Mar Biol (2007) 152:835–843
DOI 10.1007/s00227-007-0734-9

RESEARCH ARTICLE

Altered c-Fos expression demonstrates neuronal stress in mummichog, *Fundulus heteroclitus*, exposed to *Pfiesteria shumwayae* and *Chaetoceros concavicornis*

J. D. Salierno · J. D. Shields · A. Z. Murphy · G. E. Hoffman · A. S. Kane

Received: 20 February 2007 / Accepted: 17 May 2007 / Published online: 26 June 2007

Abstract To better understand sublethal effects of harmful algal blooms (HABs) on fish, mummichog, *Fundulus heteroclitus* (L.), were exposed in the laboratory to varying, environmentally relevant densities of *Pfiesteria shumwayae* (Glasgow et Burkholder, CCMP 2089, dinoflagellate) and *Chaetoceros concavicornis* (Mangin, CCMP 169, diatom). Two experiments were conducted during the spring of 2003 and 2004 to quantitatively examine the effects of acute (2 h) *P. shumwayae* and *C. concavicornis* algal exposure on mummichog brain activity using c-Fos expression as a marker of altered neuronal activity. Brains from HAB-exposed fish were removed, sectioned, and stained using immunocytochemistry prior to quantifying neuronal c-Fos expression. Fish exposed to *P. shumwayae* and *C. concavicornis* showed increased c-Fos expression compared to unexposed control fish. A significant dose-response relationship was observed, with increased labeling in brains of fish exposed to higher cell densities for both HAB species tested ($P \leq 0.01$). Increased labeling was found in the telencephalon, optic lobes, midbrain, and portions of the medulla. The greatest increases in expression were observed in the telencephalon of *P. shumwayae*-exposed fish, and in the telencephalon and optic lobes of *C. concavicornis*-exposed fish ($P \leq 0.01$). These increases in c-Fos expression are consistent with other physical and chemical stress exposures observed in fish. Neuronal stress, evidenced by c-Fos expression, demonstrates a sublethal effect of exposure and changes in brain activity in fish exposed to HAB species.

Communicated by J.P. Grassle.

J. D. Salierno · A. S. Kane (✉)
Aquatic Pathobiology Center,
Department of Epidemiology and Preventive Medicine,
University of Maryland School of Medicine,
Baltimore, MD 21201, USA
e-mail: kane@ufl.edu

J. D. Shields
Department of Environmental and Aquatic Animal Health,
Virginia Institute of Marine Science, P.O. Box 1346,
Gloucester Point, VA 23062, USA

A. Z. Murphy
Department of Biology, Georgia State University,
Atlanta, GA 30303, USA

G. E. Hoffman
Department of Anatomy and Neurobiology,
University of Maryland School of Medicine,
655 W. Baltimore Street, Baltimore, MD 21201, USA

Introduction

Pfiesteria shumwayae and *Chaetoceros concavicornis* are species of algae that form harmful algal blooms (HABs) and play a role in fish kills along the United States coastline. *Pfiesteria shumwayae*, a member of the *Pfiesteria* complex, has been associated with ulcerative lesions in fish along the mid-Atlantic coast of the United States (Burkholder et al. 1995; Glasgow et al. 1995; Burkholder and Glasgow 1997; Glasgow et al. 2001; Lovko et al. 2003). Recently, *P. shumwayae* was assigned to a new genus, *Pseudopfiesteria*, based on morphology and phylogenetic rDNA analysis (Litaker et al. 2005). However, the genus was then reclassified back to *Pfiesteria* after further molecular phylogenetic analysis (Marshall et al. 2006). In the mid-1990s, fish kills in North Carolina and Maryland estuaries co-occurring with the presence of *Pfiesteria* spp. brought HABs to the forefront of public attention in the mid-Atlantic of the USA (Burkholder et al. 1992; Noga

et al. 1996; Law 2001; Magnien 2001). In addition, *Pfiesteria* spp. have been discovered in other estuaries on the East and Gulf coasts of the USA, as well as in New Zealand and Europe (Rublee et al. 1999; Glasgow et al. 2001; Jakobsen et al. 2002; Lewitus et al. 2002; Rhodes et al. 2002).

It remains unclear whether all strains of *P. piscicida* or *P. shumwayae* are toxic to fish, but knowledge regarding their physical attraction and subsequent attachment to fish with a peduncle organelle, which leads to epithelial damage, is clearly recognized (Vogelbein et al. 2001; 2002; Miller and Belas 2003). Physical exposure to *P. piscicida* and *P. shumwayae* is known to damage fish gill lamellae, and cause epidermal skin lesions (Noga et al. 1996; Burkholder and Glasgow 1997; Glasgow et al. 2001). Direct physical contact between *P. shumwayae* (CCMP 2089 and CAAE 101272) and fish is the most consistent mechanism mediating fish mortality in laboratory exposures (Gordon and Dyer 2005). Indeed, laboratory studies have shown that *P. shumwayae* (CCMP 2089) directly attaches to fish skin, gill, olfactory organs, and oral mucosa, causing extensive tissue damage and mortality (Vogelbein et al. 2001; Berry et al. 2002; Vogelbein et al. 2002). Later studies, however, suggest that toxicity of *Pfiesteria* spp. to fish can vary depending on algal strain and assay variability (Burkholder et al. 2005). Metal availability may also play a role in toxigenesis since a metal-containing, organic-ligated toxin has recently been isolated from *P. piscicida* (CCMP1921) (Moeller et al. 2007).

Fish mortality resulting from exposure to species of *P. piscicida* and *P. shumwayae* has been well documented in the laboratory. Less is known, however, regarding fish kills in the field due to the complex assemblage of potential etiologies and mitigating stress factors. For example, infection with opportunistic pathogens, including fungi and bacteria, combined with immune system changes, may play synergistic roles in fish mortality in the wild (Blazer et al. 1999; Dykstra and Kane 2000; Law 2001; Vogelbein et al. 2001; Kiryu et al. 2002, 2003; Flewelling et al. 2005).

