

Activity of the antimicrobial polypeptide piscidin 2 against fish ectoparasites

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Abstract

The antiparasitic effects of piscidin 2, an antimicrobial polypeptide (AMPP) first isolated from mast cells of hybrid striped bass, were tested against three protistan ectoparasites of marine fish (the ciliates *Cryptocaryon irritans* and *Trichodina* sp., and the dinoflagellate *Amyloodinium ocellatum*) and one ciliate ectoparasite of freshwater fish (*Ichthyophthirius multifiliis*). *I. multifiliis* was the most susceptible parasite, with all theronts killed at $6.3 \mu\text{g mL}^{-1}$ piscidin 2. The most resistant parasite was *Trichodina*, where a few cells were killed at $12.5 \mu\text{g mL}^{-1}$, but several were still alive even at $100 \mu\text{g mL}^{-1}$. *C. irritans* was of intermediate sensitivity, with some theronts killed at $12.5 \mu\text{g mL}^{-1}$ and all killed at $25 \mu\text{g mL}^{-1}$. High parasite density apparently exhausted the piscidin 2 before it could attain its maximal effect, but surviving parasites were often visibly damaged. The lower efficacy of piscidin 2 against marine parasites compared with the freshwater ciliate might be related to the inhibitory effects of high sea water cation levels. The tissue concentration of piscidins estimated in healthy hybrid striped bass gill ($40 \mu\text{g mL}^{-1}$) suggests that piscidin 2 is lethal to the parasites tested at physiological concentrations and is thus an important component of innate defence in fish expressing this type of AMPP.

Keywords: antimicrobial, antiparasitic, fish, innate defence, piscidins, polypeptides.

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Introduction

The immune system of fish relies heavily on innate defences (Iwama & Nakanishi 1996). The humoral component of this response includes antimicrobial polypeptides (AMPPs). AMPPs are host-produced peptides and small proteins that have been isolated from all major classes of vertebrates as well as many invertebrates (e.g. shrimps, crabs and oysters) and are probably present in all living organisms. These factors display powerful, typically broad-spectrum antimicrobial activity and are increasingly considered to play a critical role in defence against pathogen invasion (Schröder 1999; Gallo, Murakami, Ohtake & Zaiou 2002; Zasloff 2002). AMPPs have been isolated or otherwise identified from an increasingly large number of fish, including many commercially important species, and are typically expressed at major portals of pathogen entry, such as the epithelial surfaces of skin, gills and gastrointestinal tract (Noga & Silphaduang 2003). In those sites, they act rapidly and non-specifically against a broad range of potentially invasive micro-organisms, delaying or preventing their colonization.

Among the most potent and broad-spectrum AMPPs are the piscidins, a family of linear, amphipathic peptides that have a highly conserved phenylalanine-rich, histidine-rich amino terminus (Silphaduang & Noga 2001). Originally localized in mast cells of gill, skin and gut of hybrid striped bass, *Morone saxatilis* ♂ × *Morone chrysops* ♀, and its parental species (Silphaduang & Noga 2001), there is recent evidence that piscidins also occur in a number of other species of fish and may be present in other types of immune cells (Silphaduang, Colorni & Noga 2006).

Hybrid striped bass can live in full strength sea water (Harrell & Webster 1996) although one of its parents, the striped bass, *M. saxatilis* (Walbaum), is anadromous and thus spends its life in both fresh water and sea water. Assuming that the euryhalinity trait is passed on to the hybrid, host defences in this species might be expected to defend against both marine and freshwater pathogens (Silphaduang & Noga 2001; Lauth, Shike, Burns, Westerman, Ostland, Carlberg, Van Olst, Nizet, Taylor, Shimizu & Bulet 2002). Because of the prominent presence of piscidins in the skin and gill, we explored the possible activity of one of the most potent piscidins (piscidin 2) against several common and important protozoan ectoparasites that affect a wide array of fish, including hybrid striped bass.

Trichodinids are bell- or saucer-shaped peritrichous ciliates (order Mobilida, family Trichodinidae), commonly occurring worldwide on the skin and gills of fish in both freshwater and marine environments. They glide on or temporarily adhere to the epithelia, feeding on bacteria and mucus. On a stressed or otherwise debilitated fish, these ciliates can proliferate massively. Their tactile stimuli cause irritation leading to mucus hyperproduction and hyperplasia. Their repeated adherence and suction activity eventually damage the epithelium and severely erode the gills (Lom 1995).

Responsible for marine white spot disease, *Cryptocaryon irritans* (order Prorodontida, family Cryptocaryonidae) is a holotrichous ciliate that parasitizes marine fish in tropical, subtropical and temperate seas. The infective stage (theront) burrows into skin, eyes and gills, developing into the parasitic stage (trophont) that actively feeds on the epithelial layers causing acute irritation, hyperplasia, respiratory distress and osmoregulatory imbalance (Colorni 1985; Colorni & Burgess 1997).

