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Susceptibility of two fishes (*Oreochromis niloticus* and *Cyprinodon variegatus*) to *Pfiesteria shumwayae* and its associated toxin: Influence of salinity

Andrew S. Gordon *, Elaine P. Gordon, Brian J. Dyer

Department of Biological Sciences, Old Dominion University, Norfolk, VA 23529-0266, USA

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Abstract

Researchers examining the mechanisms of ichthyotoxicity of *Pfiesteria shumwayae* have come to different conclusions about the role of toxin in this process. Some attribute fish mortality solely to direct attack by these pedunculate dinoflagellates on exposed fish tissue while others have provided evidence for a role of a soluble toxin. Detection of toxin, especially in low concentrations, is a function of the sensitivity of the selected bioassay methods and the various groups addressing this question have utilized different methods. One notable difference in fish bioassay methods utilized to detect *Pfiesteria*-associated toxin (PfTx) is the species of fish tested. Studies that have not detected PfTx in bioassays generally have used *Cyprinodon variegatus* (sheepshead minnow) as the test fish while those that have detected toxin generally used *Oreochromis* spp. (Tilapia). In this study response of these two fish species was compared to determine their relative sensitivity to physical attack by *P. shumwayae* and to PfTx. The results indicate that *Oreochromis niloticus* is more susceptible to *P. shumwayae* and its associated toxin than *C. variegatus* and implicate differences in the ability these species to osmoregulate as a contributing factor for this phenomenon. Salinity stress enhanced susceptibility of *O. niloticus* to PfTx and thus improved the sensitivity of the bioassay. The observation that salinity stress enhances toxicity to *O. niloticus* provides additional information regarding the mechanism of PfTx toxicity although the conditions utilized are not representative of the natural habitat of these freshwater fish.

Keywords: *Pfiesteria*; Toxin; Fish bioassay; Salinity; Osmoregulation

1. Introduction

The mechanism of ichthyotoxicity in bioassays containing *Pfiesteria* spp. remains a controversial topic. While most researchers agree that *Pfiesteria* spp. can kill fish, the involvement of soluble toxin has been disputed (Berry et al., 2002; Vogelbein et al., 2002). And in bioassays where soluble toxin has been observed its source has been questioned due to the complex nature of aquarium based bioassays (Drgon et al., 2005). Strain variability, culture history, and assay methods utilized for detection of soluble toxin are critical factors influencing conclusions regarding the mechanism of ichthyotoxicity by *Pfiesteria* spp. (Burkholder et al., 2005). Consequently it is important to understand the relative sensitivity of available assays and test organisms utilized in various studies to examine *Pfiesteria* toxicity.

A toxin from *Pfiesteria*-containing cultures (PfTx) has been isolated and partially purified (Burkholder et al., 2005, 2001b; Moeller et al., 2001). The activity and a mode of action for this bioactive substance have been examined using a reporter gene assay (Fairey et al.,

* Corresponding author. Tel.: +1 757 683 3595; fax: +1 757 683 5283.
E-mail address: agordon@odu.edu (A.S. Gordon).

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1999; Kimm-Brinson et al., 2001; Melo et al., 2001). Partial structural information has been proposed for this compound but the complete structure is still unknown (Moeller et al., 2003). This toxin has been demonstrated to be present in extracts from *Pfiesteria piscicida* and *Pfiesteria shumwayae* strains, including CCMP 2089 (Burkholder et al., 2005), a strain of *P. shumwayae* that has been reported to be non-toxigenic (Vogelbein et al., 2002; Berry et al., 2002). While *Pfiesteria* cultivated in the presence of fish produce higher concentrations, toxin has recently been demonstrated in bacteria and fungi–free conditions (Burkholder et al., 2005). *Pfiesteria* cultures that have been maintained in the laboratory for extended time generally have been observed to become less toxigenic (Burkholder et al., 2005, 2001a).