Behavioral alterations have been observed and documented in HAB-exposed fish from the wild and in the laboratory. Atlantic menhaden, *Brevoortia tyrannus*, were observed swimming erratically and writhing near the surface during blooms of *Pfiesteria*-like dinoflagellates in the field (Burkholder et al. 1995; Magnien 2001). In the laboratory, several fish species exposed to *P. piscicida* and *P. shumwayae* demonstrated loss of equilibrium, disorientation, lethargy combined with periods of hyperactivity, general depression, decreased respiration, wavering, fin twitching, and settling to the bottom of the aquaria (Burkholder et al. 1995; Lewitus et al. 1995; Noga et al. 1996; Berry et al. 2002; Gordon et al. 2002). These alterations have only been documented at concentrations lethal to fish, with the mechanism(s) of action driving these behaviors unknown.

Blooms of *Chaetoceros* spp. on the Northwest coast of the USA have been linked to numerous fish kills, with mortality occurring through direct physical contact between the algae and the gills of fish. *Chaetoceros* is a genus of diatoms found in temperate coastal waters, and can cause finfish mortalities at concentrations as low as 5 cells ml^{-1} in salmonid species (Bell et al. 1974; Yang and Albright 1992). Strains of *C. concavicornis* form long chains of bullet-shaped cells that contain hollow, silicate spines (setae) studded with smaller spines (spinules) along their length (Yang and Albright 1992). Upon contact, these spines penetrate and break off on the secondary lamellae of fish gills causing hyperplasia, hypertrophy, and partial or complete fusion of secondary lamellae (Yang and Albright 1992). Fish mortality has been attributed to microbial infections associated with damaged gill tissue, including hemorrhage and suffocation from excess mucus production (Bell 1961; Yang and Albright 1994). In addition, *Chaetoceros* spp. blooms exacerbate mortality associated with opportunistic diseases such as vibriosis and bacterial kidney disease. Exposure to low cell counts of *Chaetoceros concavicornis* is associated with neutropenia, lymphocytopenia, and thrombocytopenia in chinook (*Oncorhynchus tshawytscha*) and coho (*Oncorhyncus kisutch*) salmon, with as few as 15 cells ml^{-1} leading to mortality in juveniles (Yang and Albright 1994).

The present study investigated the effect of acute, sublethal exposures to *P. shumwayae* (CCMP 2089) and *C. concavicornis* (CCMP 169) on neuronal activity in the mummichog, *Fundulus heteroclitus*, an ecologically important, common species of estuarine killifish. This was accomplished through quantification of c-Fos protein expression in the brains of mummichog exposed to these harmful algal species.

The *c-fos* gene and its protein product c-Fos, are expressed in neurons as a result of neuronal stimulation (Herdegen and Leah 1998). Specifically, c-Fos is expressed rapidly and transiently when neuronal activity changes rapidly, and it is induced by transynaptic activity and neuronal stress. Altered c-Fos expression is associated with a stress response and related to neuronal survival, short term memory, and locomotory behaviors in many vertebrates (Bosch et al. 2001; Cheng et al. 2002; Sadananda and Bischof 2002; Espana et al. 2003). The goal of the present study was to demonstrate potential alterations in mummichog brain activity in vivo resulting from *P. shumwayae* and *C. concavicornis* exposure, visualized through changes in c-Fos expression. In addition to general changes in brain activity, knowledge of the specific regions within the brain that are activated during stress can offer valuable insights into the neural control of fish behavior.

Materials and methods

Exposures

The *Pfiesteria shumwayae* (Glasgow et Burkholder) exposure was conducted in May 2003 at the Virginia Institute of Marine Science, College of William Mary, and the *Chaetoceros concavicornis* (Mangin) in March 2004 at the Aquatic Pathobiology Center, University of Maryland College Park. Mummichog (total length 74 ± 8.1 mm) used in these experiments were collected from a reference site in Solomons, Maryland, treated for ectoparasites, and acclimated in the laboratory for at least 4 weeks prior to exposure. Laboratory holding conditions were 22.5°C (±2°), pH 8.0 (±0.5), and salinity 6 PSU, with water quality parameters measured at the conclusion of the experiment (Tables 1 and 2). Fish were fed pelleted fish chow (38% protein, Ziegler Bros., Gardners, Pennsylvania) and observed daily for general health. Fish were fasted 24 h prior to exposure and were not fed during the exposure.

Pfiesteria shumwayae exposure

P. shumwayae were cultured in the presence of *Tilapia* spp. following the methods of (Vogelbein et al. 2001, 2002). The culture originated from a Shields reference tank (1049-5E, strain CCMP #2089), which was started on April 19, 2002 when subcultures from the original isolation of CCMP 2089 were split into new tanks. The culture was grown with 25–30 *Tilapia* spp. individuals' day^{-1} for 203 days prior to experimentation. The culture had been killing fish with 60–100% mortality every day for 18 days prior to its use in the experiment; and it had been consistently killing fish for 110 days prior to its use. A fraction assay using larval fish (Vogelbein et al. 2002) was used to assess the presence of a toxin on days 153 and 202. The assays indicated that the cultures were killing fish from micropredation and not from toxicity (Vogelbein et al. 2002; Lovko et al. 2003). The culture continued to kill fish at 100% mortality day^{-1} until day 215 when fish were removed in preparation for the exposures.