Ichthyophthirius multifiliis (order Hymenostomatida, family Ichthyophthiriidae), while only very distantly related to *Cryptocaryon*, is a freshwater ciliate that shares with the latter an extraordinary similarity in morphology, life cycle and pathogenesis. *I. multifiliis* has a cosmopolitan distribution, and can infect virtually any freshwater fish, from the subarctic to the tropics, making ichthyophthiriosis one of the most common and lethal protozoan infections in both ornamental and foodfish aquaculture (Colorni in press).

Amyloodinium ocellatum (order Blastodiniales, family Oodiniaceae) is a dinoflagellate, fully adapted to parasitic life and one of the most

devastating skin and gill parasites in temperate and tropical mariculture (Paperna 1980; Noga & Levy 2006). The infective stage (dinospore) attaches to the skin and gill epithelium of fish by means of rhizoids, rapidly turning into the parasitic feeding stage (trophont). After growing for several days, the trophonts detach from the fish, encyst on the substrate and start dividing (tomonts). Reproduction culminates with the release from each tomont of many infective, highly motile dinospores that begin anew the life cycle of this parasite.

Cryptocaryon irritans, *I. multifiliis* and *A. ocellatum* are responsible for significant economic losses in the farming of ornamental and food species of fish. The complexity of their life cycles makes their eradication very difficult. Treatment in ponds or large systems is rarely cost-effective or ecologically desirable (e.g. using copper salts whose therapeutic dosage and host tolerance threshold are often dangerously close). Thus, alternative means of managing these parasites are needed. Understanding how host-produced AMPPs such as piscidin 2 protect against these pathogens might provide a new avenue for their management.

Materials and methods

Test peptide

Piscidin 2 used in this study was synthesized via Fmoc chemistry on a Rainin Symphony instrument (Rainin Instrument LLC, Oakland, CA, USA) that provides an instrument cleavage of the peptide from the resin. After synthesis, piscidin 2 was purified via analytical reversed phase high performance liquid chromatography (RP-HPLC) using a C₁₈ column (4 mm × 50 mm, 3 micron particle size, 120 Å pore size support; YMC: Waters Corporation, Milford, MA, USA) with an acetonitrile gradient that was eluted at 1 mL min⁻¹ where buffer A was 0.05% trifluoroacetic acid (TFA) in water and buffer B was 80% acetonitrile in 0.05% TFA in water. The peptide was detected by its absorbance at 210 nm. Mass spectrometry of an aliquot of purified piscidin 2 was carried out on a mass spectrometer (Micromass ToFSpec SE: Waters Corporation) that was operated in positive ion mode and equipped with a nitrogen laser (337 nm), a reflectron, delayed extraction and a post-acceleration detector. The purified peptide was lyophilized from 0.05% TFA/acetonitrile solution and stored desiccated under argon gas until reconstitution in solvent.

Sources of parasites

Trichodina sp. was obtained from spontaneous cases of heavily infected devilfish, *Pterois miles* (Bennett), captured in baited traps in the Gulf of Eilat (North Beach area, Red Sea, Eilat, Israel). After killing by rapid decapitation, gill lamellae having attached parasites were harvested from infected fish and used immediately for testing.

Cryptocaryon irritans was obtained from a spontaneous infection in gilt-head sea bream, *Sparus aurata* L., cultured at the Israel Oceanographic and Limnological Research-National Center for Medicine, Eilat. The infection was maintained according to a protocol developed at NCM (Colorni 1992). Juvenile bream (2–3 g) were challenged for one to several hours using freshly excysted theronts. The fish were then moved into a collector with a funnel-shaped bottom resting on a beaker. The tomites that collected at the bottom of the beaker were gently dislodged with a fine paint brush, separated from debris, disinfected by a 10-min immersion in a 100 ppm elemental iodine (Sigma Chemical, St Louis, MO, USA) solution, rinsed thoroughly, transferred into wells of 24-well tissue culture plates with sterile-filtered sea water and incubated at 24 ± 1 °C. Only theronts that had excysted overnight were used for each experiment or for reinfection of fish.

Ichthyophthirius multifiliis was obtained from spontaneous cases of heavily infected aquarium fish at a local (Raleigh, NC, USA) retailer. The infection was maintained on channel catfish, *Ictalurus punctatus* (Rafinesque), fingerlings by continuously adding naïve fish to a 10-L aquarium holding infected fish. After killing, *I. multifiliis* trophonts were scraped off the skin of infected fish and transferred to 2 mL of filter-sterilized aquarium water in a polystyrene Petri dish. Trophonts were then incubated in filter-sterilized aquarium water to allow encystment into tomites and subsequent division into tomites. After ~24-h incubation at 25 °C, theronts were harvested and used immediately for each experiment.

