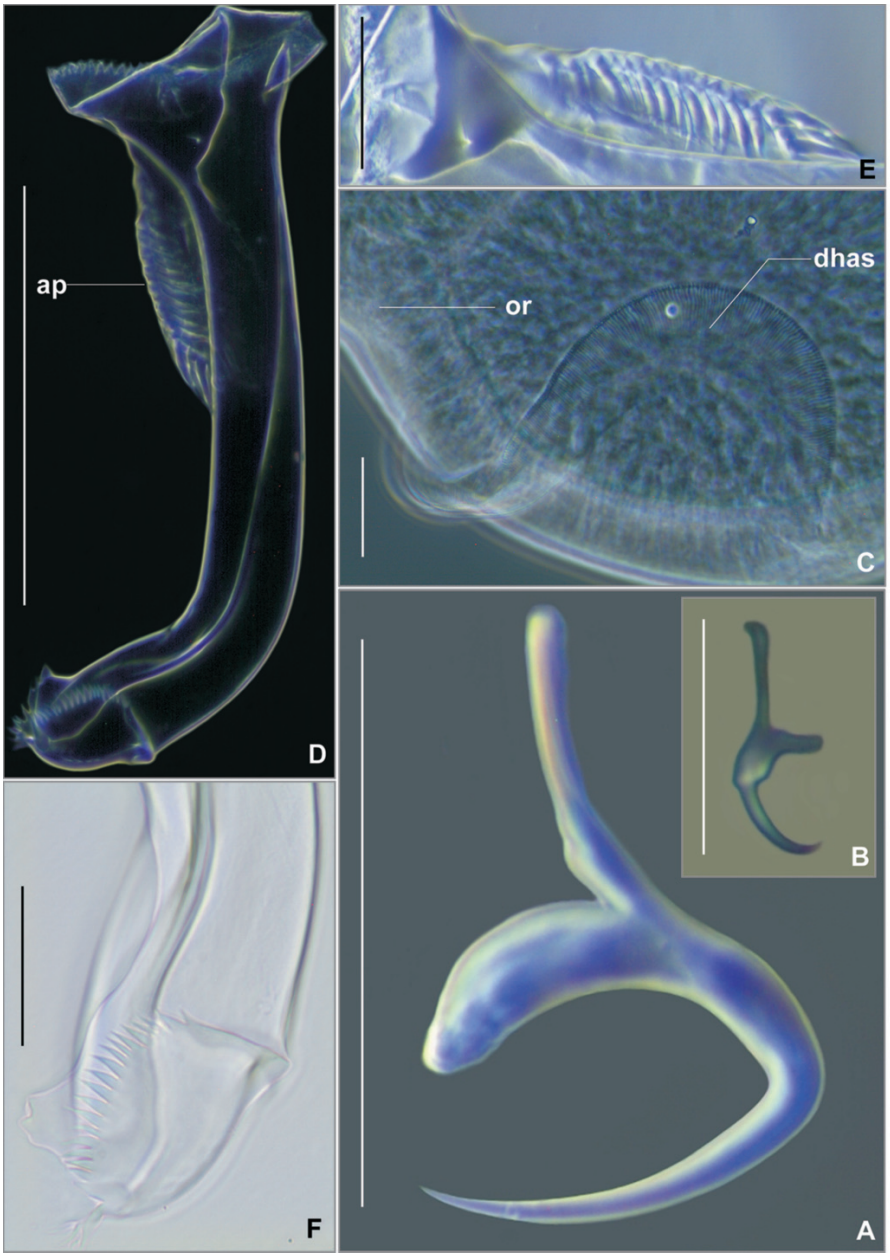

Fig. 2. Sclerotised male copulatory organ and haptoral armature of *Heterocotyle tokoloshei* sp. nov. **A** – hamulus; **B** – marginal hooklet; **C** – dorsal haptoral accessory structure (dorsal view); **D –** male copulatory organ; **E** – accessory piece; **F** – distal region of male copulatory organ. Abbreviations as in Figure 1. Scale bars: A = 50 µm, B = 10 µm, C = 30 µm, D = 100 µm, E and F = 20 µm

Vouchers: 2 bottles containing all remaining worms in either 10% BNF (SAMCTA 29480) or analytical-grade absolute ethanol appropriate for future molecular investigation (SAMCTA 29481).