Mummichog were acclimated in five 38-l aquaria for 4 days prior to exposure, 8 fish aquarium^{-1}, 40 total. Unfiltered cultures were added to the respective aquaria to achieve five cell densities: 0, 1×10^3, 2×10^3, 2.6×10^4, and 7×10^4 cells ml^{-1}. These cell densities fall within environmentally relevant concentrations, as well as those of other laboratory studies (Burkholder et al. 1995; Lewitus et al. 1995; Burkholder et al. 2001a; Vogelbein et al. 2001, 2002). Cell densities were determined based on the methods of Lovko et al. (2003). Cultures were added gently, and aeration was not supplied during exposure to minimize the potential for the dinoflagellates to encyst during the acute 2 h exposure period. The 2 h exposure period was based on pilot studies with *P. shumwayae* and pervious work with mummichog (Salierno et al. 2006). Water quality parameters were measured and maintained throughout the exposures based on vessel loading (Table 1). One-min behavioral observations were conducted every 20 min during the 2 h exposure and any alterations were recorded.

Table 1 Water quality parameters in aquaria following a 2-h exposure of mummichogs to *P. shumwayae*

Treatment (cells ml^{-1})	Temperature (°C)	pH	NH_3 (ppm)	NO_2^- (ppm)
0	22	7.8	0.011	0.4
1×10^3	22	7.8	0.006	0.2
2×10^3	22	7.4	0.045	0.3
2.6×10^4	22	7.8	0.056	0.7
7×10^4	22	7.9	0.140	2.0

Table 2 Water quality parameters in aquaria following a 2-h exposure of mummichogs to *C. concavicornis*

Treatment (cells ml^{-1})	Temperature (°C)	pH	NH_3 (ppm)
0	24.5	8.31	0.038
8×10^2	24.5	8.38	0.038
5×10^3	24.5	8.44	0.069

Chaetoceros concavicornis exposure

Mummichog were exposed to *C. concavicornis* (CCMP 169) under static conditions in 4-l glass aquaria, containing 2-l of water, to three cell densities: 0, 8×10^2, and 5×10^3 cells ml^{-1} without aeration. Aeration was not provided in order to maintain consistency with the *P. shumwayae* exposures. Cells were added to the water in the respective aquaria in which the fish had been acclimated for 24 h (6 fish treatment^{-1}, 24 total), and cell densities were determined using a Neubauer hemacytometer. Diatoms within each chain of *C. concavicornis* were counted as individuals, e.g., a chain of four individuals was counted as four. One-minute behavioral observations were conducted every 20 min throughout the 2 h exposure, and any alterations were recorded. Water quality parameters were measured and maintained throughout the exposures (Table 2).

Tissue collection and preparation

After exposure to either *P. shumwayae* or *C. concavicornis* fish were deeply anesthetized with buffered MS-222. Fish were then perfused through the heart with ice cold heparinized PBS. After perfusion, the dorsal aspect of the cranium was dissected away leaving the brain exposed, and the fish

were then preserved whole in 10% neutral buffered formalin containing 2.5% acrolein for 3 h. Brains were then removed from the crania and transferred to a solution of 30% sucrose where they remained until embedding (Salierno et al. 2006). Gill and skin samples were taken from three fish per treatment and processed for general histology (Profet et al. 1992).

Brains were embedded in egg gel molds and post-fixed in 4% paraformaldehyde overnight. They were then sunk in 30% sucrose, frozen with dry ice, and sectioned on a cryomicrotome at a thickness of 25 µm. All sections were collected, placed in a cryoprotectant antifreeze solution, and stored at −20°C until processed for immunocytochemistry (Watson et al. 1986).

c-Fos immunocytochemistry

Sections were processed free floating and in parallel, such that all sections were treated the same. Every third brain section was selected, rinsed in potassium phosphate buffered saline (KPBS), and incubated in sodium borohydride for 20 min to remove any residual acrolein. To enhance signal resolution and reduce background staining, an antigen retrieval procedure was followed using a sodium citrate buffer (Salierno et al. 2006). Sections were then immediately rinsed with KPBS, immersed into the polyclonal primary antibody (sheep anti-c-Fos, 1:1,000 in KPBS containing 0.4% Triton-X, Chemicon, Temecula, California), incubated at room temperature for 60 min, and then at 4°C for 48 h. A subset of sections from a pilot study processed with the primary antibody alone demonstrated that background staining was minimal.

After incubation in the primary antibody, sections were immersed in the secondary antibody (biotinylated rabbit-anti sheep, IgG 1:600 in KPBS with 0.4% Triton-X, Vector, Burlingame, California), and incubated for 60 min at room temperature. Sections were then immersed into avidin–biotin complex (Vector Stain ABC kit, 45 µl of avidin and 45 µl of biotin per 10 ml of KPBS with 0.4% Triton-X), and incubated at 25°C for 60 min. For visualization of c-Fos expression, sections were incubated in nickel-DAB chromogen (0.002 g 3,3 diaminobenzidine, Sigma-Aldrich, 0.25 g nickel sulfate, and 8.3 µl of H_2O_2/10 ml of sodium acetate) for 20 min and rinsed with the sodium acetate and KPBS. After staining, sections were stored in KPBS at 4°C until mounting (Salierno et al. 2006).

The following six regions of the mummichog brains were selected for analysis based on consistent c-Fos expression observed in prior experiments: the anterior telencephalon (area ventralis telencephali pars ventralis [Vv] and dorsalis [Vd]); the posterior telencephalon (diencephalic ventricle [DiV] and anterior parvocellular preoptic nucleus [PPa]); two regions in the optic tectum: anterior and posterior periventricular grey zone, (L1 & L2); the midbrain tegmentum (ventrolateral nucleus of the torus semicircularis [TSvl]; the nucleus lateralis valvulae [NLV]); and the rhombencephalon (medial longitudinal fascicle [MLF]) (Wulliman et al. 1996; Salierno et al. 2006). Once specific brain sections had been selected based on c-Fos expression patterns, specific regions within the sections were quantified for c-Fos expression.