Amyloodinium ocellatum was propagated continuously *in vitro* using a walking catfish, *Clarias batrachus* (L.), gill cell line (G1B) as host cells, as described previously (Noga 1992). A Red Sea isolate of *A. ocellatum* was used for all studies. This isolate was established in cell culture from infected striped bass obtained from a local (Eilat) private farm and has been continuously propagated *in vitro*

since May 2000. Stocks of G1B cells (ATCC #CRL 2536, American Type Culture Collection, Manassas, VA, USA) were propagated in a modified Ham's F-12 medium at 25 °C in 75-cm² flasks.

Exposure methods

Effect of piscidin 2 on the motile infective stages

The *C. irritans*, *I. multifiliis* and *A. ocellatum* cells were counted by transferring an aliquot of the stock suspension to at least three wells of a 96-well flat-bottom microtitre plate (Barloworld Scientific, Staffordshire, UK). The parasites were then killed and stained with a drop of Lugol's iodine and counted under an inverted microscope either directly in the plate or after being transferred to a haemocytometer. The number of parasites in the stock suspension was then adjusted by dilution with filtered water (40‰ Red Sea water for *C. irritans* theronts and *A. ocellatum* dinospores, or fresh water for *I. multifiliis* theronts) to produce the final concentrations for testing: 150 ± 50 per 100 µL (*C. irritans* theronts), 5–10 per 100 µL (*I. multifiliis* theronts) or either 200 or 15–20 per 100 µL (*A. ocellatum* dinospores). One hundred microlitres of parasite suspension was added to a 96-well, U-bottom polypropylene plate (Costar 3790, Corning, NY, USA). *Trichodina* adhered closely to the gill and either dislodging or concentrating the parasites was not feasible without damaging them. Thus, an approximately 5-mm long, apical fragment of gill lamella with 10 ± 5 adherent parasites was added to each well having 100 µL of 40‰ Red Sea water.

Twofold serial dilutions of piscidin 2, to produce final concentrations ranging from 100 to 0.4 µg mL⁻¹ in the wells, were prepared in diluent. Diluent consisted of 0.01% acetic acid (analytical grade, Frutarom, Haifa, Israel) with 0.2% bovine serum albumin (BSA; Sigma A9543) in endotoxin-free water (Biological Industries, Kibbutz Beit Haemek, Israel). Eleven microlitres of either a piscidin 2 dilution or one of two controls (sea water/fresh water only or diluent only) was then added to the 100 µL suspensions of parasites in triplicate wells. The salinity of the final test solution was 36‰.

For all parasites, observations of motility and changes in cytological appearance were made under a stereomicroscope or an inverted phase contrast microscope, at 5-min intervals during the first 30 min of exposure, every 10 min during the second 30 min, every 15 min for the second

60 min and every 20–30 min for the next 2 h (4 h total). The PC_{min} (minimum protozoacidal concentration) was defined as the lowest piscidin 2 concentration where at least one parasite died (defined as irreversible immobilization and/or lysis), while the PC_{100} (100% protozoacidal concentration) was defined as the lowest piscidin 2 concentration at which all parasites died. When the PC_{min} or PC_{100} varied among replications, the value is presented as a range. All PC_{min} and PC_{100} determinations were made after 4 h exposure to piscidin 2.

Effect of piscidin 2 on Amyloodinium dinospore infectivity

To explore the effect of piscidin 2 on the ability of *A. ocellatum* to infect host cells, we used a standard *in vitro* assay of dinospore infectivity (Noga 1992). A confluent G1B monolayer was gently trypsinized, resuspended in cell culture medium, and the cells were counted. Cells were then seeded into a 96-well tissue culture plate and allowed to grow to confluency. The G1B monolayers were then adapted to an artificial seawater solution (IO2/HBSS) before use in experiments. Infective dinospores were produced using standard procedures (Noga 1992). Upon excystment, dinospores were counted as described above, adjusted to a concentration of approximately 35 dinospores per 100 μL , and inoculated into replicate wells having final piscidin 2 concentrations of 100, 50, 25, 12.5, 6.25, 3.12 or 1.56 $\mu\text{g mL}^{-1}$. Controls included diluents (0.01% acetic acid (HAc) + 0.2% BSA) or IO2/HBSS only. The salinity of the final test solution was 20‰. The numbers of differentiated dinospores (i.e. trophonts and tomonts) present in each well were counted under phase contrast microscopy after incubation for 48 h at 25 °C. Five experiments were carried out in triplicate. The number of differentiated dinospores in the diluent control was considered as the 100% reference in each test, and the numbers of differentiated dinospores (i.e. trophonts and tomonts) in the treatment wells were thus expressed as a percentage of the sham-treated control (diluent only added).

Estimation of piscidin concentration in tissue

The concentration of piscidin in tissue was estimated by comparing the antibacterial activity of gill extract from healthy fish with that of a dilution series of piscidin 2 standards. A pool of gill extract

from 10 healthy hybrid striped bass (15–18 months old, 30–40 cm long and 0.5–1.5 kg) was prepared as described previously (Noga, Fan & Silphaduang 2001). A dilution series of pure synthetic piscidin 2 was prepared as described for the antiparasitic assays.