Description: Based on 16 whole flattened adult specimens and 1 proteolytic digest of the sclerotised male copulatory organ and haptor. Total body (Fig. 1) excluding haptor 1077 ± 181.8 (768–1428, n = 16) long, 455 ± 75.5 (307–583, n = 16) wide at widest point. Haptor oval 419 ± 44.0 (343–502, n = 16) long, 515 ± 67.4 (377–606, n = 16) wide consisting of 1 central and 8 peripheral loculi. Sinuous ridge single on inner and outer ring and 3 posterior septa, double on 2 lateral radial septa and triple on 3 anterior radial septa (1/2/3 configuration; see Chisholm and Whittington 1996). Hamulus (Figs 1, 2A) 55 ± 2.1 (52–58, n = 10) long with robust guard. Hamulus handle embedded in tissue of septum between posterior and posterolateral loculi (Fig. 1). Fourteen marginal hooklets (Fig. 2B) 10 ± 0.3 (9–10, n = 8) long, distributed in the marginal membrane as illustrated (Fig. 1). Dorsal surface of haptor with 4 accessory structures with sclerotised, striated anterior edge (Figs 1, 2C). Lateral extremity of each sclerotised striated edge folded around adjacent region of outer-ring septum (Figs 1, 2C).

Mouth ventral, subterminal. Small distinct dark masses of unknown function on either side of mouth (Fig. 1). Anterior region with 3 glands; 1 anteromedian and 2 anterolateral glands containing granular secretion (Fig. 1). Anterolateral glands inconspicuous, posterior to mouth opening (Fig. 1). Lateral glands not observed, possibly obscured by vitellarium. Pharynx 141 ± 25.2 (101–191, n = 16) long, 100 ± 19.1 (64–147, n = 16) wide. Glands present either side of pharynx. Intestinal caeca lacking diverticula, running full length of body, ending blindly. Eyespots in form of dispersed pigment granules distributed in 2 areas (Fig. 1); 1 pair of concentrated granules lateral to anterior region of pharynx, and loosely dispersed granules anterior to pharynx. Pair of excretory bladders positioned lateral to posterior region of pharynx, midway between outer body wall and intestinal caeca (Fig. 1).

Testis lobed (Fig. 1). Vas deferens originates from left side of testis, runs anteriorly, dorsal to transverse vitelline duct, passing between outer wall of vagina and intestinal caecum (Fig. 1). Path of vas deferens to where it swells to form seminal vesicle obscured by vagina and extensive male accessory glands. Seminal vesicle positioned right of posterior region of ejaculatory bulb, posterior to male accessory glands, narrows, entering ejaculatory bulb at its base (Fig. 1). Ejaculatory bulb muscular, ovoid, 197 ± 32.3 (136–247, n = 16) long, 103 ± 27.2 (46–145, n = 16) wide (Fig. 1); extensive network of male accessory glands enters base (Fig. 1). Anterior region of ejaculatory bulb narrows to meet proximal region of distinct sclerotised male copulatory organ 238 ± 21.3 (189–260, n = 17) long (Figs 1, 2D). Comb-like sclerotised accessory piece 87 ± 6.3 (78–98, n = 15) long, originates from proximal region of male copulatory organ (Figs 1; 2D, E). Shaft of male copulatory organ straight, then curved at distal end. Distal region of male copulatory organ armed with approximately 20 small spines (Figs 1; 2D, F).

Ovary loops right intestinal caecum dorsoventrally, narrows abruptly to form loosely curved oviduct which meets common vitelline duct near base of seminal receptacle (Fig. 1). Ovovitelline duct enters oötype. Oötype 191 ± 47.8 (126–287, n = 16) long (Fig. 1). Mehlis' glands not observed. Tetrahedral egg 90 ± 7.8 (74–100, n = 10) side length with long polar filament; measured from free eggs laid by adults. Vaginal pore on left side of body at level of seminal vesicle (Fig. 1). Vagina 204 ± 18.7 (180–222, n = 6) long; large, sack-like with longitudinal folds internally, with thick, non-sclerotised walls (Fig. 1). Proximal region of vagina narrows to form area with small, tightly packed lateral sclerites, then leads to duct entering small seminal receptacle 37 ± 7.3 (28–48, n = 8) long (Fig. 1). Spermatophores not observed. Vitellarium extends from posterior end of pharynx to posterior region of body proper (Fig. 1).