Data collection and analysis

Images from each brain section were viewed using a Nikon Eclipse 800 microscope through a 10× objective lens with consistent illumination. Digital images were captured through the microscope using a digital camera (Photometrics SenSys, BioVision Tecnologies, Exton, Pennsylvania) connected to a Macintosh G4 computer. Smaller, specific regions of interest within the brain sections were outlined, based on previous data with mummichog, and imported into NIH-Image for area calculation (Fig. 1; Salierno et al. 2006). Calculation of c-Fos stain area was consistently quantified by "black" level-adjustment thresholding in Adobe Photoshop™ in order to limit the images to contain only areas of DAB staining. This level of threshold was determined optimal to consistently visualize areas of staining, based on preliminary experiments and previous studies (Salierno et al. 2006). The black areas of stain were subsequently analyzed in NIH-Image and normalized by the total section area. Previous studies demonstrated that sections in which c-Fos positive nuclei were hand counted were in agreement with the densitometry method.

The goal was to quantify alterations in c-Fos expression within regions of the mummichog brain as exposure algal concentrations increased. Therefore, the percentage of Fos-positive nuclei within the brain regions of interest as an indicator of regional c-Fos expression was then calculated and compared across the varying algal concentrations. Concentration was treated as a continuous variable and data were arcsin square root transformed to reduce the variance, and to meet the assumptions of the ANOVA procedure prior to analysis. A two-way ANOVA was then used to analyze the effect among different algal concentrations and region of the brain on c-Fos expression (PROC MIXED, SAS, vs. 9.1, Cary, North Carolina). In the event of a significant F-value, mean c-Fos expression values within brain regions were compared by algal concentration (t-values, $P \leq 0.05$). P-values represent the comparison of the algal density with c-fos expression within specific brain regions.

Results

There was strong punctuate staining of neurons expressing c-Fos activity with exposure to *P. shumwayae* and

C. concavicornis. Differential expression in c-Fos expression was observed across brain regions, with the largest increases in labeling in exposed fish occurring in the telencephalon and optic lobes (Fig. 1). High variance in expression was observed among brain regions. Exposed fish displayed higher variability compared to controls, but this difference was not significant (Levene Test $P > 0.05$). Differences between experiments using the two HABs may reflect differences in procedures in the two laboratories, while variability within experiments likely reflects differences in responses of individual fish. However, exposures to both algal species were similar with significant increases in c-Fos activity in the same regions of the brain compared to non-exposed fish.

Pfiesteria shumwayae exposure

All brain regions investigated displayed greater labeling in exposed than in non-exposed fish, when exposed to increasing concentrations of *P. shumwayae* ($F = 25.9$, $P \leq 0.001$, Fig. 2a). c-Fos expression increased in the anterior and posterior telencephalon as *P. shumwayae* densities increased ($t = 4.49$ and 2.43, $P = 0.0001$ and 0.016 respectively, Fig. 2a). c-Fos expression in the anterior optic lobe region also increased as *P. shumwayae* densities increased ($t = 1.72$, $P = 0.087$, Fig. 2a). There were no alterations in c-Fos labeling in the midbrain tegmentum or rhombencephalon (brain stem) ($t = 0.960$ and 1.19, $P = 0.641$ and 0.426 respectively, Fig. 2a).

Behavioral alterations were observed during exposure, including "wavering" in the water column and tetany of the fins, as previously described (Berry et al. 2002). No histological differences were observed in the gill or skin samples between exposed and non-exposed fish.

Chaetoceros concavicornis exposure

Fish exposed to *C. concavicornis* showed increased c-Fos expression compared to control fish ($F = 9.06$, $P \leq 0.05$, Fig. 2b). Increased labeling was observed in the anterior and posterior telencephalon, anterior optic lobe, and posterior optic lobe ($t = 3.37$, 2.55, 2.62, and 2.67, $P = 0.001$, 0.012, 0.010, and 0.009 respectively, Fig. 2b). Empirical observations of c-Fos labeling from exposed fish suggested increased labeling in all regions of the brain compared with controls. In addition, there was a dose-response relationship with increasing densities of *C. concavicornis* associated with increased c-Fos expression.