The samples were then run on a 'bug blot' as described previously (Seo, Crawford, Stone & Noga 2005). Briefly, a native acid-urea polyacrylamide gel electrophoresis (AU-PAGE) gel (100 mm wide \times 75 mm long \times 1 mm thick) was prepared having 12% acrylamide; 37.5/1 (w/w) acrylamide/bis solution, 4.8 M urea, 5% HAc, 0.48% (v/v) N, N, N', N'-tetramethylethylenediamine (TEMED) and 0.22% (w/v) ammonium persulphate (APS). The gel was polymerized at room temperature overnight. The APS and TEMED were removed by pre-running the gel with 5% HAc for 60 min at 150 V with reversed polarity (lower chamber-cathode) using a Mini PROTEAN II Electrophoresis Cell (Bio-Rad Laboratories, Richmond, CA, USA). No stacking gel was used. Samples were mixed (1:1) with sample solution (3.0 M urea in 5% HAc) and electrophoresed with 5% HAc at 150 V for 50 min at room temperature.

Following electrophoresis, the gel was washed by rinsing twice with 0.01 M phosphate buffer, pH 6.8, for 12–10 min each time. After washing, the gel was placed on a plate having a suspension of *Escherichia coli* D31 and incubated for 3 h at 37 °C to allow protein in the gel to diffuse into agarose containing bacteria. Following overnight incubation of the bacterial plate at 37 °C, clear zones were observed where bacterial growth was suppressed by diffusion of piscidin into the agarose.

Statistical analyses

Data were analysed using the JMP IN[®] 5.1 statistical software (SAS Institute Inc., Cary, NC, USA). Analysis for significant difference between means was tested on the arcsine transformation of the '% of control' value (one-way ANOVA, Tukey–Kramer HDS, $\alpha = 0.05$). Parameters in figures and text are presented as average \pm SEM (Sokal & Rohlf 1995).

Photomicrography

Photographs of selected treatments were made with a TMS inverted phase contrast microscope (Nikon, Tokyo, Japan).

Table 1 Lethal concentrations of piscidin 2 against protozoan ectoparasites exposed under high or low cation concentrations

Parasite	Concentration ($\mu\text{g mL}^{-1}$)		Concentration (mM)		
	PC_{\min}	PC_{100}	Na	Ca	Mg
<i>Trichodina</i>	12.5–100	> 100	469.8	10.6	51.8
<i>Cryptocaryon</i> theront	12.5	25	469.8	10.6	51.8
<i>Amyloodinium</i> dinospore (200 per 100 μL)	12.5	> 100	469.8	10.6	51.8
<i>Amyloodinium</i> dinospore (20 per 100 μL)	6.3	12.5	469.8	10.6	51.8
<i>Ichthyophthirius</i> theront	6.3	6.3	0	0	0

Results

Piscidin 2 was lethal to the infective stages of all four parasites but the minimum protozoacidal concentration (PC_{\min}) varied among the different parasites (Table 1). *Trichodina* sp. was the most resistant parasite and also had the greatest variability in response to piscidin 2. Because of this variability, the dose-response assay was repeated eight times. The PC_{\min} ranged from 12.5 to 100 $\mu\text{g mL}^{-1}$, but in only one of the eight replications were any parasites dead at 12.5 $\mu\text{g mL}^{-1}$. Parasite deaths (sudden immobilization followed by rapid lysis and disintegration) typically occurred within the first 60 min of exposure and usually at 50 $\mu\text{g mL}^{-1}$ piscidin 2 or higher. There were always some surviving parasites at the final observation period (4 h), so the PC_{100} was consistently > 100 $\mu\text{g mL}^{-1}$.

For *I. multifiliis* theronts, both the PC_{\min} and the PC_{100} were 6.3 $\mu\text{g mL}^{-1}$. All theronts died within 5 min at 100 $\mu\text{g mL}^{-1}$ and within 10 min at 6.3–12.5 $\mu\text{g mL}^{-1}$. After 4-h exposure to piscidin 2, there was no lysis of any theronts at 3.1 $\mu\text{g mL}^{-1}$, but all theronts were visibly less active compared with those in control wells.

The PC_{\min} for *C. irritans* theronts was 12.5 $\mu\text{g mL}^{-1}$ and the PC_{100} was 25 $\mu\text{g mL}^{-1}$. At 50 $\mu\text{g mL}^{-1}$, most theronts died within 15 min, and even lower doses (as low as 12.5 $\mu\text{g mL}^{-1}$) also killed some parasites within 15 min; at 25 $\mu\text{g mL}^{-1}$, the great majority were dead within 60 min (Fig. 1). A few theronts typically survived long after most others had lysed and disintegrated. However, these theronts were clearly abnormal, as indicated by their shape ('swollen' appearance) and displacement of cytoplasmic contents (Fig. 2). Swelling appeared to begin at the antapical (basal) end of the cell.