Egg incubation and hatching

Tetrahedral eggs of *H. tokoloshei* are laid singly and in this case became entangled with each other and detritus forming large mats. Eggs incubated in a LD 12:12 light regime at 18°C contained larvae with eyes and marginal hooklets after 3 days. Spontaneous hatching commenced on day 5 and ended on day 8 with a 100% hatching success in both replicates.

Remarks

Heterocotyle tokoloshei can be distinguished from all known species of *Heterocotyle* by the unique morphology of the distal end of the male copulatory organ with 20 small spines (Figs 1; 2D, F), the morphology of the male copulatory organ accessory piece (Figs 1; 2D, E) and the small area of sclerotisation at the base of the proximal region of the vagina (Fig. 1).

Proteolytic digestion of the haptor revealed that the striated edge of all 4 sclerotised dorsal haptoral accessory structures wraps around the outer ring septum opposite the anterior and anterolateral loculi (Figs 1, 2C). The dorsal haptoral accessory structures resisted digestion for the most part, however, the sclerotised edge is thin and eventually disintegrates if left for too long in the digestion fluid. In addition, the degree of digestion of the sinuous ridge on the septa was equal to other non-sclerotised parts of the haptor and it is therefore likely that the ridge is not sclerotised as noted previously for other members of *Heterocotyle*.

Praziquantel treatment

When the ray was bathed in 20 mg/l praziquantel for 12 h, 3084 worms were recovered. It was clear that this treatment did not eliminate all of the parasites because numerous eggs were found in the bottom of the tank 24 h post-treatment. The stingray was then anaesthetised for approximately 1 h with a concentration of 2-phenoxyethanol at 0.15 ml/l (Vaughan *et al*. 2008b) and intubated with praziquantel at 150 mg/kg. Twelve h post-treatment a large number of eggs and approximately

392 000 worms were recovered. Twenty-four h post-treatment no eggs and 3383 parasites were collected. No further eggs or parasites were found 48 h – 10 days post-treatment.

Discussion

Heterocotyle tokoloshei is the first reported *Heterocotyle* species from South Africa and the first published account of a pathogenic *Heterocotyle* species. The host stingray's general health did not improve after the removal of the massive infection with *H. tokoloshei* and died in the quarantine facility a month after the completion of the treatment. Information on the treatment of monocotylid monogeneans is currently limited. Chisholm and Whittington (2002) tested the efficacy of various praziquantel treatment protocols against 4 monocotylid species infecting the gills (3 species) and nasal tissue (1 species) of the Giant Shovelnose Ray, *Glaucostegus typus* (Bennett, 1830), (syn. *Rhinobatos typus*). Two 40 h bath treatments of praziquantel at 5 mg/l, 48 h apart were successful in eliminating all 4 monocotylid species (Chisholm and Whittington 2002). Exposure of monocotylids *in vitro* to 20 mg/l praziquantel for 2 h proved ineffective and worms were able to make a full recovery and produce viable eggs. However, monocotylids exposed to 20 mg/l died after *in vitro* exposures of 12 and 14 h (Chisholm and Whittington 2002). Although Chisholm and Whittington (2002) indicated that *G. typus* could not withstand long periods of exposure to high levels of praziquantel (2 h at 20 mg/l at 25°C; 6 h at 10 mg/l at 26.3°C), it is likely that the distress encountered in their experimental rays was due to deteriorating water quality and not praziquantel exposure. Experimental tanks with volumes of 50 l containing 30 l of praziquantel-treated seawater were used for maintaining individual rays between 45 cm and 60 cm in total length. Filtration could not be used and water temperatures ranged between 23.8°C and 26.5°C (Chisholm and Whittington 2002). These conditions are conducive to a rapid decrease in water quality and toxic build-up of ammonia and nitrite (Randall and Tsui 2002, Kroupova *et al*. 2005).

In the present study the large female *D. brevicaudata* was exposed initially to a praziquantel bath at 20 mg/l for 12 h in a quarantine holding tank with an approximate volume of 4000 l. Although over 3000 individual *H. tokoloshei* were recovered from the bottom of the holding tank after this bath treatment, the presence of masses of eggs 24 h later indicated that it had been unsuccessful in completely removing all parasites.