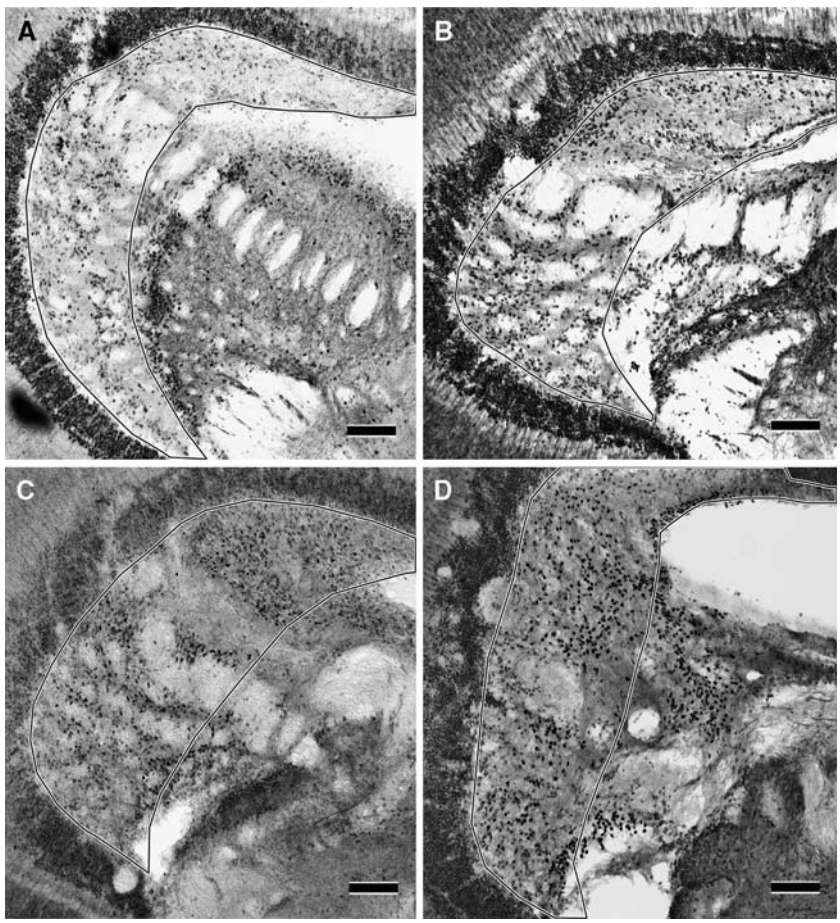

Fig. 1 *Fundulus heteroclitus*, **a**, **b** show representative optic tecta from non-exposed (0 cells ml^{-1}) and *P. shumwayae*-exposed (2×10^3 cells ml^{-1}) mummichog, respectively. **c**, **d** show representative optic tecta from non-exposed (0 cells ml^{-1}) and *C. concavicornis*-exposed (5×10^3 cells ml^{-1}) fish, respectively. Note differences in c-Fos expression represented by black punctuate nuclei in exposed brains (**b** and **d**). *Outlines* represent regions of the tecta that were quantitatively analyzed and normalized by observation area. Scale bar = 100 μm

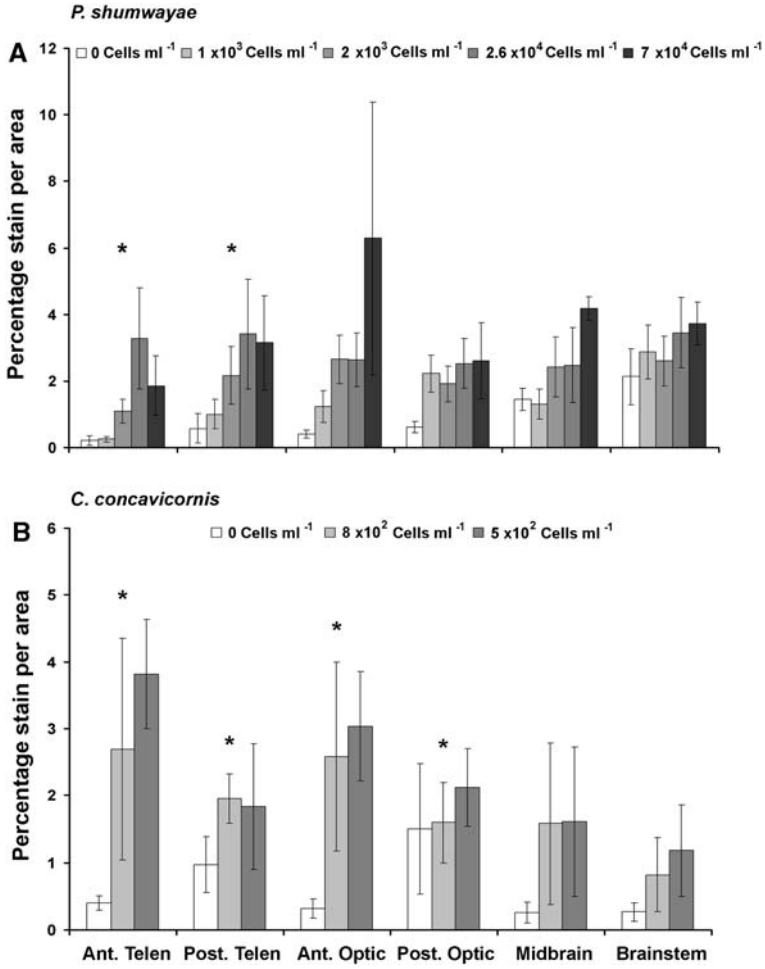

Fig. 2 *Fundulus heteroclitus*. c-Fos expression in mummichog brains exposed to varying densities of **a** *P. shumwayae* (0, 1×10^3, 2×10^3, 2.6×10^4, and 7×10^4 cells ml^{-1}, $N = 8$) and **b** *C. concavicornis* (0.8×10^2 and 5×10^3 cells ml^{-1}) (mean ± SE, $N = 6$). *Ant. Telen* anterior telencephalon, *Post. Telen* posterior telencephalon, *Ant. Optic* anterior optic lobe, *Post. Optic* posterior optic lobe, *Midbrain* midbrain tegmentum, *Brainstem* rhombencephalon. The *y*-axis equals percentage of c-Fos expression per area (mm^2). **a** Significant increases in c-Fos expression occurred in the *Ant* and *Post Telen* regions of *P. shumwayae*-exposed fish ($P = 0.0001$, and 0.0161, asterisk respectively). **b** Significant increases in c-Fos expression occurred in the *Ant. Telen and Post. Telen*, and the *Ant*. and *Post*. *Optic* regions of *C. concavicornis*-exposed fish ($P = 0.0011$, 0.0122, 0.0101, and 0.0088, asterisk, respectively)

In contrast to the *P. shumwayae* exposures, no behavioral alterations were observed in *C. concavicornis*-exposed fish. As with *P. shumwayae*-exposed fish, no histopathology was observed in the gills or skin from the *C. concavicornis*-exposed fish when compared with controls.

Discussion

Exposure of mummichog to varying densities of *P. shumwayae* and *C. concavicornis* resulted in increased neuronal c-Fos expression. Increases in c-Fos expression were observed in several regions of the mummichog brain, with the largest increases in labeling in exposed fish occurring in the telencephalon and optic lobes. These findings are consistent with other studies utilizing c-Fos expression to examine effects of stress exposure in fish (Bosch et al. 1995, 2001; Baraban et al. 2005; Salierno et al. 2006; Shimomura-Umemura and Ijiri 2006). The increases observed in this study are indicative of changes in neuronal activity resulting from exposure to *P. shumwayae* and *C. concavicornis* in vivo. This is consistent with in vitro exposures to *P. piscicida* organic and residual water fractions of mammalian and teleost cell lines, which demonstrated increased *c-fos*-luciferase expression (Fairey et al. 1999). Both HAB species investigated in this study are known to be harmful to fish and both can cause mortality at high cell densities.