Swimming behaviour of these theronts was also very slow and anomalous.

When *A. ocellatum* dinospores were exposed to piscidin 2 at a density of 200 dinospores per 100 μL , the PC_{\min} was 12.5 $\mu\text{g mL}^{-1}$ and the PC_{100} was > 100 $\mu\text{g mL}^{-1}$. While concentrations of piscidin 2 less than 12.5 $\mu\text{g mL}^{-1}$ were non-lethal, at least 6.25 $\mu\text{g mL}^{-1}$ still affected the swimming behaviour of the dinospores. At concentrations lower than 6.25 $\mu\text{g mL}^{-1}$ of piscidin 2, there were no visible effects. Dinospores clearly appeared distressed in 12.5–50 $\mu\text{g mL}^{-1}$ exposures, displaying abnormally slow movement; most had settled to the bottom of the well within 5 min of exposure. Within 20 min exposure to 50 $\mu\text{g mL}^{-1}$ piscidin 2 and within 60 min exposure to 25 $\mu\text{g mL}^{-1}$ piscidin 2, most appeared dead, lying motionless on the bottom of the well. However, the following day (approximately within 12–24 h of initial exposure) several dinospores, including those at the highest exposure (100 $\mu\text{g mL}^{-1}$) had recovered motility. When *A. ocellatum* dinospores were exposed to piscidin 2 at a 10-fold lower density (20 dinospores per 100 μL), the PC_{\min} was 6.3 $\mu\text{g mL}^{-1}$ and the PC_{100} was 12.5 $\mu\text{g mL}^{-1}$. None of the dinospores that were immobilized ever regained motility.

In the *A. ocellatum* infectivity assay, 25–100 $\mu\text{g mL}^{-1}$ piscidin 2 significantly inhibited the differentiation of dinospores into trophonts/tomonts, while there was no significant effect on dinospore differentiation compared with controls at 12.5 $\mu\text{g mL}^{-1}$ piscidin 2 or lower (Fig. 3a). However, regression analysis of these data showed a strong correlation between piscidin 2 concentration and infectivity along the entire dose-response gradient from 100 to 3.12 $\mu\text{g mL}^{-1}$ (Fig. 3b).

On the bug blot, comparison of the clearing zone of 15 μL of gill extract with a dilution series of piscidin 2 showed that the activity in the gill extract was equivalent to a piscidin 2 concentration of at least 150 ng in the gill sample (Fig. 4). Since the gill extract was a 1:4 dilution of gill tissue, this corresponded to an estimated piscidin 2 tissue concentration of 40 $\mu\text{g mL}^{-1}$.

Discussion

The great majority of studies of the biological activity of piscine AMPPs have focused on their antibacterial or antimycotic properties (see Noga & Silphaduang 2003 for a review). However, some

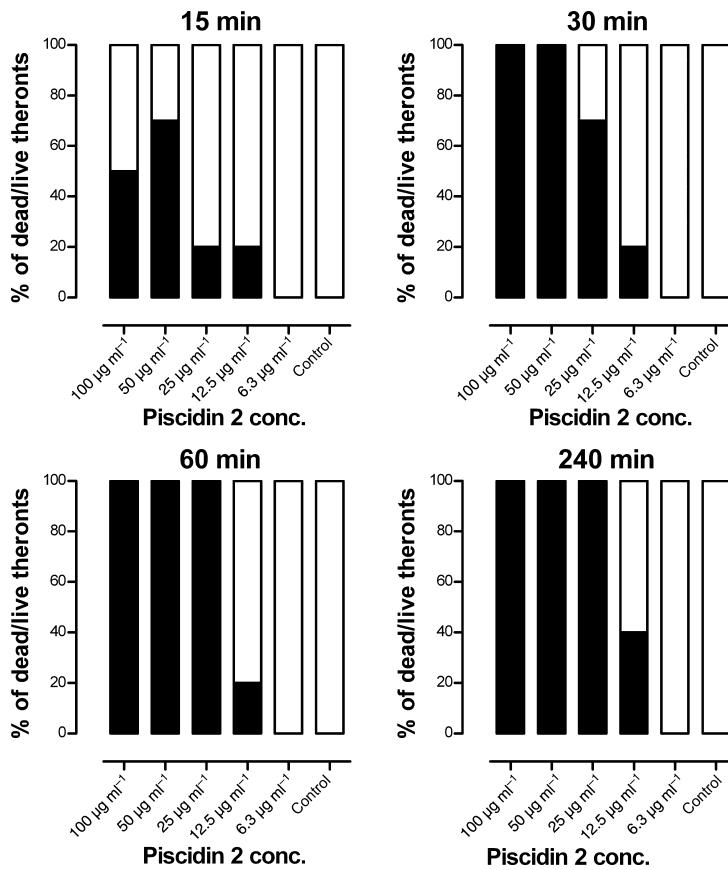


Figure 1 Time-course of the effect of piscidin 2 on survival of *Cryptocaryon irritans* theronts. Bars: black, mortality; empty, survival. The two control groups (HAc/BSA diluent, sea water) both had zero mortality and are thus represented together in a single bar. Each bar represents seven replicates (three wells per replicate).