Although it is apparent that multiple factors can affect the outcome of experiments examining *Pfiesteria* ichthyotoxicity, several studies that have not found soluble toxin have relied primarily on *Cyprinodon variegatus* (sheepshead minnow) as the test organism (Berry et al., 2002; Vogelbein et al., 2002; Lovko et al., 2003) while those that did detect soluble toxic activity in fish bioassays have primarily utilized *Oreochromis* spp. (Tilapia; Gordon and Dyer, 2005; Gordon et al., 2002). So while *Pfiesteria* toxin at sufficiently high concentration can kill many fish species including *C. variegatus* and *Oreochromis* spp. (Burkholder et al., 1995; Moeller et al., 2001), when toxin concentration is low the choice of test organism may be critical for detection of toxin.

We have noted that *Oreochromis niloticus* exposed to toxic *Pfiesteria* cultures may exhibit symptoms identical to those observed when these fish are subjected to a lethal increase in salinity. These symptoms include loss of equilibrium and of ability to properly control buoyancy (personal observation). This observation prompted us to examine the interaction between salinity stress and sensitivity of fishes to *Pfiesteria*-associated toxin.

In the present study we compared the relative sensitivities of *C. variegatus* and *O. niloticus* in bioassays containing *P. shumwayae* and in cell-free filtrates derived from the bioassay aquariums. In addition we investigated the effect of salinity on toxicity to both fishes in cell-free filtrates.

2. Materials and methods

Pfiesteria shumwayae strain (CAAE 1024C = 101272) was obtained from the Center for Applied Aquatic Ecology (CAAE) at North Carolina State University, Raleigh, NC. It was maintained on Rhodomonas (CCMP768 Bigelow Laboratories) prey (Marshall et al., 2000) and placed into aquarium bioassays containing *O. niloticus* (Burkholder et al., 2001c). Filtrates (0.2 μm) were prepared by tangential flow filtration as described previously (Gordon et al., 2002). Toxicity of filtrates was tested using the ''cup'' assay (Gordon et al., 2002) for *O. niloticus* (sex-reversed male; AquaSafra, Bradenton FL). Briefly the assay involves placing individual juvenile fish in 100 mL of filtrate contained in aerated plastic cups ($n = 10$) with mortality tallied at 24 and 48 h. Filtrate toxicity was also tested using *C. variegatus* larvae (7–10 day old, Aquatic Biosystems, Fort Collins CO) in polystyrene six-well plates (Falcon). Fifteen millilitres of filtrate was placed into each well of replicate plates and five *C. variegatus* larvae were placed into each well. Two plates were prepared using filtrate from *P. shumwayae*-inoculated aquariums and two using filtrate from control aquariums. Mortality was tallied at 24 and 48 h.

In experiments where the influence of salinity on toxicity of cell-free filtrates was tested, salinity of the filtrate was adjusted by addition of a saturated solution of Instant Ocean salts (Aquarium Systems, Mentor, OH) and measured using a refractometer (SR5 Aquatic Ecosystems, Apopka, FL). *O. niloticus* were acclimatized to the target salinity for at least 4 days (this amount of time was required since it is a halotolerant freshwater fish) and *C. variegatus* larvae were acclimatized 24–48 h prior to being exposed to salinity-adjusted filtrate. Controls consisted of filtrates from control aquariums adjusted to equivalent salinity.

Two different sets of experiments were done to examine the influence of salinity on the susceptibility of fishes to PfTx. The first set of experiments utilized *O. niloticus* as the test fish. In each experiment in this series a toxin-containing filtrate and a filtrate prepared from control aquariums containing *O. niloticus* but not *Pfiesteria* were prepared as described above. The filtrates were adjusted to the target salinity and pre-acclimatized fish were exposed to the toxic and control filtrates (10 fish to each condition) in the cup assay described above. These experiments were performed at salinities of 14, 20 and 32 ppt for both control and toxic filtrates. Data were expressed as percent mortality. The second set of experiments compared the response of *O. niloticus* and *C. variegatus* to PfTx in filtrates at two salinities (15 and 25 ppt). *O. niloticus* was exposed to toxic and control filtrates as in the first set of experiments and *C. variegatus* larvae were exposed in six-well plates as described above. Experiments with