In both algal exposures, the most notable increases in c-Fos expression occurred in the telencephalon and optic lobes of exposed fish. Densities of *P. shumwayae* and *C. concavicornis* as low as 1×10^3 cells ml^{-1} resulted in increased c-Fos expression in the anterior telencephalon and anterior optic lobes, and in the anterior telencephalon and periventricular gray zone of the optic tectum, respectively. In addition, as densities of both *P. shumwayae* and *C. concavicornis* increased, c-Fos expression also increased. These increases in expression in the telencephalon and optic tectum are consistent with changes seen in fish exposed to transport stress and HAB neurotoxins (Salierno et al. 2006). Further, increases in neuronal stress in the optic tectum and telencephalon may have deleterious effects on behavior, potentially compromising responses to stimuli and survival.

Mortality has typically been the observed endpoint in previous fish bioassays with *P. shumwayae* (Noga et al. 1996; Burkholder and Glasgow 1997; Burkholder et al. 2001a; 2001b; Berry et al. 2002; Vogelbein et al. 2002; Lovko et al. 2003). The dominant and most consistent cause of death is believed to be direct contact of *P. shumwayae* with fish (Vogelbein et al. 2002; Lovko et al. 2003). The present study, however, focused on the effects of sublethal exposure of fish to environmentally relevant densities of *P. shumwayae*. Although notable changes in CNS activity, visualized through c-Fos expression, were observed, no histological alterations were observed in the gills or skin of mummichog from this 2 h exposure to *P. shumwayae* (data not shown). The absence of histopathology in this acute exposure contrasts with the skin pathologies in mummichog exposed for > 24 h (Lovko et al. 2003).

Alterations in fish behavior resulting from exposure to *Pfiesteria*-like dinoflagellates have been observed and documented in both field and laboratory studies (Burkholder et al. 1995; Berry et al. 2002). However, the neural mechanisms controlling these behaviors remain poorly understood. Mummichog exposed to *P. shumwayae* in the present study exhibited alterations in behavior similar to those reported for tilapia (Berry et al. 2002). In addition to altered behavior, mummichog exposed to densities of *P. shumwayae* as low as 1×10^3 cells ml^{-1} displayed significantly greater c-Fos induction in neurons compared with control fish. This is the first report of neurological alterations in fish resulting from sublethal exposure to *P. shumwayae*. It is unclear, however, whether increases in c-Fos result from physical stress alone, a toxin, or a synergism between the two. Recently, a metal-containing organic-ligated toxin has been identified and characterized in *P. piscicida* (CCMP1921) (Moeller et al. 2007). However, the toxin is labile and the structure appears to be dependant on the organic matter present in the environment (Moeller et al. 2007). There is no direct evidence to support that *P. shumwayae* produces any toxic compounds. Nevertheless, exposure of mummichog to a soluble toxin in the present study cannot be ruled out and requires further investigation.

The cell densities of *P. shumwayae* used for this study are similar to, and in the range of densities recorded from, the field and from other laboratory studies. In North Carolina estuaries, densities of *Pfiesteria* spp. can range from 50–35 $\times 10^3$ cells ml^{-1}, with some samples reaching 1×10^5 cells ml^{-1} (Burkholder et al. 1995; Glasgow et al. 1995). In the Chesapeake Bay, *Pfiesteria* spp. densities ranged between 3×10^2 and 9×10^2 cells ml^{-1} in the Pocomoke river in 1997 (Glasgow et al. 2001). Laboratory bioassay exposures using *P. shumwayae* (CCMP 2089 and CAAE 101272) have ranged from 15 to 25 $\times 10^3$ cells ml^{-1}, with mortality occurring at densities greater than 3×10^2 cells ml^{-1} (Lewitus et al. 1995; Gordon et al. 2002; Vogelbein et al. 2002; Lovko et al. 2003; Gordon and Dyer 2005).

This study demonstrated that fish exposed to the HAB species *P. shumwayae* and *C. concavicornis* had significantly increased c-Fos expression, an indicator of neuronal stress. Additionally, these changes in brain activity resulting from *P. shumwayae* and *C. concavicornis* exposures are quantifiable and are not accompanied by histopathological changes in the skin and gills. Regional brain increases in c-Fos expression associated with *P. shumwayae and C. concavicornis* provide novel insights into neuronal responses to HAB exposure in fish. These alterations in neuronal activity in response to stress exposure in the laboratory may have deleterious effects in fish exposed to HABs in the wild. Results indicate that c-Fos expression describes a novel effect of sublethal exposure to *P. shumwayae* and *C. concavicornis*, and may serve to link higher-level alterations, including behavior, with neuronal stress.

Acknowledgments Portions of this study were supported by the U.S. Environmental Protection Agency, Science to Achieve Results (STAR) program (#R82-8224), the Maryland Department of Health and Mental Hygiene, and the Centers for Disease Control and Prevention. The authors thank N. Snyder, A. Miller, and C. Squyars, for their assistance with the brain processing and data collection. We also thank R. Andersen, Bigelow Marine Laboratory, for providing the *C. concavicornis* cultures. Reported experiments complied with regulations set by the Institutional Animal Care and Use Committee of the University of Maryland (Protocol R-00-36B) and VIMS (IACUC protocol 0129, IBC protocols 9906 & 0206).