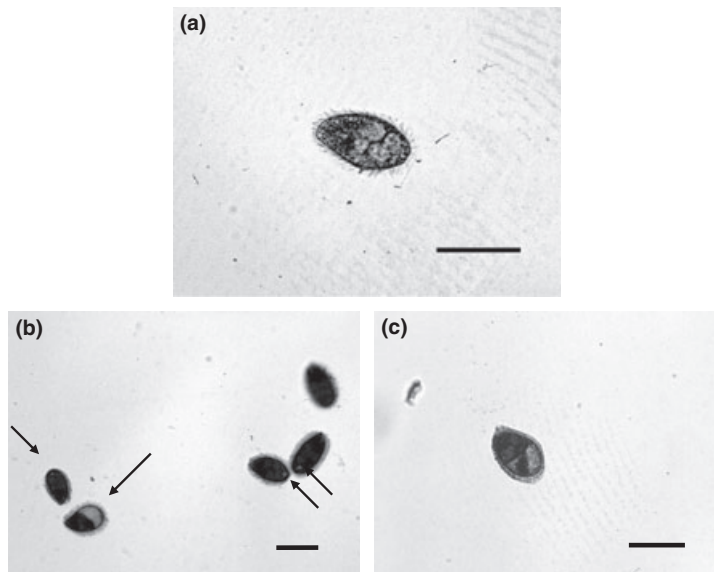


Figure 2 Effect of piscidin 2 on the morphology of *Cryptocaryon irritans* theronts. All theronts were fixed with Lugol's iodine (bars = 50 µm). (a) Normal unexposed theront (approximately 7 h after adding diluent). Note the normal morphology; the typical, four-beaded macronucleus is clearly visible. (b, c) Piscidin 2-exposed theronts (approximately 7 h after adding 12.5 µg mL⁻¹ piscidin 2). Note that some theronts appear swollen (long arrow), with the cytoplasm marginated to the apical end of the cell by a large vacuole that presumably formed as a consequence of osmotic imbalance because of membrane failure. Other theronts (shorter arrows) have an apparently incipient vacuolization at their antapical pole.

prior work has shown that another AMPP, histone-like protein, can also have potent activity against at least one important fish parasite (*A. ocellatum*).

Histone-like proteins are broad-spectrum AMPPs that have been isolated from skin, gills, gut and immune organs of many fish, including hybrid

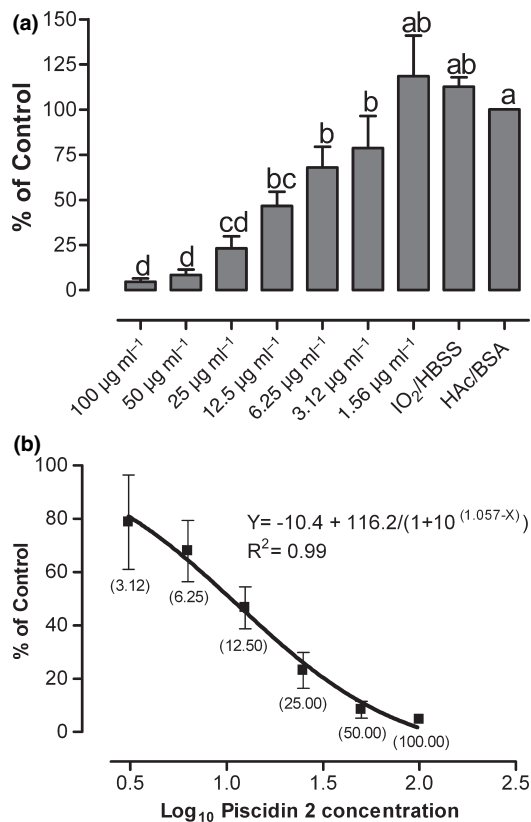


Figure 3 (a) Effect of piscidin 2 on the infectivity of *Amyloodinium ocellatum* dinospores. Data shown are the pooled mean from a total of 18 replications (six tests × three wells per concentration). There was no significant difference among any experiments prior to pooling the data. Values (mean ± SE) with different letters are significantly different ($P < 0.05$). IO₂/HBSS, artificial seawater control; HAc/BSA, acetic acid/bovine serum albumen diluent control. (b) Dose–response regression analysis on a semi-logarithmic scale of mortalities of *A. ocellatum* dinospores (presented as % of control) as a function of piscidin 2 concentration. Numbers in parenthesis represent actual piscidin concentrations in µg mL⁻¹.

striped bass (Noga, Fan & Silphaduang 2002), channel catfish (Robinette, Wada, Arroll, Levy, Miller & Noga 1998), rainbow trout, *Oncorhynchus mykiss* (Walbaum), (Fernandes, Kemp, Molle & Smith 2002; Noga *et al.* 2002) and Atlantic salmon, *Salmo salar* L. (Richards, O’Neil, Thibault & Ewart 2001). Histone-like protein-1 (HLP-1, related to histone H2B) and histone-like protein 2 (related to histone H1) are potently lethal to *A. ocellatum* (Noga *et al.* 2001, 2002) at concentrations that are well within the concentrations measured in epidermal tissues *in vivo* (Robinette & Noga 2001).