In their *in vivo* experiments using various praziquantel concentrations, Chisholm and Whittington (2002) noted differences in the susceptibility of different monocotylid size classes to the same concentrations. These differences were attributed to the ability of the monocotylid developmental stages to make use of different host microhabitats. Chisholm and Whittington (2002) observed that smaller developmental stages of the gill monogeneans withdrew deeply between the gill lamellae as a reaction to initial contact with the praziquantel, possibly shielding them from full exposure. This may be why the initial praziquantel bath treatment in the present study failed. However, it is also likely that hyperplasia of the gill lamellae in reaction to a large infection of *H. tokoloshei* could have further inhibited the effective exposure to praziquantel in solution.

In teleosts, oral praziquantel treatments at 150 mg/kg have successfully removed skin and gill monogeneans (see Hirazawa *et. al*. 2004, Williams *et al*. 2007). Until now, oral application at this dosage in elasmobranchs has not been investigated. Janse and Borgsteede (2003) presented results of both a bath treatment and oral application of praziquantel to treat captive spotted eagle rays *Aetobatus narinari* (Euphrasen, 1790) infected with monocotylids they misidentified as *Clemacotyle australis* Young, 1967 (likely *Dendromonocotyle torosa* Chisholm et Whittington, 2004; see Whittington and Chisholm 2008) and an unidentified hexabothriid species. Orally administered praziquantel at 10–40 mg/kg (note the exact concentrations are difficult to interpret from their results) did not remove the parasites infecting *A. narinari*, but limited short-term success was achieved with bath treatments.

We determined that a single dose oral treatment of praziquantel administered at 150 mg/kg by intubation was highly successful in removing all remaining parasites by 24 h post-treatment. However, because the praziquantel was administered by intubation while the ray was anaesthetised with 2-phenoxyethanol at 0.15 ml/l, it is unclear if the praziquantel was solely responsible for the success of this treatment. Pironet and Jones (2000) found that a 1.5 h freshwater bath alone removed 91.5% of the monogeneans identified as *Haliotrema* Johnston et Tiegs, 1922 (Dactylogyridae) from *Glaucosoma hebraicum* Richardson, 1845, whereas a 1.5 h freshwater bath in combination with 300 mg/l 2-phenoxyethanol removed 100% of the monogeneans. Pironet and Jones (2000) also noted that *Haliotrema* seemed sensitive to 2-phenoxyethanol, because the majority of the monogeneans became detached after 10 min in approximately 300 mg/l of 2-phenoxyethanol.

In addition to its treatment properties, this protocol can also be used as a non-invasive means of prospecting stingrays suspected to be infected with *Heterocotyle* species as well as other monogeneans. As stated above, we cannot know how much the 2-phenoxyethanol contributed to the success of our protocol but it is likely that the oral dosages of praziquantel (10–40 mg/kg) tested by Janse and Borgsteede (2003) to remove monogeneans from *A. narinari* were too low. Clearly, controlled experiments are needed to determine the isolated effects of 2-phenoxyethanol and oral praziquantel doses by intubation against monogeneans on elasmobranchs.

Monogeneans have a direct life-cycle and produce eggs which are resistant to treatment (Whittington and Chisholm 2008). It is therefore important to consider biological data to correctly time follow-up treatments in public aquarium exhibits where monocotylids have already become established (Vaughan and Chisholm 2009). Here, information on egg hatching, potential hatching cues, and development time to sexual maturity should be used to determine an optimum treat-

ment strategy to break the life-cycle. Using a one-off treatment or randomly generated follow-up strategies may only provide temporary relief, as re-infections usually result. In our opinion, preventing monogeneans from becoming established in public aquarium exhibits through the implementation of adequate quarantine protocols is both easier and more responsible. Therefore, the following preventative quarantine protocol was designed for new acquisitions of *D. brevicaudata* at the Two Oceans Aquarium based on the effect of the oral application of praziquantel and the subsequent egg development and hatching data collected for *H. tokoloshei* at 18°C. Upon arrival, stingrays are weighed and isolated in the quarantine area in a holding tank maintained at 18°C. Praziquantel at 150 mg/kg is administered orally (intubated under anaesthetic of 2-phenoxyethanol at 0.15 ml/l). The bottom of the holding tank is siphoned daily to check for parasites and eggs and the oral dosage of praziquantel re-administered on day 10–12 to coincide with the recruitment of any new larvae.

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