References

Baraban SC, Taylor MR, Castro PA, Baier H (2005) Pentylenetetrazole induced changes in zebrafish behavior, neural activity and *c-fos* expression. Neuroscience 131:759–768

Bell GR (1961) Penetration of spines from a marine diatom into gill tissue of lingcod (*Ophiodon elongatus*). Nature 192:279–280

Bell GR, Griffioen W, Kennedy O (1974) Mortalities of pen-reared salmon associated with blooms of marine algae. Northwest Fish Cutlure Conference, Seattle, WA, pp 58–60

Berry JP, Reece KS, Rein KS, Baden DG, Haas LW, Ribeiro WL, Shields JD, Snyder RV, Vogelbein WK, Gawley RE (2002) Are *Pfiesteria* species toxicogenic? Evidence against production of ichthyotoxins by *Pfiesteria shumwayae*. Proc Natl Acad Sci USA 99:10970–10975

Blazer VS, Vogelbein WK, Densmore CL, May EB, Lilley JH, Zwerner DE (1999) *Aphanomyces* as a cause of ulcerative skin lesions of menhaden from Chesapeake Bay tributaries. J Aquat Anim Health 11:340–349

Bosch TJ, Maslam S, Roberts BL (1995) A polyclonal antibody against mammalian Fos can be used as a cytoplasmic neuronal-activity marker in a teleost fish. J Neurosci Methods 58:173–179

Bosch TJ, Maslam S, Roberts BL (2001) Fos-like immunohistochemical identification of neurons active during the startle response of the rainbow trout. J Comp Neurol 439:306–314

Burkholder JM, Glasgow HB (1997) *Pfiesteria piscicida* and other *Pfiesteria*-like dinoflagellates: behavior, impacts, and environmental controls. Limnol Oceanogr 42:1052–1075

Burkholder JM, Noga EJ, Hobbs CH, Glasgow HB (1992) New phantom dinoflagellate is the causative agent of major estuarine fish kills. Nature 360:768–768

Burkholder JM, Glasgow HB, Hobbs CW (1995) Fish kills linked to a toxic ambush-predator dinoflagellate—distribution and environmental-conditions. Mar Ecol Prog Ser 124:43–61

Burkholder JM, Glasgow HB, Deamer-Melia N (2001a) Overview and present status of the toxic *Pfiesteria* complex (Dinophyceae). Phycologia 40:186–214

Burkholder JM, Marshall HG, Glasgow HB, Seaborn DW, Deamer-Melia NJ (2001b) The standardized fish bioassay procedure for detecting and culturing actively toxic *Pfiesteria*, used by two reference laboratories for Atlantic and Gulf Coast states. Environ Health Perspect 109:745–756

Burkholder JM, Gordon AS, Moeller PD, Mac Law J, Coyne KJ, Lewitus AJ, Ramsdell JS, Marshall HG, Deamer NJ, Cary SC, Kempton JW, Morton SL, Rublee PA (2005) Demonstration of toxicity to fish and to mammalian cells by *Pfiesteria* species: comparison of assay methods and strains. Proc Natl Acad Sci USA 102:3471–3476

Cheng SB, Kuchiiwa S, Nagatomo I, Akasaki Y, Uchida M, Tominaga M, Hashiguchi W, Kuchiiwa T, Nakagawa S (2002) 2,3,7,8-Tetrachlorodibenzo-p-dioxin treatment induces c-Fos expression in the forebrain of the Long-Evans rat. Brain Res 931:176–180

Dykstra MJ, Kane AS (2000) *Pfiesteria piscicida* and ulcerative mycosis of Atlantic menhaden - Current status of understanding. J Aquat Anim Health 12:18–25

Espana RA, Valentino RJ, Berridge CW (2003) Fos immunoreactivity in hypocretin-synthesizing and hypocretin-1 receptor-expressing neurons: effects of diurnal and nocturnal spontaneous waking, stress and hypocretin-1 administration. Neuroscience 121:201–217

Fairey ER, Edmunds JSG, Deamer-Melia NJ, Glasgow H, Johnson FM, Moeller PR, Burkholder JM, Ramsdell JS (1999) Reporter gene assay for fish-killing activity produced by *Pfiesteria piscicida*. Environ Health Perspect 107:711–714

Flewelling LJ, Naar JP, Abbott JP, Baden DG, Barros NB, Bossart GD, Bottein MYD, Hammond DG, Haubold EM, Heil CA, Henry MS, Jacocks HM, Leighfield TA, Pierce RH, Pitchford TD, Rommel SA, Scott PS, Steidinger KA, Truby EW, Van Dolah FM, Landsberg JH (2005) Red tides and marine mammal mortalities. Nature 435:755–756

Glasgow HB, Burkholder JM, Schmechel DE, Tester PA, Rublee PA (1995) Insidious effects of a toxic estuarine dinoflagellate on fish survival and human health. J Toxicol Environ Health 46:501–522

Glasgow HB, Burkholder JM, Mallin MA, Deamer-Melia NJ, Reed RE (2001) Field ecology of toxic *Pfiesteria* complex species and a conservative analysis of their role in estuarine fish kills. Environ Health Perspect 109:715–730

Gordon AS, Dyer B (2005) Relative contribution of exotoxin and micropredation to icthyotoxicity of two strains of *Pfiesteria shumwayae* (Dinophyceae). Harmful Algae 4:423–431

Gordon AS, Dyer BJ, Seaborn D, Marshall HG (2002) Comparative toxicity of *Pfiesteria* spp., prolonging toxicity of *Pfiesteria piscicida* in culture and evaluation of toxin(s) stability. Harmful Algae 1:85–94

Herdegen T, Leah JD (1998) Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. Brain Res Rev 28:370–490

Jakobsen KS, Tengs T, Vatne A, Bowers HA, Oldach DW, Burkholder JM, Glasgow HB, Rublee PA, Klaveness D (2002) Discovery of the toxic dinoflagellate *Pfiesteria* in northern European waters. Proc R Soc Lond Ser B-Biol Sci 269:211–214