Unlike piscidin 2, HLP-1 specifically targets the trophont stage and has no apparent effect on dinospores (Noga *et al.* 2001). Because both piscidins and HLPs are present in the skin and gill of some fish, this might function to enhance resistance by targeting more than one stage in the parasite’s life cycle.

Piscidin 2, as is typical of other lytic peptides, can act very quickly, disrupting the permeability of the microorganism membrane and leading to irreversible damage and lysis. Zasloff (1987) observed rapid (within seconds) swelling of contractile vacuoles of the free-living ciliate *Paramecium caudatum* when exposed to the AMPP magainin in low osmolality (fresh water) medium. Others have observed lysis with exposure of other parasites to various AMPPs, including *Trypanosoma cruzi* trypomastigotes to cecropin B and its analogues (Jaynes, Burton, Barr, Jeffers, Julian, White, Enright, Klei & Laine 1988), *T. brucei rhodesiense* trypomastigotes to stomoxyn (Boulanger, Munks, Hamilton, Vovelle, Brun, Lehane & Bulet 2002), malaria (*Plasmodium gallinaceum*) sporozoites to insect defensins (Shahabuddin, Fields, Bulet, Hoffmann & Miller 1998) and *Leishmania donovani* promastigotes to temporin A & B (Mangoni, Saugar, Dellisanti, Barra, Simmaco & Rivas 2005).

The rapid lethal effect of piscidin 2 was most apparent with *I. multifiliis*, which was the most sensitive parasite to piscidin 2 exposure. Part of this greater sensitivity might be due to the low cation concentrations in fresh water. Sea water has very high concentrations of both monovalent and divalent cations (Enmar, Stein, Bar-Matthews, Sass, Katz & Lazar 2000), which are typically very inhibitory to most polypeptide antibiotics (Jensen, Hamill & Hancock 2006).

Salinity in the Gulf of Eilat is very high (40.5–40.8 ‰) but piscidin 2 had potent antiparasitic activity at 36 ‰ salinity, which has very high Na⁺ (470 mM), Ca⁺⁺ (11 mM) and Mg⁺⁺ (52 mM) levels. Nonetheless, compared with most other AMPPs, piscidins are quite active in salt (Noga & Silphaduang 2003), as reflected in the activity of piscidin 2 against all the marine parasites that we tested. Lauth *et al.* (2002) also found that piscidins 1 and 2 were still highly active against *Staphylococcus aureus* in the presence of high concentrations of monovalent (up to 504 mM Na⁺) and divalent (up to 7.2 mM Ca⁺⁺ and 10.2 mM Mg⁺⁺) cations.

Piscidin 2 was most effective against marine parasites within the first few minutes of exposure,

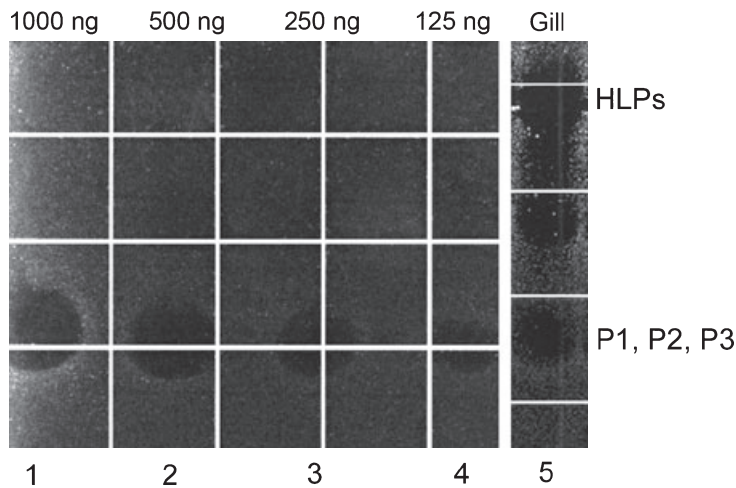


Figure 4 Bug blot of hybrid striped bass gill extract and a dilution series of piscidin 2. Lanes 1–4, 1000, 500, 250 and 125 ng piscidin 2; Lane 5, 15 μL of gill extract showing migration of histone-like proteins (HLPs) and piscidins 1, 2 and 3 (P1, P2 and P3).