Kiryu Y, Shields JD, Vogelbein WK, Zwerner DE, Kator H, Blazer VS (2002) Induction of skin ulcers in Atlantic menhaden by injection and aqueous exposure to the zoospores of *Aphanomyces invadans*. J Aquat Anim Health 14:11–24

Kiryu Y, Shields JD, Vogelbein WK, Kator H, Blazer VS (2003) Infectivity and pathogenicity of the oomycete *Aphanomyces* invadans in Atlantic menhaden *Brevoortia tyrannus*. Dis Aquat Org 54:135–146

Law M (2001) Differential diagnosis of ulcerative lesions in fish. Environ Health Perspect 109:681–686

Lewitus AJ, Jesien RV, Kana TM, Burkholder JM, Glasgow HB, May E (1995) Discovery of the phantom dinoflagellate in Chesapeake Bay. Estuaries 18:373–378

Lewitus AJ, Hayes KC, Willis BM, Burkholder JM, Glasgow HB, Holland AF, Maier PP, Rublee PA, Magnien R (2002) Low abundance of the dinoflagellates, *Pfiesteria piscicida*, *P. shumwayae*, and *Cryptoperidiniopsis* spp., in South Carolina tidal creeks and open estuaries. Estuaries 25:586–597

Litaker RW, Steidinger KA, Mason PL, Landsberg JH, Shields JD, Reece KS, Haas LW, Vogelbein WK, Vandersea MW, Kibler SR, Tester PA (2005) The reclassification of *Pfiesteria shumwayae* (Dinophyceae): *Pseudopfiesteria*, gen. nov. J Phycol 41:643–651

Lovko VJ, Vogelbein WK, Shields JD, Haas LW, Reece KS (2003) A new larval fish bioassay for testing the pathogenicity of *Pfiesteria* spp. (Dinophyceae). J Phycol 39:600–609

Magnien RE (2001) The dynamics of science, perception, and policy during the outbreak of *Pfiesteria* in the Chesapeake Bay. Bioscience 51:843–852

Marshall HG, Hargraves PE, Burkholder JM, Parrow MW, Elbrachter M, Allen EH, Knowlton VM, Rublee PA, Hynes WL, Egerton TA, Remington DL, Wyatt KB, Lewitus AJ, Henrich VC (2006) Taxonomy of *Pfiesteria* (Dinophyceae). Harmful Algae 5:481–496

Miller TR, Belas R (2003) *Pfiesteria piscicida*, *P. shumwayae*, and other *Pfiesteria*-like dinoflagellates. Res. Microbiol. 154:85–90

Moeller PDR, Beauchesne KR, Huncik KM, Davis WC, Christopher SJ, Riggs-Gelasco P, Gelasco AK (2007) Metal complexes and free radical toxins produced by *Pfiesteria piscicida*. Environ Sci Technol 41:1166–1172

Noga EJ, Khoo L, Stevens JB, Fan Z, Burkholder JM (1996) Novel toxic dinoflagellate causes epidemic disease in estuarine fish. Mar Pollut Bull 32:219–224

Profet EB, Mills B, Arrington JB, Sobin LH (1992) Laboratory methods in histotechnology. Armed Forces Institute of Pathology, Washington

Rhodes LL, Burkholder JM, Glasgow HB, Rublee PA, Allen C, Adamson JE (2002) *Pfiesteria shumwayae* (Pfiesteriaceae) in New Zealand. N.Z. J Mar Freshwat Res 36:621–630

Rublee PA, Kempton JW, Schaefer EF, Burkholder JM, Glasgow H, Oldach DW (1999) PCR and FISH detection extends the range of *Pfiesteria piscicida* in estuarine waters. Virginia J Sci 50:325–336

Sadananda M, Bischof HJ (2002) Enhanced fos expression in the zebra finch (*Taeniopygia guttata*) brain following first courtship. J Comp Neurol 448:150–164

Salierno JD, Snyder NS, Murphy AZ, Poli M, Hall S, Baden DG, Kane AS (2006) Harmful algal bloom toxins alter c-Fos protein expression in the brain of killifish, *Fundulus heteroclitus*. Aquat Toxicol 78:350–357

Shimomura-Umemura S, Ijiri K (2006) Effect of hypergravity on expression of the immediate early gene, *c-fos*, in central nervous system of medaka (*Oryzias latipes*). Adv Space Res 38:1082–1088

Vogelbein WK, Shields JD, Haas LW, Reece KS, Zwerner DE (2001) Skin ulcers in estuarine fishes: a comparative pathological evaluation of wild and laboratory-exposed fish. Environ. Health Perspect 109:687–693

Vogelbein WK, Lovko VJ, Shields JD, Reece KS, Mason PL, Haas LW, Walker CC (2002) *Pfiesteria shumwayae* kills fish by micropredation not exotoxin secretion. Nature 418:967–970

Watson RE, Wiegand SJ, Clough RW, Hoffman GE (1986) Use of cryoprotectant to maintain long-term peptide immunoreactivity and tissue morphology. Peptides 7:155–159

Wulliman MF, Rupp B, Reichert H (1996) Neuroanatomy of the zebrafish brain: A topological atlas. Birkhauser Verlag, Basel

Yang CZ, Albright LJ (1992) Effects of the harmful diatom *Chaetoceros concavicornis* on respiration of rainbow-trout *Oncorhynchus mykiss*. Dis Aquat Org 14:105–114

Yang CZ, Albright LJ (1994) The harmful phytoplankter *Chaetoceros concavicornis* causes high mortalities and leukopenia in chinook salmon (*Oncorhynchus tshawytscha*) and coho salmon (*Oncorhyncus kisutch*). Can J Fish Aquat Sci 51:2493–2500