gradually losing lethal effect. This was probably at least partly because of peptide instability in sea water. Decreased lethality was also reflected in the presence of sublethal changes in parasite behaviour and/or morphology. The most striking changes were observed with *C. irritans* theronts, where effects ranged from dead, quickly disintegrating theronts, to moribund theronts (lying on the bottom and weakly moving their cilia), to swollen theronts (that were still motile); these were often present in the same well at the same time. Occasionally, theronts developed a prominent vacuole. This vacuole was presumably because of osmotic imbalance from plasma membrane damage. At least one other marine ciliate parasite, *Miamensis avidus*, is hyperosmotic to the environment, using its contractile vacuole to regulate cell volume by expelling water that enters passively (Kaneshiro, Dunham & Holz 1969). Interestingly, the swelling in *C. irritans* appeared to start at the basal end and then proceeded anteriorly. However, no pore (e.g. cytophyge) which piscidin 2 might render more permeable has ever been detected in *C. irritans* (Colorni & Diamant 1993).

Trichodina sp. appeared to be the parasite most resistant to piscidin 2 but also was most variable in its susceptibility. This might have been due to the need to expose the parasite while still attached to host tissue, which contained varying amounts of blood, which is known to inhibit the activity of some AMPPs (Boman 1995). Thus, *Trichodina* sp. might be more susceptible than it appears.

At a relatively low density (20 dinospores per 100 μL), *A. ocellatum* dinospores were rapidly and permanently immobilized by piscidin 2. However,

at higher density (200 dinospores per 100 μL), a small number survived and later resumed motility. Immobilization may be a means of temporarily conserving energy for cell repair. However, it would not be necessary for piscidin 2 to kill the parasites to be effective: if they are immobilized, they are not infective and thus the practical effect is the same, and is energetically more efficient as a much lower AMPP concentration is required.

Density-dependent antimicrobial activity has been well documented for other AMPPs and appears to be due to the need for a minimum concentration of peptide that must interact with the pathogen membrane in order to cause cell damage (Boman 1995). Thus, an excess number of targets exhaust the available peptide. Even 20 dinospores per 100 μL is a very high parasite density unlikely to be encountered in most natural infections. For example, experimental challenge of tomato clownfish, *Amphiprion frenatus* Brevoort, with 2 dinospores per 100 μL is 100% lethal within 5 days (Cobb, Levy & Noga 1998). Although our densities were well above that of a typical natural exposure, it is still possible that low piscidin 2 concentrations (not showing an apparent effect in our tests) might affect behaviour (e.g. motility) and thus dinospore infectivity. The strong correlation of infectivity with piscidin 2 concentration even at very low peptide levels suggests that this might occur.

Using the bug blot, we estimated that the gill tissue concentration of piscidin 2 was approximately 40 $\mu\text{g mL}^{-1}$. This concentration is only an estimate because piscidin 2 co-migrates on AUPAGE with both piscidin 1 and piscidin 3 (Silphaduang & Noga 2001). Piscidin 2 is very

similar to piscidin 1, varying only by a single conservative amino acid substitution at position 18 and having an identical antimicrobial spectrum. Piscidin 3 has slightly weaker antimicrobial activity than piscidins 1 or 2 but still is quite potent (Silphaduang & Noga 2001). The antiparasitic activity of piscidin 3 is undetermined, although it is inhibitory to all bacterial pathogens affected by piscidins 1 and 2 (Silphaduang & Noga 2001). Thus, our estimated concentration of piscidin 2 in gill tissue strongly suggests that piscidin 2 or related piscidins are expressed at a concentration that is well within the inhibitory range for all the parasites that we tested, especially for parasites that have very intimate contact with host tissues. Some have argued recently that the AMPP concentrations measured in mammalian tissues are usually well below those needed to inhibit pathogens *in vitro* (Bowdish, Davidson & Hancock 2005), calling into question their biological significance as antibiotics in these animals. However, our data suggest that the concentrations of piscidins are well within the range required to be directly lethal to not only parasites but also to other important pathogens, such as bacteria, because the minimum inhibitory concentration of piscidins 1, 2 and 3 for many fish bacterial pathogens ranges from 0.8 to 12.5 µg mL⁻¹ (Silphaduang & Noga 2001).

Piscidins or related peptides appear to be present in many higher teleosts (both freshwater and marine) of the order Perciformes, including moronids, striped bass, *M. saxatilis*, white bass, *M. chrysops* (Rafinesque), European sea bass, *Dicentrarchus labrax* (L.), sciaenids, spot, *Leiostomus xanthurus* Lacepède, croaker, *Micropogonias undulatus* (L.), serranids, grouper, *Epinephelus niveatus* (Valenciennes), siganids, rabbitfish, *Siganus rivulatus* Forsskål, belontids, gourami, *Trichogaster leeri* (Bleeker), cichlids and Nile tilapia, *Oreochromis nilotica* (L.) (Silphaduang *et al.* 2006). Thus, our data suggest that piscidin 2 or related peptides have the potential to function as an important antimicrobial defence in a number of fish in marine as well as freshwater environments.

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